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ORIGINAL PAPER

Establishment of cryopreserved gene banks of European chestnut and cork oak

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Abstract Cryopreservation of selected genotypes of European chestnut and cork oak was carried out in two laboratories in a project involving conservation of field collections. Plant material was selected on the basis of disease resistance (chestnut), growth habit, phytosanitary performance and cork quality (cork oak). The cryopreservation technique comprised of vitrification of shoot apices isolated from in vitro stock shoot cultures (chestnut) and somatic embryos (cork oak). Forty-three out of 46 chestnut genotypes assayed survived the freezing process, but only 63% recovered their capacity to produce new shoots. After completion of multiplication and rooting steps, the surviving shoots produced plants that were morphologically identical to those derived from non-supercooled material. All 51 cork oak genotypes withstood freezing and were able to produce new somatic embryos through a process of secondary embryogenesis. Multiplication and germination of the recovered embryos enabled production of plants that were morphologically identical to those derived from non-supercooled material. In light of the results obtained, long-term cryopreservation of these species is feasible, thereby ensuring conservation of valuable genotypes during field evaluation.

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Introduction

Cryopreservation is a technique that ensures safe, long-term conservation of genetic resources of plant species with recalcitrant seeds, of vegetatively propagated species and of biotechnology products such as somatic embryos, cell lines and genetically transformed material. The technique was implemented at the end of the twentieth century and could be used today for routine cryostorage as long as some important factors were taken into consideration (Reed 2001). Tissue culture procedures are usually required to multiply supercooled material via axillary shoots or somatic embryogenesis, and were improved for use with tree species in recent years (Nehra et al. 2005). In addition, production of transgenic tree species and molecular breeding procedures require functional cryopreservation protocols (Häggman et al. 2001). Cryopreservation is increasingly applied on a large-scale basis to woody plants (Reed et al. 1998; Häggman et al. 2008), and as regards forest species, it is most commonly applied to conifers. In the commercial exploitation of conifers, this technique is now routinely applied to embryogenic lines awaiting field testing results (Park et al. 1998; Sutton 2002). In hardwood forest species, the most practical example appears to be the cryopreservation of a core collection of 444 elm (*Ulmus* spp.) clones in a large project involving conservation of elm genetic resources (Harvengt et al. 2004).

European chestnut (*Castanea sativa* Mill.) and cork oak (*Quercus suber* L.) are characteristic species in important Mediterranean ecosystems, each covering areas larger than

2 million ha (Conedera et al. 2004; Knapic et al. 2008). The economic, ecological and social impacts of these agroforestry systems cannot be overstated (Bounous 2005; Silva and Catry 2006). Root rot or ink disease (caused by *Phytophthora* spp.) and chestnut blight (caused by *Cryphonectria parasitica*) are the most important diseases that affect European chestnut, and an upsurge of ink disease has been observed in recent years, probably due to the drier, warmer winters than usual, which have placed the trees under hydric stress (Turchetti and Maresi 2005). In addition to variable climate conditions, fires, abandonment of cultural practices, etc., the decline in productivity of cork oak stands is also associated with pathogens, especially *Botryosphaeria stevensii* (that causes dieback and trunk canker) and *Phytophthora cinnamomi* (Luque et al. 2002; Alves et al. 2004).

Between 2000 and 2005, the Agroforestry Department of the company Transformación Agraria SA (TRAGSA) carried out a survey on ink disease–tolerant and ink disease–resistant chestnuts in an area (18,000 ha) heavily affected by the disease, in Galicia (NW Spain). A total of 206 candidate genotypes were selected (Rodríguez et al. 2005) and are currently being analyzed to determine disease resistance/tolerance and are undergoing molecular characterization. Some 199 genotypes have been established in vitro, through axillary shoot development, by use of previously described procedures (Vieitez et al. 2007), and so far 130 genotypes have been stabilized under in vitro conditions. In addition, between 2002 and 2007, the same Department surveyed several elite stands of cork oak in Extremadura (SW Spain). A total of 106 genotypes were selected under criteria of productivity, health status, cork quality and genetic diversity, and somatic embryogenic lines were established according to protocols previously established for juvenile (Bueno et al. 1992) and mature (Hernández et al. 2003) material. To date, 33 embryogenic lines from mature material and several hundred embryogenic lines corresponding to progenies of the other 73 genotypes have been established in vitro. Furthermore, three experimental plots have been established with these materials to determine their productivity and heritable characters by investigation into progenies.

