PRESERVATION OF *Quercus robur* GERMPLASM BY CRYOSTORAGE OF EMBRYOGENIC CULTURES DERIVED FROM MATURE TREES AND RAPD ANALYSIS OF GENETIC STABILITY

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Abstract

This study reports on the cryostorage of embryogenic lines derived from selected mature *Quercus robur* trees, following application of the PVS2-vitrification based procedure. In seven oak genotypes, embryo recovery levels ranging from 57-92% were obtained when 4-6 mg embryo clumps were precultured for 3 days on 0.3 M sucrose basal medium, treated with PVS2 solution for 60 min at 24ºC, and then immersed in liquid nitrogen (LN). Embryos of six out of seven lines were cryostored for one week and one year and used to evaluate cryopreservation tolerance, germination ability and to assess genetic fidelity by random amplified polymorphic DNA (RAPD) markers. There were no significant differences between the recovery frequencies of samples retrieved from LN after 1 week and 1 year of cryostorage. In five out of six lines, RAPD profiles of cryopreserved somatic embryos and regenerated plantlets were identical to those of the controls. Although polymorphisms were detected in only one cryostored embryo of one genotype, no genetic instability was found in the regenerated plantlets. This methodology appears to be suitable for long-term storage of this valuable germplasm, as the recovered plantlets were found to be genetically stable.

Keywords: cryopreservation, genetic fidelity, oak, plant regeneration, somatic embryogenesis, vitrification.

INTRODUCTION

Pedunculate oak (Quercus robur L.) is one of the most important broadleaf tree species in European forests. Oaks have recalcitrant seeds and this, along with difficulties in vegetative propagation (35), makes conservation of the species, especially of high-value genotypes, complicated. For such species, cryopreservation is currently the preferred method for long-term conservation of clonal germplasm (9, 18).

During the last decade, cryopreservation of embryogenic cell masses and somatic embryos has become increasingly important in woody plants and there are already examples of large scale applications (10). Cryopreservation is systematically used for storing the thousands of conifer embryogenic cell lines used in large scale clonal planting programmes (16), as well as for storing embryogenic lines of coffee, cacao and oil palm (10). Embryogenic material is cryostored by the traditional slow-cooling method (widely applied with conifer

species) or the new developed procedures commonly known as vitrification based techniques (9), so far applied to a limited number of hardwood trees (21), such as horse chestnut (22), chestnut (5) and cocoa (11).

Research into a cryopreservation technique that can be used with the genus *Quercus* has been performed with zygotic embryo axes and somatic embryos. The partial desiccation procedure followed by rapid immersion in LN has been applied to embryo axes, resulting in unorganized growth of axes in most studies. However, a relatively high recovery rate (development of root or/and shoot) of 60% was reported for embryo axes of *Q. faginea* (14), whereas in the case of *Q. robur*, a germination response of 40% was obtained following flash drying and rapid cooling of zygotic axes (4). As regards embryogenic cultures, the feasibility of cryopreserving somatic embryos of *Q. robur* (26) and *Q. suber* (42) has been reported. In the *Q. robur* work (26), desiccation and vitrification procedures were investigated with better results provided by the latter. However, only two half-sibling lines derived from very juvenile oak material were evaluated, and thus the number of genetic backgrounds was limited. For cryopreservation to be viable on a commercial scale, the process should be non-selective i.e., it must be able to be applied to a wide range of genotypes, and the cultures must remain genetically unaltered by the cryopreservation process (18). For woody plants the strong effects of maturation must be always considered and the importance of testing different clones and plant material of different ontogenetic stages (juvenile compared with mature) has also been emphasized for cryopreservation of *Fraxinus excelsior* (36). However, cryopreservation of embryogenic tissues of mature genotypes has hardly investigated in *Quercus* species (42).

Cryopreservation procedures result in the exposure of tissues to physical, chemical and physiological stresses that cause cryoinjury and therefore assessment of genetic stability should be performed to validate newly established cryopreservation protocols (17). Although the effects of cryoinjury on the genome are often unknown, any accumulative DNA polymorphisms may not be induced by cryopreservation *per se* but may be the result of the whole culture-cryoprotection-regeneration process. In general, there is increasing evidence to indicate that cryopreservation techniques are not the source of somaclonal variation in plants, and that morphological, cytological and molecular stability is maintained after cryopreservation (17, 10). However, the use of different genetic markers has also generated controversial results regarding the genetic fidelity of cryopreserved material (20). In addition, epigenetic variation in chromatin and DNA methylation of gene sequences has been observed in plants after cryopreservation, which suggests altered patterns of gene expression (17).

Among various molecular techniques suitable for generating DNA profiles, RAPD and amplified fragment length polymorphism (AFLP) fingerprinting are increasingly being used for detecting somaclonal variation in cryopreserved material of forest tree species (6, 20, 29).

