Chapter 9
Cryopreservation of Embryogenic Cultures

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9.1 Introduction

The first reports on somatic embryogenesis date back to 1958 (Reinert 1958; Steward et al. 1958) and represent one of the most important milestones in plant tissue culture, as they give clear evidence of the concept of 'totipotency' of vegetative cells, a peculiarity which makes possible the regeneration of a whole plant from a single somatic cell (Bajaj 1995). Indeed, thanks to the 'bipolar' nature of the somatic embryo, shoot and root formation are generally induced at the same time from a single regenerative act. This differentiates somatic embryogenesis from organogenesis, where *de-novo* formed 'unipolar' structures, mainly shoots, need to be stimulated with growth regulators for the induction of adventitious roots, thus producing a complete plant in two steps. Somatic embryos can be induced directly from cells of the explant cultured *in vitro* ('direct' or 'adventitious' somatic embryogenesis), or more often, from the dedifferentiated cells of a proliferative callus (indirect or induced somatic embryogenesis) after the explant tissue is artificially stimulated (i.e., with growth regulators) to develop embryogenic competence (Hartmann et al. 1990;

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*

Krikorian 2000). Embryogenic callus, established *in vitro* from the original explant, are periodically subcultured to maintain their embryogenic potential. These constitute 'embryogenic cultures' or 'embryogenic callus lines'.

The bipolar structure of the somatic embryo, as well as the developmental stages it passes through, makes it similar to the zygotic embryo (Dodeman et al. 1997; Benelli et al. 2001). However, the former evolves into a clonal plant, as it originates directly from a somatic cell without gametic fusion, while the latter produces a new genotype as a result of gametic reproduction. In addition, differently from zygotic embryos, somatic embryos are not protected by a seed coat and they are dependent on the culture medium for nutrition. The synthetic seed technology (i.e., the inclusion of a somatic embryo inside a nutrient-containing alginate bead) was developed to resemble as closely as possible the natural condition of the zygotic embryo.

Effective protocols for the induction and the establishment *in vitro* of embryogenic cultures were developed for a large number of plant species: conifers (Attree and Fowke 1991), temperate and tropical fruit trees (Akhtar et al. 2000; Jain and Gupta 2005), crops, and ornamental plants (Bajaj 1995). Advances in tissue culture technology opened the way to various and important applications of somatic embryogenesis, such as (i) the production of synthetic seeds (Redenbaugh et al. 1991; Lambardi et al. 2006) which allow easy storage, transportation and sowing of encapsulated somatic embryos, (ii) the scale-up production of plants in bioreactors (Denchev et al. 1992), (iii) the utilization in genetic transformation studies (Ellis 1993; Jackson and Linskens 2003), thus enabling the production of 'transgenic' lines carrying genes of specific interest, (iv) the production of somatic hybrids (Ozawa et al. 1996) and polyploid plants (Ezura and Oozawa 1994), (v) the elimination of viruses (Goussard and Wiid 1992), and, as reported here, (vi) the cryopreservation of plant germplasm.

Once established, embryogenic callus cultures require periodic subculturing to maintain both a high proliferative potential and the capacity of cells to develop into somatic embryo primordia. Repeated subculturing is not only labor intensive and time consuming, but also increases the risk of losing the embryogenic cultures through contamination, human errors or technical failures. The loss of embryogenic potential of culture lines and the occurrence of genetic alterations due to long-term subculturing is frequently reported (Harding 1996; Bhatti et al. 1997). Periodic re-initiation of embryogenic cultures can provide a solution to this drawback. However,

this practice is again time consuming, expensive and particularly difficult for species characterized by a specific 'window' of embryogenic competence, i.e., having explants suitable for embryogenic callus induction during a very brief period of the year (Becwar et al. 1990; David et al. 1995; Lambardi 2000). Particularly when working with woody species, once a good culture line is lost, there is never the certainty of getting another of equivalent embryogenic potential within a short time. Thus, it is of strategic importance to develop and optimize effective protocols for the maintenance of embryogenic callus cultures in semi-solid or in liquid media, with the aim of reducing the frequency of manipulations and, as a consequence, the risks of decay, loss or genetic alterations. Recent advances in the cryostorage of plant tissues and organs in liquid nitrogen (LN) (at –196°C) opened the door to safe, low-cost, long-term maintenance of embryogenic cultures. Both the controlled rate cooling method (two-step freezing), and procedures allowing the direct immersion of specimens in LN (vitrification or one-step freezing) were successfully tested with embryogenic callus lines from various plant species (Engelmann 2004; Panis and Lambardi 2005).

9.1.1 Controlled Rate Cooling

The traditional controlled rate cooling method is still the most common approach for the cryopreservation of clumps from embryogenic callus cultures. The main step in the protocols is the slow cooling of embryogenic clumps at 0.5–1°C min^{-1} to an intermediate temperature of –40°C, after which the clumps are immersed in LN. In recent years this approach allowed for the development of effective protocols for various conifer species (Table 9.1), finding practical application in Canada in the long-term storage of thousands of selected embryogenic callus lines (Cyr 2000). The technique was successfully applied to embryogenic clumps of hardwood and herbaceous species (Table 9.2 and 9.3). One disadvantage of the slow-cooling approach is that it requires a controlled-rate freezer. The major advantage is the ease of use for storing large numbers of cultures in a standardized manner. The Nalgene Freezing Container (Sigma-Aldrich) which cools at a rate of 1°C min^{-1} through the use of cooled (to –80°C) isopropyl alcohol is also successful (Martinez-Montero et al. 1998; Ford et al. 2000).

Table 9.1. Cryopreservation of embryogenic cultures from woody plants: conifers

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum recovery (%)	Reference
Abies cephalonica	CC	5°C (14d), darkness+ sucrose, 0.2M (1d) + 0.4M (1d)	10% PEG6000+ 10% glucose+ 10% DMSO 30 min, 0°C	10°C h^{-1} to -38°C	75	Aronen et al. 1999
Picea abies	CC	sorbitol, 0.4M (2d)	5% DMSO, 30 min	0.3°C min^{-1} to -35°C	NR	Nørgaard et al. 1993
Picea abies	SC	sorbitol, 0.2M (1d)+ 0.4M (1d), darkness	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Find et al. 1998
Picea abies	CC	sorbitol, 0.4M (2d)	5% DMSO, 30 min	0.3°C min^{-1} to -35°C	NR	Högberg et al. 1998
Picea glauca engelmanni	SC	none	0.4 M sorbitol + 5% DMSO	multistep cooling[2]	100	Cyr et al. 1994
Picea glauca	SC	sorbitol, 0.4M (1d)	0.4 M sorbitol + 5% DMSO, 30 min	0.3°C min^{-1} to -35°C	94	Kartha et al. 1988
Picea sitchensis	SC	sorbitol, 0.2M (1d)+ 0.4M (1d)	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Kristensen et al. 1994
Picea sitchensis	SC	sorbitol, 0.2M (1d)+ 0.4M (1d), darkness	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Find et al. 1998
Pinus caribaea 'Hondurensis'	SC	sucrose, 0.4M	0.4 M sucrose + 5% DMSO	0.5°C min^{-1} to -35°C	100	Lainé et al. 1992
Pinus patula	CC	sorbitol, 0.3M (1d)	0.3 M sorbitol+5% DMSO, 20 min, 0°C	-70°C, 2h[3]	60	Ford et al. 2000
Pinus pinaster	CC	maltose, 0.2M (1d)+ 0.4M (1d), darkness	10% PEG4000 + 10% sucrose + 10% DMSO 0°C	-80°C, 1d[3]	97	Marum et al. 2004
Pinus radiata	CC	sorbitol, 0.4M (1d) 20°C	0.4 M sorbitol +10% DMSO	-80°C, 75-90 min[3]	100	Hargreaves et al. 2002
Pinus roxburghii	CC	sorbitol, 0.3M (1d)	0.3 M sorbitol + 5% DMSO	multistep cooling[4]	70	Mathur et al. 2003
Pinus sylvestris	CC	5°C (14d) + sucrose, 0.2M (1d)+0.4M (1d)	10% PEG6000+ 10% glucose+10% DMSO, 30 min, 0°C	10°C h^{-1} to -38°C	78	Häggman et al. 1998
Picea mariana	CC	sorbitol, 0.8M (2d)	PVS2, 30 min, 0°C	Direct immersion in LN	67	Touchell et al. 2002