Cryopreservation methods for *Castanea* and *Quercus* species based on a vitrification technique (solidification of water in a non-crystalline form, Sakai et al. 1990) were described. In addition, the in vitro regeneration systems have also been well defined, making recovery of surviving rewarmed material possible under standard conditions (Hernández et al. 2003; Vieitez et al. 2007). Following these procedures, more than 90% of zygotic embryo axes of recalcitrant chestnut seeds and around 70% of somatic embryos induced from juvenile material survived storage in liquid nitrogen (LN) (Corredoira et al. 2004). Furthermore, three embryogenic transgenic lines of chestnut were successfully cryopreserved, and the stable integration of marker genes into transgenic plants regenerated subsequent to cryopreservation was also demonstrated (Corredoira et al. 2007). In oaks, embryogenic cultures of *Q. robur* originating from both juvenile (Martínez et al. 2003) and mature selected material (Sánchez et al. 2008) were cryopreserved in LN and their genetic stability demonstrated after one year of storage. Finally, the cryopreservation of embryogenic cultures from mature cork oak material was also found to be feasible, with high levels of embryo recovery recorded (88–93%) (Valladares et al. 2004). Cryopreservation of chestnut shoot apices from in vitro cultures multiplied through axillary shoots was also reported as an alternative to preservation of zygotic embryo axes and somatic embryos (Vidal et al. 2005).

The cryopreservation methods developed in the IIAG laboratories were transferred to the Agroforestry Department of TRAGSA and, in a joint venture between the two groups, it was decided to apply the techniques to the selected chestnut genotypes (through cryostorage of shoot apices) and cork oak (cryostorage of somatic embryos) currently under field evaluation. The ultimate goal is the development of the first applied germplasm bank of these important genetic resources as a backup to the field collection gene banks. The success of large-scale cryostorage of different genotypes of chestnut and cork oak in the two laboratories is reported.

Methods

Plant materials and culture conditions

Shoot cultures of 130 selected chestnut genotypes were established in vitro from crown branches of mature trees following forced flushing of branch segments collected in winter (Sánchez and Vieitez 1991; Vieitez et al. 2007). The cultures were maintained on GD (Gresshoff and Doy 1972) medium containing 0.09 M sucrose and 0.7% (w/v) Bacto agar at pH 5.6 (basal medium), and with 0.4 μM N^6-benzyladenine (BA) (proliferation medium). Shoots were subcultured every 5 weeks.

In the case of cork oak, somatic embryogenesis was induced from mature, selected cork oak genotypes following the method described by Hernández et al. (2003), whereas the embryogenic lines originating from juvenile material (embryo zygotic axes) were induced by the method described by Bueno et al. (1992). Regardless of the origin, the embryogenic lines were maintained by repetitive embryogenesis, with monthly subculture onto basal medium, consisting of macronutrients (Schenk and Hildebrandt 1972), micronutrients (Murashige and Skoog 1962),

vitamins and Fe-EDTA, 0.09 M sucrose, 0.6% agar and without plant growth regulators (Valladares et al. 2004). The pH of the medium was adjusted to 5.6.

In both chestnut and cork oak, the cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50–60 μmol $m^{-2}s^{-1}$) and a 25°C light/20°C dark temperature regime.

