Chapter 4
The Development of Encapsulation Dehydration

Florent Engelmann[1, 2], Maria-Teresa Gonzalez Arnao[3], Yongjie Wu[4] and Roosevelt Escobar[5]

[1]Institut de recherche pour le développement (IRD), UMR 1097, 911 avenue Agropolis, BP 64501, F-34394 Montpellier cedex 5, France [2]Bioversity International, Via dei Tre Denari 472/a, 00057 Maccarese (Fiumicino), Rome, Italy. [3]Universidad Veracruzana, Facultad de Ciencias Químicas. Prol. OTE. 6, No. 1009, CP 94340, Apartado Postal 215, Orizaba, Veracruz, México. [4]Changli Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences, Hebei 066600, Changli Town, China. [5]Centro Internacional de Agricultura Tropical (CIAT). Km 17 Recta Cali-Palmira, Apartado Aeréo 6713 Cali, Colombia.

4.1 Introduction

The application of cryopreservation to plants is relatively recent as the first report of successful cryopreservation was published by Sakai in 1960 with silver birch twigs, and *in-vitro* cultured flax cells were frozen by Quatrano in 1968. The first protocols developed in the 1980s included pre-treatment with cryoprotectants followed by controlled rate cooling. These protocols were based on freeze-induced dehydration (Sakai 1985; Kartha and Engelmann 1994; Engelmann 1997). Such protocols were applied to numerous species, especially from temperate origin; however, there were cases, particularly for plants of tropical origin, where such controlled cooling protocols did not produce good results (Bagniol et al. 1992; Haskins and Kartha 1980). Further research was thus carried out and at the beginning of the 1990s a set of new, vitrification-based protocols became available (Engelmann 2000, 2003). Vitrification can be defined as the transition

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
© Springer 2008

of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of crystalline ice (Fahy et al. 1984). Among these vitrification techniques a new technique termed encapsulation dehydration was developed for cryopreservation of pear and potato shoot-tips (Dereuddre et al. 1990; Fabre and Dereuddre 1990). This method is based on the technology developed for producing synthetic seeds, i.e. the encapsulation of explants in calcium alginate beads (Redenbaugh et al. 1986). Encapsulated explants are then precultured in liquid medium with a high sucrose concentration and partially desiccated before exposure to liquid nitrogen (LN). Encapsulating the explants allows exposure to extreme treatments including preculture with high sucrose concentrations and desiccation to low moisture contents (MCs) that would be highly damaging or lethal to non-encapsulated samples. Due to the extreme desiccation of explants, most or all freezable water is removed from cells, and vitrification of internal solutes takes place during rapid exposure to LN, thus avoiding lethal intracellular ice crystallization (Engelmann 1997). As a consequence, the whole or a large part of the frozen explant is kept intact after rewarming, which results in high survival, rapid and direct regrowth and reproducible results after cryopreservation (Engelmann 2000).

The encapsulation–dehydration technique was applied to other species from temperate climates including shoot tips of eucalyptus (Poissonnier et al. 1991) and grape (Plessis et al. 1991) and carrot somatic embryos (Dereuddre et al. 1991). Later, it was experimented with plants from tropical origin, notably apices of cassava (Benson et al. 1992), sugarcane (Gonzalez-Arnao et al. 1993a, b; Paulet et al. 1993) and coffee (Mari et al. 1995). The technique was successfully extended to over 70 different plant species (Table 4.1). In this chapter we present and discuss the optimal conditions of the successive steps of an encapsulation–dehydration protocol and make a brief overview of the current application of the technique.

Table 4.1 Plant species whose shoot tips (or other explants – specified in the column Plant species) were cryopreserved using the encapsulation–dehydration technique

Plant species	Reference
Acacia mangium	Sudarmonowati and Rosmithayani 1997
Actinidia chinensis	Wu et al. 2001
Actinidia chinensis	Suzuki et al. 1996
Actinidia spp.	Bachiri et al. 2001
Amygdalus communis L.	Al-Ababneh et al. 2003
Anacamptis morio seeds + fungal symbiont	Wood et al. 2000
Anthirrinium microphyllum	Gonzalez-Benito et al. 1998
Armoracia rusticana hairy root cultures	Hirata et al. 1995

