Chapter 6

New plants for Cork Oak Landscapes : Micropropagation and Genetic Stability Studies

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SUMMARY

Quercus suber L (Cork oak) is an important species in the Mediterranean region but presently this species faces a critical situation. Cork oak landscapes face many threats, including fires, forest clearance for agriculture or faster-growing plants, climate change, disease and over-grazing. For that reason, it is crucial to develop strategies allowing the propagation and input of elite plants in these landscapes. Plant biotechnology offers technologies that may overcome some of the problems not solved by conventional methods in cork oak breeding programs. In this chapter we review some of the recent most successfully strategies in cork oak propagation by non-conventional methods. Furthermore, it summarizes factors involved in the control of oak somatic embryogenesis as a powerful method for vegetative plant propagation, which includes data on cryopreservation, transformation and genetic fidelity strategies.

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CORK OAK LANDSCAPES: ECOLOGY, DISTRIBUTION AND SOCIO- ECONOMIC MAGNITUDE

Cork oak (*Quercus suber* L.) belongs to the genus *Quercus* that comprises more than 600 species, most of them characterised by their fruits (acorns). It is an evergreen tree, up to 20 m height which sometimes reaches a diameter over 1.5 m, with downy twigs, rather sparse leaf canopy, and a very thick and deeply ridged bark. The cork is a product of a secondary meristem and its extraction takes place when the individual tree reaches an age of 25-30 years. From that point onwards, the bark is stripped every 10-15 years, due to the tree's ability to produce a new meristem once the bark is removed. Cork is mainly used as beverage sealant and insulation material (Gonçalves, 2000).

The worldwide distribution of the cork oak is confined to the centre and western Mediterranean basin, including Portugal, Spain, France, Italy and North Africa. Within Mediterranean ecosystems, cork oak presents key ecological and socio-economic roles. The international importance of the species is well recognized, being a target of the operating gene conservation network of the European Forest Genetic Resources Programme, coordinated by the International Plant Genetic Resources Institute (IPGRI) and FAO (Vogiatzakis and Careddu, 2003). Portugal is the leading producer of cork with 55% of world's production, followed by Spain with 26 % (Toribio *et al.,* 2005). In Portugal, in the last few years, cork oak has suffered a severe decline, especially during drought periods. Research studies targeted to identify the causes of such decrease, showed positive correlations between general decline symptoms and variables like overexploitation, stand ageing without natural regeneration, drought, soil characteristics and anthropogenic stress. It was concluded that the multiple uses of traditional cork oak landscapes contributed to forest decay by making it more sensitive to particular climate occurrences (Ferreira, 2000).

CONVENTIONAL PRACTICES OF CORK OAK PROPAGATION

Traditionally, cork oak propagation has been done using the seeds (Bellarosa, 1991; Chalupa, 1995; Bueno *et al.,* 2000). Despite there are usually no major problems with seed's germination, forest regeneration programmes of this species are severely constrained by grazing. Furthermore, acorn production is irregular and highly dependent on climatic conditions (Bueno *et al.,* 2000) and cork oak seeds tend to lose germinating capability after a certain storage period (Chalupa, 1995). This species presents high heterozygocity, caused not only by its allogamy, but also by possible past hybridizations with other species of the genus *Quercus* (Natividade, 1990). Moreover, plants obtained by seed propagation, are genetically unpredictable, and considering the long duration of the juvenile stage (approximately 25 years), only when the plant reaches the adult stage it is possible to evaluate interest traits (Chalupa, 1995). Therefore, these features limit the establishment of seed orchards and its use in genetic improvement programs, contrarily to what is currently done for some forest species (Celestino *et al.,* 2005; Pinto *et al.,* 2008).

The high heterozygocity referred above together with the fact that most of the interest traits are related with the non-additive genetic component of the genetic variance, result that only through vegetative propagation it is possible to perpetuate the trees with valuable

interest (Pinto *et al.*, 2008). Furthermore, since selection of elite cork oaks is only possible in adult plants, the use of some of the traditional techniques of vegetative propagation to provide a reliable alternative to propagation via seed and a means of capturing genetic gains becomes more difficult. Research on conventional vegetative propagation methods has been followed since a long time, but with little success (Natividade, 1990; Bueno *et al.*, 2000). Therefore, and considering the projection of climate change scenario for Mediterranean region (IPCC, 2007) it is of utmost importance to introduce more adequate non-conventional methodologies for cork oak propagation and breeding.

Facing this scenario, forestry practices, especially those directed to the selection and propagation of elite trees may profit from the current technological advances. Research areas such as micropropagation, somatic embryogenesis, genetic engineering, marker-aided selection and molecular diagnostics, are being merged with traditional studies, to help in the identification and production of better-suited trees for forestry plantings. Also, a combination of classical and molecular research could be used to improve pest and stress resistance of selected genotypes and monitor pest attacks on trees (Klopfenstein and Kerl, 1995). In order to transfer the benefits of different biotechnologies to forestry plantings, a reliable plant regeneration technique is essential (Celestino *et al.*, 2005). These authors highlight that all these biotechnological tools are of no value if it is not possible to mass propagate clonal plants and obtain healthy individuals (either transgenic or not).

IN VITRO PLANT CULTURE

In the last decades, there has been a rapid progress in the development of in vitro culture techniques for the regeneration and clonal propagation of plants of interest (Merkle and Nairn, 2005). These in vitro culture techniques allow the development of different pathways for plant regeneration in a reproducible way (from several genotypes) and from several cell / tissues, resulting in true-to-type individuals (Kumar *et al.*, 2006). The number of forest trees propagated by in vitro culture techniques, which have been successfully used in forest breeding programs, has increased in the past years (for a review in hardwood tree biotechnology see Merkle and Nairn, 2005). Most of the successful cases refer to conifer species, but recently there have been significant advances in the biotechnology of angiosperm forest trees using these techniques (Merkle and Nairn, 2005).

In general, woody species are more difficult to propagate in vitro than herbaceous ones. This is due to a lower regenerative capacity, to the production and release of toxic compounds into the growth medium and to the extra difficulty of explants sterilization in woody species. Also, the phase change phenomena or ontogenetic aging, may contribute to their recalcitrance to in vitro propagation. Finally, due to their high heterozygocity, for many woody plants the results are more variable. (Gaj, 2004; Jiménez, 2005).

