Chapter 17
Cryopreservation of Pollen

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17.1 Introduction

Cryopreservation of pollen is required for carrying out investigations in both fundamental and applied aspects of pollen biology. 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 bio-scientific areas. Crossing desirable genotypes involves multiple and staggered plantings in order to synchronize flowering. This can be avoided when cryopreserved viable pollen is available, facilitating hybrids between genera, species and genotypes. This could effectively conserve field and greenhouse space. The international transfer of germplasm in the form of dry pollen is not generally restricted (Hoekstra 1995). Moreover, this will eliminate the need to grow plant populations to produce pollen. Pollen is usually subjected to less stringent quarantine restrictions. The importance of pollen cryopreservation in horticultural species is well documented (Ganeshan and Rajasekharan 2000a; Kobayashi et al. 1978).

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*

17.2 Physiological Status

Manipulations of the physiological status of a given plant species, cryopreservation technique and recovery steps provide information that suggests some generalities in approaching the overall problem of cryopreservation of pollen. In most species the genotype effect, stage of pollen beyond complete maturity, physiological status of plant, cold treatment and the methodology followed are the major factors influencing post cryopreservation survival. In most tropical species, dry-mature pollen grains freshly dehisced from anthers are in ideal physiological condition to be processed for cryopreservation.

17.3 Protocols for Cryopreservation

The objective of a useful pollen cryopreservation protocol is to collect dry mature pollen from the desired species and conserve using methods which allow retention of its normal function; ultimately assessed by its ability to germinate *in vivo* and effect normal fertilization (Hanna and Towill 1995). Alexander and Ganeshan (1993) and Ganeshan and Rajasekharan (1995) reviewed the work on pollen storage in fruit crops and ornamental crops. Hoekstra (1995) assessed the merits and demerits of pollen as a genetic resource. Grout and Roberts (1995) detailed the methodology for pollen cryopreservation. Barnabas and Kovacs (1997) and Berthoud (1997) stressed the importance and need for pollen conservation. Ganeshan and Rajasekharan (2000b) reviewed the current status of pollen cryopreservation research and its relevance to tropical horticulture. Rajasekharan et al. (2003) developed a user-friendly database software 'Polbase' for digitizing accessions collected and maintained in a pollen cryobank. Generally, dry pollen, as collected, requires no pretreatment. Specific needs for candidate species will be discussed in this chapter.

17.3.1 Rewarming

The usual rewarming procedure, after a desired period of cryopreservation, is to place the sample at room temperature for 30 to 60 min, without the use of a water bath or any rewarming systems.

17.3.2 Viability Assessment

Pre-and post-cryopreservation viability assessment is usually followed for pollen through *in vitro* and *in vivo* assays. The common procedures include a staining method to differentiate aborted and non-aborted pollen (Alexander 1980), the hanging drop technique (Stanley and Linskens 1974), modified cellophane technique (Alexander and Ganeshan 1989), PEG technique (Shashikumar 2006), and in vivo staining pollen tubes in pistil (Alexander 1987), in addition to assessment of fertilizing ability in the field to determine the ability to produce normal fruit and seed set.

17.3.3 Theoretical and Practical Considerations

1. Techniques of cryogenic storage are new for many tropical fruit tree species. The success rate is very high for bi-cellular pollen material from tropical regions.
2. Training in cryogenic operations, handling procedures, safety precautions, etc. are essential. Proper handling equipment, such as cryogloves, long forceps or tongs, and face guards, is essential.
3. It is generally advised not to use glass vials for cryogenic storage, as they can explode due to seepage of liquid nitrogen (LN) into the vial during storage.
4. Commercially available storage containers with a long static LN holding time and with low static evaporation rates are desirable. The canisters designed to hold vials, pouches, etc. in liquid and vapor phase are to be used. Dewars should be topped after opening. The LN level in storage cryocans will have to be monitored and filled regularly depending on the type of Dewar used.
5. 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.

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17.4 Protocols

17.4.1 Cryopreservation of Mango Pollen (Mangifera indica)

(Rajasekharan and Ganeshan 1992, 2001–2002; 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). Mature anthers are collected in clean Petri dishes from healthy panicles from mature trees between 10 and 11 AM. The anthers are brought into the laboratory and transferred to empty gelatin capsules.

