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Fundamental Cryobiology and Basic Physical, Thermodynamical and Chemical Aspects of Plant Tissue Cryopreservation

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1. Introduction

The greatest stability of *in vitro* plant materials with practical storage periods measured in decades can be achieved by cryogenic storage at ultra low temperatures. Liquid Nitrogen (LN) is the most common medium for cryostorage as it is relatively inexpensive and readily available (Withers, 1987; Panis and Lambardi, 2005). This process puts the cells in suspended animation where they can retain their viability indefinitely. Maintenance under these conditions effectively halts biological growth and development (Franks, 1985; Grout and Morris, 1987; Grout, 1990a; 1990b) because at below -140^{o}C, the rates of chemical and biophysical reaction will be too slow to affect cell survival. Consequently, material that can be brought to the ultra-low temperature and recovered from it without acquiring lethal injury may be stored for extremely long periods. The challenge is to devise a protocol that allows *in vitro* plant material to be recovered from the cryogen at high viability, and without structural and functional changes (Kartha, 1997; Withers, 1987; Grout and Morris, 1987; Grout, 1990 a, 1990 b). Preservation of viability depends upon the ability to minimize the stresses of cryopreservation and protect against the damaging consequences. A variety of plant material can be used for cryopreservation including *in vitro* cultivated material, pollen, seeds, embryos, buds and meristematic tissues.

Although cryopreservation has many advantages, freezing and thawing injuries related to membrane structure and function that would result in low survival percentages are still the limiting factors (Ashmore, 1997). Prior to freezing, the cells must be treated with a cryoprotectant solution such as glycerol, dimethyl sulfoxide (DMSO), or ethylene glycol. These substances protect the cells and their membranes from damage during the freezing process. After the cells have been exposed to the freezing medium containing the cryoprotectant, they must be dehydrated so that the water inside the cells will not form ice crystals damaging the cell. To dehydrate the cells, they are cooled very slowly prior to plunging them into LN at -196° C that will maintain this constant temperature as long as there is nitrogen in the storage tank (Touchell and Dixon, 1999). When cells are to be thawed, they are warmed rapidly and the cryoprotectant solution is removed. The cells are then cultured in an incubator where they will resume their growth and development. Thawed cells have retained their viability following freezing in LN for more than half a century. The cryopreservation of plant genetic resources has two purposes: (1) preservation

of genetic diversity and (2) preservation of selected varieties for the economic value of their characteristics. Maintenance of continuous culture is labour intensive. In addition, frequent subcultures can generate variants. This disadvantage can be limited by slow growth of *in vitro* cultures, whereby the frequency of subculture can be reduced. However, the principle inconvenience is the possible occurrence of somaclonal variants, incompatible with the aim of genetic resource conservation. Large differences in cell growth lag phase relate to the cryopreservation protocol, the cell source used, as well as variations in cell size and degree of vacuolation in the calli or cell suspensions used for preservation. Thus, there is a concern that cryopreservation and regrowth procedures might contribute to selection of cells with specific characteristics. In addition, tissue culture continues to play a vital role in the development of cryopreservation techniques. Thus, there is an increasing requirement to determine whether plants derived from cryopreservation are 'true to type' or not, to measure the extent of the 'normal phenotype' in cryopreserved plants, and to estimate the degree of closeness of cryopreserved plants to the 'true' parental genotype. These determinations may be achieved through the application of a range of analytical techniques to examine cryopreservation-derived changes at the phenotypic, histological, cytological, biochemical and molecular levels (Harding *et al.*, 2009).

2. Available plant cryopreservation protocols

Methods for the cryopreservation of a variety of *in vitro* cultured tissues such as embryogenic cells (Huang *et al.*, 1995), suspension cells (Ishikawa *et al.*, 1996), transgenic suspension cells (Cho *et al.*, 2007) have been developed. However, a general protocol for cryopreservation of plant cell cultures has not yet been developed. Also, an exact and most suitable guideline for individual specimens is not available as noted by Withers (1983). Thus, the steps involved in the process such as osmotic pre-treatment, cryoprotection, freezing, thawing and subsequent regeneration require standardization for individual species or even individual cell line (Cho *et al.*, 2007). The three most common methods are given below.