Armoracia rusticana hairy root cultures	Phunchindawan et al. 1997
Auricularia	Hirata et al. 1996
Beta vulgaris	Vandenbussche and De Proft 1996
Brassica napus microspore embryos	Uragami 1993
Camellia sinensis L.O. Kuntze	Aoshima 1997
Catharantus roseus cell suspension	Bachiri et al. 1995
Centaurium rigualii Esteve	Gonzales-Benito and Perez 1997
Ceratopetalum gummiferum	Shatnawi and Johnson 2004
Chichorium intybus	Vandenbussche et al. 1993
Chrysanthemum morifolium	Sakai et al. 2000
Citrus aurentium	Al-Ababneh et al. 2002
Citrus madurensis	Cho et al. 2002
Citrus spp.	Gonzalez-Arnao et al. 1998
Citrus spp. Embryonic axes	Santos and Stushnoff 2002
Citrus spp. Ovules and somatic embryos	Gonzalez-Arnao et al. 2003
Cocos nucifera	Hornung et al. 2001a
Coffea racemosa	Mari et al. 1995
Coffea sessiliflora	Mari et al. 1995
Cosmos atrosanguineus	Wilkinson et al. 1998
Cynodon sp	Reed et al. 2006
Dactylorhiza fuchsii seeds + fungal symbiont	Wood et al. 2000
Daucus carota somatic embryos	Dereuddre et al. 1991
Dianthus caryophyllus L.	Tannoury et al. 1995
Dianthus hybridus cv Sakuranadesiko	Fukai et al. 1994
Digitalis obscura	Sales et al. 2001
Dioscorea alata	Malaurie et al. 1998
D. bulbifera	Malaurie et al. 1998
Dioscorea spp.	Mandal et al. 1996
Ekebergia capensis	Perán et al. 2006
Eucalyptus gunnii	Poissonnier et al. 1991
Eucalyptus sp.	Pâques et al. 1997
Eucalyptus grandis x E. camaldulensis	Blakesley and Kiernan 2001
Fragaria x ananassa	Clavero-Ramirez et al. 2005
Holostemma annulare	Decruse et al. 1999
Iopomea batatas	Pennycooke and Towill 2001
Iris nigricans	Shibli 2000
Laminaria digitata L. gametophytes	Vigneron et al. 1997
Lilium	Matsumoto and Sakai 1995
Lolium sp.	Chang et al. 2000
Malus spp.	Zhao et al. 1999
Malus x domestica Borkh.	Paul et al. 2000; Wu 1999
Manihot esculenta Crantz.	Manrique 2000; Escobar-Pérez 2005
Medicago sativa L. cell suspensions	Shibli et al. 2001
Mentha spicata	Sakai et al. 2000
Microalgae	Hirata et al. 1996
Morus bombysis	Niino et al. 1992
Olea europaea somatic embryos	Shibli and Al-Juboory 2000
Oncidium bifolium seeds and protocorms	Flachsland et al. 2006
Paeonia lactiflora Pall. zygotic embryos	Kim et al. 2004
Pelargonium spp.	Grapin et al. 2003
Polygonium aviculare cell suspension	Swann et al. 1998

Poncirus trifoliata (L.) Raf. *x Citrus sinensis* (L.)	Wang et al. 2002a
Populus tremula x alba	Accart et al. 1993
Primula pubescens	Hornung et al. 2001b
Prunus dulcis	Shatnawi et al. 1999
Pyrus communis	Dereuddre et al. 1990
Pyrus communis cv Beurré Hardy	Scottez et al. 1992
Pyrus syriaca	Tahtamouni and Shibli 1999
Quercus ilex embryonic axes	Gonzales-Benito et al. 1999
Quercus suber embryonic axes	Gonzales-Benito et al. 1999
Ribes spp.	Reed and Yu 1995; Reed et al. 2005
Rhododendron simmsii Planch.	Verleysen et al. 2005
Rubus idaeus L	Wang et al. 2005
Rubus spp.	Gupta and Reed 2006
Saccharum spp.	Gonzalez-Arnao et al. 1993a, b
Saintpaulia ionantha Wendl.	Moges et al. 2004
Salix	Blakesley et al. 1996
Solanum spp.	Fabre and Dereuddre 1990
Solanum tuberosum	Grospietch et al. 1999
Sygysium francissi	Shatnawi et al. 2004
Theobroma cacao L. somatic embryos	Fang et al. 2004
Vaccinium pahalae (Ohelo) cells	Shibli et al. 1998
Vinca minor L. hairy root cultures	Hirata et al. 2002
Vitis spp.	Wang et al. 2000
Vitis vinifera L.	Plessis et al. 1991
Vitis vinifera L.	Zhao et al. 2001
Vitis vinifera L. embryogenic cell suspension	Wang et al. 2002b
Vitis vinifera L. somatic embryos	Miaja et al. 2004
Wasabia japonica	Matsumoto et al. 1995
Zoysia sp.	Chang et al. 2000

4.2 Successive Steps of the Protocol

An encapsulation–dehydration protocol comprises the successive steps listed below. Conditions for each step require optimization to achieve maximal recovery of explants after cryopreservation. The basic steps of preconditioning, preculture, encapsulation, osmoprotection and dehydration before exposure to LN apply in all cases.

4.2.1 Physiological Status of the Plant Material

The physiological status of the plant material is of paramount importance for the success of cryopreservation. Samples are generally excised from actively growing mother plants, thus ensuring that they are composed of actively dividing meristematic cells (Engelmann 1997; Escobar et al.

1997). For example, kiwi are excised 14 days and *Citrus* shoot tips 20 days after the last subculture (Bachiri et al. 2001; Gonzalez-Arnao et al. 1998), i.e. when *in vitro* plantlets are in full growth. On the opposite side, apple shoot tips were sampled on mother plants that were not subcultured for 70 days and grape for 3–4 months (Zhao et al. 1999, 2001). The hypothesis of the authors, to explain the higher recovery noted after cryopreservation of such explants in comparison with those sampled on more frequently subcultured mother plants, is that the moisture content of apices of non-subcultured plants is lower. In the case of somatic embryos, they are cryopreserved at a given developmental stage to optimize recovery. Torpedo stage carrot somatic embryos give the highest recovery (Dereuddre et al. 1991), while with *Citrus* globular, torpedo and heart stage embryos are the material of choice for cryopreservation (Gonzalez-Arnao et al. 2003).

4.2.2 Preconditioning

Preconditioning corresponds to the culture of the mother plants under conditions different from standard ones, which aim at conditioning explants to withstand cryopreservation protocols. Preconditioning can involve various manipulations of the culture conditions such as culturing mother plants at low temperature in the case of cold-tolerant species (Wu et al. 2001; Matsumoto and Sakai 1995; Hirata et al. 1996; Zhao et al. 1999; 2001; Sakai et al. 2000; Dereuddre et al. 1990), or on medium with high sucrose content (Decruse et al. 1999; Grospietch et al. 1999).

