Plant Cryopreservation for Biotechnology and Breeding

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Institute of Plant Physiology of Russian Academy of Sciences
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Chapter 3
Plant Cryopreservation for Biotechnology and Breeding

Elena Popova, Mukund Shukla, Haeng Hoon Kim, and Praveen K. Saxena

Abstract Plant biodiversity is crucial for sustaining human life on our planet. More than 50,000 species are used globally for food, feed, fiber, medicine and horticulture. A wide range of plant-based biotechnological systems such as isolated root cultures, embryogenic cell and tissue cultures and cell suspensions are used in breeding programs, forestry and the production of pharmaceuticals. Cryopreservation is an essential tool for conservation and long-term maintenance of diverse germplasms with minimal requirements for cost and labor and a low risk of loss of preserved samples. However, large-scale use of cryogenic storage to back-up plant genetic collections is hampered by unavailability of effective methodology and genotype-specific responses of diverse specimens to cryoprotective treatments. Newly developed techniques such as droplet-vitrification are more effective and user-friendlier than classical methods of cryopreservation. Cryopreservation has been successfully employed for preserving several different types of plant materials. In this chapter we review various approaches to develop and improve cryopreservation protocols for diverse plant species. Applications of modern cryopreservation methods in biotechnology-based industry as well as breeding programs are also discussed.

Keywords Cryopreservation • Gene banks • Germplasm collections • Liquid nitrogen • Long-term storage • Secondary metabolites

E. Popova (✉) • M. Shukla • P.K. Saxena
Gosling Research Institute for Plant Preservation, Department of Plant Agriculture, University of Guelph, Guelph, ON N1G2W1, Canada
e-mail: epopova@uoguelph.ca; mshukla@uoguelph.ca; psaxena@uoguelph.ca

H.H. Kim
Department of Well-Being Resources, Sunchon National University, Suncheon, Jeonnam 540-742, Republic of Korea
e-mail: cryohkim@sunchon.ac.kr

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3.1 Introduction

It has long been recognized that preserving plant biodiversity is essential for classical and modern plant breeding programs including genetic engineering (Cruz-Cruz et al. 2013; Cyr 2000; Wang et al. 2012; Withers 1987). Easy access to diverse plant germplasm is a prerequisite for breeding more productive cultivars, which in turn enhances food security (Wang et al. 2014a). The traditional approach to conservation of valuable clones and cultivars is based on plants grown in the fields. These collections are vulnerable to pests and pathogens and can be completely lost due to natural disasters such as hurricanes, floods or droughts (Panis and Lambardi 2006). Ex situ techniques including in vitro cultivation, slow growth storage and cryopreservation are now internationally recognized as major back-up options to support field collections (Engelmann 1997; FAO 2014). Recent advances in plant biotechnology and its broad application in modern agricultural and horticultural industries have raised a new demand for conserving plants and plant tissues such as undifferentiated cell and embryogenic cultures and transgenic or normal root cultures (Engelmann 2014). These cultures can be utilized for the large-scale production of pharmaceuticals and their precursors (Paek et al. 2005, 2009; Verpoorte et al. 2000; Wink et al. 2005) and can also serve as a powerful tool for the production of genetically superior clonal lines of forest and fruit trees (Cyr et al. 2001).

Cryopreservation, the storage of living materials at cryogenic temperatures (below −130 °C), is an alternative to conventional field and in vitro germplasm collection which enables plant genetic resources to be conserved safely and cost-effectively for decades with minimal requirements of space and routine maintenance (Engelmann 2004; Pence 2011; Volk et al. 2014b). More importantly, cryopreservation eliminates the need for regular renewal of the collection thus reducing the risk of genetic erosion caused by pests, diseases, weather conditions, pollution and genetic variations (Panis and Lambardi 2006; Wang et al. 2014a). It also prevents the decline in culture productivity, which is usually associated with culture duration (Reinhoud et al. 2000). Such advantages are due to the effect of extremely low temperatures (usually the materials are stored in liquid nitrogen (LN) or its vapor phase at temperatures ranging from −140 °C to −196 °C) that arrest nearly all cell division and metabolic activities of the cells. Thus, the plant samples can be stored unaltered and they remain viable for a theoretically unlimited duration (Benson 2008; Harding 2004; Wang et al. 2014a).

Cryopreservation serves modern breeding programs by providing long-term storage and easy international access to various genetic materials such as seeds, pollen and meristematic apices and buds. The consistently evolving area of phytochemical production via biotechnological methods is also supported by cryobanking of root cultures, embryogenic and non-embryogenic cell lines to ensure their genetic and biochemical stability (Fig. 3.1).

Cryopreservation of seeds is the most efficient option for many species due to ease of application and the amount of diversity conserved (Engelmann 2004; Pritchard and Nadarajan 2008). The status of seed cryobanking has been reviewed extensively (Pence 1995; Pritchard 2007; Pritchard and Nadarajan 2008; Stanwood

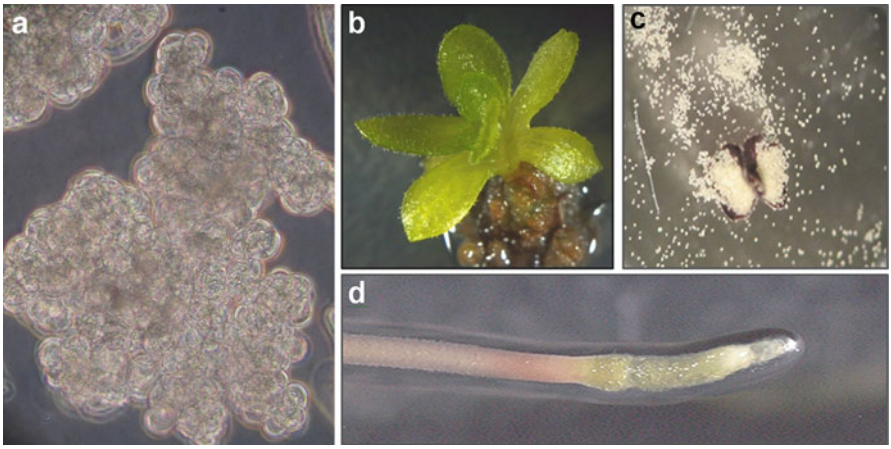

Fig. 3.1 Plant materials at various stages of cryopreservation. (**a**) Undifferentiated cells of American elm in liquid medium before adding a cryoprotectant solution; (**b**) Chrysanthemum shoot tip showing regrowth after cryopreservation; (**c**) mature pollen grains freshly dehisced from anthers of an American elm tree suitable for cryopreservation; (**d**) hairy root of *Rubia akane* showing regrowth after cryopreservation

and Bass 1981; Touchell and Dixon 1994). Cryopreservation is often addressed as the best conservation option for seedless species, species with non-orthodox seeds and for cultivars with unique attributes that can be reproduced only by vegetative propagation (Engelmann 2014; Reed 2001). Some cryopreservation techniques can be specifically tailored to eliminate pathogens from plant materials (cryotherapy) thus improving crop health and quality (Wang et al. 2009).

This chapter covers the progress of cryopreservation to conserve horticultural and some agricultural crops focusing mainly on clonally propagated species. We also give a brief review of the application of cryogenic storage to conserve cell and root cultures for industrial production of bioactive compounds. Using these examples we provide an insight into cryopreservation methodology, its evolution and technical challenges.

3.1.1 Cryopreservation of In Vitro Propagated Plant Materials: Methodology and Challenges

The theoretical bases of plant germplasm cryopreservation were created in the 1970s (Lyons et al. 1979; Mazur 1984; Meryman 1974). In the 1980s, they were translated into a methodology, which allowed cryopreservation of a significant number of species, mostly of temperate origin (Withers 1985). Following the *vitrification revolution* in the 1990s (Benson 2004), the range of cryogenic techniques increased remarkably resulting in successful cryopreservation of many tropical crops and species with

recalcitrant seeds (Engelmann and Takagi 2000; Sakai and Engelmann 2007). The number of reports on large-scale implementation of cryostorage to conserve plant genetic collections in national and industrial repositories is rising steadily (see Cruz-Cruz et al. 2013; Reed 2001, 2008). Recent examples of successful cryobanking of germplasm collections include *Musa* (Panis and Thinh 2001; Panis et al. 2005), *Allium* (Ellis et al. 2006; Kim et al. 2012a), mulberry (Atmakuri et al. 2009; Fukui et al. 2011), *Malus* (Towill et al. 2004) and potato (Kaczmarczyk et al. 2011; Sakai and Engelmann 2007). Thus, within the past three decades, substantial progress has been achieved in both the practical application of cryopreservation and the understanding of biophysical processes underlying cell response to cryogenic temperatures and cryoprotection. Various aspects of such fundamental and applied research have been described earlier (Engelmann and Takagi 2000; Fahy et al. 1990; Fuller et al. 2004; Harding 2004; Kartha 1985; Reed 2008; Towill and Bajaj 2002; Uemura and Steponkus 1992, 1999). In addition, the potential of cryopreservation in sustained production and preservation of economically important medicinal and aromatic plants has also been reviewed (Bajaj 1995; Dixit et al. 2004; Popova et al. 2011).