Cryopreservation of chestnut shoot tips

The vitrification-based procedure described by Vidal et al. (2005) was applied to 46 genotypes (Table 1) of the 130 genotypes established in vitro. The number of shoot apices cryopreserved per genotype varied from 90 to 300, and was related to the proliferation capacity of each genotype. Briefly, the protocol consists of the following steps:

1. Isolation of terminal shoot buds (10 mm long) from stock shoot cultures and transfer to Petri dishes on basal medium containing 0.2 μM BA.
2. Cold hardening (3–4°C) under dim light for 2 weeks.
3. Dissection, under stereoscopic microscopy, of shoot apices (1–2 mm) comprising the apical dome and 3–5 foliar primordia, and preculture on basal medium containing 0.2 M sucrose for 48 h at 4°C.
4. Immersion of shoot apices for 20 min in loading solution at room temperature (Matsumoto et al. 1994).
5. Dehydration with a modified (Vidal et al. 2005) PVS2 vitrification solution (Sakai et al. 1990) for 60–120 min at 0° C.
6. Suspension of the shoot apices (10–20 per vial) in 0.6 ml of PVS2 solution in cryovials and direct immersion in LN.
7. Recovery of shoot apices from cryostorage. The cryovials were rewarmed in a water bath at 40°C for 2 min, the shoot apices were washed in basal medium with 1.2 M sucrose for 20 min and then inoculated in recovery medium consisting of basal medium with 2.2 μM BA, 2.9 μM 3-indoleacetic acid (IAA) and 0.9 μM zeatin.
8. Transfer to fresh recovery medium at 24 h, 2 and 4 weeks.
9. Shoots of the genotypes exhibiting shoot recovery were multiplied and rooted according to Vieitez et al. (2007).

Effect of Supercool© on the cryopreservation of chestnut shoot apices

To evaluate the effect of the polymer on survival and shoot recovery rates, 0.5 ml of Supercool© X-1000 polymer (20% w/w solution) was added to 100 ml of the PVS2 solution before sterilization. The cryopreservation steps defined above were applied to the genotypes P029, L015, C048, P001, P020, C030, C017, P039.

Cryopreservation of cork oak somatic embryos

The cryopreservation method described by Valladares et al. (2004) was applied to 51 embryogenic lines corresponding to genotypes of both juvenile (44 genotypes) and mature origins (7 genotypes, shown in bold type in Table 3). The number of cryopreserved embryo clumps per genotype varied from 90 to 260, depending on the proliferation capacity of the embryogenic line. Briefly, the procedure consisted of the following steps:

1. Isolation of embryo clumps at globular-torpedo stages (1.5–2.5 mm) from stock embryogenic cultures 3 weeks after the last subculture.
2. Preculture of the embryo clumps on basal medium containing 0.3 M sucrose for 1–3 days.
3. Dehydration with the PVS2 vitrification solution at room temperature (Sakai et al. 1990) for 60 min.
4. Suspension of the embryo clumps (10–20 clumps to a vial) in 0.6 ml of PVS2 in cryovials and direct immersion in LN.
5. Recovery of somatic embryos from cryostorage. The cryovials were rewarmed in a water bath at 40°C for 2 min, and the somatic embryos were washed in basal liquid medium with 1.2 M sucrose (two times, 10 min each) and then inoculated in basal medium.
6. Measurement of embryogenic capacity and germination ability was assessed following the procedures described by Hernández et al. (2003) and Valladares et al. (2004), respectively.

Data collection and statistical analysis

Chestnut shoot apices that survived in any form (including calluses) 8 weeks after rewarming were recorded (survival); shoot apices showing regrowth and formation of new shoots 12 weeks after rewarming were recorded (expressed as percentage shoot recovery). In the Supercool© experiment, data were recorded 8 weeks after rewarming. In addition, the cork oak embryo recovery level was assessed (6 weeks after rewarming) as the percentage of explants that showed resumption of embryogenic capacity (embryo recovery).

