9. Cryopreservation and Germplasm Storage*

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1. Introduction

Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra-low temperatures, preferably at that of liquid nitrogen. The development of cryopreservation strategy for plant cells and organs has followed the advances made with mammalian systems, albeit several decades later. Even for mammalian systems, the discovery of chemicals with cryoprotective properties was a significant step towards the development and refinement of cryopreservation technology. A major breakthrough in this context was the finding that glycerol was capable of protecting avian spermatozoa from freezing injury (Polge et al., 1949). This generated widespread enthusiasm and renewed interest among people interested in low temperature preservation in such fields as biology and medicine. Since the early 1950's a number of low molecular weight neutral solutes have been identified as potential cryoprotectants, the most commonly recognized ones being dimethylsulfoxide (DMSO or Me_2SO) and glycerol. Dimethylsulfox-

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ide, originally used to prevent freezing damage to human and bovine red blood cells and bull spermatozoa (Lovelock and Bishop, 1959), has become a universal cryoprotectant. In recent years, considerable progress has been made in the low temperature preservation of red cells and platelets, leucocytes, bone marrow cells, protozoa, and helminth parasites of man and animals, insects and their cells and microorganisms (Ashwood-Smith and Farrant, 1980). Despite all these advances, unlike plants, most attempts to preserve animal organs at ultra-low temperature have met with limited success.

One of the earliest reports on the survival of plant tissues exposed to ultra-low temperature was made by Sakai when he demonstrated that very hardy mulberry twigs, upon induction of dehydration mediated by extracellular freezing, are capable of survival following immersion in liquid nitrogen provided the frozen samples are subsequently rewarmed slowly at an air temperature of 0 °C (Sakai, 1956). Historically, this finding had great significance in the understanding of the mechanism by which plant cells withstand such severe low temperature stress. Later, Sun (1958) achieved partial success when desiccated seedlings of *Pisum sativum* were immersed in liquid nitrogen. Although work continued on the freezing behaviour of tissues of various annual and perennial species, such studies were not extended to cultured plant cells or organs until a decade later when Quatrano (1968) first demonstrated that cultured flax (*Linum usitatissimum*) cells treated with DMSO are capable of survival after freezing to −50 °C. This is the first recorded case on the utilization of a cryoprotectant for the freezing of plant cells. This study was followed by a similar kind with cell cultures of *Ipomoea* and *Daucus carota* in which the former survived freezing to only −40 °C, while the latter survived immersion in liquid nitrogen (Latta, 1971). The experiments carried out with cell cultures of *D. carota* may thus be considered as the first successful attempt to cryopreserve cultured cells of any plant species. Since D. carota was one of the earliest species to be brought into culture, this became a model species to study cryobiology of cultured cells. Nag and Street (1973) successfully regenerated somatic embryos from a cell culture of *D. carota* which had been frozen to the temperature of −196 °C, while Dougall and Wetherell (1974) stored cell cultures of wild carrot in a frozen state. In retrospect, it is fortunate that the early investigators chose *D. carota* cell cultures, albeit unknowingly, for their research since even today certain cell cultures are extremely sensitive to freezing injury (Kartha, 1985). Since the report on the successful cryostorage of *D. carota* cell cultures, several types of material such as callus and protoplasts, meristems/shoot-tips, zygotic and somatic embryos, anthers/pollen and even whole seeds have been studied extensively from a cryopreservation perspective. Examples of successful application of cryopreservation technology to a wide array of plant material can be found in Table 1.

Table 1. List of successfully clyopreserved culture systems

Species	References
A. Meristems/Shoot-tips	
Arachis hypogaea	Bajaj (1979)
Asparagus officicinalis	Uragami et al. (1990)
Brassica napus	Benson et al. (1984)
B. oleracea	Harada et al. (1985)
Carica papaya	Towill (1990b)
Cicer arietinum	Kartha and Gamborg (1978); Bajaj (1979)
Dianthus caryophyllus	Anderson (1979); Seibert (1976) (1977); Seibert and Wetherbee (1977); Uemura and Sakai (1980); Dereuddre et al. (1988); Langis et al. (1990); Towill (1990b)
Fragaria × *ananassa*	Kartha et al. (1980); Sakai et al. (1978)
Lactuca sativa	Seibert (1977)
Lillium sp.	Bouman and de Klerk (1990)
Lycopersicon esculentum	Grout et al. (1978)
Malus domestica	Katano et al. (1983); Sakai and Nishiyama (1978); Katano (1986); Kuo and Lineberger (1985); Tyler and Stushnoff (1988a, b)
Manihot esculenta	Bajaj (1977a; 1983a); Kartha et al. (1982a); Marin et al. (1990a)
Mentha aquatica × *M. spicata*	Towill (1990a)
Pisum sativum	Kartha et al. (1979); McAdams et al. (1991)
Phoenix dactylifera	Bagniol et al. (1990)
Pyrus communis	Dereuddre et al. (1990a, b)
Ribes sp.	Sakai and Nishiyama (1978)
Rubus sp.	Sakai and Nishiyama (1978); Reed (1988)
Sambuscus racemosa	Sakai and Nishiyama (1978)
Solanum etuberosum	Towill (1981a)
S. goniocalyx	Grout and Henshaw (1978)
S. phureja	Fabre and Dereuddre (1990)
S. tuberosum	Standke (1978); Bajaj (1978); Towill (1981b; 1990b); Benson et al. (1989); Manzhulln et al. (1983); Harding (1990); Harding et al. (1991)
Trifolium T. pratense (red clover)	Yamada et al. (1991)
repens (white clover)	Thorn (1990)
Vanda hookeriana	Kadzimin (1988)
Xanthosoma sp.	Zandvoort (1987)
B. Cell suspensions	
Acer pseudoplatanus	Sugawara and Sakai (1974); Nag and Street (1975a, b); Withers (1978)
Atriplex sp.	Goldner et al. (1990)
Atropa belladonna	Nag and Street (1975a, b)
Berberis dictyophylla	Withers (1985)
B. wilsoniae	Reuff (1987)
Brassica napus	Weber et al. (1983)
B. campestris	Langis et al. (1989)
Brunefelsia dentifolia	Pence (1990)
Capsicum annum	Withers and Street (1977)
Catharanthus roseus	Kartha et al. (1982b); Chen et al. (1984a, b); Mannonen et al. (1990); Schrijnemarkers et al. (1990)

Table 1. Continued

Species	References
Citrus sinensis	Kobayashi et al. (1990); Sakai et al. (1990; 1991)
Datura innoxia	Hauptman and Widholm (1982); Weber et al. (1983)
D. stramonium	Bajaj (1976)
Daucus carota	Nag and Street (1973; 1975a, b); Bajaj (1976); Dougall and Whitten (1980); Hauptman and Widholm (1982); Weber et al. (1983); Withers and Street (1977)
Dioscorea deltoidea	Butenko et al. (1984)
Digitalis lanata	Deitrich et al. (1982); Seitz et al. (1983)
Glaucium flavum	Withers (1985)
Glycine max	Bajaj (1976); Weber et al. (1983)
Hyoscyamus muticus	Withers (1985)
Malus domestica	Dereuddre and Kartha (1984)
Musa sp.	Panis et al. (1990)
Myrtillocactus geometrizans	Haffner (1985)
Nicotiana plumbaginigolia	Maddox et al. (1982)
N. sylvestris	Maddox et al. (1982)
N. tabacum	Bajaj (1976); Hauptman and Widholm (1982); Maddox et al. (1982); Withers (1985); Schrijnemakers et al. (1990)
Onobrychis viciifolia	Withers (1985)
Oryza sativa	Cella et al. (1982); Sala et al. (1979); Finkle et al. (1982); Finkle and Ulrich (1982); Ulrich et al. (1984a); Kuriyama et al. (1989); Schrijnemakers et al. (1990); Meijer et al. (1990)
Panax ginseng	Butenko et al. (1984); Seitz and Reinhardt (1987); Mannonen et al. (1990)
Papaver somniferum	Friessen et al. (1991)
Pennisetum americanum	Withers (1985)
Petunia hybrida	Schrijnemakers et al. (1990)
Picea abies	Galerne and Dereuddre (1987); Gupta et al. (1987)
P. glauca	Kartha et al. (1988)
Pinus taeda	Gupta et al. (1987)
Populus sp.	Binder and Zaerr (1980a)
Pseudotsuga menziesii	Binder and Zaerr (1980b)
Puccinella distans	Jekkel et al. (1990)
Rhazya orientalis	Withers (1985)
R. stricta	Withers (1985)
Rosa 'Paul's Scarlet'	Withers and King (1980)
Saccharum spp.	Chen et al. (1979); Finkle and Ulrich (1979); Finkle et al. (1983); Ulrich et al. (1979); Gnanapragasam and Vasil (1990)
Solanum melongena	Withers (1985)
Sorghum bicolor	Withers and King (1980)
Tabernaemontana divaricata	Schrijnermakers et al. (1990)
Triticum aestivum	Chen et al. (1985)
T. monococcum	Withers (1985)
Vinca minor	Caruso et al. (1987)
Zea mays	Withers and King (1980); Shillito et al. (1989)
C. Callus	
Gossypium arboreum	Bajaj (1982)