LN, liquid nitrogen; NR, not reported; [1] CC, clumps of callus (developmental stage not reported); SC, cells from suspension cultures. [2] Slow cooling was achieved as follows: 0.3°C min^{-1} to -3°C → 15°C min^{-1} to -8°C → 25°C min^{-1} to -32°C → 0.3°C min^{-1} to -35°C.[3] Slow cooling was achieved by incubating the samples in a freezer at -70 or -80°C before plunging them into LN.[4] Slow cooling was achieved as follows: 0.3°C min^{-1} to -35°C → 25°C min^{-1} to -50°C.

Table 9.2. Cryopreservation of embryogenic cultures from woody plants: hardwoods, shrubs and palm trees

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum recovery (%)	Reference
Slow cooling						
Citrus deliciosa	CC		10% DMSO, 30 min, 4 °C	0.5°C min^{-1}	NR	Pérez et al.1999
Citrus sinensis	SE			0.5°C min^{-1} to -42 °C	5	Marin and Duran-Vila 1988
Citrus sinensis	HSE		10% DMSO	0.5°C min^{-1} to -42 °C	31	Marin et al. 1993
Citrus ssp.	CC		10% DMSO, 30 min, 0 °C	0.5°C min^{-1} to -40 °C	100	Pérez et al. 1997
Elaeis guineensis	SC	4°C (1h)	1M glucose + 10% DMSO, 1h, 4 °C	0.5°C min^{-1} to -40 °C	70	Chabrillange et al. 2000
Hevea brasiliensis	CC		1M sucrose + 10% DMSO, 1h, 0 °C	0.2°C min^{-1} to -40 °C	70	Engelmann and Etienne 2000
Musa spp.	SC		180 g l^{-1} sucrose + 7.5% DMSO	1°C min^{-1} to -40 °C	NR	Panis et al. 1990
Direct immersion in LN						
Aesculus hippocastanum	TSE	4°C (5d), darkness	2M glycerol + 0.4M sucrose, 30 min, 25°C + PVS2, 90 min, 0 °C		94	Lambardi et al. 2005
Citrus sinensis	SC		60% PVS2, 5 min, 25°C + PVS2, 3 min, 0 °C		84	Sakai et al. 1990
Citrus ssp.	SE	sucrose, 0.7M (1d)	encapsulation + dehydration, 5h, LF		100	Gonzàlez-Arnao et al. 2003
Elaeis guineensis	CC	sucrose, 0.75M (7d)	dehydration, 16h, SG		93	Dumet et al. 2000
Fraxinus angustifolia	CSE	4°C (10h) + sucrose, 0.5M (1d)	encapsulation + dehydration, 1h, SG		31	Tonon et al. 2001
Magnifera Indica 'Zihua'	SC	sucrose, 0.5M (1d) 25°C	PVS3[2], 20 min, 25 °C		94	Wu et al. 2003
Manihot esculenta	CC	sucrose, 0.3M (21d)	dehydration, 16h, LF		95	Danso and Ford-Lloyd 2004
Olea europaea 'Canino'	CC	4°C (4d), darkness	2M glycerol + 0.4M sucrose, 30 min, 25°C + PVS2, 90 min, 0 °C		38	Lambardi et al. 2002
Olea europaea 'Nabali'	CC	30°C (1d)	encapsulation + 0.4M sucrose + 2M glycerol, 60 min PVS2, 3h, 0 °C		64	Shibli and Al-Juboory 2000
Prunus avium	CC	multistep sucrose preculture [3] (until 20% MC)	dehydration, LF		89	Grenier-de March et al 2005
Quercus robur	GSE-HSE	sucrose, 0.3M (3d)	PVS2, 60 min, 25 °C		70	Martínez et al. 2003
Quercus robur	CC	multistep sucrose preculture [3]	dehydration, 4-5h, LF		NR	Chmielarz et al. 2005
Quercus suber	GSE	sucrose, 0.3M (3d)	PVS2, 60 min, 0 °C		93	Valladares et al. 2004

(LF, laminar flow; MC, moisture content; NR, not reported; SG, silica gel); [1] CC, clumps of embryogenic callus (developmental stage not reported); CSE, clumps or isolated somatic embryos at the cotyledonary stage; GSE, clumps or isolated somatic embryos at the globular stage; HSE, clumps or isolated somatic embryos at the heart stage; SC, cells from suspension cultures; SE, isolated somatic embryos; TSE, clumps or isolated somatic embryos at the torpedo stage. [2] PVS3: 50% sucrose (w/v) + 50% glycerol (w/v) in standard culture medium.[3] Multistep sucrose preculture was performed as follows: Sucrose, 0.25M (1d) + 0.5M (1d) + 0.75M (2d) + 1.0M (3d).

Table 9.3. Cryopreservation of embryogenic cultures from herbaceous species: crops and cut-flowers