4.2.3 Preculture

Preculture corresponds to the culture of the explants for several hours or days after excision and before encapsulation. Sugarcane and coffee apices are cultured overnight on standard solid medium after excision (Paulet et al. 1993; Gonzalez-Arnao et al. 1993a; Mari et al. 1995). Kiwi, strawberry, chrysanthemum and wasabi shoot tips are cultured from one to several days on agar-based medium with a high sucrose concentration (Bachiri et al. 2001; Clavero-Ramirez et al. 2005; Sakai et al. 2000). *Citrus madurensis* shoot tips are first cultured on solid medium with 0.1 M sucrose, then on medium with 0.3 M sucrose and 0.5 M glycerol (Cho et al. 2002). Black currant shoot tips are either cold acclimated for 2 weeks or cultured for 7 days on 0.75 M sucrose solid medium before encapsulation (Reed et al. 2005).

4.2.4 Encapsulation

For preparation of alginate beads the plant material is suspended in calcium-free liquid basal medium with 3% sodium alginate (low viscosity, 250 cps) (Gonzalez-Arnao and Engelmann 2006). This solution generally contains sucrose at the level used in the normal culture medium. In some cases, explants may also be encapsulated in a medium with a slightly higher sucrose concentration. The mixture is dropped with a pipette into liquid culture medium containing a high concentration of calcium chloride (usually 100 mM $CaCl_2$). This induces the polymerization of alginate in presence of an elevated concentration of calcium, thereby producing beads around the explants. These spherical beads are usually 4 or 5 mm in diameter and contain one apex or more. They are held in the calcium solution for 20–30 min after the last bead is formed to guarantee a good polymerization. Bead polymerization can be controlled visually since beads are translucent immediately after their formation and become progressively opaque as polymerization progresses. Beads should be transferred to the sucrose preculture medium only when they are completely opaque, i.e. when polymerization seems to be complete.

4.2.5 Osmoprotection

Osmoprotection corresponds to the treatment of encapsulated explants in medium with sucrose for several hours or days immediately before desiccation and cryopreservation. Beads containing the explants are placed in Erlenmeyer flasks in agitated liquid medium with between 0.50 and 1.25 M sucrose. The sucrose concentration most commonly employed is 0.75 M. Sucrose treatment durations vary from 16 to 18 h as for sugarcane (Paulet et al. 1993; Gonzalez-Arnao et al. 1993a) to 7–10 days in the case of coffee (Mari et al. 1995) and yam (Malaurie et al. 1998). With some plant materials osmoprotection of samples directly in a medium with high sucrose concentration is toxic and produces very low regrowth. In such cases a progressive increase in sucrose concentration by daily transfers of the plant material in medium with higher concentration may result in reducing the toxic effect of the high osmotic stress and in increasing regrowth. Gradual increase of sucrose by 0.25 M incremental steps ensured over 80% survival for shoot tips of grape for 1 M final sucrose concentration and 70% for 1.5 M (Plessis et al. 1991), whereas direct exposure to these media led to very low recovery. Recovery of apple shoot tips was maximal after daily increase of the sugar concentration with the following sequence: 0.1 M/0.3 M/0.7 M/1.0 M (Zhao et al. 1999). Some plant materials are treated with a

mixture of osmoprotectants. Lily shoot tips were protected with a mixture of 0.8 M sucrose and 1 M glycerol (Matsumoto and Sakai 1995) and *Citrus madurensis* apices with 2 M glycerol and 0.6 M sucrose (Cho et al. 2002). In an alternative treatment for cryopreservation of encapsulated explants they are treated with highly concentrated vitrification solutions (Sakai 2000) and frozen directly in LN without air dehydration. This technique, termed encapsulation vitrification, is described in detail by Sakai (see Chap 3).

4.2.6 Dehydration

After osmoprotection beads are rapidly surface dried on sterile filter paper to remove any remaining liquid medium and are submitted to physical dehydration by evaporation at room temperature. Two desiccation methods can be employed: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel. Desiccation under the laminar flow can produce variable desiccation rates depending on the airflow rate, air temperature, and relative humidity. By contrast desiccation in airtight containers with silica gel provides reproducible conditions from one experiment to the next and is, thus, highly recommended, especially in tropical countries where the relative humidity is usually very high (Gonzalez-Arnao and Engelmann 2006). In general, the bead water content that ensures highest regrowth after cooling in LN is around 20%, which corresponds to the amount of unfreezable water in the samples. At such water contents, only glass transitions are recorded by differential scanning calorimetry when samples are plunged in LN (Sherlock et al. 2005). This value may vary depending on the species and the type of samples. For example, bead water content was 27% for pear shoot tips encapsulated in larger alginate beads (Scottez et al. 1992), 33% for encapsulated meristems of apple and mulberry (Niino and Sakai 1992), and microspore embryos of oilseed rape survived best at 18–20% MC (Uragami 1993).

4.2.7 Cryopreservation

After dehydration, beads are placed in 1 or 2 ml polypropylene sterile cryotubes for cryopreservation. In most cases vitrification is employed by direct immersion of the cryotubes in LN. In some cases, including grape (Plessis et al. 1991; Zhao et al. 2001), potato (Fabre and Dereuddre 1990) and *Citrus* (Gonzalez-Arnao et al. 1998) shoot tips, controlled rate cooling using a programmable freezer followed by immersion of samples in LN was required to obtain higher regrowth after cryopreservation. For storage,

cryotubes are usually immersed in LN or held in LN vapors in storage tanks.