Table 1. Continued

Species	References
G. hirsutum	Bajaj (1982)
Hordeum vulgare	Hahne and Lorz (1987)
Lavandula vera	Wanatabe et al. (1983, 1990)
Medicago sativa	Finkle et al. (1979, 1983); Cachita et al. (1990)
Oryza sativa	Finkle et al. (1979, 1983)
Phoenix dactylifera	Tisserat et al. (1981); Ulrich et al. (1979)
Populus euramericana	Sakai and Sugawara (1973)
Saccharum spp.	Ulrich et al. (1979); Ling et al. (1987)
Triticum aestivum	Chen et al. (1985)
Trifolium spp.	Cachita et al. (1990)
Ulmus americana	Ulrich et al. (1984b)
D. Protoplasts	
Atropa helladonna	Bajaj (1988)
Bromus inermis	Mazur and Hartman (1978)
Datura innoxia	Hauptman and Widholm (1982)
Daucus carota	Hauptman and Widholm (1982); Mazur and Hartman (1978); Takeuchi et al. (1980, 1982); Weber et al. (1983)
Glycine max	Weber et al. (1983); Takeuchi et al. (1982)
Hordeum vulgare	Takeuchi et al. (1982)
Nicotiana tabacum	Bajaj (1982)
Oryza sativa × *Pisum sativun*	Bajaj (1983b)
Secale cereale	Langis and Steponkus (1990b)
Triticum aestivum	Takeuchi et al. (1982)
T. aestivum × *P. sativum*	Bajaj (1983b)
Zea mays	Withers (1980)
E. Somatic embryos	
Asparagus officinalis	Uragami et al. (1989)
Citrus sinensis	Marin and Duran-Vila (1988)
Coffea arabica	Bertrand-Desbrunais et al. (1999)
Daucus carota	Withers (1979); Dereuddre et al. (1991a, b)
Elaeis guineensis	Engelmann et al. (1995); Engelmann and Duval (1986); Engelmann and Dereuddre (1988)
Manihot esculenta	Sudarmonowati and Henshaw (1990)
Xanthosoma	Zandvoort (1987)
F. Zygotic embryos	
Camellia sinensis	Chaudury et al. (1991)
Carya sp.	Pence and Dresser (1988)
Cocos nucifera	Bajaj (1984); Chin et al. (1989)
Elaeis guineensis	Grout et al. (1983)
Hevea brasiliensis	Normah et al. (1986)
Hordeum vulgare	Withers (1982)
Howea fosteriana	Chin et al. (1988)
Juglans regia	de Boucaud et al. (1991)
Lycopersicon esculentum	Grout (1979)
Manihot esculenta	Marin et al. (1990b)
Musa sp.	Mora et al. (1991)
Oryza sativa	Bajaj (1981)
Picea glauca (cotyledons)	Toivonen and Kartha (1989)

Table 1. Continued

Species	References
Triticum aestivum	Withers (1982)
Veitchia merrillii	Chin et al. (1988)
Zea mays	de Boucault and Cambecedes (1988)
G. Pollen embryos	
Arachis hypogaea	Bajaj (1983c)
A. villosa	Bajaj (1983c)
Atropa belladonna	Bajaj (1977b)
Brassica campestris	Bajaj (1983c)
B. napus	Bajaj (1983c)
Citrus spp.	Bajaj (1984)
Nicotiana tabacum	Bajaj (1977b)
Oryza sativa	Bajaj (1981)
Triticum aestivum	Bajaj (1983c)

2. The Rationale for Cryopreservation

Preservation of both plant and animal germplasm is an integral component of sustainable agriculture systems. From a crop improvement perspective, preservation of all valuable germplasm is being accorded a high priority (IBGR, 1985). It is estimated that of the over 240,000 species of angiosperms in the world, 3000 or more have been used for food at one time or another. Twelve major crops supply the world's population with food indicating man's dependence for food on only a few species of the several thousand angiosperms on earth (Wehner, 1988). Therefore a great deal of emphasis has been placed on the need to preserve plant genetic resources as a means of maintaining biodiversity.

Cultivated crops, on the basis of their mode of propagation, can be broadly grouped into vegetatively propagated and seed-propagated species. Both groups represent an enormous array of annual and perennial species. Amongst the vegetatively propagated species, some exhibit a high degree of heterozygosity and do not produce seeds. Such plants are clonally propagated through various vegetative means such as tubers or cuttings which generally have a limited life span. In addition to being labour intensive and expensive, this practice also increases the accompanying risks associated with field maintenance such as exposure to pathogens, pests, climatic perturbations and human error. Although the most economical means of germplasm storage for seed-propagated species is in the form of seeds, this is not always feasible because of the following reasons: (i) some crops do not produce viable seeds, (ii) some seeds remain viable for a limited duration only and are recalcitrant to storage by reduction of moisture content and temperature, (iii) seeds of certain species deteriorate rapidly due to seed-borne pathogens, and (iv) some seeds are very heterozygous and, therefore, not suitable for main-

taining true-to-type genotypes (Kartha, 1982, 1985). Accordingly, an effective approach to circumvent the above problems may be the application of cryopreservation technology. It is now well established that one of the disciplines of plant tissue culture, meristem culture, is an extremely valuable technique with proven record of applications in clonal propagation, retention of genetic fidelity among propagules and elimination of viral pathogens (see chapter 3). These unique attributes make plant meristems ideal candidates for cryogenic preservation and establishment of virus-free germplasm banks and also in their international exchange (Kartha, 1985). In recent years, cryopreservation of zygotic embryos, embryo axes, and even whole seeds is also gaining popularity for the preservation of recalcitrant and tropical species.

Plant cell cultures have also become subjects of intensive research with emphasis on biotechnological applications such as genetic engineering, induction and isolation of mutants, selection of novel variants, screening for resistance against various stress factors, production of compounds of pharmaceutical value and creation of novel species of plants by somatic cell hybridization. As in the past, increasing use of cell cultures is also being made in addressing academic questions in physiology, biochemistry, genetics, and pathology, to name a few. It is now well known that cell lines subjected to any biotechnological manipulations become 'unique germplasm' in their own right and assurance of their uniqueness thus becomes a priority issue. Maintenance of cell or callus cultures in a continued state of division by repeated subculture on appropriate nutrient media is the practice often followed in several laboratories, unfortunately even today. Often, such a practice results in increased ploidy, accumulation of spontaneous mutations, decline and/or loss of morphogenic potential and biosynthetic capacity for product formation, reversion of valuable mutants to wild types, and most importantly, unintentional selection of undesirable phenotypes. Storage of cells in liquid nitrogen (−196 °C) may well alleviate these problems since at such an ultra-low temperature, all metabolic activities of cells are arrested and, consequently, no genetic changes would occur as a function of passage of time. The advantages offered by cryopreservation in preserving both the genetic stability and induced variability in plant cell cultures and organs alone are sufficient justifications to consider cryopreservation as an adjunct activity in any plant cell culture laboratory.

3. Cryopreservation Components

3.1. *Candidates for Cryopreservation*

An array of plant material could be considered for cryopreservation as dictated by the actual needs vis-a-vis preservation. These include meristems, cell, callus and protoplast cultures, somatic and zygotic embryos, anthers,

pollen or microspores and whole seeds. Most of the developmental research on cryopreservation was focused on meristems or shoot-tips, cell cultures and somatic and/or zygotic embryos with the objective of preserving clonally propagated species, experimental cell lines and problem crop species (recalcitrant) where conventional storage strategies are inadequate. It is not the intent of this chapter to cover the work carried out with all types of such material since detailed information is available in a number of publications (for a comprehensive treatment of the subject matter, see Withers, 1985; Kartha, 1985). This chapter, would instead concentrate on the technology of cryopreservation and discuss various factors which influence the survival of different culture systems at ultra-low temperature.