Procedure
1. Gelatin capsules containing 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 LN and stored for desired durations.
3. Canisters are capped with perforated lids to prevent the vials from floating out into the Dewar during refilling of LN.

Retrieval and post-storage fertility assessment
Anthers are warmed to ambient temperature, by initially holding the canister over LN vapor phase for 15 min, then pouches with anthers are removed from the canister and held at room temperature. Field pollinations are carried out or pollen germinated.

Mango pollen germination medium composition
1. Boric acid 100 ppm
2. Sucrose 15% w/v
3. Polyethylene glycol 10% w/v

17.4.2 Cryopreservation of Papaya Pollen (Carica papaya *and* Carica cauliflora)

(Ganeshan 1986a)

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 and 10 AM. Species-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 is assessed in terms of germinability *in vitro*. Pollen is germinated in hanging drops of medium (6% sucrose in distilled water) on depression slides at 25±2°C (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in germination medium.

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

Retrieval and post-storage fertility assessment
Anthers are warmed to ambient temperature by initially holding the canister over LN vapor phase for 15 min, then pouches with anthers are removed from the canister. Field pollinations are carried out or pollen is germinated on slides.

17.4.3 Cryopreservation Citrus Pollen (Citrus limon)

(Ganeshan and Alexander 1991; Ganeshan and Sulladmath 1983; Rajasekharan et al. 1995)

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.

Procedure

1. Pollen collections are usually made on a bright sunny day between 8 and10 AM.
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.

Precautions: 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 flowers depending on insect activity.
5. Do not 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 germination medium.

Germination medium

Prepared in deionised, double distilled water.
20% Sucrose
100 ppm H_3BO_3
300 ppm $Ca(NO_3)_2$ 4 H_2O
200 ppm Mg SO_4 $7H_2O$
100 ppm KNO_3
pH 7.3

Cryopreservation

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 into LN contained in the cryoflask.

Retrieval and post storage viability/fertility assessment

Pollen samples are held at ambient temperature for 10–15 min prior to a viability test or field pollination.

Pollen samples are viable if:

1. Germination *in vitro* is 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 60–80% of fresh pollen.

17.4.4 Cryopreservation of Grape Pollen (Vitis vinifera, *and* Vitis labrusca)

(Ganeshan 1985)

Pollen collection
The method of Olmo 1942.

1. Tap the 2nd or 3rd dehiscing inflorescences onto clean Petri dishes.
2. Blow out the floral debris and retain the yellow pollen mat.
3. Consolidate the collected pollen using a clean razor blade.
4. Pack the pollen into gelatin capsules or butter paper pouches.

Viability assessment
Pollen viability is tested by its ability to germinate in an artificial medium. Fresh pollen samples are cultured *in vitro* following hanging drop technique (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in germination medium. The germination medium consists of 20% sucrose solution in which pollen germinates profusely. Hanging drop cultures are incubated for 5 h at 25±2°C after which pollen growth is arrested by staining with a drop of Alexander's stain (1980). Pollen whose tube lengths are measuring more than the grain diameter are considered viable.

Cryopreservation
Storage at –196°C is accomplished by direct immersion in LN after precooling to –20°C.

Retrieval and post-storage fertility assessment
After warming pollen samples the vials are kept at ambient temperature for 30 min before taking to field. Fertility of pollen cryopreserved in LN is tested by controlled field pollinations on established male-sterile lines.

17.4.5 *Cryopreservation of* Gladiolus *Pollen*

(Rajasekharan et al. 1994)

Pollen collection and processing for cryostorage

1. The flowers are 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 are harvested and brought to the laboratory. Petals are carefully separated and pollen grains are extracted by scraping the mature anthers which are about to dehisce, with a blunt needle, passing traversely along the lobe of the anther.
3. Bulked pollen samples are transferred to empty gelatin capsules, packed in laminated poly aluminum pouches, sealed airtight.
4. Lower gradually into canisters of a LN cryobiological system.

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

Viability assessment

Germinate pollen on cellophane strips soaked in pollen germination medium (Rajasekharan et al. 1994). Field pollinations may be used to confirm viability.