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum Recovery (%)	Reference
Slow cooling						
Asparagus officinalis	SC	sucrose, 0.8 M (2d), 24°C		0.5 °C min^{-1}	NR	Jitsuyama et al. 2002
Cyclamen persicum	SC	sucrose, 0.6 M (2d)	0.6 M sucrose + 10% DMSO, 1h	-20 °C, 2 h[2]	NR	Winkelmann et al. 2004
Festuca spp.	SC	sorbitol, 0.33 M (3d)	0.5 M sorbitol + 10% DMSO	multistep cooling [3]	71	Wang et al. 1994
Ipomoea batatas	CC	multistep sucrose preculture [4]	encapsulation + dehydration, 4h in LF	10°C min^{-1} to 0 °C + 0.5 °C min^{-1} to -40 °C	70	Bhatti et al. 1997
Ipomaea batatas 'Kokei-14'	CC	ABA, 3 mg l^{-1} (3d)	10% DMSO + 10% sucrose ± 5% glycerol	0.3°C min^{-1} to -30° C	80	Shimonishi et al. 2000a
Lolium spp.	SC	sorbitol, 0.33 M (3d)	0.5M sorbitol +10% DMSO	multistep cooling [3]	63	Wang et al. 1994
Oryza sativa	SC	mannitol, 60 g l^{-1}	1 M DMSO + 1 M glycerol + 2 M sucrose, 60 min, 0°C	-25 °C, 2 h	98	Jain et al. 1996
Saccharum spp.	SC	sorbitol, 0.33 M (3d)	0.5 M sorbitol + 0.64 M DMSO	0.5 °C min^{-1} to -40 °C	92	Gnanaparagasam and Vasil 1990
Saccharum officinarum	CC	sucrose, 0.5 M (1h), 0°C	10% DMSO	0.4-0.5 °C min^{-1}	90	Martinez-Montero et al. 1998
Direct immersion in LN						
Asparagus officinalis	SC	sorbitol, 0.3 M+ sucrose, 0.2M(16h), 22 °C	0.5 M sorbitol + 12% EG (5 min), 22°C + 85% PVS[5] (5 min), 0°C		65	Uragami et al. 1989
Cucumis melo	SE	ABA, 10 mg l^{-1} (3d)	dehydration, LF		65	Shimonishi et al. 2000b
Doritaenopsis (Orchid)	SC	Sucrose, 0.1 M + ABA, 1 mg l^{-1} (7d) 25 °C	2M glycerol + 0.4M sucrose, 15 min, 25°C + PVS2, 1h, 0°C		64	Tsukazaki et al. 2000
Ipomoea batatas	CC	multistep sucrose preculture [3]	dehydration, 2.5h, SG		89	Blakesley et al. 1996
Iris nigricans	CSE	sucrose, 0.75 M(3d), 22 °C +0.75 M (1d), 30 °C	encapsulation dehydration, 4h, in LF		60	Shibli 2000
Oryza sativa	SC	sucrose, 60 g l^{-1} (10d) + sorbitol, 0.4 M (1d)	25% PVS2, 10 min + 100% PVS2		45	Huang et al. 1995
Oryza sativa 'Taipei 309'	SC	mannitol, 60 g l^{-1} (3d)	CM[6], 1h, 0°C		NR	Lynch et al. 1995

(EG, ethylene glycol; LF, laminar flow; LN, liquid nitrogen; NR, not reported; RT,; SG, silica gel); [1] CC, clumps of embryogenic callus (developmental stage not reported); CSE, clumps or isolated somatic embryos at the cotyledonary stage; SC, cells from suspension cultures; SE, isolated somatic embryos.[2] Slow cooling was achieved by incubating the samples in a freezer at -70 or -80°C before plunging them into LN. [3] Slow cooling: 1°C min^{-1} to -10°C; -10°C for 15 min;1°C min^{-1} to -40°C; -40°C for 60 min. [4] Multistep sucrose preculture: 0.1M (3d), 0.4M (3d), 0.7M (2d). [5] PVS: 22% (w/v) glycerol + 15% (w/v) ethylene glycol + 15% (w/v) propylene glycol + 7% DMSO. [6] CM, Cryoprotectant Mixture: 2M sucrose + 1M DMSO + 1M glycerol + 0.09M L-proline in H_2O.

9.1.2 Direct Immersion in Liquid Nitrogen

Recent advances in cryotechnology have opened the way to a simplified approach for the long-term storage of embryogenic callus clumps, allowing direct immersion in LN. Vitrification-based and encapsulation-dehydration protocols were developed for embryogenic cultures from various important plant species (*Citrus* spp., *Olea europaea*, *Fraxinus* spp., *Quercus* spp., *Oryza sativa*).

A summary of cryopreservation protocols developed for embryogenic cultures is reported in Table 9.1 (conifers), Table 9.2 (hardwoods, shrubs and palm trees) and Table 9.3 (crops and cut-flowers). Eleven successful protocols are also described in detail at the end of the chapter. Protocols were selected to cover embryogenic cultures from several groups of plants as well as the full range of techniques available, i.e., conifers (*Picea mariana*, *Pinus pinaster*), hardwoods (*Aesculus hippocastanum*, *Quercus suber*), fruit (*Prunus avium*, *Citrus* spp.) and palm (banana) trees, flower (*Cyclamen persicum*) and crop (*Saccharum officinarum*, *Ipomoea batatas*) species.

9.2 Application of Cryopreservation to Embryogenic Cultures

Cryopreservation of embryogenic cultures is a relatively recent application of cryogenic technology. Only in the early 1990s were the first examples of detailed and successful protocols published. They described the cryopreservation of embryogenic suspension cultures of *Musa* spp. (Panis et al. 1990) and *Citrus sinensis* (Sakai et al. 1990). Sakai's work on *C. sinensis* reported the development of Plant Vitrification Solution no. 2 (PVS2) to induce cell vitrification, a mixture of cryoprotectants which became a milestone in the history of plant cryopreservation. It was soon evident that the possibility of storing valuable embryogenic culture lines in LN could allow the long-term maintenance of their embryogenic potential, making them available only when necessary and avoiding the drawbacks resulting from repeated subculturing (contamination, genetic alteration, decrease of callus morphogenicity).

Cryopreservation of embryogenic cultures is a multi-step process, including preconditioning, preculturing, cryoprotectant treatments, storage in LN, rewarming and recovery of callus, all of which play an important role in achieving a successful protocol. An error in any of these steps, or the choice of an embryogenic culture line at an unsuitable stage of development,

may prevent the recovery of proliferative activity of the stored callus clumps. The technical challenge lies in optimizing each of these parameters for embryogenic cultures from a wide diversity of plant species.

9.2.1 Developmental Stage

The growth stage of embryogenic cells of suspension cultures can strongly influence survival after cryopreservation. In *Citrus* suspension cultures, embryogenic cells at the early lag phase and at the stationary phase are large and highly vacuolated, thus having high water content. On the contrary, rapidly growing cells in the late lag and early to mid-exponential phase are small and contain dense cytoplasm, with fewer vacuoles and relatively low water content (Pérez 2000). Cells characterized by dense cytoplasm and few vacuoles have low water content, hence tolerating cryopreservation treatments much better. Lainé et al. (1992) reported that, among the cryopreserved embryogenic cell suspensions of *Pinus caribaea*, only rapidly growing cultures could be recovered successfully after cryostorage. However, in contrast to the above general statement, Find et al. (1998) reported that embryogenic suspension cultures of *Picea abies* and *Picea sitchensis* showed maximum survival when cells were harvested at the stationary growth phase.

Embryogenic callus cultures of conifers are generally induced from the suspensor cells of immature zygotic embryos, cultured on 2,4-D-containing gelled medium. During proliferation the lines contain groups of densely cytoplasmic embryonic cells (the heads of the embryo primordia), subtended by elongated highly vacuolated suspensor cells (Stasolla and Yeung 2003). The suspensor cells have high water content and usually die during freezing. The embryonic head cells have a dense cytoplasm and, following appropriate cryoprotective treatments, they survive storage in LN (Häggman et al. 2000).