However, despite the promising results and achievements cited above, the success in the large-scale utilization of cryopreservation for long-term conservation of the existing germplasm collections has been limited. The major difficulty lies in the response of plant material to pre- and post-cryopreservation treatments, which is usually genotype and material specific. To date, there is no uniform method that can be applied with no or only minor modifications to a broad range of plant materials. It is therefore not surprising that some researchers are skeptical about the possibility of developing such method(s) for taxonomically unrelated or even related species.

The most popular and widely applicable cryopreservation methods developed so far include programmed (or slow) freezing, vitrification, encapsulation-dehydration, encapsulation-vitrification and the recently developed droplet-vitrification (see: Reed 2008; Sakai and Engelmann 2007 for method description). These methods exploit different approaches to explant dehydration, which is an essential step in cryopreserving hydrated living materials (Mazur 1984). Sufficient dehydration is required to achieve vitrification of protoplasm, i.e. transition of water from the liquid phase directly into an amorphous or glassy state, thereby avoiding the lethal formation of intracellular ice (Fahy et al. 1984).

3.1.2 Programmed Freezing

Programmed freezing is based on freeze-induced dehydration. Samples pretreated in cryoprotectants (usually dimethylsulfoxide [DMSO], ethylene glycol [EG] and sucrose alone or in low-concentration mixtures) are dehydrated while frozen slowly (0.3–1 °C/min) to −40 °C to −70 °C, then plunged directly into LN. The major disadvantages of this method are the requirement of an expensive program freezer and relatively long exposure of samples to subzero temperatures, which can be deleterious for cold-sensitive species.

3.1.3 Vitrification

The vitrification method originally developed by Sakai in the late 1980s has been successfully applied to more than 200 plant species (Matsumoto and Niino 2014). In vitrification, samples are dehydrated osmotically in a sequence of *loading* (LS) and *vitrification* (VS) solutions on ice or at ambient temperatures. The loading step, also known as *osmoprotection*, is performed via sample exposure to cryoprotectant solutions of moderate concentrations (35–45 %), usually composed of glycerol and sucrose. This step serves to prepare samples to the following extensive dehydration with highly concentrated (up to 100 %, w/v) vitrification solutions (*cryoprotection* step). The most commonly used vitrification solutions are PVS2 (Plant vitrification solution 2) which consists of, w/v, 30 % glycerol, 15 % ethylene glycol, 15 % DMSO and 0.4 M sucrose (Sakai et al. 1990) and PVS3 comprising, w/v, 50 % glycerol and 50 % sucrose (Nishizawa et al. 1993). Modifications of these two basic solutions have also been tested with promising results (Kim et al. 2009; Suzuki et al. 2008; Turner et al. 2001). Dehydrated samples are sealed in cryo-ampoules or polypropylene straws and rapidly immersed in LN. Thus, in contrast to a programmed freezing, in vitrification the most important step is pre-freezing osmotic dehydration/cryoprotection rather than freezing per se.

3.1.4 Droplet Vitrification

For droplet vitrification, samples are osmoprotected as in vitrification but cryopreserved on aluminum plates or foil stripes in microliter drops of vitrification solutions. This ensures better contact of explants with liquid nitrogen thus facilitating cooling and rewarming of samples (Towill and Bonnart 2003) which results in improved regrowth for a number of species, for example, chrysanthemum (Halmagyi et al. 2004), garlic (Kim et al. 2006a) and potato (Kaczmarczyk et al. 2011).

3.1.5 Encapsulation

Based on the technology of artificial seeds, encapsulation (Fabre and Dereuddre 1990) employs encapsulation of plant materials in Ca-alginate beads followed by air desiccation (encapsulation-desiccation) or dehydration VS (encapsulation-vitrification) combined with rapid immersion in LN. Though more laborious than *classic* vitrification, this technique is useful when cryopreserving small, fragile or extremely desiccation-sensitive materials (Escobar-Pérez 2005; Gupta 2014; Hirata et al. 2002; Matsumoto and Sakai 1995; Mikula 2006).

Generally, regardless of the cooling procedure, rewarming of samples rapidly in a water bath at approx. 40 °C was beneficial for their recovery (Sakai and Engelmann 2007).

3.2 Developing a Cryopreservation Protocol: Empirical and Systematic Approaches

Cryopreservation of clonally-propagated plant material is a complicated process comprising multiple steps such as explant selection and excision, preconditioning, osmoprotection and cryoprotection with LS and VS, freezing, storage, rewarming and recovery (Fig. 3.2). For successful regrowth, not only each step of the cryopreservation protocol should be optimized but also the physiological state of explants should be considered. Manipulation with growth regulators, cold acclimation and the addition of ABA to the culture medium are the most common procedures to enhance physiological tolerance of donor cultures and explants to stress conditions associated with cryopreservation (Burchett et al. 2006; Chetverikova 1999; Popova et al. 2009; Uemura and Steponkus 1999). Concentrations of penetrating and non-penetrating cryoprotectants as well as duration and temperature of cryoprotection treatment can be varied in order to dehydrate samples, while avoiding or reducing cytotoxicity (Kim et al. 2009; Sakai and Engelmann 2007). Preculture of plant materials with amino acids (proline), osmotically active chemicals such as sugars and sugar alcohols (mannitol, sorbitol) is a common and a very effective way to improve post-cryopreservation regrowth (Baskakova et al. 2003; Butenko et al. 1984; Carpentier et al. 2007; Popova et al. 2010; Ramon et al. 2002; Reinhoud et al. 2000; Zhu et al. 2006) though it needs to be tested and optimized for every species.

Oxidative stress is believed to be one of the major factors causing cell injury in cryopreserved samples (Benson 1990; Benson and Bremner 2004). A relatively new approach to enhance regrowth of cryopreserved tissues is the addition of antioxidants (reduced glutathione, ascorbic acid, melatonin, etc.) to cryoprotective solutions or to the culture medium used for pretreatment and recovery. For example, the addition of glycine betaine, ascorbic acid, glutathione and ABA into PVS2 improved post-cryopreservation regrowth of *Arabidopsis* seedlings (Ren et al. 2014). Vitamin E and ascorbic acid added during the pretreatment, loading, unloading and regrowth steps enhanced regrowth and reduced malondialdehyde formation in shoot tips of cryopreserved blackberry (Uchendu et al. 2010a). Lipoic acid, glutathione and glycine betaine also showed beneficial effect when used with the same plant under similar conditions (Uchendu et al. 2010b). Melatonin supplied at preculture and recovery steps significantly improved regrowth of American elm shoot tips cryopreserved by vitrification (Uchendu et al. 2013). Survival of cryopreserved *Rhodiola crenulata* callus increased following a 5-day pretreatment with 0.1 μM melatonin and was associated with enhanced peroxidase and catalase activity of melatonin-treated cells compared to the control (Zhao et al. 2011). All these findings suggest that the antioxidants may play an essential role in reducing oxidative stress associated with the cryopreservation process even with thoroughly optimized steps of the protocol.

