Brainstorming meeting and training cum demonstration on

Cryopreservation and in vitro conservation in Horticultural Crops

Organized by
Society for Promotion of Horticulture

In collaboration with
Indian Institute of Horticultural Research

Sponsored by
Bioversity International

Date: 21 & 22nd February 2014
Venue: IIHR, Bengaluru
The conservation of a broad genetic pool of wild and domesticated species for future generations is essential for sustainable food production. Classically, seed and in-field collections were the only reliable option for the ex situ long-term preservation of plant biodiversity. The application of tissue culture technology for the preservation of plant tissues and organs at both low and at cryogenic temperature, however, has greatly evolved in recent years.

Cryopreservation refers to the conservation at ultra-low temperatures (often in liquid nitrogen at -196°C) of cells, tissues and organs from in vitro culture (axillary and apical buds, embryogenic callus, somatic embryos), as well as from in vivo collected material (seeds, embryonic axes and dormant buds). This technique allows the storage of plant material at low cost, for unlimited time periods and in genetical and health stable conditions. Moreover, cryopreservation proved to be a promising approach to eliminate pathogens from diseased material (cryotherapy). The conservation at low (above freezing) temperature (“slow or minimal growth storage”) has also repeatedly proved to be a convenient option for medium-term conservation of shoot cultures, produced in commercial micropropagation laboratories, allowing a significant extension of the interval between subcultures.

Many Horticultural plant species have been successfully cryopreserved through the development of various cryopreservation methods. Long term cryogenic preservation in these crops was accomplished and for the first time in India, a POLLEN CRYOBANK was established at Indian Institute of Horticultural Research, Bangalore which was featured in the LIMCA BOOK of RECORDS-2001. Since then research activities have spread over the past 30 years and we thought that it is time to take a retrospect and prospect. The division of Plant Genetic Resource of IIHR, Bangalore organising a brainstorming session and training cum demonstration in vitro conservation and cryopreservation in horticulture crops with the following objectives:

- To assess use of cryopreservation and tissue culture techniques for conservation and management of PGR in horticulture crops
- Enhance the use of in vitro conservation and cryopreservation protocols for crop germplasm
- Equip participants with essential knowledge necessary for developing and using in vitro & cryopreservation technique.
The Brainstorm meeting followed by training cum demonstration is to be organized on 21 & 22th of February, 2014 which will include in depth analysis of basic research in the plant cryopreservation and in vitro conservation as well as reports on state-of-the-art methods, research and successes achieved with selected horticultural crops. The meeting will focus on presentations by experts in the field, followed by discussions and interactions.

Also this program is being organized for the benefit of crop based horticulture institutions, NAGS partners identified for horticulture crops and coordinating centres under the Horticulture Division of ICAR with an intention to strengthen PGR activities in horticulture.

**Registration fee:**

- General: Rs. 1000/-
- Students/SPH members: Rs. 500/-

Demand draft/Cheque drawn in favour of SPH, Bengaluru payable at Bengaluru along with filled in Registration form may kindly be sent to the Secretary, Society for Promotion of Horticulture, IIHR, Hessaraghatta Lake PO, Bengaluru-560 089 for registration.

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REGISTRATION FORM

Brainstorming session and training cum demonstration on

*Cryopreservation and in vitro conservation in
Horticultural Crops*

Organized by IIHR, in association with SPH

Date: 21 & 22\textsuperscript{nd} Feb 2014
Venue: IIHR, Bengaluru

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ABSTRACTS
Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops
Cryobanking of plant germplasm at Cryogenebank at NBGPR

Rekha Chaudhury and S.K. Malik

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Cryopreservation using liquid nitrogen (temperatures from -180°C to -196°C) reduces cell and tissue deterioration in storage by virtually halting metabolism and hence is an important tool for non-lethal ‘infinite’ storage of biological materials with reportedly not causing any change in viability, vigour and genetic makeup. Cryopreservation is the only technique available presently, for long-term storage of vegetatively propagated species (in the form of shoot tips, meristems, somatic embryos and cells) and difficult-to-store non-orthodox seed species (comprising intermediate and recalcitrant seeds), for pollen to meet breeders need and for dormant buds of deciduous temperate species to ensure clonal materials storage. All types of explants like seeds, embryos, embryonic axes, pollen and dormant buds are to be finally packed in airtight containers, polypropylene cryovials before cryostorage. Pollen desiccated to suitable moisture content can be stored in aluminum packets, gelatin capsules or polypropylene cryovials. The size of the cryovial to be used will depend upon the size as well as the quantity of the seeds/embryonal axes/pollen grains to be stored. Large seeds can be stored in heat-sealable polyolefin tubing with cork stoppers or in goblets in sleeves.

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. A minimum of 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.

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. 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 inventories which 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. 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. Replenishment of LN is required about two times per week to maintain the temperature of the cryotank between –160 to –180° C. 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 water bath is required. 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.
In Vitro and Cryopreservation Research at JNTBGRI

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Conservation of plant biodiversity is a much debated subject with multidisciplinary approach in biological sciences. They comprises diversity of genetic material and are vital part of global biodiversity, a biological and chemical goldmine and a major life-supporting system for all life forms on earth. Over a millennia, by trial and error, knowingly or unknowingly, by intuition or intention, man had always experimented with plants and learned on them for food, fiber, medicine and a variety of other products. Additionally important is the indirect ecosystem services provided by these plants. For hundreds of years, plants are associated with the cultural, economic and social fabric of many ethnic communities across the world. In a globalised economy, however, biodiversity including plant forms the feed stock of biotechnology and unless we harness the benefits of biodiversity in a non-invasive and sustainable platform that biotechnology provides and convert the bio-wealth into economic benefits we may be subject to intellectual and technological slavery. In other ways, we need to look upon wild plants very differently from what we were doing all those years as a source of genes, enzymes, proteins, phytopharmaceuticals, flavors, aroma compounds, cosmetic agents and so on. Unless the biodiversity rich nations prospect their resources, add value to them and use sustainably and conserve the genetic resources for future use, they may not have stand in the open world scenario.

The distribution of bioresources on the planet earth is not uniform. The tropics that harbor the vast majority of the world’s biota are thought to hold the key for the future. India situated in the tropics of cancer is a known mega biodiversity centre having 8% of the global biodiversity in 2.4% land area and ranks 10th among the plant rich countries of the world and 4th among countries of Asia. The country is also home for two (eastern Himalayas and Western Ghats) of the world’s 25 hotspots. The huge treasure of Indian plant resources with over 45,000 known species represent 11% of the earth flora. About 33% of flowering plants and 29% of all the plants are endemic to the country. Nearly 1500 out of 17,000 higher plant species are threatened at some level or other and are likely to become extinct in the next 50 years. There for in order to achieve our goals of conserving the bioresources into commercially useful products and process, we need to conserve the country’s’ indigenous biodiversity includes maintenance of ecological process and life supporting systems.

Right from the beginning, online with mandate of the JNTBGRI, R and D activities of the Biotechnology and Bioinformatics Division is to develop strategies and practical schemes for effective conservation, characterization and documentation of native plant resources using modern technologies for sustainable utilization. On the basis of the mandate, major objectives
of the Division are (1) **ex-situ** conservation of threatened plant taxa through micropropagation, reintroduction and restoration (2) to develop appropriate culture system for bioproduction of plant specific compounds (3) bioprospecting of medicinal aromatic plants and other RET species (4) to establish cryopreservation facility and standardise procedure for long term preservation of shoot tips, seeds and embryos of RET species and pollen and seeds of orchid species (5) to develop patentable process for production of high value- low volume bioactive components from medicinal plants (6) to develop bioinformatics tools, software, databases, gathering, sharing and in silico analysis of plant drugs for drug development and (7) resort to commercial multiplication of plants for technology demonstration for income generation and human resource development through training and capacity building.

The work done on the conservation through In Vitro and cryopreservation of RET species of different category (MandAP, Trees, Orchids, Wild Banana, Rattan and Bamboos) at JNTBGRI will be discussed with the success and its implementation.
In vitro and cryo conservation technologies in Spice crops

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Abstract

India is a rich repository of spices. The genetic resources of most of the spices are conserved either in seed gene banks and or in field repositories. In case of vegetatively propagated perennials the germplasm is conserved in clonal field repositories. Storage of germplasm in seed banks is not practical in some crops as they are vegetatively propagated and seeds are recalcitrant and heterozygous. India is the centre of diversity for many spices. However disturbances in their natural habitats have resulted in loss of these valuable materials. Biotechnological approaches like micropropagation, in vitro conservation, synseed technology, cell culture, pollen and DNA banking have great significance in conservation of spice genetic / genome resources. Conservation of the germplasm in in vitro gene bank and cryo bank is a viable method and a safe alternative to augment the conventional conservation strategies.

Conservation of genetic resources

In vitro conservation

In vitro conservation involves maintenance of explants in a sterile, pathogen-free environment and is widely used for conservation of species that produce recalcitrant seeds, or do not produce seeds. For short-and medium-term storage, the aim is to increase intervals between subcultures by reducing growth. This is achieved by modifying environmental conditions, including culture medium to realize slow-growth conservation. Factors like temperature, culture medium, use of osmoticum, and physiological state of ex-plant, culture vessel, minimization of evaporation loss, encapsulation and desiccation influenced slow growth. Conservation of pepper, cardamom, herbal spices, vanilla and ginger germplasm in in vitro gene bank by slow growth was standersised by various workers. Protocols for in vitro conservation by slow growth of black pepper and its related species viz., P.barberi, P.colubrinum, P.betle and P.longum are available. In general its done by maintaining cultures at reduced temperatures, in the presence of osmotic inhibitors, at reduced nutrient levels, or by minimizing evaporation loss by using closed containers. Black pepper cultures could be maintained in half strength WPM supplemented with 15 gl⁻¹ each of sucrose and mannitol for one year with 85% survival. In P.barberi full strength WPM with 25 gl⁻¹ sucrose and 5 gl⁻¹ mannitol was suitable for storage of cultures up to one year with 80% survival. Shoot tips of P.longum and P.colubrinum could be stored up to one year in full strength WPM with 20 gl⁻¹ sucrose and 10 gl⁻¹ mannitol with 75% and 70% survival respectively. P.betle cultures could be stored in half strength WPM supplemented with 20 gl⁻¹ sucrose for one year. Technology for in vitro conservation of vanilla and Zingeberaceous crops like ginger, turmeric, Kaempheria, cardamom was also standarized.
Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops

Tissue cultures of cardamom could not be stored under low temperatures (Nirmal Babu et al., 1994). Normal culture vessel allowed comparatively better gaseous exchange but there is faster rate of moisture depletion in culture media and drying up of cultures between 120 and 180 days depending upon species. Use of screw caps, polypropylene caps or aluminum foil as vessel closures minimized moisture loss, helped in increased longevity of cultures and the subculture period could be prolonged up to 30 days (Nirmal Babu et al., 1994, Geetha et al., 1995, Geetha 2002). Sealing culture tube with parafilm helped in reducing the chance of evaporation and consequent dehydration. Longevity of culture was reported earlier in ginger and turmeric (Balachandran et al., 1990). Normal culture room temperatures of 22 ± 2 ºC are suitable for storage of spices germplasm (Dekkers et al., 1991, Nirmal Babu et al., 1994; Geetha et al., 1995).

