Somatic Embryogenesis and Cryopreservation in Forest Species: The Cork Oak Case Study

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1. Introduction

1.1 General concepts

It is widely accepted that propagation and conservation strategies are crucial in any forest breeding program and that plant biotechnology has powerful tools to propagate and preserve selected genotypes (Jain, 1999; Park et al., 1988; Park, 2002). In particular, in vitro techniques have demonstrated to be essential in a large number of agricultural and forest breeding programs, allowing large scale clonal propagation of elite genotypes (Pinto et al., 2008) or of endangered forest species (Brito et al., 2009). In vitro techniques are also essential in breeding programs involving genetic improvement (e.g. using techniques such as somatic hybridization or genetic transformation) or involving long term germplasm conservation by cryopreservation (Benson, 2008; Fernandes et al., 2008).

Somatic embryogenesis offers advantages in improving forest species over other in vitro propagation methods (Park, 2002), namely: a) somatic embryos simultaneously possess both the shoot and root meristems; so a distinct rooting stage, which usually involves stressing procedures, is not necessary; b) during somatic embryogenesis, embryos/clusters are often formed faster and potentially at extremely high numbers per explant; c) somatic embryogenesis is amenable to automation, which means it may become cheaper than other clonal propagation techniques (Pinto et al., 2002; 2008); d) finally, a robust and efficient protocol of somatic embryogenesis will allow that embryogenic clonal lines can be preserved for long periods (e.g., in liquid N_2) while corresponding plants are transferred to field conditions and are monitored for their characteristics.

The combination of somatic embryogenesis and conservation strategies in breeding programs allows that one may develop high-value forest clonal varieties merely by recovering from N_2 those genotypes/clones that showed best characteristics in the field. These selected genotypes can later be used for advanced breeding programs and commercial forestry (Park, 2002).

From the exposed advantages, it is generally accepted among breeders that breeding programs using in vitro clonal strategy require as a pre-requisite that a cloning technique (preferably somatic embryogenesis) is well established. Then it also requires the optimization of long-term genetic testing methodologies of clonal lines and long term

conservation. The combination of these strategies will then enable large-scale production and disposition of tested clonal lines in industrial forest management. For example, micropropagation processes are well refined for spruce and larch species to support their commercial application (http://cfs.nrcan.gc.ca/ factsheets/conifersomatic). However, for most pine species, it is much more difficult to obtain somatic embryogenesis, though interesting advances are in course (e.g. Park et al., 2010). Similarly, the application of this technology to most forest dicotyledonous species, as is the case of *Quercus* genus, has demonstrated to be difficult due to species general recalcitrance to in vitro culture (Santos, 2008).

1.2 *Quercus suber* in vitro cloning: A reliable protocol?

Cork oak (*Quercus suber* L.) belongs to Fagaceae, an important family of forest trees in the Northern hemisphere dominating temperate forests and Mediterranean ecosystems. In particular, cork oak is an abundant species in the Atlantic and West Mediterranean countries where it is an important component of Mediterranean ecosystems (Pinto et al., 2002). Cork is the bark of the oak, which is a natural, renewable and sustainable raw material product of economic interest for a range of applications. Due to its enormous economical importance, intense research has been focused on cork valuable material and, more recently, on cork oak germplasm-conservation programs (Fernandes et al., 2008).

In the last decades, studies were done to improve protocols of cork oak in vitro micropropagation and conservation (namely cryopreservation). In particular, the currently available rates of success in cork oak plant regeneration by somatic embryogenesis (Fernandes et al., 2011; Lopes et al., 2006; Loureiro et al., 2005; Pinto et al., 2001; 2002) and in cork oak material cryopreservation (Fernandes et al., 2008; Fernandes, 2011) are highly encouraging for researchers and breeders to consider the integration of these strategies in breeding and conservation programs of this species (Figure 1).

Recently, it was also established a Portuguese consortium to identify and characterize cork oak ESTs Gene responses to several biotic and abiotic stresses as well as to developmental conditioning are currently being screened and data will be of upmost importance to cork oak researchers and breeders (http://www.fct.pt/apoios/projectos/consulta/ projectos.phtml.en).

For long it has been assumed that *Quercus* species have, at some extent, recalcitrant responses to micropropagation in general, and to somatic embryogenesis in particular. Most common strategies for cork oak micropropagation use stem cuttings or use juvenile material or leaves for somatic embryogenesis (Santos, 2008; Fernandes et al., 2008). When utilizing material selected from adult field trees as explant sources, the use of greenhouse forced sprouts instead of directly collected field material is strongly advised.

