

***In Vitro* Conservation and Cryopreservation of Plant Genetic Resources: A Review**

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Abstract: *In vitro* conservation of plant genetic resources is becoming a complementary approach to the conventional conservation methods. *In vitro* conservation save plant material for short, medium or long-term in small space and under controlled conditions. It is cost-effective and stored material can be simply exchanged between countries. Slow-growth and cryopreservation are discussed in this paper with emphasis on the involved factors during the preparation of plant material or through storage. Survival and regrowth of preserved material are discussed.

Key words: Cryopreservation · germplasm storage · long term conservation · medium term conservation · preservation

INTRODUCTION

Sexually propagated plants may be stored as seeds and are not in danger if compared with vegetatively propagated plants [1, 2]. Clonally propagated plants are usually maintained in botanical gardens. Some of those plants are usually preserved by continuous multiplication of tubers, roots, cuttings or bulbs. Such a procedure is laborious and exposes plants to pests and environmental stresses. Tissue culture has proved to be a useful tool for storage of vegetatively propagated commercial crops like potato, palm, forest trees, fruit trees and other species [3, 4].

Conservation of plant genetic resources via tissue culture had an immense effort by research work in the last three decades [5-8]. Storage of shoot tips or meristem derived explants under slow rate of growth has a significant use in the international germplasm resources units. This procedure makes germplasm available at any times for international distribution [6]. Several types of plant materials have been used for *in vitro* preservation of clonally propagated crops. Meristem derived explants such as shoot tips and buds were mostly suggested for their genetic and generative stability [9, 10].

***IN VITRO* PRESERVATION**

Slow growth *in vitro* preservation: The main objective of germplasm preservation is to limit the number of

subcultures and maintain the genetic diversity of a species in a sterile condition without endangering plant stability [8, 11]. In slow growth *in vitro* preservation, germplasm is cultured under normal growth or growth limiting conditions. The growth rate of *in vitro* cultures can be limited by various methods including incubation at reduced temperature and/or low light intensity, manipulation of the nutritive elements in the cultures medium and the use of osmotic agents and growth retardants [8, 12].

Preservation using osmoticums: Osmotic agents are materials that reduce the water potential of cells. The addition of osmotica to the culture has been proved to be efficient in reducing growth and increasing the storage life of many *in vitro* grown tissues of different plant species [13, 14]. According to the hypothesis for turgor-driven growth and cell expansion [15], high levels of osmotic agents in the medium would act against the creation of a critical turgor pressure, which must be established before cell expansion can occur. This stress condition will inhibit both callus growth and shoot formation [16].

Mannitol, sucrose, sorbitol [17], tributyl-2,4 dichlorbenzylphosphonium chloride (Phosphon D), malic hydrazide, succinic acid-2, 2-dimethyl hydrazide (B-995), CCC and ancymidole [4] were reported to be good materials to lengthen the storage life of *in vitro* grown tissues.

Sucrose is a major component of most tissue culture media. It functions as both a carbon/energy source and osmotic agent [11, 13]. Sucrose can be used to reduce plant growth *in vitro* [8, 18]. However, the growth of explant is dependent on sucrose concentration [19]. Microplant elongation was decreased in potato [19] and wild pear [20] with increasing concentration of sucrose. For tobacco callus cultures, 30 g l⁻¹ reduced the capacity of the culture to form shoots which was completely inhibited when sucrose level reached 150 g l⁻¹ [16].

Mannitol can also be used as an osmotic agent [21]. It is a sugar alcohol, which is produced as a primary photosynthetic product by some plants and can be metabolized by them [8, 20]. The use of mannitol did not support tissue growth in tobacco [22], but reduced shoot growth of chrysanthemum [8] and bitter almond [23]. Sorbitol is another sugar alcohol that inhibited shoot growth of *in vitro* grown chrysanthemum when it was added to the proliferation media [23]. Shibli *et al.* [23] reported that elevated sucrose, sorbitol or mannitol reduced growth of bitter almond microshoots significantly and extended the subculture interval to four months when cultures were kept at room temperature.

