18

Germplasm Conservation

Ever since primitive man learned the art of farming and realised the economic utility of plants, he started saving selected seeds or vegetative propagules from one season to the next. In effect, he was practising a type of germplasm conservation and management. Conservation of forest resources was taught and decreed in parts of India and China as far back as 700 B.C. (Swaminathan 1983). The primitive cultivars and their wild relatives constitute a pool of genetic diversity (germplasm or gene bank) which is invaluable for breeding programmes. However, the population and needs of man have steadily increased over time, leading to persistent exploitation of natural resources. The pressures created by increasing human population on the demand for more food, achievement of greater yields of major crop plants, developing pathogen resistance and utilisation of more marginal lands have led to a search for replacement of conventionally grown plants by varieties with superior traits. The introduction of superior varieties is made possible either by exploring the vast resources of wild species, or through the evolution of new cultivars as a result of the application of various scientific processes. Thus, continual introduction of new cultivars has paved the way for gradual exclusion of traditionally used other genotypes. Indiscriminate clearing of forests and conversion of agricultural lands resulting from industrialisation, urbanisation and measures taken for economic development affect the ecosystem adversely. In these processes, depletion of naturally occurring plant genetic resources is reported to the extent that nearly 2000-60,000 higher plant species became endangered, rare and on threshold of extinction (Bajaj 1987, 1995, Staritsky 1997). Many of these species happen to be the agricultural crops (FAO 2009). Indubitably, some of the valuable gene pools might be lost unless co-ordinated efforts are made towards the conservation of genetic stocks all over the world. Realising the danger of erosion of genetic resources the UN Conference on Human Environment held in Stockholm, in 1972, recommended conservation of the habitats that are rich in genetic diversity. Two years later, in 1974, the Consultative Group on International Agricultural Research (CGIAR) established the International Board for Plant Genetic Resources (IBPGR), subsequently International Plant Genetic Resources Institute (IPGRI), with the objective of providing necessary support to the collection, conservation and utilisation of the plant genetic resources anywhere in the world.

18.1 Modes of Conservation

The principle of any technology designed for germplasm conservation should be to preserve the maximum possible genetic diversity of a particular plant or genetic stock for future use. Diversity in plants is discernible at the level of species, varieties and individuals. Special genetic stocks may include the material (mutant or breeder lines with identified gene or gene combinations) developed and used in ongoing breeding programmes. It has been estimated that the survival of approximately 9,000 wild species of crop plants from tropical regions are some way threatened (see Grout 1990). This further highlights the need for a positive approach to conservation of endangered plants. When new cultivars replace the primitive or conventionally used agricultural crops, it becomes especially important that the displaced crop be properly documented and conserved. According to UNEP (United Nations Environment Programme, 1995) over 15000 threatened species are conserved internationally in botanic gardens. Global climatic changes and natural hazards also affect the natural plant habitats, thereby contributing to relatively rapid changes in agricultural strategies. Access to maximum genetic diversity in this situation would obviously make a major difference to the speed and appropriateness of responses.

18.1.1 *In situ* Conservation

This method of conservation mainly aims at preservation of land races of plants with wild relatives in which genetic diversity exists and/or in which the weedy/wild forms present hybridise with related cultivars. These are evolutionary systems that are difficult for plant breeders to simulate and should not be knowingly destroyed.

The *in situ* conservation of habitats has received high priority in the world conservation strategy programmes launched since 1980. Institutional arrangements, especially in countries of the developing world, have been emphasised. This mode of conservation has some limitations, however. There is a risk of the material being lost due to environmental hazards. Further, the cost of maintaining a large proportion of available genotypes in nurseries or fields may be extremely high.

18.1.2 *Ex situ* Conservation

Ex situ conservation is the chief mode for preservation of genetic resources, which may include both cultivated and wild material. Generally seeds or *in vitro* maintained plant cells, tissues and organs are preserved under appropriate conditions for long-term storage as gene banks. This requires considerable knowledge of the genetic structure of populations, sampling techniques, methods of regeneration and maintenance of varietal gene pools, particularly in cross-pollinated plants.

Germplasm conservation in the form of seeds is most convenient since seeds occupy a relatively small space. Their transport to various introduction centres and gene banks is also economical. Initially, the IPGRI's activities on biodiversity conservation focused on the storage of germplasm as seedbanks which include storage of seeds under different conditions, namely as (a) *Base Collections* for securing conservation at low temperature, (b) *Active Collections* for evaluation, distribution and other use, (c) *Working Collections* for materials used by breeders, and (d) *Field Genebanks* for materials difficult to store as seeds. To meet these objectives, guidelines for "Collecting Plant Genetic Diversity" were published by IPGRI (*see* Guardino et al. 1995) and around 10,000 accessions maintained worldwide for food and agriculture (FAO 2009)

Seeds are characterised as *orthodox*, *recalcitrant* or *intermediate* (Ellis 1984) depending on their storage behaviour. Mature orthodox seeds are desiccation tolerant with low moisture content (20% or less on a wet basis) and can further be dried (5% or less moisture content) without loss of viability. These seeds remain viable for many years and their longevity can be further increased by storing at low temperature or cryopreservation. Recalcitrant seeds are sensitive to desiccation and cannot be dried below critical moisture level. About 118 species from 46 families of flowering plants are known to produce recalcitrant species (Pence 1995).

Seeds of some species (*Coffea arabica, Carica papaya, Elaies guineensis* and *Oreodoxy regia*) are desiccation tolerant but sensitive to low temperature. Such seeds, termed *intermediate* are, therefore, unsuitable for long-term storage by conventional drying (Ellis et al. 1991).

Drawbacks in conservation by seeds are: (a) loss of viability over the passage of time (recalcitrant and intermediate seeds) and susceptibility to insect or pathogen attack, (b) inability to maintain distinct clones except for inbred and apomict species and (c) non-applicability to vegetatively propagated crops (*Dioscorea, Ipomoea,* potato etc.)

Contrarily, *in vitro* methods are the most useful for conservation of vegetatively propagated plants, species with recalcitrant seeds and genetically engineered materials (Bajaj 1995, Krishnapillay 2000). The potential advantages of *in vitro* conservation include: (a) requirement for little space for preservation of a large number of clonally multiplied plants, (b) maintenance of the material in an environment free of pests or pathogens, (c) protection against dangers of natural environmental hazards, (d) availability of nucleus stock to propagate a large number of plants rapidly whenever necessary and (e) minimising the obstacles generally imposed by quarantine systems on the movement of live plants across national boundaries since they are raised and maintained in an aseptic environment. The present chapter deals only with the application of plant cell and tissue cultures in germplasm conservation.

18.2 Materials Used for Conservation

The materials stored *in vitro* may be isolated protoplasts, cells from suspension or callus cultures, meristem tips, propagules at various stages of development or organised plantlets. Even seeds are the materials stored *in vitro*. The cultured cells or shoots can be maintained by serial subcultures at 4-8 weekly intervals for virtually unlimited periods.

The storage of germplasm by repeated cultures has some disadvantages. These include the risk of material loss due to human error or the failure in maintenance of *in vitro* security resulting in invasion by a pathogen. Genetic instability is also affected during serial subculturing of the plant material. This is particularly observed in materials in an undifferentiated phase (cell or callus cultures). The genetic changes may range from additions or deletions of gene sequences (*see* Chapter 14).

Maintenance of the material as plantlets and subsequent propagation from their nodal cuttings reduces the risk of genetic instability. Prolonged storage of somatic embryos also ensures a high degree of genetic stability. A basic requirement for practical feasibility of a plant tissue culture method in germplasm conservation is to reduce the frequency of subcultures to the bare minimum. This can be mainly achieved by cold storage, freeze-preservation (currently termed *cryopreservation*), low-pressure and low-oxygen storage. Applications and limitations of various plant source materials in *in vitro* conservation are discussed in Razdan and Cocking (1997, 2000).