Cryopreservation

For long-term conservation of problem species, cryopreservation is the only method currently available. Visible progress is made in the development of new cryopreservation techniques and protocols are established for over 100 different plant species. An array of plant materials could be considered for cryopreservation as dictated by actual needs vis-à-vis preservation. These include meristems, cell, callus and protoplast cultures, somatic and zygotic embryos, anthers, pollen or microspores and whole seeds. Plant germplasm stored in liquid nitrogen (–196ºC) does not undergo cellular divisions. In addition, metabolic and other physical processes are stopped at this temperature. As such, plants can be stored for very long periods and both the problem of genetic instability and the risk of loosing accessions due to contamination or human error during subculturing are overcome. Most cryopreservation endeavours deal with recalcitrant seeds, in vitro tissues from vegetatively propagated crops, species with a particular gene combination (elite genotypes), pollen and dedifferentiated plant cell cultures. Care must be taken to avoid ice crystallisation during freezing process, which otherwise would cause physical damage to tissues. Existing cryogenic strategies rely on air-drying, freeze dehydration, osmotic dehydration, addition of penetrating cryoprotective substances. Their routine utilisation is still restricted almost exclusively to conservation of cell lines in research laboratories. At this (–196ºC), temperature all cellular divisions and metabolic processes are virtually halted and consequently, plant materials can be indefinitely stored without alteration or modification. Cryopreservation of seeds of black pepper, cardamom, capsicum, anise and a series of Piper species in liquid nitrogen (LN₂) was reported. Plants could be successfully regenerated from cryopreserved seeds.

Technology for cryopreservation of black pepper, cardamom, ginger, turmeric and vanilla germplasm - using vitrification, encapsulation and encapsulation and vitrification methods – are available. Cryopreservation of encapsulated shoot buds of endangered species of Piper and vanilla were reported. Cryopreservation technology for celery and coriander somatic embryos using sucrose preculture and air desiccation is available.

Pollen Storage

Pollen storage has considerable value supplementing the germplasm conservation strategy by facilitating hybridisation between plants with different time of flowering and to transport pollen across the globe for various crop improvement programmes in addition to
developing haploid or homozygous lines. Pollen might represent an interesting alternative for long-term conservation of problematic species. An advantage is that pests and diseases are rarely transferred by pollen (excepting a few virus diseases). This allows safe movement and exchange of germplasm as pollen. Freeze preservation of capsicum pollen in liquid nitrogen (–196°C) for 42 months and Technology for cryopreservation of vanilla pollen for conservation of haploid genome as well as assisted pollination between species that flower at different seasons and successful fertilisation using cryopreserved pollen was developed.

**Recovery**

The conserved materials of all the species showed normal rate of multiplication when transferred to multiplication medium after storage. The normal sized plantlets when transferred to soil established with over 80% success. They developed into normal plants without any deformities and were morphologically similar to mother plants. RAPD profiling of these conserved plants also showed their genetic integrity.

**Preservation of cell lines**

The use of tissue culture for the biosynthesis of secondary metabolites particularly in plants of pharmaceutical significance holds an interesting alternative to controlled production of plant constituents. This technique is all the more relevant in recent years due to the ruthless exploitation of plants in the field leading to reduced availability. Thus conservation of cell lines is another approach to conserve genetic resources for upplanting material production when required and also for production of cell products through bioreactors. In fennel technology for conservation of embryogenic cell line suspensions at 4°C for up to 12 weeks produced normal plants upon transfer to normal laboratory conditions was also reported.

**DNA Storage**

Concurrent with advancements in gene cloning and gene transfer, there has been development of technology for removal and analysis of DNA. DNAs from nucleus, mitochondria and chloroplast are now routinely extracted and immobilized onto nitrocellulose sheets where DNA can be probed with numerous cloned genes. In addition, rapid development of polymerase chain reaction (PCR) now means that one can routinely amplify specific oligonucleotides or genes from entire mixture of genomic DNA. These advances, coupled with prospect of loss of significant plant genetic resources throughout world, have led to establishment of DNA bank for storage of genomic DNA. Conserved DNA will have numerous uses. DNA banking is yet to catch up in spices. It seems likely that integrity of DNA would decrease with age of specimens. The most important disadvantage is, it does not allow regeneration of live organisms.
Important references


Present status and future prospects of palm genetic resource conservation by cryopreservation

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Plant genetic resources comprise diversity of genetic material contained in traditional varieties, modern cultivars, wild relatives of crops and other wild species. For conservation, characterization and utilization of genetic diversity in palms like coconut, arecanut, date, and oil palm, a number of field gene banks were established and maintained nationally and internationally. However, the field gene banks are faced with many threats, both biotic and abiotic. Complementary conservation strategies are therefore to be evolved for a long term conservation genetic resources of these crops. Cryopreservation is the only available cost effective technique for long term conservation of palm genetic resource since most of them are of with recalcitrant/intermediate seed type or propagated vegetatively by offshoots as in case of Date palm. Under cryogenic storage, cell divisions and metabolic activities of the tissues are completely arrested and thus can be stored indefinitely without alteration or modification, which serves as a base collection. Most of the palm seeds and embryos displayed different degrees of desiccation tolerance. While most of the seeds are desiccation intolerant and recalcitrant, the embryos to some extent are desiccation and freezing tolerant. This unique attribute of palm zygotic embryo was exploited for its utility in long term conservation. At Central Plantation Crops Research Institute, four different cryopreservation methods viz., air desiccation (Karun et al., 2005), pregrowth desiccation (Sajini et al., 2006), encapsulation-dehydration, and solution based vitrification (Sajini et al., 2011) were tested on coconut zygotic embryos for the recovery of plantlets. While considering the operational simplicity and the equipments required the solution based vitrification method with a success rate of 22.5% using PVS3 was found to be most suitable for cryostorage of coconut zygotic embryos. Preliminary studies involving storage effect of embryos in liquid nitrogen for successful plantlet recovery also favors this method. The feasibility of cryopreservation of plumular tissues by droplet vitrification method was also tested and plantlet retrieval was found to be 20%. Successful cryogenic storage of somatic/zygotic embryos was also reported in other palms like date, arecanut and oil palm. Another strategy for long term conservation of coconut genetic diversity is in the form of cryopreserved pollen(Karun et al., 2005) When coconut pollen subjected to four year cryogenic storage was used for artificial pollination, nut setting was observed to be normal in West Coast Tall (WCT) and Chowghat Orange Dwarf (COD). Studies also indicated that the coconut pollen retained viability and fertility even after six years of cryogenic storage. (Karun et al., 2014). Procedure for collection, desiccation and cryopreservation of arecanut pollen was standardized at CPCRI. There are also reports on cryogenic storage of oilpalm pollen for longer periods of above 8 years (Tandon et al., 2007).
CRYOPRESERVATION OF GENETIC RESOURCES IN POTATO

*(Solanum tuberosum* L.)*

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The cultivated potato (*Solanum tuberosum* L.) is a highly heterozygous and tetraploid crop. The botanical seed of this crop, therefore, constitutes a segregating population and can only be used for conserving the total gene pool. However, the selected individual genotypes (cultivars, valuable parental lines etc.) have to be conserved vegetatively. Consequently, traditional method of conservation through tubers in fields/glasshouses is employed to maintain the germplasm. In this method, since the tubers need to be grown in the fields/glasshouses every year, the approach is labour-intensive, requires lot of space, and exposes the germplasm collection to various biotic and abiotic stresses.

In vitro approaches have been successfully adopted for conservation of potato genetic resources. Most of the national potato germplasm repositories including the nodal repository at the International Potato Centre, Lima, Peru employ minimal growth method for conservation of potato microplants. The method, however, requires frequent transfers, and thus is highly labour-intensive. Moreover, several workers have raised concerns about the safe use of this technique for maintaining genetic integrity of the conserved genetic resources, because of the possibility of in vitro-induced variations. A practical solution to these problems is cryopreservation. Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra low temperatures (-196 °C). The technique involves selection of pathogen-free explants; pre-growth on high osmotic medium to enhance stress tolerance; cryoprotectant treatment (cryoprotective dehydration/encapsulation and dehydration/vitrification); freezing (rapid/slow/step-wise); storage at ultra-low temperatures generally in liquid nitrogen (-196 °C); thawing; and recovery of plants. The greatest stability of in vitro plant materials, with practical storage periods measured in decades, can be achieved by cryogenic storage at ultra-low temperatures in liquid nitrogen. Liquid nitrogen is most widely used non-hazardous cryogen (-196 °C). Exposure of tissues or cells to -196 °C effectively halts biological growth and development. Consequently, material that can be brought to the ultra-low temperature may be stored for extremely long periods and recovered from it without any lethal injury. Storage in liquid nitrogen does not induce ploidy changes in dihaploids of *Solanum tuberosum* (Ward et al., 1993) or alter the ability of plants derived from cryopreserved shoot tips to produce tubers (Harding and Benson, 1994). The duration of storage in liquid nitrogen also does not affect the survival rate of potato shoot tips (Bajaj, 1985).

Bajaj initiated work on cryopreservation of potato germplasm in liquid nitrogen as early as 1977. He successfully cryoconserved potato shoot tips employing ultra-rapid freezing...
method using a cryoprotective solution containing dimethylsulfoxide (DMSO), glycerol and sucrose. During almost the same time important progress in potato cryopreservation was made by Towill (1981), who, using Solanum etuberosum as a model system, obtained a very high level of survival (about 71%) following a two-step freezing procedure. In this modified cooling approach, the meristems were first subjected to -40 °C with a cooling rate of 0.3 °C/min, and then immersed in liquid nitrogen. A simple freezing method for cryopreservation of potato shoot tips was developed by Schäfer-Menuhr et al. (1996). This approach, better known as 'droplet method', is essentially ultra-rapid freezing in principle. In this method, the shoot tips are cut off and trimmed to 2-3 mm long and 0.5-1 mm thick, incubated overnight at 23 °C in MS medium supplemented with 30 g/l sucrose and phytohormones as recommended by Towill (1983), and on the following day they are transferred to cryoprotective solution (pre-culture medium + 10% DMSO). After 2 hrs incubation in cryoprotective solution at room temperature, 2.5 μl droplets of cryoprotectant are pipetted into sterilized pieces of thick aluminum foils. One shoot tip is transferred onto each drop and the foils are dropped directly into liquid nitrogen. For cryogenic storage, the foils are transferred to 2 ml pre-cooled cryovials. Although on an average, 80% shoot tips survived, but only 40% shoot tips recorded regeneration and resumed normal shoot growth.

Towards the beginning of 1990s, a new procedure for cryopreservation of potato shoot tips was developed, in which pre-freezing-dehydration is replaced by dehydration at room temperature (Fabre and Dereuddre, 1990; Sakai et al., 1990). In general, this technique is known as vitrification. Vitrification is the phase transition of water from a liquid into a non-crystalline or amorphous phase, a glass, by an extreme elevation in viscosity during cooling. In contrast to classical techniques based on freezing, in vitrification-based procedures, cell dehydration is performed prior to freezing by exposures of samples to concentrated cryoprotective solutions and/or air desiccation followed by rapid cooling. Vitrification-based procedures offer practical advantages in comparison with classical freezing techniques. Out of different vitrification-based procedures identified, the most successful technique applied to potato shoot tips is the encapsulation-dehydration method. In this method, the shoot tips are encapsulated in alginate beads, pre-cultured in sucrose-enriched medium, air dehydrated, and directly cooled in liquid nitrogen. Initially this technique gave low and irregular survival rates in Solanum phureja and was unsuccessful with other Solanum species and cultivars. Bouafia et al. (1996) proposed an improved procedure for cryopreservation of Solanum shoot tips by encapsulation-dehydration. This involves encapsulation of axillary shoot tips in alginate (3% w/v) beads, pre-culture with sucrose-enriched (1 M sucrose) medium for 2 days, air-drying of alginate beads, rapid cooling in liquid nitrogen and slow re-warming. Survival rates above 60% were obtained for each cultivar tested. The main advantage of the encapsulation-dehydration procedure is that pre-culture with sucrose, a widely employed cryoprotective compound is combined with desiccation as cryoprotective treatment thus avoiding the use of DMSO, glycerol, ethylene glycol, polyethylene glycol etc. which are conventionally used as cryoprotective substances. Shoot tips cryopreserved by encapsulation-dehydration technique regenerated directly without significant callus formation. The encapsulation-dehydration procedure has only
limitation that it is highly time-consuming and labour-intensive. In comparison, vitrification employing chemical additives has the practical advantage that this is comparatively easy. This method consists of placing explants in the presence of a highly concentrated cryoprotective solution, then freezing them rapidly. Although this approach has been experimented with cell suspensions, shoot apices and somatic embryos of a wide range of plant species, it is least investigated in potato. At Central Potato Research Institute, Shimla an improved vitrification procedure was developed for cryopreservation of potato shoot tips (Sarkar and Naik, 1998e). Shoot tips (0.5-0.7 mm) were pre-cultured on liquid MS medium supplemented with 0.3 M sucrose plus 0.2 M mannitol over filter paper disc for 2 days, loaded/dehydrated with 20% and 60% PVS2 (Sakai et al., 1990) solutions for 30 min and 15 min, respectively, and vitrified with concentrated PVS2 in liquid nitrogen. About 50% vitrified shoot tips resumed normal shoot growth and developed into plantlets. Post-thaw culturing of cryopreserved shoot tips on medium containing elevated level of sucrose (0.2 M) induced multiple shoot formation. This is highly advantageous for multiplication of clones, which show little survival after cryopreservation. The details of the procedure developed at CPRI for cryopreservation of potato shoot tips are given in following protocol.