In the last decades, there have been enormous efforts in optimizing in vitro conditions for oaks, particularly cork oak (e.g. Toribio *et al.*, 1998; Pinto *et al.*, 2002; Hernández *et al.*, 2003a), from induction to acclimatization. Obviously, there are several factors that may affect the regeneration capacity of the explant: factors determined by the donor plant conditions (e.g., age, physiological status, type of explant and its position in the plant, period of collection of plant material and genotype) and those related to

environmental conditions of the culture (e.g., composition of culture medium, type and concentration of growth regulators, container volume, quality and quantity of light and temperature) (e.g., Gaj, 2004; Jiménez, 2005).

In the particular case of woody species with industrial interest, there are two main pathways to in vitro propagate large numbers of plants: micropropagation by nodal / axillary cuttings and somatic embryogenesis (SE):

MICROPROPAGATION BY AXILLARY SHOOTS

The micropropagation by axillary shoots has proved to be an effective methodology in several woody species (e.g., *Populus* sp., *Eucalyptus* sp., *Robinia pseudoacacia*, *Liquidambar styraciflua*, *Prunus* sp., *Juglans* sp., and *Ulmus*; for a review see Merkle and Nairn, 2005). Unfortunately, in cork oak, only few reports were based in this classic micropropagation methodology.

In vitro culture in *Quercus* was subject to major developments in the 1970's and 1980's. Since then it was possible to micropropagate several species of *Quercus*, such as *Quercus robur* (from nodal and apical segments; Chalupa, 1995) and *Quercus rubra* (from nodal segments; Bellarosa, 1989). Concerning, cork oak, the first report dates to 1952, when Jacquiot obtained callus from in vitro cultures of cambial tissue (Jacquiot, 1952 *vide* Bellarosa, 1989). Later, Bellarosa (1981) obtained subcultures of axillary shoots from in vitro cultures of cork-oak zygotic embryos and Pardos (1981 *vide* Bellarosa, 1989) produced cork oak cultures from nodal segments of 12 month-old seedlings. In 1988, Deidda and co-workers, reported plantlet regeneration from axillary shoots of seedlings (Deidda *et al.*, 1988). However, in three works little success was achieved in the rooting process. The first report of successful micropropagation from adult material is attributed to Manzanera and Pardos (1990) who reported in vitro plantlet multiplication using apical buds and nodal stem segments from different explant sources (young seedlings, stump sprouts and sprouts from old trees). The authors suggested that media with low concentrations of ions, such as Sommer's or Heller's, are more suitable for the growth and proliferation of explants of cork oak (Manzanera and Pardos, 1990). The plant regeneration from adult trees opened the perspective to propagate selected trees with desirable characters. When field material of is used, one of the most important and limitative factors in micropropagating this species are the phytosanitary conditions of the mother plant and the process of disinfection. Although these authors had no problems with contaminants, a high percentage of toxic compounds were released into the growth medium and consequently a large amount of plant material was lost. Later, Romano and Loução (1992) used Gresshof and Doy (1972) medium to initiate cultures from axillary / terminal buds, and were able to obtain similar results to Manzanera and Pardos (1990). However, the difficulties of the micropropagation process, mainly culture browning and bacterial contamination, were highlighted by the authors. By the same time, Gonçalves and Rainho (1992) were able to obtain plantlets from axillary shoots of 4-9 month old cork oak after inoculation on MS medium (Murashige and Skoog**,** 1962). More recently El-Kbiach *et al.* (2004) tested different macronutrient formulas and proposed that the combination of MS micronutrients and WPM macronutrients improved caulogenesis and shoot multiplication. Furthermore, several studies have already highlighted the importance of cytokinins (e.g., BAP: 6-benzylaminopurine) in

culture development (Manzanera and Pardos 1990; Pinto 2002) and the relevance of adding low levels of auxins (e.g., NAA: naphthaleneacetic acid) to the medium to improve shoot multiplication, especially in vegetative material of adult origin (Manzanera and Pardos 1990).

After *in vitro* culture establishment, shoot proliferation and elongation, the next critical stage is rooting. In this stage, the type of auxin is fundamental to promote rhizogenesis. In cork oak, continuous exposure of low concentrations of IBA (Indole-3-butyric acid) or the dipping of the base of the shoots in a concentrated solution of IBA gave the best rooting results (Manzanera and Pardos, 1990; El Kbiach *et al.*, 2004; respectively).

Other factors, such as carbon source, also affect the rates of cork oak shoot proliferation and in vitro rooting (Romano *et al.*, 1995). While sucrose (3%) allowed the best shoot elongation rates, enabling an effectively higher number of shoots during media transfer, 4% glucose was the best carbon source during rooting phases. Romano and Loução (2003a) also highlighted the importance of darkness during the first week of rooting which resulted in a remarkable enhancement of the rooting percentage, number of roots developed per shoot and length of the longest root.

In a more conservation oriented study, Romano and Loução (1999) described a simple system for in vitro conservation of cork oak shoot cultures. The authors were able to store cork oak shoots *in vitro* on a multiplication medium (Gresshof and Doy medium containing 1 mg l^{-1} of BAP) at 5 ± 1°C, without any further subculture, for two years at dark conditions. These authors also suggested that this procedure could be a promising technique for medium-term conservation of cork oak's germplasm.

Considering the acclimatization phase, the available information is scarce. Romano and Loução (2003a) reported the acclimatization of cork oak well-rooted plantlets in a mixture of peat and vermiculite and incubation in a growth chamber with high humidity. Hardening of plantlets under high humidity during four weeks was found to be essential for successful acclimatization. After two months, plants were transferred to the glasshouse and the percentage of surviving plantlets after six months ranged from 60% to 72%. In a similar strategy, El-Kbiash *et al.*, (2004) 92% reported that rooted plants were successfully potted on horticultural substrate and incubated in a chamber with a high relative humidity.