4.2.8 Rewarming

With the encapsulation–dehydration technique, rewarming is usually carried out slowly at room temperature since samples are sufficiently dehydrated before freezing and there is no risk of ice recrystallization upon warming (Gonzalez-Arnao and Engelmann 2006). Thus regrowth becomes independent of the rewarming method. For slow rewarming, beads are taken out of the cryotubes and placed in open Petri dishes in the laminar air flow cabinet for about 5 min or they are transferred directly onto the recovery medium. In cases where rapid warming is performed the cryotubes are stirred in a water bath for 2–3 min at 40°C as in the case of lily and wasabi (Matsumoto and Sakai 1995; Matsumoto et al. 1995), 1 min at 45°C for blackberry (Gupta and Reed 2006) or at 25°C as for mulberry and chicory (Niino et al. 1992; Vandenbussche et al. 1993). In some cases the beads are rehydrated with the addition of liquid culture medium for 5–10 minutes (Chang et al 2000; Gupta and Reed 2005; Reed et al. 2006).

4.2.9 Recovery

Regrowth generally takes place on standard semi-solid culture medium. Explants are transferred under standard conditions as in the case of citrus, apple and almond shoot tips (Gonzalez-Arnao et al. 1998; Zhao et al. 1999; Shatnawi et al. 1999). The composition of the recovery medium may be transitorily modified to eliminate the phenolic compounds produced by dead cells either by adding activated charcoal to the medium as in the case of sugarcane apices (Paulet et al. 1993; Gonzalez-Arnao 1996) or to stimulate proliferation of frozen explants by modifying the growth regulator content of the medium as with sugarcane and yam apices (Paulet et al. 1993; Malaurie et al. 1998). In some cases it is necessary to extract the explants from the beads and to place them directly on the recovery medium to ensure their regrowth, as in the case of microspore embryos of oilseed rape (Uragami 1993), apices of grape (Plessis et al. 1991) and mulberry (Niino et al. 1992). A softer medium is used in some cases to facilitate shoot regrowth (Gupta and Reed 2005; Reed et al. 2006). The environmental conditions are also important for regrowth. It is beneficial to perform the post-warming recovery in the dark for a short period (around 1 week) for organized structures such as meristems (Gonzalez-Arnao and

Engelmann 2006), to prevent or decrease detrimental photo oxidation of cryopreserved samples (Benson 1990).

4.2.10 Viability Assessment

The ultimate viability assessment after a cryopreservation experiment is the direct production of new tissues from the cryopreserved explants. Direct regrowth without intervening callus formation is required to maintain genetic stability. Most plants recovered from encapsulation dehydration grow without the production of callus.

4.3 Current Development and Use of Encapsulation Dehydration

Encapsulation dehydration was successful with over 70 plant species (Table 4.1). However there are few cases where this technique has been tested on a large number of accessions of the same species or species of the same genus. Sugarcane apices of 15 commercial varieties representing a broad genetic diversity were successfully cryopreserved with recovery ranging between 24 and 91% (Gonzalez-Arnao 1996). Apple shoot tips from over 20 commercial cultivars were frozen using the encapsulation–dehydration technique (Wu et al. 1999; Zhao et al. 1999). Shoot tips of 25 genotypes in nine *Rubus* species and nine *Rubus* hybrids representative of the diversity in the genus *Rubus* were successfully cryopreserved with recovery of 60–100% using the encapsulation–dehydration protocol (Gupta and Reed 2006). A wide range of *Ribes* germplasm, including 9 species and 19 cultivars, was tested with encapsulation dehydration with a mean regrowth of 58% (Reed et al. 2005). More than 35 accessions of Bermudagrass (*Cynodon*) were successfully stored by encapsulation dehydration (Reed et al. 2006). The most advanced development and application by far of encapsulation dehydration is with cassava apices. CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia) is responsible for the maintenance of the world germplasm collection of cassava (which includes 5941 accessions). The core collection, which represents 630 clones, was cryopreserved using encapsulation dehydration (Manrique 2000; Escobar Pérez 2005). Around 75% of these clones display recovery above 30%. A safety cryopreserved duplicate of this collection is being established.

4.4 Genetic Stability

The effect of encapsulation–dehydration cryopreservation on the stability of plants was assessed after regrowth for several species. No modifications were revealed at the morphological, agronomical, chromosomal, biochemical and/or molecular levels for sugarcane (Gonzalez-Arnao 1996), apple (Hao et al. 2002), yam (Sangeeta et al. 2002; Sonali-Dixit et al. 2005), kiwi and grape (Zhai et al. 2003).

4.5 Conclusions

Encapsulation dehydration is a very efficient cryopreservation technique which is simple to implement. One of its advantages from a practical point of view is its user-friendliness. Encapsulated explants are very easy to manipulate throughout the freezing protocol. Encapsulation dehydration was successfully implemented with a large number of species, both from temperate and tropical origin. It was applied to a broad number of genotypes within the same species in several cases, thereby demonstrating its efficiency and practicality. Cryopreservation represents the only safe, efficient and cost-effective long-term storage option to facilitate the conservation of genetic resources of plant species. Encapsulation dehydration is one more technique to place in the hands of researchers or genebank curators to meet germplasm conservation goals.