**Protocol: Cryopreservation of Potato Shoot Tips**

**Vitrification procedure**

1. Establish disease-free in vitro stock cultures of potato genotypes following standard micropropagation method.
2. Dissect shoot tips about 0.5-0.7 mm long from 25-day-old in vitro plantlets and pre-culture on filter paper discs on PVS-1 medium (half strength MS medium supplemented with 0.3 M sucrose, 0.2 M mannitol and 8.7 μM GA₃) for 2 days under a 16-h photoperiod (approx. 40 μmol m⁻² s⁻¹ light intensity) at 24 °C.
3. Place 20 pre-cultured shoot tips in a 5 ml test tube, and load the shoot tips with 3 ml of 20 % PVS-2 (30 % w/v glycerol, 15 % ethylene glycol and 15 % dimethyl sulfoxide in MS medium supplemented with 0.4 M sucrose) solution for 30 min at 24 °C.
4. Remove the solution by using Pasteur pipettes, add 3 ml of 60 % PVS-2 solution, and incubate in ice bath for 15 min.
5. Remove the solution by using Pasteur pipettes, treat the shoot tips with 3 ml of ice cold concentrated PVS-2 for 5 min, and transfer them to 1 ml cryotubes (5 shoot tips/cryotube).
6. Insert the cryotubes in aluminum canes, seal the canes with appropriate cryo-sleeves and plunge the sealed canes directly into liquid nitrogen in a LN₂ inventory storage container.

**Thawing and post-thaw culturing**

1. Warm the cryotubes in a water bath for 1 min at 35 °C, flush the contents of the cryotubes with dilution medium (MS + 1.2 M sucrose), and expel the contents into a 5 ml test tube.
2. Remove the diluents using Pasteur pipettes, add 4 ml of fresh dilution medium, and incubate for 30 min at room temperature.

3. Carefully pipette out dilution medium from the test tube, and transfer the shoot tips to culture medium using a Pasteur pipette.

4. Plate the vitrified shoot tips on semisolid MS medium containing 0.2 M sucrose, 5.8 μM GA₃, 1.0 μM BA and 6.0 g l⁻¹ agar in 90 mm Petri dishes (10 shoot tips/Petri dish).

5. Incubate the cultures under 16-h diffuse light (approx. 6 μmol m⁻² s⁻¹ light intensity) at 24 °C.

6. After 1 week, transfer the shoot tips onto standard shoot tip medium based on MS supplemented with 0.09 M sucrose, 2.9 μM GA₃, and 6.0 g l⁻¹ agar, and incubate under a 16-h photoperiod (approx. 40 μmol m⁻² s⁻¹ light intensity) at 24 °C.

7. Subculture the regenerated plants through single node cuttings.

Potato is one of the few plant species used for developing basic cryopreservation technique and for understanding the principles underlying cryogenic storage. However, comparable results were not obtained in potato cryopreservation using conventional freezing techniques, thereby limiting routine use of cryogenic technique for conservation of potato genetic resources. Recent developments in the field of vitrification-based cryopreservation, especially encapsulation-dehydration and chemical-vitrification, have added new dimensions to potato cryopreservation research. These techniques are simple, inexpensive and produce reproducible results. Hopefully, vitrification-based procedures can be used routinely for cryopreservation of potato germplasm in the near future.
Literature cited


In-vitro conservation of potato germplasm

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The cultivated potato, Solanum tuberosum L., is a tetraploid (2n = 4x = 48) and exhibits complex tetrasomic inheritance. It is highly heterozygous and segregates on sexual reproduction. Elite parental lines and cultivars of potato are thus maintained through vegetative propagation in order to maintain their genetic integrity. Maintenance of potato germplasm through field clonal propagation is time-consuming, requires large amounts of space and is labour-intensive. This also exposes the plants to diseases, pests, and risks of loss due to abiotic stresses and natural calamities. Therefore, throughout the world, potato gene banks prefer to conserve elite parental lines and clones as in vitro propagated microplants under disease-free tissue culture conditions. When grown under optimum propagation conditions (MS medium with 30 g l$^{-1}$ sucrose, 16-h photoperiod, 22–25°C), the microplants require subculturing after 4–8 weeks. In order to reduce the frequency of subculturing, growth of the microplants is restricted by employing growth retardants or osmotic stress in combination with a reduced energy source, low temperatures, and low light intensity. The use of low temperatures (6–8°C) and 16-h photoperiod (15–30 µmol m$^{-2}$ s$^{-1}$ light intensity from cool white fluorescent lamps) is almost universal in potato gene banks for conservation. By this method, in-vitro plantlets can be conserved for 2-3 years depending on the genotype. In sub-tropics, where maintenance of low temperature is problematic and expensive due to high demand on energy, plantlets could be conserved at normal propagation temperatures (25°C) using MS medium with osmoticum (4% sorbitol, 2% sucrose) for 12 months without sub-culture. Plantlets so conserved are normal looking unlike those conserved at low temperature.

Microplants for in vitro conservation of germplasm are usually raised by using shoot tips or nodal cuttings as the explant. These shoot cuttings or microplants can also be induced to produce microtubers by incubating them under suitable conditions. Though microtubers are convenient for handling, storage and transport of germplasm, their use for conservation of germplasm is not common. We found that microtubers induced on MS medium supplemented with 60–80 g l$^{-1}$ sucrose and 10 mg l$^{-1}$ of BA (N$^6$-benzyladenine) could be stored at 6–8 °C in diffused light for 12 months. In order to increase the shelf-life of microtubers, efforts were made to enhance microtuber dormancy by adding abscisic acid (ABA) to the medium, as ABA is known to be generally higher in dormant tubers. However, an exogenous supply of ABA adversely affected both microtuber production and microtuber dormancy. The potential of the microtubers for in-vitro conservation of potato germplasm was studied by culturing microtubers on Murashige and Skoog (MS) medium with 40 g l$^{-1}$ sucrose and 20 g l$^{-1}$ mannitol at 6 ± 1°C under a 16-h photoperiod. Both freshly harvested microtubers and microtubers stored for 9 months at 6 ± 1°C were tested. The results showed that microplants from freshly harvested microtubers could be maintained satisfactorily without subculture for 15 months, whereas those raised from 9-month-old microtubers required subculture after 3 months. Microplants from microtubers produced in the absence of ABA had better...
condition. After 18 months of incubation, the surviving microplants from freshly harvested microtubers produced new microtubers, pointing to a possible microtuber-to-microtuber cycle for the conservation of potato germplasm.

Apprehensions are expressed about the genetic integrity of the material conserved in-vitro. In slow-growth in-vitro conservation, plantlets are grown under sub-optimal conditions. Due to this they show signs of stress and have bush like appearance with thin stems and reduced or nil leaves. Continuous maintenance under these conditions may lead to physiological as well as genetic changes in the material. In the microtuber to microtuber cycle of conservation, microtubers were used to conserve the potato genotypes on slow growth conservation medium. The plants so raised could be conserved for one year and these plants again produced new microtubers in conservation. The genetic stability of the material conserved was studied using RAPD, SSR ISSR and AFLP markers. Complete similarity of mother plants and its derived microtubers after conservation of 1 year on slow-growth medium was observed. This showed that this method of in-vitro conservation of potato germplasm is safe and true-to types genotypes can be maintained by this method.

Further Reading

Pollen Cryopreservation Research at IIHR

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Cryopreservation is defined as conservation of viable cells, tissues, organs and organisms at ultra-low temperatures, usually in liquid nitrogen to a minimum temperature of -196°C. This long-term conservation method is increasingly used in the management of crop plant genetic resources and is also an important component of many plant biotechnology programs. Cryogenic storage is also used to conserve germplasm derived from wild relatives, ancient and modern cultivars and biotechnologically derived genotypes.

Storing pollen for future use is an important tool in species that are wild crop relatives, for conservation of genetic resources. The mature male gametophyte of most plant species is desiccation tolerant which render them ideally suitable for storage. A major impact of pollen cryopreservation in crop plants will be to maintain genetically diverse stocks of pollen collected from wild relatives, land races, trait specific accessions etc., for future conservation measures, a strategy which can also accomplish conservation of nuclear genetic diversity (NGD) to conserve genetic variation expressed through pollen. This is the only variability that could be tapped under natural situations to render more effective ecosystem services, especially when information on their breeding behavior is less known. Long term pollen cryopreservation has been attempted successfully in many species for conservation of NGD. The potential of plant cryopreservation can only be fully exploited by effective technology transfer to gene banks and culture collections. Whilst some cryo-conservation methods are dependent upon the use of relatively expensive and specialist cryogenic facilities, others involve simplified methods, which are suitable for use in less well-resourced laboratories.

Cryopreservation is thus now an accessible conservation option for a wide range of users and it has the potential to support both small- and large-scale laboratories and conservation centers. Cryopreservation of pollen is the method of choice for which no elaborate tissue culture methods are needed. It is cheaper than field maintenance of accessions, circumventing the risk of loss caused by diseases, climate changes and other forms of genetic erosion at the haploid stage. The biological material kept in a cryogenic state is said to be in a state of suspended animation with minimal metabolic changes affecting the conserved material.

Cryopreservation research at IIHR

With the support of a ICAR sponsored FAO UNDP project during the early eighties, IIHR was able to establish facilities, train manpower and initiate research programs for carrying out research in the area of cryopreservation in horticultural crops. During the last 25 years technology has been developed for long-term cryopreservation of Nuclear Genetic diversity (NGD). Long term cryopreserved pollen in citrus, papaya, grape, mango, tomato, eggplant, onion, capsicum, rose, gladiolus, gerbera, carnation and RET species of medicinal
plants are maintained in liquid nitrogen. The pollen cryobank is managed by periodic replenishment of the cryogen, for maintaining a constant cryogenic temperature throughout the storage duration. The technology developed is globally acceptable and will be useful for production of hybrid seeds, besides its use in gene banks and biotechnological interventions.

At the pollen cryobank of IIHR, which was featured in the LIMCA BOOK OF RECORDS in 2001 as the first of its kind in India, information pertaining to 650 pollen samples of more than 45 species belonging to 15 families is stored in a database. The software support provided for this database was FoxPro version –2. For easy management of data one main database and three sub-databases were created. Genus, species and cultivar information is available in a separate database. Information pertaining to longevity, viability and fertility of pollen after cryopreservation, media used for pollen germination, year wise listing of collected samples, year-wise list of samples cryopreserved in different years are available within the database, which is menu driven and user friendly.

