PRIYADARSHINI INSTITUTE ENGINEERING AND TECHNOLOGY

NAGPUR

DEPARTMENT OF BIOTECHNOLOGY

Germ-plasm Conservation

By-

Dhiraj Powar

Contents

Germplasm

- A **germplasm** is a collection of **genetic resources** for an organism.
- For plants, the germplasm may be stored as a seed ,stem, Callus, Whole plant in nurseries.
- In case of animals- Genes, Body parts stored in gene bank/cryobank.

Germplasm conservation

- Plant germplasm is genetic source material in the form of Seeds, Cultured cells ,Callus, Pollens .
- The in-situ /ex-situ preservation of these material is known as "**Germplasm conservation".**
- Germplasm provide the raw material (genes) which the breeder uses to develop commercial crop varieties.

What is the need of Preservation ?

- Preservation/Conservation of plant biodiversity is an important issue.
- Storage of Economically important , endangered, rare species and make them available when needed.
- The conventional methods of storage failed to prevent losses caused due to various reasons.

Methods of Germplasm conservation

1. In-situ Preservation
2. Ex-situ Preservation

In-situ Preservation

1. Preservation of the germplasm in their natural habitat
2. The conservation of domesticated and cultivated species in the farm or in the surroundings.
3. However, there is a heavy loss or decline of species, populations and ecosystem composition, which can lead to a loss of biodiversity, due to habitat destruction and the transformations of these natural environments; therefore, *in situ* methods alone are insufficient for saving endangered species.

Ex-situ preservation

1. To maintain the biological material outside their natural habitats.
2. Storage in seed banks, field gene collections, in vitro collections and botanical gardens
3. Ex situ conservation is a viable way for saving plants from extinction, and in some cases, it is the only possible strategy to conserve certain species
4. *In vitro* conservation is especially important for vegetatively propagated and for non-orthodox seed plant species

- In vitro techniques used to achieve medium-term conservation allow the storage of biological material from several months to 2–3 years without subculture, depending on the technique used and on the plant material.
- Growth reduction is generally attained by modifying the culture medium and/or the environmental conditions.
- Modifications of the culture medium can include dilution of mineral elements, reduction of sugar concentration, changes in the nature and/or concentration of growth regulators and addition of osmotically active compounds

Disadvantages of Ex-situ Conservation

- Some plants do not produce fertile seeds.
- Loss of seed viability
- Seed destruction by pests, etc.
- Poor germination rate.
- This is only useful for seed propagating plants.
- It’s a costly process.

Cryopreservation

- Cryopreservation is a non-lethal storage of biological material at **ultra-low temperature**.
- At the temperature of liquid Nitrogen (-196 degree) almost all metabolic activities of cells are ceased and preserved .
- Cryopreservation is the only technique that ensures the safe and cost-efficient long-term conservation of various categories of plants, including non-orthodox seed species, vegetatively propagated plants, rare and endangered species and biotechnology products.

Principle of Cryopreservation

- Storage of Biomaterial at ultra low temperature by means of slow freezing.
- In all cryopreservation processes, water removal plays a central role in preventing freezing injury and in maintaining post-thaw viability of cryopreserved material.
- There are two types of cryopreservation protocols that basically differ in their physical mechanisms:

1. Classical cryopreservation
2. Vitrification

Steps/Techniques Involved In Cryopreservation

Selection Of Plant Material

Pregrowth

Addition Of Cryoprotectants

Vitrification

Cryoprotective Dehydration

Encapsulation & Dehydration

Freezing

1.Rapid Freezing

2.Slow Freezing

3.Stepwise Freezing

Storage

Thawing

Determination of Survival or Viability

Examples

- *C. roseus* cells are precultured in medium containing 1M sorbitol before freezing. (Chen et al., 1984)
- Rapid freezing-cryopreservation of shoot tips of potato , strawberry , brassica species.
- Slow Freezing -cryopreservation of meristems of peas , potato , cassava , strawberry etc.
- The Stepwise freezing gives excellent results in strawberry and with suspension cultures.

Classical cryopreservation

- In this procedure, cooling is performed in the presence of ice.
- It involves cryoprotection by using different cryoprotective solutions combined or not with pregrowth of material and followed by slow cooling (0.5–2.0°C/min) to a determined prefreezing temperature (usually around −40°C),rapid immersion of samples in liquid nitrogen, storage, rapid thawing and recovery.
- They are generally operationally complex, since they require the use of sophisticated and expensive programmable freezers.
- Cryopreservation following classical protocols induces a freeze-dehydration process using a slow freezing regime.

- As temperature decrease slowly, ice is initially formed in the extracellular solution and this external crystallization promotes the efflux of water from the cytoplasm and vacuoles to the outside of the cells where it finally freezes.
- Therefore, cell dehydration will depend on the cooling rate and the prefreezing temperature set up before immersion of samples to liquid nitrogen.
- Classical cryopreservation techniques have been successfully applied to undifferentiated culture systems of different plant species, such as cell suspensions and calluses.
- Successful cryopreservation of apices from tropical species, such as cassava (Manihot esculenta), is an exceptional example.

Vitrification

- In this procedure, cooling normally takes place without ice formation
- The process where formation of ice cannot take place because of the Concentrated aqueous solution which permit ice crystal nucleation. Instead, water solidifies into an amorphous ‘glassy’ state.
- The vitrification-based procedures involve cell dehydration prior to cooling by exposure of samples to highly concentrated cryoprotective media (usually called plant vitrification solutions, PVS) and/or by air desiccation.

