



# Cryopreservation of Plant Species :

Practical Approaches from Handling to Cryobanking

**Rekha Chaudhury and S.K. Malik**





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**Rekha Chaudhury  
S.K. Malik**

**ICAR-National Bureau of Plant Genetic Resources  
(Indian Council of Agricultural Research)  
New Delhi**

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# PREFACE

In view of realization that cryopreservation, at  $-196^{\circ}\text{C}$ , is the only current alternative for long-term conservation of non-orthodox seeds, studies have been carried out routinely for ascertaining desiccation and freezing tolerance of whole seeds and other explants like zygotic embryos and embryonic axes (wherever whole seeds do not survive cryo-exposure) of several Indian plant species at ICAR-NBPGR. Pollen, dormant buds and genomic resources are yet other explants strongly advocated to be cryoconserved. Cryopreservation is widely adopted in the interest of ensurance of maximum genetic stability which is the highest priority of any genebank. At ultra-low temperatures, all metabolic processes are virtually suspended, thus the plant material can be stored indefinitely without alteration or modification. With advancement in research it has been possible to successfully cryopreserve zygotic embryos and embryonic axes of non-orthodox (intermediate and recalcitrant) seeds using new techniques of cryopreservation. In view of this, a Teaching Manual as a handy reference for use by students and early career researcher has been brought out as one of the mandated activities of PG School, ICAR-IARI. Considerable text has been generously drawn from our own published materials and in few cases from materials in public domain. Pictorial guide included of our experiments would be most helpful in conduct of day-to-day practicals.

*In the reproductive cycle, seeds have several contrasting functions: they represent the next generation of plants when they germinate and develop into a seedling; they are also the insurance policy or lottery ticket to ensure that a period of hardship can be survived.*

*Elzenga & Bekker 2017 (Plant Biology 19: 3-5)*

**Rekha Chaudhury and SK Malik**  
Tissue Culture & Cryopreservation Unit,  
NBPGR, New Delhi



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## 1.1 Cryopreservation

Cryopreservation, as defined time and again by various investigators, refers to freezing/ stabilizing cells at cryogenic temperatures, usually below  $-100^{\circ}\text{C}$ . Cryopreservation reduces cell and tissue deterioration to a miniscule by virtue of halting metabolism and hence ensures theoretically 'infinite' periods of storage (long-term conservation), and reportedly causes no change in viability, vigour and genetic makeup of the conserved materials proving an important tool for non-lethal storage of biological materials. The low temperatures are created by use of cryogen like liquid nitrogen in liquid ( $-196^{\circ}\text{C}$ ) or in vapour ( $-150^{\circ}\text{C}$  to  $-180^{\circ}\text{C}$ ) phase in specially designed cryotanks and there is no dependence on mechanical system and electricity.

### Basis of cryopreservation

The critical parameter in developing a cryopreservation procedure is the removal of the intracellular water fraction, which is capable of conversion to ice crystals during freezing or rewarming (Plate 1). However, cells may get injured during two processes i.e., dehydration (removal of water) and intracellular freezing. Hence processing of materials for cryostorage needs to be done, whereby both these injuries are avoided to the maximum limit and cells survive. Cryopreservation is the only technique available presently, for long term storage of vegetatively propagated species and non-orthodox seed species (Kartha, 1985; Bajaj, 1995; Chaudhury & Malik, 2016). Cryopreservation research for conservation of plant germplasm was initiated in India in the 1970's. At National Cryogenebank of NBPGR diverse non-orthodox and orthodox seed species, dormant buds and pollen have been successfully cryostored since 1987 (Chaudhury & Malik, 2014).

### New cryotechniques

These are based on the vitrification (glass formation) phenomenon where cell desiccation is performed either by exposure of samples to concentrated cryoprotective solutions or by air desiccation. Methods in use are:

*Desiccation:* It consists of dehydrating the plant material to a desired moisture level in air before rapid freezing by direct immersion in liquid nitrogen. This technique has been applied mainly to somatic embryos, zygotic embryos or embryonic axes of various species, as well as dormant winter buds of few temperate species.

*Pregrowth-desiccation:* This technique consists of growing / initiating the growth of the plant material for different durations (hours to weeks depending on the material) on media supplemented with cryoprotectants, partially desiccating the material before rapid freezing in liquid nitrogen. It has been successfully applied to somatic embryos of oil palm, coffee, pea and melon and zygotic embryos of coconut.

*Encapsulation-dehydration:* It is based on the technology developed for the production of synthetic seeds. Specimens are encapsulated in calcium alginate beads (Fabre & Dereuddre, 1990). These

are then pre-grown in liquid medium with high sucrose concentration. After removal from liquid medium beads are partially dehydrated to different levels and rapidly frozen. This technique has been applied mostly to shoot apices, axillary buds and somatic embryos.

*Vitrification:* Developed by Sakai *et al.* (1990) vitrification involves treatment (loading) of sample with cryoprotective substances followed by dehydration with highly concentrated vitrifying solution, rapid freezing and thawing, removal of cryoprotectant (unloading) and recovery. Vitrification procedures have been developed for more than 30 different species using protoplasts, cell suspensions, apices, embryonic axes and somatic embryos.

*Encapsulation-vitrification:* This technique is a combination of encapsulation-dehydration and vitrification procedures developed by Matsumoto *et al.* (1995). The alginate beads containing the explants are subjected to freezing by vitrification. It has been observed that recovery rate of apices frozen using this techniques was 30 per cent higher than with the encapsulation-dehydration technique.

*Droplet vitrification:* This technique has been developed by Scha fer-Menuhr (1996) and applied to potato, asparagus and apple apices. Apices are pretreated with liquid cryoprotective medium in vitrification, then placed on an aluminum foil in minute droplets of cryoprotectant and frozen slowly (apple) or rapidly (potato) in liquid nitrogen.

*V-cryoplate method:* This method has been developed by Yamamoto *et al.* (2012) and is based on use of a cryoplate for PVS2-vitrification dehydration of explants in beads. Shoot tips are pre-cultured in 0.3 M sucrose, and attached to small wells dug in cryoplates, encapsulated using alginate beads and then treated with loading solution (2M glycerol + 0.6-1 M sucrose) for 15-30 min. Explants are then dehydrated with PVS2 on this plate and frozen. Later shoot tips attached to cryoplates are transferred to solution of 1M sucrose for rapid thawing and unloading for 15 min. at 25 C and plated on a suitable culture medium.

*D-cryoplate method* This method has been developed by Niino *et al.* (2013). The steps followed are similar to the ones in V-cryoplate method except that after treating with loading solution explants are dehydrated in laminar flow cabinet for suitable period.

### ***Standardization of desiccation protocols***

Rate of desiccation of explant is a very critical step in cryoprocessing of germplasm. To achieve different dehydration rates and depending upon the type of material following methods can be used:

- a. Air drying – Whole seeds
- b. Silica gel drying- Whole seeds, embryos, dormant buds, pollen
- c. Laminar air flow drying- Embryos, embryonic axes, shoot tips, meristems and dormant buds.
- d. Flash drying- Embryos, embryonic axes
- e. Chemical dehydration (cryoprotectants) - Embryonic axes, shoot tips, meristems, dormant buds.

Desiccation of seeds, dormant buds and pollen with higher moisture content is usually carried out on charged silica contained in air tight desiccators (Plate 2, Fig A) and for faster rates under vacuum (Plate 2, Fig B). Regular monitoring of the sample should be done to ensure a high viability

and low moisture content. Excessive desiccation can lead to loss in viability. For desiccation of seeds and buds, the sample is tied in muslin cloth, labeled properly and then kept in silica for drying.

The embryonic axes usually have a very high moisture content as they are excised from sterilized seeds, which are washed 3-4 times with sterilized distilled water. Desiccation of embryonic axes can be achieved by air desiccation in laminar air flow or through use of cryoprotectants (vitrification) or encapsulation-dehydration depending upon the species and the methodology followed. The apical and axillary meristems can also be used as explant for cryostorage of many species and are processed using vitrification or encapsulation-dehydration methods.

It is essential to have a strategy to examine the effect of different parameters on survival when a new system is handled for cryopreservation. It may be essential to develop new methods or optimize standard protocols for specific plant species or tissue types. For this, optimal factors for additives and procedures like pregrowth, dehydration, cryoprotection, vitrification, encapsulation and recovery growth would need to be worked out.

For vitrification, autopipettes, disposable pipette tips, a vial stand, sterile filter papers, sterile petriplates and cryoprotectants like DMSO (Dimethylsulfoxide), ethylene glycol and glycerol are required. For encapsulation, alginic acid (sodium salt), calcium chloride and an orbital shaker with or without temperature and light control with 80-100 rpm speed are required. For fast desiccation flash drier (especially designed equipment) is used.

### ***Standardization of freezing protocol***

The rate of freezing plays an important role in successful cryopreservation of tissues since it affects the amount and rate of formation and size of ice crystals, as well as the injury due to solution effects that occur during freezing. Slow freezing permits the efflux of cellular water and facilitates extracellular freezing as a consequence of the imposed reduction in temperature. The resultant protective dehydration is attained at temperatures of about  $-30$  to  $-40^{\circ}\text{C}$  and is governed by the cooling rate, the type of cryoprotectant and the permeability of the membrane to water. Different types of explants may require different cooling rates, however, a uniform cooling rate of  $0.3$  to  $1^{\circ}\text{C min}^{-1}$  from ambient temperature is effective for a wide variety of explants. For freezing, only two options are available *viz* rapid freezing by direct plunging in liquid nitrogen or slow freezing using a programmable freezing device.

Slow freezing, in use since last 30 years, has been successful with several temperate fruit species. The protocol involves pregrowth of samples on medium with cryoprotectants including DMSO or non-penetrating chemicals followed by slow dehydration in a cryoprotectant solution at intermediate concentration. The freezing rate is usually less than one degree per minute from  $0^{\circ}\text{C}$  to the terminal prefreezing temperature of  $-35^{\circ}\text{C}$  to  $-40^{\circ}\text{C}$ , followed by a rapid immersion in LN. Crystallization occurs in the intercellular spaces and the cytoplasm probably vitrifies. Rewarming of the samples should be rapid.

### ***Programmable freezing***

To achieve uniform, controlled cooling rates, a programmable rate freezing apparatus (Plate 2, Fig C) is used. Variable freezing rates are chosen as per needs. A programmable freezer has a freezing chamber cooled by LN supplied from a pressurized LN vessel. With the use of temperature probes

(connected to the sample and the chamber) and precise computer programming, it is possible to investigate a wide range of cooling parameters. With an appropriate output device (e.g. printer, chart recorder), the temperature at which the extracellular ice is formed, releasing the latent heat of crystallization can be determined. The freezer is microprocessor-controlled and is capable of giving freezing rates in the range of  $0.1^{\circ}\text{C min}^{-1}$  to  $100^{\circ}\text{C min}^{-1}$ . Thus, the unit consists of a freezing chamber, a microprocessor (computer), a monitor (recorder) and a pressurized liquid nitrogen vessel. On achieving the terminal temperature between  $-35$  to  $-40^{\circ}\text{C}$ , samples are to be held for 30-60 min before plunging in LN.

### **Low cost freezers**

Simple low cost freezers are available that provide a cooling of approximately  $1^{\circ}\text{C min}^{-1}$ . These are plastic freezers and have two partitions. The lower partition contains iso-propanol and the upper is empty and used for holding the cryovials (Plate 2, Fig. D). The vials containing the explants are placed on the rack. The lid is closed and kept in a deep freezer at  $-40$  to  $-80^{\circ}\text{C}$ . Such freezers e.g 'Frosty' are available from commercial firms dealing with plasticware and cryoware.

### **Freezing using an alcohol bath**

The earliest record of use of simple freezing unit consisting of a methanol bath has been in 1980. Plant cell suspensions were frozen slowly using this method. Later on, methanol has been replaced by iso-propanol in such freezing units. Later use of ethyl alcohol for controlled freezing was reported. According to this method, freezing can be performed using a home-made ethanol bath, consisting of a polypropylene container filled with 700 ml of ethanol precooled at  $0^{\circ}\text{C}$ . Cryotubes are inserted in holes pierced in a thin polypropylene plate floating on top of the ethanol, which allows immersion of the cryotubes in the coolant. The ethanol bath is then placed in a  $-40^{\circ}\text{C}$  freezer, thus allowing an average cooling rate of  $0.4$ - $0.6^{\circ}\text{C min}^{-1}$  between  $0^{\circ}\text{C}$  and  $-40^{\circ}\text{C}$ . Crystallisation is induced manually in the cryoprotective medium at a temperature intermediate between the nucleation and the crystallisation temperature of the cryoprotective medium. For this, the base of the cryotubes is briefly put in contact with LN. Once the temperature reaches  $-40^{\circ}\text{C}$ , they are maintained for two hours and then immersed rapidly in LN.

### **Storage of explants in cryotanks**

When developing routine cryopreservation methods for liquid nitrogen tolerant materials like orthodox seeds, the strategy should be to examine the response of seeds to initial LN exposure. Once it is established that survival values (tested after 18-24 h of LN storage) are fairly uniform for crop groups or taxa, routine application can be taken up. Simple procedure of desiccating orthodox seeds to 5-7% moisture content followed by placing them in cryotanks in LN, which will cool the seeds slowly is sufficient to ensure their infinite storage.