Pollen germination medium composition

15% Sucrose
300 mg1^{-1} Ca $(NO3)_2$ 4 H_2O
200 mg 1^{-1} Mg SO_4 $7H_2O$
100 mg 1^{-1} KNO_3
100 mg 1^{-1} Boric acid

17.4.6 Cryopreservation of Rose Pollen

(Rajasekharan and Ganeshan 1994)

Pollen collection and processing for storage

1. Pollen of previously bagged flowers (which are due to open the following day) is collected on a bright sunny day.
2. On the day of collection bags are removed between 10 and 11 AM and the blooming flowers are brought into the laboratory.
3. After separating the petals, pollen grains are collected by gently tapping the flower hips onto butter paper.
4. Collections from flowers of a given cultivar are bulked before assessing viability *in vitro*.

Viability assessment

Assess viability by germination of pollen in a hanging drop culture. Field pollinations can also be used to assess viability.

Germination medium

15% Sucrose
150 g l^{-1} $Ca(NO_3)_2$ $4H_2O$
200 mg1^{-1} $MgS0_4$ $7H_2O$
100 mg 1^{-1} KNO_3
100 mg l^{-1} H_3Bo_3

Cryopreservation

1. The pollen samples carefully transferred into gelatin capsules enclosed in a laminated aluminum pouch.
2. The pouches with the capsule are sealed air tight and stacked in the canisters of a cryobiological storage system, and lowered gradually into LN.

Retrieval and post-storage fertility assessment

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

17.4.7 Cryopreservation of Asclepiadaceae Pollinia—General procedures

(Shashikumar 2006)

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

Procedure for extraction and processing asclepiadaceous pollinium for cryostorage

1. Collect coronary corolla of asclepiadaceous members at 10–11 AM.
2. Dissect the coronary corolla using fine needles. Pullout corposculum of each pollinium using fine-pointed forceps.
3. Collect the pollinia on a clean butter paper.
4. Immediately transfer pollinia to gelatin capsules.
5. Place the gelatin capsules inside aluminum pouches and seal.
6. Label the pouches with inventory information.
7. Place the pouches inside the canisters and close the lid.
8. Immerse the canisters slowly into the LN containers.

Retrieval of pollinia from cryogenic containers

1. Gradually lift canisters from the cryogenic containers.
2. Retrieve the aluminum pouches and allow the pouch to warm to room temperature.

Viability assessment

1. Viability in pollinia is assessed using the hanging drop method (Shivanna and Rangaswamy 1993) with 4–5 pollinia in a drop of germination medium. Boric acid 100 ppm, sucrose 20%, pH 7.0
2. Incubate the slides in a humidity chamber (RH-90%) for 4–5 h.

Qualitative and quantitative estimation of germinated pollinia extracted from asclepiadaceous members

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

1. Transfer germinated pollinia into a 5 ml test tube containing 1–2 ml of pollen germination medium.
2. Agitate test tube using a single tube mixer ('Rotex' or 'Vortex') for about 5–10 min. (This process loosens the compact mass of germinated pollen, further rupturing the pollinial wall).
3. Transfer the mixture onto 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.)

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 problems for counting.

17.4.8 Cryopreservation of Tropical Orchids

(Anonymous 2006)

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 grains are unicellular and held together by a viscous fluid. Sometimes the pollinial tetrads are organized into many granular packets, prolongations of which form the caudicle as in *Pecteilis, Habenaria, Satyrium*. Especially in those cases the pollen are more tightly packed without much surrounding fluid. Even though information on the nature of pollen is scanty, it is known that both binucleate and trinucleate are present among orchids.

Pretreatment

Protocols for pollinia cryopreservation of orchids with pollen with surrounding viscous fluid packed in pollinia are described below. A short treatment in vitrification solution formulated by Sakai et al. (1990) is preferred for cryopreservation. The treatment includes exposure to an osmotic loading solution containing 2 M glycerol and 0.4 M sucrose prepared in BK medium (Brewbaker and Kwak 1963) for 15 min and PVS2 solution containing 30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose in BK medium for 5–20 min. Simple desiccation under laminar airflow is also effective in some species.

Cryopreservation

Successful cryopreservation of pollinia typically uses direct immersion of a vial (1–2 ml) containing the PVS2-treated pollinia into LN. For

experimental purposes, overnight exposure in LN is more than sufficient to assess successful vitrification and LN tolerance of treated pollinia. Dried pollinia are also cryopreserved through vitrification.