2.1 Classical slow-cooling/freezing protocol

The function of controlled, slow-cooling in cryopreservation is to allow cryodehydration to progress without intracellular freezing, removing water from cells to a point where their contained solutions will not form ice crystals when taken to the final cryogen temperature. Cell injuries during freezing are effectively decreased by the addition of various cryoprotectants that reduce the cell size and lower the freezing point to prevent the formation of ice crystals in the cells (Jain *et al.*, 1996). Slow-cooling rates minimize thermal stress from non-uniform temperature distribution, and cryoprotectants contribute to reduce stress by changing the microstructure of the ice formed (Gao *et al.*, 1995).

2.2 Encapsulation/dehydration

In this method, explants (usually meristems or embryos) are first encapsulated in alginate beads (which can contain also mineral salts and organics), thus forming "synthetic seeds" ("artificial seeds" or "synseeds"). They are then, treated with a high sucrose concentration, dried down to a moisture content of 20-30% (under airflow or using silica gel) and subsequently rapidly frozen in LN. Due to the extreme desiccation of explants, most or all

freezable water is removed from cells and vitrification of internal solutes takes place during rapid exposure to LN, thus avoiding lethal intracellular ice crystallization (Engelman, 1997).

2.3 Vitrification

It consists of first preculturing the explants in a dilute solution of a permeating cryoprotectant (so called as loading phase), followed by a vitrification solution (so called as dehydration phase). Since the osmotic strength of the vitrification solution is very high (>8 osmol) and the duration of application fairly short, the main function is to dehydrate the sample, concentrating the permeable components and other cytoplasmic contents within the cell (Towill, 2002). Vitrification-based procedures involve removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid-freezing which results in vitrification of intra cellular solutes, *i.e.* formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. These techniques are less complex and do not require a programmable freezer, and are more appropriate for complex organs like embryos and shoot apices. Hence, are suited for use in any laboratory with basic facilities for tissue culture. Engelmann (2000) described seven vitrification-based procedures in use for cryopreservation: encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation, and droplet freezing, which have been reported to be successfully used for a number of different plant species.

Slow freezing causes extracellular ice to form, thus dehydrating the cell and preventing damage by intracellular ice formation. However, if the freezing is carried out too slowly and dehydration is excessive, the cell will suffer from "solution effects" damage (Withers, 1984; Mazur, 1984). This has been ascribed to pH alterations, intracellular solute concentration, dehydration, membrane alterations and protein denaturation and intracellular ice formation at supraoptimal rates (Towill, 2002). Solution based vitrification is very time-consuming, and processing large numbers of explants is often difficult. The encapsulation procedure allows more propagules to be handled at one time, and timing is not very critical; although it usually requires several hours to attain the desired level of desiccation. If air drying is used, the duration will differ depending on the relative humidity of the atmosphere. While two step cooling systems require a suitable apparatus, vitrification procedures eliminate the necessity for expensive and sophisticated slow-cooling equipments by allowing tissues to be cryopreserved by direct immersion in LN. Explants like shoot apices have high moisture content and require specialized treatments to prevent lethal ice crystal formation during freezing. Vitrification reduces the requirement for extensive manipulation of the apices that may result otherwise in physical damage to the tissues (Towill and Jarret, 1992). However, as vitrification solutions cause extreme desiccation of shoot apices, care must be taken during washing procedures to prevent rapid deplasmolysis. To reduce cellular damage, washing solutions should be isotonic with the thawed tissues, which is usually achieved by the manipulation of the sucrose concentration in the washing medium (Touchell and Dixon, 1999). The key to high survival rates in the slow-cooling method is to carefully control the cooling procedure, whereas in the vitrification method, the cryoprotectant exposure must be carefully controlled. It is reasonable to expect that no single method will give high levels of survival for every genotype of a species after cryogenic exposure. Since a method cannot be devised for each genotype, once a useful procedure is identified the next step would be to apply it to different genotypes to determine overall utility.