3.2. *Pretreatments and Cryoprotection*

Plant cells contain high amounts of cellular water and freezing of plant cells implies conversion of some or all of their liquid water to ice, whereas thawing is reversal of this transition. In plant tissues, the majority of free water is involved in solvation and thus available for freezing, whereas bound water is associated with macromolecular constituents of the cells in a structural and/or functional role (Mazur, 1969; Levitt, 1980). Although freezing of plant cells involves both free and bound water, freezing of the latter has been considered to be more harmful to survival (Levitt, 1980). Therefore, any mechanism by which less of free water is made available for freezing will provide greater chances of survival of the cells or tissues post-freezing. Plant species which are capable of withstanding severe freezing stress in nature have evolved mechanisms to accomplish this by allowing the water to either remain supercooled or by permitting the outflow of free water to extracellular space by exosmosis where they freeze resulting in the promotion of freeze- induced dehydration. The freeze-induced dehydration further depresses the freezing point of the remaining cellular water thus facilitating the cells/tissues to withstand exposure to even lower temperatures including that of liquid nitrogen. On the other hand, in the case of cryopreservation, since most of the experimental systems (meristems, shoot-tips, cultured cells etc.) contain high amount of cellular water and hence are extremely sensitive to freezing injury, protection from freezing and thawing injury has to be imposed artificially. This involves various strategies such as the use of cryoprotectants or other manipulations as explained below.

3.2.1. *Cryoprotectants*

Since cryopreservation involves essentially three steps, specimen treatments and freezing, storage at ultra-low temperature, and thawing, an ideal cryoprotectant should protect cells from all the factors which would affect the viability of the frozen biological sample during all these stages. Since viability

loss is not expected to occur at ultra-low temperatures, protection of the cells during freezing and thawing becomes of paramount importance. A number of compounds such as glycerol, DMSO, ethylene glycol, polyethylene glycol, sugars and sugar alcohols either alone or in combination protect living cells against damage during freezing and thawing. Such compounds can lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates. Although the exact mechanism of action of the cryoprotective compounds is still poorly understood, the colligative properties of the cryoprotectants can minimize the deleterious action of excessive electrolyte concentration resulting from removal of water and conversion of water to ice (Nash, 1966). The neutral solutes prevent the attainment of critical injurious level of solute concentration in a colligative manner by reducing the solute accumulation at a given level of dehydration to which the biological system is exposed (Lovelock, 1953; Meryman et al., 1977). The neutral cryoprotectants may also interact directly with the membrane systems to counteract the deleterious effect of freeze concentration (Santarius, 1971; Volger and Heber, 1975) or afford protection by eliciting a membrane configuration which is inherently more resistant to freezing stress (Williams and Meryman, 1970). Heber and co-workers (Heber and Santarius, 1964; Heber et al., 1971; Volger and Heber, 1975) and Santarius (1971, 1973) while demonstrating the cryoprotective properties of sugars, amino acids, organic acids and low molecular weight proteins showed that these compounds prevent freeze-induced uncoupling of photophosphorylation. Lineberger and Steponkus (1980) presented evidence to support that differential cryoprotection is afforded to chloroplast thylakoids against freeze-induced uncoupling of cyclic photophosphorylation by equimolar concentrations of glucose, sucrose, and raffinose. This differential protection effect appears to be due to nonideal activity-concentration profiles exhibited by sugars during freezing. For a detailed treatment of the role played by cryoprotective compounds in the viable freezing of plant tissue see Finkle et al. (1985).

The cryoprotectants generally used for freezing biological specimens fall into two categories, permeating and non-permeating. The most commonly used permeating additives are dimethylsulfoxide (DMSO) and glycerol. Dimethylsulfoxide permeates into cells more rapidly than glycerol, and therefore, requires shorter treatment duration. In regard to the toxicity, at equimolar concentration glycerol is less toxic to cells than DMSO while DMSO is superior to glycerol in cryoprotection of plant cells and organs. Here again, the permeation capability of glycerol is species-specific and temperature dependent. For example, at 22 °C, glycerol does not enter the cells of *Haplopappus gracilis*, but does enter *Acer saccharum*, while certain sugars like sucrose do not penetrate either type of cell (Towill and Mazur, 1976). On the other hand, the penetration rate of glycerol at 20 °C was twice that at 0 °C (Mazur and Miller, 1976). The non-permeating compounds include sugars, sugar alcohol and high molecular weight additives, polyvinylpyroli-

done, polyethylene glycol (PEG), dextran, hydroxyethyl starch/etc. Most of the cryoprotectants exhibit varying degrees of cytotoxicity at higher concentrations. Generally a concentration of 5–10% for DMSO and 10–20% for glycerol is adequate for most material. In instances where application of a single cryoprotectant does not result in survival, a mixture of cryoprotectants has been beneficial (Chen et al., 1984a, b; Finkle and Ulrich, 1979; Finkle et al., 1985; Withers, 1985). Ulrich et al. (1979) demonstrated the beneficial effect of using a mixture of cryoprotectants involving PEG, glucose, and DMSO (10–8–10%) in the successful cryopreservation of sugarcane callus. In subsequent studies several other cell or callus lines have been cryopreserved using the same mixture (Finkle et al., 1985). Prior to the discovery of a sorbitol – DMSO system (Chen et al., 1984a), the most effective cryoprotectant mixtures applicable to a variety of cell cultures were 0.5 M DMSO + 0.5 M glycerol + 1 M sucrose, and 0.5 M DMSO + 0.5 M glycerol + 1 M proline (Withers, 1985). It is to be borne in mind that the exposure duration of cryoprotectants to cells needs to be such that the concentration applied does not cause sudden plasmolysis which in itself could be a major cause of injury to the osmotic responsiveness of the cells. Although opinions differ as to the method of application of cryoprotectants to the cells, it is preferable to apply them very gradually in an ice bath at 4 °C in order to minimize any injury. Similarly, the removal of cryoprotectants from the thawed samples should also be a gradual process to alleviate the problems associated with deplasmolysis.

3.2.2. *Preculture Strategies*

Where the application of cryoprotectants, singly or in combination, at the nontoxic level becomes inadequate to ensure survival following cryopreservation, certain physiological modifications have been attempted with various types of culture systems to overcome the problem.

Incorporation of cryoprotectants into the culture medium followed by an incubation of isolated shoot-tips or meristems prior to their freezing has been the practice of choice. Pea and strawberry meristems precultured on respective shoot regeneration media supplemented with 5% DMSO for a period of two days and frozen at slow cooling rates exhibited enhanced survival (Kartha et al., 1979, 1980). The same method also resulted in enhanced survival in case of potato meristems (Manzhulin et al., 1983). Substituting 5% glycerol for DMSO only marginally enhanced the survival of strawberry meristems over the controls (Kartha et al., 1980). The DMSO preculture effect on the survival of pea meristems was further studied recently combining the preculture duration and the terminal freezing temperature. It appears that for pea shoot-tips immersed in liquid nitrogen (LN_2), the terminal freezing temperature had no effect whereas the number of days of preculture in 5% DMSO supplemented medium did influence the survival, with a 2-day preculture duration being the optimum. In pea shoot-tips not

immersed in LN_2 the number of days of preculture did not influence survival whereas the terminal freezing temperature had an effect (McAdams et al., 1991). Higher rates of survival and plant regeneration were also obtained when shoot meristems of white clover (*Trifolium repens*) were precultured on agar-B5 medium supplemented with 5% DMSO and 5% glucose at 4 °C for two days prior to freezing by slow cooling (Yamada et al., 1991).