Rewarming

To avoid de-vitrification the LN-treated pollinia need to be rewarmed rapidly. The vials are removed from LN and dropped into a 40°C water bath. The rewarmed pollinia are washed in a solution of 1.2 M sucrose prepared in BK medium. Pollinia cryopreserved through simple drying 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 on a cavity slide, adding one to two drops of diluted fluorescein diacetate solution and observing under a fluorescent microscope after a few minutes. Germination is determined by plating samples of rewarmed and washed pollinia in two–three drops of BK medium containing 0.5–5% sucrose onto cavity slides and incubating at 24±2°C for 12–24 h.

Protocols for specific species

Cymbidium bicolor pollinia simple drying

C. bicolor is an epiphytic orchid with two pollinia that has pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen are binucleate and easily germinate in BK medium (Brewbaker and Kwak 1963) with 0.5–5% sucrose.

Prepare in advance

1. Sterilized BK medium containing 0.5% sucrose.
2. 0.02% methyl blue in lacto-phenol: lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Harvest freshly opened flowers
4. Assess germination capability in BK medium with 0.5% sucrose

The procedure

1. Isolate pollinia from flowers using a fine tip forceps.
2. Put the pollinia in a sterile Petri dish and place it open in a laminar airflow cabinet for 30–75 min.
3. Collect the dried pollinia in 1 ml cryovial, place on cane and submerge in LN.
4. Warm in a water bath at 40°C for 2 min.
5. For an *in vitro* germination assay, place dried and LN treated pollinia in cavity slides, add two–three drops of BK medium containing 0.5% sucrose and place in a humid chamber for 12 h. 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 germination percentage assessed.

A 75 min desiccation under laminar airflow is required to achieve about 76% *in vitro* germination after cryopreservation (desiccation control was 87%). However, 30–60 min desiccation may also be tried to determine optimum period, considering genotype, seasonal and regional differences.

Arundina bambusifolia pollinia silica gel drying

Arundina bambusifolia is a terrestrial orchid with two pollinia with unicellular pollen compactly packed and surrounded by a viscous fluid. It easily disperses in liquid media. It is more convenient to use a drying protocol rather than vitrification. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium containing 0.5–5% sucrose.

Prepare in advance

1. Germination: sterilized BK medium containing 0.5% sucrose
2. Methyl blue 0.02% in lacto-phenol: lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Harvest freshly opened flowers

The procedure

1. Isolate pollinia from flowers using a fine tip forceps.
2. Put the pollinia in aluminum foil boats and place 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 1 ml cryovial. Place on cane and submerge in LN.
4. Warm in a 40°C water bath for 2 min.
5. For *in vitro* germination assay, place dried and LN treated pollinia in cavity slides, add two–three drops of BK medium containing 0.5% sucrose and place in a humid chamber for 12 h 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 60 min desiccation with charged silica gel is required to get sufficient dehydration to achieve about 80% *in vitro* germination after cryopreservation (desiccation control is 79%). However, a 60–120 min desiccation may also be tried to determine the optimum period, considering genotypic, seasonal and regional differences.

Dendrobium ovatum pollinia vitrification

Dendrobium ovatum is an epiphytic orchid with two pollinia that have pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen tetrads will easily disperse through gentle tapping. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium with 0.5–5% sucrose.

Prepare in advance

1. Germination: sterilized BK medium containing 0.5% sucrose
2. Methyl blue (0.02% in lacto-phenol): lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Osmotic loading solution: BK medium containing 0.4 M sucrose and 2 M glycerol
4. PVS2: BK medium with (w/v) glycerol 30%, ethylene glycol 15%, DMSO 15% and 0.4 M sucrose
5. Rinsing solution: BK medium containing 1.2 M sucrose
6. Harvest freshly opened flowers

The procedure

1. Isolate pollinia from freshly opened flowers.
2. Put the pollinia in 1 ml loading solution in a 2 ml cryovial and kept at room temperature (24±2°C) for 15 min. Do the experiment in two vials, one for control and another for LN exposure.
3. Transfer the pollinia into 1 ml-chilled PVS2 in a 2 ml cryovial and keep for 5–10 min.
4. Place the tube for LN treatment on a cane and submerge in LN.
5. To warm, plunge into a 40°C water bath for 2 min.
6. Rinse the control samples in rinsing solution immediately after PVS2 treatment and rinse the LN-treated samples after rewarming. Remove 1 ml from the vial and add 1 ml of rinsing solution and hold for 2 min. Repeat five times without waiting.
7. Transfer pollinia to a piece of sterile filter paper to drain.
8. For *in vitro* germination: place pollinia in cavity slides and add two–three drops of germination medium. Place in a humid chamber for 12 h. Pollinia may be stained in a drop of lacto-phenol blue and tapped for dispersing pollen.
9. A 5 min PVS2 treatment results in 78% *in vitro* germination that is similar to the control (81%). Up to 10 min, PVS2 exposure may give satisfactory results, considering genotype, seasonal and regional differences.