2.4 Other protocols

The other available methods are the pre-culture/dehydration used by Dumet *et al.* (1993), where clumps of somatic embryos of oil palm were dissected from standard cultures and pre-grown on 0.75M sucrose for 7 days or further dehydrated under air flow and silica gel for few hours before immersing in LN. In another preculturing method given by Panis *et al.* (1996), the proliferating meristems of banana were pre-cultured for 2-4 weeks on MS medium with 0.3-0.5M sucrose, then excised and plunged into LN. In the droplet freezing method by Schäfer *et al.* (1997), apical shoot tips of potato were first incubated in DMSO, then transferred into 2.5ml droplets placed on small leaflets of aluminium foil and immersed in LN. All these techniques have been used for only a limited number of plant species.

3. Cryoprotective-agent

In theory, ultra low temperature, such as -196^{o}C in LN, maintains tissue quality. In practice, the water-rich plant tissues require additional compounds to prevent ice crystal damage during cryopreservation. Different chemicals and treatments are applied to protect and to recover the plant materials during and after storage in LN. Such a chemical should sustain the viability of biological material during freeze thaw cycle and must not be toxic to tissues at the concentration required adequately to lower the freezing point.

There are two types of cryoprotectants: (i) low molecular weight compounds which penetrate the cell with ease, *e.g.* glycerol and DMSO, and (ii) compounds which penetrate the cell slowly e.g. sucrose, PVP, dextran etc. These compounds protect the cell surface membrane by reducing growth rate and size of ice crystals and by lowering the effective concentration of solutes in equilibrium with ice inside and outside the cell. This increases membrane permeability which aids water removal from the cell and facilitates protective dehydration during early stages of freezing. Most commonly used cryoprotective agents include glucose, DMSO and polyethelene glycol. Glucose serves as a dehydrating agent. DMSO (toxic at higher temperature) passes through the cell membrane readily and protects the cells during the process of freezing. Polyethylene glycol (PEG) gives a water stress to the cells so that ice formation is reduced and may also diminish the harmful effects of DMSO on the freeze stressed cells. Desferrioxamine, an iron-chelating agent, has also been used as a cryoprotectant (Benson *et al.*, 1995). When establishing a cryopreservation protocol, it is also important to determine if cryoprotectants impair cell growth and development. However, published information on the toxicity of cryoprotectants prior to the cooling process is limited. In some species, cryoprotectants cause a temporary loss of semi permeability of membranes (McLellan *et al.*, 1990), while, Pushkar *et al.* (1976) and Moiseyev *et al.* (1982) found that in the presence of low molecular weight PEGs, the activity of enzyme system was decreased. The cryoprotectant mixture of Withers and King (1980), containing DMSO (0.5M), glycerol (0.5M) and sucrose (1.0M), has been most widely used particularly in studies with rice cells and has not been found to be toxic to the rice cells or inhibit its regeneration.

4. Regeneration of plants after cryopreservation

Regeneration is an important criterion for most of the cryopreserved materials. Moreover, the viability rate of *in vitro* cultured and cryopreserved cells must be high to avoid the growing of particular types of cells (Menges and Murray, 2004). Since elite genotype