The developed micropropagation by stem cutting is efficient with juvenile and, less, mature genotypes. Briefly, after disinfection with sodium hypochloride, explants (with 1-2 apical and/or lateral buds) are inoculated on WPM ("Woody Plant Medium", Lloyd and McCown, 1980) medium containing benzylaminopurine (BAP 0.5 mg/L) and naphthalene acetic acid (NAA 0.1 mg/L). After multiplication and elongation, shoots are exposed to an indol-butiric acid shock for rooting. Plants in this stage are then ready for acclimatization (Pires et al., 2003; Figure 2a).
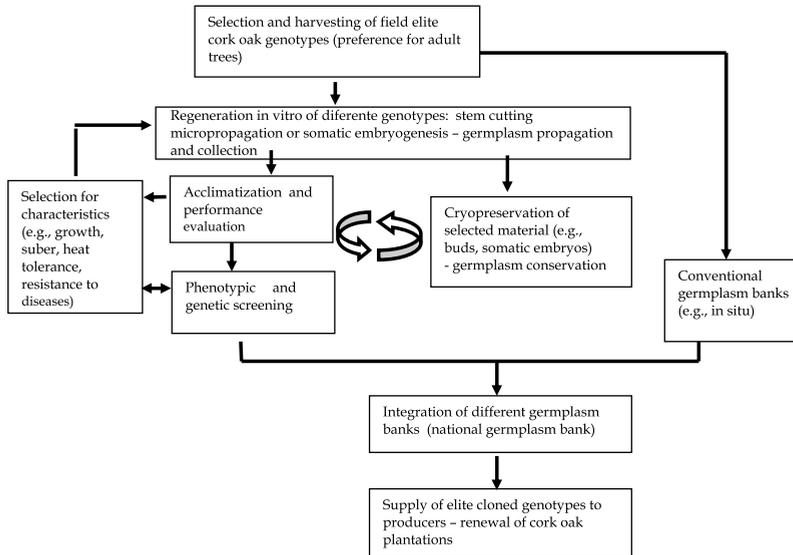
Fig. 1. Schematic representation of a proposed strategy of integrating cork oak micropropagation and cryopreservation technologies in Portuguese breeding programs of this species (adapted from Santos, 2008).

As reported above, somatic embryogenesis is a regeneration strategy with enormous potential for breeding programs. Somatic embryos were developed in *Q. canariensis* (Bueno et al., 1996; 2000), *Q. rubra* (Vengadesan & Pijut, 2009), *Q. serrata* (Ishii et al., 1999; Takur et al., 1999), *Q. robur* (Cuenca et al., 1998; Endemann et al., 2002; Wilhelm et al., 1999), *Q. acutissima* (e.g., Kim, 2000) and *Q. petrea* (Chalupa, 2005). However, not only most studies use juvenile sources of explants (e.g., zygotic embryos and seedlings), but also plant conversion frequencies are still low, supporting the recalcitrance of these species.

Q. suber somatic embryogenesis was obtained first from juvenile plants (e.g., Bueno et al., 1996; 2000; Pinto et al., 2001) and later from leaf explants of mature plants (e.g., Hernandez et al., 2003; Lopes et al., 2006; Pinto et al., 2002; Santos et al., 2007) (Figure 2b,c).
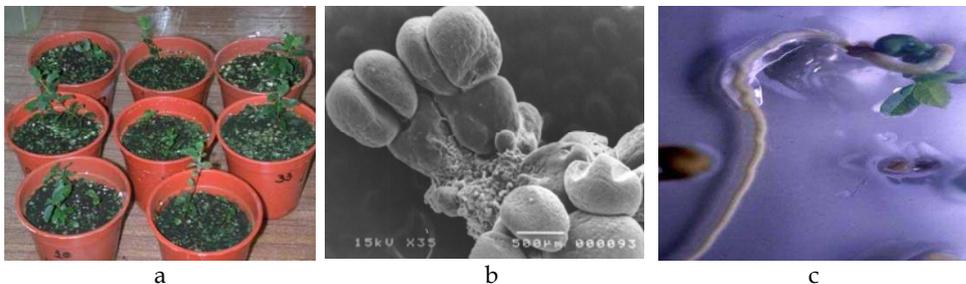