Preculturing isolated meristems or shoot-tips for a specific duration on shoot regeneration medium without the addition of cryoprotectants has also been found to enhance survival. For example, *Solanum goniocalyx* (Grout and Henshaw, 1978) and *S. etuberosum* (Towill, 1981a) shoot-tips exhibited enhanced survival if the freshly isolated explants were precultured in light on the regeneration medium for a period of 2 to 3 days prior to cryoprotectant treatment and freezing. Benson et al. (1989) carried out detailed investigations on pre- and post-freeze light regimes on the survival of *S. tuberosum* shoot-tips. They concluded that post-freezing survival was significantly affected by different light combinations and two different cultivars tested exhibited different pre-light requirements. However, other than the physiological alterations brought about by various light combinations, the exact manner in which light conditions influence cryosurvival still remains to be elucidated. Sucrose is the major carbon source in all plant tissue media and it is a well known cryoprotectant. Obviously, it is no surprise that several attempts have been made to utilize sucrose in the preculture media as well as the cryoprotectant during the actual freezing of plant material. Somatic embryos of oil palm (*Elaeis guineensis*) exhibited excellent survival if cryopreserved following 7-day preculture on nutrient medium enriched with 0.75 M sucrose (Engelmann et al., 1985; Engelmann and Duval, 1986; Engelmann and Dereuddre, 1988). Similar effect has also been noted for the cryopreservation of somatic embryos of coffee (Bertrand-Desbrunais et al., 1988) and *Picea abies* (Galerne and Dereuddre, 1987) and sweet orange (Marin and Duran-Vila, 1988). Regrowth rates of close to 100% were obtained with cryopreserved apical shoot tips isolated from 2-month old carnation stems precultured on medium supplemented with 0.75 M sucrose and treated with 5% DMSO or more (Dereuddre et al., 1988). An interesting aspect in this study was the finding that the resistance of axillary shoot tips to freezing decreased as a function of their distance from the apex implying that preculture in sucrose-supplemented medium alone cannot counteract the inherent developmental and physiological differences found in explants derived from the same plant.

Cell suspension cultures have been extensively used for cryobiological research because of their ease of availability, handling and maintenance. However, cells present in any suspension exhibit an array of phenotypes, physiological variations, differences in mitotic oscillations, ploidy and cell cycle. From a cryobiological prospective then, it is always possible to find certain fraction of the cell population in a stage condusive to freezing. In other words, cryopreservation of cell cultures is relatively easier to ac-

complish as compared to organized structures such as meristems where structural conformity has to be maintained in order to ensure faithful regeneration of plantlets. Although extensive studies have not been carried out to examine the role of heterogeneity brought upon the culture by the variables mentioned above, it is now apparent that cells have to be maintained in an active state of division often accompanied by frequent subculture. Highly vacuolated and quiescent cells are extremely sensitive to cryoprotectant treatment and freezing and such cells are naturally eliminated during the specimen preparation stage (Kartha et al., 1988). In attempts to enrich the cell population most resistant to freezing, Sugawara and Sakai (1974) observed high viability of *Acer pseudoplatanus* cells when cells at their late lag phase or early division phase were used for cryopreservation. In a subsequent study with cell cultures of the same species, Withers (1978) obtained enhanced post-freezing survival and mitotic index from the cells newly entered into Gl phase of the cell cycle. Sala et al. (1979) and Withers and Street (1977) presented evidence to indicate that the viability and regrowth potential of cryopreserved cells are enhanced when cells in late lag and early- to mid-exponential phases are selected for experimentation. The sensitivity of cells in early lag phase or stationary phase to freeze-thaw stress is attributable to the increase in cell size, vacuolar volume and water content whereas the resistance of small sized cells to freezing stress and thereby enhanced survival may relate to their dense cytoplasm. Therefore, one approach to enhance the post-freezing survival of cells is to reduce the cell volume and water content by inducing partial dehydration in prefreezing steps. Supplementation of culture medium with such osmotically active compounds as mannitol, sorbitol, sucrose, and proline increases freezing resistance. Mannitol has been found to be beneficial in reducing the mean cell volume of cells of *Acer pseudoplatanus* and *Capsicum annuum* and increasing their post-freezing survival (Withers and Street, 1977). A preculture strategy of using 3 to 6% mannitol has been successfully used for the cryopreservation of a number of cell cultures such as *Nicotiana tabacum*, *N. plumbaginifolia* (Maddox et al., 1982/83), soybean and sycamore (Pritchard et al., 1986a, b) and *Digitalis lanata* (Diettrich et al., 1982, 1985). Pritchard et al. (1986a, b) examined osmotic stress as a pregrowth procedure for cryopreservation of sycamore and soybean cell suspensions. Addition of 6% mannitol or sorbitol to liquid culture medium decreased the water potential by – 0.93 MPa. Sycamore cells grown to exponential phase in such media exhibited increased levels of total and soluble protein and respiratory activity, but decreased free proline. Soybean cells showed increased respiratory activity and free proline levels, but total protein levels remained unaffected. Water relation studies indicated that sycamore cells are capable of greater osmotic adjustment than soybean cells, and that mannitol uptake does not contribute significantly to that effect. The differential behaviour in osmotic adjustment may be attributable to the differences noticed in responses of cells to mannitol treatment and post freezing survival. For example, preculturing in mannitol supplemented medium did

not enhance the survival of *N. sylvestris* (Maddox et al., 1983) or alkaloid-producing cell cultures of *Catharanthus roseus* (Chen et al., 1984a).

Sorbitol has been used either as an osmotic agent in the preculture medium or as the sole cryoprotectant during the freezing process. Weber et al. (1983) successfully cryopreserved cell cultures of *Glycine max*, *Datura innoxia*, *Brassica napus*, and *Daucus carota* and cell culture-derived protoplasts of *G. max*. Similarly, sorbitol has been successfully used as the sole cryoprotectant in the cryopreservation of *Coleus blumei* cell cultures (Reuff et al., 1988). However, cell cultures of alkaloid producing *Catharanthus roseus* could not be cryopreserved using sorbitol as the only cryoprotectant (Chen et al., 1984a), but they could be successfully cryopreserved by preculturing the cells in nutrient medium containing 1 M sorbitol for 6–20 hours followed by freezing using 1 M sorbitol and 5% DMSO. In this study, equimolar concentrations of $CaCl_2$ and KCl, in addition to being toxic to cells, were ineffective in their cryoprotective efficacy. Although considerable survival was noticed subsequent to preculturing the cells in media supplemented with 1 M concentrations of glucose, trehalose, and sucrose, the overall survival and recovery was much superior when sorbitol was used as the preculturing agent.

In order to understand the mode of action of cryoprotectants, the freezing behaviour of DMSO and sorbitol solutions and the alkaloid producing *C. roseus* cells treated with DMSO and sorbitol, alone or in combination, were examined by nuclear magnetic resonance (NMR) and differential thermal analysis (DTA). Incorporation of DMSO or sorbitol into the liquid medium had a significant effect on the temperature range from initiation to completion of ice crystallization. Compared to control, less water crystallized at temperatures below −30 °C in DMSO-treated cells. Similar results were obtained with sorbitol-treated cells, except sorbitol has less effect on the amount of water crystallized below −25 °C. There was a close association between the percent unfrozen water at −40 °C and per cent survival after freezing in liquid nitrogen. These studies indicated that in the alkaloid-producing *C. roseus* cells, the amount of liquid water at −40 °C is critical for successful cryopreservation and the combination of DMSO and sorbitol was effective in preventing this critical level of water from freezing (Chen et al., 1984b).

Attempts to cryopreserve an embryogenic cell culture of white spruce (*Picea glauca*) using DMSO as the only cryoprotectant were unsuccessful, and DMSO was found to cause 35–40% viability loss. However, successful cryopreseration could be accomplished following the sorbitol preculture strategy. Additionally, sorbitol was found to counteract the toxicity caused by DMSO (Kartha et al., 1988). The same procedure has been successfully extended to the cryopreservation of an embryogenic cell culture of a sugarcane (*Saccharum*) hybrid leading to efficient plant regeneration (Gnanapragasam and Vasil, 1990). The sorbitol – DMSO system is also applicable to embryogenic cell cultures of other conifer material such as *Picea glauca* and *Laryx* species (Kartha, unpublished).

Other additives to preculture media to enhance post-cryopreservation sur-

vival include various amino acids including proline (Butenko et al., 1984). Withers and King (1979) tested a number of amino acids for cryoprotection, and found that proline is only one of those that can cryoprotect plant cells. They suggested that proline might act as a membrane stabilizer. Hellergen and Li (1981) while trying to elucidate the mode of action of proline on the protection of *Solanum tuberosum* suspension cultures frozen to −14 °C reported that proline might exert its effect by the removal of excessive intracellular water by osmotic gradient. If this is the case, any potent osmoticum should be able to do the same since sucrose at about the same concentration acted in the same way as proline (Hellergren and Li, 1981). From the above discussion it becomes very clear that appropriate preculture strategies coupled with judicious selection and application of cryoprotectants can definitely increase cryosurvival.