18.3 Methods of *in vitro* Conservation

The methods used for conservation of plant germplasm *in vitro* basically fall into two categories: (a) slow-growth systems, (b) cryopreservation (Monette 1995). Application of these methods invariably depends on the plant material to be used for storage.

18.3.1 *Slow-growth Systems*

Cultures grown under modified conditions enabling them to be stored for longer periods before transfer to fresh medium constitute slow-growth systems. Modifications in culture environment can be brought about by temperature reduction or manipulation of chemical constituents (using ABA or high sucrose content) of growth media. Slow-growth strategies are generally applicable for "medium-term storage" (Reed 1995, Reed and Chang 1997). Cold storage (reduced temperature under refrigerated conditions) is the most predominant method of medium-term storage and provides a back-up collection for use in plant distribution. This method is described in detail under Section 18.4. The disadvantage in slow growing cultures is the accumulation of variant cells over the passage of time which could be prevented using synthetic seed technology (*see* Section 18.6).

18.3.2 Cryopreservation

The principle underlying cryopreservation basically involves bringing the plant cell and tissue cultures to a non-dividing or zero metabolism state by subjecting them to superlow temperature in the presence or absence of cryoprotectants. In this technique the plant material is frozen and maintained at the temperature of liquid nitrogen (LN), which is around –196°C or –150°C in the vapour phase. Cryopreservation is an established method of storing sperm cells in order to use them for artificial insemination during animal breeding programmes. The progress made in the area of plant cryobiology since 1975, has shown that entire plants can now be regenerated from cells, meristems and embryos frozen and stored in for almost indefinite lengths of time. This procedure has been successfully applied for germplasm conservation of a wide range of plant species, such as cassava, pea, chick-pea, rice, wheat peanut, coconut, oilpalm, strawberry and sugarcane and other agrihorticulture crops (*see* Bajaj 1987, 1995; Razdan and Cocking 1997, 2000). Zygotic embryo or embryonic axes excised from orthodox, intermediate, or recalcitrant seeds of almost a hundred species growing in temperate and tropical climates from a range of crop, fruit and tree species can be successfully cryopreseved (Engelmann 2011). These studies have generated much enthusiasm to employ the cryogenic method as a meaningful tool for long-term conservation of desirable and diverse genetic stocks. Cryopreservation is most useful for long-term storage of plant germplasm since cells at ultralow temperature do not divide and remain genetically stable.

Cryopreservation of living plant cells demands considerable technical skill. It is essential that cells during freeze-preservation be protected against cryogenic injuries. Formation of ice crystals inside the cell can cause the rupture of organelles and the cell itself. Increase in concentrations of intracellular solutes to toxic levels is another potential source of cell damage. Cells may also suffer the loss of vital solutes through leakage during the freezing process. The physiological conditions of the material, prefreezing treatments and application of cryoprotectants considerably affect the viability of cells frozen to cryogenic temperatures. Cryopreservation broadly involves four steps: (a) *freezing*, (b) *storage*, (c) *thawing and* (d) *reculture*.

18.3.3 Freezing

Numerous protocols for Cryopreservation of plant cells have been described. The sensitivity of cells to low temperature varies with the species (*see* Finkel and Ulrich 1983). However, the general practice is to suspend the material in the culture medium and after treating with a suitable cryoprotectant transfer it to sterile polypropylene ampoules with screw-caps and freeze by one of the following methods:

(A) *Slow-Freezing Method*

In this method the material is frozen at slow cooling rates of 0.5-4°C min^{-1} starting from 0°C until the temperature reaches –100°C, and finally transferred to LN. The slow-freezing procedure has proved especially successful with cells from suspension cultures (Kartha 1987).

(B) *Rapid-Freezing Method*

This method is simple as the vials containing the plant material are directly lowered into a tank filled with LN. The temperature decreases rapidly, at the rate of –300°C to 11000°C min^{-1}. Dry ice (CO_2), used instead of LN, exerts a similar effect (Nitzsche 1983).

Seibert 1976 and Reed 1992, used the rapid-freezing procedure in which isolated shoot apices were frozen by first pouring LN in the freezing vial and then dipping it in a flask of LN. Isolated shoot apices of *Dianthus caryophyllus* and strawberry, maintained at –196°C for 2 months, remained viable and regenerated whole plants when cultured. The survival of shoot tips following this ultrarapid cooling procedure has been explained on the basis that the critical temperature zone for ice crystal growth is passed so rapidly that ice crystals of a lethal size are not formed inside the cells. The rapid-freezing method has been successfully applied for germplasm conservation of a large number of species (see Bhojwani and Razdan 1983, Withers 1985, Bajaj 1990a, b).

The water content of the samples is critical in deciding whether to use the slow- or rapid-freezing method. Small specimens with a low water content are appropriate materials for rapid-freezing method. Shoot apices of strawberry and *Solanum goniocalyx* are materials with small cells potentially able to regenerate plants after rapid freezing. The main drawback in following this procedure is that some plant materials do not survive rapid freezing by direct immersion in LN and, therefore, step-wise freezing becomes necessary.

(C) *Step-wise Freezing Method*

This method combines the advantages of both slow- and rapid-freezing procedures. The plant material is cooled step-wise (ca 1°C-5°C min^{-1}) to an intermediate temperature, maintained at that temperature for 30 min, and then rapidly cooled by plunging it into LN. The step-wise freezing method can be used for freeze-preservation of a wide range of plant materials, which include shoot apices, buds and suspension cultures. In the initial slow freezing, ice is formed outside the cells and the unfrozen protoplasm of the cells loses water due to the vapour deficit pressure between the supercoiled protoplasm and the external ice. Compared to rapid freezing method, strawberry shoot tips showed enhanced (60-80%) survival rate using this method (Sakai 1997).

(D) *Dry-Freezing Method*

Materials dehydrated by drying in an oven or under vacuum demonstrate remarkable resistance to cryogenic damage. The basic idea for the dry-freezing method originated from the fact that non-germinated dry seeds are able to survive freezing at superlow temperatures in contrast to water-imbibing seeds which show susceptibility to cryogenic injuries. Similarly, dehydration of cells under vacuum also leads to a better survival of plant organs after freezing at –196°C. However, there is a dehydration optimum which varies according to species and tissues.

Different types of freezing units have been used to freeze plant materials (Fig. 18.1). In particular, programmed freezing units (Palmer R 201 and R 202; Palmer Products, Windmill Road, Sunbury-on-Thames, England) have been recommended to achieve greater control on the rates of freezing for handling a large number of specimens at one time. A simple set-up of LN Dewar flask may also work satisfactorily besides being economical. In this, the cooling rates can be determined at various distances above the level of LN inside the flask. Ampoules containing plant material can then be exposed to a predetermined distance in the flask to achieve the desired cooling rate. Once the prefreezing temperature has been achieved, the ampoules can be lowered into the LN and stored. A routine cryopreservation procedure developed by Withers and King (1980) is described in Fig. 18.2.