All types of explants are to be finally packed in airtight containers before cryostorage. Glass containers can shatter when warmed from sub-zero temperatures, so polypropylene screwcap cryovials are generally used. Polypropylene cryovials of different sizes (1ml, 2ml, 5ml, 50ml) (Plate 3, Fig A) designed for low temperature works are commercially available. Pollen desiccated to suitable moisture content can be stored in aluminum packets, gelatin capsules or polypropylene cryovials. For rapid rates of cooling, cell suspensions are placed in stainless steel or silver hypodermic tubing or glass capillaries. The size of the cryovial to be used will depend upon the size as well as the quantity of

the seeds/ embryonic axes/ pollen grains to be stored. Large seeds can be stored in heat-sealable polyolefin tubing with cork stoppers or in goblets in sleeves.

There are no guidelines regarding the minimum number of seeds to be cryostored per accession. As far as possible large samples should be stored depending upon the reproductive biology of the species, as there would be greater chances that rare and potentially useful genes would be represented in the stored samples. Preferably 2,000 seeds may be stored for self-pollinated species and 4,000 for cross-pollinated species. For explants like embryos, embryonic axes, meristems, shoot tips and pollen, there is no standard recommendation for the minimum number of explants to be stored. It usually depends upon the availability of the material, percentage survival and on the plan of retesting.

Once the storage protocol is established, a part of the sample should be stored for long-term, and should be treated as 'base collection'. It should not be meant for distribution, exchange or retesting. Another part of the sample should be stored separately as 'test sample', which could be utilized for providing material to breeders or other researchers or could be used for routine viability retesting.

Successful cryopreservation depends upon the use of good equipment and an efficient inventory system. A good liquid nitrogen storage tank should be self contained, vacuum insulated vessel with the LN reservoir and samples in the same cavity. A consistent temperature will thus be maintained by this. Storage of germplasm in vapour above the LN is preferred by most workers since it is relatively safe for working personnel.

Most storage tanks have access from the top, therefore a vertical inventory scheme would be needed. Inventories are mainly metallic (steel or aluminum) in which boxes are fitted which can hold the cryovials. Small seeds, excised embryonic axes, shoot apices and meristems can be easily stored in large numbers in 1 ml/2ml polypropylene cryovials. A 650 liters capacity LN tank can hold up to 50,000 of 1 ml cryovials. If germplasm of a large number of species is to be handled i.e storing a range of seed sizes, then a flexible inventory system would be required. Aluminum canisters can be very useful for holding the germplasm since 1ml, 2ml or 5ml cryovials mounted on aluminum canes or else 50ml vials and polyolefin tubings can be effectively stored in them (Plate 3, Fig A).

### ***Maintenance of cryostored germplasm***

Liquid nitrogen boils off continuously as heat infiltrates into the cryotanks through the sidewalls and the access port on the top. Additional loss of LN occurs when warm samples are placed in the tank. Each tank has a static holding time, which is the maximum time for which a tank can hold a particular quantity of LN, after which more LN has to be replenished. This is dependent on the rate of evaporation and the capacity of the tank. Evaporation rate of LN for most of the tanks is 0.5 to 1.5% of its capacity per day and accordingly, replenishment of LN is required about two times per week to maintain the temperature of the cryotank between -160 to -180° C.

### ***Retesting of viability***

Post-cryopreservation handling procedures and regeneration protocols need also be fully standardized and readily available to ensure high survival rates. It is essential to monitor the viability of explants after regular intervals to ensure that no deterioration is there over the time. For thawing of samples, a 37°C-40°C water bath is required.

### 1.1.1 Seed storage behaviour

Based on seed storage behavior, seeds are classified as orthodox, intermediate and recalcitrant (Ellis *et al.* 1990; Chaudhury & Malik, 2016). The categorization of seeds is primarily based on their desiccation and chilling sensitivity. Recalcitrant and intermediate seeds, together termed non-orthodox, cannot be dried below a critical moisture content (10 to 40%) and cannot withstand chilling temperatures without substantial loss in viability. Yet another method to define them is in relation to the seed's tolerance of dehydration across water sorption regions and appropriate temperature for storage (Pritchard, 2004). Seed longevity of intermediate seeds ranges from few months to years while recalcitrant seeds from weeks to months.

#### Distinguishing characteristics of Orthodox and Non-orthodox seeds

Orthodox	Non-orthodox (Intermediate & Recalcitrant)
Can be dried to 5%/ less Moisture Content (MC) with no loss of viability	Can be dried upto 30% MC with minimal loss of viability but at lower moisture significant loss of viability is apparent
MC homogenous in seed lot	MC varies within seed lot and cotyledon/EA
Generally small size	Large seed/fruit size
Extended life	Short life span
Tolerant to desiccation and freezing	Sensitive to desiccation and freezing
Generally Annual crops	Invariably Perennial trees from moist tropics, temperate & aquatic habitats
Undergo maturation drying	No maturation drying
Exhibit dormancy in few cases	Metabolically active on shedding
Fleshy coverings absent	Seeds covered with fleshy or juicy ariloid and impermeable testa
Desiccation sensitivity remains unaltered with storage time	Become increasingly desiccation sensitive with storage time

### 1.1.2 Cryogenebanking of orthodox seeds

Initiation of cryobanking of prioritized orthodox seed species (medicinal plants, varieties, registered germplasm, genetic stocks, wild species, threatened and endangered plants) as a pilot project in 1986 has led to a full fledged cryogenebank conserving mainly non-orthodox seed species, besides selected orthodox seed spp. at NBPGR. Diverse accessions of orthodox seeds of diverse crops have been successfully maintained at temperatures between -170 to -180°C (Anonymous, 2015). Retesting of viability of cryoconserved seed germplasm has shown that original viability values of diverse germplasm has been ensured as tested maximum upto 24 years of cryostorage.

### 1.1.3 Cryogenebanking of non-orthodox seeds

Difficult-to-store non-orthodox (intermediate and recalcitrant) seeds are abundantly found grown in moist ecosystem of tropics, subtropics and in aquatic and riparian environments. Seeds are



subjected to high humidity during seed development, maturation and at harvest. Due to these conditions seeds do not undergo maturation drying as a final, preshedding development phase. Short-, medium- and long-term seed storage can only be recommended when seed storage behaviour and developmental stage when the seeds exhibit tolerance to desiccation is known (Chaudhury and Malik, 2004). Recalcitrant seeds at various developmental stages are reported to have varying degree of tolerance to desiccation (Chandel *et al.*, 1995a). This also varies among species (Hong and Ellis, 1996). For example, immature/partially mature embryos of jackfruit and litchi have been found to be more adaptable to manipulation than mature embryos/embryonic axes (Chaudhury, 2000). Gradual increase and decrease in desiccation tolerance with development and with initiation of germination have been observed in both non-orthodox and orthodox species. Rapid and careful handling, vitrification and use of embryonic axis have been found effective in cryopreservation for tea, black pepper, cardamom, almond, citrus, trifoliate orange, neem and other tropical fruit species. The non-orthodox seeded species can be designated as highly recalcitrant (e.g. mango, litchi, jackfruit, mahua, jamun, mangosteen etc.), moderately recalcitrant (papaya, several Citrus species, black pepper, banana etc.) and weakly recalcitrant (some Citrus species, bael, custard apple etc.). This classification is based on the degree of desiccation sensitivity, hydrated storage life span and chilling sensitivity. On these basis seed storage behavior of more than 30 species of several indigenous tropical and temperate species have been categorized. Cryopreservation of desiccation-sensitive tissues, like that of non-orthodox seeds are only possible once the moisture content of explants is reduced to an optimal level with reasonably high viability and is able to survive freezing stresses as low as -196°C. Various desiccation techniques for seeds and embryonic axes, namely air desiccation, pregrowth-desiccation, vitrification and encapsulation-dehydration are in use at cryolab of NBPGR (Malik *et al.*, 2012). Cryoprotocols of vitrification and encapsulation have been successfully attempted in embryonic axes of *Artocarpus heterophyllus*, *Litchi chinensis*, *Poncirus trifoliata* and *Citrus* species (Malik and Chaudhury, 2006).

#### 1.1.4 Cryogenebanking of pollen

Pollen grains are simple, haploid male gametes that can be easily collected and stored in viable conditions for a sufficiently long time for use throughout the year. They can be cultured on a rather simple medium with germination occurring within a few hours. Due of these advantages, pollen are favorite systems for studies on range of biological problems (Shivanna and Rangaswamy, 1992; Chaudhury *et al.*, 2010).

Pollen conservation is complementary to seed or vegetative propagule and hence pollen banks are generally established as supplements to the genebanks. Pollen storage is resorted mainly to serve plant breeders who attempt hybridization between plants cultivated/grown in different geographic regions, or showing non-synchronous flowering. In addition pollen is stored for:

- Eliminating the need to continuously grow male lines used in plant breeding.
- Facilitating supplementary pollination for improving the yields particularly in orchard species.
- Obviating the variability incidental to daily collection of pollen samples.
- Facilitating the identification of self-incompatibility alleles.

Keeping in view the technical, practical and economic aspects of cryopreservation, strict criteria are to be followed for selecting germplasm for cryostorage. The germplasm, which can be effectively cryostored in conventional seed genebanks for prolonged duration beyond 100 years, should not be stored in cryobanks. However, the priority for what to conserve in cryobank depends upon the available infrastructure and needs of the country or the institution. The cryobank may follow specific need-based priorities for better management.

### **2.1 Non-orthodox seeds : need for cryostorage**

- Non-orthodox seeds (comprising intermediate and recalcitrant seeds) are large sized and are shed with high moisture levels. They are invariably desiccation-and freeze-intolerant and lose viability after being dried below a critical limit, usually between 12-30% moisture. At these moisture levels, the seeds cannot be subjected to subzero temperatures since they undergo freeze injury.
- Majority of species producing non-orthodox seeds are trees or other plant forms where seed regeneration in the field is highly impractical mainly due to long gestation period. Several tree species and wild species producing even orthodox seeds can be considered for cryostorage in view of apparently there being no need for regeneration if optimal cryoprotocols ensuring longest survival are ensured.
- There are no defined procedures for conservation of the germplasm of non-orthodox-seeded species. The curator and genebank managers must determine the post-harvest biology and accordingly devise best methodology.

#### **2.1.1 Harvest of fruits and seeds**

- Fruits/seeds may be collected from trees marked at the time of flowering (Plate 3, Fig B, C).
- Harvest the fruits directly from tree avoiding fallen fruits.
- Maturity of fruits may be divided into three developmental stages while keeping optimum gap in days after anthesis (DAA)
  1. Immature- fruits with seeds just after expansion of cotyledons
  2. Partially mature- fruits with seeds having fully expanded cotyledons
  3. Fully mature- fruits with seeds having maximum dry weight
- Seed storage behavior of species, if already known, would help in planning for species-specific collection trips. Information on floral biology, fruit maturity, ecology and seed biology must be gathered for optimizing chances of success.
- Whole fruits of consistent maturity status should be collected from the parent plants to ensure high quality. Preliminary studies would determine which stage of fruit maturity is the best.



- An adequate number of seeds would be required for determining the appropriate protocols for cryopreservation and to utilise in long-term cryostorage. Passport data collection is a very important activity.
- Invariably many of the fruits bearing non-orthodox seeds have pulp and high water content with chances of contamination and hence fruits may be surface decontaminated prior to transport. Fallen (abscised) material showing signs of weathering must be avoided for minimising infections

### **2.1.2 Post harvest handling and transport**

- For effective conservation, rapid transport of whole fruits instead of seeds, seed extraction on the day of experimentation and quick processing for storage within a week of extraction are required. Delay in transport and handling adversely affects the storage behaviour.
- For retention of best seed quality, fruits should be transported to the lab by courier/ speed post and in the best possible conditions, enclosed in moisture-retaining bags or containers.
- Seeds may also be sensitive to chilling and elevated temperatures hence transport temperature must not be too low or too high.
- The soft and succulent fruits would deteriorate and get infected by the time they are received in the lab even when sent by fastest mode. Hence seeds from half of the fruits may be extracted, coated using bavistin powder, packed in sawdust/ charcoal/ peatmoss and should be transported to reach the laboratory within 48 h of extraction.

### **2.1.3 Laboratory handling**

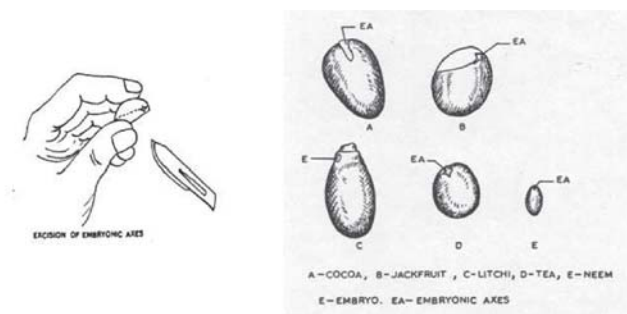
- Fruit characters, as per IPGRI descriptors, like color, firmness, shape, surface features, fresh weight, dimensions and other physical features should be recorded for distinguishing developmental stages and correlating with physiological maturity of fruits and seeds.
- For water content determination, a minimum of ten seeds must be used (on an individual seed/ embryo/axis basis/ bulk) and for viability testing 20 to 50 explants may be used.
- The part of the seed most appropriate for conservation must be decided after understanding the morphology and physiology of the whole seed and axes, and ascertaining the ability of explants to regenerate. In large seeded species like coconut and oil palm, excised embryos/ plumules can be taken up and in litchi, jackfruit, *Madhuca* spp. and other similar types, embryonic axes prove to be the explants of choice.
- Seeds should not be retained in fruits for too long as it could lead to vivipary.

### **2.1.4 Extraction and excision of explants in laboratory**

- Seeds should be extracted from fruits using muslin cloth or paper towel (but not washed as moisture would increase) and cleaned thoroughly to remove any fruit part that may cause infection. Extracted seeds should be used up for experimentation within few hours to few days.
- If temporary storage is required, seeds may be stored in sawdust, charcoal or after treating with fungicides like Bavistin or Thiram powder at temperatures between 15 to 20°C. This

practice will ensure high survival and pathogen free laboratory storage with retention of initial moisture content.