On account of the abundance of protocols available worldwide for various plant species and materials (Engelmann 2014; Reed 2008), one will have a broad selection of options while attempting to cryopreserve a new species, genotype or a cell line. A

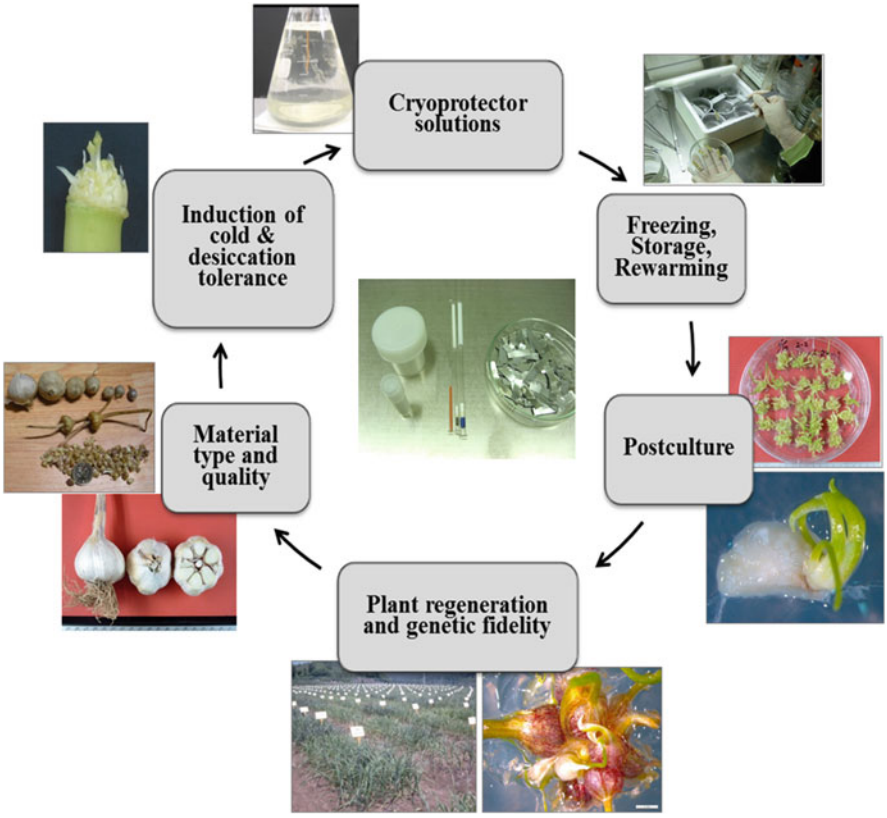

Fig. 3.2 A simplified scheme of cryopreserving clonally propagated germplasm: cryobanking of *Allium* collection at National Agrobiodiversity Center, South Korea as an example. Photograph in the center of the illustration represents various containers/holders, ampoules, straws and aluminum foil strips used for cryopreservation

simple, time-saving approach would involve the use of an existing protocol developed for a closely related species with further modification and optimization of major steps, if needed, to improve post-cryopreservation recovery. Unfortunately, in many studies none of the existing protocols results in desirable regrowth after cryogenic storage. In this case, the researcher has two options. The first obvious and commonly used strategy is to develop the protocol de novo by optimizing preculture conditions, composition and concentration of cryoprotectant solutions, cooling and rewarming rates, and conditions during recovery with a hope that the process will enhance the regrowth. Also referred to as the *experimental* or *trial-and-error* approach, this blind screening of multiple conditions is time and labor consuming. Often this approach is impractical and too expensive to justify, particularly for the curators of small but valuable collections in botanical gardens or private companies. A common consequence of failures in this approach is discontinuation of cryopreservation research on such recalcitrant species, cell lines or cultivars (Popov, Redington, personal communication).

An alternative to *blind* screening is a systematic evaluation of the impact of each step of the cryopreservation protocol on the recovery and physiological state of the samples. This includes cytological and ultrastructural investigation of cells (Bachiri et al. 2000; Miao et al. 2005; Micuła et al. 2005; Salma et al. 2014), measuring the kinetics of cryoprotectant influx/efflux (Kim et al. 2004a, b), determination of the amount and physical state of intracellular water (Fang et al. 2009; Volk and Walters 2006), thermal analysis for understanding water and lipid behaviour during cooling and rewarming (Kim et al. 2009; Towill and Bonnart 2003), biochemical assays (Ramon et al. 2002; Zhu et al. 2006; etc.) and gene expression (Ren et al. 2013; Volk et al. 2014a). In the most desirable scenario these analyses are performed systematically using selected model systems which exhibit different tolerance to cryopreservation treatment as shown for shoot tips of *Arabidopsis*, garlic and mint and root culture of madder (Kim et al. 2012a, b, 2014; Volk et al. 2014a). A representative example of such a multi-stage investigation is the protocol development for large-scale cryopreservation of *Allium* field collection at the National Agrobiodiversity Center in Suwon, South Korea. Major steps of this process are shown in Table 3.1 and Fig. 3.2. As a result of this study, a total of 1158 *Allium* accessions have been cryopreserved during 2005–2010 using the droplet-vitrification technique with a mean post-cryo regeneration percentage of 65.9 % (Kim et al. 2012a).

It is quite obvious from the scope of a systematic development of a cryopreservation protocol (Table 3.1) that this approach is ambitious, extensive and must be reinforced by long-term planning and strong financial support. The cryobanking of the whole national *Allium* collection (Fig. 3.2) was achieved through combined efforts and investments on behalf of enthusiastic members of the cryopreservation team and field conservatory groups, government support and over 10 years of extensive research employing a range of biochemical and molecular tools including analytical chemistry (HPLC, Differential Scanning Calorimetry), electron microscopy, RT-PCR, immuno-assay, etc. These methods require specialized equipment, chemical reagents of high purity and qualified personnel, which may be financially and technically restrictive for small laboratories, particularly in developing countries (Reed 2001).

In view of these limitations we designed a simplified approach to protocol development based on differentiation of samples according to their size and permeability to cryoprotectants as well as their sensitivity to chemical and osmotic toxicity of vitrification solutions modified from those commonly employed in PVS2 and PVS3 (Kim et al. 2009). The plant material is classified according to its response to a number of standardized treatments with progressively increasing total concentration of cryoprotectants. The consequent steps are rapid freezing of samples in drops of vitrification solution on aluminum foil strips (droplet vitrification), rewarming by plunging into pre-heated (40 °C) 1.2 M sucrose solution and further unloading for a proper duration depending on the materials. The treatments with better survival results can be incorporated into standard protocol which can be further adapted for different genotypes.

Table 3.1 Systematic development of a cryopreservation protocol for cryobanking *Allium* collection at the National Agrobiodiversity Center (Suwon, South Korea) using clove apices and bulbil primordia from immature inflorescences

Step	Factors investigated	Year
1. Revealing the most critical factors that affect regeneration after cryostorage; developing the *standard protocol*	Origin and size of starting material; explant size	2001–2002
	Cold acclimation and preconditioning	2001–2003
	Cryoprotectant treatment: duration, composition of cryoprotectant solutions;	2002–2003
	Air dehydration before cryoprotection;	
	Unloading	2002–2003
	Recovery (the effect of growth regulators)	2003
	Cooling and rewarming methods[a]	2005–2006
2. Investigating the impact of cryoprotection	Thermal behavior of explants and cryoprotectant solutions during cooling and rewarming	2002–2003
	Analyzing the dynamics of cryoprotectant influx/efflux (HPLC)	2002–2003
	Investigation of genetic stability of recovered plants (field performance, AFLP)	2002–2003
3. Large scale implementation of the standard protocol	Application protocol to diverse genotypes; Genotype-specific adjustments of protocol for sensitive varieties	2005–2010
	Revealing duplicate accessions and filling the gaps in the collection	2005–2010
4. Cryotherapy	Elimination of garlic virus A, onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV) due to cryotherapy	2007–2010

Source: Modified from Kim et al. (2012a)

[a]In 2001–2005, the majority of samples were cryopreserved using a vitrification protocol. Beginning 2005, a newly-developed droplet vitrification procedure was adopted as it generally produced higher recovery after cryopreservation

This approach was successfully tested with embryogenic cultures of the Korean forest species *Kalopanax septemobilis* (Shin et al. 2012) and hairy root cultures of six medicinal plants, which showed high sensitivity to both osmotic and chemical toxicity of loading and vitrification solutions (Kim et al. 2012b). The same principle was later applied to formulate an efficient cryopreservation protocol for chrysanthemum shoot tips which are also very sensitive to chemical toxicity but relatively tolerant to osmotic action of cryoprotectant mixtures (Lee et al. 2011).

