In vitro cryopreservation techniques

The know-how

Neelam Sharma, R Gowthami and Ruchira Pandey
In vitro propagation: An essential pre-requisite

Limitations
• Material availability
• Technical know-how
CRYOPRESERVATION

Storage of germplasm under suspended growth in liquid nitrogen

Advantages
- Indefinite storage
- Genetic stability
- Less space requiring
- Minimal inputs
- Retention of active constituents

Limitations
- Explant availability
- Recalcitrance to in vitro techniques
- Standardization for individual genotypes
There are two potential sources of cell damage during cryopreservation.

- Formation of large ice crystals inside the cell.

- Intracellular concentration of solutes increase to toxic levels before or during freezing as a result of dehydration.
  - Dehydration of highly hydrated cells to avoid lethal intracellular ice formation and consequent cell death
  - Can be achieved by inducing freeze dehydration or vitrification
  - Metabolic activity suspended
CRYOPRESERVATION TECHNIQUES

CLASSICAL TECHNIQUES
- Freeze induced dehydration
  - Involves slow cooling using a programmable freezer
  - Cost intensive
- More successful with dormant buds, cell suspensions, calli and shoot tips of temperate species (apple, pear, etc)

NEW TECHNIQUES
- Vitrification-based (vitrification of intracellular aqueous compartment)
  - Elimination of most water following exposure to highly concentrated cryoprotectant solution or air dessication
  - Cost effective
- Widely applicable to shoot tips, embryos and embryonic axes of both temperate and tropical species
CRYOPRESERVATION TECHNIQUES

Cryopreservation

Classical
(freeze-induced dehydration)

New techniques
(Vitrification based)

Controlled cooling procedure involves:
• Pre-growth
• Cryoprotection
• Freezing (0.1-2.0°C/min) down to -40°C
• Storage in Liquid nitrogen
• Thawing
• Regrowth

• Vitrification
• Encapsulation-vitrification
• Dehydration
• Encapsulation-dehydration
• Pregrowth
• Pregrowth-dehydration
• Droplet freezing
• V/D plate method
Vitrification is a process in which ice formation cannot take place because the aqueous solution is too concentrated to permit ice crystals nucleation. Instead, water solidifies into an amorphous ‘glassy’ state.
## NEW CRYOPRESERVATION TECHNIQUES

<table>
<thead>
<tr>
<th>Cryopreservation method</th>
<th>Explant</th>
<th>Procedure</th>
</tr>
</thead>
</table>
| **Pregrowth**<br>(Musa: 0.5 M Suc 3 wks) | • Meristems  
• Shoot tips/buds  
• Somatic embryos | • Pregrowth of explants in cryoprotectants  
• Cryopreservation and storage  
• Regrowth and regeneration of plants |
| **Pregrowth-dehydration**<br>(Asparagus (NS), Cassava, Coconut)(ZE)) | • Shoot tips/buds  
• Nodal cuttings  
• Somatic embryo  
• Zygotic embryos | • Pregrowth of explants in cryoprotectants  
• Dehydration in laminar airflow or silica gel  
• Cryopreservation and storage  
• Regrowth and regeneration of plants |
| **Dehydration**<br>(Banana) | • Zygotic embryos and embryonic axes of non-orthodox seed species | • Explant dehydration using silica gel or air flow for 60-360 min (250°C)  
• Cryopreservation and storage  
• Thawing and regeneration |
<table>
<thead>
<tr>
<th>Cryopreservation method</th>
<th>Explant</th>
<th>Sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitrification</strong></td>
<td>Meristems, Shoot tips/buds, Nodal cuttings, Somatic embryos, Zygotic embryos, Cell suspensions</td>
<td>&gt;60</td>
</tr>
<tr>
<td><strong>Encapsulation-vitrification</strong></td>
<td>Meristems, Shoot tips/buds, Nodal cuttings, Somatic embryos, Zygotic embryos, Cell suspensions</td>
<td>12</td>
</tr>
<tr>
<td><strong>Encapsulation-dehydration</strong></td>
<td>Meristems, Shoot tips/buds, Nodal cuttings, Somatic embryos, Zygotic/microspore embryos, Cell suspensions</td>
<td>~70</td>
</tr>
<tr>
<td><strong>Droplet vitrification</strong></td>
<td>Meristems, Shoot tips/buds, Nodal cuttings, Somatic embryos, Zygotic embryos, Cell suspensions</td>
<td>Meristems of Asparagus, yams, Potato, musa, alliums</td>
</tr>
</tbody>
</table>
CRYOPRESERVATION

1. Pregrowth
2. Cryo-protection
3. Freezing
4. Thawing
5. Regrowth
Enhance tolerance to cryopreservation stress, particularly by increasing resistance to dehydration and desiccation.