References

Accart F, Monod V, Poissonnier M, Dereuddre J, Pâques M (1993) Cryoconservation d'apex de *Populus tremula* x *alba* cultivés *in vitro* transformés génétiquement ou non. Ann Recherches Sylvicoles 1993: 23–29

Al-Ababneh SS, Karam NS, Shibli RA (2002) Cryopreservation of sour orange (*Citrus aurantium* L.) shoot tips. *In Vitro* Cell Dev Biol-Plant 38: 602–607

Al-Ababneh SS, Shibli RA, Karam NS, Shatnawi MA (2003) Cryopreservation of bitter almond (*Amygdalus communis* L.) shoot tips by encapsulation-dehydration and vitrification. Adv Hortic Sci 17: 15–20

Aoshima Y (1997) Cryopreservation of multiple shoots of tea (*Camellia sinensis* L.O. Kuntze) by alginate encapsulation-dehydration. Tech Bull Shizuoka Tea Exp Stat 21: 13–21

Bachiri Y, Gazeau C, Hansz J, Morisset C, Dereuddre J (1995) Successful cryopreservation of suspension cells by encapsulation-dehydration. Plant Cell Tissue Organ Cult 43: 241–248

Bachiri Y, Song GQ, Plessis P, Shoar-Ghaffari A, Rekab T, Morisset C (2001) Routine cryopreservation of kiwifruit (*Actinidia* spp) germplasm by encapsulation-dehydration: importance of plant growth regulators. CryoLetters 22: 61–74

Bagniol S, Engelmann F, Michaux-Ferrière N (1992) Histo-cytological study of apices from *in vitro* plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. CryoLetters 13: 405–412

Benson EE (1990) Free radical damage in stored plant germplasm. International Board for Plant Genetic Resources, Rome.

Benson EE, Chabrillange N, Engelmann F (1992) A comparison of cryopreservation methods for the long-term *in vitro* conservation of cassava. In: Abst. Society for Low Temperature Biology Autumn Meeting, Stirling, 8-9 September 1992.

Blakessey D, Kiernan RJ (2001) Cryopreservation of axillary buds of a *Eucalyptus grandis* x *Eucalyptus camaldulensis* hybrid. CryoLetters 22: 13–18

Blakesley D, Pask H, Henshaw GG, Fay MF (1996) Biotechnology and the conservation of forest genetic resources: *In vitro* strategies and cryopreservation. Plant Growth Regul 20: 11–16

Chang Y, Barker RE, Reed BM (2000) Cold acclimation improves recovery of cryopreserved grass (*Zoysia* and *Lolium* sp.) CryoLetters 21: 107–116

Cho EG, Hor YL, Kim HH, Ramanatha Rao V, Engelmann F (2002) Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: Importance of pregrowth and preculture conditions. CryoLetters 23: 325–332

Clavero-Ramirez I, Galvez-Farfan J, Lopez-Aranda JM, Gonzalez-Benito ME (2005) Apex cryopreservation of several strawberry genotypes by two encapsulation-dehydration methods. CryoLetters 26: 17–24

Decruse SW, Seeni S, Pushpangadan P (1999) Cryopreservation of alginate coated shoot tips of *in vitro* grown *Holostemma annulare* (Roxb.) K. Schum, an endangered medicinal plant: Influence of preculture and DMSO treatment on survival and regeneration. CryoLetters 20: 243–250

Dereuddre J, Scottez C, Arnaud Y, Duron M (1990) Effets d'un endurcissement au froid des vitroplants de poirier (*Pyrus communis* L. cv. Beurré Hardy) sur la résistance des apex axillaires à une congélation dans l'azote liquide. C R Acad Sci Paris 310 Sér III: 317–323

Dereuddre J, Blandin S, Hassen N (1991) Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen. 1. Effects of preculture. CryoLetters 12: 125–134

Engelmann F (1997) *In vitro* conservation methods. In: Ford-Lloyd BV, Newburry JH, Callow JA (eds) Biotechnology and Plant Genetic Resources: Conservation and Use. CAB International, Wellingford, pp 119–162

Engelmann F (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (eds) Cryopreservation of Tropical Plant Germplasm—current Research Progress and Applications. JIRCAS, Tsukuba/IPGRI, Rome, pp 8–20

Engelmann F (2003) Current research status and utilization of plant cryopreservation. In: Proceedings of the International Workshop on Cryopreservation of

Bio-genetic Resources. International Technical Cooperation Center, RDA, Suwon, Korea, 3-5 June 2003, pp 19–40

Escobar RH, Mafla G, Roca WM (1997) A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Rep 16: 474–478

Escobar-Pérez R.H. (2005) Aspectos logísticos de manejo y determinación de la estabilidad genética de materiales crioconservados de yuca (*Manihot esculenta* Crantz). MSc. Thesis. Facultad de Ciencias Agropecuarias, Universidad Nacional de Colombia-Sede Palmira. Colombia

Fabre J, Dereuddre J (1990) Encapsulation-dehydration: A new approach to cryopreservation of *Solanum* shoot-tips. CryoLetters 11: 413–426

Fahy GM, MacFarlane DR, Angell, CA, Meryman HT (1984) Vitrification as an approach to cryopreservation. Cryobiology 21: 407–426

Fang JY, Wetten A, Hadley P (2004) Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. Plant Sci 166: 669–675

Flachsland E, Terada G, Scocchi A, Rey H, Mroginski L, Engelmann F (2006) Cryopreservation of seeds and *in vitro*-cultured protocorms of *Oncidium bifolium* Sims. (Orchidaceae) by encapsulation-dehydration. CryoLetters 27: 235–242

Fukai S, Togashi M, Gor M (1994) Cryopreservation of *in vitro* grown *Dianthus* by encapsulation-dehydration. Tech Bull Fac Agric Kagawa Univ 46: 101–107

Gonzalez-Arnao MT (1996) Desarrollo de una técnica para la crioconservación de meristemos apicales de caña de azúcar. PhD Thesis, Centro Nacional de Investigaciones Cientificas, Cuba

Gonzalez-Arnao MT, Engelmann F (2006) Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. CryoLetters 27: 155–168