Preservation at low temperature: Storage under low temperature is one of the major tissue culture techniques used for preservation of genetic resources [8]. Elimination of disease problems and reduction of genetic modification as well as low labor and space requirements are major achievements of cold preservation of plant material [24, 25]. Under such condition, accumulation of unsaturated lipids on the cell membrane would cause cell membrane thickening and retard cell division and elongation [26].

Cold preservation depends on ecology and geographical origin of plants, but usually temperature in range of 0-5°C is employed with cold tolerant species [27]. In general, tropical and subtropical plants are less tolerant to cold temperature than temperate plants [28]. For instance, coffee (*Coffea* spp.) was successfully preserved at 20°C [29], while apple germplasm was preserved at 1-4°C [18]. *In vitro* raspberry (*Rubus* spp.) [30] and mint (*Mentha* spp.) [31] cultures were also best preserved at 4°C with a 12-h photoperiod. Pear (*Pyrus communis*) is usually preserved at temperature between 4-10°C [32], although shoots can be also preserved at 1-4°C for 1-5 years depending on the genotype [33]. Microshoots of *in vitro* grown wild pear were also preserved at 8°C for 12 weeks [20]. Shoot tips of apricot (*Prunus armeniaca*) were successfully preserved at 3°C for 24 weeks [34], while potato germplasm was preserved

at 10°C [35]. Meristem cultures of some root and tuber crops and shoot-tips of banana (*Musa* spp.) were preserved for up to 18 months at 18°C [36]. Cold treatment of donor carnation plants (*Dianthus caryophyllus* L.) at 4°C for 3 days or more resulted in doubling in the percentage of excised, frozen shoot apices which survived freezing and a 6-to-7-fold increase in the percentage which formed leaf primordia or shoots [37].

Plant species differ in their requirement for light or dark during storage. Most cold preservation protocols were performed under either low light intensity or complete darkness [36, 38], but still several plant species were cold preserved under light conditions [39]. *In vitro* cultures of coffee [29], citrus [40] and potato [19] were preserved successfully under light conditions while apple rootstocks [18] and apricot shoot tips [34] were best preserved in the dark.

Several types of plant materials including shoot tips, nodal segments and rooted shoots are used for low temperature preservation of plant such as apple and pear [18]. Shoot tips were most successful for *in vitro* preservation due to their high survival and regrowth percentage and high genetic stability [24]. Callus, embryo and cell suspension cultures can also be used for cold preservation of plant materials [41].

CRYOPRESERVATION

Cryopreservation is a long-term storage of biological material in liquid nitrogen (LN) at -196°C [42]. During cryopreservation, cell division and metabolic and biochemical processes are arrested [43, 44] and thus the cells are allowed to retain their properties unchanged [44, 45] for an indefinite period of time. Also, cryopreservation offers maximum stability of phenotypic and genotypic characteristics of the stored germplasm [27].

The development of cryopreservation for plant cells and organs has followed the advances made with mammalian species. The first report on survival of plant tissues to exposure to ultra-low temperatures was made by Sakai in 1956 when he demonstrated that very hardy mulberry (*Morus* spp.) twigs could withstand freezing in liquid nitrogen after dehydration mediated by extra-organ freezing [27, 47]. In recent years, several new cryopreservation techniques have been developed which allow to apply cryopreservation to larger range of tissue and organs, in varying infrastructural situations [48-50].

Shoot tips, 1-3 mm long, are widely used due to their high genetic stability, high survival and regrowth percentages. Shoot tips are characterized by their small

dense and actively dividing cells and it would insure rapid multiplication rates after thawing. Also the low water content of shoot tip cells would propose a strong reason for choosing them as a basic plant material for cryopreservation [27]. Embryogenic cell suspensions of grapevine (*Vitis vinifera* L.) were successfully cryopreserved by encapsulation-dehydration and subsequently regenerated into plants [50, 51]. Scottez *et al.* [52] found that during encapsulation-dehydration, axillary shoot tips gave higher survival and regrowth percentages than did apical shoot tips.