Each experiment consisted of 12 cryotubes with 10–20 explants each per experiment. In the Supercool© experiment, 12 explants/cryotube and 4 cryotubes/treatment were used per genotype. For shoot recovery (chestnut) and embryo recovery (cork oak) assessment, at least 30 explants/genotype were utilized (see Tables 1 and 3). The results were subjected to analysis of variance by comparison of group

Table 1 Shoot recovery percentage (±SE) of European chestnut (*Castanea sativa*) shoot apices of 46 genotypes, after exposure to PVS2 vitrification solution and subsequent immersion in liquid nitrogen (LN)

Genotype	Shoot tips remaining in LN	Shoot tips rewarmed	Shoot recovery ± SE (%)
C004	150	30	17 ± 3.3*hijk
C007	70	37	5 ± 2.7cdef
C009	170	30	0 ± 0ab
C012	200	32	0 ± 0ab
C017	134	36	0 ± 0ab
C028	110	30	0 ± 0ab
C030	392	320	2 ± 1.2abc
C031	168	72	0 ± 0a
C032	80	34	6 ± 3.1cdefg
C035	140	39	0 ± 0ab
C042	60	30	0 ± 0ab
C047	168	93	12 ± 2.1fghi
C048	144	48	33 ± 9.2lmn
C063	80	30	0 ± 0ab
C064	166	54	14 ± 0.8ghij
C086	100	30	33 ± 6.7lmn
C088	110	34	0 ± 0ab
C092	130	38	5 ± 2.4bcde
L010	140	34	6 ± 2.9cdefg
L015	192	72	21 ± 4.7ijkl
L016	140	31	23 ± 3.7ijklm
P001	166	69	25 ± 6.8jklm
P002	90	31	16 ± 3.1hijk
P007	190	30	0 ± 0ab
P010	160	30	30 ± 10.0klmn
P011	184	31	19 ± 4.1hijkl
P014	140	37	0 ± 0ab
P015	100	31	42 ± 1.8no
P016	69	30	7 ± 3.3cdefg
P018	170	30	37 ± 8.8mno
P019	80	30	0 ± 0ab
P020	192	48	4 ± 2.2abcd
P022	100	30	0 ± 0ab
P024	168	56	14 ± 3.9ghij
P026	110	30	0 ± 0ab
P028	100	30	0 ± 0ab
P029	224	72	11 ± 2.8efgh
P034	160	32	53 ± 10.6o
P035	140	30	0 ± 0ab
P039	192	49	0 ± 0a
P040	120	38	5 ± 2.6cdef
P042	110	33	12±3.1efghij
P044	110	31	29 ± 4.8klmn
P047	120	42	10 ± 2.4defghi
P049	120	59	13 ± 3.8ghij
P051	170	31	10 ± 0.3efghi

Assessment was made 12 weeks after rewarming and plating on recovery medium

* Means with the same letter are not significantly different at the $P = 0.05$ level according to the Kruskal–Wallis test

Table 2 Shoot recovery percentage (±SE) of chestnut shoot apices after exposure to PVS2 solution and PVS2 plus Supercool© (PVS2 + SC) and subsequent immersion in liquid nitrogen. Data recorded after 8 weeks on recovery medium

Clone	PVS2	PVS2 + SC	*F*-test
P029	9.2 ± 2.1a	28.8 ± 3.6b	$P < 0.01$
L015	8.3 ± 3.4	16.7 ± 3.4	ns
C048	22.2 ± 2.8a	48.2 ± 5.8b	$P < 0.05$
P001	22.7 ± 5.3	25.5 ± 12.6	ns
P020	0 ± 0	7.5±3.8	ns
C030	4.2 ± 4.2	0 ± 0	ns
C017	0 ± 0	0 ± 0	–
P039	0 ± 0	0 ± 0	–

Within each row, means with the same letter are not significantly different at the $P = 0.05$ level. *F*-test: ns, not significant

means using the Dunnet test (Tables 2 and 3) or the non parametric Kruskal–Wallis test (Table 1) at the $P = 0.05$ level. Percentage data were subjected to arcsine transformation prior to analysis.

Results

Cryopreservation of chestnut shoot tips

One hundred and thirty chestnut genotypes were successfully established in vitro and are currently maintained by regular subculture under the previously defined protocols (Vieitez et al. 2007). However, maintenance of in vitro cultures of the remaining 76 genotypes (to complete the field collection of 206 selected genotypes) is not yet possible. The failure may be related to both the poor reactivity exhibited for these genotypes, as the explants for initiating the in vitro cultures were collected from very old trees lacking shoots with juvenile characteristics, and to the loss of cultures due to endogenous contamination. In a first approach and following the 9-step protocol described in Methods, a total number of 8303 shoot tips, corresponding to 46 genotypes of

European chestnut, were excised from in vitro shoot cultures and cryopreserved in the two laboratories participating in the project (Table 1). Currently, 6189 shoot apices remain immersed in LN (from 3 years to several months of storage, depending on the genotype), whereas the other 2114 shoot tips were rewarmed for analysis of survival and shoot recovery. The supercooled material can be rewarmed at any time for further testing.