Gonzalez-Arnao MT, Engelmann F, Huet C, Urra C (1993a) Cryopreservation of encapsulated apices of sugarcane: effect of freezing procedure and histology. CryoLetters 14: 303–308

Gonzalez-Arnao MT, Engelmann F, Urra C, Lynch PT (1993b) Crioconservacion de meristemos de plantas *in vitro* de caña de azucar mediante el metodo de encapsulacion/deshidratacion. Biotecnol Apl 10: 225–228

Gonzalez-Arnao MT, Engelmann F, Urra C, Morenza M, Rios M (1998) Cryopreservation of *Citrus* apices using the encapsulation-dehydration technique. CryoLetters 19: 177–182

Gonzalez-Arnao MT, Juarez J, Ortega C, Navarro J, Duran-Vila N (2003) Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique. CryoLetters 24: 85–94

Gonzales-Benito ME, Perez C (1997) Cryopreservation of nodal explants of an endangered plant species (*Centaurium rigualii* Esteve) using the encapsulation-dehydration method. Biodivers Conserv 6: 583–590

Gonzales-Benito ME, Herradon E, Martin C (1999) The development of a protocol for the encapsulation-dehydration and *in vitro* culture of embryonic axes of *Quercus suber* L. and *Q. ilex* L. Silvae Genet 48: 25–28

Gonzalez-Benito ME, Nuñez-Moreno Y, Martin C (1998) A protocol to cryopreserve nodal explants of *Anthirrhinium microphyllum* by encapsulation-dehydration. CryoLetters 19: 225–230

Grapin A, Dumet D, Holota H, Dorion N (2003) Cryopreservation of Pelargonium shoot tips by encapsulation-dehydration: effect of sucrose concentration, dehydration duration and genotype. Acta Hortic 623: 225–230

Grospietch M, Stodulkova E, Zamecnik J (1999) Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. CryoLetters 20: 339–346

Gupta S, Reed BM (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. CryoLetters 27: 29–42

Hao YJ, Liu QL, Deng XX (2002) Effect of cryopreservation on apple genetic resources at morphological, chromosomal and molecular levels. Cryobiology 43: 46–53

Haskins RH, Kartha KK (1980) Freeze preservation of pea meristems: cell survival. Can J Bot 58: 833–840

Hirata K, Phunchindawan M, Kanemoto M, Miyamoto K, Sakai A (1995) Cryopreservation of shoot primordia induced from horseradish hairy root cultures by encapsulation and two-step dehydration. CryoLetters 16: 122–127

Hirata K, Phunchindawan M, Tukamoto J, Goda S, Miyamoto K (1996) Cryopreservation of microalgae using encapsulation-dehydration. CryoLetters 17: 321–328

Hirata K, Mukai M, Goda S, Ishio-Kinugasa M, Yoshida K, Sakai A, Miyamoto K (2002) Cryopreservation of hairy root cultures of *Vinca minor* (L.) by encapsulation-dehydration. Biotechnol Newsl 24: 371–376

Hornung R Domas R, Lynch, PT (2001a) Cryopreservation of plumular explants of coconut (*Cocos nucifera* L.) to support programmes for mass clonal propagation through somatic embryogenesis. CryoLetters 22: 211–220

Hornung R, Holland A, Taylor HF, Lynch PT (2001b) Cryopreservation of auricula shoot tips using the encapsulation/dehydration technique. CryoLetters 22: 27–34

Kartha KK, Engelmann F (1994) Cryopreservation and germplasm storage. In: Vasil IK, Thorpe TA (eds) Plant Cell and Tissue Culture. Kluwer, Dordrecht, pp 195–230

Kim HM, Shin JH, Sohn JK (2004) Cryopreservation of zygotic embryos of herbaceous peony (*Paeonia lactiflora* Pall.) by encapsulation-dehydration. Korean J Crop Sci 49: 354–357

Malaurie B, Trouslot MF, Engelmann F, Chabrillange N (1998) Effect of pretreatment conditions on the cryopreservation of *in vitro* cultured yam (*Dioscorea alata* and *D. bulbifera*) shoot apices by encapsulation-dehydration. CryoLetters 19: 15–26

Mandal BB, Chandel KPS, Dwivedi S (1996) Cryopreservation of yam (*Dioscorea* spp.) shoot apices by encapsulation-dehydration. CryoLetters 17: 165–174

Manrique N (2000) Respuesta varietal de 95 genotipos de la colección núcleo de yuca (*Manihot esculenta* Crantz) a la crioconservación usando la técnica de Encapsulación-deshidratación. BSc. Thesis. Universidad Nacional de Colombia, Sede Palmira. Colombia

Mari S, Engelmann F, Chabrillange N, Huet C, Michaux-Ferrière N (1995) Histocytological study of coffee (*Coffea racemosa* and *C. sessiliflora*) apices of *in vitro* plantlets during their cryopreservation using the encapsulation-dehydration technique. CryoLetters 16: 289–298

Matsumoto T, Sakai A (1995) An approach to enhance dehydration tolerance of alginate-coated dried meristems cooled to –196°C. CryoLetters 16: 299–306

Matsumoto T, Sakai A, Takahashi C, Yamada K (1995) Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. CryoLetters 16: 189–196

Miaja ML, Gambino G, Vallania R, Gribaudo I (2004) Cryopreservation of *Vitis vinifera* L. somatic embryos by vitrification or encapsulation-dehydration. Acta Hortic 663: 599–603

Moges AD, Shibli RA, Karam NS (2004) Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *In Vitro* Cell Dev Biol-Plant 40: 389–395

Niino T, Sakai A (1992) Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry. Plant Sci 87: 199–206