Use of cryopreservation may enable conservation of mature elite individuals for prolonged periods, for gene conservation purposes. The aim of the present study was to define a cryopreservation protocol, based on the vitrification procedure, for somatic embryos of different genotypes of selected mature oak trees. A further objective was to assess the clonal fidelity of cryostored somatic embryos and regenerated plantlets by use of random amplified polymorphic DNAs, i.e. RAPD markers.

MATERIALS AND METHODS

Plant material and culture conditions

Somatic embryos were taken for cryopreservation from seven embryogenic lines initiated from expanding leaves of epicormic shoots of seven *Quercus robur* trees, each more than 100 years old (40). These trees (CR-0, CR-7, B-13, B-17, B-18, T-42 and Sainza) were located in three selected stands in northwestern Spain. The lines used were maintained for more than two to three years by secondary embryogenesis, with sequential subculture at 5-week intervals onto a proliferation medium consisting of MS (27) mineral salts and vitamins, 0.09 M sucrose

and 0.6% Vitroagar (Hispanlab) (basal medium), supplemented with 0.44 μM N^6-benzyladenine (BA), 0.27 μM 1-naphthaleneacetic acid (NAA) and 500 mg/l casein hydrolysate. All media were of pH 5.7 and had been autoclaved at 121ºC for 20 min. Samples consisting of 4-6 mg clumps of globular or heart-shaped secondary embryos were dissected from each of the stock embryogenic lines and used for cryopreservation experiments.

All cultures were incubated under 16 day/8h dark photoperiod by cool-white fluorescent light (50-60 μmol m^{-2} s^{-1}), with a 25ºC light/20ºC dark temperature regime.

Cryopreservation

Harvested somatic embryo clumps from the seven embryogenic lines were precultured for 3 days on basal medium containing 0.3 M sucrose and were then placed, 10 explants to a vial, in 2 ml cryovials with 1.8 ml of PVS2 vitrification solution consisting of 30% w/v glycerol, 15% w/v dimethylsulphoxide (Me_2SO), 15% w/v ethylene glycol in basal liquid medium containing 0.4 M sucrose (31). Line T-42 was only used in a preliminary experiment, where the effect of PVS2 application for 30-120 min at 0ºC and 24ºC (room temperature) was tested. In a second experiment, the six lines (CR-0, CR-7, B-13, B-17, B-18, and Sainza) were used and PVS2 solution was applied at 24ºC for 0, 60 or 90 min. After PVS2 treatment, the embryo clumps were resuspended in 0.6 ml of PVS2 and were then plunged in LN and maintained for 24 h, after which they were re-warmed by 2 min immersion in a 40ºC water bath and the PVS2 solution drained off. The samples were then washed with basal liquid medium supplemented with 1.2 M sucrose, with two changes of medium, each for 10 min, before being placed on filter paper discs on proliferation medium (recovery medium) in 9 cm diameter Petri dishes. After 24 h, the embryos were transferred, without any filter paper, to fresh proliferation medium, where they remained until they were evaluated for recovery ability 8 weeks later. Uncooled samples precultured in medium containing 0.3 M sucrose and treated with PVS2 solution (treated controls) were also included. At least three ten-explant replicates per treatment were used in each experiment, and each experiment was perfomed twice. In a further experiment, the cryopreservation of 300 somatic embryo clumps of each of the six embryogenic lines (20 cryotubes containing 15 clumps each) was considered. The samples were treated as above with exposure to PVS2 for 60 min, and uncooled-PVS2-treated control groups were also included. After one week and one year of cryostorage, the material was retrieved from LN and used for evaluating embryo recovery and germination ability, as well as for assessing genetic fidelity.

Germination experiments were carried out with cryopreserved material of all six lines. Eight weeks after thawing, recovered embryos were transferred to maturation medium consisting of basal medium with half-strength macronutrients and no casein hydrolysate, but supplemented with 6% sorbitol and 0.09 M sucrose (33). After 4 weeks in maturation medium, 8-10 mm embryos were transferred to germination medium (which differed from the maturation medium in that it contained 0.44 μM BA and no sorbitol), where they remained for 8 weeks.

Data collection and statistical analysis

Eight weeks after thawing, embryo recovery was estimated in terms of the percentage of the total explants that showed resumption of embryogenesis with visible cotyledonary embryos. The number of new cotyledonary embryos developed from globular or heart-shaped original clumps was also recorded. In the experiment on temperature of PVS2 application, the fresh weight of embryogenic explants was also assessed. In germination experiments, the number of cryostored embryos that had germinated (radicle and/or epicotyl development at least 1 cm long) was recorded after 8 weeks of culture in germination medium.

The results were subjected to analysis of variance followed by mean comparison with the least significant difference (LSD) test at the $p \leq 0.05$ level. Percentage data were subjected to arcsine transformation prior to analysis (data shown in Tables are all untransformed).

