In Vitro Conservation and Cryopreservation

Sandhya Gupta, Anang Pal and Ramesh Chandra
Tissue Culture and Cryopreservation Unit, ICAR-NBPGR, New Delhi

In vitro conservation is the method used mainly for conserving vegetatively propagated crops. The conventional method for conserving such germplasm is in the field genebanks, where the germplasm is under the threat of loss due to the attack of pest and pathogen or due to natural disaster. Therefore, In vitro conservation is important to have a back up of important genetic resources under in vitro genbank or cryo-genebank. Cryopreservation is the only available method for long-term conservation of germplasm may it be in-vitro grown shoot apices, vegetative propagules, or explants from non-orthodox seeds and embryonic axes.

Methods of In Vitro Conservation

Normal Growth
The objective of this method is to maintain germplasm cultures under normal growing conditions. Though it involves frequent subculturing which is laborious. However, the method is advantageous as the cultures are available for immediate multiplication and distribution and it avoids requirement of low temperature facility or the application of stresses.

Plant Material
In vitro cultures of mulberry (*Morus* spp.)

Procedure:
- Cut in vitro grown-shoots into small segments bearing single node each.
- Transfer nodal segments onto MS medium with low BAP in the glass test tubes (1 segment per tube).
- Incubate all the cultures at culture room.

Slow Growth
Slow growth method has largely been used for various vegetatively propagated crops. The objective of slow growth method of in vitro conservation is to reduce proliferation/ and multiplication, thus consequently, reducing the growth of cultures so as to increase the shelf-life of cultures. Further, the period between subcultures increases while the genetic stability of germplasm can be ensured. There are several methods like low temperature incubation, various enclosure types, modified media etc., are being used for slow growth in vitro conservation.

Procedure:
- Transfer the explants onto MS medium in the glass test tubes
- Close the tubes with either cotton plug or polypropylene caps to study effect of different enclosures. Incubate all the cultures at culture room.
- For low temperature effect, store the cultures at appropriate low temperature for example temperate crops at 4°C, tropical crops 15-20 °C.
- To study effect of modified media on culture growth various growth retardants (abscisic acid 5-10 mg/l or dimethylamino succinamide 50 mg/l or maleic hydrazide 10 mg/l) or osmotica (sucrose or mannitol) are included in the media.
- Periodically observe and assess survival of cultures and growth including the ability of cultures to resume growth on fresh medium.

Cryopreservation of shoot tips

Cryopreservation of shoot tips/ meristem is being used for long-term storage of various vagetatively propagated germplasm. Several techniques such as encapsulation-dehydration, vitrification, encapsulation vitrification, droplet vitrification, V cryoplate and D cryoplate are being employed for cryopreservation of shoot apices. Although, both apical and axillray shoot tips can be used for cryopreservation, actively growing apical shoot tips usually respond better.

I. Encapsulation-Dehydration Technique-Protocol

Plant material
In vitro cultures of blackberry (*Rubus* spp.)

Stock Cultures
Multiply micropropagated plantlets of *Rubus* species on medium containing MS mineral salts and vitamins with double EDTA-Fe, 2 mg/l N6 benzyladenine (BA), 0.1 mg/l indole 3 butyric acid (IBA), 0.1 mg/l GA3 3.5 g/l agar, 1.45 g/l gelrite and 30 g/l sucrose at pH 5.7 in test tubes. Grown the cultures at 25ºC with a 16 h light/8 h dark photoperiod.

Cold acclimation
Cold acclimatize 3 weeks old cultures at 22ºC with 8 h light (10 µE.m-2.s-1)/-1ºC 16 h dark) for 4 weeks.
Shoot tip Excision
Excise shoot tips with 2 to 3 pairs of leaf primordial (about 1 mm long) from cold acclimated shoots.