Regarding the performance of cork oak micropropagated plantlets after transference to ex vitro conditions, the scenario is not different and to our knowledge only one study has been performed. Romano and Loução (2003b) characterised some aspects of the foliar anatomy of cork oak, and measured the rates of water loss by different types of leaves during the acclimatization period. The results are very similar to those reported for other species where acclimatization was followed (for a review see Hazarica, 2006). Leaves of in vitro grown plants presented open stomata and collapsed guard cells, while acclimatized leaves presented closed stomata. Also, a shade-leaf structure with large intercellular air spaces and a low mesophyll cell density, but with a differentiated palisade cell layer was observed in transverse sections of in vitro leaves. Leaves from acclimatized plants showed

a sun-leaf structure with small intercellular air spaces, high cell density and two or three palisade cell layers. During acclimatization, leaf thickness increased, as well as, cell compactness and differentiation. Stomatal density, aperture and guard cell protuberance decreased during the acclimatization period, while trichome density increased (Romano and Loução 2003b).

SOMATIC EMBRYOGENESIS: A POWERFUL TOOL IN FOREST BIOTECHNOLOGY

Somatic embryogenesis (SE) is the process by which somatic cells differentiate into somatic embryos. Somatic embryos morphologically resemble zygotic embryos. They are bipolar and bear typical embryonic organs, the radicle, hypocotyl and cotyledons. However, they develop through a different pathway (Merkle *et al.*, 1995; Dodeman *et al.*, 1997; von Arnold *et al.*, 2002). Compared with other in vitro propagation methods, somatic embryogenesis offers several advantages. A separate rooting step is not required, as somatic embryos have both a shoot and a root meristem; SE usually form propagules faster and in much larger numbers per explant; the SE process can be automated, bearing the potential to become cheaper than other clonal propagation techniques currently in use (Park *et al.*, 1998a); and it allows the cryopreservation of the embryogenic clonal lines, while the trees obtained from these lines are tested in the field (e.g., Park 2002). This last point is of great importance for advanced breeding programs and commercial forestry as they can directly benefit from the use of elite clones. In the area of forest biotechnology, this propagation method is nowadays regarded as a system of choice for genetic manipulations and mass propagation of superior forest tree genotypes.

The induction of SE in the *Quercus* genus dates already to 1982, when Srivastava and Steinhauer reported the obtainment of SE from zygotic embryos in *Quercus lebani* (Srivastava and Steinhauer, 1982). After this work, several others in different species followed, most of which using the same source material: *Quercus cautisima* (Kim *et al.*, 1997), *Q. alba* (Gingas and Lineberger 1989), *Q. rubra* (Rancillac *et al.*, 1996), *Q. serrata* (Sasamoto and Hosoi 1989), *Q. pubescens* (Féraud-Keller *et al.*, 1989), *Q. cerris* (Ostrolucka and Petronova 1991), *Q. petraea* (Jörgensen 1993; Chalupa, 1995), *Q. robur* (Chalupa 1995; Manzanera *et al.*, 1996), *Q. canariensis* (Bueno *et al.*, 1996) and *Q. ilex* (Féraud-Keller and Espagnac 1989; Mauri and Manzanera 2003). In the particular case of *Quercus suber*, the majority of the studies also refer to the induction of SE from zygotic or very young material. Induction of SE from immature/young tissues was achieved from: cotyledons of mature zygotic embryos (Toribio 1986), nodal segments (El Maâtaoui and Espagnac 1987; El Maâtaoui *et al.*, 1990), immature zygotic embryos (Bueno *et al.*, 1992; Manzanera *et al.*, 1993) and leaves of young plants (Fernández- Guijarro *et al.*, 1991; Fernández-Guijarro *et al.*, 1995). Despite the lack of knowledge about the future value of plants obtained from zygotic embryos these protocols are a good starting point for future studies using adult recalcitrant material (Merkle *et al.*, 1997).

WHERE ARE WE STANDING IN THE CASE OF CORK OAK

Induction from mature tissues

Since the last reviews of Chalupa (1995), Manzanera *et al.* (1996) and Wilhelm *et al.* (2000), considerable progress has been achieved in the micropropagation through SE

in the *Quercus* genus, especially because reproducible protocols using mature tissues have been finally obtained.

Many differences can be noticed when comparing the protocols for SE initiation from juvenile tissue with those using adult material. The most blatant difference concerns the success rates of the induction stage: while in juvenile tissues it is common to obtain a 100% induction frequency, using mature tissues the success is much lower.

The preliminary studies published by Fernández Guijarro (1997) and Toribio *et al.* (2000) showed the possibility of inducing somatic embryos from leaves of mature cork oak trees. This has been further confirmed by Pinto *et al.* (2002), Hernández *et al.*, (2003a), Toribio *et al.* (2004, 2005) and Hernández (2007), who were able to clonally propagate and obtain emblings (SE-derived plants) from different selected trees. It is also possible to induce SE from floral structures, but in this case it is important to distinguish between somatic and gametic embryogenesis. Bueno *et al.* (1997) were able to produce haploid *Q. suber* plants from anthers by combining a starvation treatment with a mild heat shock at 33 ºC for 5 days, followed by culture at 25 ºC in a simple agar medium without plant growth regulators (PGRs).

In this book chapter we will concentrate in the SE reports where plant regeneration was induced from selected mature cork oak trees. These new findings open the possibility to apply SE to genotypes of interest, essential for cork oak plant breeding programs. Similarly to other species, plant regeneration by somatic embryogenesis in cork oak can be achieved following several phases: induction of embryogenic response, multiplication, maturation, conversion and acclimatization. In our opinion this last phase should be considered in the plant regeneration protocol if the main goal is the production of high quality plants.

Induction

The type of explants, the genotype, the culture medium and the growth regulators have a major influence in the induction of embryogenic cultures and plant conversion. The stress caused by growing conditions may favour the initiation of the embryogenic response by triggering the appropriate signal for genetic reprogramming and expression of totipotency of the somatic cells (von Arnold *et al.*, 2002; Fehér *et al.*, 2003; Jimenez, 2005).