Table 3.2 summarizes cryopreservation strategies and optimized pretreatment conditions for the selected species as revealed by applying this rationalized approach to the droplet vitrification method. The following recommendations emerged from the modification of the initial protocol developed for highly tolerant species while transferring it to more sensitive plant materials:

Table 3.2 Strategies and optimized procedures applied for cryopreservation of various species with different explant properties and different sensitivity/tolerance to chemical and osmotic stress of cryoprotectant solutions

Strategies or procedures	Plant species			
	Allium sativum	*Kalopanax septemlobus*	*Chrysanthemum morifolium*	*Rubia akane*
Size and type of the material	Bulbil primordia, clove apices 1.5×3.0 mm	Embryogenic callus, 40 mg fr. wt.	Shoot tips, axillary buds 1.2×1.5 mm	Root apices, 7–10 mm
Tolerance or sensitivity to osmotic stress	Very tolerant	Tolerant	Tolerant	Very sensitive
Tolerance or sensitivity to chemical stress	Very tolerant	Tolerant	Sensitive	Very sensitive
Strategy for developing cryopreservation protocol	Sufficient dehydration through increasing duration of exposure to highly concentrated VS at room temperature	1. Loading is important for regrowth	1. Improve dehydration tolerance through preculture with step-wise increasing sucrose concentrations	1. Use prolonged preculture with moderate sucrose concentrations to induce desiccation tolerance
		2. Avoid DMSO and EG or Reduce their concentration in VS and incubation temperature (ICE)	2. Loading is crucial for regrowth and composition of LS is important	2. Loading is crucial for regrowth and composition of LS is important
				3. Use very short (up to 20 min) incubation with VS
			3. Avoid DMSO and EG or Reduce temperature of VS incubation (ICE)	4. Avoid DMSO and EG or Reduce their concentration in VS and incubation temperature (ICE)
Optimized cryopreservation protocols				
1. Preculture	0.3 M suc, 2–3 days, 10 °C	No	0.3 M suc (31 h) → 0.5 M (17 h) → 0.7 M (7 h)	0.3 M suc (48 h) → 0.5 M (5 h)
2. Loading	No (optional)	Any LS, 20 min, 24 °C	C4, 40 min, 24 °C	C4, 30 min, 24 °C
3. Dehydration	PVS3, 150–180 min, 24 °C	PVS3, 40 min, 24 °C or A3-80 %, 40 min, 0 °C	PVS3, 60 min, 24 °C or A3, 55 min, 0 °C	B5, 15 min, 24 °C or A3-70 %, 20 min, 0 °C
4. Freezing	Foil (droplet)	Foil (droplet) or ampoules	Foil (droplet)	Foil (droplet)

(continued)

Table 3.2 (continued)

Strategies or procedures	Plant species			
	Allium sativum	*Kalopanax septemlobus*	*Chrysanthemum morifolium*	*Rubia akane*
5. Rewarming	37–40 °C, 30 s	37 °C, 60 s	37 °C, 30 s	37 °C, 30 s
6. Unloading	0.8 M suc, 40 min	0.8 M suc, 30 min	0.8 M suc, 40 min	0.8 M suc, 30 min
Regrowth after cryopreservation	77–100 %, depending on genotype	95–100 %	82–85 %	84–89 %

According to Kim et al. (2012a), Lee et al. (2001), Shin et al. (2012)
A3: 37.5 % glycerol + 15 % DMSO + 15 % EG + 22.5 % sucrose, w/v; A3-80 %: 33.3 % glycerol + 13.3 % DMSO + 13.3 % EG + 20.1 % sucrose, w/v; A3-70 %: 29.2 % glycerol + 11.7 % DMSO + 11.7 % EG + 17.4 % sucrose, w/v; B5: 40 % glycerol + 40 % sucrose, w/v; C4: 17.5 % glycerol + 17.5 % sucrose, w/v; DMSO: dimethylsulfoxide; EG: ethylene glycol; PVS3: 50 % glycerol + 50 % sucrose, w/v; Suc: sucrose; VS: vitrification solution

(a) Step-wise preculture with gradually increasing sucrose concentration can improve explant tolerance to further dehydration caused by VS. However, highly sensitive tissues (hairy roots) may not survive exposure to sucrose beyond 0.5 M even for a few hours.
(b) Loading step and composition of loading solution are essential for better survival of sensitive materials after cryopreservation.
(c) The use of DMSO and EG in cryoprotectant solutions should be avoided or minimized, and the cryoprotection treatment performed on ice to reduce chemical toxicity.
(d) Total concentration of cryoprotectants should be reduced as compared to those in standard PVS2 and PVS3.

Although these findings may not be universally applicable, they can be taken into consideration when developing cryopreservation protocols for new genotypes and plant materials.

3.3 Cryopreservation of Horticultural Plants: Chrysanthemum and Lily as Model Plants

Among horticultural plants, chrysanthemum and lily are often favored by breeders due to the ease of the selection and a broad variety of genotypes (Teixeira da Silva 2003). They are commonly propagated vegetatively by bulb division, stem cuttings or by in vitro culture of adventitious shoots and nodal segments (Martín and González-Benito 2009). These plants represent successful models of the application of biotechnological tools in horticultural industry (Teixeira da Silva 2004). Horticultural markets worldwide consistently demand the introduction of new

varieties replacing the existing popular genotypes. This transition renders *old-fashioned* genotypes redundant and the maintenance of their nuclear stock economically unviable. Despite the loss of commercial interest, these genotypes represent a source of genetic diversity, which is essential for plant improvement by conventional and molecular breeding (Martín and González-Benito 2009). In search of a cost-effective option for long-term storage, cryopreservation of lilies and chrysanthemum has been frequently attempted during the past decades. Collectively, these studies have laid the foundation for the development of simple and effective protocols for cryopreserving horticultural species.

Meristematic organs are the most favorable material for cryopreservation due to their high genetic stability and potential use in cryotherapy (Wang et al. 2014a). Cryopreservation of chrysanthemum shoot tips excised from greenhouse (Fukai 1990; Fukai and Oe 1990; Fukai et al. 1991) and in vitro (Martín and González-Benito 2005; Martín et al. 2011; Sakai et al. 2000; Zalewska and Kulus 2013) plants has been reported using programmed freezing and, later, vitrification and encapsulation-dehydration techniques with post-cryogenic regrowth ranging from 35 % to 85 %. All these methods, however, have certain disadvantages that hinder their routine implementation for cryopreserving diverse cultivars. Abnormal development was frequently observed for apices cryopreserved by programmed freezing, presumably as a result of a pretreatment with DMSO (Fukai and Oe 1990; Fukai et al. 1991). Cold hardening of in vitro donor plants for a minimum of 3 weeks was essential for shoot tip recovery after exposure to LN regardless of the cryopreservation procedure used.

A significant step towards simplifying the cryopreservation procedure for chrysanthemum was made by Halmagyi et al. (2004) who replaced cold-hardening of donor plants by 24 h incubation of the excised apices in the medium supplemented with 0.5 M sucrose. Precultured apices showed 80 % regrowth when cryopreserved in 4-µl drops of full-strength or diluted PVS2 stuck to aluminum foil strips. By contrast, programmed freezing, ultra-rapid freezing on top of the hypodermic needle and encapsulation-dehydration tested in the same study resulted in regrowth below 50 %. The only restriction of the new protocol was a narrow time range of explant exposure to vitrification solutions: 5–10 min in full-strength PVS2 or 10–20 min in 60 % PVS2. Exceeding the exposure time by 5 min caused nearly 20 % loss of post-cryopreservation regrowth (Halmagyi et al. 2004).