Many plant species including pear, apple, mulberry, citrus and herbaceous plants have been successfully preserved in LN [53-55]. Cell suspension could be used at the exponential phase of growth [56], in which cells are small in size and have relatively small vacuoles and low water content [27, 57]. Also small cell aggregates have a higher freeze-tolerance than large cell aggregates [21]. The embryo and the embryonic axis of some recalcitrant seeds are too large to survive without serious structural damage after exposure to liquid nitrogen [27, 58-60]. This problem could be overcome either by using immature embryos or by using zygotic embryo [49]. Embryonic axis of *Camellia japonica* was cryopreserved more easily than somatic embryos, due to the differences in the degree of differentiation and water content [61]. Organs are not recommended for freeze preservation, due to their large size and the fact that they contain different type of cells. These different cells require different protocols to be preserved without damaging the organ [27]. Somatic embryos were successfully cryopreserved in some plant species including black iris [62] and olive [11].

Organized tissues such as shoot tips are preferred over cell and callus cultures for preservation of germplasm of many plant species [63] due to high genetic stability and high survival and regrowth percentages [45]. A key element to the successful development of any cryopreservation protocol is the selection of the optimal physiological state at which explants should be used for freezing [64, 65]. One of the most important requirements for successful cryopreservation is avoiding formation of ice crystals inside the cells during freezing and thawing. This could be achieved by various pretreatments including cold acclimation, exposure to abscisic (ABA), immersion in concentrated sugar solutions, or extensive bead dehydration [27, 62, 63].

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 major limiting factors [27]. In addition, the inability to put general guidelines for

cryopreservation for all plants had made it impossible to state a cryopreservation protocol as every plant has its own unique needs for cryopreservation.

Since there is no single protocol or method for wide range of species or even genotypes [21], several cryopreservation protocols for germplasm conservation have been developed [21, 27, 66]. The first protocol is the classical two-step freezing technique in which the tissue is pretreated and cooled slowly at a controlled rate for tissue dehydration followed by rapid freezing in LN [27]. The adjustment of freezing rate and prefreezing temperature allows the modification of the amount of residual intracellular water and thus reduces the damage caused by crystallization of this water [67]. However, this technique is time consuming and complex and requires an expensive cooling apparatus that would give the required cooling temperature accurately [21, 27]. Ice formation could also occur mostly during the slow cooling step that would kill the cell. Moreover, this technique provides low survival percentage [21, 27, 69]. The new techniques, which are based on vitrification, have been developed over the past 20 years [21, 27, 70, 71]. Included among these are encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet freezing [21, 27]. In all of these methods a very rapid freezing process is used, with samples being plunged directly into liquid nitrogen once the pretreatment stages have been completed [21]. As a result, the internal solutes vitrify and deleterious intracellular ice formation is avoided [27].

Since the freezing process in these methods is extremely simple, a programmable freezer is not required and the cryopreservation can be achieved using simple equipment. In addition, larger organ structures may be more readily cryopreserved when compared with the use of classical approaches [21, 27].

Encapsulation-dehydration: Encapsulation-dehydration includes encapsulation of plant material in calcium alginate beads, followed by pregrowth treatment in a medium containing high levels of sucrose ranging from 0.3 M to 1.5 M for at least one day [21, 27, 72, 73]. The alginate beads are then dehydrated before freezing using either air-drying in a laminar flow hood or by exposure to silica gel [27]. Encapsulation of plant material with calcium alginate induced a short delay only in the development of meristems [21]. Encapsulation-dehydration is widely used because it is applicable to many plant species [62, 72]. Encapsulation-dehydration was described to have many advantages over vitrification [21]. Vitrification method

involves very delicate steps to handle compared to Encapsulation-dehydration [27]. Also cryoprotectant solution used in vitrification is complex and it is composed of cryoprotectant cocktails while sucrose is the most used in Encapsulation-dehydration method [49]. Toxicity caused by cryoprotectants especially DMSO is a major problem in vitrification and is eliminated by Encapsulation-dehydration method as sucrose is not toxic [21, 27]. Also Encapsulation-dehydration is not expensive and does not need expensive cooling apparatus (compared to two-step freezing) and avoid ice formation during cooling [74].