In preliminary studies of SE induction in cork oak (Toribio *et al.*, 2000; Hernandez *et al.*, 2001), it was observed that the basic protocol developed to induce SE from leaves of young seedlings (Fernandez-Guijarro *et al.*, 1995) was also suitable for obtaining embryogenic lines from leaves of adult cork oak trees. However, the induction frequency was low and not all the tested genotypes formed somatic embryos. These protocols were optimized by Hernandez *et al.* (2003a), who were able to induce SE in several genotype from expanding leaves of epicormic shoots after those having been forced to sprout from segments of branches (in a growth chamber) collected from several hundred years-old cork oak trees. However, contamination was common problem, despite of the highly controlled conditions. This drawback was a matter of study by Toribio *et al.* (2005), who revealed that the putative endogenous contamination is influenced by genotype, collection date and, mainly, by the time of harvesting, with leaves harvested from recently sprouted shoots

becoming less contaminated than leaves collected from older epicormic shoots. A major step forward has been achieved by Pinto *et al.* (2002), who was able to successfully induce SE from leaves of mature trees collected directly from the field.

Although there are different protocols available to induce SE from mature leaves (mostly differing in medium composition and the type of plant growth regulators (PGRs)) they share some similarities, namely, the necessity for both an auxin and a cytokinin to induce SE process. The most widely used auxin sources are NAA or 2,4-D (2,4-dichlorophenoxyacetic acid), while BAP, or zeatin are the most frequently used cytokinins. Pinto *et al.* (2002) reported SE in leaf explant calluses obtained from a 60-year-old tree and induced on MS medium supplemented with 4.5 mM 2,4-D and 9.0 mM zeatin, 3% sucrose, 0.3% gelrite. Later on, Hernandez *et al.* (2003a) suggested the induction of SE following a two-stage method (as described in Fernandez-Guijarro *et al.* 1995), but with an extra preconditioning in vitro phase. The culture medium used at preconditioning consisted of Gamborg's macronutrients diluted by half, Murashige and Skoog (1962) (MS) micronutrients, vitamins and Fe-EDTA, supplemented with 1% sucrose and 0.6% agar, (without PGRs). The primary medium for induction consisted of Schenk and Hildebrandt (1972) macronutrients, MS micronutrients, vitamins and Fe-EDTA, supplemented with 3% sucrose, 0.6% agar, and 10 ìM BAP and 50 ìM NAA. The secondary medium for induction had the same composition as the primary one, but the concentrations of PGRs were reduced to 0.5 ìM BAP and 0.5 ìM NAA. Furthermore, these authors verified that SE induction frequency was significantly higher when the exposure to growth regulators was increased from seven to 30 days and that only in expanding leaves with less than 15 mm length it was possible to induce somatic embryos suggesting the existence of a development window that could be more prone to induction. In these protocols, a period in the dark of approximately 30 days, after which the cultures were transferred to the light, was reported (Pinto *et al.*, 2002; Hérnandez *et al.*, 2003a). Wilhlem *et al.* (2002) highlighted the required period for somatic embryos formation was shorter when juvenile tissues were used as explants in comparison to mature explants. Pinto *et al.* (2002) reported a 3-months period to observe embryogenic structures, whereas Hérnandez *et al.* (2003a) reported SE after 2-months in culture. Later, Toribio *et al.*, (2005) published detailed and reliable protocols for complete plant regeneration of cork oak by SE.

The differences in SE performance among genotypes support a genotypic effect as suggested by Merkle *et al.* (1997). The influence of genotype on morphogenic processes is well documented, including the genetic control of SE induced in zygotic embryos in other woody plants (Park *et al.*, 2002; Lopes *et al.*, 2006; Pinto *et al.*, 2008). Significant variances in the embryogenic response due to the cork oak family influence were detected by Fernandez-Guijarro *et al.* (1996). Toribio *et al.* (1998) and Hernández *et al.* (2003a) revealed a significant influence of individual adult cork oak trees on SE initiation frequency. Fernandez-Guijarro (1997) highlighted that some phases of this process show an additive genetic control, particularly the induction of SE in zygotic embryos, being amenable to genetic improvement.

Independently of the explant source (leaves or zygotic embryos), cork oak somatic embryos show the same developmental pattern. Somatic embryos appeared almost directly

on the surface of leaves, without a defined pattern of organization, and they soon started to show secondary embryogenesis The full expression of embryogenesis occurred when the leaves were transferred to PGR-free medium, in which more leaves showed their embryogenic ability, and somatic embryos their potential to multiply themselves by repetitive embryogenesis (e.g., Pinto *et al.,* 2002, Hérnandez *et al.,* 2003a; Toribio, 2005; Hérnandez, 2007).

REPETITIVE SOMATIC EMBRYOGENESIS

In dicotyledonous woody species as cork oak, repetitive embryogenesis (SE) is a phenomenon whereby new somatic embryos are initiated from other somatic embryos (Toribio 2005). The process of SE is normally not synchronized (Toribio 2005), therefore, a broad spectrum of different developmental stages can be found simultaneously. In an optimized procedure, this phenomenon can be automated by the use of liquid culture and bioreactors (Park 2002). In cork oak, secondary embryogenesis takes place continuously on media without PGRs (merely by monthly subculture to fresh medium), giving a recurrent process (repetitive SE) that can last for years without an apparent decline of multiplication ability. Additionally, similarly to what occurs in other species, the primary SE in *Q. suber* is less efficient than the secondary SE (Pinto *et al.,* 2002; Hernández *et al.,* 2003a; Pinto *et al.,* 2008b). Other relevant feature of this process is that embryos may reach their full cotyledonar stage without any specific treatment of differentiation, allowing its easy isolation, with new embryos being formed from previous somatic embryos mostly following a multicellular budding pattern (Toribio *et al.,* 2005; Hernández, 2007). Hérnandez *et al.* (2003a) reported that the initially induced embryos multiplied themselves (repetitive SE) on a MS medium lacking PGRs for more than 2 years without a decline and highlighted that, from a practical point of view, the number of somatic embryos produced per leaf of mature trees, and even the frequency of induction, are not relevant features of the protocol, considering the high rate of proliferation that is further achieved by repetitive SE. Fernandes *et al.* (2008) reported a five years period of maintenance of the embryogenic lines initially induced by Pinto *et al.,* (2002). These oak embryogenic cultures could be stored at low temperature for four months without loss of their proliferative ability, which permitted to reduce the maintenance costs by increasing the gaps between subculture periods.