Fig. 2. Micropropagation from field mature cork oak trees: a) Acclimatized plants micropropagated by stem cuttings; b) Scanning electon microscopy of two cotyledonary somatic embryos; c) converted embling (Adapted from Pires et al., 2003; Pinto et al., 2002; Santos, 2008).
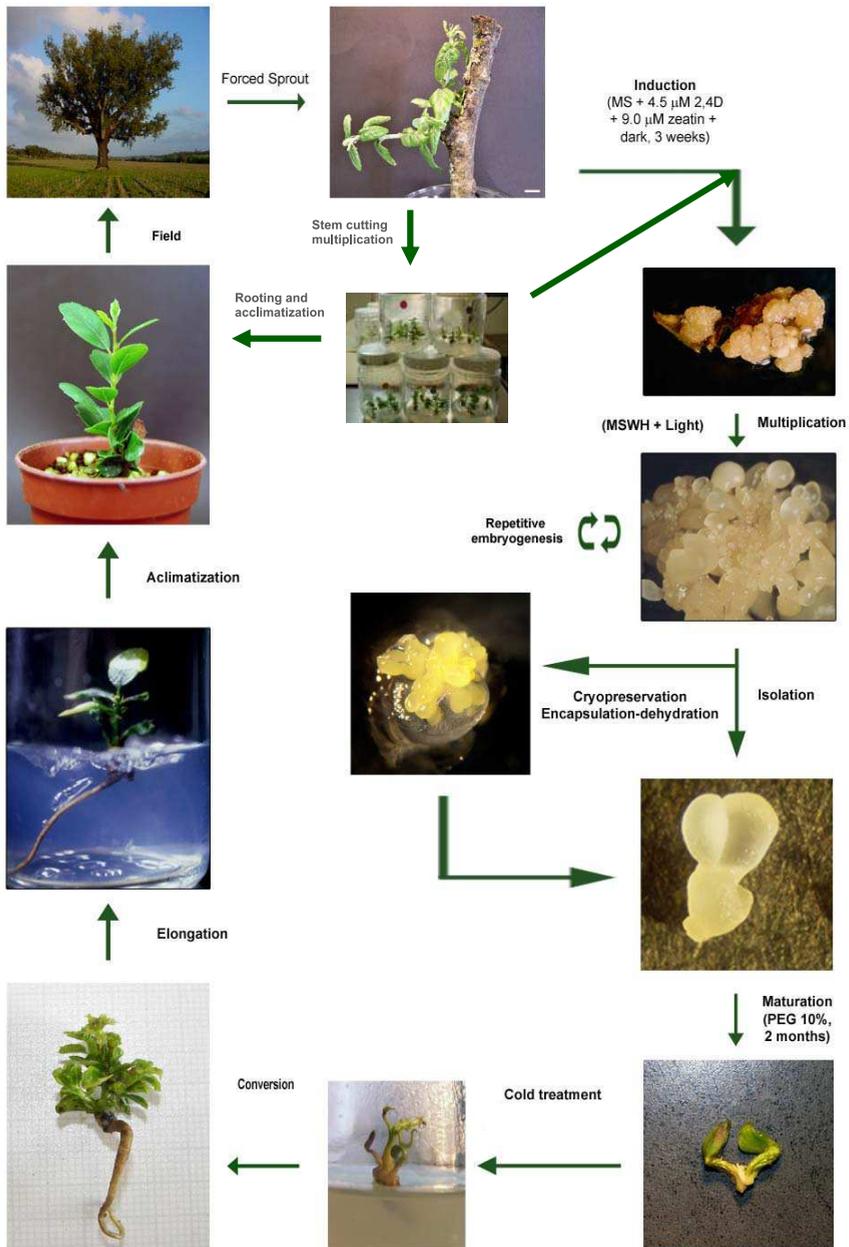
Fig. 3. Enhanced protocol of our group for cork oak somatic embryogenesis. MS medium - Murashige and Skoog, 1962; MSWH - MS medium with no growth regulators. (Adapted from Fernandes, 2011).

The initially protocol developed by Santos and collaborators (Pinto et al., 2002; Lopes et al., 2006) was, however, not sufficiently efficient for large scale propagation and for immediate transfer to industrial breeding programmes of cork oak. Meanwhile, those and other authors reported the deficient maturation of somatic embryos during somatic embryogenesis, as the main cause for low conversion rates in this species (Chalupa, 2005; Fernandes et al 2011; Hernández et al., 1999).

Hernández et al. (2003) highlighted that an adequate reserve deposition in the embryo tissues seems to be necessary for their adequate maturation. Efforts were made since then to manipulate physical conditions in order to promote the adequate accumulation of reserves (Fernandes, 2011; Santos, 2008). Fernández-Guijarro et al. (1995) reported that somatic embryos from cork oak young seedlings increased maturation under light followed by storage at 4 °C, and that controlled starvation could benefit synchronization.

Santos (2008) and Fernandes (2011) compared the accumulation profiles of carbohydrate, lipid and protein reserves during the maturation of cork oak somatic embryos and the zygotic counterparts. Assuming that the accumulation of reserves that occurs in zygotic embryos may be ideal for embryos maturation, Fernandes (2011) also compared the accumulation profiles of somatic embryos exposed to different conditions such as polyethylene glycol (PEG), abscisic acid (ABA) and cold, and defined the condition that led to an accumulation profile closer to the one of the zygotic embryos.

From those analyses, the authors proposed an improvement to the initial somatic embryogenesis protocol developed by Pinto et al. (2002) (see Figure 3). In the improved protocol clusters of somatic globular embryos are isolated and transferred to MS medium (Murashige & Skoog, 1962) with PEG. After maturation, cotyledonar embryos are transferred to MS medium and submitted to chilling (4° C). Conversion is then achieved on woody plant medium (WPM) medium supplemented with BAP 0.5 mg/L and NAA 0.1 mg/L. After some weeks plants are acclimatized with success (Fernandes, 2011).

In conclusion, the inclusion of cold and osmotic stress in the protocol improved somatic embryos maturation and consequent conversion in approximately 70% of the genotypes. However, it was evident a genotype dependence in this process, with responsiveness ranging from very-good/in most genotypes to null, in few genotypes (Fernandes, 2011) (Figure 3).

1.3 Current challenges for the SE process

The loss of embryogenic competence is one of the major drawbacks of long term micropropagation potocols (e.g., Brito et al., 2009). In particular, embryogenic masses were maintained for long periods may dedifferentiate and lose their embryogenic potential. In cork oak this phenomena has originated two types of calluses under the same conditions: embryogenic (EC) and non-embryogenic (NEC) and these last calluses rarely regain embryogenic ability (Santos, 2008).

In vitro functional changes during embryogenesis imply changes in explant cells from differentiated and quiescent (G_0) stage to dedifferentiated dividing (G_1-S-G_2/M) stages, and later an evolution to embryogenic states. All these transitions imply changes in gene expression, and in cell cycle dynamics, where growth regulators, namely auxins and cytokinins, are crucial players (Gahan, 2007).