Enhance survival but do not offer cryoprotection.

It may also be inclusive of preconditioning treatments with cryoprotectants conferring resistance to LN.
CRYOPROTECTANTS

01
A chemical or mixture of chemicals to protect tissues under ultra low temperature

02
Sucrose, glucose, polyethylene glycol, etylene glycol

03
Vitrification solution contains 2 or more of components

04
Duration of exposure very critical

Cryoprotectants acts like antifreeze; they lower freezing temperature, increase viscosity and prevent damage to the cells

Control of dehydration and prevention of chemical toxicity during dehydration:
Critical and indispensable for successful cryopreservation
Regrowth of the plants from stored tissues or cells is the only test of survival of plant materials.

Fluorescien diacetate (FDA) staining, Triphenyl tetrazolium chloride (TTC)

Ex vitro/in vitro pollen germination

Growth measurement by cell number

01

02

03

04
EXPLANTS FOR CRYOPRESERVATION

- Seeds
- Dormant buds
- Shoot tips/meristem
- Somatic embryos
- Pollen
- Embryo & embryonic axis
- Cell suspensions
Bacopa monnieri shoot tips isolation

*In vitro* cultures

Carefully remove the shoots and place in the sterile petri dish under stereomicroscope in a laminar air flow cabinet.

Apical shoots

Remove leaves one by one till the glassy apical dome with two leaf primordia and where the apical domes can be easily observed.

Shoot tips on preculture medium

Shoot tips (1–2 mm long and 0.5 – 1.0 mm thick)
Uragami et al., 1989 in Asparagus cell culture
24 weeks shoot donor plants

Isolation of explant

Preculture of explants for 2 days in MS +0.3 M sucrose

PVS2 dehydration at 0°C

Ex vitro establishment

Regrowth on MS+0.2 mg/L BA

Thawing and treatment with unloading solution (1.2M sucrose) for 20 min.

LN freezing
VITRIFICATION: *Bacopa monnieri*

- Proliferating shoot cultures on MS + 0.2 mg/l BA
- Isolation of shoot tips and preculture on high sucrose medium
- Dehydration of shoot tips using PVS2 (30% glycerol + 15% ethylene glycol + 15% DMSO + 0.4 M sucrose) for 35 min at 0°C
- LN (+) Cryovials plunged in LN for minimum of 1 hr
- LN (-) Control
  - Remove PVS2 and replace with unloading solution (1.2M sucrose) for 20 min
- Thawing - (Remove cryovials from LN and place in waterbath at 42°C for 2 min)
- Explants cultured on regrowth medium

Eighth International Training Course on ‘In vitro and Cryopreservation Approaches for Conservation of Plant Genetic Resources’, ICAR-NBPGR, New Delhi. Nov 5-19, 2019
VITRIFICATION IN GARLIC

Isolated shoot tips

Remove the outer layers of cloves

Remove the shoot bases with few layers using borer

Subject the material to sterilization and wash off the sterilant using distilled water under Laminar flow

Isolate the shoot tips under microscope

Cryoprotective dehydration of shoot tips

Rapid cooling in liquid nitrogen

Rapid warming

Inoculate the shoot tips onto the medium for pregrowth

Regrowth

Plating of explants on regrowth medium

Removal of cryoprotectants & treatment with US for 20 min

Vitrification in Garlic

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ENCAPSULATION-DEHYDRATION TECHNIQUE

Cryopreservation of Yam by Encapsulation-Dehydration Technique

(a) Explant

(b) Preculture - I

(c) Encapsulation
Alginate with shoot tips is sucked into the pipette

(d) Preculture - II

16 h at 25°C with MS liquid medium + 0.3 M sucrose

3% Na-alginate in Ca²⁺-free MS medium + 0.4 M sucrose

100 mM CaCl₂

72 h in 0.75 M sucrose

(e) Air drying

Dry silica gel

(f) Rapid cooling

Rapid at 40°C water

5°C water

or

or

(g) Rewarming

Slow at 25°C (room/culture room temperature)