Niino, T, Sakai A, Yakuwa H (1992) Cryopreservation of dried shoot tips of mulberry winter buds and subsequent plant regeneration. CryoLetters 13: 51–58

Pâques M, Poissonnier M, Dumas E, Monod V (1997) Cryopreservation of dormant and non-dormant broad-leaved trees. Acta Hortic 447: 491–497

Paul H, Daigny G, Sangwann-Norreel BS (2000) Cryopreservation of apple (*Malus* x *domestica* Borkh.) shoot tips following encapsulation-dehydration or encapsulation-vitrification. Plant Cell Rep 19: 768–774

Paulet F, Engelmann F, Glaszmann JC (1993) Cryopreservation of apices of *in vitro* plantlets of sugarcane (*Saccharum* sp.) using encapsulation/dehydration. Plant Cell Rep 12: 525–529

Pennycooke JC, Towill LE (2001) Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* {L.} Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration CryoLetters 22: 381–389

Perán R, Berjak P, Pammenter NW, Kioko JI (2006) Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species *Ekebergia capensis*, Sparrm. CryoLetters 27: 5–16

Phunchindawan M, Hirata K, Sakai A, Miyamoto K (1997) Cryopreservation of encapsulated shoot primordia induced in horseradish (*Armoracia rusticana*) hairy root cultures. Plant Cell Rep 16: 469–473

Plessis P, Leddet C, Dereuddre J (1991) Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot-tips of grape vine (*Vitis vinifera* L. cv. Chardonnay). C R Acad Sci Paris 313 Sér III: 373–380

Poissonnier M, Monod V, Pâques M, Dereuddre J (1991) Cryopreservation in liquid nitrogen of *Eucalyptus gunnii* shoot tips grown *in vitro* following encapsulation and dehydration. Ann Rech Sylv AFOCEL1991 1992: 5–23

Quatrano RS (1968) Freeze-preservation of cultured flax cells utilizing dimethyl sulfoxide. Plant Physiol 43: 2057–2061

Redenbaugh K, Paasch BD, Nichol JW, Kossler ME, Viss PR, Walker KA (1986) Somatic seeds: Encapsulation of asexual plant embryos. Biotechnology 4: 797–801

Reed BM, Yu X (1995) Cryopreservation of *in vitro*-grown gooseberry and currant meristems. CryoLetters 16: 131–136

Reed BM, Schumacher L, Dumet D, Benson E (2005) Evaluation of a modified encapsulation-dehydration procedure incorporating high sucrose pretreatments for the cryopreservation of *Ribes* germplasm. *In Vitro* Cell Dev Biol-Plant 41: 431–436

Reed BM, Schumacher L, Wang N, D'Achino J, Barker RE (2006) Cryopreservation of bermudagrass germplasm by encapsulation dehydration. Crop Sci 46:6–11

Sakai A (1960) Survival of the twig of woody plants at –196°C. Nature 185: 393–394

Sakai A (1985) Cryopreservation of shoot-tips of fruit trees and herbaceous plants. In: Kartha KK (ed) Cryopreservation of Plant Cells and Tissues. CRC Press, Boca Raton, Florida, pp 135–170

Sakai A (2000) Development of cryopreservation techniques. In: Engelmann F, Takagi H (eds) Cryopreservation of Tropical Plant Germplasm—Current Research Progress and Applications. JIRCAS, Tsukuba/IPGRI, Rome, pp 1–7

Sakai A, Matsumoto T, Hirai D, Niino TI (2000) Newly developed encapsulation-dehydration protocol for plant cryopreservation. CryoLetters 21: 53–62

Sales E, Neubauer SG, Arrillaga I, Segura J (2001) Cryopreservation of *Digitalis obscura* selected genotypes by encapsulation-dehydration. Planta Medica 67: 833–838

Sangeeta A, Mandal BB, Sonali-Dixit S, Srivastava PS (2002) Molecular, phenotypic and biosynthetic stability in *Dioscorea floribunda* plants derived from cryopreserved shoot tips. Plant-Sci 163: 971–977

Santos IRI, Stushnoff C (2002) Cryopreservation of embryonic axes of *Citrus* species by encapsulation-dehydration. Plant Genet Resour Newsl 131: 36–41

Scottez C, Chevreau E, Godard N, Arnaud Y, Duron M, Dereuddre J (1992) Cryopreservation of cold-acclimated shoot tips of pear *in vitro* cultures after encapsulation-dehydration. Cryobiology 29: 691–700

Shatnawi M, Engelmann F, Frattarelli A, Damiano C, Withers LA (1999) Cryopreservation of apices from *in vitro* plantlets of almond (*Prunus dulcis* Mill.). CryoLetters 20: 13–20

Shatnawi MA, Johnson KA (2004) Cryopreservation by encapsulation-dehydration of "Christmas bush" (*Ceratopetalum gummiferum*) shoot tips. *In Vitro* Cell Dev Biol-Plant 40: 239–244

Shatnawi MA, Johnson KA, Torpy FR (2004) *In vitro* propagation and cryostorage of *Sygysium francissi* (Myrtaceae) by the encapsulation-dehydration method. *In Vitro* Cell Dev Biol-Plant 40: 403–407

Sherlock G, Block W, Benson EE (2005) Thermal analysis of the plant encapsulation-dehydration cryopreservation protocol using silica gel as the desiccant. CryoLetters 26: 45–54

Shibli RA (2000) Cryopreservation of black iris (*Iris nigricans*) somatic embryos by encapsulation-dehydration. CryoLetters 21: 39–46

Shibli RA, Al-Juboory KH (2000) Cryopreservation of 'Nabali' olive (*Olea europaea* L.) somatic embryos by encapsulation-dehydration and encapsulation-vitrification. CryoLetters 21: 357–366