In cork oak, most of the secondary embryos are formed next to the root pole, and more rarely in the cotyledons (El Maâtaoui *et al.,* 1990; Bueno *et al.,* 1992; Fernandez-Guijarro *et al.,* 1995; Puigderrajols *et al.,* 1996; Bueno *et al.,* 2000; Pinto *et al.* 2002; Hernández *et al.,* 2003a). This fact seems to be a common feature in the *Quercus* genus (Wilhelm 2000). In general, a typical somatic embryo at the cotyledonar stage of cork oak is a bipolar structure flanked by two cotyledons that may appear to have a transparent to opaque appearance according to the degree of maturation (Fernandez- Guijarro *et al.,* 1995; Pinto *et al.,* 2002; Hernández *et al.,* 2003a). The occurrence of abnormal morphologies in the somatic embryos, such as the presence of one or more than two cotyledons or even fused embryos or merged cotyledons, have been frequently reported in this species (e.g., Puigderrajols *et al.,* 1996; Pinto *et al.,* 2002; Hernández 2003b). However, such occurrence, was not reflected at the next stages of the SE process, as the plants derived

from these SE showed no morphological variability when compared with those obtained from conversion of normal dicotyledonary embryos (Pinto *et al.*, 2002). Few studies have dealt with the genetic control of the repetitive embryogenesis process and similarly to the induction phase this factor seems to play an important role in secondary SE. Hernández *et al.* (2003b) referred that when embryogenic masses were broken into small pieces and sub-cultured, their growth rate, measured as the increase in relative fresh weight, was very high and strongly influenced by genotype, which explained 21% of the total variance. The genotype also affected the number of detachable embryos produced by the embryogenic clusters, accounting for 14% of the total variance.

There are same controversies concerning the direct or indirect origin of secondary somatic embryogenesis in cork oak. El Maâtaoui *et al.* (1990) did not recognize the presence of callus prior to the appearance of secondary somatic embryos and, thus considered secondary somatic embryos as having a direct origin. Contrarily, Puigderrajols *et al.* (1996) referred that secondary SE has an indirect origin since it occurs after an initial proliferation of callus on the area next to the root pole. In support of the latter view Pinto *et al.* (2002) observed the formation of a mass next to the root pole of initially isolated embryos, precisely in the area where later on the secondary embryos appeared. Ultrastructural studies of the secondary SE process revealed the occurrence of both pathways: the multicellular pathway from a compact mass of proliferation and the unicellular pathway from isolated cells in friable callus (Puigderrajols *et al.* 2001). The origin of these secondary somatic embryos is extremely relevant to the genetic uniformity of regenerated plants, as a multicellular origin may result in the formation of genetically variable plants (chimeras). A unicellular origin is undoubtedly the most desirable pathway for applications that involve the cloning of SE, such as genetic transformation. The protocols should be optimized in order to synchronise the SE process and promote the unicellular pathway of regeneration in favour of the multicellular one (Puigderrajols *et al.*, 1996, 2001).

MATURATION OF SOMATIC EMBRYOS

Has highlighted above, the recurrent secondary embryogenesis is the basis of the great potential of this technique for mass clonal production of somatic embryos. However, if the production of individual plants is desired, this process has to be arrested and the individual embryos should be left to mature (Toribio *et al.*, 2005).

Maturation may, sometimes, occur spontaneously by the end of the subculture period. A low availability of nutrients and consequently some degree of starvation have been pointed as the inducing factors of spontaneous maturation in cork oak (Toribio *et al.*, 2005). In this case secondary embryogenesis ceases and somatic embryos enlarge and become white opaque. These somatic embryos can then be selected for the germination/conversion phase (Toribio *et al.*, 2005). However, to use SE as a mass vegetative propagation technique of high value trees for forest planting, this maturation stage needs to be controlled and improved.

Abscisic acid (ABA) is a well-known PGR involved in the maturation process, promoting the accumulation of reserve substances, and reducing the frequency of abnormal morphologies and secondary embryogenesis (Gaj 2004). In cork oak, some experiments

aimed to control the recurrent cycle by the addition ABA to the culture medium were inconclusive, i.e., no visible effects of ABA on the arrest of repetitive embryogenesis were detected (Pinto *et al.,* 2002; Toribio *et al.,* 2005). However, other studies, suggested that the addition of ABA was important to promote SE maturation (Bueno *et al.*, 1992; Garcia Martin *et al.*, 2005). Garcia Martin *et al.* (2005) found that the addition of 1 ìM ABA to the culture medium promoted somatic embryo maturation and increased both fresh and dry matter, without affecting the relative water content. However, these works differed from the formers, as the source explants for SE induction were leaves of a 2-month old *Q. suber* seedling. Alternative strategies for SE maturation include partial desiccation under high humidity conditions (Toribio *et al.,* 2005) and starvation treatments (Fernandez-Guijarro *et al.* 1994, 1995). In detail, the latter strategy consisted on an alternate culture of somatic embryos on (i) a medium that increased embryo proliferation and (ii) a low salt medium inhibiting embryo formation, which would partially synchronize embryo development. Moreover, maturation under light followed by storage at 4 °C for at least 30 days was found to be very important in switching embryos from an embryogenic pathway to a germinative one. Under these conditions 15% of the embryos showed coordinated root and shoot growth and 35% formed either shoots or roots (Fernandez-Guijarro *et al.,* 1995). Unfortunately, few studies provided detailed data on the effect of maturation treatments on the subsequent steps of germination and conversion.

GERMINATION AND CONVERSION OF SOMATIC EMBRYOS

One of the main problems of cork oak regeneration by SE is the low rates of somatic embryos conversion into plants. This low conversion is common in the genus (Wilhelm 2002) and, as referred by Chalupa (1995), it is common that the development of SE is blocked after the formation of the cotyledons, which may acquire a green colour, but do not proceed into the next developmental stage. According to Fernandez-Guijarro *et al.,* (1995) from the moment that somatic embryos are programmed to origin more embryos, the normal development is blocked, and only after this programme is interrupted, somatic embryos can proceed to germination.