Using embryogenic and non embryogenic calluses of adult cork oak genotypes, our group reported differential distribution of cells staged in G_1, S and G_2 phases according to callus

and growth regulators type (Fernandes, 2011) confirming that cell cycle dynamics during somatic embryogenesis suffers exogenously-induced alterations, and in particular, it is conditioned by growth regulators (Gahan, 2007). We also found that using the two different somatic embryogenesis protocols available (Fernandes, 2011; Pinto et al., 2002), not only cell cycle dynamics changed with time during the process, but also genotypes with different somatic embryogenic competences had different cell cycle dynamics (Fernandes, 2011). Curiously, responsive genotypes showed cell cycles with similar progression profiles (Fernandes, 2011).

Considering the key players regulating cell cycle dynamics, cyclins are among the most important. In a broad sense, D-type cyclins are thought to regulate the G_1-to-S transition, A-type cyclins, the S-to-M phase control, and B-type cyclins regulate both the G_2-to-M transition and intra-M-phase control (Gahan, 2007). The cyclin D (CYCD)/retinoblastoma pathway is believed to be involved in controlling both the commitment of cells to the mitotic cell cycle and decisions involving cell growth, differentiation, and cell cycle exit (Dissmeyer et al., 2009; Cools & Veylder, 2009). Key genes for growth and cell division are regulated by E2F transcription factors, which are inactive when bound by retinoblastoma. The phosphorylation of retinoblastoma is initiated by CYCD-containing cyclin-dependent kinases (CDKs) and is completed by cyclin E–CDK2, resulting in the dissociation of retinoblastoma from E2F factors, triggering the passage of cells from G_1- to S-phase (Gahan, 2007). This key role for the G_1 exit pathway results in it being the primary and predominant cell cycle control point. However, cyclin E–CDK2 is rate-limiting for entry into S-phase and can trigger S-phase in the absence of RB phosphorylation. CYCD3;1 are the best studied examples and expression of their genes is regulated by extrinsic signals, such as sucrose availability. CYCD3;1 expression is also regulated by plant hormones (Dewitte et al., 2003; for review see Dewitte & Murray, 2003).

The E2F family plays a critical role in organizing cell cycle progression by coordinating early cell cycle events with the transcription of genes required for entry into S-phase (e.g., Inzé, 2000). Two major classes of genes possess characteristic E2F binding sites, the first class encodes essential enzymes in the pathways for nucleotide and DNA synthesis that are co-ordinately up-regulated in late G_1. The second class corresponds to genes for regulators of cell cycle progression. Genes from both classes respond to ectopic expression of E2Fs from the first sub-group, namely those that can induce entry into S-phase (Dewitte & Murray, 2003; Gahan et al., 2007).

The cell cycle involves a complex network of regulating molecules. So the control of all these classes of checkpoints regulators is under study in cork oak embryogenic (EC) and non-embryogenic (NEC) tissues (Santos, 2011, unpublished data). Understanding and controlling these checkpoints will become a powerful tool to both better understand the embryogenic per se and to manipulate the developmental stages of embryogenic process.

2. Cryopreservation

2.1 General principles

Public and private efforts have been made to protect and conserve germplasm by preserving the genetic material of selected genotypes (e.g., Engelmann, 2000). As in other species, cork oak germplasm preservation can be done in situ (in the field and in natural environment),

which demands large areas and is susceptible to environmental hazards. Alternatively preservation may be done ex situ (for general review see Li & Pritchard et al., 2009). In particular, in vitro preservation allows that in a small area, large amounts of genotypes are multiplied and maintained under controlled conditions where environmental influences are minimal. However, precocious ageing as well as somaclonal variation and genetic instability may arise after long term culture (Brito et al., 2009).

Alternatively, cryopreservation is the storage of living materials at extremely low temperatures using usually liquid nitrogen (−196 °C), and is an ideal strategy for plant germplasm preservation (Benson, 2008; Feng et al., 2011; Wang & Perl, 2006). This preservation strategy allows not only the preservation of material in small volumes (involving low maintenance requirements) but also, by reducing to residual values the cell metabolism, it allows that cells are stored for long periods, with low probability of genetic instability occurrence (Feng et al., 2011). Cryopreservation therefore allows: the conservation of plant material minimizing occurrences of genetic instability, contaminations and diseases; the preservation of endangered, rare or selected genotypes. Cryopreservation is already being applied to several plant species including forest woody species (e.g., Sakai et al., 2008). Also different plant materials have been used in this preservation strategy: shoot tips, cell cultures, embryos and seeds (Feng et al., 2011).

For cryopreservation to be useful in breeding programs, it is necessary to develop the cryogenic technique *per se*, and to ensure that robust and efficient regeneration protocols are available. Freezing and thawing stages require that cells are structurally and functionally cryoprotected. This may happen naturally (e.g. some naturally dehydrated material) but usually it is induced artificially with treatment with cryoprotectants that influence ice formation and activity of electrolytes present in the solution. Ideally, cryoprotectants should have low or no cytotoxicity. Cryoprotectants may be: a) permeating compounds, such as dimethylsulphoxide (DMSO, used usually in the range of 5-10%) that has a rapid entrance rate and so requires short incubation periods; another permeating compound is glycerol (used often in the range of 10-20%); b) non-permeating compounds such as sugars, sugar alcohols, polyethylene glycol (PEG). Often mixtures of cryoprotectant compounds are preferred to improve their efficacy (e.g., combinations of PEG : glucose : DMSO). Finally other strategies as cold hardening or ABA treatment may increase the freezing resistance and survival rates of cells.