(E) *Vitrification*

Water content in cells of the material to be preserved is critical in deciding whether to use slow- or rapid-freezing methods. To maintain viability of cells with more water content after cooling to the temperature of LN, it is essential to avoid intercellular freezing. This can be accomplished either by *drying or vitrification.* Materials dehydrated by drying in an oven or under vacuum demonstrate resistance to cryogenic damage. The drying process is not

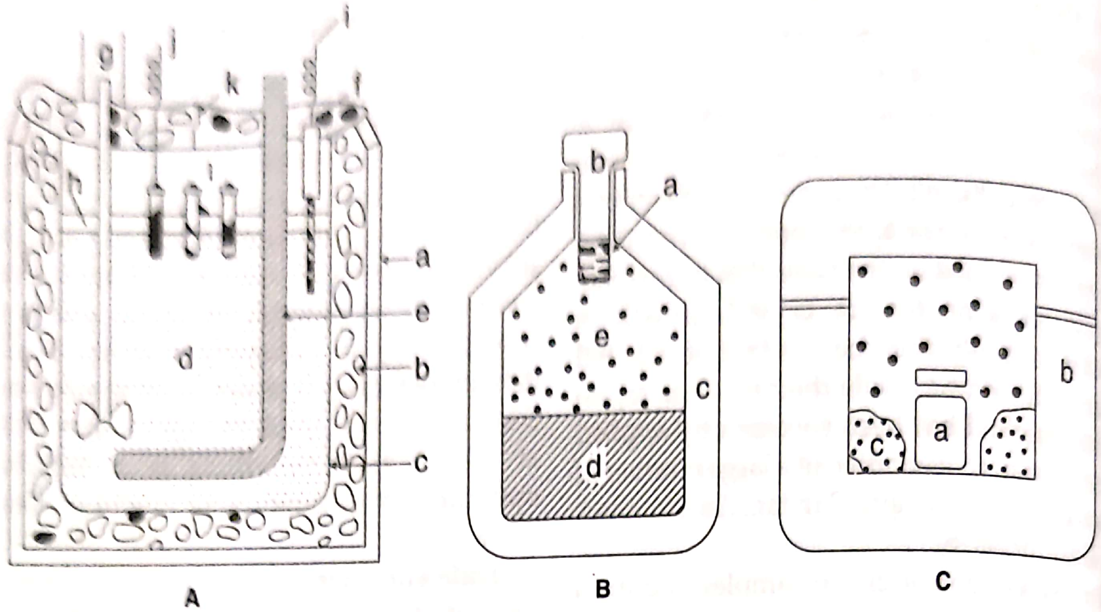

Fig. 18.1 Improvised freezing units designed for preservation of cell and tissue cultures. A: the unit consisting of an electrically cooled alcohol bath on the surface of which the specimens are floated (a—plastic bin; b—polystyrene packing material; c—glass beaker or metal cannister; d—alcohol such as methylated spirit; e—dip cooler; f—thermostat to cooler; g—stirrer; h—polystyrene raft; i—specimen ampoule; j—thermometer for recording specimen temperature; k—bag of polystyrene packing material). B: Unit used to achieve slow cooling by exposing the specimens to cold nitrogen vapour above the level of liquid nitrogen. The specimen ampoules (a) are attached to the plug (b) of a Dewar vessel (c). The vessel contains liquid nitrogen (d), which generates a cold temperature (e). C: A simple unit in which specimens are kept in a vacuum flask (a), later placed inside an insulated cabinet (b), containing solid CO_2 (c). An electrically cooled refrigerator running at ca –70°C would also be suitable (adopted from Withers 1984).

always successful as there is a dehydration optimum which varies according to species. Another potential procedure that prevents cells from cryogenic injuries and successfully applied to most of the plant materials for *in vitro* conservation is vitrification.

Vitrification is an effective mechanism against freezing wherein a highly concentrated cryoprotective solution supercools to very low temperatures and solidifies into metastable glass without undergoing crystallisation (Fahy et al. 1984). The metastable glass is exceedingly viscous and stops molecular diffusion inside the cells. In the vitrification method, cells or meristems are sufficiently dehydrated with a highly concentrated vitrification solution at 25° or 0°C without causing injury prior to immersion in LN. Vitrification solutions PVS2 (Sakai et al. 1990) and PVS3 (Nishizawa et al. 1993) have been developed which are glycerol-based and less toxic. The complete vitrification procedure involves: (a) loading cells or meristems with an intermediate concentration of suitable cryoprotectant, (b) dehydrating cells and meristems by exposure to highly concentrated vitrification solution (e.g., PVS2 or PVS3). (c) transfer of cells and meristems to minicryotubes (1.8 ml) followed by proper sealing and immersion in LN, (d) rapid thawing in water bath at 40°C, and (e) removal of vitrification solution and reculture for shoot or plantlet regeneration.

The vitrification procedure has been particularly successful with the cryopreservation of species and cultivars in a range of herbaceous and woody plants (*see* Fig. 18.3).

(F) *Encapsulation/Dehydration*

Cryopreservation by vitrification requires careful control of the procedures for dehydration and cryoprotectant permeation in order to prevent

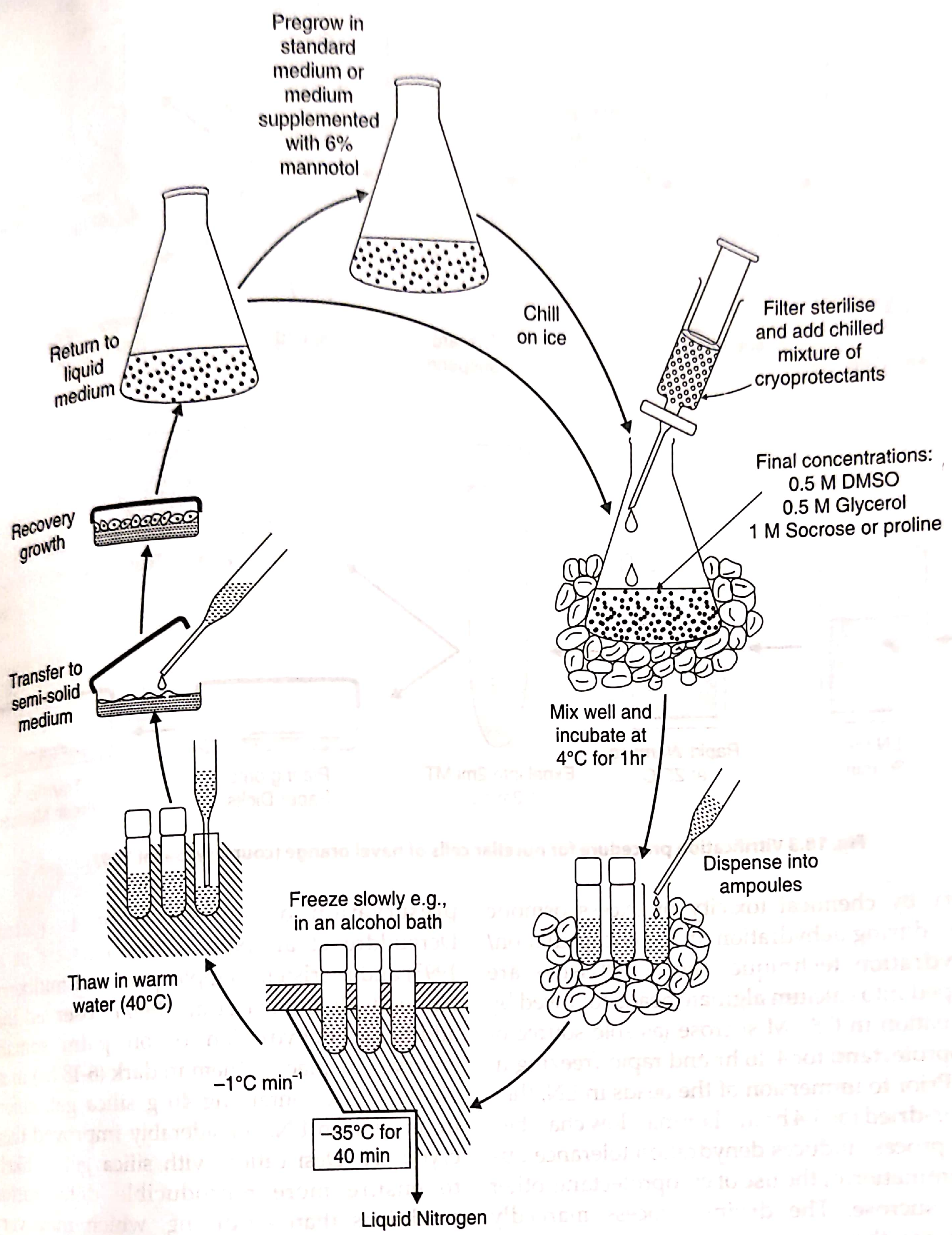

Fig. 18.2 Routine procedure for cryopreservation of cell cultures developed by Withers and King (1980). The appropriate freezing unit to maintain the frozen material is shown in Fig. 18.1 A.