Encapsulation - dehydration cryopreservation methods are based in a successive osmotic and evaporation dehydration of plant cells [70]. Dehydration techniques allow more flexibility when handling large sample numbers because the processing is less time-critical than with vitrification [75]. Survival and regrowth of the cryopreserved plant material is dependent on preculture duration and residual water content after desiccation [27]. The water content of the empty beads is determined by drying it in oven at 100°C or 90°C for 16 h [52]. Hirata *et al.* [76] found that root tips of horseradish (*Armoracia rusticana*) which encapsulated in calcium alginate beads with 0.5 M glycerol and 0.3 M sucrose and proper dehydration up to 33% moisture content was cryopreserved successfully. Optimal survival was achieved with cryopreserved shoot tips excised from preconditioned stock shoots of 'Troyer' citrange (*Poncirus trifoliata* X *Citrus sinensis*), with 0.22 M sucrose and dehydrated to 17.1% moisture content [50].

The highest survival or regrowth rates were also obtained with cryopreserved shoot tips excised from 5-week cold acclimated bitter almond and pretreated with 0.75 M sucrose and then dried for 4 or 6 h [74]. Gonzalea-Amao *et al.* [77] also obtained high survival rate when encapsulated sugarcane apices were pretreated with 0.75 M sucrose and desiccated to 20-25% moisture content. Similar observations were reported in Spanish plant (*Antirrhinum microphyllum*) nodal explants [78], almond apices [72]. Root tips of horseradish (*Armoracia rusticana*) encapsulated in calcium alginate beads containing 0.5 M glycerol and 0.3 M sucrose with proper dehydration up to 33% moisture content were cryopreserved successfully [76]. Somatic embryos of coffee (*Coffea canephora*) were also successfully cryopreserved using encapsulation-dehydration techniques by Hatanaka *et al.* [79].

Wang *et al.* [49] found that the viability of dehydrated cells of grapevine (*Vitis vinifera* L.) was 96% at the beginning of dehydration (0 h); it slowly decreased

to 84% as the water content fell from 67.7 to 20.6% and then declined sharply to 42% at a water content of 16.2%, after 10 h of dehydration under laminar air flow, the highest viability (78%) was recorded with 20.6% water content after 6 h of dehydration, any further dehydration reduced viability.

Vitrification: Vitrification techniques have been developed over the past ten years for different plant species [57] in which tissues are dehydrated by high osmoticum concentration to avoid the risk of ice formation during cryopreservation and thawing [8]. It consists of three major phases: the loading phase, dehydration with highly concentrated vitrification solutions and the unloading phase [27]. Loading phase involves treatments of samples with cryoprotectants or diluted vitrification solutions. By using a highly concentrated vitrification solution, samples will be dehydrated [69] this method is simple, does not need expensive cooling apparatus and can be applied to a wide range of plant material [80-82].

Plant vitrification solution (PVS2) is an aqueous cryoprotectant solution in which living systems can be cooled slowly to below the glass transition temperature without appreciable ice formation either intra or extracellularly [83]. It exhibit protective properties, as well as increasing the osmotic potential of the external medium [84, 85], water will then tend to flow out of the cells and dehydration of tissues will occur [21]. Cryoprotectants must be non-toxic at proper concentrations, have low molecular weight, readily miscible with water and have the ability to penetrate cells rapidly [86]. Among those materials the most used cryoprotectants are dimethyl sulphoxide (DMSO), glycerol, ethylene glycol (EG), polyethyl glycol (PEG), amino acids and sugars [7] Most vitrification solutions employed are derived from those elaborated by Sakai *et al.* [75], which comprise 22% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) polypropylene glycol, 7% (w/v) DMSO and 0.5 M sorbitol. Another PVS2 stock solution which contains 30% (w/v) glycerol, 15% (w/v) DMSO, 15% (w/v) ethylene glycol in standard liquid medium containing 0.15 M sucrose was reported by Benson [58]. The duration of contact between explants and the vitrification solution is a critical parameter, in view of their high toxicity [26]. The dehydration period generally increases with the size of the explant used [21, 27].

Performing the dehydration step at 0°C instead of room temperature allows to reduce the toxicity of vitrification solutions and thus to broaden the window of exposure duration ensuring survival of samples [21, 27].

This also allows to manipulate a large number of samples at the same time [27]. Unloading starts after rapid warming, whereby vitrification solution is drained out of the cryogenic vials and replaced with sucrose at elevated concentrations [21, 27]. Unloading aims at removing progressively the vitrification solution by adding liquid medium containing 1.2 M sucrose or sorbitol to dilute the vitrification solution and reduce the osmotic shock [26].