The developmental stage of embryogenic callus cultured in steady state is not well investigated for hardwood and herbaceous species, in terms of recovery from cryostorage, and the results are not consistent. Valladares et al. (2004) used a simple vitrification protocol to compare the post-thaw survival of globular, early-torpedo, and cotyledonary embryos of *Quercus suber*, and 90% of the embryogenic clumps of globular embryos could be successfully cryopreserved. However, contrasting results were obtained when isolated globular embryos were used. The recovery percentage achieved was the highest with one embryogenic line, and the lowest with the other, hence far from providing clear information on the aspect of better adaptability to cryostorage of somatic embryos at different

stages of development. Working with *Citrus sinensis*, Marin et al. (1993) chose only the smallest (0.5–1 mm) morphogenetic structures of the embryogenic callus culture, consisting of heart-shaped embryos which had the highest survival rate after cryostorage. Callus clumps of *Aesculus hippocastanum*, containing a prevalence of embryogenic masses at an advanced stage of somatic embryo maturation (i.e., the torpedo stage), gave optimum regrowth of healthy and proliferating embryogenic callus, in this performing better than callus clumps prevalently made up of globular, heart shaped and cotyledonary somatic embryos (Lambardi et al. 2005). In *Iris nigricans*, 2–4 mm somatic embryos had higher survival than smaller (1–2 mm) or larger (4–6 mm) ones (Shibli 2000). Medium and large somatic embryos of *Cucumis melo* showed higher survival than smaller ones (Shimonishi et al. 2000b).

9.2.2 Preconditioning of Embryogenic Callus

With few exceptions, cryopreservation protocols for embryogenic cultures report an initial preconditioning phase, during which the samples undergo moderate dehydration on media containing sugars (e.g., sucrose, glucose) or sugar alcohols (sorbitol). During preconditioning, callus cells are subjected to mild osmotic stress which induces metabolic changes and enhances chilling and desiccation tolerance. In addition the absorbed sugar stabilizes the membranes by replacing water and forming hydrogen bonds with the phospholipids (Turner et al. 2001). However, if applied at relatively high concentrations (i.e., over 1.0 M), sugar itself may become toxic to the mass of cells, particularly when the duration of the preconditioning period is prolonged (Wu et al. 2003). A progressive exposure of explants to increasing sugar concentrations, instead of a direct exposure to a large dose, plays a key role in avoiding toxic effects (Bhatti et al. 1997; Find et al. 1998; Chmielarz et al. 2005).

9.2.3 Preculture

Because of their high water content, cells of embryogenic cultures are extremely sensitive to cryoinjury. Often, sugar preconditioning alone does not dehydrate callus samples sufficiently to withstand the freezing process, and additional cryoprotection is required. Protocols include one or more additional preculture treatments which precede either slow cooling to –40°C, followed by storage in LN, or direct immersion in LN.

9.2.4 Cryoprotectants

Treatment with cryoprotectants is typical of controlled cooling procedures. Two kinds of cryoprotectants are used for this purpose, those that penetrate the plasma membrane (e.g., DMSO and glycerol), and the non-penetrating compounds (e.g. polyethylene glycol (PEG) and sucrose). DMSO alone, at a concentration of 5–10%, is often reported as the only cryoprotectant (Martinez-Montero et al. 1998; Find et al. 1998; Pérez et al. 1999). Other authors prefer combinations of cryoprotectants at lower concentrations, considering this approach more beneficial than a single cryoprotectant at high concentration. For instance, in *Picea abies* and *Pinus taeda* (Gupta et al. 1987), *Pinus sylvestris* (Häggman et al. 1998) and *Abies cephalonica* (Aronen et al. 1999) the replacement of DMSO with a mixture of PEG, glucose and DMSO resulted in better recovery of proliferating embryogenic callus after cryopreservation.

A variety of cryoprotectant treatments are used when the direct immersion of callus clumps in LN is pursued. The most common approach is the treatment of specimens with PVS2 for periods ranging from 30 to 90 min. Due to possible toxic effects of the PVS2 solution which can compromise embryogenic cell viability, the incubation time is a fundamental parameter which must always be optimized. Using the encapsulation-vitrification technique, longer incubation times with PVS2 are required. In *Olea europaea* (cv Nabali) encapsulated callus clumps were incubated in PVS2 at 0°C for 3 h before the beads were directly plunged into LN (Shibli and Al-Juboory 2000). As an alternative to the use of the PVS2 vitrification solution, physical dehydration is another common approach to the direct immersion in LN of naked or encapsulated callus clumps. The dehydration to which the explants are subjected depends on the method applied, ranging from 4 (*Iris nigricans*; Shibli 2000) to 16 h (*Manihot esculenta*; Danso and Ford-Lloyd 2004) when dehydration is carried out under the sterile air flow of a laminar cabinet, or from 1 (*Fraxinus angustifolia*; Tonon et al. 2001) to 16 hours (*Elaeis guineensis*; Dumet et al. 2000) with silica gel.

9.2.5 Cooling Rates

Reports of cryopreservation protocols based on controlled rate cooling show that cooling at 0.2–0.5°C min^{-1} to about –40°C is effective in the protection of embryogenic cultures of a large variety of plant species from intracellular ice formation (Tables 9.1–9.3). Slow cooling of specimens is generally obtained by using a programmable freezer. However, some authors (Martinez-Montero et al. 1998; Ford et al. 2000) report the use of

Nalgene Freezing Containers, where a cooling rate of 1°C min^{-1} is achieved with the use of chilled isopropyl alcohol in a standard freezer. As an alternative, incubating the embryogenic lines at –20°C (Winkelmann et al. 2004) or at –70/–80°C (Jain et al. 1996; Ford et al. 2000; Hargreaves et al. 2002; Marum et al. 2004) for 1 to 24 h, before plunging the samples into LN, also drops the temperature gradually enough to prevent cryoinjury.

9.2.6 Warming and Rinsing

Cryoinjury can occur not only during the ultra-freezing process, but also during sample thawing, due to recrystallization, (Meryman and Williams 1985). Recrystallization can be avoided by rapid thawing of samples in a water bath at 35 to 45°C. In *Citrus* spp. warming at room temperature is as effective as fast warming for recovery of viable isolated somatic embryos (Marin and Duran-Vila 1988; González-Arnao et al. 2003).

Cryoprotective solutions are rapidly drained off the cryovials to prevent prolonged exposure. Cultures are rinsed with a hormone-free liquid medium containing a high concentration of an osmoregulating agent (generally, 1.2 M sucrose) and finally plated on an appropriate medium. Rapid removal of the cryoprotective solutions may also cause deplasmolysis injuries to the cells (Finkle and Ulrich 1982; Wang et al. 1994). This can be avoided by gently transferring embryogenic samples on to a filter paper placed on a semi-solid culture medium that is replaced after a few days (Cyr et al. 1994; Marum et al. 2004; Valladares et al. 2004).

9.3 Genetic Stability

Some peculiarities of cryogenic technology (e.g., the blocked metabolism of cells and the absence of subcultures) reduce the risks of genetic and epigenetic alterations to a minimum. Presently there is no clear evidence that morphological, cytological or genetic alterations are a consequence of cryopreservation (Harding 2004). Following cryopreservation, embryogenic cultures of *Picea glauca engelmanni* derived from 12 full-sib families, were examined using a single DNA fingerprinting probe, and no genetic variation was evidenced (Cyr et al. 1994). Similarly, no sign of somaclonal variation was detected in embryogenic cultures of *Citrus sinensis* (Marin et al. 1993), *Picea glauca* (Park et al. 1994), *Picea mariana* (Isabel et al. 1993), or *Pinus sylvestris* (Häggman et al. 1998) after the lines were recovered from storage in LN.

Some threats to genetic stability arise from the common practice of using DMSO as cryoprotectant at concentrations of up to 10%. However, in an embryogenic culture of *Abies cephalonica* (Aronen et al. 1999), intraclonal genetic variation detected in the unfrozen embryogenic line (17% of the RAPD profiles) was attributed to a 30-min pretreatment of callus clumps with 10% DMSO. However, as DMSO-treated cryopreserved lines did not show any signs of instability, it was hypothesized that cryopreservation indirectly removed the mutagenic potential of DMSO by eliminating a high proportion of cells bearing genetic alterations.