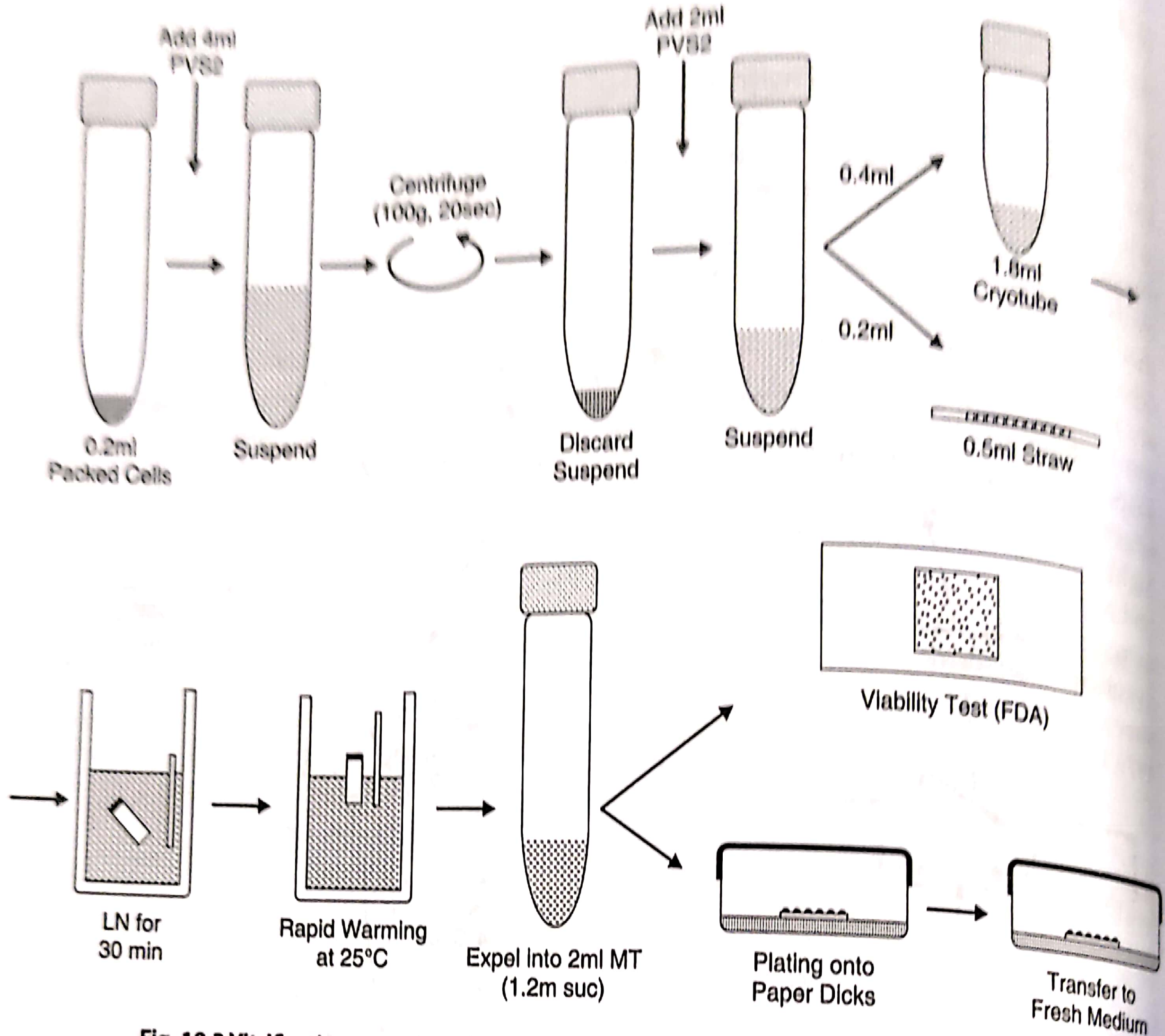

Fig. 18.3 Vitrification procedure for nucellar cells of navel orange (courtesy: Sakai 1997).

injury by chemical toxicity or excess osmotic stress during dehydration. In the encapsulation/dehydration technique, the cells/tissues are trapped into calcium alginate beads followed by incubation in 0.85 M sucrose (as sole source of cryoprotectant) for 4-16 hr and rapid freezing in LN. Prior to immersion of the beads in LN, they are air-dried for 3-4 hr in a laminar flow chamber. This process induces dehydration tolerance due to elimination of the use of cryoprotectants other than sucrose. The drying process markedly increases, the sugar concentration in the beads, which results in the glass transition during cooling to -196°C (Dereuddre et al. 1991).

The encapsulation/dehydration technique has found increasing application in cryo-preservation of somatic embryos (carrot, Dereuddre et al. 1991; coffee, Dussert et al. 1993) and meristems (apple, pear and mulberry; Niino 2000). Dumet et al. (1997) observed that additional dehydration of oil palm somatic embryos by placing them in dark (6-18 hr) in an air-tight box containing 40 g silica gel, before immersion in LN, considerably improved their cryobility. Desiccation with silica gel is likely to ensure more reproducible dehydration conditions than air-drying, which may vary depending on humidity and speed of airflow inside the chamber.

The encapsulation/dehydration method for Cryopreservation of coffee apices is given in Fig. 18.4.

18.3.4 Factors Affecting Freezing

(A) Physiological Condition of Material

A close correlation is found between the cell cycle of the plant material and its freezing potential. The relatively small and cytoplasmically rich (meristematic) cells of the late lag phase or early exponential phase have the highest freeze tolerance. It is generally recommended that materials should be subcultured and cryopreserved only when most of the cells are in the late lag or exponential phase. This can be readily determined in the case of cell suspensions. In comparatively larger tissues (shoot apices, young embryos and plantlets), highly vacuolated cells are severely damaged and regrowth occurs from the actively dividing meristematic cell component. Cells of the mature zygotic embryos do not seem to recover and their survival may require special treatment (Withers 1979, Dumet et al. 1997).

(B) Prefreezing Treatments and Cryoprotection

Various studies have shown that freshly harvested cells or tissues may not survive supercooling and require to be conditioned by their brief culture (pre-growth) before freezing. Prefreezing treatment of this type proved beneficial for potato and banana shoot apices only when they were cultured for 48 hr in the presence of cryoprotectant 5% dimethylsulphoxide (DMSO, Grout and Henshaw 1978, Thorpe and Yeung 2011). The process of hardening is also important as a prefreezing treatment in tissue culture. Plants grown at low temperature (4°C) for 3 days to a week before taking shoot apices for cryopreservation are reported to considerably enhance the survival frequency of the excised tissues. During the hardening process some molecular alterations occur in the plasma membrane which include changes in linkage groups within proteins, sugars and similar substances (Nitzsche 1983).