- In case whole seeds are not being processed for cryopreservation, embryos or embryonic axes may be excised from the seeds. The exact location of the embryonic axes within the seed should be ascertained.
- Seeds may be sterilized using sodium hypochlorite (2-2.7%) for 10 min followed by water washings. Embryos and embryonic axes, being deep seated, generally do not carry any infection and hence, a 8-10 min treatment with a sterilant is sufficient. The concentration of the disinfectant and the duration of the treatment may be altered as required.



- While excising embryonic axes (EA), the time taken to handle a number of samples should be minimized so that there are no major differences in moisture contents between axes.

### 2.1.5 Determination of moisture content and air-desiccation

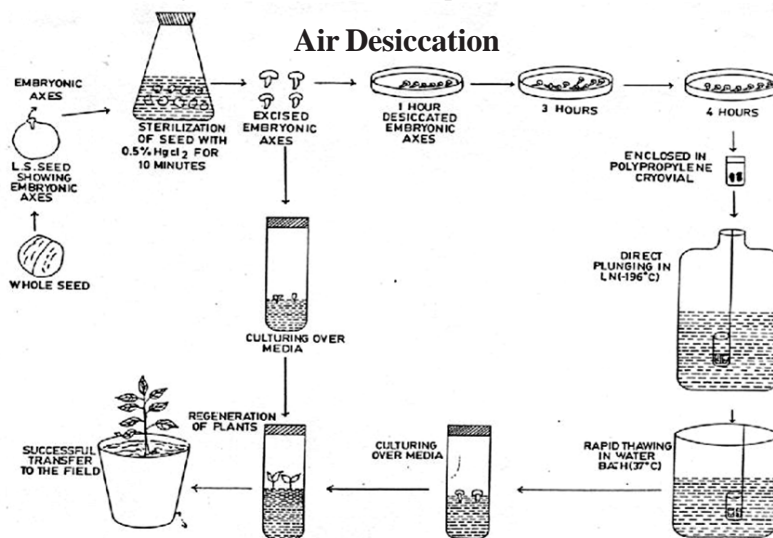
- Moisture content can be measured using low constant or high constant temperature oven method (ISTA, 1999). The weight of the sample container moisture bottles (Plate 3, Fig D) should be comparable to the explant to be weighed.
- In case of very small explants like embryonic axes, buds and pollen, small vials made of aluminum foil can be used instead of glass weighing bottles.
- Whole seeds and embryos can be desiccated in the desiccator/ vacuum desiccator filled with charged silica gel. The duration of desiccation needs to be standardized depending upon the initial and desired moisture levels. After each desired desiccation level, explants may be packed in the cryovials and maintained in the desiccator till the germination and freezing steps are to be undertaken.
- In case of non-orthodox seeds, freezing can be attempted once they show the ability to tolerate desiccation down to 10-14% moisture content.
- The sensitivity to desiccation of seeds, embryos and embryonic axes can be evaluated by assessing the relation between the moisture content and the germinability of explants. The moisture content at which survival of seeds, embryos and embryonic axes is highest is the critical moisture content of that explant.
- For Air-desiccation freezing excised embryonic axes may be kept in batches of 20-25 in the sterile air flow of laminar flow cabinet immediately after excision. Axes may be desiccated for 1 to 5h

depending upon the size of axes, the initial and desired moisture content levels. After each desiccation interval, moisture content and viability of embryonic axes need to be determined. Viability of desiccated axes should be determined by culturing them in the prescribed culture media.

- If germinability of embryonic axes is lost on desiccation to moisture content range of 10 to 25%, then a higher or lower rate of drying or a different stage of maturity of explants may be explored. Alternative culture media may be tested for germination.

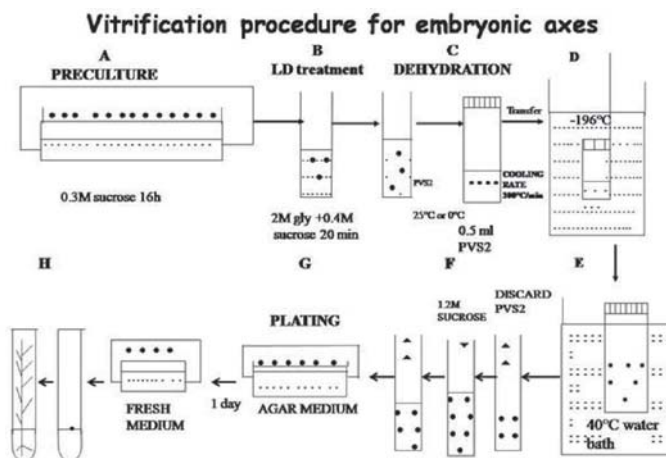
### 2.1.6 Storage in liquid nitrogen

- Freezing should be attempted at the critical moisture contents or between 10 to 25% moisture content, whichever is the lowest.
- A sufficient number of explants must be enclosed in cryovials or in sterile pieces of aluminum foil before immersion in liquid nitrogen. The freezing rate can be modified using various methods and containers. Thawing is to be performed in a water bath at the temperature of +37 to 40°C.
- Regrowth of explants is to be assessed by using *in vitro* culture methods for embryonic axes and embryos and ISTA methods for whole seeds. In case recovery growth is not upto the desirable level, the necessary changes in media and germination procedures must be made for optimum results.
- Production of morphologically abnormal seedlings should be noted as it may indicate stress of desiccation and / freezing or incorrect excision procedures.



- Occasionally, seeds of particular species may not respond well to NaOCl and/or to the commonly-used benomyl-based fungicides. In these cases other alternatives such as dilute solutions of 0.5-2% (w/v) calcium hypochlorite, 0.1-1% (w/v) mercuric chloride or 70% ethanol may be used.
- Embryo/axis are always limited in number when large experimentations are to be done. Hence fewer individuals per accession are usually cryostored. Additional explants will have to be added each year to the Cryobank.

### 2.1.7. Vitrification of embryonic axes :



### 2.1.8 Viability tests

#### 2.1.8.1. Pertiplate germination

Fresh fruits must be cut open to extract the seeds which are washed thoroughly. The seeds and embryos are placed between two sheets of moistened paper in plastic Petri plates (11 cm diameter) and incubated at  $27 \pm 2^\circ\text{C}$  with 16/8 h light/dark photoperiod. The extrusion of the shoot and/or root indicate germination of the embryo and isolated embryonic axis (Plate 4, Fig A, B).

#### 2.1.8.2. *In vitro* recovery

The embryonic axes, fresh as well as after different treatments, in most cases are cultured on Murashige and Skoog (MS) macro- and micro-nutrients, vitamins, iron,  $1 \text{ g l}^{-1}$  activated charcoal and  $0.17 \text{ g l}^{-1} \text{ NaH}_2\text{PO}_4$  supplemented with  $1 \text{ mg l}^{-1}$  each of 6-benzylaminopurine (BAP) and  $\alpha$ -naphthalene acetic acid (NAA), as defined by Chin *et al.* (1988). Cultures are maintained at  $25 \pm 2^\circ\text{C}$  with a 16 h photoperiod under a light intensity of  $35 \mu\text{E m}^{-2} \text{ s}^{-1}$ . Growth is assessed after 3 weeks of culture (Plate 4, Fig B) and plantlets subsequently transferred to field (Fig C). In specific cases other *in vitro* media can be standardized as per need. Several steps are involved in whole seeds cryobanking (Plate 5).

#### 2.1.8.3. TTC test

In the TTC assay, also named as tetrazolium test, 2,3,5-Triphenyl tetrazolium chloride (TTC) is used to differentiate between metabolically active and inactive tissues. The white compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism), while it remains white in dead areas where these enzymes have been either denatured or degraded. Explants like seeds, embryos, embryonic axes are incubated in TTC solution 0.2-0.6% (w/v) TTC in either water / phosphate buffer ( $0.05 \text{ M Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ , pH 7.4). Dormant buds are incubated in this solution along with a 0.05% (v/v) wetting agent (Tween 20), and incubated in darkness at  $30^\circ\text{C}$  overnight. Thereafter, explants are rinsed using distilled water and observed under a stereoscopic microscope. TTC staining indicates viability.

# 3

## POLLEN CRYOBANKING: Practical Considerations

### 3.1 Pollen collection and transport to lab

- Collection and handling of pollen grains in viable condition is a primary requirement for any experimental study on pollen. Generally, pollen collected soon after anther dehiscence gives optimal response.
- Flowers with freshly dehiscent anthers may be collected early morning and brought to the laboratory (Plate 6, Fig A). Pollen collections are to be made on a bright sunny day between 8 - 10 A.M and to be preferred during peak of flowering period.
- Flowers are to be harvested at peak anthesis and brought to the laboratory.
- Dehiscent anthers are gently tapped with a needle over a butter paper sheet while holding the petals back. When anthers are not properly dehiscent they are to be left under a table lamp for dehiscing.
- Pure pollen free of anther debris is collected on clean butter paper.
- Whenever pollen are sticky they can be extracted using organic solvents.

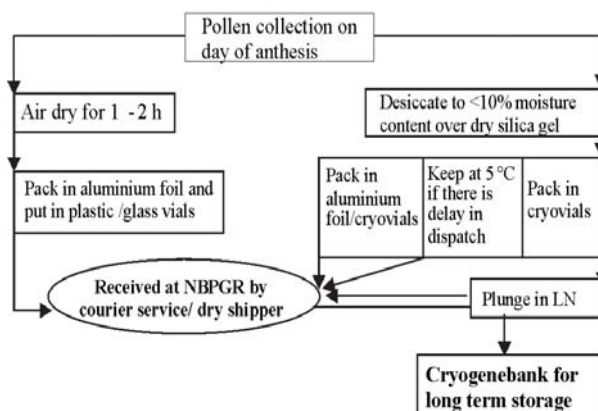
### 3.2. Viability tests

- *In vitro* germination is the most commonly used viability testing method in pollen physiology. This technique provides a simple experimental method to study the physiology and biochemistry of pollen germination and pollen tube growth.
- Within a few hours pollen tubes grow *in vitro* and data are to be quantified (Plate 6, Fig B, C). The composition of a germination medium to obtain optimal response has to be empirically formulated for each species. Liquid nutrient medium needs to be prepared in deionised, double distilled water with pH 7.3.
- Compositions of 2 different commonly used pollen culture media are as follows-

Chemicals	Brewbaker and Kwack's Medium	Robert's medium
Sucrose	10%	20%
Boric acid	100 mg l <sup>-1</sup>	10 mg l <sup>-1</sup>
Calcium nitrate	300 mg l <sup>-1</sup>	-
Magnesium sulfate	200 mg l <sup>-1</sup>	-
Potassium nitrate	100 mg l <sup>-1</sup>	100 mg l <sup>-1</sup>
Calcium chloride	-	362 mg l <sup>-1</sup>
Tris	-	60-130 mg l <sup>-1</sup>

- A small amount of pollen is to be placed with a needle on a glass slide. A drop of pollen germination medium is placed on the pollen and disbursed uniformly in it by needle tip. In several cases semi-solid media is used for *in vitro* germination. After the prescribed duration of incubation (generally 3-6h) in a humid chamber, record the germination percentage under the microscope for qualitative and quantitative viability estimates. Pollen showing tube lengths longer than the pollen diameter are scored as viable with 400-500 pollen in two replicate drops scored in total.
  - Pollen germinability after cryostorage is to be assessed using the same protocols used for fresh pollen. Images invariably are recorded using compound microscope.
- (i) *In vitro* germination has been commonly used for testing the viability. It provides a simple experimental method to study the physiology and biochemistry of pollen germination and pollen tube growth.
- (ii) Tetrazolium (TTC) test is also conducted.

#### Collection and storage of Pollen in cryobank



- (iii) Fluorochromatic Reaction (FCR) test is additionally conducted to test the presence of esterases in the cytoplasm and to check membrane intactness. In this test pollen grains are mounted in fluorescein diacetate (FDA) solution, where the non-polar non-fluorescent FDA enters the pollen cytoplasm. Esterases of cytoplasm hydrolyze FDA and releases fluorescein, which is polar and fluorescent. Fluorescein accumulates in the cytoplasm of viable grains and gives green or yellowish green fluorescence within 1-2 min (Plate 6, Fig D). Stock of FDA is prepared as 2 mg/l in acetone. To 2 ml of 10% sucrose solution FDA stock is added drop-wise till a slight turbidity appears. This solution is to be used up within 5-10 min of preparation.
- (iv) Fertilizing ability of cryostored pollen has been observed in selected cases and fruit set quantified. The best method to test the viability is by field pollination and quantifying it by seed set percentage.

### 3.3 Determination of moisture content and desiccation

- Moisture content can be measured using low constant temperature oven method described earlier. The weight of the sample container should be comparable to the explant to be weighed. Small aluminum foil cups may be used instead of glass weighing bottles.
- Desiccation of pollen with higher moisture content is usually carried out on charged silica contained in air tight desiccators for 2-3 hours. Regular monitoring of the sample should be

done to ensure a high viability and low moisture content. Excessive desiccation can lead to loss in viability.

### **3.4 Storage in LN**

- Pollen desiccated to suitable moisture contents can be stored in aluminum packets, gelatin capsules or cryovials.
- Pollen in sufficient quantities can be easily packed in cryovials of 1 ml capacity and stored at  $-196^{\circ}\text{C}$  (under liquid nitrogen) by placing into a canister and lowering in a cryotank. After 24 h, the cryovials may be shifted to the vapor phase of LN at temperatures between  $-170^{\circ}\text{C}$  to  $-180^{\circ}\text{C}$ . Eight to ten replicates of each pollen sample need to be cryostored.
- For thawing samples may be removed from storage and kept at room temperature for 30 minutes prior to a viability test.