DNA extraction and RAPD analysis

RAPD analyses were conducted on six above-mentioned embryogenic lines (Tables 2 and 3), to evaluate the genetic stability of somatic embryos cryostored for one week and for one year, as with the non-cryostored embryos (controls) collected from the same stock cultures used for the cryopreserved material. In addition, PVS2-treated but not cryopreseved embryos (treated controls) were included in the analyses to test for any possible mutagenic effects caused by the potentially toxic compounds of the vitrification solution. For each embryogenic line, 40 somatic embryos, i.e. ten embryos per treatment, were used (controls, treated controls, cryostored for one week and cryostored for one year). The genetic fidelity of plantlets generated from one-year cryostored embryos of lines CR-0, CR-7, B17 and Sainza was also evaluated by including ten plantlets from each line in the analyses.

Genomic DNA was extracted from approximately 100 mg of tissue per somatic embryo or plantlet with a DNeasy Plant Minikit (QUIAGEN), and stored at -20ºC until further use.

For each genotype, the reliability of 40 arbitrarily chosen 10 base primers for RAPD analyses was initially tested with Kits A and S (Operon Technologies). The primers OPA-6, -11, -12, -16, -19 , OPS-2, -6, -10 and -15 were discarded from all lines, and primer OPS-4 was also discarded from lines B18 and CR-0, as they failed to generate clear and reproducible RAPD profiles (for PCR conditions, see below). At least two independent PCR runs were performed per sample and selected primer, and only fragments amplified with similar staining intensity in each of the runs were considered in subsequent analyses. For each primer, RAPD bands were scored as present or absent after visual inspection of gel photographs, and the molecular weight of each fragment was estimated using Bio-RAD Quantity One software.

PCRs were carried out in a Hybaid Omnigene Thermocycler with a 25 µl reaction mixture containing dNTPs (100 µmol/l each), bovine serum albumin (0.4 g/l, from Sigma Chemical Co.) decanucleotide primer (0.2µmol/l), 20 ng of genomic DNA, and 0.4 U of BioTaq DNA polymerase in the buffer provided by the manufacturer of the enzyme (Bioline). The amplification reactions consisted of one cycle at 94ºC for 1 min, 45 cycles of 10 s at 94ºC, 15 s at 36ºC and 90 s at 72ºC, and a final step of 7 min at 72ºC. The amplification products were separated on 1.4% agarose gel containing 0.15 µg/ml ethidium bromide in 0.5 x TBE buffer. The sizes of the amplification products were estimated by use of a 100 bp ladder (Invitrogen); bands were visualized under UV light for photography and capture of digitalized images.

RESULTS AND DISCUSSION

Cryopreservation

All seven embryogenic lines were successfully cryopreserved. Two to three weeks after the embryo clumps were thawed, emergence of new globular embryos from the original explants was evident. Direct regrowth of pre-existing embryos did not occur but recovery was observed through secondary embryogenesis from surviving cells of the original embryo clumps. A few samples exhibited survival by initiating callus masses with no further development of somatic embryos, and they were not considered for embryo recovery data.

With the aim of defining the correct dehydration conditions, incubation of the embryo clumps with PVS2 at 0ºC and 24ºC was compared with line T-42. After cryostorage, analysis of variance indicated that there was no significant main effect difference for duration of PVS2 treatment, whereas temperature of PVS2 exposure was statistically significant both on embryo recovery and fresh weight. Analysis of combined data from the different PVS2 periods showed that significantly ($P \leq 0.01$) higher cryopreservation tolerance was recorded when PVS2 was applied at 24ºC regardless of exposure periods, although the differences were less

pronounced with 120 min application, for which lower recovery rates were achieved (Fig.1). Similar trends ($P \leq 0.01$) were observed for fresh weight of cryopreserved samples, with values, for incubations of 30 and 60 min at 24ºC, that were approximately twice the values obtained at 0ºC. In this study, fresh weight may be related to embryo production as no callus is formed in embryogenic explants following post-thaw culture in recovery medium. Use of a lower temperature, 0ºC, during the dehydration by a vitrification solution has been reported to have a positive effect as it minimizes the risk of toxicity, especially with species that are relatively sensitive to the dehydration procedure (38). The use of chilled vitrification solution (0ºC) is described in a considerable number of protocols for cryopreservation of shoot apices (30, 36) and also embryogenic cultures (21, 42, 22) of broadleaf trees. However, in the present study, incubation at 24ºC gave rise to better results, suggesting that PVS2 penetration at 0ºC did not provide sufficient protection to withstand cryostorage within the tested periods. In addition, the application of vitrification solution at 24ºC (room temperature) is a more practical system for the PVS2-vitrification procedure in oak.