By expanding the scope of this work, we achieved cryopreservation of chrysanthemum shoot tips using the droplet-vitrification method and rationalized approach described in a previous section. A series of 14 vitrification solutions with varied concentrations of penetrating and non-penetrating cryoprotectants were tested with *Chrysanthemum morifolium* apices excised from 4-week-old in vitro plants (Fig. 3.3). As Fig. 3.3 shows, chrysanthemum apices were moderately sensitive to osmotic stress produced by glycerol and sucrose and very sensitive to toxic action of permeating cryoprotectants DMSO and EG. Increasing DMSO and EG concentration by only 10 % caused a 60 % decrease in regrowth of apices after 30 min exposure with or without cryopreservation while reducing toxic chemicals by 10 % slightly increased the regrowth. Further studies revealed the crucial importance of the load-

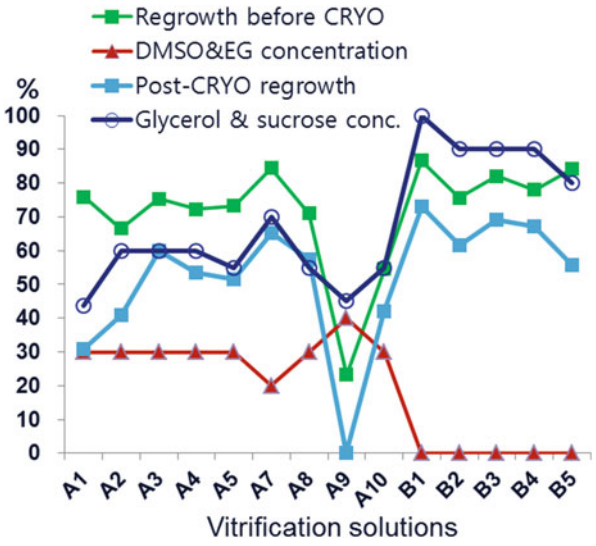

Fig. 3.3 Effect of glycerol and sucrose versus DMSO and EG concentrations in vitrification solutions on regrowth of *Chrysanthemum morifolium* shoot apices before and after cryopreservation (CRYO). Apices excised from 4-week-old in vitro plants were step-wise precultured with 0.3, 0.5 and 0.7 M sucrose for 31, 17 and 7 h, respectively, loaded for 40 min then dehydrated in vitrification solutions A1-A10 for 30 min or B1-B5 for 150 min. Solutions of A series were modified from commonly employed PVS2 (A1) and contained glycerol, sucrose, DMSO and EG. Solutions of B series were based on PVS3 (B1) and contained only glycerol and sucrose (see Table 3.3 for the composition of the solutions)

ing step and composition of the loading solution for post-cryopreservation regrowth of chrysanthemum apices. Thermal analysis of cryoprotected apices during freezing and rewarming suggested that loading had little effect on thermal behavior of the explants. Most likely, it primarily served to mitigate the osmotic stress caused by further exposure to VS. Step-wise preculture of apices in liquid medium supplemented with 0.3, 0.5 and 0.7 M sucrose to induce dehydration tolerance also improved regrowth (Table 3.2). The resulting protocol employing step-wise preculture, loading, exposure to PVS3 or A3 vitrification solution, freezing using aluminum foil strips, rapid rewarming and recovery on GA_3 containing medium resulted in 80–90 % regrowth of two chrysanthemum cultivars (Table 3.2, Lee et al. 2011).

Wang et al. (2014b) developed an alternate procedure for cryopreservation of *Chrysanthemum morifolium*. This new protocol employed shoot tips dissected from 12-day-old nodal segments, one-step preculture with 0.5 M sucrose for 24 h, loading for 20 min and cryoprotection with PVS2 for 30 min at 0 °C followed by freezing in LN using aluminum foil strips. This procedure is highly effective, user-friendly and has been tested in six chrysanthemum genotypes resulting in 43–83 % regrowth. In comparison, programmed freezing method suggested by Fukai et al. (1991) was successful with 3 chrysanthemum cultivars, 12 species and 2 interspecific hybrids, with high variation of regrowth rates of 9.4–100 %.

Table 3.3 Composition and total concentration of the alternative vitrification solutions used in systematic protocol development for *Allium*, chrysanthemum, lily and other species mentioned in the study

Vitrification solution	Composition (%, w/v)	Total concentration (%)	Remarks
A1	Glycerol 30.0 + DMSO 15 + EG 15 + sucrose 13.7	73.7	Classical PVS2
A2	Glycerol 42.5 + DMSO 15 + EG 15 + sucrose 17.5	90.0	
A3	Glycerol 37.5 + DMSO 15 + EG 15 + sucrose 22.5	90.0	
A4	Glycerol 32.5 + DMSO 15 + EG 15 + sucrose 27.5	90.0	
A5	Glycerol 35.0 + DMSO 15 + EG 15 + sucrose 20.0	85.0	
A7	Glycerol 37.5 + DMSO 10 + EG 10 + sucrose 32.5	90.0	
A8	Glycerol 30.0 + DMSO 15 + EG 15 + sucrose 25.0	85.0	
A9	Glycerol 30.0 + DMSO 20 + EG 20 + sucrose 15.0	85.0	
A10	Glycerol 40.0 + DMSO 15 + EG 15 + sucrose 15.0	85.0	
B1	Glycerol 50 + sucrose 50	100.0	Classical PVS3
B2	Glycerol 50 + sucrose 40	90.0	
B3	Glycerol 45 + sucrose 45	90.0	
B4	Glycerol 40 + sucrose 50	90.0	
B5	Glycerol 40 + sucrose 40	80.0	

Source: Modified from Kim et al. (2009)

The majority of studies on cryopreservation of lily germplasm utilized meristems or adventitious shoot primordia regenerated on the scales of in vitro or field grown bulbs (Bouman and de Klerk 1990; Chen et al. 2011; Matsumoto and Sakai 1995). First experiments involved preculture of explants with 10 % sucrose at low temperature (5 °C) followed by direct freezing in LN in cryovials or by programmed freezing (Bouman and de Klerk 1990), but resulted in poor regrowth of <8 %. Vitrification method employed later by the same authors was more successful: 10 min dehydration of precultured and loaded explants with PVS2 was sufficient to achieve 80 % regrowth after cryopreservation. Duration of PVS2 exposure could be extended to 1 h without significant effect on the regrowth (Bouman et al. 2003). Cryopreservation of roots was also achieved effectively though conversion of regenerated roots into plants was not high (Bouman et al. 2003). These results were in agreement with those reported earlier by Matsumoto et al. (1995) who reported the highest post-cryopreservation recovery of lily apices after exposure to PVS2 for 20 min at 25 °C or for 110 min at 0 °C. Chen et al. (2011) obtained highest regrowth of cryopreserved lily apical meristems excised from adventitious buds after 90 min exposure to PVS2.

Despite a noticeable tolerance of lily apices to osmotic and chemical action of cryoprotecting solutions, optimization of preculture procedure, loading duration

and composition of loading solution were found to be important for an efficient cryopreservation (Chen et al. 2011; Matsumoto et al. 1995). Droplet vitrification improved regrowth for two of three cultivars as compared to classical vitrification in cryovials (Chen et al. 2011). Another effective cryopreservation protocol employed two-step pretreatment of excised juvenile corms followed by incubation in alternative loading and vitrification solutions (Fig. 3.4). This method resulted in vigorous regrowth of approximately 60–70 % explants after cryopreservation. In all cases, cold hardening of excised meristems or bulb scales with adventitious buds improved regrowth after cryopreservation.

Yin et al. (2014) used shoot tips of adventitious shoots to test different preculture regimes, loading solutions and PVS2 exposure durations for cryopreservation of lily. The optimized protocol implied preculture with 0.5 sucrose for 24 h and incubation in loading solution containing 0.4 M sucrose and 2 M glycerol for 20 min followed by a PVS2 treatment for 4 h at 0 °C.

When these protocols were tested with different genotypes, they resulted in comparable regrowth. Bouman et al. (2003) reported maximal regrowth from 19 % to 93 % for 10 genotypes using vitrification. Meanwhile, Chen et al. (2011) showed regrowth of 43 %, 65 % and 84 % for three lily cultivars cryopreserved by droplet vitrification. Yin et al. (2014) recorded 42.5–87.5 % regrowth for six genotypes tested with the same method.