LN (-196°C) at least 1 h

For 4-8 h

(h) Recovery growth - I

16 h in dark on MS medium + BAP 1.5 + NAA 0.2 + GA₃ 0.2 mg l⁻¹ lined with filter paper

(i) Recovery growth - II

2 weeks in dark on medium as in Recovery growth-I

(j) Recovery growth - III

Fully grown plantlet (MS medium + NAA 0.15 mg l⁻¹)

3 weeks in 16h/day light on MS medium + BAP 1.0 + NAA 0.5+GA₃ 0.3mg l⁻¹

Fabre Dereuuddre 1990- Shoot tips of potato

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Shoot tip (0.8-1.2mm) from 6-8 wk old plant
Preculture on 0.3M sucrose

Encapsulation - Dehydration

- Encapsulate in 0.3% ca. alginate+0.4M sucrose
- Preculture in 0.75M sucrose for 3 days
- Dehydrate to MC 25%, 4 hr air drying
- FP soaked in MS+1.5 mg/l BA+0.2mg/l NAA+ 0.2mg/l GA3
- Transfer to fresh medium in dark (15 days) → Low light (10 days) → Full light on MS + 2.0mg/l zeatin

Vitrification

- Loading soln (2M glycerol + 0.4M sucrose) 25 C
- PVS2 for 90 min at 0C
- LN freezing
- Thawing at 40 & sucrose washing
- Recovery medium (15 days) (MS+1.5 mg/l BA+0.2mg/l NAA+ 0.2mg/l GA3)
- Transfer to MS 0.5mg/l Zeatin

In both techniques, shoot tips regenerated directly
Preculture of shoot tips

Source of explants

Isolation of shoot tips

Preculture of shoot tips

Shoot tips in 4% (w/v) sodium alginate solution

Explants in alginate solution dispensed drop wise in CaCl2 soln.

PVS2 dehydration

Matsumoto et al., 1995-
Shoot tips of *Wasabi japonica*

Sprouted bead

Regrowth

Treatment with unloading solution (1.2M sucrose) for 20 min.

Thawing

LN freezing

ENCAPSULATION-VITRIFICATION TECHNIQUE
VITRIFICATION, DROPLET VITRIFICATION, CRYOPLATE METHOD

**Cryopreservation of Yam by Vitrification Technique**

- **(a) Explant**
- **(b) Preculture**
- **(c) LS treatment**
- **(d) Dehydration with PVS2**
- **(e) Rapid cooling**
- **(f) Rapid warming**
- **(g) Unloading (Washing)**

**Method**

<table>
<thead>
<tr>
<th>Method</th>
<th>Cooling rates</th>
<th>Warming rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrification</td>
<td>100-200° C/min</td>
<td>80-140° c/min</td>
</tr>
<tr>
<td>DV, V plate and D plate</td>
<td>4000-5000° C/min</td>
<td>3000-4500° c/min</td>
</tr>
</tbody>
</table>

**Cooling rates**

- Vitrification: 100-200° C/min
- DV, V plate and D plate: 4000-5000° C/min

**Warming rates**

- Vitrification: 80-140° c/min
- DV, V plate and D plate: 3000-4500° c/min

**Droplet vitrification**

**Cryoplate method**
DROPLET VITRIFICATION IN MINT

In vitro plants → Shoot tips (0.5-1mm) → Preculture in 0.3 M sucrose solution overnight → Cryoprotection in solution A (2h), then in PVS 2 (20 min)

3 months after rewarming

2 and 4 weeks after rewarming

Rewarming at 40 °C, Some seconds

Flüssiger Stickstoff (LN)

Droplets of PVS 2 (2 μl) on aluminium foile

Shoot tip (2mm) isolation & preculture

Dehydration of shoot tips using PVS3 for 120-150 min.