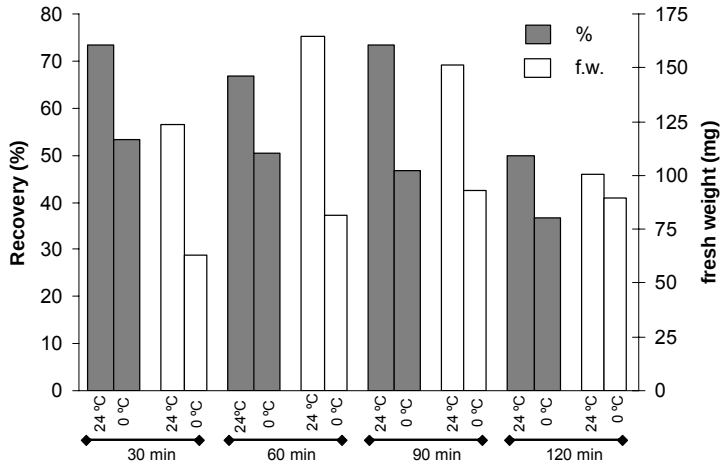

Fig. 1. Influence of temperature during exposure to PVS2 vitrification solution for various periods on embryo recovery (%, dark bar) and mean fresh weight (f.w., white bar) of somatic embryo clumps of *Q. robur* (genotype T-42) after inmersion in liquid nitrogen and subsequent 8 weeks culture in recovery medium. Each value represents the mean of three 10-explant replicates.

Non cryopreserved embryos of the six embryogenic lines tested (Table 1) showed good tolerance to the sucrose preculture and to the vitrification solution applied for 60 and 90 min at 24ºC, and there was no significant reduction in the rates of embryogenic development, which were generally higher than 80% (Table 1, treated controls). When stored in LN untreated controls, all embryos died, with exception of CR-7 and B-17 genotypes, which retained only a minimal post-thaw survival. Explants which only received the sucrose preculture treatment (0 min PVS2) exhibited a certain degree of recovery after cryostorage, with the exception of B-18 line, which did not survive. Sucrose has a well-known cryoprotective action, and the present study confirms the role of sucrose preculture in vitrification and cryopreservation tolerance (13, 11, 42). Sugars may act as an osmoticum and desiccate tissues; however, it was also suggested that accumulation of sucrose inside the tissues helps in maintaining cell viability during cryopreservation by preventing gross freeze injury to cell membranes (41).

In the six oak genotypes, cryopreservation of somatic embryos resulted in recovery rates ranging from 53% to 92% following application of PVS2 for 60 or 90 min (Fig. 2A). These treatments significantly (P≤0.05) increased the frequency of embryo recovery with respect to sucrose precultured embryos (0 min PVS2), although there were no significant differences in the results obtained with these two times of exposure to PVS2. Incubation for 30 min was not considered in this experiment (Table 1) owing to the lower efficiency of this treatment in the cryopreservation of oak embryogenic lines of juvenile origin (26). Similarly, in the T-42 genotype (Fig. 1) the fresh weight of recovered explants was lower after incubation with PVS2 for 30 min than after 60 and 90 min incubation.

Table 1. Embryo recovery percentages (%) after cryopreservation of somatic embryos of six embryogenic lines originated from *Q. robur* mature trees. The samples were precultured in 0.3 M sucrose medium, exposed to PVS2 vitrification solution for various periods with (LN +) or without (LN -; treated controls) subsequent immersion in liquid nitrogen. Mean number of somatic embryos per embryogenic explant is indicated in parenthesis. Assessments were made 8 weeks after thawing.

Embryogenic line		Untreated control	PVS2 (min)			
			0	60	90	120
CR-7	LN -	86.7 (5.8)	91.7 (5.3)	95.0 (5.8)	90.8 (4.2)	
	LN +	11.7 (1.6)	**A**21.7 (2.3**a**)	**B**81.7 (5.0**b**)	**B**90.0 (5.2**b**)	
CR-0	LN -	98.3 (6.6)	96.7 (8.6)	95.0 (7.7)	83.2 (7.2)	
	LN +	0.0 (-)[a]	**A**8.3 (2.8**a**)	**B**73.3 (5.6**b**)	**B**71.2 (5.3**b**)	
B-13	LN -	98.3 (8.6)	96.7 (8.8)	93.3 (7.1)	96.7 (8.3)	
	LN +	0.0 (-)	**A**31.7 (3.9**a**)	**B**91.7 (9.4**b**)	**B**75.0 (10.0**b**)	
B-17	LN -	93.3 (11.6)	92.0 (9.2)	83.3 (11.4)	81.7 (10.3)	
	LN +	3.3 (1.5)	**A**5.0 (1.7**a**)	**B**75.0 (6.2**b**)	**B**68.3 (6.3**b**)	
B-18	LN -	96.7 (6.6)	91.5 (7.5)	86.7 (4.8)	86.7 (4.7)	60.0 (n r)[b]
	LN +	0.0 (-)	**A**0.0 (-)	**B**56.7 (3.7**a**)	**B**58.2 (4.4**b**)	**B**50.0 (n r)
Sainza	LN -	93.3 (8.7)	96.7 (8.3)	75.0 (6.7)	80.0 (6.1)	66.7 (7.6)
	LN +	0.0 (-)	**A**6.7 (2.0**a**)	**B**62.6 (8.6**b**)	**BC**53.0 (5.3**b**)	**C**35.0 (6.1**b**)

[a] There were no recovered explants, [b] Not recorded

Within each LN+ row, values with different letters indicate significant differences at P≤0.05 level in the embryo recovery percentages (capitals) and embryo number (low case letters), according to LSD test.