The cryopreserved shoot apices turned blackish within 24 h after rewarming and cultured in recovery medium, then recovered their green color within 10 days, and in the most responsive genotypes resumed growth within 2–3 weeks (Fig. 1a). In these genotypes, shoot growth was normal 8–12 weeks after rewarming, although the response also depended on the genotype (Fig. 1b). Most chestnut genotypes (93%) subjected to vitrification and cryopreservation withstand supercool in LN. Forty-three genotypes survived in some form although in 17 genotypes, the shoot apices did not develop into healthy shoots but rather into calluses with no further shoot formation. The 29 genotypes that formed new shoots (63% of the genotypes tested, Table 1) were cultured on proliferation medium and produced new shoots with recovery values ranging from 2 to 53%, allowing regeneration of plants. The genotypes able to produce new shoots after LN treatment were capable of proliferating in a similar way as their non-cryopreserved but treated (covering all steps of the cryopreservation procedure except the immersion in LN) counterparts. The shoots subjected only to the action of the PVS2 solution exhibited shoot recovery rates ranging from 50% to 100%, according to the genotype (data not shown). These values were always higher, within the same genotype, than the shoots immersed in LN. No differences between the cryopreserved-derived shoots, the shoots treated with the PSV2 solution but not immersed in LN and the shoot stock cultures were observed in terms of shoot multiplication capacity and rooting rates, and the morphological characteristics were the same in both supercooled (Fig. 1c) and non-supercooled plants.

Addition of the polymer Supercool© to the PVS2 vitrification solution improved survival of rewarmed shoot tips of the 8 genotypes tested, and the survival rate was 100% in all genotypes, except C030 (36%) and P020 (92%). The shoot recovery capacity (Table 2) also increased in 5 out of 8 genotypes tested, with the rates depending on the genotype. These results indicate increases in shoot recovery rates in most of the genotypes tested, although in only two of them the differences are statistically different. In genotypes C030, C017 and P039, the addition of Supercool© was unsuccessful as the recovery frequencies remained at 0%. The causes of the failure may be related to the genotype rather than to the cryopreservation process.

Cryopreservation of cork oak somatic embryos

Stock embryogenic cultures 3 weeks after the last subculture (Fig. 1d) were used for the isolation of embryo clumps subjected to cryopreservation. Samples of all cork oak genotypes tested withstood supercool in LN and all were capable of producing new embryos (Table 3). The somatic embryo clumps sometimes appeared necrotic several hours after rewarming but, 10 days after transfer to basal medium, reactivation of growth and new embryogenic structures were evident. These structures emerged from surviving cells of initial explants through a mechanism of repetitive or secondary embryogenesis (Fig. 1e).

Following the 6-step procedure defined in Methods, a total of 8810 embryo clumps corresponding to 51 selected cork oak genotypes were subjected to cryopreservation. Currently, a total of 6850 embryo clumps remain in the cryogenic tanks and 1960 were used for recovery experiments (Table 3). As with chestnut, the remaining samples can be rewarmed at any time for further tests. Although there were clear genotypic differences in the rates of recovery of the embryos (18–100% in cryopreserved samples), these were not related to the origin (juvenile/mature) of the plant material used to initiate the embryogenic lines. For example, the genotype of mature origin DII3A/02 exhibited an embryonic recovery of 100%, whereas in the Rozo 1A(66/02) genotype (of seedling origin), only 25% embryonic recovery was obtained.