Encapsulation-dehydration procedure
Encapsulation-Encapsulated excised shoot tips in alginate beads composed of 3% (w/v) low viscosity alginic acid in liquid MS medium without calcium, pH 5.7 and polymerize them for 20 min in MS medium with 100 mM CaCl2 and 0.4 M sucrose.
Osmotic Dehydration-Pretreat beads in liquid MS medium with 0.75 M sucrose for 20 h on a rotary shaker (50 rpm).
Air Dehydration-Blot dry the beads on sterile filter paper and desiccate for 6 h [approx. 20% moisture content] in a glass Petri dish under laminar flow at ambient temperature of ~25ºC and at 35 ± 2 % relative humidity (%RH).
Freezing-Place the dried beads in 1.2 ml cryovials (10 beads/cryovial) and plunge directly into liquid nitrogen (LN).
Thawing-The vials were rewarmed in 45ºC water for 1 min and in 25ºC water for 2 min.
Regrwoth-Transfer the beads onto recovery medium (multiplication medium with no IBA) in 24-cell plates for regrowth, one shoot tip per cell.

Moisute Content-Weigh the beads and oven dry at 103 ± 2ºC for 17 ± 1 h. Weigh again. Calculate the moisture content (MC%) by [(Fresh Weight-Dry Weight)/Fresh Weight] x 100. 'Fresh weight' represents the weight of beads before oven drying but after all pretreatments (i.e. step 1 to 3).

Assesment of Survival and Regrowth-Record the data on regrowth 4 week after rewarming.

II. Vitrification Technique-Protocol

Plant material
In vitro cultures of blackberry (*Rubus* spp.)

Stock Cultures
Multiply micropropagated plantlets of *Rubus* species on medium containing MS mineral salts and vitamins with double EDTA-Fe, 2 mg/l N6 benzyladenine (BA), 0.1 mg/l indole 3 butyric acid (IBA), 0.1 mg/l GA3 3.5 g/l agar, 1.45 g/l gelrite and 30 g/l sucrose at pH 5.7 in test tubes. Grown the cultures at 25ºC with a 16 h light/8 h dark photoperiod.

Cold acclimation
Cold acclimatize 3 weeks old cultures at 22ºC with 8 h light (10 μE.m-2.s-1)/-1ºC 16 h dark) for 4 weeks.

Shoot tip Excision
Excise shoot tips with 2 to 3 pairs of leaf primordial (about 1 mm long) from cold acclimated shoots.

Vitrification procedure

Pretreatment-Pretreat excised shoot tips for 48 h on MS medium containing 5% dimethyl sulfoxide (DMSO) (v/v) with 3.5 g agar and 1.75 g/l gelrite.
LS treatlment- Transfer shoot tips into 1.2 ml cryo-vials and treat with 1 ml loading solution (LS) (2M glycerol in 0.4 M sucrose solution) for 20 min at 25ºC.
PVS2 treatlment- Remove LS and add PVS2 cryoprotectant solution [(v/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in liquid MS medium with 0.4 M sucrose at pH 5.8] into cryovials for 20 min at 25ºC.
Freezing- Submerge vials in LN for 1 h.
Thawing-The vials were rewarmed in 45ºC water for 1 min and in 25ºC water for 2 min.
Regrowth- Immediately rinse the shoot tips twice in liquid MS medium containing 1.2 M sucrose. Plant the shoot tips on recovery medium (multiplication medium with no IBA) in 24-cell plates for regrowth, one shoot tip per cell.
Assesment of Survival and Regrowth-Record the data on regrowth 4 week after rewarming.

Cryopreservation of Non-Orthodox Seeds and Embryonic Axes

Objectives

- To understand the handling methods of the perishable fruits and seeds of non-orthodox seeds to retain maximum viability.
- To devise best methods to reduce moisture of seeds and embryonic axes to optimal levels.
- To suitably apply the virtification based methods for successful cryopreservation.

The following parameters are to be investigated before taking up cryopreservation of any species:

1. Confirm the seed storage behavior of species through the available literature. Find out information on floral biology, fruit maturity, ecology and seed biology. If seed storage behavior is known and information is available, plan for species-specific collection trips.
2. The part of the seed most appropriate for conservation has to be decided after understanding the morphology and physiology of the whole seed and axes, and ascertaining the ability of explants to regenerate. In large seeded species like coconut and oil palm, excised embryos can be taken up and in litchi, jackfruit, *Madhuca* spp. and other similar types, embryonic axes prove to be the explants of choice.