Cold is considered the most efficient treatment to trigger the conversion process, being widely used, not only in *Quercus* but also in conifers (Haggman *et al.,* 1999). In cork oak, several studies used this strategy to promote germination and conversion of somatic embryos (Bueno *et al.,* 1992; Manzanera *et al.,* 1993; Fernandez-Guijarro *et al.,* 1995; García-Martin *et al.,* 2001; Pinto, 2002). In the particular case of Garcia-Martin *et al.* (2001), improved germination rates (best results close to 100%) were obtained not only by chilling somatic embryos for two months, but also by increasing the sucrose concentration on the culture medium to high levels (15%). In Pinto (2002), it was referred that cold significantly increased embryo conversion and decreased the number of secondary somatic embryos formed per initial explant, and that germination and/or conversion mainly occurred in embryos in a more advanced stage of evolution. Concerning the use of sucrose, it is suggested that the lack of sufficient reserves in the cotyledons to support the advanced stages of germination, mainly the growth of a large tap root before leaves are functional, may be responsible for the low percentages of conversion that are usually reported.

After germination, González-Benito *et al.* (2002) suggested that a low concentration

of BAP (0.04 ìM) allowed an appropriate radicle elongation in all germinating somatic embryos, and that higher concentrations of this PGR has opposite effects. It was also verified that the application of BAP increased caulinar apex elongation and maintained an active growth of the plantlets, although no significant effect on the percentage of shoots with normal morphology was observed. However, once again, genotype affected all the variables recorded during conversion.

PLANTLET ACCLIMATIZATION

At present, cork oak in vitro plant regeneration is well developed to fulfil low-level demands (e.g., conservation purposes, genetic assays, and plant development studies), as only a limited number of plants are usually obtained (Toribio *et al.,* 2005). This results not only from the problems verified in the stages mentioned above (e.g. maturation, germination / conversion), but also from the difficulty of the acclimatization step, in which many plantlets are lost. In general, in vitro conditions contribute to the formation of emblings with physiological and anatomical characteristics that need to be gradually acclimatized to greenhouse and / or field environments (for review see Hazarica 2006). Since the last revision in *Quercus* (Wilhelm 2000), considerable efforts have been made to optimize plant regeneration in oaks, but the process of acclimatization to the soil substrate still remains the major bottleneck for large scale production.

In cork oak, Bueno *et al.* (1992) reported that thirty-one plantlets developed normally and were transferred to soil and acclimated to greenhouse conditions, but no details on the survival rates were given. Manjon *et al.* (1998) reported that when *Q. suber* emblings were transferred to substrate they stop growing, and after some time died. In search of a solution to these problems, Diez *et al.* (2000) studied mycorrhizal associations in cork oak and observed that in vitro mycorrhization with *Scleroderma polyrhizum* and *Pisolithus tinctorius* increased the formation of secondary roots and the survival rate after acclimatization of plants obtained from somatic embryos. Later on, Hernández *et al.* (2003b) referred that after transferring the plantlets to ex vitro conditions bearing high relative humidity conditions, nearly half of them ceased growing and, after a decrease in relative humidity, 67% died. Only 33% of the plants that survived the change to low humidity were completely acclimatized. This means that, in the whole conversion process and depending on the genotype, only a few of the somatic embryos reached the state of completely acclimatised plants.

A recent strategy of acclimatization consists in transplanting the emblings to 180 ml forest containers, filled with substrate (pine bark:peat:sand, 3:1:1, v / v / v) and designed to avoid malformations in root growth, under ex vitro conditions. These emblings were placed in a growth chamber and were covered with inverted glass beakers to guarantee a high humidity. After two months, the beakers were removed 1 h / day during one month. Finally, the plants were moved to a nursery, under shade conditions (Hernández *et al.*, 2003b; Toribio *et al.,* 2005).

Most of the studies that reported emblings regeneration and acclimatization just provide the number of surviving plants, and only a few works gave details about their morphology and field performance. The ability to induce SE from leaves of adult trees opens the possibility to compare the performance of the parent trees and their progenies

at the same age and place of test. Celestino *et al.* (2007) reported a field test using emblings regenerated from five selected cork oak trees and from young plants of their half-sib progenies. They were planted in the field together with zygotic seedlings of these families, and the performance of plantlets of somatic vs. zygotic origin and plantlets obtained from SE of mature trees vs. SE of juvenile seedlings was compared. The effect of genotype and kind of progeny were studied using a complete factorial design; therefore the field trial comprised 15 treatments arranged in five completely randomised blocks. The authors observed that all the plants originated from seeds survived, while almost half of somatic seedlings died after the winter and needed to be replaced. Almost 70% of these plants survived after the following summer. There were no differences between somatic seedlings of mature or juvenile origin. Zygotic seedlings doubled their height relatively to the somatic ones after one year in the field, and, within somatic seedlings, those of mature origin grew slightly more than those originated from juvenile explants. Also, no apparent morphological alterations were detected among the three kinds of progenies. However, no complementary studies at the physiological level were performed that could sustain the obtained results. This is indeed, an important gap in the current knowledge of the acclimatization process in cork oak.

To improve this propagation system and overcome its main constraints, particularly the maturation stage and the low germination frequency, it will be fundamental to increase our knowledge in the developmental physiology aspects governing the SE production process. In order to optimize these and the further steps, one should focus on comparative physiological, biochemical and molecular studies of both the zygotic and somatic embryos development. As referred by Wilhelm (2000), due to the limited data available on plant performance, many questions cannot be answered and more studies concerning plant physiology during acclimatization and field trials are needed.

EVALUATION OF GENETIC FIDELITY

The commercial use of any micropropagation technique implies the assessment of emblings performance in the field and the analysis of their genetic stability (Park 1998). The economic consequences of somaclonal variation can be enormous in forest trees, due to their long life cycles. Therefore, plants presenting somaclonal variation should be detected and eliminated from the subsequent stages of the breeding program, in order to guarantee the success and acceptance of a large-scale micropropagation scheme of a particular plant species.