# 4

## DORMANT BUDS CRYOBANKING: Practical Considerations

### 4.1 Buds collection and transport to laboratory

- Fields are to be visited during peak winter months when leaves are fallen and buds are totally compacted. Preferably twigs be collected after trees have remained under snow for 5-7 days constantly.
- For collection of buds 60 cm long twigs of last one year's growth having dormant buds are to be harvested from plants growing at the field genebank in winter seasons from December to February. These buds need to be wrapped/packed in cotton bags and airlifted at low temperatures from field to laboratory. Twigs cut ends need to be wax sealed to retain original moisture.

### 4.2. Handling in laboratory

- On receipt the twigs are to be wrapped in plastic bags and stored in refrigerator at 10-15°C temperature for maximum of 10-30 days.
- Dormant buds should be extracted initially under the stereo microscope. Morphology of the buds should be studied in advance for quick and complete excision of explant for various experimentations.

### 4.3. Extraction and excision of explant in laboratory

- Buds are chipped off from the main stem using the sharp blade so that no woody tissue remains attached to it. External scales about in 5-7 numbers on the buds need removal.
- For processing nodal sections, pieces of bud sections about 35 mm long each with a healthy bud are cut from the well acclimated twigs/scion.
- For testing the viability 10-15 buds were washed with Tween 20 for 15 min followed by washing with running tap water.
- The 10-15 buds are surface sterilized in 0.1%  $\text{HgCl}_2$  for 10 minutes followed by 3 washings with autoclaved distilled water. These buds were then cultured *in vitro* in suitable culture medium supplemented with suitable cytokinin.

### 4.4. Determination of MC and air desiccation

- Moisture content of fresh and desiccated buds is measured using low constant temperature oven method described earlier. A 10-12 buds are cut to small pieces and used up for MC determination by drying at 103°C + 2°C temperature in an oven for 17 h.
- For processing the excised buds for desiccation, non-sterile buds are kept above silica gel in desiccators of about 4 litre capacity maintained at 27°C.
- Nodal sections may be placed in wire drying rack or meshes and kept exposed to low temperatures in refrigerator.



- Buds processed for desiccation are left non-sterile and are sterilized only before culturing *in vitro*.
- Depending on the initial moisture contents, the target moisture contents between 17 to 24% may be achieved by desiccating the chipped buds for 3 to 7 h and nodal sections for 2-3 days.

#### 4.5. Storage in LN

- Programmable freezing : A programmable freezer has a freezing chamber cooled by LN supplied from a pressurized LN vessel. The freezing rate of 0.5 to 1 degree per minute is given from 0°C to the terminal prefreezing temperature of -30°C to -40°C. Samples are held at this temperature for 30 min followed by a rapid immersion in LN.
- Step-wise freezing (Plate 7): Dormant buds predesiccated to varying moisture contents are subjected to freezing after enclosing in 2 ml cryovials. The slow step wise freezing is achieved by sequentially lowering the temperature at -5°C/day using deep freezers up to terminal temperature of -30°C. For this, the cryovials/ polyolefin tubings enclosing the explants are shifted sequentially at 5°C, -5°C, -10°C, -15°C, -20°C, -25°C and -30°C keeping at each of the temperatures for a minimum of 24h.
- Samples are to be held at terminal temperature of -30 to -40°C for 24-48 h followed by a rapid immersion in LN.
- Simple fast freezing may be achieved by direct plunging of pre-desiccated buds in liquid nitrogen without any pre-freezing.
- The excised buds are always enclosed in cryovials whereas nodal sections are sealed in heat shrinkable polyolefin tubes with birch plugs in both ends for cryostorage.
- Buds / nodal sections may be rehydrated in moist peat moss for various periods ranging from 2-5 h at room temperature.
- For *in vivo* recovery, bud chipped from the bud sections need to be grafted to a suitable rootstock in field and record the bud emergence and regrowth.
- In case of test of *in vitro* recovery 4-7 outer scales are to be further removed from the buds before culturing.
- Thawing is done by 2 methods, slow by keeping LN retrieved cryovials in air at ambient temperature for about 40 minutes or fast by plunging in water bath maintained at 38°C for 3-5 minutes.
- Glass containers can shatter when warmed from sub-zero temperatures, so polypropylene screwcap cryovials are generally used. Polypropylene cryovials of different sizes (1ml, 2ml, 5ml, 50ml) designed for low temperature works are commercially available.

## **IN VITRO PLANTLET REGENERATION FROM SEEDS/ AXES/ DORMANT BUDS : Applications for Cryostorage of Non-orthodox Seed Species**

Cryobanking of diverse germplasm at National Cryogenebank at NBPGR in the form of embryos, embryonic axes ( Plate 8) , shoot tips, meristems, dormant buds etc necessitates the *in vitro* regeneration into healthy plants before and after storage for non-orthodox seed species (Chaudhury & Malik, 2014). Embryonic axes, being small and highly meristematic are preferred explants for storage. Apomictic seeds and polyembryonic seeds of certain tropical fruits, being true-to-type are also preferable explants for storage. Dormant buds from temperate deciduous fruits and nuts are the materials of choice for cryobanking in view of their amenability to cryopreservation procedures and to maintenance of clonal integrity. Work on these diverse explants belonging to more than 400 species are underway at NBPGR. Cryopreservation of embryonic axes, especially of large seeded non-orthodox seed species, instead of whole seeds economizes on storage space. After cryostorage for various periods, the *in vitro* recovery methods mainly using MS medium with various hormone combinations have been standardised for tea, jackfruit, litchi, almond, citrus species, neem, walnut, oak, etc. the explants are required to be placed in optimal conditions to trigger rapid and direct (i.e. without callus) growth. The cultures are usually placed in the dark or under reduced light for several days to reduce deteriorative photo-oxidation phenomena. The composition of the culture medium is also often transitorily modified usually by changing the nature and / or concentration of growth regulators. After few days or weeks the cultures are brought back to standard culture conditions. In addition rehydration of desiccated explants by different methods is resorted to avoid any imbibitional injuries. Role of  $\text{Ca}^+$  and  $\text{Mg}^+$  ions and ammonium ions has been found to be prominent in recovery growth of conserved germplasm. Modifications, even when minor, in the *in vitro* culture conditions have led to improvement in the recovery rate for several species. In case of *Garcinia* species and *Calophyllum* sp. a simple and effective method has been developed for rapid regeneration of plantlets via adventitious bud differentiation. Mature apomictic ‘seeds’ of *Garcinia indica* are ideal explants for *in vitro* establishment and multiplication of selected superior clone for obtaining true-to-type plants. In some seed species, *in vitro* recovery of embryonic axe excised from cryostored seeds has been found essential as the cotyledons and endocarp were found to impede the growth of viable embryonic axes. In all the cases the aim has been to recover plants, that have been stored at ultra-low temperatures of  $-196^\circ\text{C}$  for various periods, bring back to room temperature without any damages- structural and physiological and obtain plantlets without an intervening callus to ensure genetic integrity of the conserved germplasm. In case of cryoconserved mulberry dormant buds, genetic stability has been assessed using ISSR markers in the *in vitro* raised plants and no variation was detected when compared to fresh controls.

# 6

## TECHNICAL ASPECTS: Cryostorage in Large Capacity Cryotanks

### 6.1 Cryotanks and sample storage

- Most storage tanks have access from the top, therefore a vertical inventory made of metallic (steel or aluminum) may be used in which boxes are fitted which can hold the cryovials.
- Small seeds, excised embryonic axes, pollen, dormant buds, shoot apices and meristems may be stored in large numbers in 1 ml/2ml polypropylene cryovials. A 650 liters capacity LN tank can hold up to 50,000 of 1 ml cryovials.
- If germplasm of a large number of species is to be handled i.e storing a range of seed sizes, then a flexible inventory system would be required. Aluminum canisters can be very useful for holding the germplasm since 1ml, 2ml or 5ml cryovials mounted on aluminum canes or else 50ml vials and polyolefin tubings can be effectively stored in them.
- Liquid nitrogen boils off continuously as heat infiltrates into the cryotanks through the sidewalls and the access port on the top. Additional loss of LN occurs when warm samples are placed in the tank. Evaporation rate of LN for most of the tanks is 0.5 to 1.5% of its capacity per day and accordingly, replenishment of LN is required about two times per week to maintain the temperature of the cryotank between  $-160$  to  $-180^{\circ}$  C.
- The size of the cryovial to be used will depend upon the size as well as the quantity of the seeds/ embryonic axes/ dormant bud/pollen grains to be stored. Large seeds can be stored in heat-sealable polyolefin tubing with cork stoppers.
- Storage in liquid phase of nitrogen may lead to ingress of LN inside the vial unless there is an impermeable seal. This could facilitate cross-contamination from other vials or contamination *via* inoculum present in the LN.
- While thawing cryovials containing LN inside, it can be hazardous to the personnel because LN escapes dangerously under pressure. Ideally the cryovials should be suspended above the LN at temperatures of about  $-160^{\circ}$ C. Depletion of LN in a cryostorage vat or LN freezer would lead to irretrievable loss of all samples.
- There are no guidelines regarding the minimum number of seeds to be cryostored per accession. As far as possible large samples should be stored depending upon the reproductive biology of the species, as there would be greater chances that rare and potentially useful genes would be represented in the stored samples. For explants like embryos, embryonic axes, buds and pollen, there is no standard recommendation for the minimum number of explants to be stored. It usually depends upon the availability of the material, percentage survival and on the plan of retesting.
- Once the storage protocol is established, a part of the sample should be stored for long-term, and should be treated as 'base collection'. It should not be meant for distribution, exchange or retesting. Another part of the sample should be stored separately as 'test sample', which could

be utilized for providing material to breeders or other researchers or could be used for routine viability retesting.

### **Liquid phase storage**

- **Advantages:** Offers a uniform temperature
- **Preferred explants :** Meristems, shoot apices and dormant buds
- **Problems:**
  - LN may enter the storage tubes/cryovials.
  - There is risk of sample contamination and cross contamination with microbes.
  - On warming the LN expands and may cause small explosion.
  - Evaporation would be more-increasing the cost/vial

### **Vapour phase storage**

- **Advantage:** Above problems are avoided
- **Preferred explants:** Seeds-orthodox and intermediate, embryos, embryonic axes and pollen
- **Problems:** Temperature gradient would be present below the lid.

## **6.2 Sample storage containers**

- All types of explants are to be finally packed in airtight containers before cryostorage.
- Glass containers can shatter when warmed from sub-zero temperatures.
- Polypropylene screwcap cryovials of different sizes (1ml, 2ml, 5ml, 50ml) depending upon the size as well as the quantity of the seeds / embryonic axes / dormant buds/ pollen grains are used.
- Pollen desiccated to suitable moisture content can be stored in aluminum packets or gelatin capsules.
- Large seeds and twigs are to be stored in heat-sealable polyolefin tubing with cork stoppers or in goblets in sleeves.

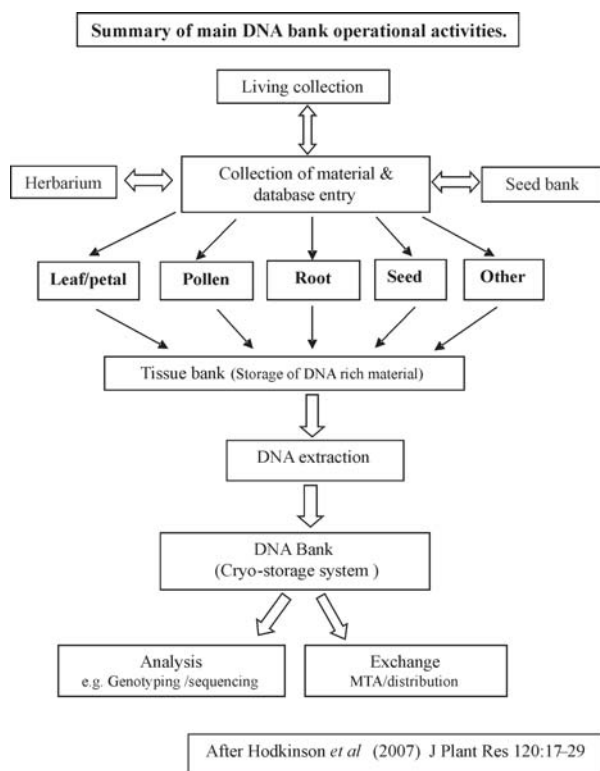
## **6.3 Safety considerations**

- LN must be used in a well ventilated room to avoid the risk of suffocation.
- Handling and storage tanks must be vented to prevent explosion
- Cold resistant gloves, safety grasses and closed top shoes must be used.
- Oxygen monitoring equipment should be installed where large capacity cryotanks are maintained.
- Closed circuit camera should be installed in the cryobank.

# 7

## DNA BANK

DNA bank is a particular type of genetic resource bank that preserves and distributes the DNA samples and provides associated information. DNA bank offers tremendous opportunities of practical and academic value. Idea of DNA as a genebank resource has emerged out from the availability of the genomic resources as a spin off from analysis of DNA extracted from numerous plant species in laboratories across the world. DNA are also available as amplification products of PCR reactions. Other biotechnology experiments require construction of DNA libraries, i.e. collection of segments of DNA containing several copies of the part of genome including clones of cDNA, cosmid, PAC (plasmid-derived artificial chromosomes), BAC (bacterial artificial chromosomes), YAC (yeast artificial chromosomes) etc. All these DNA forms are important resources for use in several applications, viz. characterizing the source material, understanding genetic and evolutionary relationships between taxa, functional analysis of genes, comparative genomics and plant breeding. DNA storage is a relatively new technique that is rapidly increasing in terms of importance. DNA from nucleus, mitochondria and chloroplast are extracted for storage. The technique is relatively simple and cost-effective. The regeneration of entire plants from DNA cannot be envisaged at present, but DNA can be stored and single and small number of genes is expected to be utilized using biotechnological techniques.