Although genetic conservation through pollen storage does not accomplish whole genome conservation, a breeder involved in genetic enhancement of a given crop can have access to a Pollen Cryobank facility, for sourcing NGD inputs in his genetic amelioration program. Besides the already existing role of pollen cryobanks in breeding, there are many promising applications, which have come to focus with the recent advances in allied bioscientific areas.
In vitro conservation in Horticultural crops

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Tissue culture techniques are of great interest for collection, multiplication and storage of plant germplasm. The method allows propagating plant material with high multiplication rates in an aseptic environment. Virus-free plants can be obtained through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of plant germplasm. The miniaturization of plants allows reduced space requirements and considerable saving in labour costs for the maintenance of germplasm accessions.

A major limiting factor affecting the increased use of in vitro conservation has been the concomitant improvement of routine tissue culture techniques and the development of simple cryoprotection methods that enhance recovery processes especially after cryogenic storage. When one method of conservation is subject to unavoidable hazards, as with FGBs, an alternative method should also be used as a backup conservation method. The role of in vitro conservation techniques in the overall conservation strategies should be indicative of the fact that it should complement other conservation strategies within the total program of a given crop, accession cluster, species or population. The methods chosen should be carefully considered taking into account the feasibility, practicality, economy and security of the material to be conserved. In vitro collections of species could be maintained at the same or separate site, but should have clear linkages with the field gene banks.

The methods employed vary according to the storage duration required. For medium-term conservation, the aim is to reduce growth, thus increasing intervals between subcultures. For long-term conservation, cryopreservation, i.e. conservation at ultra-low temperature, usually in liquid nitrogen (-196°C), the aim is to establish base collections conserved to posterity. At this temperature, all metabolic processes are reduced to a bare minimum. Plant material can thus be conserved without any genetic alteration for extended periods without the need for frequent regeneration.

At IIHR, protocols have been optimized for 23 horticultural crops, which are being conserved under normal and reduced culture conditions. Vitro-plants of Jackfruit accessions have been successfully conserved for 4 years under standard culture conditions prior to first subculture. While more jackfruit accessions are accessed in vitro, 4 citrus accessions are maintained in vitro and conservation attempts has resulted in maintaining vitro plants under reduced culture conditions for 6 months. Protocols for conserving plant diversity for 22 RET species of medicinal plants have been optimized. A review of the current status of specific plant species will be discussed along with protocols developed.
Banana conservation strategies- current status and future plan

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Cultivated bananas are parthenocarpic in nature and major approaches adopted as vegetatively propagated crop comprise conservation in field, in vitro and cryo gene banks and ex situ conservation in the form of seeds is not feasible, except for wild bananas. However, the prevalence of viral diseases poses major constraint for the safe handling of banana germplasm in field conservation and germplasm exchange programmes.

The NRCB, Trichy after thorough morpho-molecular characterization, has successfully established a core banana collection of 363 accessions (313 indigenous and 50 exotic) which included different genomes namely AA (26), AAA (26), AAAA (1), BB (20), AB (23), AAB (99), ABB (103), ABBB (7), Rhodochlamys (5), lesser known species (3) are being maintained in field genebank. The plants in the field are replanted every four years. Though it provides easy access to germplasm accessions for characterization and evaluation and direct control over germplasm, but plants are highly vulnerable to pests, diseases and natural calamities. Further, it requires expensive labour, inputs and land for maintenance. Hence, complementary strategies like in vitro and cryopreservation have been attempted in conservation of banana germplasm in the recent past.

At NRCB, Trichy, virus indexed core collection accessions maintained in the field genebank were regenerated in vitro using shoot tip explants. Though the tissue culture protocols are variety specific, currently protocols are available for varied genomic groups which are being exploited with minor modifications. Most of the accessions of core collection as well as other germplasm (420 accessions in total) are also being maintained under in vitro at NBPGR, New Delhi. To bring down the maintenance cost, low cost tissue culture protocols have been successfully developed and used at NRCB, Trichy and NBPGR, New Delhi. The successful use of low cost alternatives in the tissue culture media without affecting the genetic fidelity has been demonstrated through field evaluation and molecular characterization.

Cryopreservation of Musa germplasm is done at NRCB and NBPGR in a collaborative mode. NRCB provides virus indexed cultures/suckers to NBPGR while NBPGR has standardized cryopreservation protocols using vitrification technique and proliferating meristems as explants. Till date, 60 accessions have been successfully cryopreserved and remaining is envisaged to be cryopreserved in a phased manner. The success of cryopreservation technique in banana has also been proved through field evaluation of cryopreserved germplasm accessions and their molecular characterization for genetic fidelity.

The other ex situ conservation strategies and future areas are: conservation of pollen, seed and embryogenic cell lines. Also, models need to be developed for in situ conservation,
especially for wild relatives and species. Banana is one of the vegetatively propagated crops, where multiple conservation strategies are essentially required and the strategies are discussed.
Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops
PROTOCOLS

Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops

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Cryopreservation of Mango Pollen (*Mangifera indica* L.)

(Rajasekharan, and Ganeshan 2001-02 and Shashikumar 2006)

**Pollen Collection**

Anthers are collected for pollen collection and processing for storage (There is only one anther which produces viable pollen, dehisced anthers are ash colored)

1. Mature anthers were collected in clean petri dishes from healthy panicles of trees from the mature trees between 10 and 11 AM.
2. The anthers were brought into the laboratory and transferred to empty gelatin capsules,

**Viability assessment**

**Checklist for *in vitro* pollen germination**

Items needed to prepare germination medium and slide kit

1. Tools: dissecting needles, forceps, camel hair brush
2. Petri dishes
3. Filter paper disks
4. 100ppm boric acid
5. Sucrose 15% W/V
6. Poly ethylene glycol (PEG, Mol. Wt. 10,000)
7. Cellophane 1 cm² (non-water proof)
8. Sterile strainers
9. Sterile double distilled water
10. Glass micro slides
11. Glass cover slips 1.2 cm²
12. Tissue Paper
13. Slide holder box
14. Compound microscope
15. Any other adhesive synthetic gum
Pollen germination medium composition

1. Boric acid 100 mg l\(^{-1}\)
2. Sucrose 15% W/V
3. Poly ethylene glycol 10 % W/V

Prepare in advance:

1. Soak 1 cm\(^2\) Cellophane strips in pollen germination medium for 30 minutes Place each soaked cellophane strip on a glass micro slides using forceps
2. Remove excess germination medium from the slides using tissue paper
3. Allow the slides to dry for two hours
4. Store the dried slides for later use in a slide holder box
5. Place the water soaked filter paper as lining inside petri plates to create humidity chamber

Procedure:

This is a 30 minutes procedure once the slide kit is prepared.

1. Extract pollen from anthers using forceps or camel hairbrush or dissecting needles.
2. Transfer Adequate quantity of pollen on to a cellophane of previously prepared slide
3. Spread the pollen in to thin layer using camel hair brush
4. Place the slides in a petri plate using glass rods as a support to the slides
5. Incubate the slides for two to three hours in petri plate under humid (100 % RH, 26 ±2\(^{\circ}\)C) condition
6. Observe the pollen for germination and tube growth under the compound microscope
7. Stain the pollen using Alexander stain and place the 1.2 cm\(^2\) cover glass
8. Slides can be made paramagnet after applying Quick Fix for counting aborted, un-germinated and germinated pollen.

Possible Problems:

Three replicates are made along with controls; some species requires standardization of optimum humidity.

1. Insufficient moisture in humidity chamber
2. Physiological status of pollen
3. Uneven spreading of pollen on cellophane
Cryopreservation

1. Cryocans
2. Canisters
3. Liquid Nitrogen
4. Sealing Unit

Procedure

1. Gelatin capsules containing pollen borne anthers are enclosed in laminated poly aluminum pouches and sealed airtight.
2. Pouches are stacked in canisters of a Cryobiological System (Mach SM 43 MVE, USA) and lowered gradually into liquid nitrogen and stored for desired durations.
3. For smaller quantities of anthers, butter paper can be used instead of gelatin capsules.

The canisters were capped with perforated lids in order to prevent the vials from floating out into the dewar during refilling of liquid nitrogen. The cryoflask was maintained in the laboratory at 22±2°C

Retrieval and post-storage fertility assessment

Field pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, etc.

Procedure:

1. Anthers were thawed from frozen state to ambient temperature,
2. By initially holding the canister over liquid nitrogen vapour phase for 15 minute,
3. After which the laminated pouches with anthers were removed from the canister.
4. Field pollinations were carried out with cryopreserved ‘Alphonso’ pollen hermaphrodite flowers on the panicles which were
5. Emasculated and covered with polythene bags a day prior to crossing.
6. On the following day, pollen was applied on to the receptive stigma using a camel hairbrush, by squeezing the anthers with the help of forceps.
7. After crossing the emasculated flowers with fresh and cryopreserved pollen, the panicles were again covered by polythene bags in order to avoid contamination by open pollinations.

8. Observations were recorded on fruit set after 14 days till maturity.
Cryopreservation of Papaya Pollen (*Carica papaya* and *Carica cauliflora*)

(Ganeshan 1986)

**Pollen collection**

Pollen of *Carica papaya* L. ‘Washington’ and *Carica cauliflora* L are to be collected in petri dishes from plants bearing male flowers between 9.00 and 10.00 a.m. Specie-wise bulked pollen samples are to be transferred to empty gelatin capsules or butter paper pouches, individually packed and in turn transferred to laminated aluminum pouches.

**Viability assessment**

Viability of fresh and cryopreserved pollen was assessed in terms of germinability *in vitro*. Pollen was germinated in hanging drops of medium (6% sucrose) at 25±2°C.

**Checklist for *in vitro* pollen germination**

1. Cavity slides
2. Cover glass
3. Petri dishes
4. Butter paper
5. Needles
6. Fine tweezers
7. Petroleum Jelly
8. Non-adsorbent Cotton
9. Blotting sheet
10. Germination medium (freshly prepared)

Pollen germination medium: 6 per cent sucrose in double distilled water.

**Procedure**

1. Collected pollen on butter paper is consolidated for spreading on a drop of medium placed on a cover glass smeared on the edges with petroleum jelly.
2. Pollen is uniformly spread on the drop of medium with the help of a fine needle. A cavity slide is covered over cover glass holding the drop of medium with pollen.
3. The cavity slide is placed with the cover glass facing up on a Petri dish containing pre-moistened blotting sheet and covered with the lid containing pre-moistened
blotting sheet and transferred to an incubator maintaining a temperature of 25°C ± 2°C.

4. After incubating the cultures for 4 h, pollen tube growth was arrested by staining with a drop of Alexander’s stain (1980).

5. A pollen grain was considered as viable if tube length was greater than the grain diameter.

6. More than 500 pollen grains were scored for each replication using a Leitz-Neo Promar projection microscope.

**Cryopreservation**

1. Pollen in gelatin capsules or butter paper pouches sealed using poly aluminum pouches are loaded to canisters and cryopreserved.

2. Cryopreservation was accomplished in a Mach-SM-43 cryobiological system (MVE, U.S.A) by direct immersion in liquid nitrogen.

3. Pollen samples stacked in canisters were kept completely immersed in liquid nitrogen throughout the desired storage period.

4. The canisters were capped with perforated lids in order to prevent the vials from floating out into the Dewar during refilling of liquid nitrogen.

5. The cryoflask was maintained in the laboratory at 22°C ± 2°C

**Retrieval and post-storage fertility assessment**

Field pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.
Procedure

1. Fertility of pollen cryopreserved in liquid nitrogen tested by controlled field pollinations.

2. Unopened female flowers of were bagged 1 day prior to pollination in order to avoid contamination.

3. Cryostored pollen, after thawing to room temperature, was taken to the field and applied on the receptive stigma of the female flower using a painting brush followed by immediate bagging.

4. For suitable controls, fresh pollen was applied on stigmas of previously bagged female flowers, following bagging.

5. Fruit and seed set were recorded for all the flowers pollinated after allowing normal fruit development and maturity.
Cryopreservation Citrus Pollen (*Citrus limon* Burm.)