Plant cryopreservation strategies may include slow or rapid freezing approaches. The first is based on physico-chemical changes during the process, namely associated with apoplastic ice crystal formation while cytoplasm may remain free from intra-cellular ice formation. Slow freezing decreases therefore the osmotic potential of the cytoplasm contributing to the cell desiccation. Rapid freezing is achieved by immersion of the cryoprotectant-treated samples in N_2, leading to an ultra-fast cooling that prevents the formation of ice crystals inside the cell (e.g., Sakai et al., 2008).

Some variants involve vitrification that includes a cell dehydration step prior to storage in N_2 (Sakai et al., 2008). This may rely on the ability of concentrated solutions of cryoprotectants to become viscous to very low temperatures, without ice formation. Consequently, during the vitrification process plant cells are dehydrated and the cytoplasm vitrified during freezing, which allows that ice crystals are rarely formed. Vitrification has already been applied to a large number of species (Panis et al., 2005; Sakai et al., 2008).

Nonetheless, during this process, usually complex and toxic solutions with high osmotic potential, such as the PVS2, are used for cryoprotection (Fernandes et al., 2008). Also the duration of the successive steps of a vitrification protocol is in general very short, hampering the simultaneous treatment of a large number of samples.

Some alternatives to vitrification-based techniques were developed, namely encapsulation-dehydration strategies (e.g., Engelmann et al., 2008). Encapsulation-dehydration is based on concepts related with artificial seeds. Concisely, plant tissues, such as shoot tips or somatic embryos, are covered by for example alginate. Then, they are dehydrated (using exposures to highly concentrated solutions, and/or to air in a flow chamber), before being transferred to N_2. This strategy has the advantage of using less toxic compounds such as glycerol than in other vitrification methods, thus minimizing stress conditions (e.g. Volk et al., 2006). It also has the advantage of easy and inexpensive manipulation, not requiring expensive instruments, as occurs in controlled freezing (Fernandes et al., 2008).

This method has been applied to many species, such as, for example, mulberry (Niino & Sakai, 1992), *Prunus* sp. (Brison et al., 1992), sweet potato (Feng et al., 2011; Hirai & Sakai, 2003), persimmon (Matsumoto et al., 2001), apple (Niino & Sakai, 1992; Paul et al., 2000), lily (Bouman & Klerk, 1990) and even grapevine embryogenic cell suspensions (Wang & Perl, 2006) or pear (Niino & Sakai, 1992; Scottez et al., 1992). In several assays as in those with *Robinia pseudoacacia*, it was demonstrated that encapsulation-dehydration originated better results than vitrification (Verleysen et al., 2005). Recently we have also demonstrated that encapsulation-dehydration was the most efficient method of cryopreservation of *Quercus suber* somatic embryos (Fernandes et al., 2008).

2.2 *Quercus suber* cryopreservation

Propagation of cork oak presents several drawbacks as it has a high heterozigocity, often leading to individuals with high probability of instability and genetically distinct from parents, therefore leading to high numbers of undesired genotypes (Lopes et al., 2006). Moreover, seeds are only stored for short periods as they rapidly loose viability. This recalcitrance jeopardises the development of conservation and improvement programs in this species. As in most forest species, *Quercus suber* conservation approaches consist mainly in agro-forest sustainable systems, and scarce strategies using biotechnological approaches have already succeeded. Valladares et al. (2004) highlighted that highly interesting individuals may be maintained with vegetative propagation.

The cryopreservation of seeds or embryos seems therefore to have huge potential as an innovative preservation strategy, in particular in species with recalcitrance. González-Benito et al. (2002) examined different factors included in the cryopreservation protocols for *Quercus ilex* and *Q. suber* embryonic axes. The authors demonstrated that temperature of in vitro incubation played an important role, mostly for *Q. ilex* axes. *Q. suber* axes were sensitive to desiccation and cooling.

With respect to *Quercus* sp. somatic embryos, Martinez et al. (2003) and Valladares et al. (2004) successfully cryopreserved embryogenic cultures of *Q. robur* and *Q. suber*, using the vitrification method. As reported above, highly toxic cryoprotectants are used in most classical vitrification processes. To overcome these negative effects, recently our group (Fernandes et al., 2008) used a less toxic variation to the classical vitrification technique, called the encapsulation-dehydration method, to cryopreserve *Q. suber* material.