The new embryos produced after cryopreservation were proliferated according to previously defined procedures (Valladares et al. 2004), and the capacity of the embryos to multiply (recorded as the number of new somatic embryos produced per explant) and the germination ability were assessed. No differences between the cryostored and non-cryostored explants were observed, and the plants derived from supercooled (Fig. 1f) somatic embryos were morphologically indistinguishable from those derived from non-supercooled embryos.

Discussion

The European Forest Genetic Resources Programme (EUFORGEN, http://www.biodiversityinternational.org/networks/euforgen) emphasizes the need for the conservation and sustainable use of forest genetic resources. The different conservation strategies for forest biodiversity are well defined (Häggman et al. 2008) and, among these, cryopreservation may be considered as a complementary strategy for storage of plant cells, tissues, seeds and embryos. Cryopreservation is normally viewed as a secondary storage method designed as a secure backup to living collections (Reed 2008). In keeping with this idea,

Table 3 Embryo recovery percentage (±SE) of somatic embryo clumps of 51 cork oak (*Quercus suber*) genotypes after exposure to PVS2 vitrification solution with subsequent immersion in liquid nitrogen (LN)

Genotype	Embryo clumps remaining in LN	Embryo clumps rewarmed	Embryo recovery ±S.E. (%)
ADB1A (12/06)	150	84	50 ± 7.3* bcdef
ADB1A (2/06)	150	84	87 ± 5.3klmnop
Cab3A/07	**100**	**31**	**90 ± 0.3hijklmn**
Cab5A/07	**140**	**31**	**94 ± 5.6mnopq**
Carc3A (45/06)	150	32	78 ± 2.8ghijklm
Carre1B (625/06)	140	33	36 ± 5.2abc
Carre1C/08	**130**	**30**	**90 ± 5.8mnopq**
Chap3A (743/04)	200	43	95 ± 5.6pq
Chap3A (752/04)	110	30	100 ± 0.0q
CM2A (638/06)	130	32	94 ± 3.2opq
CM2A (639/06)	100	30	100 ± 0.0q
CM3B (361/06)	130	30	67 ± 8.8defghij
CM3B (365/06)	150	30	100 ± 0.0q
CM3B (685/06)	140	30	100 ± 0.0q
CM4A (472/06)	100	30	33 ± 3.3abc
DGF1A (719/06)	120	30	93 ± 3.3mnopq
DGF1A (746/06)	100	30	100 ± 0.0q
DGF2A (812/06)	160	102	61 ± 7.6cdefgh
DGF3A (1151/06)	140	32	91 ± 0.3jklmnop
DGF4A (938/06)	140	30	100 ± 0.0q
DGF4A (939/06)	70	30	90 ± 5.8mnopq
DGF5A/09	**155**	**33**	**85 ± 5.2jklmnop**
DI1A (1200/03)	180	62	73 ± 3.2fghijk
DI1A (832/03)	160	63	81 ± 5.9jklmn
DI1A (665/03)	90	30	100 ± 0.0q
DI3A (744/03)	200	44	90 ± 4.7nopq
DI3B (1364/03)	90	30	70 ± 5.8efghijkl
DI5A (827/03)	140	43	88 ± 2.3lmnop
DII3A (597/04)	160	51	100 ± 0.0q
DII3A/02	**110**	**30**	**100 ± 0.0q**
DII5A (1437/03)	80	30	100 ± 0.0q
DII6A (468/04)	140	31	35 ± 2.9abc
DII6A (470/04)	160	36	81 ± 7.1jklmno
DII6A (472/04)	80	31	42 ± 4.1bcde
DII6B (554/04)	160	39	56 ± 4.4cdefg
DII6B (566/04)	160	40	55 ± 3.3cdefg
DII6B (576/04)	160	40	18 ± 3.1a
DII6B/07	**140**	**32**	**44 ± 3.1bcde**
Rop3A (670/02)	150	30	100 ± 0.0q
Rop3A (681/02)	130	33	82 ± 4.3hijklmno
Rop5A (451/03)	110	31	35 ± 2.9abc
Rop5A (452/03)	110	30	40 ± 5.8abcd
Rozo1A (66/02)	60	32	25 ± 8.7ab
Rozo2A (41/02)	200	40	77 ± 10.2jklmno
Rozo5A (233/04)	150	30	87 ± 13.3mnopq
Rozo5A (234/04)	110	32	59 ± 2.6cdefghi
Rozo6A (140/04)	100	30	86 ± 0.9jklmno
Rozo6A (142/03)	160	42	79 ± 8.1ijklmn
Rozo6A (1619/03)	140	70	90 ± 3.1mnop
SL1A (412/04)	120	30	83 ± 12.0klmnop
SL2A	**150**	**31**	**71 ± 17.3ghijklmn**