De Verno et al. (1999) found genetic alterations in embryogenic cultures of *Picea glauca*, 2 and 12 months after they were re-established in culture after cryopreservation. However, as plantlets regenerated soon after callus recovery from cryostorage did not evidence any sign of genetic variation, the authors concluded that the observed genetic instability was presumably a consequence of repeated subculturing rather than of the cryopreservation procedure itself.

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9.4 Protocols

9.4.1 Controlled Rate Cooling of Pinus pinaster *Embryogenic Cultures*

(Marum et al. 2004)

Checklist for slow cooling approach
Items needed for cryopreservation

1. Forceps/tools
2. Sterile filter paper discs (55 mm)
3. Transfer pipette, pipette
4. Ice, LN
5. Cryovials and markers
6. Cryobox or cryocane for immersion of the cryovials in LN
7. Nalgene Freezing Container (Sigma-Aldrich) and isopropanol

Prepare in advance

1. Embryogenic cell masses, harvested 7 days after transfer to fresh proliferation medium and suspended in 20 ml hormone-free medium (corresponding to a suspension density of 150 mg ml^{-1}) inside a 50 ml flask
2. Nalgene Freezing Container filled with isopropyl alcohol and kept at –80°C for 24 h
3. Autoclaved filter paper discs
4. Filter sterilized maltose stock solution (4 M) in liquid MS (Murashige and Skoog 1962) medium
5. PSD cryoprotectant: 10% (w/v) PEG (polyethylene glycol) 4000 + 10% (w/v) sucrose + 10% (v/v) DMSO in liquid proliferation medium, filter sterilized
6. Proliferation medium: MS medium with 200 mg l^{-1} casein hydrolysate, 400 mg l^{-1} glutamine, 10 mg l^{-1} pantothenic acid, 20 g l^{-1} sucrose, 0.7% agar and 5 μM 2,4-D in Petri dishes.

The procedure

1. Carefully disaggregate the embryogenic masses with a transfer pipette and vigorously shake the flasks to break up into a fine suspension.
2. Add maltose stock solution drop wise over a period of 30 min to a final concentration of 0.2 M and incubate on a rotary shaker at 100 rpm in the dark for 24 h.
3. Add maltose stock solution drop wise over 30 min to a final concentration of 0.4 M and incubate on a rotary shaker at 100 rpm in the dark for an additional 24 h.
4. Add PSD cryoprotectant drop wise over a period of 30 min to a final concentration of 5% of the components (i.e., in a 1:1 ratio with the suspension culture).
5. Dispend aliquots of 1.8 ml of the pretreated embryogenic suspension into cryovials.
6. Transfer the cryovials to the Nalgene Freezing Container filled with isopropyl alcohol, previously kept at –80°C for 24 h.
7. Place the container at –80°C and hold for 24 h.
8. After 24 h, directly plunge the cryovials into LN.
9. Transfer the cryovials to a 45°C water bath and hold until the samples thaw completely, and then transfer to an ice bath.
10. Plate the contents of the cryovial on an autoclaved filter paper disc on proliferation medium in a 90 mm Petri dish and incubate in the dark at 22±2°C.
11. After 1 h, transfer the filter paper with the suspension culture to fresh medium and incubate for 18–24 h in the dark at 22±2°C.
12. Transfer the filter paper with the suspension culture to fresh medium again and subculture at 2-week intervals.

9.4.2 Vitrification of Embryogenic Cultures of Picea mariana

(Touchell et al. 2002)

Checklist for vitrification
Day 1: Items needed for preculture

1. Forceps/tools
2. Preculture medium

Day 3: Items needed for cryopreservation

1. Forceps/tools
2. Sterile pipettes or syringes for solutions (one for each)
3. 1.2 ml cryovials and markers
4. Nalgene® Labtop Cooler (0°C model) or ice
5. PVS2 (modified) and rinsing solution
6. Embryogenesis induction medium for recovery

Prepare in advance

1. Preculture medium: Semi-solid LM medium (Litvay et al. 1981), containing 0.8 M sorbitol and 3 g l^{-1} Phytagel in Petri dishes
2. PVS2 (modified): 30% (v/v) glycerol, 15% (v/v) ethylene glycol, 15% (v/v) DMSO in ½ strength LM medium
3. Nalgene Labtop Cooler (0°C model) placed at –20°C to chill
4. Rinsing solution: Liquid ½ strength LM with 1.0 M sucrose
5. Embryogenesis induction medium: ½ strength LM with 1 g l^{-1} casein hydrolysate, 500 mg l^{-1} glutamine, 9.1 µM 2,4-D, 4.5 µM 6-benzyladenine, 30 µM sucrose, 3 g l^{-1} Phytagel

The procedure
This is a one-day procedure, following a 48 h preculture.

1. Transfer the embryogenic masses (3 mm diameter) onto preculture medium and incubate for 48 h.
2. Using a sterile pipette or a syringe, put 1 ml of PVS2 (modified) into a cryovial. The ratio of PVS2 to tissue should be about 10:1.
3. Place 10 embryogenic masses in the cryovial. Place the cryovials in Nalgene Cooler (0°C) for 30 min.
4. Directly immerse the cryovials in LN.
5. Warm the samples in a 40°C water bath for 2 min.
6. Drain off the PVS2 solution and add rinsing solution three times for 5 min each.
7. Recover the embryogenic masses on induction medium in the dark at 25°C and subculture at 2–3 week intervals.

9.4.3 Controlled Rate Cooling of Embryogenic Callus of Citrus *ssp.*

(Pérez et al. 1997)

Checklist for cryopreservation
Items needed for cryopreservation

1. Forceps/tools
2. Sterile pipettes for solutions (one for each)
3. 2 ml cryovials and markers
4. Cryobox or cryocane to hold the cryovials
5. Cooling bath and pure methanol as coolant
6. Cryoprotective solution
7. Rinsing solution
8. Solid MS medium for recovery

Prepare in advance

1. Cryoprotective solution: Liquid MS (Murashige and Skoog 1962) with 10% (v/v) DMSO
2. Rinsing solution: Liquid MS
3. Solid basal MS medium for recovery (7 g l^{-1} agar)
4. Cooling bath containing pure methanol

The procedure

1. Add 1.8 ml cryoprotective solution to the cryovials.
2. Transfer the cell cultures (150–200 mg of loose cells from callus) to the cryovials containing liquid medium and DMSO. Resuspend and hold at 4°C for 30 min.
3. Place the cryovials in cooling bath containing pure methanol (cooling rate of about 0.5°C min^{-1}) to –40°C, then immerse in LN.
4. Rewarming: immerse the cryovials in 37°C water bath for 5 min.
5. Remove the cryoprotection solution from the cryovials.
6. Rinse cells 3 times with 1.8 ml rinsing solution.
7. Transfer the cells to fresh-gelled MS medium and keep at 27±1°C and a 16 h photoperiod (43µmol m^{-2} s^{-1}).

9.4.4 Encapsulation Dehydration of Citrus Somatic Embryos

(Dereuddre et al. 1990) Adapted by M.T. Gonzalez-Arnao and N. Duran-Vila (Gonzalez-Arnao et al. 2003).