(Ganeshan and Alexander, 1991)

**Pollen collection**

The species handled for pollen studies include *Citrus limon*, *Citrus aurantifolia*, *Citrus sinensis* and *Poncirus trifoliata*.

**Inventory**

Petri dishes, butter paper, forceps, muslin cloth fixed to a 10 cm. cylindrical ring with the help of a firm rubber band, clean razor blade, etc.

**Procedure**

1. Pollen collections are usually made on a bright sunny day between 8 - 10 A.M.
2. Staminate flowers are harvested at peak anthesis and the dehiscing anthers are gently caressed over the muslin cloth fixed to the cylindrical ring, which acts as a sieve.
3. Dehiscing anthers are carefully removed from pistillate flowers and pollen is extracted over the muslin cloth sieve.
4. Pure pollen is extracted in clean Petri plates or butter paper.

The following points should be noted:

1. Quality of pollen collected depends on the correct stage of anthesis/anther dehiscence
2. Pollen should be free of anther debris
3. Do not collect pollen from infected or insect damaged flowers
4. Decide upon bagging of inflorescence depending on insect activity
5. Do not venture to collect pollen on a rainy day or if it had rained overnight.
6. Do not force out pollen from anthers.

**Viability assessment**

Pollen collections are subjected to viability indexing by germination *in vitro* by the hanging drop technique (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in a drop of liquid medium consisting of a carbohydrate source, inorganic and organic compounds.
Cavity slides, cover glasses (22 mm square), microslides, petroleum jelly, large petri plates, filter paper discs, incubator, double distilled water, needle, forceps, "Quick fix" (epoxy resin) Prepared in deionised, double distilled water.

20% Sucrose,

100 mg l⁻¹ H₃BO₃,

300 mg l⁻¹ Ca(NO₃)₂.4H₂O,

200 mg l⁻¹ MgSO₄.7H₂O,

100 mg l⁻¹ KNO₃,

pH 7.3.

1. Apply petroleum jelly to the edges of a new cover glass.
2. Place this cover glass on the worktable so that the edge on which petroleum jelly is applied faces the roof.
3. Place 25 to 50 μl drop of nutrient medium on the center of the cover glass assuming the shape of a small bubble.
4. Add pollen grains with the help of a needle carefully, filling the drop with optimum quantity of pollen.
5. Invert a cavity slide over the cover glass with the cavity over the drop.
6. Reverse the cavity slide and place in a petri plate fixed with moistened filter paper disc cut to its exact size.
7. Place the cavity slide over a microslide in such a way that the cover glass with the hanging drop over the cavity is well clear of its surrounding area, intact and hanging. Cover petri plate with the top lid (also fixed with moistened filter paper) and keep in an incubator at 25 ± 2°C dark, for 18 to 20 hours.
8. After the prescribed duration of incubation, the hanging drop preparation is separated carefully from the cavity slide.
9. After removing the residual petroleum jelly from the slides of the cover glass, a drop of Alexander’s differential stain (Alexander 1980) is added over the drop with germinated pollen and mixed slowly.

10. Place a clean microslide over the stained drop. The drop is flattened with the cover glass sticking to the underneath of the microslide.

11. Remove excess stain and seal the edge of the cover glass microslide interface with a thin layer of quick acting adhesive (Quick fix).

12. The slide with germinated pollen is ready for observations under the microscope for qualitative and quantitative viability estimates.

Noteworthy points are:

1. Prepare medium fresh
2. Use only new cover glass
3. The medium on the cover glass should be just optimal for it to hang on the cover glass when inverted
4. Add just enough stain to the drop containing germinated pollen. If excessive stain is added, the germinated pollen drifts away outside the cover glass when the drop is flattened, leading to errors in quantitative estimation.

Cryopreservation
Inventory
Butter paper, glass vials, silica gel, gelatin capsules, polyethylene aluminum laminated pouches, head sealer, domestic refrigerator, freezer, liquid nitrogen storage cryoflask filled with liquid nitrogen.

Pollen samples are packed in glass vials/gelatin capsules/butter paper packets depending on the method of storage.

Cryogenic temperatures (-196°C) afford much more prolonged duration storage, beyond two years ad demonstrated by Japanese workers (Kobayashi et al.,1978), Ganeshan and Alexander (1991) reported cryogenic preservation of lemon pollen for 3.5 years and beyond (Ganeshan and Rajasekharan,1999).
Storage in liquid nitrogen:

1. Pollen samples are packed either in gelatin capsules or butter paper packets, sealed air tight in polyethylene aluminum laminated pouches and lowered into a canister of a cryoflask.
2. The canister is capped with a perforated lid and plunged slowly in liquid nitrogen contained in the cryoflask.
3. Frequent refilling of the cryoflask with liquid nitrogen at least once every 10 to 15 days, ensures a constant cryogenic temperature.
4. For proper ease of operation, samples to be cryopreserved for a long duration are located at the bottom of the canister and those that are to be retrieved after a short duration, at the top of the canister.

Retrieval and post storage viability/fertility assessment

Pollen samples retrieved from cryogenic temperatures have to be carefully pulled out of the canister with the help of a one foot long blunt forceps, held at ambient temperature for 10-15 minutes prior to a viability test or field pollination.

Pollen samples are viable if:

1. Germination profiles in vitro are as good as fresh pollen
2. Germination time taken is not more than that of fresh pollen (pollen vigor)
3. Estimated germination is in the range of 60 to 80 per cent of fresh pollen.

Field pollinations with cryopreserved pollen for fertility assessment

Inventory

Breeder's crossing kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.

Procedure

1. Pollen samples retrieved from cryostorage are transported to field locations in an icebox and used in crosses with compatible seed parents in order to obtain an estimate of fertility, fruit and seed set.
2. Cryostored pollen is applied to pre-bagged emasculated perfect flowers at peak stigma receptivity with the help of a painting brush, followed by bagging to avoid contamination with stray pollen.

3. For comparative fertility estimates, crosses with fresh pollen must be carried out as far as possible at least initially.

4. If the pollen parent in question is not in flowering, crosses may be repeated with the fresh pollen over the same seed parent within a short period.

Observations to be recorded in field are:

1. Number of crosses made with fresh and stored pollen
2. Number of fruits set to maturity
3. Number of seeds per fruit
4. Number of aborted seeds

Seeds set through cryostored pollen are germinated and observation of any marker characters transmitted by pollen parent (for example, trifoliate leaf character in F1 population of crosses involving pollen of *Poncirus trifoliata*) would confirm that the stored pollen was fertile and has transmitted the character conserved to the next generation (Rajasekharan et al., 1995)
Cryopreservation of Grape Pollen (*Vitis vinifera, Vitis labrusca*)

(Ganeshan 1985)

Pollen collection

Pollen is collected from plants in well established vineyards of Bangalore Blue, Bangalore Purple, Black Champa, Queen of Vineyards, Thompson Seedless, Perlette, Kishmish Charni, Kishmish Long, Kishmish Beli, Beauty Seedless, Arkavathi, Arka Hans, Pandhari Sahebi, Anab-e-Shahi, using the method described by Olmo (1942).

The methodology followed includes:

1. Tapping of 2/3rd dehiscing inflorescence on to clean Petri dishes
2. Blow out the floral debris and retain the yellow pollen mat collected
3. Consolidate the collected pollen using a clean razor blade
4. Packing the pollen into gelatin capsules or butter paper pouches

Viability assessment

1. Pollen viability is tested by its ability to germinate in an artificial medium.
2. Fresh pollen samples are cultured in vitro following hanging drop technique as described in detail for citrus.
3. The germination medium consists of 20% sucrose solution in which pollen germinated profusely.
4. Hanging drop cultures are incubated for 5 h at 25 ± 2°C after which pollen growth was arrested by staining with a drop of Alexander's stain (1980).
5. Slides with germinated pollen are scanned and scored using a Leitz Neo-Promar projection microscope.
6. Pollen whose tube lengths are measuring more than the grain diameter were considered viable.

Cryopreservation

1. Storage at -196°C was accomplished by direct immersion in liquid nitrogen after pre-cooling to -20°C.
2. Laminated aluminum pouches containing gelatin capsules/butter paper pouches with pollen samples are stacked in canisters of a Mach-SM-43 cryobiological system (MVE, USA) and immersed in liquid nitrogen.

3. Complete immersion of samples throughout the storage duration was ensured by frequent refilling of the cryoflask with liquid nitrogen and capping the canisters with perforated lids, in order to prevent the stored samples from floating out into the Dewar.

4. The cryoflask is maintained in the laboratory at 22 ± 2°C.

**Retrieval and post-storage fertility assessment**

Field pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.

Procedure

After thawing pollen samples drawn out of the liquid nitrogen cryoflask during each viability assay, the vials are kept at ambient temperature for 30 min before taking to field. Fertility of pollen cryopreserved in liquid nitrogen is tested by controlled field pollinations on established male sterile lines. Cryostored pollen, after thawing to room temperature, is taken to the field and applied on the receptive stigma of the female flower using a fine brush or clean index finger followed by immediate bagging.

Fruit and seed set are recorded for all the flowers pollinated after allowing normal fruit development and maturity.
Cryopreservation of Gladiolus Pollen

(Rajasekharan et al. 1995)

Pollen collection and processing for cryostorage

1. The flowers were tied with thread at the bud stage in order to prevent contamination by stray pollen and to obtain pure samples.
2. On the day of collection, the flowers were harvested and brought to the laboratory. Petals were carefully separated and pollen grains were extracted by scraping the mature anthers, which were about to dehisce, with a blunt needle, passing traversely along the lobe of the anther.
3. Bulked pollen samples were transferred to empty gelatin capsules, packed in laminated poly aluminum pouches, sealed airtight,

Precaution: Care was taken not to scrape off the tapetal tissue, which could contaminate the pure pollen.

Viability assessment

Checklist for in vitro pollen germination

Items needed to prepare germination medium and slide kit

1. Tools: dissecting needles, forceps, camel hair brush
2. Petri dishes
3. Filter paper disks
4. Cellophane 1 cm² (non-water proof)
5. Sterile strainers
6. Sterile double distilled water
7. Glass micro slides
8. Glass cover slips 1.2 cm²
9. Tissue Paper
10. Slide holder box
11. Compound microscope
12. Adhesive synthetic gum

Pollen germination medium composition

The nutrient medium consisted of 15% sucrose supplemented with

300 mg L⁻¹ Ca(NO₃)₂ · 4 H₂O,

200 mg L⁻¹ MgSO₄ · 6H₂O,

100 mg L⁻¹ KNO₃ and

100 mg L⁻¹ Boric acid.

Prepare in advance.

1. Soak Cellophane 1 cm² strips in pollen germination medium for 30 minutes
2. Remove excess germination medium from the cellophane strip using tissue paper

Procedure:

1. Place the booklets with cellophane in 80 mm Petri dish
2. The top sheet of the filter paper carefully removed to expose the cellophane surface,
   blotted with a strip of dry filter paper.
3. Pollen was carefully dusted over the cellophane
4. The Petri dishes were then covered with their complementary lids
5. Wrapped tightly using a thin polypropylene film
6. Incubate at 25±2°C in the dark for a duration of 4-6 hours,
7. After incubation the cellophane strips were carefully separated from the booklets
8. Place on a clean micro slide, exposing the germinated pollen present on the
   cellophane to the stain.
9. Staining was done using a drop of versatile stain (Alexander, 1980).
10. A cover slip was placed over the stained cellophane strip after which the preparations
    were sealed with an epoxy resin, for microscopic examination
11. In each preparation, more than 400-500 pollen grains were scored for their viability,
    using a Leitz Neo-Promar projection microscope.
12. Pollen grains, whose tube lengths were longer than the grain diameter, were considered as having germinated.