3.2.3. *Cold Acclimation*

Plant species with inherent capacity to withstand severe freezing stress have evolved various mechanisms; one such mechanism being cold hardening or cold acclimation. These species achieve cold hardening by responding to such early environmental cues as low temperature, reduced photoperiod or a combination thereof (Levitt, 1980). The underlying physiological and molecular mechanisms of freezing tolerance of plants are not well understood. Hardy plants undergo a series of metabolic and physiological changes during cold acclimation. It is believed that these changes, which are promoted by low temperature, enable the plants to survive severe freezing stress. Preferential synthesis of some metabolites during cold hardening such as proteins, sugars and nucleic acids has been suggested to be induced by low temperature (Brown, 1978; Kacperska-Palacz, 1978; Levitt, 1980). Marked increases were also found in the content of total soluble sugars, reducing sugars and ATP in winter wheat during cold hardening (Perras and Sarhan, 1984). In subsequent studies, Perras and Sarhan (1989) showed that cold hardening induced important changes in the soluble protein patterns depending upon the tissue and cultivar freezing tolerance. For example, a 200 kilodalton protein was induced concomitantly in the leaves, crown and roots of cold acclimated winter wheat. Guy and Haskell (1987) have also found three high molecular weight induced proteins (160,117 and 85 kD) in spinach during cold acclimation. On the other hand, appearance of several small polypeptides (11 to 38 kD) were found in acclimated alfalfa seedlings (Mohapatra et al., 1987). Similarly, during induction of freezing tolerance in cell suspension cultures, low molecular weight polypeptides (20 to 48 kD) were detected in *Brassica napus* (Johnson-Flanagan and Singh, 1987) and *Bromus inermis* (Robertson et al., 1987).

Taken together, all the physiological and metabolic alterations that occur during the cold acclimation process influence the freezing survival of plants either directly or indirectly. In this context, two scenarios have been developed on the exploitation of the cold acclimation process in the context

of cryopreservation; the first involves utilization of explants from plants which are naturally acclimated and the other is the artificial induction of cold acclimation provided the experimental plants are capable of doing so, and use such material for cryopreservation. Both approaches have successfully resulted in cryopreservation of various types of plant material. Sakai and Nishiyama (1978) employed winter vegetative buds of apple for cryopreservation. They found that in hardy shoots of apple prefrozen at the temperature range from −30 to −50 °C, little or no injury was observed in the leaf buds and cortex following immersion in LN_2 and that the buds stored for almost two years still remained alive. The same approach, according to them, was applicable to hardy buds of other fruit crop species such as gooseberry, currant, raspberry and pear. Similarly, shoot-tips aseptically isolated from dormant apple buds survived immersion in LN_2 following pre-freezing below −10 °C without the application of cryoprotectants.

The process of cold acclimation is influenced by seasonal changes and, therefore, explants isolated from donor plants at various time periods may have a profound effect on their cryosurvival. Two examples amply illustrate this point. Apices prepared from brussels sprouts (*Brassica oleracea*) from late July to early November showed no tolerance to freezing to −30 °C and LN_2 while those obtained on 25 November or 16 December survived freezing to LN_2 temperature. Apices preconditioned by low temperature in autumn or winter could survive freezing in LN_2, whereas those isolated from plants growing in summer and which had experienced no low temperature could not survive freezing (Harada et al., 1985). In the case of apple, shoot tips suspended in distilled water survived freezing slowly to −40 °C if taken from January to middle of March and their freezing tolerance (FT) decreased to −20 °C from late March to early April. Again from early May to late October, the FT increased to −40 °C (Katano, 1986).

The second approach involves acclimating the donor plants or *in vitro* cultures to low temperature prior to explant isolation for freezing. Cold treatment of donor carnation plants at 4 °C for 3 days or more resulted in a doubling of the percentage of excised, frozen shoot apices which survived freezing and a 6 to 7-fold increase in the percent that formed leaf primordia and shoots (Seibert and Wetherbee, 1977).

Studies on cold acclimating *in vitro* cultures have been attempted. Cultures of apple and Saskatoon berry plants (*Amelanchier alnifolia*) subjected to a 10 week short photoperiod, low temperature hardening treatment, including a −3 °C exposure followed by 5–7 days at 2 °C were 4 to 8 °C hardier than untreated shoot cultures (Caswell et al., 1986). A correlation has been established between cold acclimation and the level of dehydration attained by dormant apple vegetative buds in relation to cryopreservation in studies carried out by Tyler and Stushnoff (1988a, b). They found that the survival of apple buds could be increased if the tissue was sufficiently dehydrated prior to cryopreservation. Buds collected early in the cold-acclimating period suffered injury as a result of dehydration, but the percent survival of the

dehydrated buds, after storage in LN_2 was greater than non-dehydrated buds. As cold acclimation progressed, the buds became more resistant to dehydration stress and the survival increased after cryostorage. Other studies on the effect of cold acclimation of *in vitro* plants on the survival of cryopreserved shoot tips are those by Kuo and Lineberger (1985) again with apple, Reed (1988) with *Rubus* spp. and Dereuddre et al. (1990a) with *Purus* spp.

Callus and cell cultures have also been used to study the effect of cold acclimation on cryopreservation. For example, Sakai and Sugawara (1973) showed that callus derived from the cambial area of poplar twigs survived freezing down to −120 °C or even the temperature of liquid nitrogen, after cold acclimation. Similarly growth at 2 °C increased the freezing tolerance of cultured pear cells (Wu and Wallner, 1985). In a study on the cryopreservation of winter wheat suspension culture and regenerable callus, it was found that cold hardening or ABA treatment before cryopreservation increased freezing resistance and improved survival in liquid nitrogen (Chen et al., 1985). While cold acclimation is an excellent way of enhancing cryosurvival, its application is limited to those species which exhibit this trait for low temperature acclimation. It is doubtful if tropical species would respond in a similar way.

3.2.4. *Application of Abscisic Acid*

Abscisic acid (ABA), a naturally occurring plant hormone, is implicated in eliciting an array of physiological and biological responses in plants such as stomatal control, water relations, photosynthesis, growth, dormancy, embryo maturation and adaptation to stress (for a review see Zeevaart and Crelman, 1988). It is also know as a 'stress hormone'. In recent years, ABA has been demonstrated to induce freezing resistance in plants (Irving, 1969), plant parts (Chen et al., 1983) and in plant tissue cultures (Chen and Gusta, 1983). It has been shown that tissue cultures from five species capable of cold hardening increased their cold hardiness upon treatment with ABA while species that could not harden, did not (Chen and Gusta, 1983). ABA was also effective in circumventing the requirements for both low temperature and lengthy acclimation periods in the induction of freezing tolerance in an embryogenic microspore-derived cell suspension culture of winter rape (*Brassica napus* cv. Jet Neuf). A higher level of freezing tolerance was induced when cells were cultured for 7 to 8 days in 50 μM ABA and 13% sucrose at 25 °C (Orr et al., 1986). *Bromus inermis* cell suspension cultures treated with 75 μM ABA for 7 days at 25 °C survived slow cooling to −60 °C and over 80% of the cells in ABA-treated cultures survived immersion in LN_2. As discussed earlier, cold hardening or ABA treatment of winter wheat suspension culture before cryopreservation increased the freezing resistance and improved the survival of the cells in LN_2 (Chen et al., 1985). The exact mechanism by which ABA imparts this effect remains to be elucidated.

3.3. *Freezing Methods*

3.3.1. *Slow Freezing*

Various physicochemical events occur during freezing of biological specimens at slow cooling rates. Mazur (1969) identified the events to which a cell is subjected during freezing and thawing. With temperature reduction the cell and its external medium initially supercool followed by ice formation in the medium. The cell membrane/wall act as a physical barrier and prevent the ice from seeding the cell interior at temperatures above ca −10 °C and thus the cells remain unfrozen but supercooled. As the temperature is further lowered, an increasing fraction of extracellular solution is converted into ice resulting in the concentration of extracellular solutes. Since the cells remain supercooled and its aqueous vapour pressure exceeds that of frozen exterior, the cell equilibrates by loss of water to external ice (dehydration). Slowly cooled cells reach equilibrium with the external ice by efflux of water and remain shrunk provided the cell is sufficiently permeable to water. In such cases, intracellular ice formation, considered to be one of the most important factors responsible for causing freezing injury, will not occur. This phenomenon is utilized in devising cryopreservation techniques by slow freezing.