- Cooling rate may be rapid or ultra-rapid, depending on how samples are immersed into liquid nitrogen.
- Vitrification *per se* is a physical process, defined as the transition of the liquid phase to an amorphous glassy solid at the glass transition (Tg) temperature .
- This glass may contribute to preventing tissue collapse, solute concentration and pH alterations during dehydration.
- Therefore, the freeze-induced dehydration step characteristic of classical procedures is eliminated and the slow freezing regime is replaced by a rapid or ultra-rapid cooling process, characteristic of the vitrification-based protocols.

Case study of Mint Cryopreservation

- For initiating in vitro shoot cultures, apical shoots cut from in vivo explants collected in the field were surface-sterilized in 70% ethanol for 30 s, followed by sodium hypochlorite solution with 2% active chlorine for 5 min, and then rinsed twice in sterilized distilled water.
- After surface sterilization, shoot tips with basal parts were excised from the shoots and cultured on 60 ml solid MS 6 media in culture bottles containing 0.088 M sucrose and 0.4% (w/v) gallon gum without growth regulators.
- After 30 days, shoots were transferred on solid MS medium as stock plants, which were subcultured every 2 months on MS medium. Cultures were incubated at 25±1°C with a 16 h light/8 h dark photoperiod under white fluorescent light in culture flasks (standard conditions).
- Then, shoot tips with basal parts were dissected from the shoots and precultured for 1 day at 25°C on the MS medium with 0.088 M or 0.3 M sucrose.

1. Pour sodium alginate solution (2 µl) in the wells of the aluminum plate using a micropipette. The alginate solution contains 2% (w/v) sodium alginate in calcium-free MS basal medium with or without 0.4 M sucrose.
2. Place the precultured shoot tips in the wells one by one with the tip of a scalpel blade and slightly press the shoot tips to make them fit in the plate's wells.
3. Pour the calcium chloride solution drop wise on the section of the aluminum plate where the shoot tips are located until they are covered and wait for 15 min to achieve complete polymerization. The calcium solution contains 0.1 M calcium chloride in MS basal medium with or without 0.4 M sucrose.
4. Remove the calcium solution from the cryo-plate by sucking it gently with a micropipette.
5. Place the cryo-plate with attached shoot tips in a 25 ml pipetting reservoir filled with 20 ml loading solution (LS). Shoot tips are osmoprotected at 25°C for 30 min. The LS solution contains 2 M glycerol + 0.6 M to 1.2M sucrose in liquid MS basal medium.
6. Remove the cryo-plate from LS and place it in a 25 ml pipetting reservoir filled with PVS2 (about 20 ml). Shoot tips are dehydrated at 25°C for 10 min to 60 min.
7. After dehydration, transfer the cryo-plate in a 2 ml cryotube, which is held on a cryo-cane, and directly plunge it in LN where it is kept for at least 30 min.

- For regrowth, cryotubes were retrieved from LN. The cryo-plates with shoot tips were transferred from the cryotubes and immersed in 2 ml 1 M sucrose solution in MS basal medium contained in another 2 ml cryotube.
- Shoot tips were incubated in this solution for 15 min at room temperature and then transferred on solid MS medium.
- Post-rewarming regrowth(regrowth level) was evaluated after 2 weeks of culture at 25°C under standard conditions by counting the number of explants that developed normal shoots

Plant material	Gene bank/country
Seeds of 1200 accessions from 50 different species mainly of endangered medicinal plants	The National Bureau for Plant Genetic Resources (NBPGR), New Delhi, India
Seeds of more than 110 accessions of rare or threatened species	Kings Park and Botanic Garden, Perth, Australia
Seeds of coffee involving 450 accessions	IRD Montpellier, France
Dormant buds of apple involving 2200 accessions	National Center for Genetic Resources (CNGR), Fort Collins, USA
Dormant buds of mulberry involving 420 accessions	National Institute of Agrobiological Resources (NIAR), Yamagata, Japan
Shoot-tips of banana involving 630 accessions	INIBAP International Transit Center, Catholic University of Leuven, Belgium
Shoot-tips of cassava involving 540 accessions	International Center for Tropical Agriculture (CIAT), Cali, Colombia
Pollen of 13 pear cultivars and 24 *Pyrus* species	National Center for Genetic Resources (CNGR), Fort Collins, USA
Pollen of more than 700 accessions of traditional Chinese flower species	College of Landscape Architecture, Beijing Forestry University, Beijing , China
More than 1000 callus strains of species of pharmaceutical interest	Phytera, Sheffields, UK
Several thousand conifer embryogenic cell lines for large-scale clonal planting programs	Sylvagen, Vancouver, Canada
Embryogenic cell lines of coffee and cacao	Biotechnology Laboratory of the Nestlé Company, Notre Dame d'Oé, France

Table :Large-scale application of cryopreservation techniques to different plant germplasm.

Major advantages are :

1. Once the material is successfully conserved to particular temperature it can be preserved indefinitely.

2. Once in storage no chance of new contamination of fungus or bacteria.

3. Minimal space required.

4. Minimal labor required.

Limitation of Germplasm

- The expensive equipment needed to provide controlled and variable rates of cooling/warming temperatures can however be a limitation in the application of in vitro technology for large scale germplasm conservation.

- Formation of ice crystal inside the cell should be prevented as they cause injury to the cell.

- Sometimes certain solutes from the cell leak out during freezing.

- Cryoprotectant also effect the viability of cells.

APPLICATIONS

- It is ideal method for long term conservation of material.
- Disease free plants can be conserved and propagated.
- Recalcitrant seeds can be maintained for long time.
- Endangered species can be maintained.
- Pollens can be maintained to increase longitivity.
- Rare germplasm and other genetic manipulations can be stored.


THANK YOU