Shibli RA, Smith MAL, Shatnawi MA (1998) Pigment recovery from encapsulated-dehydrated *Vaccinium pahalae* (ohelo) cryopreserved cells. Plant Cell Tissue Organ Cult 55: 119–123

Shibli RA, Haagenson DM, Cunningham SM, Berg WK, Volenec JJ (2001) Cryopreservation of alfalfa (*Medicago sativa* L.) cells by encapsulation-dehydration. Plant Cell Rep 20: 445–450

Sonali-Dixit S, Ahuja Ghosh S, Mandal BB, Srivastava PS (2005) Metabolic stability of plants regenerated from cryopreserved shoot tips of *Dioscorea deltoidea* - an endangered medicinal plant. Sci Hortic 105: 513–517

Sudarmonowati E, Rosmithayani (1997) Cryopreservation of *Acacia mangium* shoot tips. In: Jenie UA (ed) Proc Indonesian Biotech Conf: Challenges of Biotechnology in the 21st Century, Bogor, June 17-19 1997, vol. 2, pp 733–742

Suzuki M, Niino T, Miura N, Akihama T (1996) Viability of cryopreserved shoot tips from kiwifruit seedlings at different growth stages *in vitro*. Bull Fac Agric Meiji Univ 107: 27–35

Swann TW, Deakin EA, Hunjan G, Souch GR, Spencer ME, Stafford AM, Lynch PT (1998) Cryopreservation of cell suspensions of *Polygonum aviculare* using traditional controlled rate freezing and encapsulation/dehydration protocols, a comparison of post-thaw cell recovery. CryoLetters 19: 237–248

Tahtamouni RW, Shibli RA (1999) Preservation at low temperature and cryopreservation in wild pear (*Pyrus syriaca*). Adv Hortic Sci 13: 156–160

Tannoury M, Vintejoux C, Dereuddre J (1995) Cryoconservation par encapsulation et déshydratation d'apex d'oeillet (*Dianthus caryophyllus* L.) cultivés *in vitro*. Acta Bot Gallic 142: 415–424

Uragami A (1993) Cryopreservation of cultured cells of vegetables. In: Cryopreservation of Plant Genetic Resources. Technical Assistance Activities for Genetic Resources Projects Ref N°6. Japan International Cooperation Agency, Tokyo, pp 107–135

Vandenbussche B, De Proft MP (1996) Cryopreservation of *in vitro* sugar beet shoot-tips using the encapsulation-dehydration technique: Development of a basic protocol. CryoLetters 17: 137–140

Vandenbussche B, Demeulemeester MAC, De Proft MP (1993) Cryopreservation of alginate-coated *in vitro* grown shoot-tips of chicory (*Cichorium intybus* L.) using rapid freezing. CryoLetters 14: 259–266

Verleysen H, Bockstaele (van) E, Debergh P (2005) An encapsulation-dehydration protocol for cryopreservation of the azalea cultivar "Nordlicht" (*Rhododendron simsii* Planch). Sci Hortic 106: 402–414

Vigneron T, Arbault S, Kaas R (1997) Cryopreservation of gametophytes of *Laminaria digitata* (L.) Lamouroux by encapsulation-dehydration. CryoLetters 18: 93–98

Wang QC, Tanne E, Amir A, Gafny R (2000) Cryopreservation of *in vitro* grown shoot tips of grapevine by encapsulation-dehydration. Plant Cell Tissue Organ Cult 63: 41–46

Wang QC, Batuman O, Li P, Bar J, Gafny R (2002a) Cryopreservation of *in vitro*-grown shoot tips of "Troyer" citrange (*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* Osbeck.) by encapsulation-dehydration. Plant Cell Rep 20: 901-906

Wang QC, Gafny R, Sahar N, Ilan S, Munir M, Tanne E, Avihai P (2002b) Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. Plant Sci 162: 551–558

Wang Q, Laamanen J, Uosukainen M, Valkonen JPT (2005) Cryopreservation of *in vitro*-grown shoot tips of raspberry (*Rubus idaeus* L.*)* by encapsulation-vitrification and encapsulation-dehydration. Plant Cell Rep 24: 280–288

Wilkinson T, Wetten A, Fay MF (1998) Cryopreservation of *Cosmos atrosanguineus* shoot tips by a modified encapsulation/dehydration method. CryoLetters 19: 293–302

Wood CB, Pritchard HW, Miller AP (2000) Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and storage temperature. CryoLetters 21: 125–136

Wu Y, Engelmann F, Zhao Y, Zhou M, Chen S (1999) Cryopreservation of apple shoot tips: Importance of cryopreservation technique and of conditioning of donor plants. CryoLetters 20: 121–130

Wu Y, Zhao Y, Engelmann F, Zhou M (2001) Cryopreservation of apple dormant buds and shoot tips. CryoLetters 22: 277–284.

Zhai ZY, Wu YJ, Engelmann F, Chen RZ, Zhao YH (2003) Genetic stability assessments of plantlets regenerated from cryopreserved *in vitro* cultured grape and kiwi shoot tips using RAPD. CryoLetters 24: 315–322

Zhao Y, Wu Y, Engelmann F, Zhou M, Zhang D, Chen S (1999) Cryopreservation of apple shoot tips by encapsulation-dehydration: effect of preculture, dehydration and freezing procedure on shoot regeneration. CryoLetters 20: 103–108

Zhao Y, Wu Y, Engelmann F, Zhou M (2001) Cryopreservation of axillary buds of grape (*Vitis vinifera*) *in vitro* plantlets. CryoLetters 22: 321–328