selection is on the basis of both *in vitro* and *ex vitro* evaluations, the process from somatic embryo initiation to mass seedling production can be long. A consequence of this extended time period is that embryogenicity may diminish or even be irrevocably lost in a few weeks or months (Breton *et al.*, 2006). A way around this problem is to cryopreserve tissues while they are at their peak productivity, shortly after embryogenic tissue induction and when enough tissue is available (Kong and Aderkas, 2011). Thus, a relatively simple and reproducible protocol for the regeneration of plants from the cryopreserved explant is essential for long-term preservation/conservation. Plant regeneration and its frequency are dependent on various factors like: (1) genotypes, (2) age and physiological state of the culture preserved, (3) state of differentiation i.e., isolated cells or well-differentiated tissue and organs, (4) water content of cells of the explant, (5) concentration and duration of treatment with the cryoprotectant, (6) method and rate of freezing and (7) method of thawing and culture including combinations of plant growth regulators used during regeneration (Withers, 1983; Bajaj and Sala, 1991; Tsukara and Hirosawa, 1992). The pregrowth phase of plant cells is considered as very important stage for attaining successful cryopreservation. During this period, various changes may occur at the cellular level including a decrease in cell and vacuole size, changes in the flexibility and thickness of cell walls and alteration of metabolic activities (Withers, 1978). Tissue survival is mainly affected by pretreatment i.e., the longer the pretreatment, the higher the survival percentage. Embryogenicity is also affected by the temperature of the pretreatment. Lower temperatures prevent embryos from maturing and, thus extend embryogenic tissue recovery (Kong and Aderkas, 2011). After recovery from LN, explants contain living, weakened and killed cells. Undifferentiated suspensions, which consist of large vacuolated cells, are also prone to severe cryoinjury compared with embryogenic cultures and apical organs, which contain small cytoplasmic-rich meristematic cells (Wang *et al.*, 2002). In addition, suspension cells are sensitive to environmental stresses, such as dehydration, high osmotic pressure, and low temperatures. Therefore, the assessment of the condition of a specimen both quantitatively and qualitatively after the various stages in the cryopreservation procedure is one of the most important aids to the development of a freeze preservation protocol. Viability of the explants/cells after cryopreservation can be assessed by the flourescein diacetate (FDA) or triphenyltetrazolium chloride (TTC) test. The FDA gets converted to flourescein as a result of esterase activity. Cells with an intact plasma membrane fluoresce green in ultraviolet light as the larger molecules of fluorescin are unable to pass through the membrane. The TTC reduction is based on the mitochondrial respiratory efficiency of cells that converts the tetrazolium salt to insoluble formazon, which is extracted and measured spectrophotometrically.

The first report on survival of plant tissues on exposure to ultra low–temperatures was made by Sakai, when he demonstrated that very hardy mulberry (*Morus sp.*) could withstand freezing in LN after dehydration by extra organ freezing (Sakai,1956). Huang *et al.* (1995) achieved success in plant regeneration from cryopreserved suspension cells of rice. Cornejo *et al.* (1995) discovered that cryopreservation did not affect the ability of rice cells to integrate and express foreign genes. Yang *et al.* (1999) reported developing an efficient protocol for regeneration of a model rice variety Taipei 309 from long-term storage. Anther-derived rice (*O. sativa* L. sp. japonica variety) plants were obtained after cryopreservation by an encapsulation/dehydration technique. Eighty percent of the plantlets developed into normal plants after being transferred to greenhouse conditions. Histological observations showed that the origin of the plants was not modified by the cryopreservation process (Marassi *et al.*, 2006).