Checklist for Alginate Bead Cryopreservation
Day 1: Items needed

1. Tools
2. Sterile Petri dishes
3. MS medium in Petri dishes to hold somatic embryos.
4. *In vitro* cultured ovules
5. Alginate solution: 3% alginate, low viscosity, in 0.4 M sucrose calcium-free MS (Murashige and Skoog 1962) medium
6. Calcium chloride solution: 100 mM in MS medium
7. Sterile 250 ml beakers for calcium chloride solution
8. Small sterile beakers for alginate solution
9. Sterile cut pipette tips to make beads (Tip capacity 100-1000µl, approximate cut diameter of the tips: 4 mm depending on the size of tissues)
10. Sterile strainers
11. Preculture medium: MS with 0.75 M sucrose in 125 ml flasks

Day 2: Items needed to dry beads and cryopreserve

1. Sterile strainer
2. Sterile 250 ml beaker
3. Dry filter paper for draining beads
4. Sterile Petri dishes for drying beads (one for each 15 beads)
5. Tools and sterile Petri dishes
6. Cryovials (1.8 or 2 ml) and markers
7. Petri dishes with recovery medium
8. Dewar flask and LN

Prepare in advance

1. Somatic embryos induced *in vitro* from ovules isolated from immature fruits and maintained on MS medium with 0.7% agar under a 16 h light/8 h dark photoperiod at 25°C for 16 h
2. MS medium with 0.7% agar in Petri dishes
3. Alginate solution: It is very difficult to dissolve alginate, so heat medium and add alginate slowly under agitation. Do not boil the solution to dissolve alginate.
4. Calcium chloride solution (100 mM) (in a flask)

5. Sterile pipettes with cut tips for forming beads
6. Sterile 50 ml beaker for alginate solution, sterile 250 ml beakers for forming beads in calcium chloride and for draining beads
7. Preculture medium (75 ml in 125 ml flasks)
8. Sterile 250 ml flask for 0.75 M sucrose solution
9. Sterile sieves or tea strainers for removing beads from solutions
10. Petri dishes with sterile filter paper for draining beads
11. Sterile Petri dishes for holding beads during dehydration
12. Sterile cryovials

The procedure

This is a 2-day procedure once the dissection of embryos is accomplished.

1. Dissect embryos on MS medium plates.
2. Suspend tissues in alginate solution in a small sterile beaker.
3. Mix apices with alginate solution and using a pipette pick up a single embryo and drop into a 250 ml beaker of 100 mM calcium chloride to make beads. Avoid air bubbles in the alginate.
4. Leave the beads in the solution for 20 min to firm up.
5. Place beads in 0.75 M sucrose MS in 125 ml flasks on a shaker for 18–20 h.
6. Drain beads and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
7. Place surface-dried beads in open sterile Petri dishes and expose them to the air current of the laminar flow cabinet for ~4–5 h (20–25% moisture content in the beads, fresh weight basis). They should not touch each other or they will not dry properly. There should be no extra moisture in the dish.
8. Place 10 beads in a cryovial and plunge in LN one at a time, hold under the surface for 30 s, or place on cane and immerse.
9. Warm slowly by placing the encapsulated apices in open Petri dishes and exposing to the laminar flow for 2 min. Place beads on semi-solid medium in Petri dishes.
10. For regrowth, place dishes in the dark for 1 week and then transfer to standard growth conditions.

Possible problems

Some plants are very sensitive to sucrose. Controls should include:

(1) dissection, (2) encapsulated embryos, (3) encapsulated embryos after sucrose treatment (4) Dried beads at 20–25% moisture content.

9.4.5 Controlled Rate Cooling of Banana Embryogenic Cultures

(Panis et al. 1990)

Checklist
Items needed for cryopreservation

1. Graduated centrifuge tube
2. 2 ml cryovials and markers
3. Cryobox or cryocane for storage
4. Teflon tape for sealing cryovials
5. Sterile pipettes for replacing culture medium with cryoprotectant
6. Automatic pipette pump
7. Beaker and sterile water at 40°C for thawing
8. Methanol and methanol bath for the slow cooling of cells
9. Thermometer in the methanol bath
10. Cryoprotectant
11. Recovery medium in 90 mm Petri dish

Prepare in advance

1. Methanol bath
2. Cryoprotectant: 180 g l^{-1} sucrose and 7.5% DMSO in liquid MS
3. Growth medium: ½ MS (Murashige and Skoog 1962), 10 mg l^{-1} ascorbic acid, 5 µM 2,4-D, 1 µM zeatin
4. Recovery medium: MS, 10 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 1 µM 6-benzyladenine (BA), 2 g l^{-1} Gelrite

The procedure

1. Settle 10-day old embryogenic suspension cells in a graduated centrifuge tube and remove culture medium.
2. Add cryoprotectant gradually (over a period of 1 h) at room temperature until a final settled cell volume of 30% is obtained.
3. Transfer 1.5 ml to 2 ml cryotubes and seal with Teflon tape.
4. Cool cryovials in a stirred methanol bath (1°C min) to –7.5°C.
5. Immerse cryovials in LN for 3 s to initiate ice crystallization, then continue to cool cryovials to –40°C and hold 30 min.
6. Plunge the cryovials into LN.
7. Rewarm rapidly in a beaker of 40°C sterile water for 1.5 min.
8. Plate cells recovery medium and place in the dark for 1 week before moving to standard growth room conditions.

9.4.6 Vitrification of Aesculus hippocastanum *Embryogenic Callus*

(Lambardi et al. 2005)

Checklist for vitrification
Items needed for cryopreservation

1. Forceps, tools, sterile Petri dishes, cell dissociation sieve
2. Sterile pipettes or syringes for solutions (one for each)
3. 2 ml cryovials and markers, cryobox or cryocane
4. Nalgene Labtop Cooler (0°C model) (or ice)

Prepare in advance

1. Osmotic loading solution: 2 M glycerol and 0.4 M sucrose in MS (Murashige and Skoog 1962) medium
2. PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO (v/v), 0.4 M sucrose in MS medium
3. Rinsing solution (Liquid MS with 1.2 M sucrose)
4. Proliferation medium: MS medium with 50 mg l^{-1} arginine, 500 mg l^{-1} proline, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D)
5. Embryogenic cultures (containing mostly torpedo stage embryos), precultured on proliferation medium and maintained for 5 days at 4°C in the dark for cold acclimation.
6. Nalgene Labtop Cooler (0°C) placed at –20°C (or an ice bath)

The procedure

This is a 1-day procedure following cold acclimation of the cultures.

1. Drain the callus and transfer to the 2 ml cryovials (5 samples, 100–180 mg each).
2. Add osmotic loading solution (1 ml), hold for 30 min at 25°C.
3. Remove the loading solution, add PVS2 and hold for 90 min in the cooler or on ice to maintain the solution at about 0°C.
4. Remove the PVS2 and add 0.6 ml of fresh PVS2.
5. Immerse the cryovials directly in LN.
6. Rapidly warm in a 45°C water bath for 50 s.
7. Drain PVS2, add rinsing solution and hold 20 min at 25°C.
8. Recover the callus samples by filtration with a sterile sieve.
9. Plate the samples on the proliferation medium and maintain in the dark at 23±1°C for 3 weeks.
10. At 3 weeks transfer the embryogenic callus to maturation conditions (hormone free MS medium, 16 h photoperiod) to induce the completion of somatic embryo development.