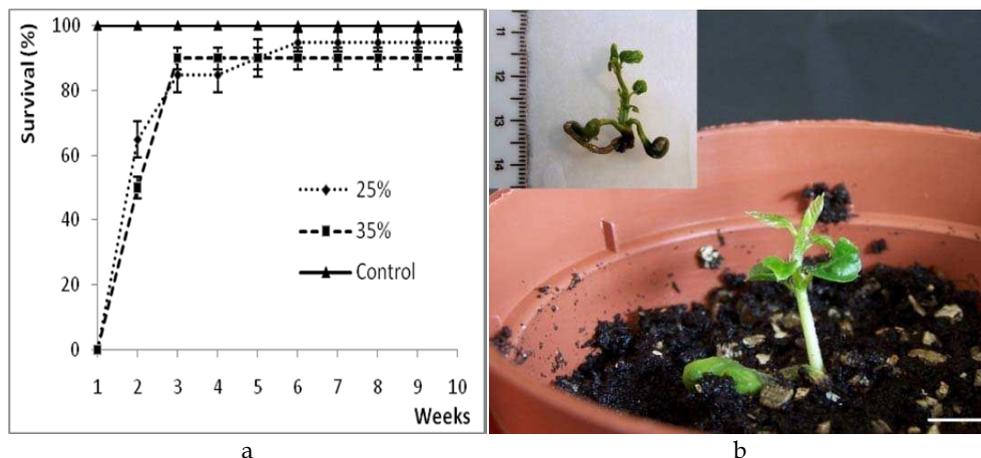a   b

Fig. 4. a) Percentage of survival of cork oak cells after 10 weeks (for two protocols of cryopreservation used CRY25 and CRY35, corresponding to the final % content of water). b) Acclimatized plant obtained from cryopreserved somatic embryos that were recovered and cultivated on MSWH for maturation and conversion (adapted from Fernandes et al., 2008).

In this standard protocol for cork oak, somatic embryos or embryogenic clusters derived from mature trees and previously maintained on MS medium without growth regulators (MSWH) are used as samples. For encapsulation, embryos/clusters were separated and loaded in the alginate plus $CaCl_2$ solutions, forming the beads (3–4 mm), each one contained one embryo/cluster. They were then pre-cultivated on sucrose-enriched standard liquid medium (MS_{WH} with 0.7M sucrose) for 3 days. Beads were then desiccated by drying in the airflow of a laminar flow cabinet, and carefully weight loss was monitored for water content calculation. Two final water content (WC) values were assessed: 25% (CRY25) and 35% (CRY35). Afterwards, beads were placed in cryotubes (10 per vial), and immersed in N_2 for 24 h. Samples' thawing was done by incubating the cvryotubes at 38 °C (2 min) and incubating in detoxification solution (1 h). Beads were transferred to solid standard medium (MS_{WH}) for regeneration (Figure 4a, b; Fernandes et al., 2008).

This cryopreservation technique developed by Fernandes et al. (2008) for cork oak somatic embryos is simple, effective and non-toxic for the species. Also, survival rates in encapsulated-dehydrated (but non-frozen) cork oak samples achieved 90%. We also demonstrated that cryopreserved somatic embryo derived clones were able to recovery, leading to plants morphologically normal and that had genetic stability.

3. Screening of genetic variation in cloned and cryopreserved material

In vitro regenerated plants may exhibit somaclonal variation as a result from genetic or epigenetic modifications (Fourré et al., 1997; Isabel et al., 1996). It is generally accepted that morphological, cytological and molecular variations may be generated by the imposed stress during in vitro or cryopreservation processes. These induced variations are conditioned by the genotypes used and/or by the techniques/protocols used. Theoretically,

any protocol of plant cloning or germplasm conservation should lead to no somaclonal variation.

Genetic variability of in vitro material or of cryopreserved samples may be assessed by several techniques. Molecular and genetic techniques when used individually give a limited perspective of the occurrence of somaclonal variation, but if combined may provide an interesting and complementary toolbox of markers for "true-to-typeness" evaluation (e.g., Santos et al., 2007).
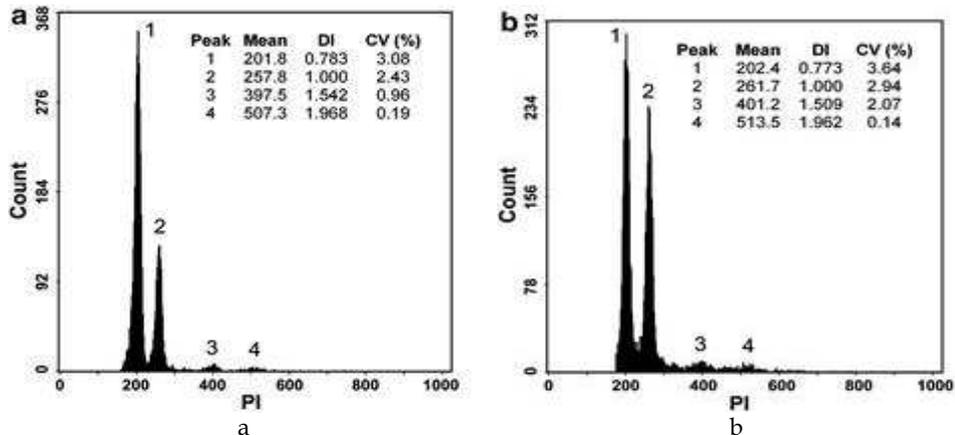

Fig. 5. Flow cytometry histograms of relative propidium iodide (PI) fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Quercus suber* and from the reference standard *Glycine max* (2C=2.50 pg DNA): **a)** leaves of the mother plant; **b)** somatic embryo. Histogram *peak 1:* nuclei at G_0/G_1 phase of *Q. suber*; *peak 2:* nuclei at G_0/G_1 phase of *G. max*, *peak 3* nuclei at G_2 phase of *Q. suber*, *peak 4* nuclei at G_2 phase of *G. max* (adapted from Loureiro et al., 2005).