5. Variation in cryopreserved derived plant

The genome "quality" reflects its organization and structure, and the genome "flexibility" reveals the complex functionality including capability to response to intracellular and exogenous signals. The measure of tolerance of the genome to exogenous factors depends on the genome "flexibility" generating genetic variation (Skyba and Cellarova, 2009). Zhang and Hu (1999) and Moukadiri *et al.* (1999a) suggested that phenotypic variations seen in some of the regenerated plants were mainly due to tissue culture induced variations rather than effect of cold storage that were revealed by flow cytometric analysis (Moukadiri *et al.*, 1999a) and randomly amplified polymorphic DNA (RAPD) markers (Moukadiri *et al.*, 1999b). However, differences in band intensities among some but not all bands might indicate structural rearrangements in DNA caused by different types of DNA damage (Danylchenko and Sorochinsky, 2005) that might not be readily detected using the given system. The RAPD technology has previously been used successfully to detect occurrence of genetic alterations (Finkle *et al.*, 1985; Harding, 1997; Aronen *et al.*, 1999; Ahuja *et al.*, 2002; Urbanova´ *et al.*, 2005; Castillo *et al.*, 2010), but this approach possesses limits with reproducibility, and it is currently being replaced by techniques such as Amplified Fragment Length Polymorphisms (AFLPs) and/or Simple Sequence Repeats (SSRs, microsatellites). These techniques are now being used to consider more carefully the issue of genetic fidelity after cryo-procedures, especially in the breeding of long-living conifers (Salaj *et al.*, 2010), where genetic changes might be substantially expressed only later on, in mature trees.

Phenotypic and DNA variation among putative plant clones is termed somaclonal variation. Somaclonal variation caused by the process of tissue culture is also called tissue culture-induced variation to more specifically define the inducing environment (Kaepler *et al.*, 2000). Somaclonal variation is a likely reflection of response to cellular stress in other situations as well. Therefore, understanding the mechanism of tissue culture variation will be useful in defining cellular mechanisms acting in the process of evolution, and in elucidating the mechanism by which plants respond to stress. Epigenetic processes are likely to play an important role in these mechanisms. Primary regenerants (R0) are often more variable than their progeny. Examples of aberrant phenotypes in regenerated plants include abnormal leaf structures and variant floral morphology (Kaeppler *et al.*, 2000) and change in kernel color of *O. rufipogon* seeds in R1 plants (Zeliang *et al.*, 2010). Qualitative mutation is frequent among tissue culture regenerants and the summation of protein assays, DNA studies and specific mutant analyses suggests that single-base changes or very small insertions/deletions are the basis of these changes (Kaeppler *et al.*, 1998).

However, there is no convincing evidence for genetic alterations due to cryoprotectant effects in cryopreservation experiments where concentrations of protectants are relatively low, exposure time are short and reduced temperatures are likely to have an ameliorating effect. It has also been suggested that freezing damage is related, in part, to free radical effects, and that both DMSO and glycerol provide an element of protection against these agents by acting as free radical scavengers (Benson, 1990). Although one of the benefits of cryostorage is the maintenance of germplasm in a genetically secure environment, very little work has been conducted on the genetic fidelity of shoot material or plants recovered after cryostorage. Most workers relied on observations of phenotype for confirmation of stability in morphogenic cultures. Phenotypic abnormalities became more common as the number of

reports of regeneration increased, suggesting that many genetic changes are not incompatible with regeneration (Withers, 1984). The climate of opinion on genetic stability in tissue culture is also changing due to the recognition of 'somaclonal variation' as an important phenomenon associated with *in vitro* works (Scowcroft and Larkin, 1983; Scowcroft, 1984). The need to screen for and to report on induced genetic variation is now widely recognized. In the case of *Manihot spp* (Withers, 1984) and *Saccharum spp* (Ulrich *et al.*, 1979), cryopreservation led to an apparent loss of totipotency. However, the reported phenomenon is different from the time-related loss of totipotency observed in long-term callus cultures. There is increasing evidence that, provided that the cryopreservation technique applied ensures the greatest possible maintenance of the integrity of the stored specimen, there will be no modification at the phenotypic, biochemical, chromosomal or molecular level due to cryopreservation (Engelmann, 1997). In a study on *Cosmos atrosanguineus* cryopreservation by Wilkinson *et al.* (2003), the use of AFLP gave no indication of any variation among the tested regenerants and any growth abnormality observed directly after regeneration was not carried over to the later growth stages. Moreover, the nature and location of cell damage appeared to be dependent on the pre-growth cryoprotectant, freezing protocol and species under study.