Encapsulation-vitrification: The encapsulation-vitrification technique is a combination of encapsulation-dehydration and vitrification procedures whereby samples are encapsulated in alginate beads, then submitted to freezing by vitrification [21, 27]. Hirai *et al.* [87] found that osmoprotected *in vitro* grown meristems of strawberry (*Fragaria ananassa* Duch.) with 2.0 M glycerol plus 0.4 M sucrose produce more shoot formation than using 0.4 sucrose only. In case of carnation, encapsulated apices were pregrown for 16 h with progressively more concentrated sucrose solution, then incubated for 6 h in a vitrification solution containing ethylene glycol and sucrose and frozen either rapidly or slowly, where maximum survival was 100 and 92% after rapid and slow cooling, respectively [27].

The encapsulation-vitrification method is easy to handle, saves greatly the time needed for dehydration [87] and the recovery growth is much earlier than encapsulation-dehydration techniques [11, 87]. Hirai *et al.* [87] observed that encapsulated-vitrified meristems of *Fragaria ananassa* cooled to -196°C produced higher shoot formation than encapsulated dried meristems. Encapsulation-vitrification has also been [11, 87]. As a cryogenic protocol with high potential for large-scale cryopreservation.

Factors affecting cryopreservation: The cryopreservation procedure comprises a number of steps including preculture in media with osmotically active compounds, treatment with cryoprotective agents, cooling and storage at -196°C, thawing, post-thaw treatments and recovery of growth [8]. Each step influences the success of cryopreservation [57].

i. Physiological conditions of plant material: The capacity to survive storage in LN is dependent upon many factors including genotype, physiological status and pre- and post- freezing manipulations [21, 27]. The type of explant as well as its physiological state when entering storage can influence the duration of storage achieved [26]. Type and nature of cells determine the ability of cells to withstand freezing stress [27, 70]. The

survival of cryopreserved shoot tips of taro (*Colocasia esculenta*) increased gradually as the age of the donor plants increased [from 46% in 1-week-old plants to 75% in 3-week-old plants [88].

In general it is recommended to take explants from rapidly growing cultures since actively dividing cells have dense cytoplasm and little developed vacuolar system which makes them more likely to withstand freezing and remain viable [8, 27].

ii. Prefreezing treatments: Extracellular freezing is considered as effective method of dehydrating living cells [75]. Freezing tolerance of plant material can be increased by cold hardening and/or preculturing in media with high levels of osmotic agents before exposure to LN [52]. Usually, the pretreated stored plant materials show higher survival percentages after thawing than non-pretreated materials [52]. The effects of prefreezing and rewarming rates upon the survival of cortical tissues of winter mulberry twigs immersed in liquid nitrogen shows that the cortical cells frozen slowly to -120°C survived subsequent rapid rewarming by direct immersion in water at 30°C, although cortical cells from less hardy plants are well known to be sensitive to rapid thawing [75]. Cold hardening for five weeks at 5°C along with 0.3 M sucrose preculture significantly increased survival and regrowth percentages of cryopreserved shoot tips of bitter almond [7].

Pretreatment involves the cultivation of biological materials to be stored in the presence of a cryoprotective agent such as sucrose, sorbitol, mannitol, dimethylsulfoxide (DMSO) or polyethylglycol (PEG) which may have an osmotic action or may also protect membranes, proteins and enzymatic binding sites from the freezing stress [67]. The concentration and duration of exposure to the osmotic agents depend on the plant species and samples of plant material [21, 27]. The pregrowth condition must allow the decrease of as much as possible of the water level of the tissues in order to avoid the detrimental formation of intracellular ice crystals in the tissues [8, 27, 89].

Pretreatment using sucrose plays a major role in improving the resistance of apices to both dehydration and freezing in LN [62, 73]. Swan *et al.* [73] reported that encapsulation-dehydration technique, sucrose pretreatment played a major role in the tolerance of apices to dehydration and further freezing. On the other hand Mycock *et al.* [54] found that somatic embryos of date palm (*Phoenix dactylifera*) and (*Pisum sativum*) showed similar recovery after cryopreservation, irrespective of the pretreatment additive. Besides its osmotic effect, sucrose

act by stabilizing membranes and proteins during desiccation. High sugar concentration in the cell cytoplasm help to establish a vitrified state during cooling and enables cells to tolerate dehydration that can cause freezing damage [7, 56].