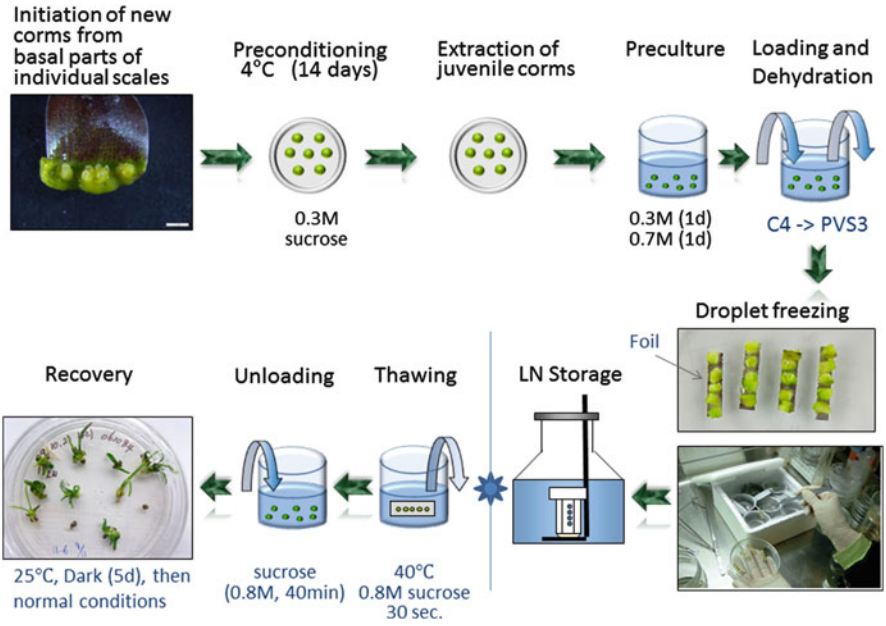

Fig. 3.4 Cryopreservation of lily by droplet vitrification using new corms developed from in vitro growth bulb scales. C4: 17.5 % glycerol + 17.5 % sucrose, w/v; PVS3: 50 % glycerol + 50 % sucrose, w/v

Despite a prominent difference in their physiology, both chrysanthemum and lily remain representative examples of how the cryopreservation methodology has evolved for horticultural plants. First attempts relied on programmed freezing with a number of randomly tested cryoprotective pretreatments. These experiments were rarely successful, mostly due to the lack of knowledge of the physiology of explant response to cryoprotection and low temperature and the experience in resolving these issues. Regrowth was improved by applying PVS-based vitrification and later its modified version, droplet-vitrification methods. These procedures were also simpler and affordable compared to programmed freezing due to exclusion of expensive equipment. Cold hardening of donor plants, once considered essential for regrowth, was recently replaced by using the explants at certain physiological state achieved by preculture with sucrose or manipulations with growth regulators in the medium. In all cases, sequential exposure to increased concentration of cryoprotectants improved regrowth of cryopreserved explants. In vitro cultured plant material remains the best source of explants for cryopreservation due to its proven sterility and availability throughout the year.

Apart from high regrowth rate obtained with number of cultivars, routine implementation of cryopreservation for conserving chrysanthemum and lily germplasm is hampered by genotype-dependent response to standard treatments (Bouman et al. 2003; Fukai et al. 1991; Yin et al. 2014). These difficulties may be overcome in the future by better understanding of mechanisms underlying the response of explants to cryoprotectant treatment and dehydration, and water behavior in cryoprotected samples. Droplet-vitrification method reported in most recent studies (Chen et al. 2011; Lee et al. 2011; Yin et al. 2014) appears to be the most promising for systematic improvement of cryopreservation of lily germplasm.

3.3.1 Genetic Stability

Cryopreservation procedures induce severe stress in plant materials and commonly results in lethal damages to the treated explant. Therefore, monitoring of genetic stability of plants regenerated from cryopreserved explants has been considered very important (Harding 2004). Fukai et al. (1991) found no difference in morphological parameters such as date of flowering, length of flower stem, number of leaves, flower weight and number of florets in 18 regenerants of *Chrysanthemum morifolium* cv. Shuhounitikara after cryopreservation using programmed freezing. Later, however, the authors reported that nearly 70 % of chimeric *Dendranthema grandiflorum* plants derived from cryopreserved shoot tips had altered flower color (Fukai et al. 1994).

Ploidy levels determined by flow cytometry analysis (FCM) remained stable in plants of three chrysanthemum genotypes regenerated from shoot tips cryopreserved by droplet vitrification as compared to control stock cultures (Lee et al. 2011; Wang et al. 2014b). Assessment of genetic stability by RAPD markers did not detect any polymorphic bands in 21 regenerants from shoot tips cryopreserved by vitrification, while one of 25 regenerants developed after encapsulation-dehydration showed a different band pattern (Martín and González-Benito 2005). More detailed investi-

gation into cryopreservation using encapsulation-dehydration techniques with both RAPD and AFLP markers showed that one of the initial steps in explant preparation, 3-day preculture with 0.3 M sucrose at 5 °C, caused modification in the RAPD profile (Martín et al. 2011). Even higher polymorphism was detected with AFLP markers (40.1 % compared to 5.78 % with RAPD). Based on these findings, the authors suggested that genetic variation in the material may be associated with pretreatment steps and not necessarily with the damage caused by the freezing-rewarming process (Martín et al. 2011). Most recently, no polymorphic bands were detected by simple sequence repeats (SSR) in regenerants of two chrysanthemum genotypes cryopreserved by droplet vitrification (Wang et al. 2014b).

Compared to chrysanthemum, analysis of the genetic stability of cryopreserved lily is scarce. In one study (Yin et al. 2014), genetic stability of the regenerants was assessed using inter-simple sequence repeat (ISSR) markers and no differences were found between plants developed in control culture and from cryopreserved adventitious shoot tips of two genotypes within 12 months after cryogenic treatment.

3.4 Cryopreservation of Cell Cultures for Secondary Metabolite Production

In vitro cultures of undifferentiated somatic plant cells are an attractive source of phytochemicals for food and pharmaceutical industries (Nosov 2012; Paek et al. 2005; Smetanska 2008). These cultures are normally heterogenic and prone to genetic instability (Nosov 1999). In the majority of cell lines, the frequency of genetic and epigenetic variations increases in the course of repetitive subcultures causing the loss of regeneration potential and changes in secondary metabolite profile (Heine-Dobbernack et al. 2008). Cryopreservation is often addressed as the only effective option to prevent culture ageing and reduce the risks of culture loss caused by contamination or technical errors (Engelmann 2004; Reinhoud et al. 2000).

The methodology of cryopreserving cultured somatic plant cells has been summarized in a number of reviews (Heine-Dobbernack et al. 2008; Nosov et al. 2014; Popova et al. 2011; Reinhoud et al. 2000; Sakai and Engelmann 2007; Withers 1985). Here, we focus mainly on the cell cultures that have been tested for industrial or semi-industrial production of secondary metabolites of potential economic importance.

Cell cultures derived from *Taxus* spp. have been largely utilizes for the commercial production of paclitaxel, a complex diterpenoid with high anti-tumor activity (Malik et al. 2011). Cryopreservation of *T. chinensis* cell culture in a cryoprotective mixture of 0.5 M DMSO and 0.5 M glycerol by programmed freezing has been reported with 40 % maximum viability after rewarming (Kim et al. 2001). The cryopreserved cell line showed lower accumulation of dry biomass as compared to control culture in the course of 40-day cultivation, however, paclitaxel production was similar to that of the control (Kim et al. 2001).

Programmed freezing was also applied for cryopreserving *Polyscias filicifolia* cell culture which is currently used for the production of bioactive food additive Vitagamal

(Titova et al. 2011). A combination of 15 % glycerol and 10 % sucrose was optimum for cryoprotection and resulted in 45 % post-cryogenic regrowth measured soon after freezing (Krivokharchenko et al. 1999). Same culture was successfully regenerated after 5 years of cryogenic storage with 25–40 % survival rates, proliferated in flasks and scaled up to 20, 75 and 630 L bioreactors for semi-industrial biomass production (Titova et al. 2007). The regenerated culture not only fully retained its specific growth traits to the level recorded before cryopreservation, but showed significantly higher productivity and growth rates compared to the same cell line maintained for 5 years under standard conditions by repetitive subcultures (Table 3.4).

The levels of diosgenine, sitosterol and stigmasterol in *Dioscorea deltoidea* cell culture were unchanged after cryopreservation by programmed freezing following preculture of cells with amino acids asparagines and alanine (Butenko et al. 1984). Cryopreservation of *Panax ginseng* and *P. quinquefolius* cell cultures was successful using programmed and direct freezing respectively (Fedorovskii et al. 1993; Joshi and Teng 2000; Mannonen et al. 1990; Seitz and Reinhard 1987). Glycerol and sucrose in moderate concentrations (up to 20 %) were efficient for cell pretreatment and cryoprotection of ginseng and *Dioscorea* similar to cell cultures of other species. Higher growth, biomass productivity and yield have been recorded for *Panax ginseng* cell suspension regenerated after two-step freezing to cryogenic temperature as compared to unfrozen culture (Joshi and Teng 2000). The ginsenoside pattern was not affected by cryopreservation (Mannonen et al. 1990; Seitz and Reinhard 1987).