6. Current status of research and development in cryopreservation

When plant cells are cryopreserved, their plasma membranes are believed to freeze first. As the plant cell is frozen, ice crystals form in the intercellular space and eventually expand with drop in temperature. This creates an osmotic pressure difference between the inside and outside of the cell; therefore, to compensate for this difference, the cell expels water. Since the freezing outside the cell is faster than the inside, ice formation occurs first outside the cell. This formation of ice in turn reduces the water content outside the cell and causes the water inside the cell to move out thereby eventually dehydrating the cells. Temperature, as a major triggering variable in low temperature exposure, affects all structures and processes in the living matter with no exception. Skyba and Cellarova (2009), using the *Hypericum peforatum* model, studied the effect of temperature on the physical and physiology aspect of the plant after cryopreservation. They concluded that, the way in which temperature was decreased affect cell viability and choice of the explants and its seasonal rhythm affect survival rate after cryopreservation. In the case of *in vitro* cultured cells, systematic studies about cryopreservation and its applicability are yet to be determined, because even cell lines of closely related species require different parameter and the same cell line may behave differently in different laboratories. Even if a cryopreservation method has been worked out, the problems of transporting the cultures or reproducing the same method in a different laboratory remain to be solved (Dobbennack *et al.*, 2009). Further, there is no demonstrated mechanism for enhancing the survival of thawed cells. It is supposed that high sugar in culture medium lowers water content and increases endogenous sugar concentrations in cells (Matsumoto *et al.*, 1998). Sugars are known to protect membranes from desiccation events that are inherent to any preservation and added between protocol and are also known to enhance glass formation during cooling.

Cereals, especially rice and barley are two well studied model crops where a variety of information is available. Cryopreservation of rice cell suspension was first reported by Sala *et al.* (1979). Various types of *in vitro* cultures of rice, such as cell suspensions, protoplast,

zygotic embryos and cultured shoot apices survive freezing in LN and retain their morphogenic potential. Rice cells suffer severe metabolic impairment after freezing in LN and show reduced uptake of glucose after freezing (Cella *et al.*, 1982) and preferentially use fructose as carbon source. A detailed study on cryopreservation of suspensions cells of Taipei309 was carried out by Lynch and Benson (1991). They suggested that successful cryopreservation depends on cryogenic technique and pre and post freezing tissue culture. In the post-freezing recovery phase, carbon source in the culture medium has been reported to be an important factor. Kuriyama *et al.* (1989) showed that viability and proliferation of thawed rice cells are depressed in presence of NH_4^+ ions. The effect is thought to be due to the inability of freshly thawed cells to control ionic gradients across plasma membranes. However, rice cells utilize NH_4^+ ions effectively once they have started recovery from cryo-injury. Freezing protocol of Withers and King (1980) has been used in majority of the studies with rice cells. Rice is normally cultured with sucrose as the sole source of nitrogen. Sucrose is rapidly hydrolyzed by actively growing rice cells to fructose and glucose, which are utilized equally. Sucrose hydrolysis is dependent on invertase activity (Amino and Tazawa, 1988), which in turn is associated with the cell wall (Schmitz and Lorz, 1990). However, long-term maintenance of freezed cells on fructose was detrimental. The direct immersion of frozen cells in liquid medium is damaging for rice suspension cells (Lynch and Benson, 1991; Huang *et al.*, 1995; Lynch *et al.*, 1995). Physiological condition of growth, cell aggregate size, embryogenicity and water content of cells has been reported to influence the cryopreservation by Lynch *et al.* (1995) and Watanabe *et al.* (1995). Their study revealed that cells from poorly cryoprotectable genotypes showed increased freezing tolerance after protoplasting (removing of cell wall).