A number of factors such as cooling rates, pretreatments and cryoprotection, type and physiological state of the experimental material, and the terminal freezing temperature (also called prefreezing temperature) influence the success of slow freezing method. If the freeze preservation equipment does not have the capability to predict the initiation of freezing the solution and mechanisms to override the supercooling of the sample, a linear cooling rate cannot be obtained. In other words, the specimen would be frozen at different cooling rates even though a precise cooling rate was originally selected. This biophysical variable can have a profound effect on the viability of the sample being frozen.

The most commonly used method of freezing meristems and cell cultures is by regulated slow cooling at a rate of 0.5 to 1.0 °C/min down to either −30, −35 or −40 °C followed by storage in liquid nitrogen. In some instances, a modification to slow cooling by holding the samples for a predetermined period of time in the temperature region of −30 to −40 °C is also practised and is referred to as 'step-wise freezing'. Meristems, cell cultures and somatic embryos of a number of species have been successfully cryopreserved following slow freezing methods (see Kartha, 1985; Withers, 1985).

3.3.2. *Rapid Freezing*

Rapid freezing is the simplest form of cryopreservation since the procedure does not require sophisticated and expensive equipment. It is believed that during rapid lowering of temperature, the cells do not have sufficient time to equilibrate with the external ice by efflux of water as explained for the

slow freezing method. However, the cells attain equilibrium by intracellular freezing which is lethal for most biological specimens. The extent of intracellular freezing or ice formation is also governed by the extent to which the cells have been dehydrated prior to their quenching in liquid nitrogen. Meristems and somatic embryos of a few plant species have been successfully cryopreserved by rapid freezing. How such cells survive is explained on the basis of the mechanism of Luyet (1937) which suggests that viability of cells may be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal crystal (ice) growth occurs (Seibert and Wetherbee, 1977, for cryopreservation of carnation meristems). In recent years, the conventional rapid freezing technique has been modified to evolve 'vitrification' effects resulting in the viable freezing of a number of culture systems (see the section 3.3.4)

3.3.3. *Droplet Freezing*

The basis of droplet freezing is identical to slow freezing. Protective dehydration of the cells is induced by regulated slow cooling avoiding the chances of intracellular ice formation. This technique was originally developed for the cryopreservation of cassava meristems (Kartha et al., 1982a).

In the droplet freezing method, the cryoprotectant solution (15% DMSO and 3% sucrose) is dispensed into droplets of 2 to 3 μl on an aluminum foil contained in a petri dish. Each cassava meristem, treated with the cryoprotectant solution, is then transferred to the droplets and frozen by programmed slow cooling at a rate of 0.5 °C/min to various sub-zero temperatures and stored in liquid nitrogen. This technique resulted in successful cryopreservation of cassava meristems. Before this technique can be adapted for cassava germplasm preservation, the plant regeneration frequency of cryopreserved meristems needs to be improved. The advantage of droplet freezing on aluminum foil could be attributed to the fact that the metal has an efficient thermal conductivity resulting in homogeneous cooling of the sample by uniform dispersion of temperature. Moreover, since a very small amount of cryoprotectant solution is used for each meristem, a uniform cooling rate occurs accompanied by less amount of extracellular ice formation, thus avoiding the excess mechanical pressure exerted on the cell wall as the ice propagates.

3.3.4. *Vitrification*

The development of vitrification techniques for the cryostorage of cultured plant cells and organs is of recent origin although Sakai as early as 1958 succeeded in obtaining the survival of hardy mulberry cortical tissues in LN_2 by vitrification. The conventional cryopreservation techniques so far discussed involved a freeze-induced cell dehydration step prior to LN_2 stor-

age. An alternate approach to cryopreservation is based on the ability of highly concentrated solutions of cryoprotectants to supercool to very low temperatures upon imposition of rapid cooling rates, to become viscous at sufficiently low temperatures, and solidify without the formation of ice. This process is known as vitrification (Rall and Fahy, 1985). If cells are capable of tolerating this severe osmotic stress to allow glass transition to occur, theoretically they should be able to survive the freezing process.

There are both advantages and disadvantages to vitrification. The advantages are that (i) it is a very simple process, and (ii) it does not require regulated cooling so that the need for expensive programmable freezing equipment is eliminated. The main disadvantage is that the highly concentrated levels of many cryoprotective agents required for vitrification may cause extreme toxicity to the cells. Toxicity of the vitrification solution is relative to its osmotic potential and therefore formulation of vitrification solutions differing in osmotic potential may alleviate the problem of toxicity (Langis and Steponkus, 1990a). The most commonly used compounds in vitrification solutions include DMSO, glycerol, ethylene glycol, polyethylene glycol, propylene glycol, sucrose, etc. In all cases where vitrification has been shown to occur, osmotic dehydration of the cells prior to their quenching in LN_2 has been found to be essential. Cryopreservation by vitrification has been successfully so far in a limited number of culture systems, such as cell cultures of *Brassica campestris* (Langis et al., 1989), navel orange (Sakai et al., 1990), somatic embryos of *Asparagus officinalis* (Uragami et al., 1989), *Daucus carota* (Dereuddre et al., 1991a, b), mesophyll protoplasts of *Secale cereale* (Langis and Steponkus, 1990b), shoot tips of mint, potato, papaya (Towill, 1990a, b) and carnation (Towill, 1990b; Langis et al., 1990). A brief description of the methodology of vitrification is given in Table 2.

3.4. *Cryostorage and Thawing Regimes*

Storage temperature should permit total immobilization of metabolic activities of cells and arrest the demand for energy inputs if long-term preservation is desired. This is accomplished at ultra-low temperatures such as that of liquid nitrogen (−196 °C). Ease of availability of liquid nitrogen makes this strategy feasible in most countries and preferable to other systems wherein any defect in the mechanical system or interruption in power supply could seriously endanger specimen viability.

The events occurring during thawing do not simply reflect freezing in reverse. If thawing is carried out at slow rates, structural changes in ice may occur which may or may not affect cell viability. Of primary importance is avoidance of recrystallization and ice crystal growth which may occur during warming. In order to avoid any potential problems, thawing is generally carried out rapidly by immersing the specimen for 1 to 2 minutes in 35 to 40 °C water bath.

Table 2. Examples of cryopreservation by vitrification

Cultures systems and references	Procedure
Cell cultures of *Brassica campestris* L. (Langis et al., 1989)	Equilibration of cells at 0 °C with 1.5 M ethylene glycol (EG); dehydration of cells at 0 °C in a concentrated solution containing 7.0 M EG + 0.88 M sorbitol + 6% (w/v) BSA; transfer of cell suspension into 0.5 ml polypropylene straw and quenching in liquid nitrogen (LN_2). Thawing in air for 10 s followed hy 10 s in an alcohol bath at 20 °C. Viability of 40% based on TTC assay. Note: To attain maximum viability, the cells were unloaded into a 1.5 osmolar sorbitol medium following thawing.
Mesophyll protoplasts of rye (*Secale cereale* L.) (Langis and Steponkus, 1990b)	Essentially the same as above.
Somatic emhryos of *Asparagus officinalis* (Uragami et al., 1989)	The vitrification solution (PVS) contained (w/v) 22% glycerol, 15% EG, 15% propylene glycol, and 7% DMSO in MS medium enriched with 0.5 M sorbitol. After initial cryoprotection with sorbitol-supplemented MS medium containing 12% EG, the cells and somatic emhryos were exposed stepwise to 85% PVS at 0 °C, loaded into 0.5 ml transparent straws and quenched into LN_2. PVS was removed and diluted stepwise after rapid thawing. Survival in the range of 50–65%; embryos developed into plantlets.
Cell cultures derived from nucellar tissues of navel orange (*Citrus sinensis*, var. *brasiliensis* Tanaka) (Sakai et al., 1990)	Cells were dehydrated with highly concentrated 60% cryoprotective solution (PVS2) containing (w/v) 30% glycerol, 15% EG, and 15% DMSO in Murashige and Tucker medium containing 0.15 M sucrose at 25 C for 5 min followed by chilling at 0 °C for 3 min. Aliquots of 0.1 ml treated cells were then loaded into 0.5 ml transparent plastic straws and quenched in LN_2. After rapid warming, the cell suspension was expelled in 2 ml of MT medium containing 1.2 M sucrose. Average survival rate was 80%. The vitrified cells regenerated plantlets.
Same cell culture as above (Sakai et al., 1990)	Procedure same as above except in that 1.8 ml plastic cryotubes were employed instead of plastic straws. The frozen cultures were stored for 40 days retaining an average survival rate of 90%. The method has been extended to six other species or cultivars of *Citrus*.
Shoot tips of Mint (Mentha aquatica × *M. spicata*) (Towill, 1990a).	Mint shoot tips were gradually exposed to a mixture containing 35% EG, 1.0 M DMSO and 10% PEG-8000 and quenched in LN_2. Rapid thawing at 35°C. Cooling and warming rates calculated at about 4800 and 9000 °C/min, respectively.