9.4.7 Cryopreservation of Embryogenic Callus of Prunus avium *by Dehydration*

(Grenier-de March et al. 2005)

Checklist for dehydration
Items needed for sucrose preconditioning and rehydration

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. MS medium + 0.25 M sucrose (day 1 and 9) in Petri dishes
4. MS medium + 0.50 M sucrose (day 2 and 9) in Petri dishes
5. MS medium + 0.75 M sucrose (day 3 and 8) in Petri dishes
6. MS medium + 1.0 M sucrose (day 5 and 8) in Petri dishes
7. Embryogenic tissues subcultured 3 weeks before cryopreservation

Items needed for dehydration in laminar air flow

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. Sterile Petri dishes with air-vented lids (100 × 20 mm) (Greiner)

Items needed for cryopreservation

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. 1.8 ml cryovials and markers
4. Cryobox or cryocane for storage of the cryovials in LN
5. Ice

Prepare in advance

1. Rinsing solution: liquid MS (Murashige and Skoog 1962) medium with 1.2 M sucrose
2. Preconditioning and rehydration medium: MS (0.5% phytagel) with 0.25 M sucrose, 0.50 M sucrose, 0.75 M sucrose, or 1.0 M sucrose in Petri dishes
3. Multiplication medium: MS salts, Morel vitamins (Morel and Wetmore 1951), 500 mg l^{-1} casein hydrolysate, 88 µM sucrose, 0.2% (w/v) phytagel, 0.54 µM NAA (naphthalene acetic acid), 0.46 µM KIN (kinetin), 0.44 µM BA (6-benzyladenine)

The procedure

This is an 11-day procedure with 7-day step-wise sucrose preconditioning and a 3-day rehydration treatment.

1. Divide the embryogenic callus (with somatic embryos at the globular stage) into clumps of 2–3 mm.
2. Culture the clumps on 0.25 M sucrose MS medium at 23°C for 1 day (20 clumps each per 10 mm Petri dish).
3. Transfer to 0.5 M sucrose MS medium for 1 day.
4. Transfer to 0.75 M sucrose MS for 2 days.
5. Transfer 1.0 M sucrose MS for 3 days.
6. Weigh an empty sterile Petri dish and record the value.
7. Transfer the clumps into the weighed Petri dish (20 clumps per Petri dish) and weigh again to calculate the fresh weight of the clumps.
8. Transfer the clumps to a sterile air-vented Petri dish and desiccate at 25°C under the sterile air of a laminar air flow cabinet.
9. Weigh the samples (with a Petri dish that was previously used for the measurement of the fresh weight) every hour and continue desiccation until their weight reaches 50–60% of the initial fresh weight.
10. After desiccation, place 20 clumps of embryogenic callus in each cryovial filled with LN.
11. Plunge the cryovials into LN.
12. Warm the 1.2 M sucrose solution in a 40°C water bath and pour into a small Petri dish (40 × 12 mm).
13. Take out the cryovials from LN and pour directly into a Petri dish containing warm rinsing solution and hold for 1 min.
14. To avoid overheating transfer the Petri dish to ice for 10 min.
15. For rehydration, transfer the samples to 1.0 M sucrose MS medium in the dark at 25°C for 12 h.
16. Transfer 0.75 M sucrose MS plates in the dark at 25°C for 12 h.
17. Transfer to 0.5 M sucrose MS plates in the dark at 25°C for 12 h.
18. Transfer to 0.25 M sucrose MS plates n the dark at 25°C for 48 h.
19. Transfer to multiplication medium in the dark at 25±1°C. Subculture at 3-week intervals and grow in normal growth room conditions.

9.4.8 Vitrification of Embryogenic Cultures of Chestnut, Common Oak and Cork Oak

By AM Vieitez based on Corredoira et al. 2004; Martinez et al. 2003; Valladares et al. 2004

Items needed

1. Tools
2. Sterile filter paper discs for draining embryo clumps
3. Cryovials and markers
4. Sterile pipettes for replacing solutions from cryovials
5. Petri dishes with sterile filter paper discs for draining
6. Specific recovery medium for each of the three species

Culture information and culture media

1. Chestnut embryogenic lines maintained by repetitive or secondary embryogenesis with subculture at 6-week intervals on MS (Murashige and Skoog, 1962) with half-strength macronutrients, 3 mM glutamine, 0.1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 0.09 M sucrose, 7 g l^{-1} agar.
2. Oak embryogenic lines maintained by secondary embryogenesis with subculture at 5-week intervals on MS, 0.1 mg l^{-1} BA, 0.05 mg l^{-1} NAA, 500 mg l^{-1} casein hydrolysate, 0.09 M sucrose, 6 g l^{-1} agar.
3. Cork oak embryogenic lines maintained by secondary embryogenesis with subculture at monthly intervals on Schenk and Hildebrandt (1972) macronutrients, MS micronutrients, vitamins and Fe-EDTA, 0.09 M sucrose and without plant growth regulators, 6 g l^{-1} agar.

Prepare in advance

1. Preculture medium: (the specific proliferation media defined above without plant growth regulators) with 0.3 M sucrose and 0.7% agar for preculture of embryo clumps (in Petri dishes)
2. PVS2: 30% glycerol, 15% ethylene glycol, 15% dimethylsulfoxide, in liquid basal MS medium with 0.4 M sucrose (50 ml in 100 ml flasks)
3. Embryogenic cultures of for isolation of somatic embryo clumps
4. Rinsing medium: Liquid MS with 1.2 M sucrose (50 ml in 100 ml flasks)
5. Recovery medium in Petri dishes: The corresponding proliferation medium defined for chestnut, oak and cork oak.

The procedure

The procedure for chestnut, oak and cork oak is very similar, and specific differences will be pointed out.

1. Isolate clumps (4–6 mg) of globular and heart-shaped embryos from stock embryogenic cultures, 3–4 weeks after the last subculture.
2. Preculture somatic embryo clumps for 3 days on 0.3 M sucrose MS medium.
3. After preculture, place embryo clumps in 2 ml cryovials (10 clumps to a vial) with 1.8 ml of ice-cold PVS2. Time of exposure at 0°C: 60 min chestnut, 30–60 min oak, and 60 min cork oak.
4. Resuspend the embryogenic clumps in 0.6 ml of fresh PVS2, place the cryovials on cane and plunge in LN.
5. To warm, immerse in a 40°C water bath for 2 min.
6. Drain off the PVS2 and replace it twice with rinsing medium for 10 min each time.
7. Place the embryogenic clumps of each cryovial onto sterilized filter paper discs and transfer to recovery medium in a Petri dish.
8. After 24 h transfer the samples to fresh recovery medium in Petri dishes without paper discs.
9. Determine somatic embryo recovery by assessing the cultures for 6 weeks (chestnut, cork oak) or 8 weeks (oak) for the frequencies of embryo clumps showing secondary embryogenesis.

Post-cryostorage embryogenesis resumption rates were 57–92% for 10 oak embryogenic lines, 88–93% for three cork oak lines and 68–70% for three chestnut lines.

Notes

PVS2 was tolerated well by chestnut, oak and cork oak embryogenic cultures. The developmental stage of somatic embryos to be cryopreserved should be considered. Clumps formed by 2–3 globular or heart-shaped embryos give a better response for withstanding storage in LN than the more differentiated (cotyledonary) embryos.