Precautions: sow optimal quantity of pollen to get good germination

**Retrieval and post-storage fertility assessment**

Requirement: pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc

**Procedure**

1. For pollinations with cryostored pollen, pouches were retrieved from a cryogenic state and thawed to ambient temperature
2. Transferring the pollen form the capsule on to a clean Petri plate
3. Flowers were emasculated and bagged for pollinations with cryostored pollen on the following day.
4. Cryostored pollen was applied to the receptive stigma of the emasculated flower using a camel-hair brush.
5. Pollinated flowers were enclosed in perforated butter paper bags to provide gas exchange and to prevent contamination with stray pollen.
6. To make a performance-related comparison, similar crosses were carried out using fresh pollen collected from flowers of the same pollen parent on the day of pollination.
7. The fruit (Capsule) and seed set were recorded for all crosses after allowing normal development and maturity.
Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops

Cryopreservation of Rose Pollen
(Rajasekharan and Ganeshan 1994)

Pollen collection and processing for storage

1. Previously bagged flowers pollen was collected on a bright sunny day, which are due to open the following day.
2. On the day of collection bags were removed between 10 00 hours and 1100 hours and the bloomed flowers were harvested and brought into the laboratory.
3. After separating the petals, pollen grains were collected by gently tapping the flower hips onto butter paper.
4. Collections from different flowers of a given cultivars were bulked before assessing viability in vitro.
5. The pooled samples appear in the form of a fine powder.

Viability assessment

1. Apply the thin film of petroleum jelly or any other sealing substance around the rim of the 22mm glass cover slip
2. Place ca.50μl drop of culture medium in the center of the clean dry cover glass.

Precaution: the volume of the culture medium drop should be such that it does not spread and come in contact with the rim or the bottom of the cavity, or with the sealing substance.

3. Add a suitable amount of pollen grains to the medium drop and mix thoroughly with the needle to obtain a homogenous pollen suspension.
4. Carefully invert the cover glass with the pollen suspension over the cavity such that pollen culture drop is suspended in the center of the cavity. Pollen grains move to the lower meniscus of the hanging drop and are exposed to the atmosphere of the cavity.
5. Apply gentle pressure around the edges of the cover glass so that the cavity becomes sealed (with the cover glass and the sealing substance) the hanging drop is ready
6. Hanging drops of medium containing pollen were incubated 25± 2 °C at for 4 h in dark, enclosed in a moist chamber providing 100% relative humidity
7. The cover slips with hanging drops were separated from the cavity slides and stained (Alexander, 1980).

8. The percentage viability was determined by counting the germinated pollen grains using a Leitz Neo-Promar projection microscope.

9. A minimum of 500 grains was scored per replicate. Pollen was considered germinated when the emerging pollen tube was longer than the grain diameter.

**Prepare in advance**

**Germination medium**

- 150 mg l\(^{-1}\) CaNO\(_3\) 4H\(_2\)O
- 200 mg l\(^{-1}\) MgSO\(_4\) 7H\(_2\)O
- 100 mg l\(^{-1}\) KNO\(_3\) and
- 100 mg l\(^{-1}\) H\(_3\)BO\(_3\)

**Cryopreservation**

1. The pollen samples carefully transferred into gelatin capsules enclosed in a laminated aluminum pouch.

2. The pouches with the capsule were sealed air tight and stacked in the canisters of a Mach SM-33 (MVE, USA) cryobiological system, and lowered gradually into liquid nitrogen (LN) (rapid freezing and direct plunging).

3. The complete immersion of sample was ensured by maintaining the level of liquid nitrogen above the sample through frequent refilling of the cryoflask.

**Retrieval and post-storage fertility assessment**

Requirement: pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc
Procedure

1. For pollinations with cryostored pollen, pouches were retrieved from a cryogenic state and thawed to ambient temperature.
2. Transferring the pollen from the capsule onto a clean Petri plate.
3. Flowers were emasculated and bagged for pollinations with cryostored pollen on the following day.
4. Cryostored pollen was applied to the stigma of the emasculated flower using a camel-hair brush.
5. Pollinated flowers were enclosed in perforated butter paper bags to provide gas exchange and to prevent contamination with stray pollen.
6. To make a performance-related comparison, similar crosses were carried out using fresh pollen collected from flowers of the same pollen parent on the day of pollination.
7. The fruit (hip) and seed set were recorded for all crosses after allowing normal development and maturity.
Cryopreservation of Pollinium in Asclepiadaceae-General procedures

(Shashi Kumar, 2006)

Pollen in asclepiadaceous members (e.g., Tylophora indica) are arranged in compact sac-like structure called pollinium, a fine needle is used to extract individual pollinia from freshly opened flowers. Pollen is binucleate, number and size of pollen in pollinium vary with species. Fresh pollinium is processed for cryopreservation. 20-30 pollinia is placed in a gelatin capsule, which is then sealed in poly aluminum pouches.

Checklist for Pollen Cryopreservation

Items needed to extract, process, pretreatment and cryostorage of pollen

1. Tools: dissecting needles, forceps, camel hair brush
2. Butter papers
3. Gelatin capsules (empty)
4. Aluminum pouches
5. Heat sealers
6. Canisters
7. Liquid nitrogen container
8. Rotex’ mixer

Items needed to assess pollen viability

1. Pollen germination medium (Boric acid 100ppm, sucrose 20%, pH-7.0)
2. Cavity slides
3. Cover slips
4. Alexander’s stain

Procedure for extraction and processing Asclepiadaceous pollinium for cryostorage:

1. Collect coronary corolla of asclepiadaceous members at 10 to 11 am
2. Dissect the coronary corolla using fine needles pullout corposculum of pollinia using fine pointed forceps
3. Collect the pollinia on a clean butter paper
4. Immediately transfer adequate quantities of pollinia in to gelatin capsules
5. Place the gelatin capsules inside aluminum pouches and seal using heat sealer
6. Label the pouches with inventories i.e.: collection date, species verities name
7. Place the pouches inside the canisters and close the lid
8. Immerse the canisters slowly into the liquid nitrogen containers

Retrieval of pollinia from cryogenic containers

1. Lift Canisters from the cryogenic containers gradually.
2. Retrieve the aluminum pouches containing pollinia.
3. Allow the pouch to warm to room temperature from cryogenic temperature
4. Cut open the pouch and use the pollinia in gelatin capsule for further experimentation

Viability assessment

1. Viability in pollinia is assessed using hanging drop method
2. Transfer 4-5 pollinium in to a drop of germination medium on a cover glass smeared with petroleum jelly on all the four corners.
3. Invert cover glass over the cavity slide so that the drop hangs in the centre of the cavity.
4. Incubate the slides in a humidity chamber (Rh-90%) for 4 to 5 hours
5. Observe geminated pollen under compound microscope.

Qualitative and quantitative estimation of germinated pollinia extracted from Asclepiadaceous members

The pollen in asclepiadace member is in a compact mass, pollinia, which pose difficulties in qualitative and quantitative estimation after germination of pollen grains. In order to overcome this difficulty, follow technique described below.

1. Transfer germinated pollinia in to a 5ml test tube containing 1 to 2 ml of pollen germination medium.
2. Agitate test tube using a ‘Rotex’ mixer for about 5 to 10 minutes. (This process loosens the compact mass of germinated pollen, further rupturing the pollinial wall)
3. Transfer the mixture on to a clean slide over a drop of Alexander’s stain.
4. Lower clean cover slip on the germinated pollen mixed in a drop of stain, gently tapping on the cover slip. (This results in individual separation of germinated pollen grains, for photomicrography as well as quantitative estimation.)
Fertility assessment

1. Select healthy mature flower buds of asclepiadace member
2. Remove corolline corona using fine forceps leaving style and stigma
4. Following day carefully place 2-3 pollinia on the stigmatic surface using fine forceps
5. Rebag flower buds with butter paper cover.
6. Carry out pollinations with fresh pollinium following the same procedure described above.
7. Open the bags after 15-20 days to record fruit drop, fruit and seed set.

Possible Problems:

1. Dissection of pollinia after extraction may result in loss of viability
2. Culturing more than 2-3 pollinia may over crowd and pose problem for counting
Cryopreservation of Tropical Orchids

(William Decruze and Ganeshan in press)

Physiological Status

Orchids in general possess pollen tetrads collected into highly organized waxy pollinia with appendages. The pollen are tightly packed in the pollen sac (pollinia) generally surrounded by a viscous fluid. In species like Vanilla pollen are unicellular and the grains are held together by a viscous fluid. Sometimes the pollinial tetrads are organized into many granular packets, prolongations of which form the caudicle as in Plectelis, Habenaria, Satyrium etc. Especially in those cases the pollen were more tightly packed with out much surrounding fluid. Even though information available on the nature of pollen is scanty, it is known that both binucleate and trinucleate are present among orchids.

Pretreatment

Protocol for pollinia cryopreservation of orchids having pollen with surrounding viscous fluid packed in pollinia is described. The pollen are already in a vitrified state and a short treatment in vitrification solution formulated by Sakai et al (1990) was preferred for the cryopreservation. The treatment include exposure to a loading solution containing 2M glycerol and 0.4M sucrose prepared in BK medium (Brewbaker and Kwack 1963) for 15min and PVS2 solution containing 30% glycerol, 15% ethylene glycol, 15 % DMSO and 0.4M sucrose in BK medium for 5-20min. Simple desiccation under laminar airflow is also effective in some species.

Cryopreservation

Successful cryopreservation of Pollinia has typically used direct immersion of a vial (1-2ml) containing the PVS2 treated pollinia sample into liquid nitrogen as one step cooling technique. For experimental purpose overnight exposure in LN is more than sufficient to assess successful vitrification and LN tolerance of treated pollinia. Dried pollinia are also cryopreserved through one step freezing.
Rewarming

To avoid de-vitrification the LN treated pollinia need to be rewarmed rapidly without any shock in the vitrified frozen state. The vials are thus carefully taken out from LN and dropped into a water bath maintained at 40°C. The rewarmed pollinia are then washed in a solution containing 1.2M sucrose prepared in BK medium before viability determinations. Pollinia cryopreserved through simple drying protocol does not require any further washing or rehydration, but can be directly used for germination assay or pollination.

Viability assessment

Immediate survival is determined by placing a small sample of thawed pollinia on a cavity slide adding one to two drops of diluted fluorescein diacetate solution and observed under a fluorescent microscope after a few minutes. Germination is determined by plating samples of rewarmed and washed pollinia in 2-3 drops of BK medium containing 0.5-5% sucrose onto cavity slides and incubating at 24±2°C for 12 to 24 hrs.
Protocols for specific species

Cryopreservation of *Cymbidium bicolor* pollinia through simple drying

*C. bicolor* is an epiphytic orchid having two pollinia with pollen tetrads compactly packed surrounded by a viscous fluid. On initial observation we could note that the pollen are binucleate and easily germinate in BK medium containing 0.5-5% sucrose.

Requirements

1. Tools (needles, forceps, fine tweezers)
2. Sterile Petri plates
3. Cavity glass slides
4. BK medium containing 0.5% sucrose
5. Modular incubator as humid chamber
6. Cryovials (1ml)
7. Water bath
8. Inverted Microscope
9. LN dewar and liquid nitrogen
10. Lactophenol blue

Prepare in advance

1. Sterilized BK medium containing 0.5% sucrose
2. 0.02% methyl blue in lacto-phenol (20ml lactic acid: Phenol 20ml: Glycerol 40ml Distilled water 20ml: 1% cotton blue 2ml)
3. Harvest freshly opened flowers
4. Assess germination capability in BK medium containing 0.5% sucrose

The Procedure

1. Isolate pollinia from freshly opened flowers using a fine tip forceps
2. Put the pollinia in a sterile Petri dish and place it open in a laminar air flow cabinet for 30-75min
3. Collect the dried pollinia in 1ml cryovial, place on cane and submerge in LN
4. Hold in LN for a minimum of 1hr.
5. Warm in a water bath at 40°C for 2min
6. Use the pollinia as such for pollination
7. For *in vitro* germination assay, take dried and LN treated pollinia in cavity slides, add 2-3 drops of BK medium containing 0.5% sucrose and place in a humid chamber (modular incubator) for 12 hrs and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A desiccation period of 75 min under laminar airflow is required to get sufficient dehydration to achieve about 76% *in vitro* germination after cryopreservation almost equal to corresponding desiccation control (87%). However 30-60min period of desiccation that may also be tried to determine optimum period considering genotype, seasonal and regional differences.
Cryopreservation of *Arundina bambusifolia* pollinia through silica gel drying

*Arundina bambusifolia* is a terrestrial orchid having two pollinia with unicellular pollen compactly packed surrounded by a viscous fluid. It easily disperses in liquid media. Hence it is more convenient to use a drying protocol rather than vitrification. The pollen easily germinates and pollen tube grows in BK medium containing 0.5-5% sucrose.