Gross genetic variations of genomic origin affect mostly the number of chromosomes and ploidy level and can be detected by chromosome counting or flow cytometry (Loureiro et al., 2005). Chromosome mutations (e.g., inversion, translocation) and genic mutations are screened by molecular markers that detect DNA sequence modifications. Among these, the most popular are RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms) or microsatellites/SSRs (simple sequence repeats). RFLPs and AFLPs are highly reproducible techniques but are costly and may be time-consuming. For easy application of SSRs, it is required that the microsatellite loci and its flanking primers are readily available for a given species, while RAPDs is simple but may have a lack of reproducibility.

Several genetic and molecular markers have been used to assess somaclonal variation in micropropagated oak plants. Loureiro et al. (2005), using flow cytometry, found no ploidy or DNA content variations in cork oak embryogenic tissues or among somatic embryos (Figure 5).

Also, Fernandes et al. (2008) using cryopreserved material, confirmed by flow cytometric analyses that the two cryopreservation procedures (CRY25 and CRY35) provided genetic

fidelity, for the parameters used: in all samples, both ploidy and DNA content were in concordance with literature data: 2C= 1.90 pg DNA (Loureiro et al., 2005; Santos et al., 2007), and changes in DNA content of non-cryopreserved and cryopreserved samples were minimal ($\leq$ 0.01 pg/2C) (Figure 6; Table 1).

Flow cytometric techniques, despite highly rapid and robust, may however not detect minor changes in DNA content, so it must be emphasised that the putative occurrence of small changes in DNA content in these kinds of assays should not be discarded.
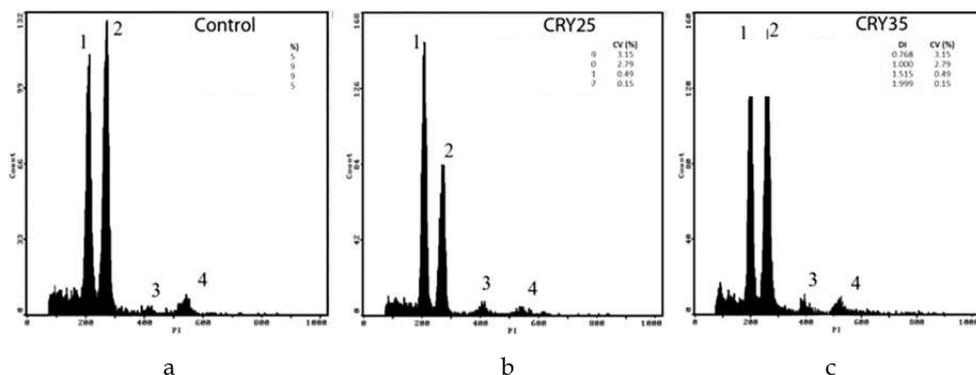

Fig. 5. Flow cytometric estimation of genome size of *Q. suber* samples of control (a) and after cryopreservation: CRY25 (b) and CRY35 (c). In all graphics peaks 1 and 3 correspond to 2C and 4C nuclei of cork oak somatic embryos, while peaks 2 and 4 correspond to the internal soy standard (adapted from Fernandes et al., 2008). PI: intensity of propidium fluorescence.

Tissue	DI	SD	2c DNA (pg)	SD	1c DNA (Mbp)[b]
Fresh	0.774[a]	0.0059	1.93	0.015	946
25% Cry[c]	0.777[a]	0.0073	1.94	0.018	950
35% Cry[c]	0.772[a]	0.0138	1.93	0.034	944

Table 1. Nuclear DNA content of *Quercus suber* L. fresh and cryopreserved embryos. The results are given as mean and standard deviation (SD) of the 2C DNA content in mass values (pg). Nuclear DNA content in Mbp is also given. [a] Mean values followed by the same letter are not significantly different according to the Tukey-Kramer multiple comparison test at $P \leq 0.01$. Note: [b]1 pg DNA = 978 Mbp. [c]Cryopreserved embryos (25% and 35 % water content) (adapted from Fernandes et al 2008).

Both works supported therefore that the protocols for somatic embryogenesis and cryopreservation developed so far for cork oak led to "true-to-typeness" (for ploidy and DNA amount parameters) and were worthy of use in cork oak breeding programs.

As explored above, molecular markers provide information on sequence mutation. This information is therefore complementary to the information provided by flow cytometry (or even by chromosome counting).
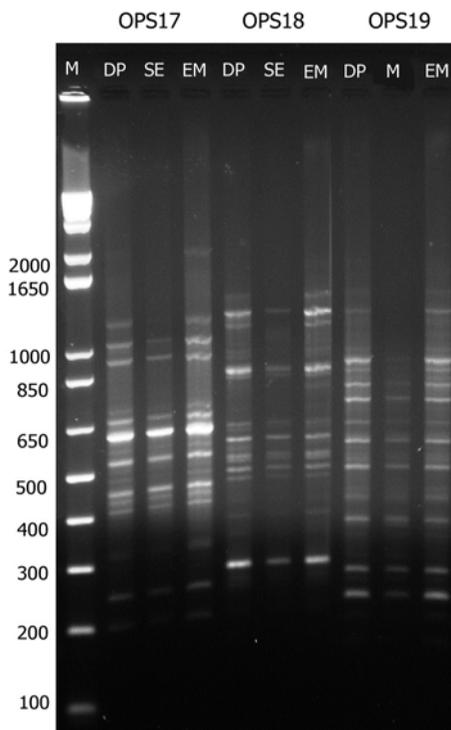
Fig. 6. DNA profiles generated by the RAPD primers OPS 17, 18 and 19, in the three different stages of the somatic embryogenesis process: donor plant (DP), somatic embryo (SE) and embling (EM). M, size marker (1Kb Plus DNA Ladder) (adapted from Fernandes et al., 2011).