Significant differences (P≤0.001) in genotype performance were also found, with B-18 and Sainza lines yielding the lowest recovery rates, which did not increase, but rather decreased in response to increasing the time of incubation with PVS2 to 120 min. The mean number of embryos per embryogenic explant also varied between genotypes (P≤0.05). Within each embryogenic line, the number of embryos recorded 8 weeks after thawing was severely reduced (P≤0.05) in samples precultured on sucrose medium but not treated with PVS2, as occurred with the embryo recovery frequencies (Table 1). Embryo number was also significantly lower (P≤0.05) in cryostored material of lines B-17 incubated with PVS2 for 60 and 90 min, and B-18 incubated for 60 min, than in untreated controls (LN-).

The definition of the more efficient incubation conditions, in terms of temperature and duration of exposure to PVS2, is critical for the PVS2-vitrification based procedure. Although similar recovery frequencies were obtained with incubation periods of 60 or 90 min, exposure for 60 min is preferable as a common protocol for all tested genotypes since it is more

practical and ensures safe storage by avoiding prolonged exposure to PVS2, and the associated risk of toxic effects (32). Embryogenic resumption rates of 60-70% were reported in two oak embryogenic lines of juvenile origin (26), which suggests that the ontogenetic state of plant material is not related to its cryopreservation tolerance, whereas the genotype appears to have a more marked influence. Similarly, genotype was more important than the mature/juvenile origin of the stock material affecting the regrowth of cryostored shoot apices of chestnut (44) and *Fraxinus excelsior* (36).

Incubation of somatic embryos for 60 min with vitrification solution was reported to produce recovery frequencies of 68% in chestnut (5) and 88-93% in cork oak (42), whereas 90 min exposure was used for obtaining 93% regrowth of cryopreserved embryogenic callus of *Aesculus hippocastanum* (22) and 38% in *Olea europea* (3). Cryopreservation by encapsulation-vitrification was also applied to somatic embryos of *Olea europea* (37) and *Fraxinus angustifolia* (39), giving rise to 58% and 27% survival, respectively. Following encapsulation-dehydration techniques, 25-72% of cocoa embryogenic samples (11) and 63-70% of coffee somatic embryos (8) were also able to withstand cryopreservation. In this study, successful results were achieved for oak with the PVS2-vitrification procedure, giving rise to levels of resumed growth higher than or comparable to those reported for cryopreservation of embryogenic cultures in broadleaf trees with vitrification based techniques. The method can be considered feasible for the different genetic backgrounds tested in this study. It is worth noting that a similar duration of exposure to PVS2, 60 min, can be used for cryopreservation of three Fagaceae species, *Castanea sativa*, *Q. suber* and *Q. robur*, in which cryopreservation of embryogenic cultures has been investigated (5, 42, 26).

Table 2. Embryo recovery percentages (%) after cryopreservation of somatic embryos of six oak embryogenic lines. The samples were exposed to PVS2 vitrification solution for 60 min with (LN +) or without (LN - ; treated controls) subsequent immersion in liquid nitrogen for one week or one year. Assessments were made 8 weeks after thawing. Germination data (%) were recorded after 8 weeks culture of recovered embryos in germination medium.

Embryogenic line	**Embryo recovery % ***			
	Treated control (LN-)	**1 week (LN+)**	**1 year (LN+)**	**Germination (%)**
CR-7	75.0±5.1	81.7±4.4	78.3±3.9	37.8
CR-0	90.0±3.4	70.0±8.2	64.2±4.4	25.5
B-13	86.7±4.5	78.8±7.9	80.2±2.8	0.0
B-17	90.0±3.4	93.0±3.1	89.2±7.3	67.9
B-18	83.3±5.1	63.3±5.1	61.7±6.2	8.3
Sainza	85.0±3.9	68.9±5.6	57.4±6.7	50.7

* Mean values ± standard errors of five to six 10-explants replicates (N=50-60 samples for treatment).

In a further experiment, embryogenic samples pretreated with PVS2 solution for 60 min at 24ºC were retrieved from LN after 1 week and 1 year of cryostorage (Table 2). Regrowth of samples with or without storage in LN (LN-; LN+) maintained similar trends with respect to genotypic variation, as shown in Table 1. In addition, there were no significant differences between recovery frequencies for the two storage times.