Desiccation-Freezing method- Protocols

A. Whole seeds / embryos dessiccation

Following steps are to be undertaken for desiccation and freezing

Step I. Fruits/seeds may be collected from trees marked at the time of flowering. Maturity of fruits may be divided into three developmental stages while keeping optimum gap in days after anthesis (DAA) to get distinct maturity stages of fruits. Three stages of maturity at which fruits/seeds should be collected are:

1. Immature fruits with seeds just after expansion of cotyledons
2. Partially mature fruits with seeds having fully expanded cotyledons
3. Fully mature fruits with seeds having maximum dry weight

Harvest the fruits directly from tree and record fruit color, firmness, shape, surface features, fresh weight, dimensions and other physical features. These parameters will be important in distinguishing developmental stages and correlating with physiological maturity of fruits and seeds.

Step II. Fresh fruits should be transported immediately after collecting to the laboratory in cloth bags/ cardboard boxes. In case it is difficult to transport fruits, freshly extracted seeds packed in sawdust/charcoal/peatmoss should be transported to reach the laboratory within 48 h of extraction.

Step III. In the laboratory, fruits/seeds may be stored in moist and mild cold conditions. Seeds should be carefully extracted from fruits and cleaned to remove any fruit part that may cause infection while storing. Extracted seeds should be used up for experimentation within few hours to few days or else may be treated with suitable fungicide if intended for storage for a week or more.

Step IV. Moisture content of seeds and embryos should be measured using the low constant temperature oven method. Small pieces of explants may be kept in hot air oven at 103±2°C for 17 h in a glass bottle or in pieces of aluminum foil. Loss of weight equivalent to the amount of water is measured. Moisture content should be calculated using the following equation:

$$\text{\% Moisture content on fresh weight basis} = \frac{\text{fresh weight before drying- fresh weight after drying}}{\text{Fresh weight of sample}} \times 100$$

Step V. After determining the moisture content, the most suitable desiccation method needs to be devised. Whole seeds and embryos can be desiccated in the desiccator/ vacuum desiccator filled with charged silica gel. The duration of desiccation needs to be standardized depending upon the initial and desired moisture levels. After each desired desiccation level, explants may be packed in the cryovials and maintained in the desiccator till the germination and freezing steps are to be undertaken.

B. Embryonic axes desiccation

Step I. In case whole seeds are not being processed for cryopreservation, embryos or embryonic axes may be excised from the seeds. The exact location of the embryonic axes within the seed should be ascertained. The method for excising embryonic axes would not be the same for all seeds. The most convenient and quick method for excising embryonic axes needs to be devised to avoid any damages and dehydration.

Step II. Seeds may be sterilized using sodium hypochlorite (2-2.7%) for 15 min. Embryos and embryonic axes, being deep seated, generally do not carry any infection. Hence, a 8-10 min

treatment with a sterilant is sufficient. The concentration of the disinfectant and the duration of the treatment may be altered as required.
While excising embryonic axes, the time taken to handle a number of samples should be minimized so that there are no major differences in moisture contents between axes.

Step III. Desiccation

Air desiccation: Excised embryonic axes may be kept in batches of 20-25 in the sterile air flow of laminar flow cabinet immediately after excision. Axes may be desiccated for 1 to 5h depending upon the size of axes, the initial and desired moisture content levels. After each desiccation interval, moisture content and viability of embryonic axes should be determined. Viability of desiccated axes should be determined by culturing them in the prescribed culture media.

Rapid desiccation: Rapid or fast drying can be achieved using a flash drier. A flash drier is a simple device in which a rapid flow of compressed dehumidified air is passed through a container at the rate of 10 lit min^{-1}. The air inlets are attached to fish tank diffusers for uniform air distribution. The container is ventilated to allow the air to pass and it is filled half with charged silica gel. Embryonic axes are suspended above the diffusers, enclosed in plastic mesh or muslin cloth.

Precautions

1. Strictly follow the guidelines of collecting and transport of fruits and seeds to the laboratory. Try to plan and complete your experiments as quickly as possible to check any loss in viability and infection in fruits/seeds.
2. While experimenting with developing seeds, try to maintain the correct stage (days after anthesis) for all replicates.
3. Time gap in excision of embryonic axes should be minimum to subject all the axes to an equal desiccation level.
4. Rate of desiccation and freezing should be standardized and uniformly maintained for all replicates.
5. Recovery media and method should be manipulated to get optimum growth.