Table 3.4 Growth characteristics of *Polyscias filicifolia* cell cultures in flasks and in 20 L bioreactors

Culture	M_{max}* (g/l)	V (%)	μ (day^{-1})	T (day)	P (g/l day)
Cell cultures in 250 ml flasks					
Initial cell culture (before cryopreservation)	14.1 ± 2.0	90 ± 4	0.19 ± 0.03	3.6 ± 0.2	0.86 ± 0.14
Cell culture regenerated after 5 years cryogenic storage	13.2 ± 2.2	91 ± 3	0.17 ± 0.02	4.1 ± 0.6	0.80 ± 0.16
Cell culture maintained for 5 years by repetitive subcultures at 24 ± 3 °C	9.0 ± 0.8	84 ± 2	0.14 ± 0.01	4.9 ± 0.3	0.54 ± 0.06
Cell cultures in 20 L bioreactors					
Initial cell culture (before cryopreservation)	13.9 ± 2.0	88 ± 4	0.17 ± 0.02	4.1 ± 0.4	0.95 ± 0.18
Cell culture regenerated after 5 years cryogenic storage	13.6 ± 1.8	89 ± 5	0.16 ± 0.02	4.3 ± 0.4	1.10 ± 0.15
Cell culture maintained for 5 years by repetitive subcultures at 24 ± 3 °C	8.8 ± 0.8	82 ± 2	0.12 ± 0.04	6.3 ± 2.1	0.58 ± 0.19

Source: Modified from Titova et al. (2011)

M_{max}: maximal biomass concentration (dry weight bases); *V*: cell viability; μ: specific growth rate; *T*: generation (doubling) time of cell culture; *P*: culture productivity on dry weight basis

Thus, on the basis of the review of aforementioned studies, one may conclude that programmed freezing is most applicable to cryopreservation of undifferentiated cell cultures. This may have been due to the decline in interest to use cell cultures as a source of bioactive metabolites in the 1990s when vitrification-based methods started to develop. Meanwhile, more recent studies demonstrated high effectiveness of two-step freezing, vitrification and encapsulation approaches for cryopreservation of embryogenic and non-embryogenic cell cultures of *Gentiana tibetica* (Mikula 2006), *Catharanthus roseus* (Samar et al. 2009), *Sapindus mukorossi* (Kim et al. 2006b) and a few other species (Heine-Dobbernack et al. 2008).

3.5 Cryopreservation of Isolated Root Cultures

In vitro cultures of adventitious or transformed (hairy) roots are widely used for the production of bioactive compounds and constitute a convenient model to study plant morphogenesis, growth, and development (Hahn et al. 2003). Conservation of hairy and adventitious roots is valued for research and development because of the high levels of their morphological uniformity, genetic stability and high regeneration ability.

Cold storage of isolated root cultures at 4 °C has been reported with high survival rate but this method is useful only for short-term conservation. For example, Yoshimatsu et al. (1996) successfully stored genetically transformed hairy root segments and tips (1 mm) on growth regulator-free medium at 4 °C for nearly 4 months. Through cryopreservation of a competent culture, it may be possible to store root material permanently and to reintroduce it into culture without any loss of morphogenic and biosynthetic capacity (Benson and Hamill 1991). Several factors such as culture age, pre-growth, cryoprotection, freezing rate and post-freeze culture conditions play important roles in recovery after cryopreservation. Primary and secondary roots showed similar post-cryopreservation regeneration (88–95 %), while root sections without apices displayed significantly lower regeneration (65 %) (Salma et al. 2014). Park et al. (2014) suggested an optimized cryopreservation process including preculture, osmoprotection, cryoprotection and unloading for high recovery of *Rubia akane* hairy roots which are very sensitive to cytotoxicity of cryoprotectant solutions.

Benson and Hamill (1991) were the first to perform cryopreservation of hairy roots of *Beta vulgaris* and *Nicotiana rustica*, using programmed and ultra-rapid freezing. Since then, cryopreservation of isolated roots of various plant species has been reported using different techniques (Table 3.5) such as programmed freezing (Teoh et al. 1996), vitrification (Jung et al. 2001), encapsulation–dehydration (Hirata et al. 2002; Lambert et al. 2009) and encapsulation– vitrification (Xue et al. 2008). Earlier few study reported cryopreservation of root cultures by conventional programmed freezing or vitrification method, however encapsulation-dehydration technique is used more frequently. In this method encapsulation provides protection against physical damage and does not require toxic cryoprotectants (Xue et al. 2008).

Table 3.5 Cryopreservation of isolated root cultures

Species	Conservation method	Survival[a]	Post-storage stability	References
Adventitious root cultures				
Hyoscyamus niger	Vitrification	93.3 %	Tropane alkaloid content retained at control level	Jung et al. (2001)
Panax ginseng	Vitrification	60 % (32.5 % actual root formation)	Production of 11 ginsenosides retained at control level or above it	Oh et al. (2009), Popova et al., unpublished data
Hairy root cultures				
Astragalus membranaceus	Enc-Vitrif.	5.6 %	Not given	Xue et al. (2008)
Ajuga reptance	Enc.-Deh.	20 %	Not given	Hirata et al. (2002)
Armoracia rusticana	Enc.-Deh.	60 %	Not given	Hirata et al. (1998)
Artemisia annua	Prog. freezing	65 %	Artemisinin content retained at control level	Teoh et al. (1996)
Atropa belladonna	Vitrification	83 %	Tropane alkaloid content retained at control level	Touno et al. (2006)
Beta vulgaris	Prog. freezing to 0 °C then directly to LN	>80 % (6 % actual root conversion)	Betacyanin and betaxanthin content retained at control level	Benson and Hamill (1991)
Eruca sativa	Ultra-Rapid	28.2 % (3.1 % actual root formation)	Not given	Xue et al. (2008)
	Enc.-Vitrif.	73.3 %	Not given	Xue et al. (2008)
Gentiana macrophylla	Enc.-Vitrif.	No regrowth	–	Xue et al. (2008)
Maesa lanceolata	Vitrification	No regrowth	–	Lambert et al. (2009)
	Enc-Deh.	90 %	Not given	Lambert et al. (2009)
Medicago truncatula	Vitrification	No regrowth	–	Lambert et al. (2009)
	Enc.-Deh.	53 %	Not given	Lambert et al. (2009)
Nicotiana rustica	Prog. freezing to 0 °C then directly to LN	23 %	Alkaloid production retained at control level	Benson and Hamill (1991)
Panax ginseng	Vitrification	60 %	Content of five ginsenosides retained at control level	Yoshimatsu et al. (1996)
Rubia akane	Droplet vitrif.	89 %	Not given	Kim et al. (2012b, 2014)
Vinca minor	Enc.-Deh.	>70 %	Vincamine content retained at control level	Hirata et al. (2002)

Modified from Popova et al. (2011)

[a]Mean survival after cryopreservation using an optimized protocol; *Enc.-Vitrif.*: encapsulation-vitrification; *Enc.-Deh.*: encapsulation-dehydration; *Prog. freezing*: programmed freezing

The droplet-vitrification technique developed by Panis et al. (2005) was proven to be very efficient with hairy roots of several species. An efficient protocol for the cryopreservation of madder (*Rubia akane* Nakai) hairy root cultures was developed using droplet-vitrification method (Kim et al. 2012b, 2014). In this method, also known as *foil vitrification*, the explants are dehydrated with highly concentrated vitrification solutions (VSs) and cryopreserved in tiny drops of VS attached onto aluminum foil strips which ensures higher cooling/rewarming rates compared to cryovials (Towill and Bonnart 2003). There is a possibility of combining different procedures. For *Dianthus caryophyllus* a combined encapsulation and droplet-vitrification method was applied with the use of V-Cryo-plate technique (Sekizawa et al. 2011). So far, the combined techniques remain less popular with root cultures but may be used widely in the future with the development of optimized procedures.

Regrowth assessment is critical to optimize the protocol for cryopreservation. The use of staining method to detect the viability without considering proliferation and elongation of explant may be misleading. Benson and Hamill (1991) reported a low percentage (3.1 %) of root development after ultra-rapid freezing of *Beta vulgaris*, even with higher (73.3 %) viability with FDA stain.