# 8

## COSTING OF CRYOGENEBANKING

Hummer and Reed (2000) indicate that, at the NCGR, Corvallis, Oregon, USA the annual maintenance cost of one temperate fruit tree accession is US\$77 in the field, US\$23 under *in vitro* slow growth storage, and only US\$1 under cryopreservation, to which US\$50–60 should be added once for cryopreserving this accession. Roca (personal communication) evaluates the annual maintenance cost of CIAT's (International Center for Tropical Agriculture, Cali, Colombia) cassava collection, which includes 5000 accessions, at around US\$5000 under cryopreservation, against US\$30 000 under *in vitro* slow growth storage.

At NBPGR cost of cryostorage works out to be \$ 1 per sample.

The costs involved in cryoconservation need to consider the following:

- a) The cost of literature review to find out which corps and habitats are under threat of extinction and hence need cryobanking
- b) Identifying the sites and deciding costs to collect fresh sample of seed, pollen, roots, dormant buds, cutting or any other plant part
- c) Sampling cost, processing of sample whether fresh or in liquid nitrogen containers, packaging, transportation
- d) Collection of information about pre- and post-harvest knowledge possessed by local communities, individual farmer breeders, knowledge experts in cultivations, protections, or processing.
- e) Describing the sample, characterizing it morphologically and by use of molecular markers
- f) Cost of long-term storage of germplasm.
- g) Cost of infrastructure needed especially cryotanks, LN station, pipelines, etc.
- h) Cataloguing and data base upgradation of information on cryostored germplasm
- i) Regular viability checks, grow out to observe field performance
- j) Designing of a duplicate set to be kept in another region with appropriate institution
- k) Cost of human resource development, upgradation of skills,
- l) Cost of germplasm supply for utilization of germplasm.

# 9

## ELECTRON MICROSCOPY AND HISTOLOGICAL TECHNIQUES AIDING CRYOBANKING

### 9.1. *In vitro* regeneration

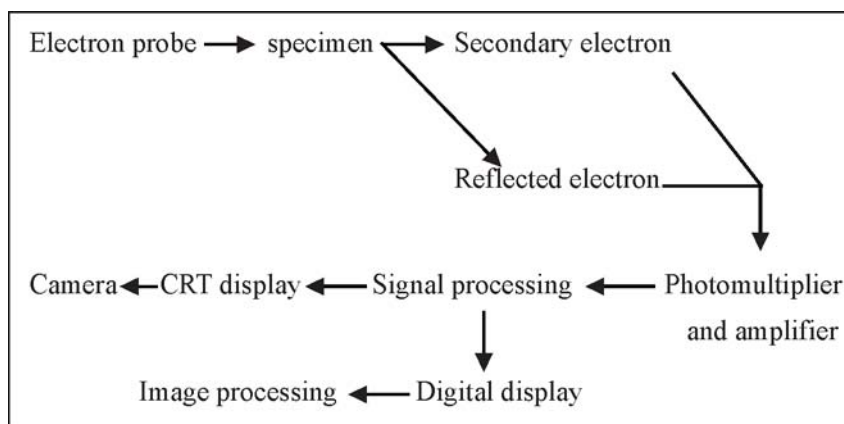
Studies on structural aspects of plant regeneration during *in vivo* and *in vitro* conditions before and after cryoexposure require detailed examination of histology and micromorphology. *In vitro* regeneration may result in *de novo* formation of organised structures like roots, shoots, buds etc. or bipolar embryos or may result in wound healing (callus formation and histogenesis). The *in vitro* organisation and morphogenesis of multicellular plants depends upon the integration and mutual interaction of the various organs, tissues and cells. To trace the emergence of organs from within the initially relatively uniform cell mass or callus, intensive anatomical examination of tissues is needed.

### 9.2 Histochemical staining

It is a technique presently being used to solve many problems in basic and applied biological research and is applied to correlate physiological and biochemical changes occurring in seeds/ EA/ tissues with morphological changes. The principles and procedures of histochemistry are almost similar to those used in biochemistry except that in histochemistry the coloured end product is precipitated within the cells. Thus with tissues containing a variety of cell types, biochemical functions of a single cell type can be studied with the cell in a normal structural and presumably functional relationship with other cell populations. Therefore, histochemical studies of tissues determine the differences between cells, which might otherwise appear to be a population of a single cell type. Histological, histochemical and ultrastructural studies also help in evaluation of structural and biochemical changes possibly taking place in the cells after desiccation and freezing.

### 9.3 Micromorphological studies using SEM

Scanning Electron Microscopy can reveal topographical details of a surface with clarity and detail, which cannot be obtained by any other means. The contrast in the micrograph is due to the topographical variations and atomic number differences in the specimen.



In general following attributes can be studied and further utilized for germplasm comparison/ characterization, phylogenetic and biosystematic studies through SEM:

- (a) Presence of lesions- their shape, size and frequency;
- (b) Epidermal cells- shape and size;
- (c) Epidermal appendages/protuberances –type, shape, structure and frequency;
- (d) Epicuticular depositions -morphology and quantity;
- (e) Structure, morphology and frequency of stomata;
- (f) Structure of anticlinal walls of seed coat cells.

**Preparation of material :** Preparation techniques will differ as per the nature of the material. However, the surface of a specimen to be investigated by SEM must:

- a. be free from foreign particles
- b. be vacuum stable
- c. remain stable after exposure to the electron beam
- d. emit sufficient number of secondary electrons.

The final result of electron microscopy will depend almost entirely on the preparation technique carried out before the specimen goes into the pressure vessel. So to meet the above requirements, biological specimens have to be passed through following steps:

**a. Washing and cleaning:** Suitable material for SEM studies should be carefully selected and washed with distilled water for cleaning the surface.

**b. Fixation and dehydration:** Fixation of specimen is must to preserve the structure and arrangement of cells without shrinkage or collapse or swelling during subsequent treatments. It also provides specimen hardening. Following steps are to be followed.

1. Fix the material in 1-2% glutaraldehyde in 0.5M phosphate buffer at 7.0 pH for 24 h at room temperature.
2. Wash with three changes of phosphate buffer.
3. Fix in 1% Osmium tetroxide in buffer for 1h at room temperature.
4. Wash with buffer thoroughly.

Dehydration of the specimen is done by passing it through 25, 50, 75 and 100% ethanol or dry acetone for 1h each. At the end two changes in 100% ethanol or dry acetone are made.

**c. Critical point drying:** After fixation and dehydration samples are dried at certain pressure and temperature, together known as critical point, at which the density of the vapour is same as that of the liquid. At critical point, the surface tension is zero because phase boundary between the liquid and gaseous states disappears. The fluids, which undergo phase change from liquid to gas at the critical point, are called the transitional fluids. Commonly used fluid in critical point drying is liquid carbon dioxide because it has more acceptable critical temperature ( $T_c$  30°C) and pressure ( $P_c$  1072 psi). Steps to be followed for CPD are given below.

1. Place dehydrated specimen in wire baskets, taking care not to allow drying ; this operation is to be carried out under the transfer solvent (acetone).
2. Fill the transfer boat with transfer liquid (acetone) and put baskets in boat.
3. Close all the valves of the chamber and run cold water to cool chamber to 20°C.



4. Load specimen boat in the chamber, close door and open inlet valve. Filling of liquid CO<sub>2</sub> gas should be rapid; open vent valve to avoid back pressure.
5. Leave inlet valve fully open (with vent valve slightly open to maintain liquid level) and open drain valve to remove substitution liquid. This flushing action should be kept up for 3-5 minutes.
6. After flushing the bulk of substitution liquid, fill the chamber and close all valves. Leave for 1 h to allow impregnation (time depends on the size of the specimen).
7. Step 5 to be repeated to fill chamber with fresh liquid CO<sub>2</sub>.
8. Close the inlet valve and allow the level of liquid to fall upto the level of boat.
9. Slowly heat up the body of apparatus using a flow of water at about 35-40°C and walls closed. Near the critical point turbulence inside the chamber becomes optically visible.
10. Carefully vent the CO<sub>2</sub> gas. Avoid condensation effects which can be caused by rapid venting or by the presence of acetone.
11. Open the specimen chamber and remove the tissue baskets.

**d. Mounting and sputter coating:** Critical point dried material is to be mounted on brass/aluminium stubs using an adhesive which is also electrically conductive and this is able to convey electrons from the specimen to the stub. For large specimens silver adhesive/liquid carbon black and for the small materials double-sided adhesive tape is used. Biological materials are poor electrical conductors and will consequently charge up when placed under electron beam. It is essential to coat such specimens with a thin layer of conducting material prior to their examination with SEM. Main reasons for coating the specimen are to improve electrical and thermal conductivity and to enhance secondary electron emission. Direct current coating is done with a thin plate of gold/gold-palladium in a commercially available ion sputter coater.

Procedure of coating is given below-

1. Fix the specimen stubs in stub holding clamps and place the ring on the stage in the sputtering chamber.
2. Mount the gold target plate on the stage.
3. Switch on the mechanical pump and establish relatively low vacuum in the sputtering chamber.
4. Run high voltage current for short duration (2-3 min.) to ionize and excite large inert molecules such as oxygen and nitrogen to a rapidly moving state. These molecules bombard the gold plate and dislodge its particles, which are then deposited on the specimen surface, forming a thin coating.
5. Shut off the vacuum pump and remove specimen stubs from the clamps.

The critical point dried material and metal-coated stubs should always be stored in a desiccator to avoid reabsorption of moisture from the air.

**e. Scanning and Photography:** Load the stub into the specimen chamber (vacuum vessel) of scanning electron microscope. Scan the specimen and photograph or store the relevant portions of image.

## 9.4. Histological and histochemical studies

The following steps are to be followed for histological and histochemical examination of tissues :

1. Selection of tissue
2. Fixation
3. Dehydration
4. Infiltration and embedding

5. Sectioning and mounting of sections
6. Staining
7. Microscopic observation

**1. Selection of tissue:** *In vitro* cultures to be studied for histological examination should be carefully examined morphologically. Regenerated tissues with the some part of mother tissue should be trimmed as much as possible in case of *in vitro* cultures. Part or the whole explant can be selected depending upon size and area to be investigated in case of cultured material, seed, embryo or embryonic axes. Culture medium should necessary be removed from the tissue from liquid culture.

**2. Fixation :** Fixation is one of the most critical steps in the processing of plant tissue. In histological studies role of fixative is to minimize the loss of intracellular material and to ensure that the tissue remains an accurate representative of the living state while for histochemical and enzyme studies additional requirement of preserving the activity of metabolite or enzyme is required.

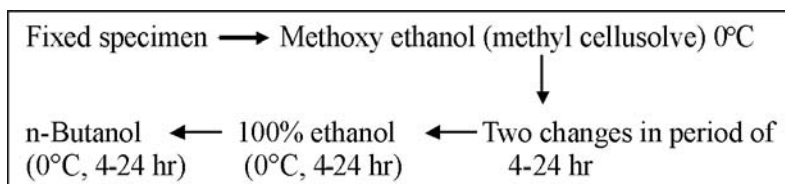
Some commonly used fixative and their constituents are :

- |                          |  |
|--------------------------|--|
| 1. FAA                   | : Formalin-acetic acid-ethyl alcohol (10:1:2)  |
| 2. Nawaschiin or craf    | : Chromic acid-acetic acid- formaldehyde (3:2:1)   |
| 3. Farmer and Carnoy     | : Ethyl alcohol-glacial acetic acid (3:1) and Ethyl alcohol-glacial acetic acid-chloroform (6:1:3) |
| 4. Glutaraldehyde-Osmium | : Primary fixation -2-6% glutaraldehyde, Secondary fixation – 0.5 to 2% Os O <sub>4</sub>          |

Fixatives for localization of some important metabolites and enzymes related to growth and differentiation in plant cells are :

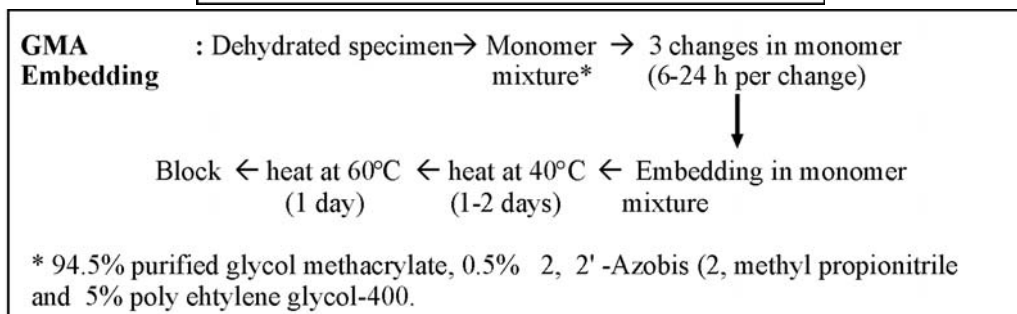
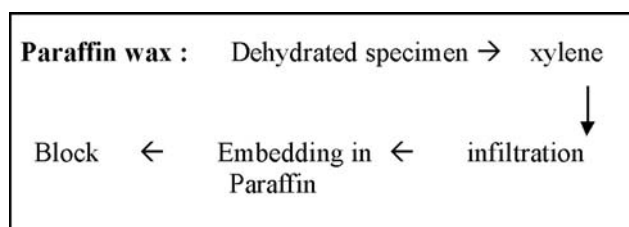
- |                         |  |
|-------------------------|--|
| DNA                     | : Cold (4°C) Carnoy's fluid  |
| RNA                     | : Fresh/Carnoy's fluid, post fix in formaline  |
| Protein                 | : Ethanol : Acetic acid (5:1) or Absolute methanol : formaldehyde: acetic acid (17:2:1)  |
| Histones                | : 10% neutral buffered formaline (Nuclear basic proteins)                                |
| Carbohydrate            | : Fresh/4% formaldehyde  |
| Lipid                   | : Formal-Calcium(1% CaCl <sub>2</sub> in 4% neutral formalin) or Glutaraldehyde - Osmium |
| Succinate dehydrogenase | : Unfixed frozen/cold (4°C) formaldehyde for 5-15 min.                                   |
| Peroxidase activity     | : 1% formaldehyde or glutaraldehyde for 2 hr.  |
| Phosphatase activity    | : Unfixed frozen/3% glutaraldehyde for 2 hr.   |
| ATP ase activity        | : Unfixed frozen/cold (4°C) 4% formaldehyde  |
| Amylase activity        | : Unfixed frozen   |

**3. Dehydration:** In this process fixed tissue is treated with a series of solutions containing progressively increasing concentrations of the dehydrating agent and decreasing concentrations of water. The most commonly used dehydrating agents are ethyl alcohol, iso-propyl alcohol, acetone and tertiary butyl alcohol. Material is passed through the graded series of dehydrating agent and finally transferred to a solvent of paraffin or plastic whichever is to be used for embedding. Feder and O' Brien (1968) suggested following series for dehydration:



Specimen dehydrated using above series may be embedded in paraffin wax, polyster wax or glycol methacryalate (a plastic).