After thawing and subsequent culture for 8 weeks in recovery medium, embryogenic samples were transferred to maturation medium, and isolated somatic embryos were then used for germination experiments. Cryopreserved embryos of the six genotypes germinated at rates ranging from 0% to 68% (Fig. 2B) and no phenotypic abnormalities were observed in comparison with non-cryopreserved embryo–derived plants. The no germination response of

B-13 embryos was also produced in uncooled material of this clone. Relevant differences in germination frequency (0-70%) have been determined for non-cryostored somatic embryos of these genotypes (43). Generally, the low plant conversion ability of different embryogenic lines is still a crucial step for oak embryogenic systems. Long-term storage of plant germplasm requires an efficient and reliable plant regeneration system following cryopreservation (11). In this respect work is being carried out to improve conversion of oak embryos into plantlets.

In conclusion, cryopreservation of oak somatic embryos was achieved by a simple procedure of PVS2-vitrification, which was successfully applied for long-term storage of material derived from seven mature trees. These results emphasize the importance of testing a range of genotypes and valuable plant material of mature ontogenetic state.

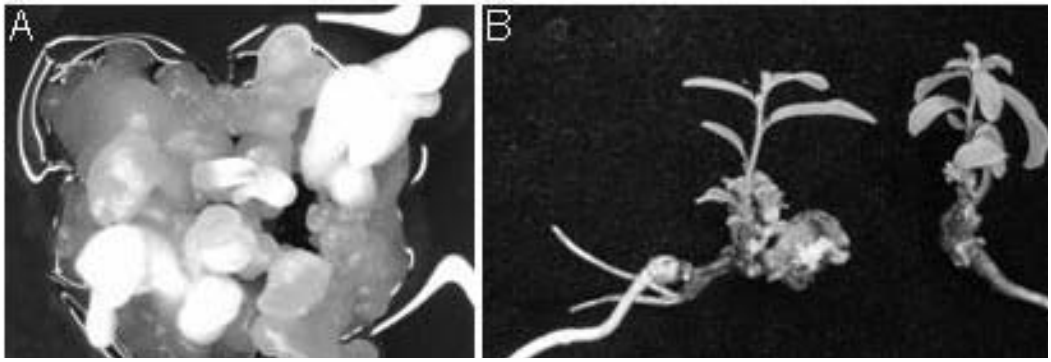

Fig.2 A. Somatic embryo recovery of line B-13 from a cryopreserved embryo clump following exposure to PVS2 solution for 60 min. B. Somatic seedlings regenerated from one-year cryopreserved embryogenic cultures derived from the *Q. robur* selected tree CR-7.

RAPD analysis

A summary of the effectiveness of RAPD markers used in the six genotypes, including the polymorphic fragments, is shown in Table 3.

Table 3. Effectiveness of RAPD markers used for the evaluation of genetic stability of *Q. robur* somatic embryos (control, treated control, cryostored for 1 week and for 1 year) originated from six genotypes, and plantlets derived from one-year cryostored embryos of genotypes CR-0, CR-7, B-17 and Sainza.

	B-17	**B-18**	**B-13**	**CR-7**	**CR-0**	**Sainza**
Number of primers used	31	30	31	31	30	31
Total band classes scored (a)	273	293	303	248	262	231
Polymorphic fragments scored (b)	0	0	0	7	0	0
Polymorphic primers %	0	0	0	3.2	0	0
Polymorphism % (b/a x 100)	0	0	0	2.8	0	0
Frequency of variation % (b/c x 100)	0	0	0	0.06	0	0
Minimum number of fragments per primer	3	4	4	3	5	3
Maximum number of fragments per primer	14	18	18	13	14	13
Average number of fragments per primer	8.8	9.8	9.8	8.0	8.7	7.5
Total number of bands scored (c)	13,650	11,720	12,120	12,345	13,100	11,500

The number of bands per primer ranged from 3 to 18, and the total number of bands amplified ranged from 231 to 313, depending on the genotype. All the primers used were capable of revealing polymorphisms among three or more genotypes. Of the total of 14 fragments amplified with primer OPS-1, 11 showed polymorphisms, and the six genotypes were able to be distinguished, which confirms that the selected primers were efficient for detecting polymorphisms (Fig 3A). Other authors have also reported the usefulness of RAPD for the identification of genotypes in oak (12), birch (29), and pomegranate (34), and also for clone and cultivar identification in hybrid poplar (28), among others. In the present study, no intraclonal variation was found within the control somatic embryos in any of the six embryogenic lines that were analyzed**,** which confirms the results reported by Valladares et al. (43), who evaluated the genetic stability of oak embryogenic cultures, and suggested the suitability of the secondary embryogenesis system for the *in vitro* maintenance and proliferation of somatic embryos. Similarly, the true-to-type cloning of adult genotypes of cork oak by secondary embryogenesis was also confirmed by Lopes et al. (24).