Somatic embryogenesis has often been regarded as a stable system; however, as referred above and as it occurs in other *Quercus* species, there is the chance that cork oak SE has a multicellular origin that increases the risk for somaclonal variation (Wilhelm, 2000). Reports in *Q. robur* (Wilhelm *et al.*, 1999; Endemann *et al.*, 2001) demonstrated that several years of continuous culture can result in tetraploidy. In cork oak, the possibility to propagate this species by secondary embryogenesis in media lacking PGRs minimizes this risk for genetic variation, but still it is important to screen the resulting emblings (at least) and compare them to the parental tree.

Chromosomic mutations like inversion, deletion or translocation and genetic mutations can be detected by molecular markers such as RFLPs (restriction fragment length

polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms) or microsatellites / SSRs (simple sequence repeats), while major genetic changes, like polyploidy and aneuploidy can be easily screened by the use of flow cytometry.

In *Q. suber*, RAPD markers were used to monitor the genetic stability of somatic embryos (Gallego *et al.,* 1997). In this study no aberration in RAPD banding pattern was found among the tested samples. Hornero *et al.* (2001) suggested that genetic fingerprinting based on amplified fragment length polymorphisms (AFLP) is more powerful and reliable than RAPDs, as it allows the direct analysis of variation at the entire DNA level with the generation of more reproducible markers. In that work, SE was induced in expanding leaves collected from mature cork oak branches that were forced to sprout. DNA was extracted from leaves of the mother plants (three different genotypes) and from somatic embryos derived from each tree. In one tree, AFLP patterns from leaves and somatic embryos were identical, but some variation was detected in somatic embryos from the other two trees. Although the level of genetic variation detected in these lines is lower than that recorded for half sibs of cork oak, its influence on phenotypic variation needs further assessment (Hornero *et al.,* 2001). More recently, the genetic stability of the SE process was also evaluated by simple sequence repeats (SSRs = microsatellites) (Lopes *et al.,* 2006). Uniform microsatellite patterns were, in general, observed within and between somatic embryos and the respective donor genotypes. For one genotype the same pattern was observed in all the analysed samples, except one, where a mutation was found (accounting for 2.5% of the tested material).

Other methodologies focused on ploidy changes must also be routinely used to assay genetic stability. Due to its precision and rapidity, flow cytometry (FCM) appears as the ideal technique to easily achieve such a goal. However, only a few works have used this technique to assess somaclonal variation in cork oak micropropagation (Bueno *et al.,* 1996; Bueno *et al.,* 2000; Loureiro *et al.,* 2005; Santos *et al.,* 2007). The most complete study so far, showed no major ploidy differences between somatic embryos and the mother plant from which they were obtained as well as ploidy level stability between somatic embryos with two cotyledons (considered as normal) and abnormal ones, presenting one or more than two cotyledons (Loureiro *et al.,* 2005).

OTHER APPLICATIONS OF SE: CRYOPRESERVATION, ARTIFICIAL SEEDS AND GENETIC TRANSFORMATION

As mentioned above, SE has a great potential for vegetative mass propagation of superior forest tree genotypes. However, the long periods that are needed for evaluating the quality of regenerated material, makes it imperative to store the source cultures for several years (Vendrame *et al.,* 2001). Cryopreservation has been used as a suitable and efficient mean for long term storage of plant tissues and cell cultures, including embryogenic cultures, which proved to be amenable to cryogenic conservation (Park 2002).

Cryopreservation techniques offer several advantages in tissue culture: it reduces labour and supply costs, it decreases the risk of culture contamination, it retains embryogenic capacity by reducing culture deterioration, it limits somaclonal variation and

it may enhance the embryogenic capacity by the elimination of highly vacuolated non-embryogenic cells (Vendrame *et al.*, 2001). Currently, cryopreservation was already successful in the conservation of different types of materials, including seeds with orthodox and intermediate storage behaviour, dormant buds, pollen, biotechnology products and apices sampled from *in vitro* plantlets of vegetatively propagated species (Engelmann, 2004). However, considering its high potential, it is expected that cryopreservation will soon become more and more used in the long-term conservation of plant genetic resources.

Shoot-tips and somatic embryos are excellent material for in vitro long-term storage (in liquid nitrogen) of ex situ plant genetic resources. Cryopreservation of organized structures has significantly progressed with the development of several vitrification-based protocols, such as encapsulation-dehydration and PVS2. These approaches allowed significant improvements in the survival and recovery after cryopreservation when compared with conventional crystallization-based protocols (Gonzalez-Arnao *et al.*, 2008).

Among forest species, cryopreservation protocols have been successfully applied to embryogenic cultures of white spruce (*Picea glauca* (Moench) Voss), silver birch (*Betula pendula* Roth) and radiata pine *Pinus radiata* D. Don), among others (for a review see Vendrame *et al.*, 2001). In oaks, there are already several studies that demonstrated the feasibility of the cryopreservation of somatic embryos. González-Benito *et al.* (2002) studied different aspects of the cryopreservation protocols used in *Quercus ilex* and *Q. suber* embryonic axes. In this study, *Q. suber* axes proved to be more sensitive to desiccation and cooling than *Q. ilex*. Only 35% of the axes survived when included into cryovials and immersed directly in liquid nitrogen, while none survived when immersed in sub-cooled liquid nitrogen (-210 °C). Later Valladares *et al.* (2004) described a protocol for cryopreservation of embryogenic lines induced from leaves of adult trees of cork oak. These authors used a simple vitrification procedure, consisting of a pre-culturing of 2-4 mg clumps (with two or three globular embryos) on semisolid medium containing 0.3 M sucrose for three days, followed by incubation in PVS2 vitrification solution at 0C (for 60 minutes) before direct immersion in liquid nitrogen, and were highly successful in the cryopreservation of three cork oak embryogenic lines (88-93% of embryo recovery). The average number of embryos produced per explant was significantly higher for cryostored embryos than for untreated stock cultures and the germination and plant regeneration rates of cultures derived from cryostored embryos, were around 60%, similar to those of non-cryopreserved stock cultures. Recently, Fernandes *et al.* (2008) described a highly successful encapsulation-dehydration procedure for cork oak somatic embryos. During this procedure, embryogenic clusters were encapsulated in an alginate bead, cultured for 3-days in 0.7 M sucrose, desiccated to 25 or 35% water content (WC), and frozen in liquid nitrogen. After thawing, cryopreserved somatic embryos had high viability and exhibited long-term survival (approximately 90% survival). Also, no morphological differences were observed between somatic embryos desiccated to 25 and 35% WC (Fernandes *et al.*, 2008). These authors highlighted that the used method minimizes the risk of tissue injury, because, unlike vitrification, it does not require toxic cryoprotectants as PVS2. As cryopreservation may also induce genetic instability (Sakai 2004), Fernandes and co-