**4. Infiltration and Embedding:** Embedding of dehydrated tissues in some matrix is must to support the tissue against the impact of cutting and also to hold each part of tissue in sections. Commonly used matrices are paraffin wax and plastic. Plastic embedding has become more common because hardness of plastic blocks provides cutting of sections even less than 1µm thickness thus improving the resolution of the specimen. The most common plastic embedding media for light and electron microscopy are glycol methacrylate (GMA) and epoxy resins. These embedding procedures enable one to obtain high-quality specimens giving good clarity, contrast and resolution for microscopic examination. Moreover, plastic does not interfere with staining procedures, therefore, its removal prior to staining is not necessary. For routine studies paraffin wax is used as per following steps:



**5. Sectioning and Mounting:** Sectioning of material embedded in paraffin or plastic blocks is done with a microtome. Thickness of section depends upon the type of tissue and the aim of study. For routine observations 5-10 µm thick sections are required. Paraffin or plastic sections in the form of ribbon or individuals are fastened to a glass slide with an adhesive prior to staining.

**6. Staining :** Staining of cellular structures is based on specific affinity between certain dyes and particular cell structures or content.

**General staining:** For the examination of general histology, paraffin sections are usually stained with Safranine, Fast Green, Heamatoxylene, and Toluidine Blue in various comb-inations or as single stain. While, GMA sections are stained with Toluidine Blue'O as described below:

- A. Stain GMA sections for 1-5 min in 0.05% Toluidine Blue'O in benzoate buffer (pH4.4).
- B. Destain in slow running water, briefly rinse slides in distilled water, dry on a hot plate (35°-40°C).
- C. Apply mountant and cover slip.

Polyphosphates, polysulphates and polycarboxylic acid are stained red or reddish purple; lignin and some polyphenols green or blue green; RNA- purple; DNA-blue or blue green.

**Histochemical staining** : Following are some important histochemical staining methods frequently in use for the study of *in vitro* cultured material .

	<b>Localization of</b>	<b>Staining technique/ Stain</b>
1.	DNA	Feulgen reaction
2.	RNA	Azure -B
3.	DNA and RNA	Methyl green pyronin - y stain
4.	Proteins	Amido Black 10B and Coomassie Brilliant Blue
5.	Nuclear proteins (histone)	Alkaline Fast Green
6.	Carbohydrates	Periodic Acid Schiff's (PAS) reaction
7.	Lipids	Sudan Black B
8.	Lipids	Benzpyrene method
9.	Callose	Aniline Blue

Several techniques for enzyme localization have been developed; these are widely applied to diverse problems in plant sciences. Localization procedures for some enzymes e.g. Succinate dehydrogenase, peroxidase, phosphatase, triphosphatase and amylase, which are frequently related to growth and differentiation in plant cells, can be checked up in detailed papers

**7. Microscopic observation:** Extensive microscopic observation and interpretation of structures obtained in sections at cellular and sub cellular levels is the most important part of histological and biochemical examination.

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**Practical on embryonic axes cryopreservation**  
*Prunus amygdalus* (Almond)  
**Air desiccation-freezing (after Chaudhury & Chandel, 1995)**

**The procedure**

1. Crack open the hard endocarp to extract the seeds.
2. Surface sterilize the seeds using 0.1% mercuric chloride for 10-15 minutes in laminar flow cabinet.
3. Rinse four times with sterile distilled water.
4. Remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with embryonic axes.
5. Make an incision at the other attached connection of embryonic axes to separate it out.
6. Use 15-20 axes for moisture content determination using low constant temperature oven method.
7. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 6.8 to 7.5% (on fresh weight basis) as determined by low constant temperature oven method.
8. Place about 10-15 desiccated axes in sterile 2 ml cryovial and plunge rapidly in liquid nitrogen (LN).
9. Hold in LN for 5-10 min or more as per designed schedule.
10. Thaw the cryovials in a water bath at +38°C for 5 min.
11. Culture the axes in MS medium containing 1mg ml<sup>-1</sup> each of BAP and NAA and 2g l<sup>-1</sup> charcoal and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>.

**Items required a day before initiating cryopreservation**

1. Sterile glass petridishes and 100 ml flasks
2. Sterile filter paper discs
3. Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
4. Sterile cryovials and cryomarkers
5. MS culture medium in glass test tubes
6. Dewar flasks containing LN

**Precautions**

1. If the seeds are too hard and it is not easy to separate out their cotyledons, they may be first soaked in water for 20 minutes.
2. The excision of attachment points of axis to cotyledons should be carefully done avoiding damages to shoot or root apex.

***Camellia sinensis* (Tea)**  
**Air desiccation-freezing (after Chaudhury *et al.*, 1991)**

**The procedure**

1. Break open the black hard endocarp to extract the seeds.
2. Surface sterilize the seeds using 0.1% mercuric chloride for 15 minutes in laminar flow cabinet.
3. Rinse four times with sterile distilled water.
4. Remove the papery brown seed coat and gently separate out the cotyledons snapping one of the attached connections with embryonic axes.
5. Make an incision at the other attached connection of embryonic axes to separate it out.
6. Use 15-20 axes for moisture content determination using low constant temperature oven method.
7. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 10 to 13.5% (on fresh weight basis) as determined by low constant temperature oven method. If the initial moisture of axes is around 50%, it may take 2-3 h of laminar flow drying to achieve target moisture content.
8. Place about 10-15 desiccated axes in sterile 1.2 ml cryovial and plunge rapidly in liquid nitrogen (LN).
9. Hold in LN for 1h or more as per designed schedule.
10. Thaw the cryovials in a water bath at +37-38°C for 5 min.
11. Culture the axes in components of Nitsch and Nitsch medium supplemented with 1mg ml<sup>-1</sup> each of BAP and Kinetin, 0.1% yeast extract and 2% sucrose (after Nakamura 1985, *Bull. Shizuoka Tea Experiment Station* 11: 1-5) and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>.

**Items required a day before initiating cryopreservation**

1. Sterile glass petridishes and 100 ml flasks
2. Sterile filter paper discs
3. Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
4. Sterile cryovials and cryomarkers
5. Nitsch and Nitsch culture medium (modified) in glass test tubes
6. Dewar flasks containing LN.

**Precautions**

The excision of attachment points of axis to cotyledons should be carefully done avoiding damages to shoot or root apex.



## *Azadirachta indica* (Neem)

### Air desiccation-freezing (after Chaudhury & Chandel, 1991)

#### The procedure

1. Collect seeds from ripe yellow neem fruits.
2. Break open the endocarp to extract the seeds just before experimentation.
3. Surface sterilize the seeds using 0.1% mercuric chloride for 10 minutes in laminar flow cabinet.
4. Rinse four times with sterile distilled water.
5. Remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with embryonic axes.
6. Make an incision at the other attached connection of embryonic axes and scoop it out.
7. Use 15-20 axes for moisture content determination using low constant temperature oven method.
8. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 11 to 16% (on fresh weight basis) as determined by low constant temperature oven method. If the initial moisture content of neem axes is about 45%, it may require 3.5 to 4 h of desiccation to achieve target moisture level.
9. Place about 10-15 desiccated axes in sterile 1.2 ml cryovial and plunge rapidly in liquid nitrogen (LN).
10. Hold in LN for 1h or more as per designed schedule.
11. Thaw the cryovials in a water bath at +37-38°C for 5 min.
12. Culture the axes in MS medium containing 0.1mg ml<sup>-1</sup> each of BAP and NAA and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>.

#### Items required medium in glass test tubes

6. Dewar flasks containing LN

#### Precautions

1. The seeds may be harvested only from ripe yellow fruits
2. The extraction process may be completed within 4-5 days of harvest to obtain optimal results in cryopreservation.

**Practical on Vitrification of embryonic axes**  
*Citrus macroptera*  
**Vitrification (after Malik and Chaudhury, 2006)**

**The procedure**

1. Collect seeds from ripe fruits, remove the seed coat from the seeds just before experimentation.
2. Surface sterilize the seeds using 0.1% mercuric chloride for 10 minutes in laminar flow cabinet. Rinse four times with sterile distilled water.
3. Gently separate out the cotyledons snapping one of the attached connections with the zygotic embryonic axes.
4. Scoop it out by making another incision at the other joining point.
5. Use 15-20 axes for moisture content determination using low constant temperature oven method.
6. Preculture the axes on basal MS medium supplemented with 0.3 M sucrose and 2M glycerol for 16 to 24h and maintain in culture room conditions.
7. Transfer 15-25 axes to 1.2 ml sterile cryovials and treat with 0.5 ml loading solution (0.4M sucrose, 2M glycerol in basal MS medium) for 20 min at 25°C.
8. Replace loading solution with 0.5 ml Plant Vitrification Solution 2 (PVS2) for 30 min at 25°C.
9. Plunge tightly closed cryovials containing axes in PVS2 solution rapidly in liquid nitrogen (LN).
10. Hold in LN for 1h or more as per designed schedule.
11. Thaw the cryovials in a water bath at  $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 1 min with vigorous shaking.
12. Immediately replace the PVS2 solution with 0.5ml unloading solution (1.2M sucrose in MS basal medium). Leave the vials at 25°C for 20 min.
13. Drain the solution and blott the axes dry on sterile filter papers.
14. Culture the axes and maintain at  $25 \pm 2^{\circ}\text{C}$  with a 16 h photoperiod under light intensity of  $35 \mu\text{E m}^{-2}\text{s}^{-1}$ .

**Items required a day before initiating cryopreservation**

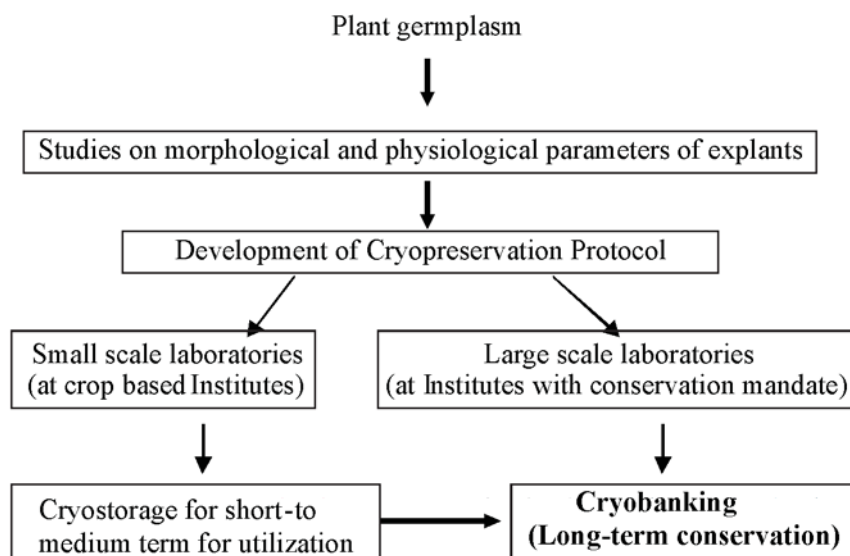
1. Sterile glass petridishes and 100 ml flasks , sterile filter paper discs
3. Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
4. Sterile cryovials and cryomarkers
5. Preculture medium- basal MS medium supplemented with 0.3 M sucrose and 2M glycerol
6. Loading solution (0.4M sucrose, 2M glycerol in basal MS medium)
7. PVS2 solution [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) Dimethyl sulphoxide]
8. Unloading solution (1.2M sucrose in basal MS medium).
9. Culture medium [MS medium macro- and micro-nutrients, vitamins, iron, supplemented with  $1\text{g l}^{-1}$  activated charcoal,  $0.17\text{g l}^{-1}$   $\text{NaH}_2\text{PO}_4$  and  $1\text{mg l}^{-1}$  each of BAP and NAA after Chin *et al.*, (1988) *Cryoletters* 9: 372-379]
10. Dewar flasks containing LN

**Precautions**

1. Wear gloves while handling PVS2 in order to avoid any contact.
2. The extraction of seeds from the ripe fruits may be completed within 4-5 days of harvest to obtain optimal results in cryopreservation.