**Requirements**

1. Tools (needles, forceps, fine tweezers)
2. Sterile Petri plates
3. Cavity glass slides
4. BK medium containing 0.5% sucrose
5. Modular incubator as humid chamber
6. Cryovials (1ml)
7. Water bath
8. Inverted Microscope
9. LN dewar and liquid nitrogen
10. Lactophenol blue
11. Charged silica gel packed in glass bottles
12. Small boats made in aluminum foil to carry pollinia to dry under silica gel.

**Prepare in advance**

1. Sterilized BK medium containing 0.5% sucrose
2. 0.02% methyl blue in lacto-phenol (20ml lactic acid: Phenol 20ml: Glycerol 40ml Distilled water 20ml: 1% cotton blue 2ml)
3. Harvest freshly opened flowers
4. Assess germination capability in BK medium containing 0.5% sucrose

**The Procedure**

1. Isolate pollinia from freshly opened flowers using a fine tip forceps
2. Put the pollinia in aluminum foil boats and place it in a glass bottle packed with charged silica gel and close it air tight. Keep the bottle at 24±2°C for 60-120 min
3. Collect the dried pollinia in 1ml cryovial, place on cane and submerge in LN
4. Hold in LN for a minimum of 1hr.
5. Warm in a water bath at 40°C for 2min
6. Use the pollinia as such for pollination
7. For in vitro germination assay, take dried and LN treated pollinia in cavity slides, add 2-3 drops of BK medium containing 0.5% sucrose and place in a humid chamber (modular incubator) for 12 hrs and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A desiccation period of 60min under charged silica gel is required to get sufficient dehydration to achieve about 80% in vitro germination after cryopreservation almost equal to corresponding desiccation control (79%). However, desiccation period beyond 60min up to 120min may also be tried to determine optimum period considering genotype, seasonal and regional differences.
Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops

Cryopreservation of *Dendrobium ovatum* pollinia through vitrification

*Dendrobium ovatum* is an epiphytic orchid having two pollinia with pollen tetrads compactly packed surrounded by a viscous fluid. The pollen tetrads will easily disperse through gentle tapping. The pollen easily germinates and pollen tube grows in BK medium containing 0.5-5% sucrose.

**Requirements**

1. Tools (needles, forceps, fine tweezers)
2. Sterile Petri plates
3. Cavity glass slides
4. BK medium containing 0.5% sucrose
5. Sterile filtration apparatus fitted with nitrocellulose membrane
6. Loading solution
7. PVS2 solution
8. Washing solution
9. Modular incubator as humid chamber
10. Cryovials (2ml)
11. Water bath
12. Inverted Microscope
13. LN dewar and liquid nitrogen
14. Lactophenol blue

**Prepare in advance**

1. Sterilized BK medium containing 0.5% sucrose
2. 0.02% methyl blue in lacto-phenol (20ml lactic acid: Phenol 20ml: Glycerol 40ml Distilled water 20ml: 1% cotton blue 2ml)
3. Loading solution: BK medium containing 0.4M Sucrose and 2M Glycerol
4. PVS2: BK medium containing; v/v glycerol 30%, ethylene glycol 15%, DMSO 15% and 0.4M sucrose.
5. Washing solution: BK medium containing 1.2M sucrose
6. Harvest freshly opened flowers
7. Assess germination capability in BK medium containing 0.5% sucrose
The Procedure

1. Isolate pollinia from freshly opened flowers using a fine tip forceps
2. Put the pollinia in 1ml loading solution taken in 2ml cryovial and keep at room temperature (24±2°C) for 15 min. Do the experiment in 2 batches one for control and another for LN exposure.
3. Transfer the pollinia into 1ml chilled PVS2 taken in 2ml cryovial and keep for 5-10 min.
4. Place the batch for LN treatment in cane and submerge in LN. Held the cane in LN at least for an hour.
5. Pull out cane without any disturbance and immediately put into a water bath maintained at 40°C and held there 2min for warming.
6. Wash the batch of control sample immediately after PVS2 treatment and LN treated sample after rewarming in washing solution. For this add 1ml of washing solution into the vials and remove 1ml from the vial and keep for 2min. Repeat the process five times with out any waiting period. Finally pick the pollinia softly using forceps and transfer to a piece of sterile filter paper to drain off the washing solution.
7. Use the drained pollinia as such for pollination
8. For in vitro germination assay, take PVS2 treated and LN treated pollinia in cavity slides, add 2-3 drops of BK medium containing 0.5% sucrose and place in a humid chamber (modular incubator) for 12 hrs and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A five-minute PVS2 treatment is sufficient to achieve 78% in vitro germination after cryopreservation almost equal to corresponding desiccation control (81%). However, up to 10 min exposure may give satisfactory results and thus may be tried to determine optimum period considering genotype, seasonal and regional differences.
Cryopreservation of *Luisia macrantha* pollinia through vitrification

*Luisia macrantha* is an epiphytic orchid having two pollinia with pollen tetrads compactly packed surrounded by a viscous fluid. The pollen easily germinates and pollen tube grows in BK medium containing 0.5-5% sucrose.

**Requirements**

1. Tools (needles, forceps, fine tweezers)
2. Sterile Petri plates
3. Cavity glass slides
4. BK medium containing 0.5% sucrose
5. Sterile filtration apparatus fitted with nitrocellulose membrane
6. Loading solution
7. PVS2 solution
8. Washing solution
9. Modular incubator as humid chamber
10. Cryovials (2ml)
11. Water bath
12. Inverted Microscope
13. LN dewar and liquid nitrogen
14. Lactophenol blue

**Prepare/ in advance**

1. Sterilized BK medium containing 0.5% sucrose
2. 0.02% methyl blue in lacto-phenol (20ml lactic acid: Phenol 20ml: Glycerol 40ml Distilled water 20ml: 1% cotton blue 2ml)
3. Loading solution: BK medium containing 0.4M Sucrose and 2M Glycerol
4. PVS2: BK medium containing; v/v glycerol 30%, ethylene glycol 15%, DMSO 15% and 0.4M sucrose.
5. Washing solution: BK medium containing 1.2M sucrose
6. Harvest freshly opened flowers
7. Assess germination capability in BK medium containing 0.5% sucrose
The Procedure

1. Isolate pollinia from freshly opened flowers using a fine tip forceps
2. Put the pollinia in 1ml loading solution taken in 2ml cryovial and keep at room temperature (24±2°C) for 15 min. Do the experiment in 2 batches one for control and another for LN exposure.
3. Transfer the pollinia into 1ml chilled PVS2 taken in 2ml cryovial and keep for 5-20 min.
4. Place the batch for LN treatment in cane and submerge in LN. Held the cane in LN at least for an hour.
5. Pull out cane without any disturbance and immediately put into a water bath maintained at 40°C and held there 2min for warming.
6. Wash the batch of control sample immediately after PVS2 treatment and LN treated sample after rewarming in washing solution. For this add 1ml of washing solution into the vials and remove 1ml from the vial and keep for 2min. Repeat the process five times without any waiting period. Finally pick the pollinia softly using forceps and transfer on to a piece of sterile filter paper to drain off the washing solution.
7. Use the drained pollinia as such for pollination
8. For in vitro germination assay, take PVS2 treated and LN treated pollinia in cavity slides, add 2-3 drops of BK medium containing 0.5% sucrose and place in a humid chamber (modular incubator) for 12 hrs and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A ten minute PVS2 treatment is required to achieve 62% in vitro germination after cryopreservation comparable to corresponding desiccation control (67%). However, 5-15 min exposure may give satisfactory results and thus may be tried to determine optimum period considering genotype, seasonal and regional differences.
Cryopreservation of *Rhyncostylis retusa* pollinia through vitrification

*Rhyncostylis retusa* is an epiphytic orchid having two pollinia with pollen tetrads compactly packed surrounded by a viscous fluid. The pollen easily germinates and pollen tube grows in BK medium containing 0.5-5% sucrose.

**Requirements**

1. Tools (needles, forceps, fine tweezers)
2. Sterile Petri plates
3. Cavity glass slides
4. BK medium containing 0.5% sucrose
5. Sterile filtration apparatus fitted with nitrocellulose membrane
6. Loading solution
7. PVS2 solution
8. Washing solution
9. Modular incubator as humid chamber
10. Cryovials (2ml)
11. Water bath
12. Inverted Microscope
13. LN dewar and liquid nitrogen
14. Lactophenol blue

**Prepare in advance**

1. Sterilized BK medium containing 0.5% sucrose
2. 0.02% methyl blue in lacto-phenol (20ml lactic acid: Phenol 20ml: Glycerol 40ml Distilled water 20ml: 1% cotton blue 2ml)
3. Loading solution: BK medium containing 0.4M Sucrose and 2M Glycerol
4. PVS2: BK medium containing; v/v glycerol 30%, ethylene glycol 15%, DMSO 15% and 0.4M sucrose.
5. Washing solution: BK medium containing 1.2M sucrose
6. Harvest freshly opened flowers
7. Assess germination capability in BK medium containing 0.5% sucrose
The Procedure

1. Isolate pollinia from freshly opened flowers using a fine tip forceps
2. Put the pollinia in 1ml loading solution taken in 2ml cryovial and keep at room temperature (24±2°C) for 15 min. Do the experiment in 2 batches one for control and another for LN exposure.
3. Transfer the pollinia into 1ml chilled PVS2 taken in 2ml cryovial and keep for 10-15 min.
4. Place the batch for LN treatment in cane and submerge in LN. Held the cane in LN at least for an hour.
5. Pull out cane without any disturbance and immediately put into a water bath maintained at 40°C and held there 2min for warming.
6. Wash the batch of control sample immediately after PVS2 treatment and LN treated sample after rewarming in washing solution. For this add 1ml of washing solution into the vials and remove 1ml from the vial and keep for 2min. Repeat the process five times with out any waiting period. Finally pick the pollinia softly using forceps and transfer to a piece of sterile filter paper to drain off the washing solution.
7. Use the drained pollinia as such for pollination
8. For in vitro germination assay, take PVS2 treated and LN treated pollinia in cavity slides, add 2-3 drops of BK medium containing 0.5% sucrose and place in a humid chamber (modular incubator) for 12 hrs and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A 20 minute PVS2 treatment is required to achieve 82% in vitro germination after cryopreservation almost equal to corresponding desiccation control (84%). However, 10-20 min exposure may give satisfactory results and thus may be tried to determine optimum period considering genotype, seasonal and regional differences.
Cryopreservation of Solanaceous Species (Tomato, Eggplant and Bell Pepper)

The procedures followed for Solanaceous species especially tomato, eggplant and bell pepper are almost similar, due to the kind of flower bearing habit.