Table 2. Continued

Cultures systems and references	Procedure
	Survival ranged from 31 to 75% and in many cases, the frozen shoot tips regenerated into shoots without or with only slight callus formation.
Shoot tips of mint, potato, carnation and papaya (Towill, 1990b)	Preliminary report. The above procedure was applied with minor modification. Initial addition of DMSO and PEG was done slowly to facilitate permeation of DMSO into the shoot tips. The vitrification solution caused considerable loss of viability to shoot tips within an hour. Brief treatment was less toxic. The final concentration of between 35–50% (w/w) of either EG or PEG were effective on retaining viability upon exposure to LN.
	Exact survival rates not given.
Shoot-tip of carnation (*Dianthus caryophyllus*) (Langis et al., 1990)	Shoot tips isolated from either fresh cuttings or cuttings stored at 5 °C in the dark up to 16 weeks (cold acclimated) were equilibrated 60 min with 8 wt% EG (1.53 osm) at 20 °C or 10 wt% DMSO (1.53 osm) at 0 °C. Further dehydration of the shoot tips was carried out in a vitrification solution composed of 50 wt% EG + 15wt%sorbitol + 6 wt% BSA, for an optimal duration of 15 min. Following dehydration, the shoot tips and the solution were frozen in heat sealed polypropylene straw (0.5 ml) in LN_2. The samples were stored in LN_2 for 34 days. The shoot tips were recovered in 1.5 osm sorbitol solution at 20 °C and after 30 min, returned to a hypotonic semi-solid regeneration medium.
	This procedure resulted in 100% survival (76/76) for shoot tips initially equilibrated with EG.
Alginate-coated somatic embryos of Carrot (*Daucus carota*) (Dereuddre et al., 1991a, b)	Although the authors did not identify this procedure as Vitrification (Dereuddre et al., 1991a), the subsequent study on thermal analysis (Dereuddre et al., 1991b) showed that vitrification has in fact occurred during freezing. Somatic embryos of carrot were encapsulated in calcium alginate using standard procedure. The encapsulated embryos were precultured for 18 h in a nutrient medium enriched with 0.3 M sucrose. Prior to quenching in LN_2, the precultured embryo beads were dehydrated at room temperature, in a laminar air flow cabinet, for 4 h.
	Survival rates of up to 92% were obtained. Resistance of somatic embryos to LN_2 was correlated with glass transition hetween −50 and −70 °C and similar glass transitions were obtained with encapsulating material and highly concentrated sucrose solution.

3.5. *Viability Assays and Regrowth Manipulations*

In the past a technique which has proven to be satisfactory for a number of cryopreserved cell cultures involved gradually washing the thawed cells with chilled nutrient medium prior to their return to culture either in liquid or semi-solid medium. It was later found to be deleterious to a number of cell cultures. For example, viability of suspension cultured sugarcane cells protected with a mixture of cryoprotectants was poorer when they were washed with a cold solution than with a 22 °C wash (Finkle and Ulrich, 1982). The washing step has been found to be deleterious to a few cell cultures such as the alkaloid – producing *Catharanthus roseus* (Chen et al., 1984a), *Zea mays*, Paul's Scarlet rose, and *Acer pseudoplatanus* (Withers and King, 1979; 1980). While a special post-thaw culturing technique had to be developed to enhance regrowth of the alkaloid-producing cultures of *C. roseus* (Chen et al., 1984a), the non-producing cell cultures of the same species could be successfully returned to either liquid or semi-solid medium immediately following post-thaw wash (Kartha et al., 1982b). The special growth technique involved plating thawed cells on to filter papers placed over regrowth medium for a specified period of time to facilitate slow diffusion of cryoprotectants and subsequently transferring the filter paper with cells to two changes of nutrient medium. This technique has since been applied to other cell cultures such as *Picea glauca* (Kartha et al., 1988), *Saccharum* sp. (Gnanapragasam and Vasil, 1990) and *Citrus sinensis* (Sakai et al., 1991b). Other manipulations included omission of ammonium ions in post-thaw culture media or incorporation of activated charcoal (Kuriyama et al., 1989).

A number of viability assays are available for rapid estimation of viability of cryopreserved cells. These include fluorescein diacetate staining (FDA), triphenyltetrazolium chloride (TTC) reduction assays and the use of a number of vital stains. The FDA and TTC are generally used to assess viability. The FDA staining technique was originally devised by Widholm (1972) and is based on the observation that only living cells are stained with FDA and emit fluorescence under UV light. In the TTC method (Steponkus and Lanphear, 1967), determination of survival is based on the reduction of TTC by viable cells by mitochondrial activity, to form formazan, a water insoluble red compound. This reduction is considered to be quantitative and the formazan is made soluble by addition of ethanol and the supernatant can thus be examined spectrophotometrically.

Other parameters such as mitotic index, cell number, cell volume, dry and fresh weight, plasmolysis and deplasmolysis, plating efficiency, leakage of electrolytes, could also be used and compared with the unfrozen control in order to assess viability. A combination of various assays is advisable for a true representation of viability.

The most convincing test for viability of frozen-thawed cells would be their ability to regrow upon return to culture without a long lag phase, and exhibit identical growth characteristics as the original culture. A long lag phase is a

direct reflection of freezing injury resulting from the use of sub-optimal freezing conditions. In case of meristems, the most reliable and convincing method of viability assessment is the direct differentiation of the cryopreserved meristems into plantlets without intervening callus. This is extremely important from the perspective of germplasm preservation which demands retention of genetic fidelity. Indirect shoot regeneration mediated by a callus phase should be viewed with caution since it could lead to undesirable somaclonal variation. Direct regeneration of plants can only be obtained if the meristem explants do not suffer extensive freezing damage. In this context, some of the earlier studies revealed extensive freezing damage to the meristems and preferential survival of leaf primordia (Haskins and Kartha, 1980; Grout and Henshaw, 1980).

3.6. *Post-Cryopreservation Stability*

Cryopreservation strategy has been proposed as the final answer to the problems of genetic stability on the grounds that storage at liquid nitrogen temperature will not permit the occurrence of any genetic drift in the preserved specimen. A limited number of examples available so far totally endorse this theory. For example, extensive field evaluations extending over 3 asexual generations of strawberry plants regenerated from cryopreserved meristems after 5 years of storage did not reveal any unique variation resulting from cryopreservation *per se*. Tissue culture-induced variations were noted however, these variants were present both in plants derived from unfrozen and cryopreserved meristems (Kartha, unpublished). Similarly plants regenerated from cryopreserved cassava meristems were field tested at CIAT (Cali, Colombia) and a number of well defined genetic markers as well as esterase isozyme profiles were employed to detect variations, if any. The results revealed no variation arising as a consequence of cryopreservation (Kartha and Roca, unpublished). Towill (1983) examined over 1000 regenerants from cryopreserved meristems of two cultivars of potato and did not detect any variation. These few examples are very encouraging and indicate that cryopreservation has the potential of being a safe method for germplasm preservation. Retention of maximum viability and plant regeneration has to be accorded a high priority from a preservation perspective. Extremely low rate of survival following cryopreservation should be viewed with extreme caution since other variables such as cryoselection might operate in this instance.