9.4.9 Controlled Rate Cooling of Cyclamen persicum *Suspension Cultures*

(Winkelmann et al. 2004)

Checklist for controlled cooling approach
Day 1: Items needed for preculture

1. 7–10 day old suspension cultures
2. Forceps/tools
3. Sterile plastic pipettes
4. Pipette pump
5. Preculture medium

Day 3: Items needed for pretreatment and cryopreservation

1. Forceps/tools
2. Sterile plastic pipettes for replacing solutions
3. Pipette pump
4. 15 ml centrifuge tubes
5. Cryovials (pre-cooled at –20°) and markers
6. Cryobox or cryocane to plunge the cryovials into LN
7. 0.2 µm filters to sterilize DMSO
8. Syringes to sterilize DMSO

Prepare in advance

1. 7–10 day old embryogenic suspension cells, cultured on suspension medium in Erlenmeyer flasks. It is important to use 7–10 day old cells as the cells are in the linear growth phase.
2. Suspension medium: ½ strength MS salts, full-strength Fe-EDTA, 250 mg l^{-1} peptone, 2 g l^{-1}glucose, 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2iP
3. Cryoprotectant: Liquid MS (Murashige and Skoog 1962) medium with 0.6 M sucrose and 10% DMSO, filter sterilized
4. Rinsing solution: Liquid MS medium with 0.18 M sucrose, 2.0 mg l^{-1} 2,4-D and 0.8 mg l^{-1} 2iP.
5. Recovery medium: Sterile filter paper on Petri dish of ½ strength MS medium with 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2-isopentenyl adenine (2iP) and 3.7 g l^{-1} Gelrite.
6. Preculture medium: suspension medium with 0.6 M sucrose
7. Cool empty cryovials to –20°C.
8. Nalgene® Freezing Container (Sigma-Aldrich) filled with isopropyl alcohol and kept at –80°C for 24 h.

The procedure

1. 7–10 days after subculture, allow the embryogenic suspension cells to settle at the bottom of the Erlenmeyer flask.
2. Replace suspension medium with preculture medium with a sterile pipette.
3. Incubate cells for 2 days at 24°C in the dark, shaking at 120 rpm.
4. Replace the preculture medium with cryoprotectant.
5. Incubate for 20 min without shaking, and then shake at 60 rpm for additional 40 min.
6. Divide suspensions into 10 ml portions and pour them into 15 ml centrifuge tubes to settle.
7. Adjust the cell density to 33% sedimented cell volume by removing the surplus supernatant medium.
8. Transfer aliquots of 1 ml to pre-cooled 1.8 ml cryovials.
9. Hold the cryovials for 2 h at –20°C in a freezer and then immerse them in LN.
10. Thaw suspension cells in a 38°C water bath for 3 min and immediately pour cells into a 100 ml flask with 10 ml rinsing medium.
11. Place flasks on a shaker (60 rpm) at 24°C, in the dark for 24 h.
12. Plate 1 ml aliquots on pieces of filter paper on Petri dishes of recovery medium and incubate at 24°C in the dark.
13. After 3 weeks, plate 100 mg samples of callus on differentiation medium (½ strength MS salts, full-strength Fe-EDTA, 250 mg l^{-1} peptone, 2 g l^{-1}glucose, 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2iP, 3.7 g l^{-1} Gelrite)

9.4.10 Encapsulation Dehydration and Controlled Cooling of Embryogenic Callus of Ipomoea batatas

(Bhatti et al. 1997)

Items needed

1. Forceps, tools, sterile Petri dishes (90 mm)
2. Small sterile Petri dishes or beakers for alginate
3. Sterile 250 ml beakers for $CaCl_2$
4. Sterile Pasteur pipettes, sieves or tea strainers
5. Sterile Petri dishes for drying beads
6. 2 ml cryovials and markers, Cryobox or cryocane

Prepare in advance

1. Preculture medium: Petri dishes with 25 ml MS with 0.1 M sucrose, 0.4 M sucrose and 0.7 M sucrose, solidified with 0.7% agar (one of each for each culture)
2. Alginate solution: Liquid MS (Murashige and Skoog 1962) medium without calcium and with 3% (w/v) Na-alginate and 0.1 M sucrose
3. Calcium chloride solution: liquid MS medium with 100 mM $CaCl_2$ and 0.1 M sucrose
4. Recovery medium: MS with 0.1 M sucrose and 0.7% agar

The procedure

This is a 9-day protocol, with an initial 8-day sucrose conditioning.

1. Excise embryogenic masses of 1–2 mm diameter.
2. Suspend in the alginate solution. With a sterile Pasteur pipette pick up an embryogenic mass and some alginate solution and drip it into the calcium chloride solution to make beads. Leave the beads in the solution for 15 min to harden.
3. Transfer beads to 0.1 M sucrose MS and culture for 3 days.
4. Transfer to 0.4 M sucrose MS and culture for 3 days.
5. Transfer to 0.7 M sucrose MS and culture for 2 days.
6. Move beads to an open sterile Petri dish and expose them to sterile airflow of a laminar flow hood for 4 h. Then transfer to 2 ml cryovials.
7. Put cryovials in a programmable freezer (10°C min^{-1} to 0°C; 0.5°C min^{-1} to –40°C). Plunge cryovials into LN. Rewarm in a 38°C water bath for 2 min.
8. Transfer beads to recovery medium and incubate at 25°C in the dark for 48 h. Remove embryogenic masses from the beads and incubate at 25°C in the light (50 µmol m^{-2} s^{-1}).

9.4.11 Controlled Cooling of Embryogenic Callus of Saccharum officinarum

(Martinez-Montero et al. 1998)

Items needed

15–25 day old callus

1. Tools
2. Preculture medium
3. 2 ml cryovials and markers
4. Thin polypropylene plate with holes to insert cryovials
5. Ethanol bath (a commercial alcohol freezing unit could also be used such as Nalgene or "Mr. Frosty").
6. 700 ml ethanol precooled at 0°C
7. Cryoprotectant
8. Recovery medium in Petri dishes

Prepare in advance

1. Ethanol precooled at 0°C
2. Preculture medium: Liquid MS (Murashige and Skoog 1962) medium with 0.5 M sucrose
3. Cryoprotectant: DMSO
4. Recovery medium: MS medium with 50 mg l^{-1} arginine, 500 mg l^{-1} proline, 1 mg l^{-1} 2,4-D and 8 g l^{-1} agar

The procedure

1. Place 15–25 day old callus pieces (3-5 mm diameter) in preculture medium for 1 h at 0°C.
2. Place 6 pieces of the pretreated callus in a 2 ml cryovial with 1.6 ml preculture medium. Add 0.2 ml DMSO drop by drop to the vial over a period of 30 min to reach 10% (v/v).
3. Set up an ethanol bath (700 ml precooled at 0°C) with a polypropylene plate on top to hold vials.
4. Insert cryovials in the ethanol bath and place ethanol bath in a –40°C freezer. The cooling rate is 0.4 to 0.6°C min^{-1}.
5. Induce nucleation at about –10°C by touching the base of each tube to LN. Resume cooling for about 2 h and when –40°C is reached hold the cryovials for 2 h more, then plunge cryovials in LN.
6. Rewarm by plunging into a 40°C water bath.
7. Transfer callus directly to recovery medium without washing.