Luisia macrantha pollinia vitrification

Luisia macrantha is an epiphytic orchid with two pollinia with pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium with 0.5–5% sucrose.

Use the same protocol as for *Dendrobium ovatum*

A 10 min PVS2 treatment is required to achieve 62% *in vitro* germination comparable to the PVS2 control (67%). However, 5–15 min exposure may give satisfactory results and thus may be tried to determine optimum period, considering the genotype, seasonal and regional differences.

Rhyncostylis retusa pollinia vitrification

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

Use the same protocol as for *Dendrobium ovatum*

A 20 min PVS2 treatment is required to achieve 82% *in vitro* germination after cryopreservation almost equal to the corresponding PVS2 controls (84%). However, 10–20 min exposure may give satisfactory results considering genotype, seasonal and regional differences.

17.4.9 Cryopreservation of Solanaceous Species

(Rajasekharan et al. 1998; Rajasekharan and Ganeshan 2003)

The procedures followed for solanaceous species, especially tomato, eggplant and bell pepper, are similar due the flower-bearing habit.

Pollen collection and processing for cryopreservation

1. Pollen extraction: carefully clip 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 30–45 min, releasing pollen.
4. Tap gently over a clean butter/waxed paper.
5. Transfer collected pollen to gelatin capsules.
6. Enclose gelatin capsules in small laminated pouches and seal.
7. Lower canisters into a LN cryoflask.

Alternative method

1. The just opened flowers are brought to the laboratory.
2. Keep flowers in an incubator with light at 25°C for 1 h.
3. Remove the style and cut the anther cone at the end using a small scissors. Hold the flower upside down and tap out the pollen.
4. Bulk the pollen sample and transfer to a gelatin capsule and seal in a laminated aluminum pouch.

Viability assessment

1. Tomato pollen is germinated by the hanging drop technique (Shivanna and Rangaswamy 1993) in BK medium with 20% sucrose.
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).*

   Germination medium

   15% Sucrose
   300 g l^{-1} $Ca(NO_3)_2$ $4H_2O$
   200 mg1^{-1} $MgSO_4$ $7H_2O$
   100 mg 1^{-1} KNO_3
   100 mg l^{-1} H_3BO_3
3. Incubate at 25±2°C for 4–6 h and stain (Alexander 1980).
4. Field pollination may be used to determine viability.

17.4.10 Cryopreservation of Allium cepa *Pollen*

(Ganeshan 1986b)

Pollen collection and processing for cryopreservation

1. Plants of *Allium cepa* are grown in insect-proof cages under field conditions. Pollen is collected from umbels in partial to full bloom.
2. Umbels are gently tapped on clean Petri dishes between 11AM and 1 PM. Pure pollen samples collected from several umbels are thoroughly mixed before filling gelatin capsules (25 mg in each capsule).
3. Capsules are individually packed in laminated aluminum pouches and sealed.
4. Cryopreservation of pollen samples was accomplished by direct immersion in LN after precooling the culture tubes at –20°C for 2 h.

Viability assessment

1. Viability of pollen was assessed in terms of germinability *in vitro* and fertility by controlled pollinations.
2. Fresh, non-frozen (control) and frozen pollen samples are germinated in 20% sucrose medium (prepared in double glass-distilled water) using a hanging drop technique (Shivanna and Rangaswamy 1993).
3. Capsules with pollen are removed from LN and brought to ambient temperature and samples drawn for viability tests *in vitro*.
4. Pollen is held for 30 min at ambient temperature before it is dusted on the medium and incubated in a humid chamber at 25°C ± 2°C for 6 h. Cultures are stained with a drop of Alexander's stain (Alexander 1980).

Fertility assessment

Controlled pollinations with cryopreserved pollen may be carried out on a male sterile line in 50–60% relative humidity conditions or under greenhouse conditions. Warm frozen samples at ambient temperature and hold for 30 min before pollinating.