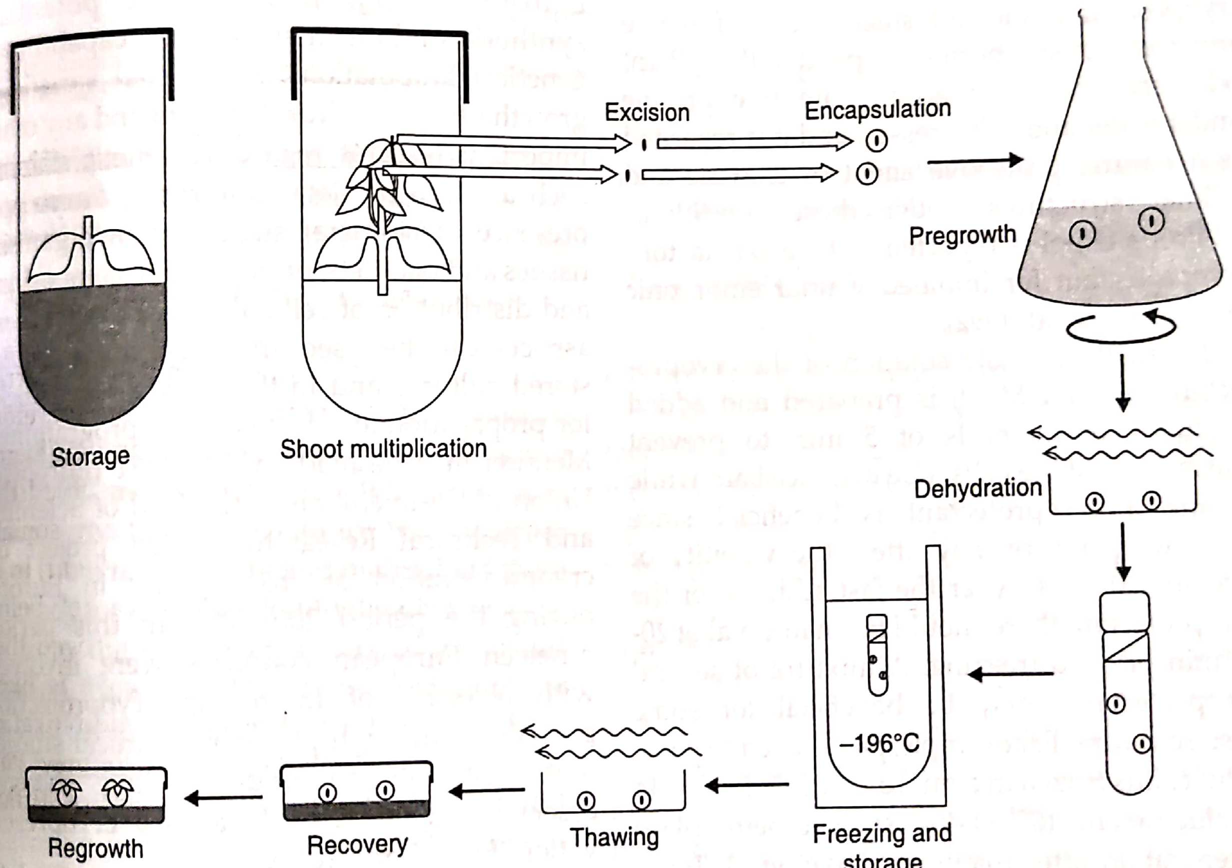

Fig. 18.4 Cryopreservation of coffee apices, as described by Mari et al. (1995).

Dehydration also increases the chances of a material to survive freezing. Fully hydrated materials, on the other hand, require rapid thawing to ensure cellular survival. The process of dehydration, however, increases the intracellular concentration of solutes and thus makes them toxic to cells. To protect cells from this toxic solution effect, *cryoprotectants* are added to the freezing mixture. Substances which in the solid state are amorphous, instead of crystalline, and dissolve in water with or without sugar generally prevent the formation of large ice crystals inside the cell due to freezing of the protoplasm. Such substances are mostly used as cryoprotectants (Uemura and Sakai 1980). Several of the cryoprotectants (Appendix 18.1) tried have different efficiencies. For plants, the most frequently used cryoprotectant is DMSO. The other compounds sometimes used successfully, either alone or in combination with DMSO, are sugar and glycerol. Withers and King (1979) found proline to be a superior cryoprotectant for cell suspensions of maize and some other species (Appendix 18.2). Plant cells are known to accumulate free proline under water and salt stresses, and it is regarded as a natural protective agent of enzyme and cellular structures under these conditions. 1,2-Propanediol is reported to be a satisfactory cryoprotectant for imbibed walnut embryonic axes (Brison et al. 1992).

Normally a dilute solution of the cryoprotectant (5-10% DMSO) is prepared and added gradually at intervals of 5 min to prevent plasmolysis of the cells. Use of an ice bath while adding a cryoprotectant is beneficial since room temperature may affect the viability of cells and tissues. After the last addition of the cryoprotectant there should be an interval of 20-30 min prior to freezing. A mixture of several cryoprotectants may be beneficial for some tissue cultures. For example, pieces of embryonic palm callus frozen in a mixture of 10% PEG, 0.44 M glucose and 10% DMSO showed better plant regeneration after thawing (Finkel et al. 1980). Prefreezing and cryoprotection treatments applied to some cell cultures are listed in Table 18.1. Droplet freezing and droplet vitrification have also proved successful for a number of species (Engelmann 2012).

18.3.5 Maintenance of Cryogenic Cultures

Maintenance of the frozen material at the desired temperature is very important. Temperatures above −130°C may allow ice-crystal growth inside the cells and, consequently, reduce their viability. Long-term storage of material frozen at −196°C will require an LN refrigerator that can accommodate about 4000 ampoules of 2 ml size. It has been estimated that 20-25 l LN are consumed per week in the refrigerator and hence a regular supply of LN is necessary to store the frozen material.

While storing large numbers of germplasm a proper documentation should be made. The documented information may include exact taxonomic classification of the material, culture history, morphogenic potential, synthetic or biotransformation capabilities, genetic manipulations, somaclonal variations, growth kinetics, culture medium and any other important features regarding genetic stability such as isozyme data, autotrophy, karyotype, presence of chimeral structures in organised tissues and plantlets, or population composition and distribution of cell cultures. Data on these aspects can be used in future exchange of stored cultures and in their sale as feedstocks for propagation and biosynthesis programmes. Mention may be made of EUCOST (European Union of Co-operation in the field of Scientific and Technical Research) funded project on cryopreservation of crop species in Europe during the period 2007-2010. In this period nineteen European countries were involved with objective of improving cryoprotection procedures through physiobiochemical studies that contribute in understanding changes associated with tolerance toward cryopreservation (Engelmann 2012).