Assessments were made 6 weeks after rewarming and plating on basal medium. Genotypes of mature origin are shown in bold type

* Means with the same letter are not significantly different at the $P = 0.05$ level according to the Dunnet test

the results reported here demonstrate that the development of cryobanks to duplicate the field collections of selected material of European chestnut and cork oak appears feasible.

Simple, efficient and reproducible protocols, as well as consistent procedures for in vitro plant regeneration are required to develop applied gene banks. In European chestnut, protocols for the cryopreservation of zygotic embryo axes, somatic embryos, transformed embryogenic lines (Corredoira et al. 2004, 2007; Vieitez et al. 2010) and in vitro-grown shoot tips (Vidal et al. 2005) are well defined. Although the most efficient system for cryopreservation makes use of embryonic axes and somatic embryos as explants for cryostorage, we have used shoot tips for this species because: (1) the seeds produced by the trees originating the chestnut field collection may be genetically diverse and, consequently, cryopreservation of their embryonic axes is of unproven genetic value and (2) the induction of somatic embryogenesis from adult chestnut material has not yet been reported (Corredoira et al. 2006). In the establishment of a cryopreserved gene bank of European elms, Harvengt et al. (2004) used supercooled (rewarmed) dormant buds as source of plant material. However, this protocol does not appear to be appropriate for chestnut as we have always found that dormant buds of this species generally perform very poorly in tissue culture. With chestnut shoot apices, 63% of the cryostored genotypes were able to recover their capacity for indefinite proliferation and plantlet regeneration at similar rates to the non-supercooled material. In the case of dormant buds of elm (Harvengt et al. 2004), the initial multiplication ability appears transient, as several clones were lost after 14 months in multiplication medium. In chestnut, the shoot

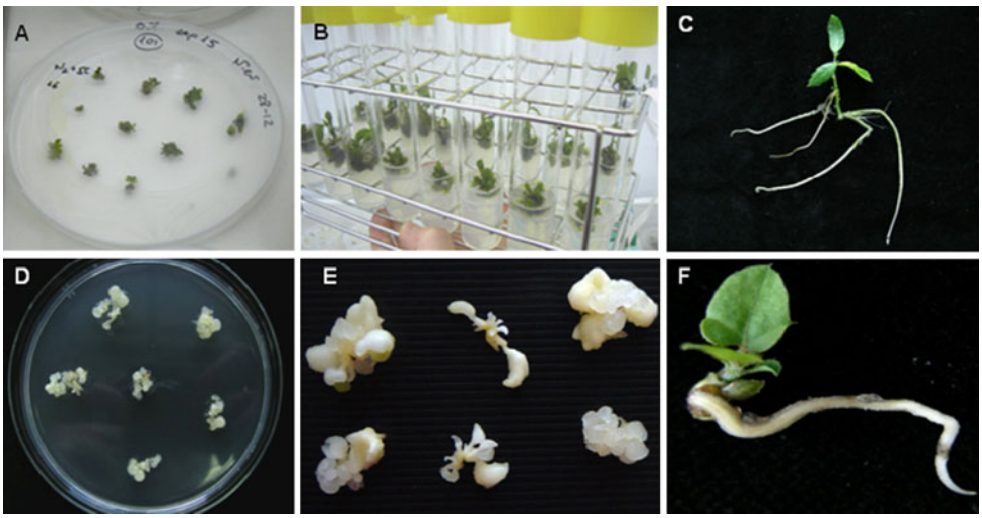

Fig. 1 Steps in the cryopreservation of European chestnut and cork oak. Shoots growing from successfully cryopreserved chestnut shoot apices at different stages of development (**a**, **b**). Rooting of elongated chestnut shoot derived from the cryopreserved shoot apex of genotype C032 (**c**). Stock embryogenic cultures from which embryo clumps at globular-torpedo stages are isolated for cryopreservation (**d**). Somatic embryo recovery following exposure to PVS2 solution and liquid nitrogen treatment (**e**). Somatic seedling regenerated from cryopreserved embryogenic cultures obtained from cork oak genotype ADB1A(2/06) (**f**)