Pollen collection and processing for cryopreservation

1. Pollen extraction was accomplished by carefully clipping the anthers from healthy flowers at the time of dehiscence
2. Place flowers in clean petri-dishes in a desiccator containing activated silica gel under ambient conditions.
3. The anthers dehisce after a duration of 30-45 minutes, releasing pollen
4. Tap gently over a clean butter paper
5. Transfer collected pollen to gelatin capsules
6. Enclose gelatin capsules in small laminated pouches and seal airtight using a mechanical sealer
7. Capsules are sealed airtight, using a heat sealer and then stacked at the bottom of the canisters and lowered gradually into a liquid nitrogen cryoflask.
9. The canisters are capped with perforated lids, to avoid samples from floating out into the cryoflask while refilling.

Alternate method:

1. The flowers just opened were brought to be brought to the laboratory
2. keep the flowers in the BOD incubator attached with fluorescent light at 25°C for dehiscing for one hour.
3. Thereafter the style was taken out using a small forceps and the anther cone was cut at the end using a small scissors and the flower was held upside down using the small forceps and the pollen was tapped out to a butter paper.
4. pollen sample to be bulked and transferred to a gelatin capsule and that in turn put it in a laminated aluminum pouch and sealed air tightly using a mechanical sealer
Viability assessment:

1. Tomato pollen is germinated by hanging drop technique as described earlier in 20 percent sucrose supplemented with Brewbaker’s salts.
2. Eggplant pollen is germinated in vitro by the improved cellophane method (Alexander and Ganeshan, 1989). *(Eggplant pollen fails to germinate in hanging drops, since pollen sinks to the bottom of the drop).*
3. The medium consisted of a carbohydrate source (15 per cent sucrose) supplemented with 300 ppm of Calcium nitrate, 200 ppm of Magnesium sulphate, 100 ppm of Potassium nitrate and 100 ppm of Boric acid.
4. The preparations were incubated at 20± 2°C for a duration of 4-6 hours, after which staining was accomplished using the versatile stain (Alexander, 1980).
5. Pollen grains whose tube lengths were greater than the grain diameter were considered as germinated. More than 300 pollen grains per replicate were scored, using a binocular microscope and percentage germination was calculated.

Retrieval and post-storage fertility assessment

Pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.

1. Flowers were emasculated a day prior to pollination and covered with butter paper bags, in order to avoid contamination.
2. Cryostored pollen was applied on the receptive stigma the next day, and then immediately covered.
3. For controls, stigma of emasculated flowers was applied with freshly collected pollen. Fruit and seed set were recorded with crosses made using fresh and cryostored pollen.
4. At least 5 effective pollinations, using cryostored pollen were accomplished for each set of experiment.
5. Fruit and seed set data was generated from each cross.
Cryopreservation of *Allium cepa* Pollen

(Ganeshan, 1986)

**Pollen collection and processing for cryopreservation**

1. Plants of *Allium cepa* L. cv. ‘Arka Niketan’ were grown in insect-proof cages under field conditions and pollen to be
2. Pollen collected from umbels in partial to full bloom.
3. Umbels were gently tapped on clean petri dishes between 1100 and 1300 hours.
4. Pure pollen samples collected from several umbels were thoroughly mixed before refilling into gelatin capsules (25 mg in each capsule)
5. Capsule were individually packed in laminated aluminum pouches.
6. Pollen, kept in such space economizing containers was maintained at a constant temperature of -196°C in liquid nitrogen with periodic refilling of the cryogen.
7. Prior to long-term storage, pollen samples were initially indexed for germination *in vitro*.
8. Cryopreservation of pollen samples was accomplished in a Mach-SM-33 cryobiological system (MVE, USA) by direct immersion in liquid nitrogen after pre-cooling the culture tubes at –20°C in a deep-freeze for two hours.
9. Complete immersion of all pollen samples in liquid nitrogen throughout storage (except when removed for testing) was ensured by placing culture tubes in canisters capped with perforated lids and lowered into the cryoflask.

**Viability assessment**

1. Viability of pollen was assessed in terms of germinability by in vitro and fertility by controlled pollinations.
2. Fresh, non-frozen (control) and frozen pollen samples were germinated in
3. 20% sucrose medium (prepared in double glass-distilled water) using a hanging drop technique.
4. Capsules with pollen were removed from liquid nitrogen and brought to ambient temperature and samples drawn for viability tests *in vitro* after cryopreservation.
5. Pollen was held for 30 min at ambient temperature before it was dusted on the medium,
6. Incubated in a humid chamber at 25°C +2°C for six hours.
7. Cultures were stained with a drop of Alexander’s stain (Alexander, 1980)
8. Slides were scored for germination using a projection microscope.
9. Pollen whose tube lengths measured more than the grain diameter were considered viable. More than 500 grains were scored for each replication.

**Fertility assessment**

Requirements: Pollination kit: Petri dishes, butter paper, forceps, polythene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.

1. Controlled pollinations with pollen cryopreserved to be carried out on a male sterile line in the temperature, 50-60% relative humidity conditions.
2. Potted plants were used in the latter two environments.
3. After thawing frozen samples
4. At ambient temperature and holding for 30 min.
5. Pollen was applied to the receptive stigmas of freshly opened male sterile flowers.
   Fresh ‘Selection-13’ pollen was used for pollinating the flowers of two CMS1 umbels for comparison at both dates.
6. Unused pollen samples were returned to liquid nitrogen after the required number of pollinations was carried out.
7. The pollinated umbels were bagged separately to avoid insect pollination. Pollinations were made on alternate days, during peak flowering under each environment using one sample of frozen pollen withdrawn from storage.
8. Fruit and seed set to be recorded for all the umbels.

**Some theoretical and practical considerations of cryogenic preservation of pollen**

1. Techniques of cryogenic storage are new to many tropical fruit tree species, of which the success rate is very high for bi-cellular pollen material, often collected from tropical regions.
2. Post- cryogenic storage survival of pollen, eliciting a high viability response can be used as a model to study the moisture kinetics involved in other plant tissues.
3. The cryogen used i.e., liquid nitrogen though inert, is hazardous by way of causing frost injuries with risk for the personnel using and those who work in the surrounding area. Training in cryogenic operations, handling procedures, safety precautions, etc. are essential.
4. Places with cryoflasks filled with nitrogen should minimize the risk of gaseous nitrogen build-up. Small volumes of liquid on expansion at ambient temperature, converts into large volumes of gaseous nitrogen, reducing the local oxygen content. This can cause drowsiness, and in extreme cases, asphyxiation.

5. Proper handling equipment, such as cryogloves, long forceps or tongs, face guards if necessary while working for long duration, are essential. It is generally advised not to use glass vials for cryogenic storage, as they can explode due to seepage of liquid nitrogen into the vial during storage.

6. Liquid nitrogen source as far as possible must be located in proximity and should be easily accessible.

**Cryogenic containers**

1. Commercially available storage containers with a long static liquid nitrogen holding time and with low static evaporation rates are desirable. The canisters designed to hold vials, pouches, etc. in liquid and vapour phase are to be used.

2. It is of cardinal importance to ensure transfers or retrievals in and out of the canisters as quickly as possible, so that samples held in storage at -196°C are not allowed to warm up significantly as a result of being relocated temporarily.

3. The liquid nitrogen level in storage cryocans will have to be monitored regularly (at least once every week and more frequently if the containers are often opened within this period). Accordingly, the cryogen level has to be replenished to levels recommended by the manufacturer to achieve the required temperature. Keeping the containers at room temperature controlled between 5 - 10°C reduces loss of the cryogen to a great extent, thus extending the time between replenishment of the cryogen.
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### Brainstorming meeting and training cum demonstration on Cryopreservation and in vitro Conservation in Horticultural Crops

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A Brief Note on Brainstorming Meeting and Training cum Demonstration on Cryopreservation and *in vitro* Conservation in Horticultural Crops held at IIHR, Bangalore from 21\textsuperscript{st} to 22\textsuperscript{nd} February 2014

The Society for Promotion of Horticulture Bangalore in collaboration with Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru and Bioversity International organized a Brainstorming Meeting and Training cum Demonstration on Cryopreservation and *in vitro* conservation in Horticultural Crop Genetic Resources on 21\textsuperscript{st} and 22\textsuperscript{nd} February 2014. The main objective of the program was to assess use of cryopreservation and tissue culture techniques for conservation and management of PGR in horticulture crops, enhance the use of in vitro conservation and cryopreservation protocols for horticulture crop genetic resources and equip participants with essential knowledge necessary for developing and using in vitro & cryopreservation techniques. The Brainstorm meeting primarily focussed on presentations by experts in the field, followed by discussions and interactions. The meeting was followed by training cum demonstration which included in depth analysis of basic research in the plant cryopreservation and in vitro conservation already developed by IIHR and other ICAR institutions under Horticulture SMD as well as reports on state-of-the-art methods, research and successes achieved with selected horticultural crops.

The inaugural session of the meeting was held in the IIHR Auditorium. Dr. S. Ganeshan, Organizing Secretary of the Meeting (Head & Principal scientist, Division of PGR, IIHR.) welcomed the Delegates, Dignitaries and Participants and gave a brief overview of two day program. Dr. C.K. Narayana, Director, IIHR delivered the opening remarks citing the importance of conservation of PGR in light of its erosion due to various kinds of human activities and
natural causes. Dr. V.A. Parthasarathy, National Project Coordinator, UNEP-GEF Programme, Bioversity International reflected on the significance and importance of cryopreservation. He emphasized the need to use state of the art technologies for genetic resources conservation.

Dr N. K. Krishna Kumar, Patron SPH & DDG (Hort.), ICAR stressed upon the need to train human resources and use cryopreservation technology in horticultural crops, which would usher in higher levels of genetic resource availability to breeders thereby paving way for development and release of new varieties and hybrids. Referring to the Coorg Honey Dew papaya variety, he said that the pollen collections made during the 80s at IIHR are still available in the Cryobank for use by breeders.

The Chief Guest Padmashree Dr. K. L. Chadha (Formerly DDG-Hort.) ICAR, released the “Compendium on cryopreservation and in vitro conservation in Horticultural crops”. In his inaugural address, he traced the historical perspective as to how the program on cryopreservation of pollen was conceived at IIHR through an FAO-UNDP project in the early 80s under his guidance as Director, IIHR.

Dr. K. Madhavi Reddy, Secretary, SPH and Principal Scientist, Division of Vegetable crops, IIHR, proposed vote of thanks, on behalf of the organization Committee.

There were two technical sessions, followed by a plenary session. In all there were 9 resource persons who were drawn from various ICAR institutes having expertise on cryopreservation & in vitro conservation. A total number of 39 scientist delegates representing different ICAR institutes under the horticulture division participated. The first technical session comprised of 4 presentations, which included an overview of Cryobanking activities going on at NBPGR, New Delhi. Cryopreservation research at JNTBGRI, Palode, IISR, Calicut and CPCRI, Kasargode. The second technical session comprised of 5 presentations, which reviewed cryopreservation & in-vitro conservation research in potato and banana followed by accomplishments made by the host institute (IIHR) over the last 3 decades.
The technical sessions were followed by plenary session which was chaired by Padmashree Dr. K.L. Chadha & Co-Chaired by Dr. N. K. Krishna Kumar, DDG (Hort.), ICAR and Dr. C.K. Narayana, Director, IIHR. The Chairmen of both the sessions summarized the presentations and the Rapporteurs presented a detailed report along with recommendations. Padmashree Dr. K.L. Chadha distributed participation certificates to all the participants.

For the benefit of the participants of the brainstorming meeting, a practical training-cum-demonstration session on pollen cryopreservation and in vitro conservation was conducted in the Division of Plant Genetic Resources. Participants were exposed to field gene bank activities for observing flowering and collection of pollen from field. The participants were also demonstrated as to how to extract pollen and perform the viability assessment using various methods. Hands-on training was given to the participants during the training. The participants were shown and explained how cryopreservation is carried out at IIHR. Protocols for testing the genetic fidelity in tissue culture conserved plants were also demonstrated. All programme ended with distribution of certificates to all the participants.

Participants in field learning pollen collection and back in the laboratory involved in germinating pollen