It is essential that the viability of the germplasm be tested periodically on some samples

Table 18.1 Examples of pregrowth and cryoprotection treatments applied to cell suspension cultures[a,b]

Species	Pregrowth	Cryoprotection
Daucus carota	Cells harvested 2 weeks after transfer to maintenance medium containing 10 mM myoinositol and 0.1% casein hydrolysate	10% DMSO applied at room temperature; no period of equilibration
Daucus carota, Nicotiana tabacum	Cells harvested after 4 days growth in standard medium and in exponential growth	10% DMSO plus 10% glycerol applied at 0°C; no period of equilibration
N. tabacum	Cells harvested after 3 days growth in presence of 6% mannitol and in exponential growth	0.5 M DMSO plus 0.5 M glycerol plus 1 M sucrose applied at ice temperature; 1 hr equilibration
N. sylvestris	Cells harvested after 3-5 days growth in presence of 6% sorbitol	0.5 M DMSO plus 0.5 M glycerol applied at ice temperature; 1 hr equilibration
Zea mays and many other species	Cells harvested after 4-7 days growth in standard medium or in presence of 6% mannitol or 10% proline	0.5 M DMSO plus 0.5 M glycerol plus 1 M sucrose or proline applied at room temperature or on ice; 1 hr equilibration
Catharanthus roseus	Cells harvested after 4 days growth in standard medium and cultured for 20 hr in presence of 1 M sorbitol	1 M sorbitol plus 0.5 M DMSO applied at ice temperature; 1 hr equilibration

[a] The treatments are listed in order of increasing modification in conditions of pregrowth and cryoprotection.
[b] After Withers (1985).

during long-term storage and properly recorded. These details would reduce the risk of exposing other samples to ambient temperature during sample removal.

18.3.6 Thawing

Cryopreserved materials are plunged into warm water at 37-40°C which gives a rapid thawing rate of 500-750°C min^{-1}. After about 90s the material is transferred to either to an ice bath or water bath at room temperature and maintained there until ready for reculture. Rapid thawing protects cells from the damaging effects of ice-crystal formation. However, if the water content of the cells had been reduced to an optimum level before freezing, the thawing rate is less critical. Measuring thermal events during cooling and re-warming using DSC (Differential Scanning Calorimetry) proved beneficial in establishing efficient cryopreservation protocol for zygotic embryos of *Parkia speciosa* and various citrus species growing in Australia (Nadarajam et al. 2008, Hamiton et al. 2009).

18.3.7 Reculture

Thawed materials are washed several times to remove the cryoprotectants so as to avoid any deplasmolytic injury to the cells. The washed material is then recultured in a fresh culture medium following the standard procedures. Some workers have strongly recommended that thawed material not be washed. This may be because certain vital substances leached from the cells during the freezing process are lost by thorough washing. In fact, the frozen and thawed cells of *Zea mays*, and somatic embryos as well as plantlets of carrot, showed faster recovery and higher survival rate when they were cultured in the presence of the surrounding medium (Withers and King 1979). An alternative means of achieving post-thaw recovery while avoiding deplasmolysis and cryoprotectant injury was explored by Chen et al. (1984). They layered thawed cells of various *Catharanthus roseus* genotypes onto a filter paper disc overlying a semi-solid medium. After 4-5 hr the filter paper bearing the cells was plated on a fresh medium where the latter resumed growth. Culturing thawed cells without washing has been found more suitable for *Digitalis lanata* as well (Diettrich et al. 1982).

Recultured frozen and thawed plant materials exhibit special requirements for better survival. Grout et al. (1978) observed that shoot tips from frozen seedlings of tomato developed directly into plantlets on the addition of GA_3 to the culture medium. In the absence of GA_3 apices formed calli which later differentiated adventitious shoots. Normally the control (non-frozen) shoot apices do not require GA_3 to directly develop into plants. Activated charcoal is also reported to enhance the overall survival of freeze-preserved and thawed plants of carrot (Withers 1979). Anthony et al. (1996, 2000) observed that supplementation of culture media with surfactant Pluronic® F-68 (0.01-0.2%, w/v) increased the post-thaw growth of cryopreserved suspension cultured cells of *Oryza sativa* cvs. Taipei 309 (Japonica rice), tarom and ryegrass (*Lolium trifolium*). Similar beneficial effects were noticed with regard to the post-thaw growth of cryopreserved cells of Indica rice variety Pusa Basmati 1 on incorporation of commercial haemoglobin solution (Erythrogen™) into the culture medium. Other factors governing post-thaw recovery of cryopreserved cells are media composition (especially NH_4^+ concentration), use of activated charcoal, and method of removing cryoprotectant during thawing (Watanabe 2000).

18.3.8 *Viability of Cryopreserved Cells and Tissues*

The techniques to determine the viability of cryopreserved cells and tissues are the same as used for cell cultures (*see* Chapter 5). Generally, the cryopreserved materials are stained with FDA, Evan's blue, or 2,3,5-triphenyltetrazolium chloride (TTC). Initially, most of the cells in a population may appear to be viable. However, often, a small proportion recovers completely and divides to regenerate complete plants. The best indicator for survival of frozen and thawed cells, therefore, is their entry into the division phase and regrowth of shoot apices/embryos in culture. The viability is then synonymous with the survival and represented as:

$$\frac{\text{No. of cells/organs growing}}{\text{No. of cells/organs thawed}} \times 100$$

18.4 Cold Storage

Germplasm conservation by storing materials in cultures at low and non-freezing temperatures (1-9°C) has also been practised for some plants (Table 18.2). At low temperatures the ageing of the plant material is slowed down but not completely stopped as in cryopreservation. The advantage in this method is that cells and tissues are not subjected to cryogenic injuries. Further subculture of the plant material is necessary but only very infrequently. Medium-term cold storage (Section 18.3.1) is not only simple, it also gives a high rate of plant material survival.

Cold storage of *in vitro* derived shoots/plants has been particularly successful in fruit-tree species. Virus-free strawberry plants could be maintained for 6 years at 4°C provided a few drops of the liquid medium were added to the cultures after every three months (Mullin and Schlegel 1976). About 800 cultivars of grape plants have been stored for over 15 years at 9°C by yearly transfer to fresh medium (*see* Morel 1975). The use of ABA and high levels of sucrose may help to prolong the interval between two subcultures. Germplasm of a wide range of species can now be stored for 6-24 months by maintaining their shoot cultures in slow growth at low or cold temperatures (Table 18.3). Plantlets at slow growth are generally stored in glass tubes or plastic boxes (Gunning and Largerstedt 1985), although use of "gas permeable-heat-sealable" polythene bags is also recommended (Reed 1991).

Table 18.2 Some examples of shoots/plants stored *in vitro* at 1-9°C[a]

...ecies	Period in storage (months)	Viability (%)
...ria × ananassa	72	100
...virginiana	72	100
...vesca	72	100
...lium multiflorum	10-11	88-100
...s corniculatus	1	90
...lus domestica	12	100
...dicago sativa	15-18	94-95
...bus sp.	14	95
...folium pratense	15-18	70-86
T. repens	15-18	89-92
	11	90-100
...is vinifera	12	?

[a] See details in Bhojwani and Razdan (1983).
? = Not known.

18.5 Low-pressure and Low-oxygen Storage

Attempts have been made to develop low-pressure storage (LPS) and low-oxygen storage (LOS) as feasible techniques for future conservation of cultured plant materials. It is also envisaged to use these procedures (Fig. 18.5) as alternatives to cryopreservation and cold storage. In LPS, the atmospheric, pressure surrounding the tissue cultures is reduced, resulting in partial decrease of the pressure created by gases in contact with the plant material. On the other hand, in LOS the atmospheric pressure (760 mm Hg) is not reduced but the inert gases (particularly nitrogen) are combined with oxygen to create low-oxygen pressure. The results obtained from plant tissue culture experiments with LPS and LOS have been found comparable regardless of the plant or species used. These are summarised in Appendix 18.3.