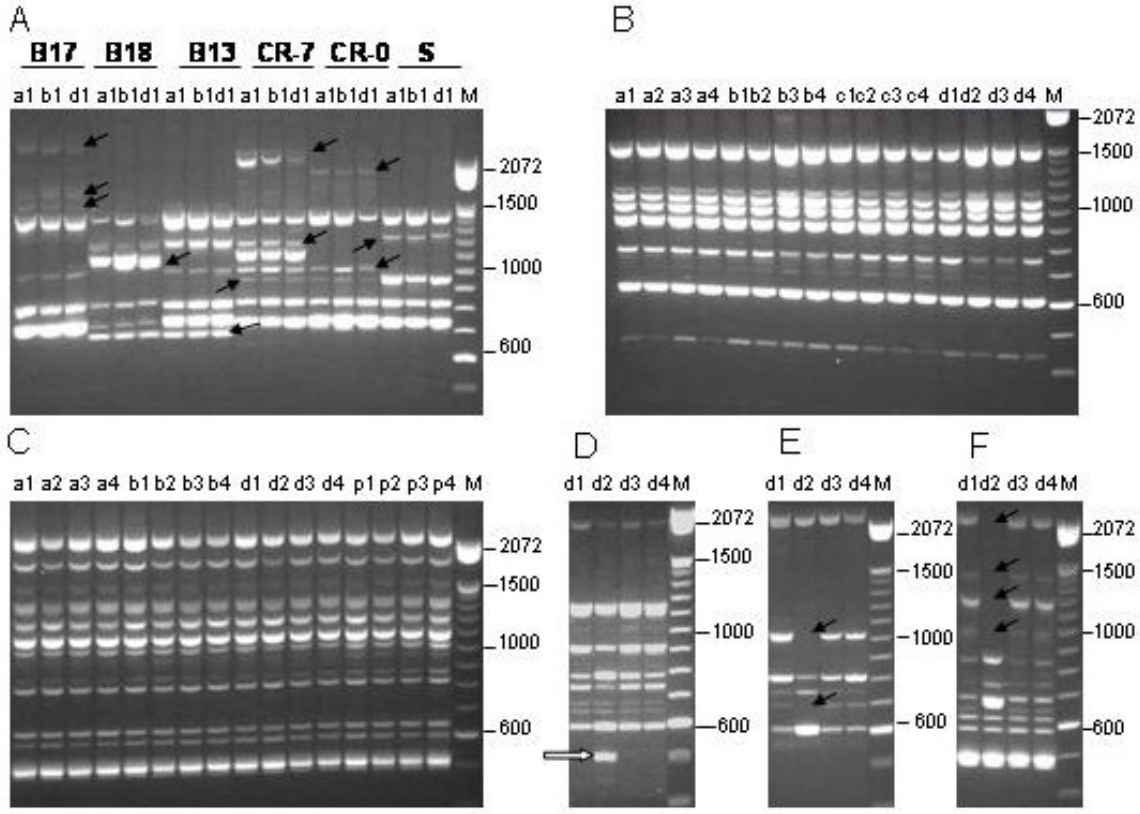

Fig. 3. RAPD profiles generated with (A) OPS-1 and DNA from oak somatic embryos (SE) of genotypes B-17, B-18, B-13, CR-7, CR-0 and Sainza (S), (B) OPA-1 and B-18 SE, (C) OPS-12 and CR-0 SE, (D, E , F) OPA-15, OPS-8 and OPS-12 and CR-7 SE. Lanes represent profiles obtained from independent samples of control SE (a1-4), treated control SE (b1-4), SE recovered after one week (c1-4) or after one year (d1-4) of cryostorage, and from plantlets (p1-4) developed from one-year cryostored SE. M:100 Base-Pair Ladder marker. A: black arrows mark the polymorphic bands among genotypes. D: white arrow shows the new band in d2 SE at 500 bp. E, F: black arrows mark the absent bands in d2 SE.

The B-18 somatic embryos examined, gave rise to a total of 11,720 bands (number of samples analyzed x number of band classes with all the primers used), all 40 embryos produced 293 possible bands and no signs of polymorphism were detected among control, treated control, one-week cryostored, and one-year cryostored embryos. The appearance of