### Laboratory Requirements for Cryopreservation

Requirements for cryopreservation laboratory dealing in plant tissues basically depend upon the crops and materials to be handled, techniques to be used and most important the aim of the studies. Initially the target of a laboratory may be the development of cryopreservation protocols and finally to process the tissues for long-term conservation. This two step approach of attempting cryopreservation technology would benefit both the researchers and curators with optimum utilization of the resources.



Selection of suitable explant and conduct of basic morphological and physiological studies on the explant would lead to development of suitable cryopreservation protocol. If the researcher is aiming to develop or optimize cryopreservation protocol for a species, it may be essential to make proper strategy based on the available information on reproductive biology and propagation method of the species.

The cryobank may follow specific need based priorities for better management. Following are the germplasm priorities followed at National Cryogenebank, NBPGR:

- Species producing intermediate and recalcitrant seeds having sizable indigenous diversity
- Vegetatively propagated species
- Threatened and endangered plant species with critically small population size
- Wild and weedy relatives of crop plants
- Genetic stocks including released varieties
- Exotic germplasm
- Medicinal and aromatic plants
- Core collections

## **Explants for cryopreservation**

Following explants are generally processed for cryopreservation of various categories of germplasm.

1. Seeds - orthodox, intermediate and recalcitrant.
2. Embryos, embryonic axes and plumules
3. Meristems and shoot apices
4. Dormant buds
5. Pollen grains
6. Genomic resources

## **Requirements for establishing a cryolaboratory**

For starting a small-scale cryolaboratory narrow mouthed cryocontainers having capacity to hold 11 to 60 liters of liquid nitrogen can be used. Such containers are available from Indo-Burma Petroleum (Nasik, India) and several international companies like MVE (USA), L'Air Liquide (France), etc. Further, after experimentation, the samples, which are to be stored for long-term, should be transported to the nearest large-scale cryolaboratory. For transportation, cryo-dryshippers of different capacities available from various international companies like Voyageur, can be used, wherein liquid nitrogen is adsorbed by the filled in porous material, thus reducing the risk of accidental spillage of liquid nitrogen during transportation.

While planning a large-scale storage strategy, one time investment is made so the workplan and workload expected in the next few decades should be kept in mind. A large-scale cryolaboratory should have at least two components – a laboratory and a cryobank. The laboratory should be fully equipped to handle diverse type of plant tissues and requirements for application of all the new techniques. Conventional thermometers cannot be used for monitoring of temperature during slow freezing thus a copper-constant thermocouple capable of recording temperature upto  $-200^{\circ}\text{C}$  should also be available for accurate work.

The cryobank should be equipped with extra-large capacity cryotanks (ranging from 650 liters to 1800 liters) equipped with electronically controlled filling system and alarm. Liquid nitrogen is highly compressed form of nitrogen gas so tends to evaporate faster. Moreover, being heavier than air it settles down near the tanks. To avoid accumulation of nitrogen vapours, the cryobank should be well ventilated and equipped with exhaust fans for removing excess nitrogen vapours.

For both types of laboratories a constant and reliable supply of liquid nitrogen is essential. For a small-scale laboratory, one can install a LN production Plant or a supplier may be hired who can regularly supply LN. Few spare tanks of LN should always be kept filled to replenish LN in storage tanks. For a large-scale laboratory where LN consumption is much more, a vertical LN station / horizontal reservoir of extra large capacity (4000-8000 lits) can be maintained near the cryobank which should be constantly filled to ensure continuous supply of LN to storage tanks. For this, a pipeline from the LN station can be laid to avoid movement of heavy tanks for replenishment.

In addition to the equipments mentioned above both types of cryolaboratories should be equipped with safety gadgets like gloves, aprons, face masks, face shields or goggles, etc. Other accessories like cryomarkers, glass markers, metallic (aluminum) holdings like canes on which the cryovials can be mounted before dipping in liquid nitrogen and heavy-duty trolleys for transportation of tanks should also be available.

Apart from the essential requirements for cryopreservation detailed above, several other infrastructure facilities are essential in case detailed studies on underlying mechanisms are intended. Traditionally, studies on freezing behavior in plant tissues have been done using Differential Scanning Calorimeter (DSC), nuclear magnetic resonance (NMR) spectrometry, micromorphological studies using scanning electron microscopy (SEM) and visual observations (Engelmann and Takagi, 2000). Lately, NMR microscopy, a novel non-invasive method to visualize the freezing behaviour in plant tissues is being employed. It is also possible to observe cells during freezing and thawing using a specialized microscope system (Cryomicroscope), in which the temperature of the microscope stage and the rate of change of temperature are accurately controlled. Depending on the aim of the studies such facilities can be built up.

## Recording of seed characteristics

*Seed morphological and physiological characters*

S.No.	Acc No.	Average seed wt (g)	dimensions L X B(cm)	Seed coat ratio	MC (%)	Viability (%)
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						

*Effect of treatments on germinability of seeds*

Accession no.	Treatment	% ge of seeds showing germination after ( ) days of putting		
		(2)	(4)	(8)
	+			
	-			
	*			
	+			
	-			
	*			

## Glossary of Terms

<b>Accession</b>	A distinct, uniquely identifiable sample of seeds/propagule representing a landrace, cultivar, breeding line or a population, which is maintained in a genebank for conservation and use
<b>Accession number</b>	A unique identifier assigned to an accession, when it is registered with a genebank. This number is never assigned again to another accession even after loss of the accession.
<b>Active collection</b>	A germplasm accession that is used for regeneration, multiplication, distribution, characterization and evaluation. Active collections are maintained in short to medium-term storage and usually duplicated in a base collection maintained in medium- to long-term storage.
<b>Agricultural biodiversity</b>	Also referred as ‘agrobiodiversity’, encompasses the variety and variability of animals, plants and micro-organisms necessary to sustain key functions of the agro-ecosystem, its structure and processes for, and in support of, food production and food security. It covers, inter alia, crop varieties, including forage and fodder plants and trees, animal breeds, including fish, molluscs, bird species and insects, as well as fungi, yeasts and micro-organisms such as algae and diverse bacteria.
<b>Arboreta</b>	The arboretum contains a collection of trees, shrubs and other plants that are used for education, research, conservation and recreation. Most of the famous arboreta are operational in United States and Europe.
<b>Biological diversity</b>	The variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part. It includes diversity within species, between species and of ecosystems.
<b>Biotechnology</b>	Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. It generally includes a number of individual techniques, inter-alia, recombinant DNA-molecule manipulation, protein engineering, cell fusion, nucleotide synthesis, monoclonal antibody use and production, product recovery, and unique fermentation techniques, e.g. Biocatalysis using immobilized enzymes
<b>Botanical Garden</b>	Botanical gardens hold living collection of plants and are generally located under different management systems in different bio-

geographical region. Several of these have other conservation facilities attached with it e.g. seed and pollen banks and tissue culture units. Conservation effort is focused on wild, ornamental and rare and endangered species.

**Centre of diversity**

A geographical area where a plant species, first developed its distinctive properties (in farmers' field or in the wild). A primary centre of diversity is the region of true origin (often referred to as the centre of origin) and secondary centres of diversity are regions of subsequent spread of a crop.

**Cryopreservation**

Cryopreservation is a process where cells or whole tissues are preserved in viable conditions by cooling to low sub-zero temperatures, such as generally  $-196^{\circ}\text{C}$ . At this temperature any biological activity is virtually halted.

**Domestication**

Domestication is the process by which plants, animals or microbes selected from the wild adapt to a special habitat created for them by humans, bringing a wild species under human management. In a genetic context, the process in which changes in gene frequencies and performance arise from a new set of selection pressures exerted on a population.

**Endemic**

Native to, and restricted to, a particular geographical region. Highly endemic species, those with very restricted natural ranges, are especially vulnerable to extinction if their natural habitat is eliminated or significantly disturbed.

**Genepool**

The sum total of all the genes and combinations of the genes that occur in a population of organisms of the same species. The term genepool in reference to wild relatives and related taxa is classified into primary, secondary, tertiary depending upon crossability.

**Genetic diversity**

The genetic variability (variety of genetic traits) within a population or a species, arising due to number and relative abundance of alleles.

**Genetic resources**

Genetic material of actual or potential economic, scientific or societal value contained within and among species. In a domesticated species, it is the sum of all the genetic combinations produced in the process of evolution. The term includes modern cultivars and breeds; traditional cultivars and breeds; special genetic stocks (breeding lines, mutants, etc); wild relatives of domesticated species; and genetic variants of wild species.

**Herbal Gardens**

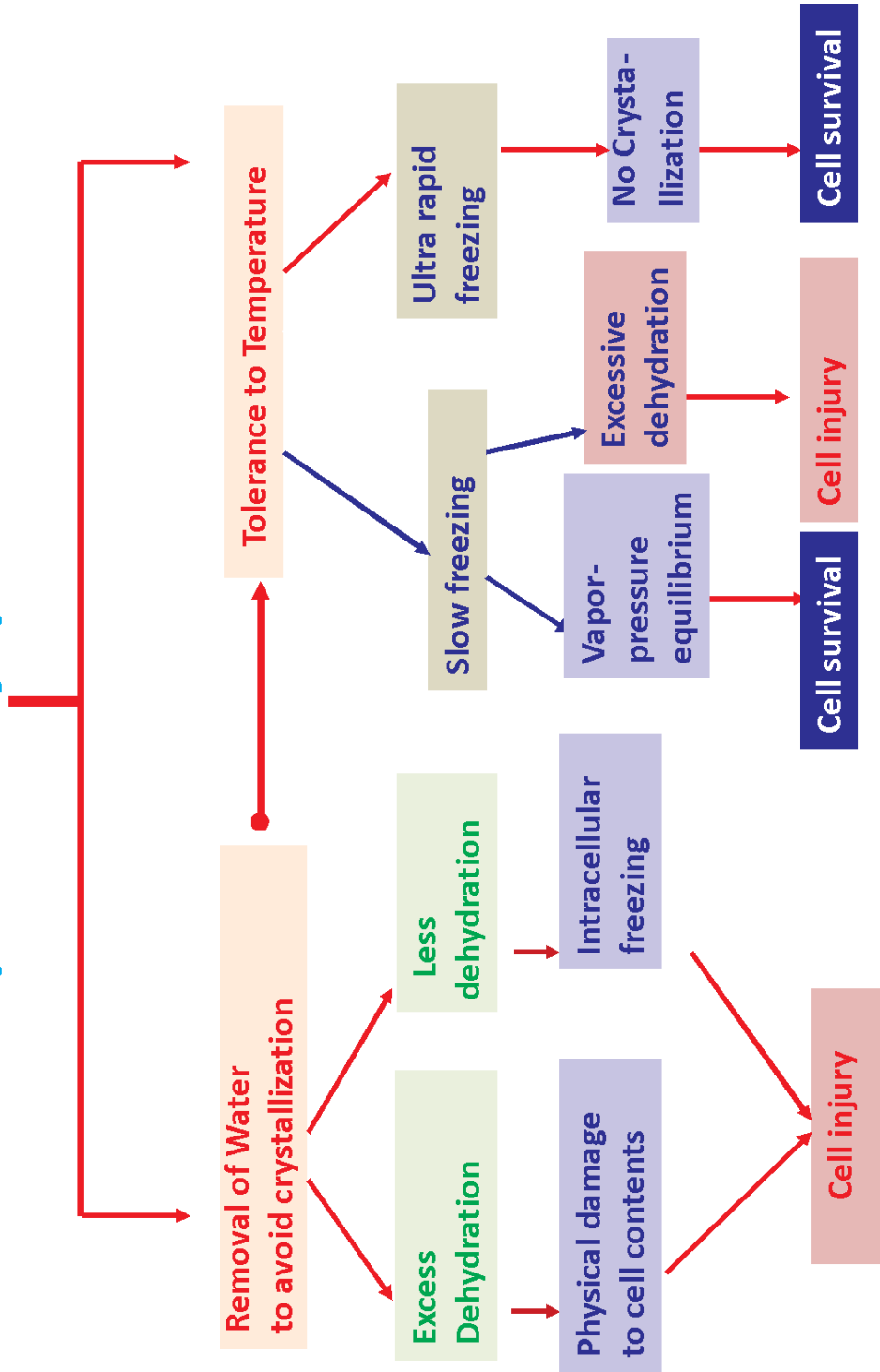
The herbal gardens have been generally established to demonstrate, propagate and conserve the medicinal and aromatic plants of particular area. Various non-governmental organizations and state forest and horti-cultural departments have established their herbal gardens in respective states.



<b>Landrace</b>	A crop cultivar or animal breed that evolved with and has been genetically improved by traditional agriculturalists, but has not been influenced by modern breeding practices. It comprises population of individuals that have become adapted to specific local environmental conditions in which it evolved and is usually genetically heterogeneous.
<b>Crop Wild Relative</b>	Defined as a wild plant species that is more or less closely related to a particular crop and to which it may contribute genetic material, but unlike the crop species has not been domesticated.
<b>Cultivar</b>	Means a variety of plant that has origin-nated and persisted under cultivation or was specifically bred for the purpose of cultivation
<b><i>Ex situ</i> conservation</b>	Means the conservation of components of biological diversity outside their natural habitats
<b><i>In situ</i> conservation</b>	Means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.
<b>Sustainable use</b>	Means the use of components of biological diversity in such manner and at such rate, that it does not lead to the long-term decline of the biological diversity thereby maintaining its potential to meet the needs.