When special lines of cell cultures are cryopreserved, it is imperative that the cells maintain the same trait after retrieval from liquid nitrogen storage. A number of examples are now available to depict that the cryopreserved cells maintain their embryogenic potential or plant regeneration capability (for a review see Withers, 1985). Cryopreservation technology has also been applied to a number of cell cultures which possess unique biosynthetic potential (for a review see Kartha, 1987). For example, 25 different anthocyanin

producing cell cultures of wild carrot produced approximately the same amount of anthocyanin after cryogenic storage as did the unfrozen controls (Dougall and Whitten, 1980). Cryopreserved cells of *Digitalis lanata* also retained biochemical activity relative to the transformation of cardenolides (Dietrich et al., 1982; Seitz et al., 1983). *Lavandula vera* callus recovered after cryopreservation retained not only the biosynthetic capacity for biotin, but also plant regeneration capability as did the original green callus cultures (Watanabe et al., 1983). Cell lines of alkaloid-producing *Catharanthus roseus* also retained their biosynthetic capability for the production of indole alkaloids (Chen et al., 1984a; Mannonen et al., 1990). Other examples of the cryopreservation of such special cell cultures (the name of the natural compound they produce are included in parenthesis) are: *Chenopodium rubrum* (betalaines) (Ziebolz and Forche, 1985); *Coleus blumei* (rosmarinic acid) (Reuff et al., 1988); *Dioscorea deltoidea* (steroids) (Butenko et al., 1984); *Eschscholtzia californica* (anthridines) (Ziebolz and Forche, 1985); *Panax ginseng* (ginsenosides) (Seitz and Reinhard, 1987); *Papaver bracteatum* (chlorophylles) (Ziebolz and Forche, 1985); *P. somniferum* (sanguinarine) (Friessen et al., 1990) and *Thalictrum rugosum* (isoquinolin alkaloids) (Ziebolz and Forche, 1985). These results indicate the potential application of cryopreservation for the long-term preservation of cell cultures in a stable condition.

4. Recent Developments in Cryopreservation

In the recent past, certain new developments have occurred in cryopreservation technology. These include (i) vitrification, (ii) simple freezing method, (iii) encapsulation–dehydration, and (iv) cryoselection. The vitrification procedure has already been discussed elsewhere in this chapter.

4.1. *Simple Freezing Method*

This method developed by Sakai et al. (1991b) for cell cultures of *Citrus sinensis* totally eliminates the need for programmable freezing and the use of DMSO as a cryoprotectant. The nucellar cells are cryoprotected with a mixture of 2 or 3 M glycerol and 0.4 M sucrose for 10 minutes at 25 °C. A 0.2 ml of this sample is loaded to a 0.5 ml transparent straw and frozen spontaneously by placing the straw in a freezer at −30 °C for 20–30 minutes prior to immersion in LN_2. The thawing is carried out using established procedures by placing the straws in a water bath at 40 °C. After thawing, the cell suspension was expelled into 2 ml of a diluent solution containing 1.2 M sucrose in a nutrient medium at 25 °C. This procedure resulted in 90% survival based on FDA and phenosafranin staining. Upon plating, the

cryopreserved cells resumed growth within 3 days and subsequently developed into plantlets via embryogenesis.

If this procedure can be successfully extended to other culture systems, it would make cryopreservation technology accessible and affordable throughout the world.

4.2. *Encapsulation-Dehydration*

Encapsulation is now commonly used in the 'synthetic seeds' technology by coating somatic embryos in alginate beads. Some preliminary conservation experiments have been carried out recently using this technique. Mulberry buds and sandalwood somatic embryos encapsulated in alginate could be stored for 45 days at 4 °C and made to resume growth after the storage period (Bapat et al., 1987; Bapat and Rao, 1988). For cryopreservation requirements, this technique was further refined by Dereuddre and his coworkers by combining a dehydration step. In the encapsulation-dehydration step, the alginate-coated shoot-tips or somatic embryos are precultured in a medium supplemented with 0.3 to 0.75 M sucrose, dehydrated in a laminar air flow cabinet for 2–6 hours and quenched in LN_2. This procedure resulted in high survival rates of encapsulated shoot-tips of *Solanum phureja* (Fabre and Dereuddre, 1990), *Pyrus communis* (Dereuddre et al., 1990b) and somatic embryos of carrot (Dereuddre, 1991a, b). Based on thermal analysis during freezing, these authors propose that this technique may allow storage of plant organs at higher temperatures than that of liquid nitrogen without the risk of ice recrystallization since vitrification is in operation during the freezing process.

4.3. *Cryoselection*

Conventional cryopreservation involves cryoprotection with or without predehydration followed by either regulated or rapid freezing and storage in liquid nitrogen. Unless the cells or tissues are derived from fully hardened donor sources and maintained in a hardened or dehydrated state, their exposure to liquid nitrogen temperature invariably results in immediate death of cells. We have developed a novel procedure by which immature embryo-derived callus of non-hardy spring wheat could be frozen to liquid nitrogen temperature without the application of cryoprotectants (Kendall et al., 1990). In this process called 'cryoselection', during the first challenge to LN_2, less than 15% of the calli survived, whereas this survival could be increased to 30 to 40% upon subsequent challenge. Survival during cryoselection was independent of the method of freezing, cooling rates and thawing regimes. Seed progeny derived from five of the 11 regenerant cryoselected lines exhibited significantly enhanced tolerance to freezing at −12 °C thus implying that

cryoselection appears to involve, at least in part, selection for genetic rather than epigenetic variants. Analysis of one callus line indicated that cryoselection did not induce significant alterations in lipid composition, adenylate energy charge, or freezing point. An increase in the soluble sugar component was detected. Changes were also detected in the protein complement of microsomal membranes and soluble protein extracts of cryoselected callus. In all, seven unique proteins ranging from 79 to 149 kilodaltons were identified. These results demonstrate that freezing tolerant callus can be isolated from a heterogeneous population by cryoselection, and factors that contribute to hardiness at the callus level are biologically stable and can contribute to tolerance at the whole plant level.

5. Conclusions and Future Prospects

Cryopreservation technology has advanced very rapidly ever since the first demonstration of successful cryopreservation of *Daucus carota* cell cultures by Latta (1971) two decades ago. The reason for this rapid progress is attributable to several factors, the most notable being the realization of the merit of cryopreservation as indispensable for germplasm preservation as well as for the safe storage of valuable experimental cell cultures. During the current decade, the dire need to maintain valuable cell cultures in a genetically stable condition with concomitant retention of organogenic or embryogenic potential has become more transparent with the advent of biotechnology. It is assumed that the future years would further hightlight the need for cryopreservation for the preservation of proprietary transgenic germplasm.

An assessment of the current status of cryopreservation technology as it relates to various culture systems reveals that cell culture cryopreservation is leading the way as compared to other organized structures such as meristems or zygotic embryos. The realization of the fact that the extent to which cells are non-lethally dehydrated prior to freezing by various strategic manipulations (preculture, cold acclimation, etc.) plays a role in improving post-freezing survival has, in fact, led to improving cryopreservation technology as well as developing novel approaches such as vitrification and encapsulation- dehydration. It is expected that these improvements in cryopreservation technology would translate into actually bringing difficult-to-cryopreserve systems into cryostorage.

Advocating cryopreservation strategy for germplasm preservation is theoretically very valid. But before doing so, it should satisfy the following rigid criteria: (i) an evaluation of germplasm should be carried out to determine the extent of inherent variability and chosing a representative sample of the variability of the species in question for cryopreservation, (ii)the regeneration capability of the cryopreserved material should be very high both before and after cryostorage, (iii) the nature of regeneration mode is unaltered after

cryopreservation, (iv) the procedure should guarantee retention of genetic stability, and (v) the procedure should not lead to inadvertent selection as a consequence of poor survival post-freezing. However, at the moment, the use of cryopreservation is limited to small laboratory collections and its use on a large scale is only exceptional. Indeed, the successful freezing of a plant material which implies the establishment and optimization of very precise conditions requires extensive research using highly sophisticated equipment. In this context, the search for less complicated freezing techniques such as encapsulation-dehydration, Sakai's simplified freezing method and vitrification is of great interest and may be helpful in certain areas. However, the resort to conventional freezing techniques may still remain obligatory in the majority of cases. Additionally, further exploration of cryoselection strategy as an adjunct to cryopreservation may aid in the genetic improvement of crops which are sensitive to low temperature.

Determination of genetic stability of cryopreserved material based on currently practised methods involving conventional phenotypic, biochemical and cytological markers could reach a higher degree of resolution if complemented with restriction fragment length polymorphisms (RFLP) or random amplified polymorphic DNA (RAPD) analyses.

Over the years, national and international bodies, public research institutes and private firms have shown increasing interest in germplasm storage and cryopreservation and this trend is expected to rise in the future as new generations of germplasm and transgenic germplasm become available.

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