In micropropagated *Q. serrate* no aberrations in the banding patterns were detected by RAPD markers (Thakur et al., 1999). Also, RAPDS were used to assess putative occurrence somaclonal variation in *Q. suber* embryogenic lines, but no molecular changes were found (Gallego et al., 1997, Sanchez et al., 2003). More recently, RAPDS were also used to evaluate genetic instability of somatic embryos of *Q. suber* obtained by the above described protocols (Figure 6; Fernandes et al., 2011).

Techniques for RAPD analyses however pose several problems of reproducibility, and give restricted information. So, other molecular analyses can provide complementary information to RAPDS. Microsatellites and AFLP are among the most used markers in *Quercus*. AFLP markers detected changes in cork oak embryogenic lines (Hornero et al., 2001). We also used AFLP to test putative genetic instability during the developed cryopreservation/somatic embryogenesis processes of cork oak (Fernandes et al., 2008). It was used six primer sets that revealed an overall high proximity value between the two vitrification-encapsulation cryopreserved (CRY25 and CRY35) and control samples. Occasionally, few extra AFLP-bands in CRY25 samples were detected and the occurrence of putative small mutations, or DNA methylation or even to subpopulation cryo-selection should not be excluded (Fernandes et al., 2008).

Locus	Allele size (bp)			
	QsG0	QsG5	QsGM1	QsGM2
QM58TGT	201	185/211	203/210	203/210
QM50-3M	276/286	285/287/292	278	282/288
QpZAG9	223/238	223/233	218/223	223/233
QpZAG15	108/123	106/120	nd	nd
QpZAG36	209/219	216/220	207/209	207/209
QpZAG110	223/233	220/238	221	221/233
QrZAG7	106/119	115/127	106/121	106/121
QrZAG11	229/263	261/273	nd	nd

Table 2. Characteristics of the microsatellite loci amplified in *Q. suber*. Allele size found in this study and allele size range and number of alleles (in parenthesis) found in other publications are also given: values for QrZAG7 and QrZAG11 are from Hornero et al. (2001*b*), values for the 6 remaining loci are from Lopes et al. (2006).

Finally, Wilhelm et al. (2005) found that that during somatic embryogenesis process of *Q. robur*, genetic instability occurred. Also, Lopes et al. (2006) confirmed genetic stability of somatic embryos and emblings derived from our somatic embryogenesis protocol (Lopes et al., 2006; Pinto et al., 2002) and after, Fernandes et al. (2008) also confirmed this stability using SSRs in material after recovering from cryopreservation (Table 2). Together with this overall genetic stability, the regenerated cork oak plants looked normal, healthy and well developed shoot. The authors concluded that the encapsulation-dehydration cryopreservation protocol used in cork oak somatic embryos was an efficient method of storage, regarding several parameters: recovery and survival rates and genetic/morphologic stability. To support the battery of protocols for molecular and genetic analyses of cork oak, Santos et al. (2007) published in detail the reliable protocol for analysis of cork oak material by SSRs and ploidy/nDNA quantification, where technical aspects and potential troubleshooting that may occur during analysis of this material are deeply discussed.

4. Concluding remarks

The utility of plant biotechnology tools in woody forest species propagation and preservations has been recognised decades ago, but only recently, it has been effectively incorporated in industrial breeding programs. Despite no robust and efficient protocol for cork oak regeneration by somatic embryogenesis is available yet, the advances observed in the last decade, together with the already available protocol for cryopreservation of this species, open perspectives for the incorporation of these two approaches in future breeding program of this species. Moreover, with the available protocols stable genotypes were obtained.

Genetic and molecular stability was assessed using complementary genetic and molecular techniques such as flow cytometry, RAPDS, AFLP and SSRs. Finally, an interesting research field will focus on the control of cell cycle progression order to control different stages of somatic embryogenesis and preservation. It is our believe that by manipulating proteins that control cell cycle phases transition (which are at the basis of differentiated/ undifferentiated cells) we´ll be able to manipulate the reversion phenomena between NEC and EC and also to better control the developmental somatic embryogenesis stages.

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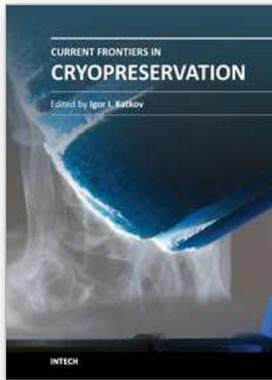
Current Frontiers in Cryopreservation

Edited by Prof. Igor Katkov

Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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