# Principles of Cryopreservation



## PLATE 2



Fig. A. Air tight desiccators containing charged silica gel



Fig B Vacuum desiccator for faster drying



Fig. C Programmable rate freezing apparatus



Fig. D Low cost freezer - 'Frosty'

### PLATE 3

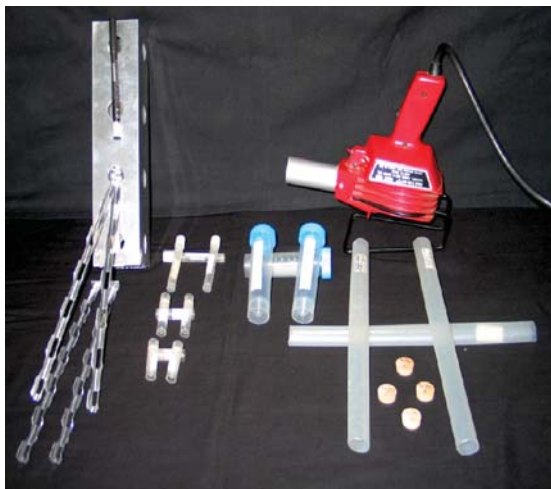


Fig. A Cryovials and polyolefin tubings along with aluminium canes and canister



Fig. B Freshly harvested pods with extracted seeds

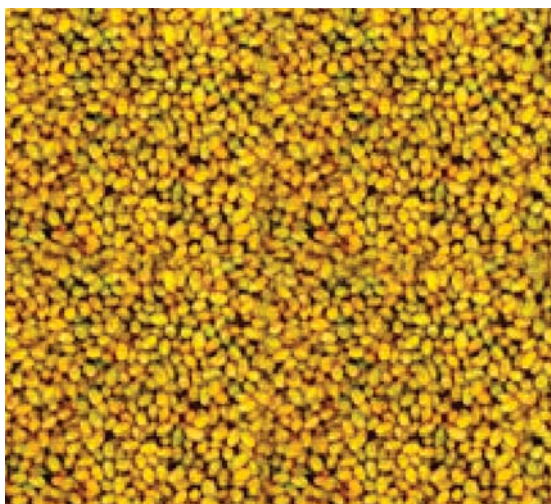


Fig. C Freshly harvested fruits from trees



Fig. D Glass moisture bottles and Aluminium cups of different sizes



## PLATE 4

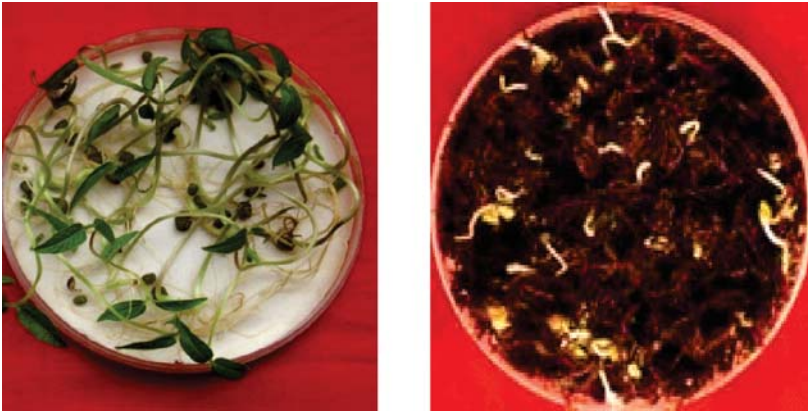


Fig. A. Viable seeds germinating in petriplates using substrata- filter paper and moss grass

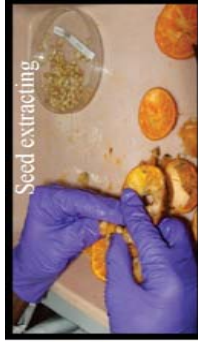


Fig. B. *In vitro* growth of plantlets raised from embryonic axes and subsequent healthy growth after 3 months of culture



Fig. C. Healthy *In vitro* plantlets after transfer to field in seed germination tray

## PLATE 5



# Desiccation and Fast Freezing method for whole seed cryopreservation



## PLATE 6

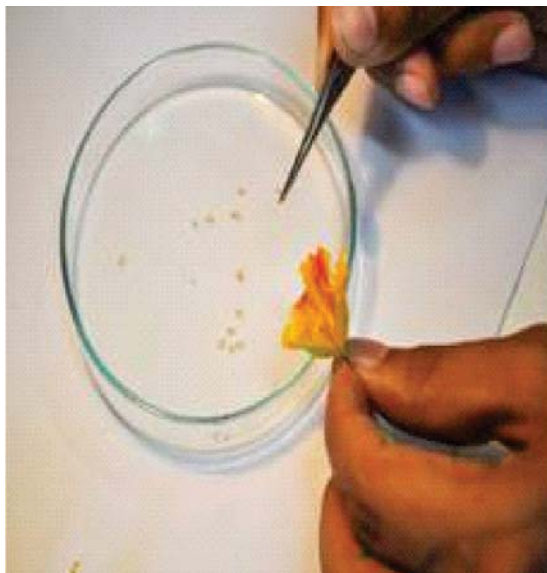


Fig. A. Collection of pollen from flowers

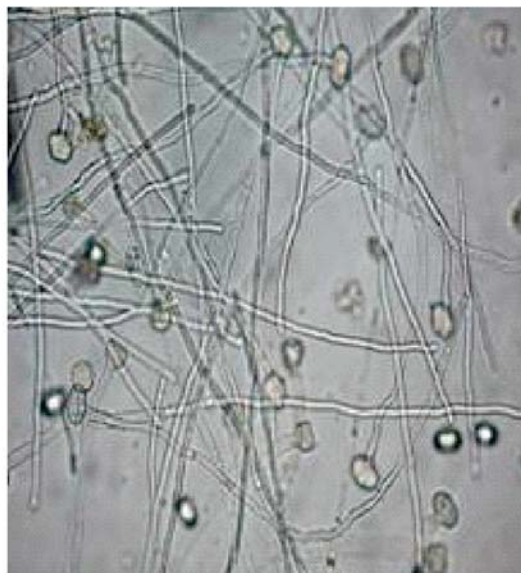


Fig. B. In vitro germination of viable pollen showing long pollen tubes

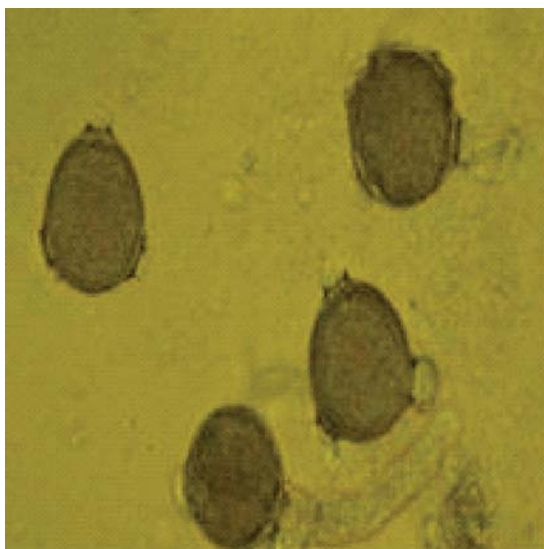


Fig. C. Close up of germinating pollen

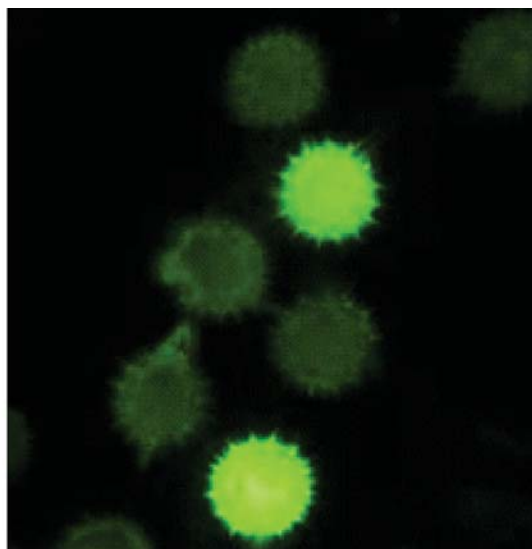


Fig. D. FDA test showing fluorescent viable pollen



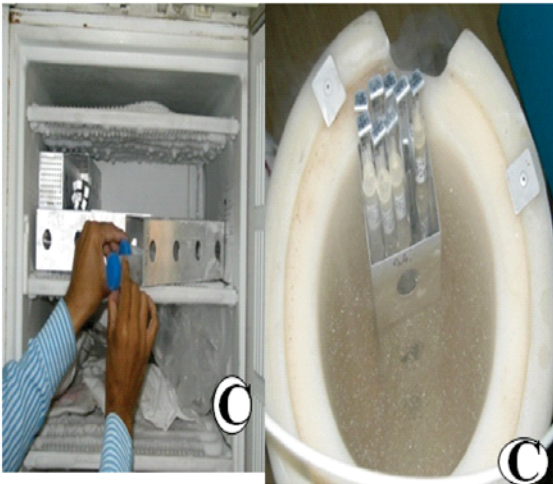
# PLATE 7

## Stepwise freezing in dormant buds of *Morus* spp.

(A)



(B)



(C)

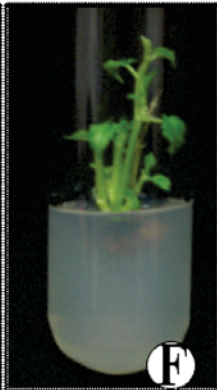
(C)



(D)



(E)



(F)

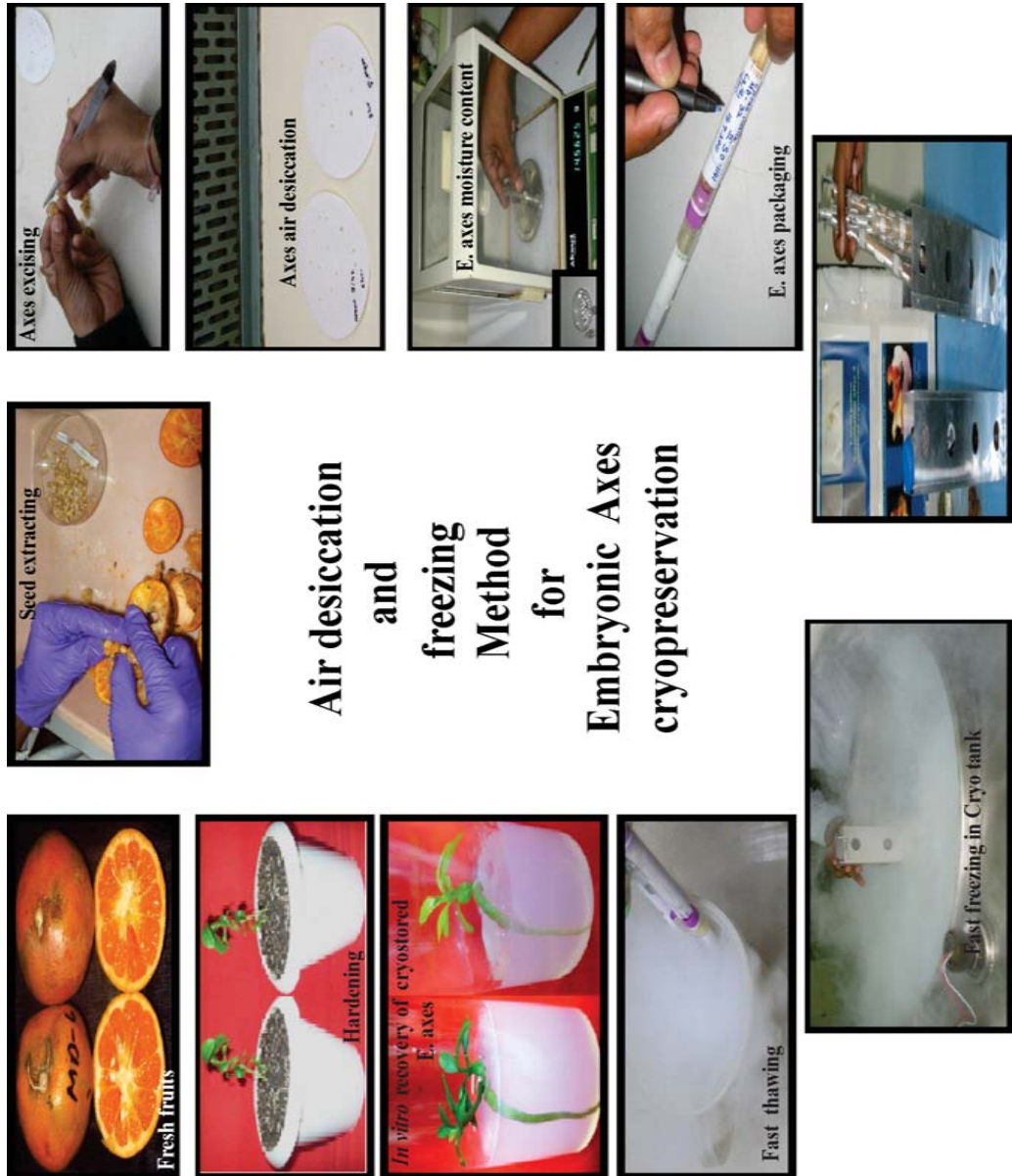


(G)



(H)

## PLATE 8





## **Tissue Culture and Cryopreservation Unit**

**ICAR-National Bureau of Plant Genetic Resources**

Pusa campus, New Delhi - 110012, India