nine monomorphic fragments amplified by OPA-1 in 16 embryos of B-18 line is shown in Fig. 3B. The results obtained for B-13 were similarly consistent, control, treated control, one-week cryopreserved, and one-year-cryopreserved embryos showed monomorphic band patterns. The RAPD profiles generated with the 40 somatic embryos examined of B-17, CR-0 or Sainza, together with the 10 plantlets regenerated from one-year cryostored embryos, were also monomorphic for each primer. The monomorphic patterns of CR-0 somatic embryos (controls, treated controls and cryostored for one year) and plantlets regenerated from CR-0 somatic embryos cryostored for one year, when primed with OPS-12 are shown in Fig. 3C. The lack of somaclonal variation was also observed in one-week cryostored embryos (data not shown). Thus, in five of the six genotypes analyzed in the present study, the RAPD profiles for each primer were highly uniform among all samples analyzed. It should be noted that the total number of RAPD bands scored per clone (more than 11,500) was higher than obtained in similar studies in which RAPD markers were used to assess the genetic stability of plants recovered from cryopreserved material (7, 25). The present results are consistent with those of Häggman et al. (15) who also reported the genetic stability of cryopreserved embryogenic cultures of *Pinus sylvestris*. The genetic stability of plants regenerated from cryopreserved shoot apices of woody species such as *Prunus* (19), grape and kiwi (46), hybrid aspen (20), apple (23), and silver birch (29) has also been shown by RAPD or AFLP markers. It was also reported that RAPD patterns of plants regenerated from cryopreserved material of *Dioscorea floribunda* (1) and *D. bulbifera* (7) were monomorphic for all the regenerated plants except one, suggesting that cryopreservation can be used for long-term conservation of germplasm in both species.

The analysis performed in the CR-7 line with 31 primers, produced a total of 248 distinct fragments and 12,345 bands (including the polymorphic bands). The amplification products were monomorphic for all the samples except for one embryo cryostored for one year (named d2). Thus, among the treated material (treated controls, cryostored embryos, and regenerated plantlets), only 2.5 % (one out of 40) showed any genetic variability. Of 1,550 total profiles (total number of samples x total number of primers used) obtained for this genotype, only three differed; these were originated by the d2 embryo, when its DNA was amplified with primers OPA-15, OPS-8 and OPS-12. The RAPD pattern obtained, with OPA-15, for sample d2 showed a new band of 500 bp that was always absent from the rest of the samples (Fig. 3D). The genetic variability detected with primer OPS-8 was visualized by two fragments of 725 and 1,050 bp that were absent from d2 embryo but always present in the other samples (Fig.3E). Finally, the variability observed with OPS-12 was due to four bands of 1,000, 1,200, 1,500 and 2,200 bp absent from d2 embryo and always present in the other samples (Fig. 3F). These results indicated a frequency of variation for this genotype of 0.06% and 2.8% polymorphism (given as number of polymorphic loci/number of total loci). In *Dendranthema grandiflora*, the RAPD pattern obtained in plants regenerated from shoot apices cryopreserved by the vitrification method were also identical to that of mother plants (25). Although, in the same study a frequency of variation of 0.10% was obtained in plants regenerated from material cryopreserved by the encapsulation-dehydration procedure, the authors suggested that this instability was not related to the cryopreservation method. The relatively high frequency of mutation was attributed to the fact that the only variant showed five differences in the band pattern, similarly to the present results, in which one embryo cryopreserved for one year showed seven polymorphic bands. On the basis of the present findings of no polymorphisms in control embryos and non-frozen PVS2 treated embryos, the variations detected in one CR-7 cryostored embryo did not appear to be caused by the tissue culture or the vitrification solution. Although the analysis performed by RAPDs in embryogenic cultures of *Abies cephalonica* revealed the mutagenic effect of Me_2SO in non frozen samples, the absence of genetic variations in Me_2SO-treated cryostored samples suggested that cryostorage probably eliminates a high proportion of mutations caused by the

Me_2SO (2). DeVerno et al. (6) found that *in vitro* culture after cryopreservation caused somaclonal variation in somatic embryogenic cultures of white spruce, although no variations were detected in trees regenerated from cryostored embryos with normal morphological characteristics; this suggests that only embryogenic tissues with unaltered genomes have the capacity to regenerate normal trees. Similarly, in the present study no variation was found in regenerated plantlets, which suggests that the possible genetic alterations in somatic embryos recovered from cryostorage prevented their conversion into plantlets. Other reports have indicated that DNA variations occurring during culture of oak somatic embryos appear to be related to a loss of their morphogenetic capacity (33, 45). The low variation detected in the present study should be considered as a marginal finding that may have arisen as a consequence of the response of cultures to exposure of LN, temperature variations or cryopreservation conditions. Although the only variant was found in a one-year cryostored embryo, analysis performed in silver birch showed no differences in the RAPD patterns among plants regenerated from meristems that were cryostored for one week or five years (29). In the present study, RAPD markers allowed us to detect a low percentage of polymorphism, however it must be considered that approximately 0,015% of the oak genome was screened. Moreover, other variations associated with changes at the epigenetic or ploidy level cannot be detected by RAPD.

The assessment of genetic fidelity of plants recovered from cryopreserved material is very important, especially in the case of long-lived forest trees. In this study, RAPD markers were adopted because of their simplicity, and they were useful for detecting polymorphisms. The results reported here show that RAPD markers allowed detection of somaclonal variation, as such variation was observed in one embryo that had been cryopreserved for one year. Moreover, RAPD analysis showed that plants regenerated from oak embryogenic tissues cryopreserved by the vitrification method can be used for clonal propagation, as they were found to be genetically stable under the experimental conditions used.

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