Low-pressure or hypobaric systems have been found useful for both short-term and long-term storage. Short-term storage is generally used to extend the shelf life of plant materials such as fruits, cut flowers, vegetables, potted plants and cuttings. Long-term storage is used for materials grown in cultures. The principles on which low-pressure systems are based include: (a) maintaining materials in an atmosphere of controlled temperatures, (b) reducing atmospheric pressure in such a manner that the partial pressures of each gas within the storage and around the cultured material are

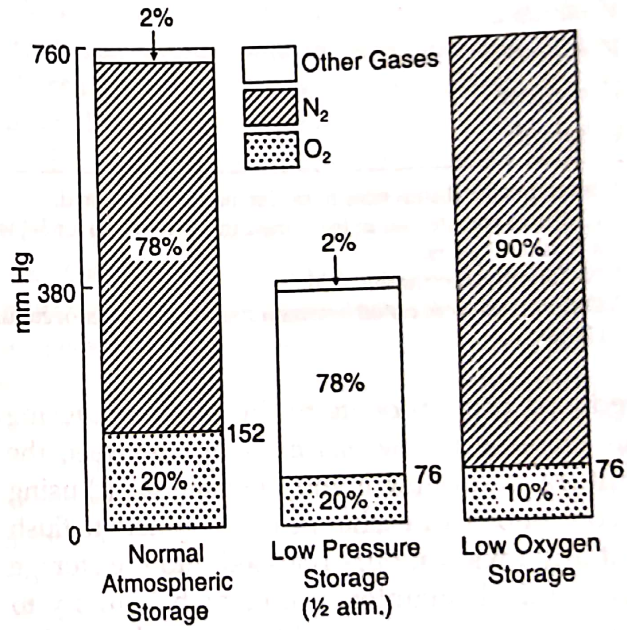

Fig. 18.5 Graphic representation of tissue culture storage under normal atmospheric pressure, low pressure and low oxygen (adopted from Bridgen and Staby 1983).

Table 18.3 Conditions for storage of shoot cultures of various species at reduced temperatures[a]

Species	Normal culture conditions	Storage conditions	Storage period
Dioscorea alata *D. rotundata* *Ipomoea batatas*	26°C; Standard medium	16-20°C; 3% mannitol	8-24 months
Solanum stenotomum *S. goniocalyx* *S. chaucha* *S. juzepejukii* *S. tuberosum* *S. curtilobum*	22°C; 16-hr photoperiod; 3% sucroce	12°C /16-hr day 6°C/8-hr night; 8% sucroce	12 months[a]
Solanum species (as above)	22°C; Standard medium; 20 ml medium	22°C; 20 mg/litre ABA; 60 ml medium	12 months[a]
Solanum species (as above)	22°C; Standard medium	22°C; 6% mannitol;	12 months[a]
S. tuberosum	20-22°C; 16-hr photoperiod 4000lx; Standard medium	10°C; 16-hr photoperiod 2000 lx; 50 mg/litre B-nine[c]	24 months[b]
Trifolium repens	25°C; 16-hr photoperiod	5°C; dark	10 months[d]
Vitis amurensis *V. berlandieri* *V. caribea* *V. champini* *V. labrusca* *V. rupestris* *V. vinifera* *V. hybrids*	26°C/15-hr day, 20°C/9-hr night	9.5°C; 15-hr photoperiod	6 months[d]

[a] Interspecific differences in performance were noted.
[b] Cultures transferred to fresh medium and placed for 3-4 weeks at 20-22°C could be returned to slow-growth conditions for a further 2 years.
[c] N-dimethylsuccinamic acid.
[d] Differences were noted between species and types of culture (proliferating shoots or single-rooted shoots). Source: Withers (1985).

reduced proportionate to the pressure causing an increase in the gas exchange between the culture environment and the material, (c) using a continuous air exchange in the system to flush out any toxic vapours released into the storage area and (d) maintenance of high humidty to preclude shrinkage, weight loss and desiccation of the material.

LPS has an added advantage of reducing the microbial activity in cultures in which the cells or tissues carry them systemically. Subatmospheric pressures also have a fungistatic effect by inhibiting spore germination, mycelial growth and sporulation of various fungi.

The partial pressures of oxygen below 50 mm Hg reduce organised and unorganised plant tissue growth. This is because low oxygen intake by cultured materials results in a decrease in CO_2 production during LOS. Consequently, the photosynthetic process is reduced in these tissues, thereby inhibiting their further growth. The idea that low partial pressures of oxygen may be advantageous for the storage of plant tissue cultures was propounded by Caplin (1959). His experiments with carrot tissue cultures demonstrated that the amount of growth under mineral oil (liquid petroleum widely used for conservation of cultures of various micro-organisms) is controlled by the supply of oxygen to the tissue. Since mineral oil prevented the diffusion of gases, therefore the level of oxygen below the oil reduced gradually due to intake by cultured tissues. This ultimately resulted in suspension of tissue growth.

The major limitation in using LOS for germplasm conservation is the long-term effects of low partial pressures of oxygen on plants. Studies on tobacco and chrysanthemums revealed that tissue or plant growth was not inhibited by either LPS or LOS treatments for the initial 6-week period, but later there was considerable difference in the growth of stored materials. The medium desiccation observed in these treatments would further limit the storage time of cultures unless there is a control mechanism for monitoring the relative humidity within the storage chamber. A comprehensive review of LPS and LOS treatments has been given by Bridgen and Staby (1983).

18.6 Synthetic Seed Technology for Short-Term Storage

Synthetic seeds are proficient tools to facilitate short-term germplasm conservation with particular objective for exchange in trade such as in floriculture, forestry, etc. Synseeds provide readily available germplasm source of mass propagation as they are capable of getting convertible into plants like conventional seeds. A high percentage (ca 80%) of germination was observed in excapsulated synthetic seed on short-term storage upto 150 days in petridishes or screw capped polythene tube (without super cooling) in case of orchid *Dendrobium* 'White Fairy' (Siew et al. 2014). More importantly, plants regenerated from synseed of the desert date palm (*Balamites aegypticum* Del) with this procedure were found genetically stable using ISSR (Inter Simple Sequence Repeat) system (Varshney and Anis 2014). For synthesis of synseeds see Section 7.5.2.

18.7 *In vitro* Genebanks

In addition to highlighting the potential applications of various germplasm resources in this chapter, *in vitro* storage ensures international exchange of germplasm which is disease-free. Aspects of establishing *in vitro* genebanks and exchange of conserved germplasms at the international level are discussed in detail by Withers (1988). It is now possible to exchange genetic resources maintained *in vitro* for cassava, potato, sweet pea, yam, and several other species. Like seedbanks (Section 18.1.2), the types of *in vitro* collections are designated as: (a) *In vitro* Base Collections (long-term storage of materials by cryopreservation and not for distribution), (b) *In vitro* Active Collections (storage of materials in slow growth for a relatively short period), which can be used for multiplication, evaluation, indexing and distribution, and (c) *In vitro* Genebanks (comprising *in vitro* base and active collections) for exchange of germplasm at the national/international level.

The principle of *in vitro* gene banks should be to preserve th,e maximum possible genetic diversity of a particular genetic stock for future use. Genetic stocks may include materials (mutant or breeder lines with identified gene or gene combinations) specially developed for use in the ongoing breeding programmes. When new cultivars replace the primitive or conventionally used agricultural groups, it becomes all the more important that germplasm of displaced crops be properly conserved either *in situ* or *in vitro*. Global climatic changes also affect the natural plant habitats or bring about rapid changes in agricultural strategies. Thus, the materials which would otherwise be discarded due to pressures on resources can be stored *in vitro* without reservation (*see* Staritsky 1997).

18.8 Applications and Limitations

Progress in the development of plant cell and tissue culture techniques for long-term germplasm conservation has been quite significant, especially in view of the level of activity and small number of workers involved. The techniques of cell culture in particular have been so refined that only a few species are likely to be recalcitrant to known procedures. During the past decade studies on the cryopreservation of cell cultures have trended from basic aspects (cryoprotection, freezing, thawing, injury, recovery etc.) to applied aspects.