Jain *et al.* (1996) reported a two step freezing protocol for aromatic rice varieties. Suspension cells were cryopreserved by pre-conditioning cells in mannitol, pretreatment in a cryoprotectant solution containing sucrose, DMSO, glycerol, proline and modified R2 medium, cooling to -25°C in a freezer followed by storage in LN. Plants were regenerated from freezed cells as well as from protoplasts isolated from re-established suspension cells. Cryopreservation by vitrification of rice calli has been reported by Wang *et al.* (1996). Watanabe *et al.* (1998) cryopreserved non-embryogenic rice callus cells by vitrification and found that the cell grew vigorously after cryopreservation in the same manner as untreated control and program freezed cells. Medium containing organic nitrogen (amino acids) source was found most suitable as pre-growth medium for suspension cells while inorganic nitrogen was required for successful post thawing recovery. Hu and Gou (1996) and Towill and Walters (2000) successfully cryopreserved pollen grain. Adventitious buds were also cryopreserved efficiently by Zhang and Hu (1999). Adding haemoglobin solution (ErythrogenTM) to post–thaw medium of indica rice (*O. sativa* L.) cells has been reported to enhance survival following cryopreservation (Forkan *et al.*, 2001). During rapid-freezing of rice embryogenic suspension cells, the addition of AFP-I (polar fish antifreeze protein) displayed protective action in the higher concentrated (but non vitrifying) cryoprotectants and detrimental effect in more dilute ones (Wang *et al.*, 2001). Pregrowth-desiccation of the suspension cells using sucrose and sorbitol in AA medium also gave 96-100% survival (Zhang *et al.*, 2001). In another study by Jelodar *et al.* (2001) the protoplast yields increased for re-established cell suspension cultures after cryopreservation compared to unfrozen control cultures. Direct immersion in LN of calli pre-treated with abscisic acid was found to be a fast and highly efficient freezing procedure that maintained the main characteristics of

the cell populations and appeared to increase their metabolic activity (Moukadiri, 2002). Marassi *et al.* (2006) developed encapsulation-dehydration technique and successfully cryopreserved anthers with this technique.

In barley, preculturing reduced the volume of vacuoles and eliminated some osmotically sensitive mitochondria. Reduction in total water content, increase in bound water ratio and accumulation of new proteins also occurred during this step. The adaptation other than in structure and physiology improved the tolerance of cells to desiccation and freezing (Wang and Huang, 2002). Antifreeze proteins first identified in polar fishes also accumulate in freezing–tolerant overwintering cereals (Antikainen and Griffith, 1997). These proteins can lower the freezing temperature noncolligatively and inhibits the growth of ice crystals. The function of these proteins during cryopreservation of rice cells was tested by Wang and Huang (1998) and Wang *et al.* (1999). They found that at the proper concentration, antifreeze proteins may enhance viability through inhibition of ice crystallization, whereas at high concentration, they may decrease the survival rates by ice nucleation.

In a study on *Panax* (Ginseng), Mannonen *et al.* (1990) preserved *P. ginseng* cultures either in LN or under mineral oil for 6 months and compared their growth behaviour and ability to produce ginsenosides after a recovery period with the cultures maintained by frequent sub cultivation during the same period. They demonstrated that neither growth kinetics nor the degree of vacuolation that occurred during growth was affected by either storage protocol. However, some changes in secondary metabolism were found with preservation under mineral oil but not with the cryogenic method (Yoshimatsu and Shimomura, 2002).

In the case of conifers, the conventional cryopreservation method of embryogenic tissue required a few key steps: (1) pretreatment with osmotic regulators, such as sugars (usually, sucrose) or sugar alcohols (such as sorbitol and mannitol), (2) cryoprotectant treatment with DMSO, and (3) a carefully controlled slow-cooling process before the immersion in LN (Klimaszewska *et al.*, 1992; Cyr, 1999; Gale *et al.*, 2007). Currently, efforts are being made to simplify this process by exploring new methods of cryopreservation in both angiosperms and gymnosperms (Touchell *et al.*, 2002; Gale *et al.*, 2008; Popova *et al.*, 2009; Yin and Hong, 2010). DMSO was found to cause genetic alteration under some conditions by Vannini and Poli (1983) and DNA damage and/or rearrangements in some cases viz., *Abies cephalonica* (Aronen *et al.*, 1999), *Solanum tuberosum* L. (Harding, 1997) and *Rubus grabowskii* (Castillo *et al.*, 2010) This effect of DMSO is usually explained by its effect on membrane permeability and function, thermostability of chromosome structure, or inhibition of DNA synthesis. The addition of abscisic acid to *in vitro* stock plants has been found to improve cryopreservability for cold hardy species (Ryyanen, 1998) but in general, cryopreservation procedures have been shown to be species specific.