workers evaluated the genetic stability of regenerated material using FCM, AFLPs and SSRs. Flow cytometry confirmed that both ploidy level and DNA content were consistent with the available literature (2C = 1.90 pg DNA; Loureiro *et al.*, 2005; Santos *et al.*, 2007) and that no significant differences between control and cryopreserved samples were detected at the DNA-sequence level.

In sum, the cryopreservation of oak's somatic embryos allows long-term preservation of germplasm until phenotypic traits are evaluated on adult trees under field conditions. However it is important to assure that embryogenic clones are maintained indefinitely in cryogenic storage without any change in genetic makeup or loss of viability (Park 2002). If such conditions are guaranteed, it is possible that in a near future cryopreservation together with the ultimate developments in SE protocols from mature trees are applied in cork oak improvement programs.

Another storage technique is the production of synthetic seeds. The first attempts to determine the optimal storage period and the optimal conditions for conversion of encapsulated somatic embryos of cork oak were published only recently by Bueno *et al.*, (2008). However, these authors used somatic embryos induced from zygotic seeds. The somatic embryos were coated with alginate for the production of synthetic seeds and their storability for commercialization was investigated. Synthetic seeds were cold stored at 4 ºC for two months without significant loss of conversion capacity. The conversion rates of synthetic seeds were higher on a medium supplemented with mineral nutrients than without. The authors referred that this method enabled the assessment of growth parameters without the risk for contamination, which opens the possibility for an automated control of culture growth for the future up scaling of plant production.

Genetic transformation offers an attractive alternative to breeding because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background. This has particular importance in woody species, since many adaptative and economic traits are under non-additive genetic control and, consequently, a specific genetic make-up has to be transferred to the offspring (Alvarez *et al.*, 2004). A prerequisite for the production of transgenic plants is the availability of a method to regenerate a complete plant from the transformed cell. Cork oak somatic embryo cultures offer an excellent starting point for genetic manipulation. These cultures present low manipulation necessities and high proliferation rates, and the maturation, conversion and acclimatization protocols to obtain plantlets from embryogenic lines are already optimized (e.g. Pinto *et al.*, 2002, Hernandez *et al.*, 2003a), which opens the possibility to manipulate and clone desired genotypes.

The reports available so far are very recent and result from the optimization of the protocols used to regenerate plants by SE. Álvarez *et al.* (2004) developed a protocol to transfer foreign chimeric genes into cork oak. These authors observed that cork oak somatic embryos obtained from recurrent proliferating embryogenic masses were susceptible to AGL1 strain (harbouring the pBINUbiGUSint plasmid,), a disarmed succinamopine-nopaline strain of *Agrobacterium tumefaciens*. Evidence of stable transgene integration was obtained by polymerase chain reaction for the *nptII* and *uidA* genes and by Southern blotting and expression of the *uidA* gene. However, the transformation efficiency (i.e.

percentage of inoculated explants producing independent transgenic embryogenic lines) was of 4%, only. The transgenic embryos were then germinated and successfully transferred to soil. Sánchez *et al.* (2005) also presented a protocol for genetic transformation of cork oak using pro-embryo masses induced from immature zygotic embryos in. These embryogenic masses were inoculated with the *A. tumefaciens* LBA4404 / p35S GUS INT / pCAMBIA 1301 strain. Transformants were selected on hygromycin supplemented medium, withthe viable embryos constituting 13% of the embryos selected on this medium during 4 months. The expression of glucuronidase, 4 months after co-cultivation, confirmed a transformation rate of 5.8%. Recently, Álvarez and Ordás (2007) published considerable improvements to the transformation system for selected mature cork oak trees. Álvarez and Ordás (2007) reported an optimized protocol for *A. tumefaciens*-mediated transformation (the transformation experiments were carried out with the disarmed *Agrobacterium tumefaciens* strain AGL1harbouring the binary vector pBINUbiGUSint or pBINUbiGUSint in combination with the ternary plasmid pBBR1MCS-5.*virGN54D*) of mature embryogenic masses. Factors such as, plant genotype, explant type and time elapsed between the last subculture and inoculation, i.e. the explant pre-culture period, were found to be very important in the success of the transformation system. The interaction between inoculum density and the co-cultivation period influenced the transformation efficiency, as well. A transformation efficiency of up to 43% was obtained andit was also found that this protocol could be applied to various genotypes.

CONCLUDING REMARKS

Up to date, plant regeneration in cork oak by SE has been obtained from different initial explants, in a process highly conditioned by the individual genotype. Presently, cork oak is one of the few forest species in which reproducible protocols for complete plant regeneration from several selected genotypes of adult trees have been obtained. The multiplication ability of this SE process is guaranteed by the occurrence of a repetitive process that dispenses the external addition of PGRs. In fact, the whole plant regeneration process can be achieved almost without the addition of PGRs. This review clearly demonstrates that that SE is probably the best plant regeneration method in addition to classical approaches. One of its main advantages is the possibility to induce and regenerate true-to-type plants from selected adult trees of high commercial value. Furthermore, the existence of optimized cryopreservation protocols for the embryogenic lines allows to preserve the embryogenic lines until the regenerated plants are tested under field conditions.

However, there still some bottlenecks, especially in the control of the repetitive SE, and on the maturation and germination / acclimatization stages of the SE process. Once these intermediate steps are optimized, the rates of plant regeneration will certainly increase to levels that fulfil the needs of the cork oak breeding and industry, allowing the large-scale micropropagation of elite trees of this species.

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