3.6 Cryopreservation of Pollen for Breeding

Pollen storage is of key importance to plant breeders and horticulturists involved in fruit and forest tree improvement. Pollen culture techniques have been used for decades to obtain haploids or homozygous diploid plants from various plant species including wheat, maize, rubber tree, apple, poplar and medicinal plants (Maheshwari et al. 1982). A consistent supply of viable pollen provided by pollen banks removes seasonal, geographical or physiological limitations of hybridization programs and supports hybrid development between genera and species. In addition, pollen storage could be critical in saving endangered or threatened plant species without harvesting plants from their natural habitats. There is a vast pool of studies providing strong evidence of a dramatic decline of pollinator species in Europe and North America which in turn leads to a significant reduction in seed production and decrement in heterozygosity of plant populations (Kearns and Inouye 1997). This negatively affects fruit production and may ultimately contribute to the complete loss of economically important genetic diversity (Hanna and Towill 1995).

Traditional pollen bank requires large field areas for maintaining plants at different stages to synchronize flowering, which is both costly and laborious. Other well-known methods of pollen banking for breeding purpose include refrigeration at 3–5 °C for short-term storage, freeze-drying and freeze-storage, vacuum-drying and cold storage in organic solvents (Bajaj 1987). However, frequently observed sharp reduction in pollen viability over the storage period is of major concern (Bajaj 1987). The majority of pollen cryopreservation studies were accomplished by conventional programmed freezing and freeze-drying methods, and were mainly limited to mature pollen. However vitrification has also been tested successfully for pollen cryopreservation in *Brassica* (Xu et al. 1997).

Generally low temperature (−20 °C) is not effective for the long-term storage of pollen whereas storage at −196 °C seems to be more reliable.

Cryopreservation of pollen has been considered the most efficient option for long-term conservation (Parton et al. 2002). Moreover, cryopreserved pollen can be easily transported across the countries as they are usually subjected to less stringent quarantine restrictions (Ganeshan et al. 2008). One of the major advantages of cryopreserving pollen is that it is much easier to handle compared to seeds or other plant tissue due to low water content, highly resistant exine and nonvacuolated cells (Bajaj 1987). Pollen cryopreservation normally does not require any expensive cryostats as pollen grains can be directly immersed in liquid nitrogen for long-term storage. They naturally contain many soluble antioxidants and enzymes. The outer layer of pollen is coated with sporopollenin, which can effectively withstand the oxidative stress during cryopreservation (Smirnova et al. 2009; 2012). Xu et al. (2014) reported that increasing generation of reactive oxygen species (ROS) during cryopreservation may help in improving pollen viability as recent study have shown that ROS may stimulate pollen germination and tube growth (Speranza et al. 2012).

Pollen viability after freezing at −180 °C was first reported by Konwlton in 1922. Since then cryopreservation of pollen has been successful with different plant species, including *Clianthus formosus* (Hughes et al. 1991), *Carica papaya* (Ganeshan 1986), *Juglans regia* (Luza and Polito 1985), *Panax ginseng* (Zhang et al. 1993), *Solanum tuberosum* (Weatherhead et al. 1978), Rose (Rajasekharan and Ganeshan 1994), *Citrus limon* (Rajasekharan et al. 1995), *Gladiolus* (Rajasekharan et al. 1994) and native orchid species (Marks et al. 2014). However, there are limited studies showing that pollen viability can be maintained beyond 1 year of storage in liquid nitrogen (Xu et al. 2014). Visser (1955) successfully germinated tomato pollen stored at −190 °C for 2 years and at −20 °C for 3 years. Successful storage of pollen obtained from *Lycopersicon hirsutum* and *L. peruvianum* at −80 °C for 10 months suggests that *Solanum* species may also be amenable to cryopreservation (Sacks and Clair 1997). However, the cryobiology of pollen is not well understood. Viability of pollen subjected to cryopreservation depends on a number of factors such as pollen moisture content, freezing and thawing procedure, physiological stage of the mother plant, flowering stage, plant vigor and genotypic differences (Crips and Grout 1984; Ganeshan et al. 2008). Aside from genotype-dependent variations in pollen storage behavior and longevity, the stage of pollen development and the method of collection may be important (Stanley and Linskens 1974). Dry-mature pollen grains freshly dehisced from anthers of healthy plants are in an ideal physiological condition for cryopreservation. Lowering the moisture content and ensuring low relative humidity at the time of collection can increase viability of cryopreserved pollen. Generally, pollen moisture content below 35 % is suitable for cryopreservation (Ichikawa et al. 1970); however, relative humidity below 50 % can be harmful and may reduce viability (Daniel 1955) although these parameters may vary from species to species. Binucleate pollen has been shown to be more tolerant to desiccation and storage due to thicker walls as compared to trinucleate pollen (Bajaj 1987).

Considerable interest exists for cryopreservation of orchid pollen as a tool to support both breeding practices and conservation of endangered species (Bernhardt and

Edens-Meier 2010; Vendrame et al. 2008). It is particularly important for the orchid industry, which is known to consistently demand novelty. In orchids, pollen grains are fused into a structure called the *pollinium*. Orchid pollen storage may resolve the issue with temporal and spatial separation between periods of sexual reproduction and can also be used for haploid plant development. Earlier conventional pollen storage methods involving low temperature and low humidity have been developed for several species (Ito 1965; Niimi and Shiokawa 1992; Towill 1985; Yates et al. 1991). Successful cryopreservation of orchid pollen using different methods has also been reported (Marks et al. 2014; Meeyot and Kamemoto 1969; Pritchard and Prendergast 1989; Seaton 1994; Shijun 1984; Vendrame et al. 2008).

3.6.1 General Steps for Pollen Cryopreservation

The first important step in the cryopreservation protocol is the collection of dry mature pollen pretested to verify the viability prior to cryopreservation. Generally pollen does not require any pre-treatment with cryoprotectant before cryopreservation. Pollen samples are kept either in gelatin capsules or butter paper pouches, sealed in airtight poly aluminum pouches, loaded to canisters and cryopreserved. The rewarming procedure of pollen samples is carried out at ambient temperature for 30–60 min followed by viability test and field pollination. Depending upon the purpose of storage, a variety of tissues such as microspore, pollen embryo and whole anthers can be stored in cryopreservation facilities.

Viability of stored pollen can be determined by using acetocarmine-fuchsin/triphenyl tetrazolium chloride, hanging drop technique, PEG technique or in vitro germination. However, fertilization and seeds setting are more effective and reliable methods of viability testing.

In addition to seeds and tissue cultures, cryopreservation of pollen is another area of considerable importance for the conservation of genetic resources. Thus, the establishment of pollen cryobanks will facilitate a regular supply of pollen, which may reduce the maintenance of orchards and nurseries and allow germplasm storage and international exchange with minimal restrictions. User-friendly database software Polbase is available for accessions stored in pollen cryobank.

3.7 Conclusion and Prospects

Climate change is a new threat for conserving global biodiversity and in combination with other human activity could lead to global mass extinctions. Current approaches to in situ conservation are not likely to be sufficient to address anticipated changes, creating an urgent need to develop new models and technologies. Long-term conservation of plants out of their natural habitat represents a number of challenges as maintaining them in the field requires extensive resources and leaves them prone to natural calamities.

Cryopreservation is regarded as the safest and most cost-effective strategy for long-term conservation of the germplasm of economically-important plant species as well as endangered plants. Development of cryopreservation methods for asexually propagated plants is of crucial importance as seeds have a limited shelf life and need to be periodically replenished. Many species produce seeds that cannot be stored for more than a couple of weeks, and some species rarely or never produce seed. During recent years, a significant progress has been made with the development of new cryopreservation techniques for non-orthodox and vegetatively propagated species. Currently available technologies offer the possibility of cryopreservation for many plants. Properly pretreated plant tissue such as dormant buds, pollen, roots can be stored in LN for an indefinite period of time. However, the efficiency of cryopreservation protocols depends on the genotypes of the plant and may vary not only for the individual plant species but also for cultivars and genetic lines. In addition, the physiological stage of plant material, its tolerance to osmotic, chemical, desiccation and cold stress, and the behavior during cooling and warming significantly affects survival and regeneration capacity of cryopreserved tissues. Thus, much more additional research is needed to further improve the amenability of cryopreservation methodologies as well as the rates of recovery of healthy and normal plants from cryopreserved tissues. In this regard, a systematic and rationalized approach based on sensitivity/tolerance of explants to various steps of the procedure may help in developing efficient commonly applicable cryopreservation protocols for new plant genotypes and species that are at high risk of extinction.

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