7. Conclusion

Cryogenic storage is often referred to as a safe system, but this is dependent on reliable procedures and subsequent handling. For propagules from clones or desiccation-sensitive seed, uncontrolled temperature fluctuations, especially above -120^{o} C may drastically affect viability. Hence, cryogenic storage tanks should be carefully monitored for temperature or LN level, along with proper handling, which is crucial for safe storage. It is believed that many biochemical (Stirn *et al.*, 1995), genetic (Wang *et al.*, 1992), and histological (Wang and

Huang, 1995) properties in relation to the embryogenic potential disappear rapidly during extended subculturing. Therefore, cryopreservation can be used to arrest the genetic instability that occurs by continual culture of embryogenic lines (Wang *et al*., 2002). Techniques of controlled freezing, vitrification, encapsulation-dehydration, dormant bud preservation and combinations of these are now directly applicable with plant genotypes representing hundreds of species (Reed, 2002). Although the stresses involved in the introduction into and recovery from storage may be considerable, they can be minimized by appropriate handling and are unlikely to be genetically influential (Withers, 1984). Cryopreservation may also aid germplasm preservation of vegetatively propagated plants maintain the morphogenetic potential of cultured cells, and facilitate regeneration from young explants. The initial investment for cryopreservation is greater, but recurrent costs are minimal. As expertise in the cryopreservation of organized cultures increases, cryopreservation is likely to be the chosen long-term storage method for shoot tips and embryos because of its advantages of lower cost and greater convenience and stability. Cryopreservation techniques have been shown to be adaptable to a variety of plant tissues, but they must be tested and adapted to each new species that is tried (Pence, 1990). Although the species-specific nature of the cryostorage protocols presents problems, the overall benefits of the process argue well for cryoconservation of endangered genetic resources. The relative costs of storing cultures in the growing state and by cryopreservation will also change over time. Further studies will also increase the applicability of the procedure to other threatened species from around the world. Recent interest in the potential for turning *in vitro* related instability to advantage in plant breeding should also improve our understanding of the phenomenon and hence its control.

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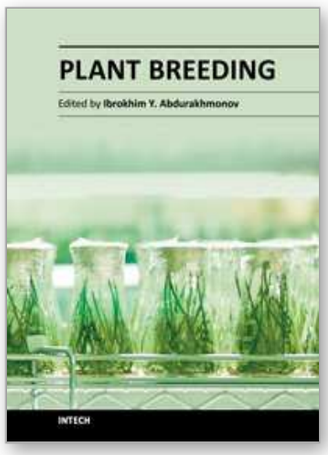

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Modern plant breeding is considered a discipline originating from the science of genetics. It is a complex subject, involving the use of many interdisciplinary modern sciences and technologies that became art, science and business. Revolutionary developments in plant genetics and genomics and coupling plant "omics" achievements with advances on computer science and informatics, as well as laboratory robotics further resulted in unprecedented developments in modern plant breeding, enriching the traditional breeding practices with precise, fast, efficient and cost-effective breeding tools and approaches. The objective of this Plant Breeding book is to present some of the recent advances of 21st century plant breeding, exemplifying novel views, approaches, research efforts, achievements, challenges and perspectives in breeding of some crop species. The book chapters have presented the latest advances and comprehensive information on selected topics that will enhance the reader's knowledge of contemporary plant breeding.

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