The evidence available so far indicates that cryopreservation is the most reliable approach to the long-term preservation of cell cultures which possess the biosynthetic capacity for synthesis and accumulation of secondary metabolites (Kartha 1987, Staritsky 1997). A brief outline of the protocol used for the cryopreservation of secondary compound-producing cell cultures is given in Table 18.4. Accumulation of secondary metabolites requires a certain degree of structural differentiation, often accompanied by increase in cellular and vacuolar volume as well as lengthening of the generation period. Therefore, the identification of appropriate growth stages most conducive to freezing becomes critical in devising an appropriate cryopreservation strategy of cells producing secondary metabolites. Recent developments regarding reculture techniques using various additives (*see* Section 18.3.7) and judicious use of cryoprotectants should enable cryopreservation of many secondary metabolite-producing cell cultures. Demonstration of post-freezing stability in terms of ploidy, growth rate, mitotic index and, most importantly, the biosynthetic activity in preserved cells (*see* Kartha 1987) further supports the prospect of using cryogenic techniques for the long-term preservation of such specialised cells.

Germplasm conservation using cell, tissue and organ cultures earlier reported in 60 species including various crops (*see* Withers 1985; Bajaj 1987, 1990a,b) has widened. From the information available, conservation is possible in both monocotyledonous and dicotyledonous families. Temperate species figure more prominently than tropical species (*see* Razdan and Cocking 1997, 2000).

In biotechnology laboratories, Cryopreservation and medium-term storage of plant materials can be of enormous value in maintenance of stock cultures and samples that await screening for future use. Almost an infinite number of replicates from germplasm *of* a large number of plant species can be stored *in vitro* using little space in contrast to the thousands of hectares of land that would be required for the same number of plants if maintained *in situ*. Thus, the material which would otherwise be discarded due to pressures on resources can certainly be stored without reservations. The principal objectives of germplasm conservation using cell and tissue cultures have been: (a) conservation of somaclonal and gametoclonal variation in cultures, (b) maintenance of recalcitrant seeds, (c) conservation of cell lines producing medicines, (d) storage of pollen for enhancing longevity, (e) conservation of rare germplasm arising through somatic hybridisation or other methods of genetic manipulations, (f) delaying the process of ageing, (g) storage of meristem culture for micropropagation, micrografting and production of disease-free plants, (h) conservation of plant material from endangered species, (i) establishment of germplasm banks and (j) exchange of information as well as germplasm at the international level. Additionally, cryopreservation has potential use in cryotherapy (*see* Chapter 15).

The expensive equipment needed to provide controlled and variable rates of cooling/warming temperatures can, however, be a limitation in the application of *in vitro* technology for large-scale germplasm conservation. It may be necessary to develop a low-cost technology to obtain the freezing equipment necessary once a protocol has been developed for preservation of a particular cell line, tissue, or plantlet. Further,

Table 18.4 Protocol used for cryopreservation of secondary metabolite-producing cell cultures[a]

Species	Methodology
Catharanthus roseus	Four-day-old cultures precultured in liquid medium with 1 M sorbitol for 6-2 hr. Frozen using 1 M sorbitol + 5% DMSO. Optimal cooling rate 0.5°C/min to –35°C; stored in liquid nitrogen (LN). Regrowth on filter paper placed over nutrient medium. No post-thaw wash.
Daucus carota	Two-week-old cultures treated with equal volume of medium containing 10% DMSO and frozen at 1°C/min to –70°C followed by storage in LN. Cells recultured after washing.
Digitalis lanato	Cells precultured for 1 week in medium with 3% mannitol; cryoprotectants: sucrose, glycerol and DMSO. Cooling rate 0.5-2.0°C/min to –60°C followed by storage in LN. Regrowth on solid medium. No post-thaw wash.
D. lanata	Six-day-old cultures pregrown in medium with 6% mannitol for 3 days. Cryoprotectants: DMSO, glycerol and sucrose. Cooling rate 1°C/min to –35°C and stored in LN. Regrowth on solid medium. No post-thaw wash.
Dioscorea deltoides	Cells precultured in medium containing asparagine (20 mM), or alanine (50 mM), or proline (20 mM), or serine (10 mM). Cryoprotectant: 7% DMSO. Cooling rate 0.5°C/min to –30°C, 9°C/min from –30 to 70°C, followed by storage in LN. Post-thaw wash.
Lavandula vera (callus)	Green callus pieces suspended in liquid medium. Cryoprotectants: 5% DMSO and 10% glucose. Cooling rate 1°C/min to –40°C followed by storage in LN. Reculture on solid medium after washing.
Panax ginseng	Cold hardening of cells by gradually increasing sucrose concentration from 3 to 25% and simultaneous decrease of culture temperature to 2°C. Cryoprotectants: sucrose-glycerine-DMSO. Cooling rate 0.5°C/min to –30°C, 9°C/min from –30 to –70°C, followed by storage in LN. Reculture after post-thaw wash.

[a] For details, refer to Kartha (1987).

distribution techniques and information networks need to be developed for materials conserved in cultures. It is hoped that in the near future the value of unique cell cultures will become increasingly recognised for conservation of higher genotypes, as is currently done with microbial cultures.

Progress with reference to the development of *in vitro* genebanks has been slow but steady although some prominent centres of *in vitro* Active Genebanks (IVAG) have been established which meet the standards of IBPGR. Notable among these are CIAT, CIP, IITA, IVIA, NCGR and IPGRI. A general tendency toward germplasm storage in seedbanks has contributed little to understanding the application of *in vitro* methods for conservation. Moreover, the efforts made for the establishment of *In vitro* Base Genebanks (IVBG) of cryopreserved materials are far from satisfactory. The expensive equipment needed to provide controlled and variable rates of cooling/warming temperatures can also be a limitation in the application of *in vitro* technology for large-scale germplasm conservation. Establishment of *in vitro* genebanks would be profitable only by creating the facilities and infrastructure necessary for worldwide movement of germplasm. The *in vitro* gene banks and crops mostly they conserve are: CIP (International Potato Centre, Peru) – potato, and tubers; CIAT (Centro de Agriculture Tropical, Colombia) – Manihot; Gatersleben Gene Bank, Germany

– potato, onion spp.; CENARGEN (National Centre for Genetic Resources and Biotechnology, Brasil) – cassava, yam, asparagus, stevia, strawberry, banana, potato and sweet pea; International Institute of Tropical Agriculture, Nigeria – *Dioscorea* spp; NBPGR (National Bureau of Plant Genetic Resources, India) – *Musa*, *Fragaria*, *Colocasia*, *Dioscorea*, *Ipomoea*, *Curcuma*, ginger, medicinal and Aromatic Plants; NCGR (National Clonal Germplasm Storage, USA) – *Humulus*, *Mentha*, *Pyrus*, *Ribes*, *Fragaria*, *Rubus*, *Vaccinium*; INIBAP (International Network for Improvement of Banana and Plantain, France) – *Musa*; Institute of Plant Breeding, University of Philippines – cassava, sweet potato, potato, yam, banana, garlic; BGRC (Braunschweig Genetic Resources Centre, Germany) – Potato (c.f. Razdan and Cocking 1997, 2000; Bhojwani and Dantu 2013). Gene Banks especially for fruits has been established w.e.f Jan 1st, 2003 at Fruit Breeding Institute, Dresden – Pillnitz, Germany, which closely integrates with national decentralized network of the Fruit Gene Bank as a collection and holding partner (Manek and Hanke 2014).