Mannitol can be also used as an osmoticum agent Swan *et al.* [70]. It is a sugar alcohol, which is usually produced as a primary photosynthetic product by some plants and can be metabolized by them. Swan *et al.* [70] found that cell fresh weight of *Helianthus tuberosus* increased after mannitol and sucrose preculture treatments, with or without cryoprotection. Sorbitol is another sugar alcohol that inhibited shoot growth, but increase cell content of solutes [8, 90].

Dehydration is another factor which affects the sensitivity of the cryopreserved plant material to freezing with liquid nitrogen (-196°C) [91]. Partial dehydration is usually achieved by using osmoticum or cryoprotectants in the medium before or during cryopreservation [92]. Sufficient dehydration before freezing is achieved by exposure of plant material to air flow under the laminar cabinet [7, 92], or by using silica gel [72]. For different duration depend on plant species. Dumet *et al.* [93] found that the recovery of the cryopreserved somatic embryos of oil palm (*Elaeis guineensis* Jacq) was markedly improved by completing the 7-day pregrowth period in 0.75 M sucrose with the additional dehydration period carried out by placing the embryos in the airflow of the laminar cabinet.

iii. Cryoprotection: The development of a simple and reliable method for cryopreservation would allow more wide spread use of the cryopreserved cultured cells, meristems and somatic embryo [7, 62, 76]. Plant materials are subjected to a cryoprotective treatment using various cryoprotective substances including DMSO, glycerol, ethylene glycol (EG), polyethylene glycol (PEG), amino acids and sugars before freezing [27, 56]. In a few exceptional cases, a single cryoprotectant (usually dimethyl sulfoxide-DMSO) is effective, however, a cryoprotectant mixture consisting of DMSO, glycerol (each at 0.5 M) and a third component such as sucrose, proline, mannitol or sorbitol (at 1.0 M), is usually far more effective [89]. Cryoprotectants must be non-toxic at the proper concentrations [8, 89], have low molecular weight, readily miscible with water and able to penetrate cells rapidly [27]. They are able to reduce the size of ice crystals and lower the freezing point of intracellular contents which enable cells to withstand very low temperatures without disruption of the cell membrane or contents [94]. Amino acids can be grouped into three

categories according to their ability to protect thylakoid membranes against freeze damage, the protective amino acids (group 1) include proline, threonine, γ -amino-butyric acid, arginine and lysine amino acids that provide intermediate protection (group 2) include serine and non-protective amino acids (group3) include valine, leucine, isoleucine, methionine, tyrosine and phenyl-alanine [71].

In all cases cryoprotectants are more effective when prepared in culture medium rather than in water, pH of the mixture should be adjusted to that of the standard culture medium, filter sterilized, chilled and then applied to the cell suspension culture. The cryoprotectant and cells are mixed thoroughly and left to incubate for approximately 1 h [89]. Sucrose plays a role in cryoprotection at the cellular level due to the colligative action of relatively small molecules which depresses the freezing point [72, 95]. Such compounds may also protect proteins or membrane phospholipids during freezing or drying by replacing the extensive shell of water molecules which is oriented around proteins [8].

iv. Cooling rate: Slow cooling is necessary to enable the process of protective dehydration to occur [89]. During slow freezing, cooling to very low temperature or prolonged exposure to low temperature before transferring the plant materials to LN may be injurious to cells because of excessive cellular dehydration and the formation of damagingly large crystals [90]. Once the pretreatment stages have been completed, rapid freezing is used by directly plunging tissue into LN [89]. As a result, the internal solutes vitrify and deleterious intracellular ice formation is avoided [27].