tips of 17 cryostored genotypes did not develop into healthy shoots (Table 1). There are at least three reasons for this failure. The first is related to the size of the shoot apex immersed in LN, which is apparently not optimal for these genotypes; this is an important aspect as it has been demonstrated in several species, including chestnut, that smaller apices provide fewer small actively dividing cells with few vacuoles, and therefore the tissue is more tolerant to dehydration than in a larger apex with many vacuolated cells (Escobar et al. 1997; Takagi 2000; Vidal et al. 2005). The second reason may be related to the genotype as it has been demonstrated that cryopreservation of the plant germplasm is genotype dependent (Reed 2000). The third reason is related to the in vitro growth performance of the cultures used as source of shoot tips for cryopreservation. Clearly, the success or otherwise of the process may be determined by poor physiological condition of the cultures. Taking these aspects into consideration, efforts are been made to achieve a successful response for recalcitrant genotypes. In this respect, the addition of the polymer Supercool©, which inhibits the formation of ice in cryoprotective solutions (Wowk et al. 2000) was found to be beneficial for the survival of several genotypes, as also demonstrated in potato shoot tips (Zhao et al. 2005). This compound will be used in further experiments.

Attempts to preserve cork oak zygotic embryo axes by partial desiccation followed by rapid immersion in LN have been unsuccessful, as only unorganized post-supercooled growth of axes was reported (González-Benito et al. 2002). However, methods for the cryopreservation of somatic embryos of different *Quercus* species, including cork oak, are well defined at the laboratory level (Wilhelm 2000; Martínez et al. 2003; Valladares et al. 2004). Somatic embryogenesis is a form of micropropagation that allows storage of the genetic reservoir contained in a field collection. In contrast to the results achieved with chestnut shoot apices, cryopreservation of cork oak somatic embryos is simpler, easier and more efficient, as embryo clumps of 100% of the embryogenic lines assayed recovered their capacity to proliferate after immersion in LN. In the first stages of development, the somatic embryo is mainly formed by small, dividing and scarcely vacuolated cells, which provide ideal target material for cryopreservation. Differentiation of the somatic embryos (size) is directly related to the capacity of the embryos to withstand the pressure of LN, and their use at the globular and/or torpedo stages is recommended for this species rather than embryos at the cotyledonary stage (Valladares et al. 2004).

The results reported in the present study show that the simple and efficient vitrification-based supercooling technology allows the development of a large-scale gene bank for long-term storage of a great proportion of the selected genotypes of European chestnut and all cork oak genotypes tested, and the procedure is being used in the two laboratories participating in the study. One concern related to these technologies is the genetic stability of the rewarmed, regenerated plants. In our experience, RAPD profiles of the regenerated plantlets of 5 embryogenic lines of *Quercus robur* cryopreserved during one year were identical to those of the controls (Sánchez et al. 2008). Furthermore, 3

transgenic embryogenic lines of European chestnut were successfully cryopreserved and the stable integration of reporter genes into plants that were regenerated subsequent to cryopreservation was demonstrated (Corredoira et al. 2007). Using RAPD analysis, no molecular differences were found in 5 cryopreserved genotypes of European chestnut in comparison with the non-supercooled parentals (Jorquera 2009). The chestnut and cork oak plants used in the present study exhibit identical morphological characters regardless of whether they were cryopreserved or not, and molecular markers (AFLPs, microsatellites) are being applied to test their genetic stability.

In light of the results achieved, the gene banks of the two species will be completed with all the genotypes available in the field collections.

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