Gonzalez-Armao *et al.* [91] reported that the survival of sugarcane apices was achieved with both rapid freezing and slow freezing using a programmable freezer, but it was generally higher after rapid cooling. In the case of cell suspension cultures, an effective dehydrating procedure is to freeze at a rate of 1°C per minute to approximately -35°C, followed by holding at that temperature for approximately 40 min [89]. Reed [85] reported that the survival rates of pear apical meristems increased as cooling rate decreased while Scottez *et al.* [51] found that the resistance of encapsulated and dehydrated plant material to LN did not depend on the cooling rate.

v. Storage: The storage duration in liquid nitrogen is not a critical factor affecting the recovery of cryopreserved plant material [21, 27]. Once the plant material is plunged into LN, the internal solutes will be vitrified and no longer will biochemical or biophysical events occur [21, 27]. It is

not possible to improvise adequate storage conditions and cryopreserved material must be held at a suitably low temperature in a vacuum-insulated refrigerator [89].

vi. Thawing: This is normally carried out rapidly, to avoid any risk of ice damage by crystallization, by agitating the ampoules in a container of sterile warm water at approximately 40°C [21, 27]. The zone of recrystallization could be passed so rapidly during rapid thawing that there would be no time for recrystallization and thus cell injury could be prevented [90]. Therefore, rapid thawing is recommended after the plant material picked out from LN to avoid any risk of ice damage by recrystallization [21, 27, 89].

Thawing can be done either at room temperature under laminar airflow cabinet [7, 91], or in a water bath [27]. Lambardi *et al.* [81] reported that the best survival of cryopreserved *Populus alba* L. shoot tips was observed after the cryovials were maintained at room temperature for 5 sec and then plunged into a water bath at 40°C.

vii. Reculture: After warming to room temperature, thawed materials have to be washed several times to remove the cryoprotectants to avoid any deplasmolytic injury to cells due to loss of membrane surface area during dehydration and shrinkage. The effect of the post-culture medium composition on the viability of dehydrated and cryopreserved cells of grape vine (*Vitis vinifera* L.) was studied by Wang *et al.* [49], all liquid media tested greatly reduced viability compared with solid media, where as the addition of activated charcoal (AC) to the solid post-culture medium promoted the viability of cryopreserved cells and promote the viability of dehydrated cells but this effect was less significant compared to solid medium devoid of AC.

Engelmann [47] demonstrated that increased survival of cryopreserved immature embryos was obtained by culturing on a modified medium supplemented with 100 mg l⁻¹ GA3. However, Withers [89] observed that incorporation of GA3 into the growth medium, either in the presence or absence of 2,4-D, failed to promote growth of the cryopreserved meristems of carrot (*Daucus carota* L).

Survival and regeneration testing: Various rapid techniques have been used to detect viable plant cells, including fluorescein diacetate (FDA) staining [27] and a triphenyl tetrazolium chloride reduction assay (TTC test) [50]. The TTC test distinguishes between survived and non-survived plant cells, tissues and organs [95], on the basis of their respiration rate. The test utilizes the activity of dehydrogenase as an index of respiration rate of the

viable plant material [27, 92]. Tetrazolium salt solution reduced to formazan by hydrogen ions released by respiration of the viable plant material. As a result, the red color will develop [73]. These vital staining techniques are usually used specifically whether the cells were from cultures of different ages, different species and different plant treatment even with toxic material if it is still a live [96]. Regrowth of the cryopreserved plant material is another indicator for plant viability [27, 72, 88]. It was indicated by cellular growth and greening of the apical regions [12], or in the form of callus [97].

GENETIC STABILITY

The combination of somatic embryogenesis, artificial seed technology and cryopreservation is considered to be a potentially fruitful new approach to the genetic conservation of problem subjects including both root and tuber crops and other clonally propagated material through to recalcitrant seed-producing species [89]. Cryopreservation ensures future availability of plant material, it also retains viability, thereby reducing the risk of any loss of genetic diversity [21, 27, 72]. Genetic stability of the regenerated plant materials has been observed [69, 70]. Shibli *et al.* [63] reported that cryopreservation by encapsulation-dehydration did not diminish the capacity of ohelo (*Vaccinium pahalae*) cells to produce anthocyanins and other flavonoids. Benson *et al.* [82] studied cryopreservability of potato shoot tips and the competence and ploidystability in the recovered plants. They found that, cytological studies revealed plant ploidy status was maintained and chromosomal abnormalities were not observed. Chen *et al.* [98] reported that alkaloid-producing cells of *Catharanthus roseus* retained their alkaloid producing capability after cryopreservation and alkaloid content maintained unchanged. Leaf morphologies of plants regenerated from cryopreserved cells of grapevine also appeared to be similar to those plants from control cells [49].

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