Placement of PVS3 droplets on Aluminum foil strips

Placement of shoot tips on PVS3 droplets on the aluminum strips

Placement of aluminum strips in cryovials in LN2

Storage in LN

Ex vitro establishment

Regenerants under culture room conditions

Regrowth

Culture of explants on regrowth medium

Remove aluminum strips from LN2 & treatment with US for 20 min

Regrowth
Isolation and preculture of shoot tips

Place sodium alginate solution on the wells of cryoplate

Place the precultured shoot tips and pour calcium chloride solution

Place cryoplates in the Loading solution and followed by PVS2 dehydration

Immerse in liquid nitrogen

Rewarm and plating

Immerse in liquid nitrogen

Place cryoplates in cryovials

V (Vitrification) cryoplate method
Mint
Yamamoto et al., 2011
**Isolation and preculture of shoot tips**

Place sodium alginate solution on the wells of cryoplate

Place the precultured shoot tips and pour calcium chloride solution

Place cryoplates to the Loading solution

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D (Dehydration) cryoplate method

*Juncus* spp (Mat rush)

Nino *et al.*, 2013, 2014

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Rewarm and plating

Immerse in liquid nitrogen

Desiccate the cryo-plates in laminar flow cabinet or in silica gel

Remove the cryo-plate from the LS and place it on paper filter in Petri dish.
Cryopreservation.....

- Banana
- Allium
- Ginger
- MAP
- Morus
- Apple
- Pear
- Rubus

Testing genetic stability.....

- Morhological
- Biochemical
- Molecular

Virus detection.....
<table>
<thead>
<tr>
<th>Institute</th>
<th>Country</th>
<th>Crop</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioversity International, Leuven</td>
<td>Belgium</td>
<td>Banana</td>
<td>DV</td>
</tr>
<tr>
<td>Crop Research Institute, Prague</td>
<td>Czech Republic</td>
<td>Potato, garlic, hops, mint</td>
<td>DV</td>
</tr>
<tr>
<td>International Center for Tropical Agriculture (CIAT), Cali</td>
<td>Colombia</td>
<td>Cassava</td>
<td>DV, ED</td>
</tr>
<tr>
<td>International Institute of Tropical Agriculture (IITA), Ibadan</td>
<td>Nigeria</td>
<td>Yam, banana, cassava</td>
<td>DV</td>
</tr>
<tr>
<td>International Potato Center (CIP), Lima</td>
<td>Peru</td>
<td>Potato</td>
<td>DV, SV</td>
</tr>
<tr>
<td>Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden</td>
<td>Germany</td>
<td>Strawberry/fruit trees</td>
<td>V, DBF</td>
</tr>
<tr>
<td>Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Genebank Department, Gatersleben</td>
<td>Germany</td>
<td>Potato, garlic, mint</td>
<td>DF, DV</td>
</tr>
<tr>
<td>National Agrobiodiversity Center (NAAS), RDA, Suwon</td>
<td>South Korea</td>
<td>Garlic</td>
<td>DV</td>
</tr>
<tr>
<td>Tissue Culture and Cryopreservation Unit, NBPGR, Delhi</td>
<td>India</td>
<td>Banana, chives, medicinal plants, berries, fruit trees</td>
<td>V, DV, SF DBF</td>
</tr>
<tr>
<td>USDA-ARS, Fort Collins and Corvallis</td>
<td>USA</td>
<td>Citrus sp, grape, garlic, mint, fruit trees (apple, pear)</td>
<td>V, DV, SF DBF</td>
</tr>
</tbody>
</table>
### STATUS OF GERMPLASM CRYOBANKED IN THE IVBG at ICAR-NBPGR

<table>
<thead>
<tr>
<th>Crop/Species</th>
<th>Total no. of accessions</th>
<th>Technique (s)*</th>
<th>Explant (s)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allium sativum</td>
<td>68</td>
<td>V, DV</td>
<td>ST</td>
</tr>
<tr>
<td>A. albidum</td>
<td>1</td>
<td>V, DV</td>
<td>ST</td>
</tr>
<tr>
<td>A. chinense</td>
<td>5</td>
<td>V, DV</td>
<td>ST</td>
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<tr>
<td>A. hookeri</td>
<td>2</td>
<td>V, DV</td>
<td>ST</td>
</tr>
<tr>
<td>A. ramosum</td>
<td>1</td>
<td>V, DV</td>
<td>ST</td>
</tr>
<tr>
<td>A. scorodoprasum</td>
<td>1</td>
<td>V, DV</td>
<td>ST</td>
</tr>
<tr>
<td>A. tuberosum</td>
<td>4</td>
<td>V, DV</td>
<td>ST</td>
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<tr>
<td>Bacopa monnieri</td>
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<td>V</td>
<td>ST</td>
</tr>
<tr>
<td>Dioscorea bulbifera</td>
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<td>V</td>
<td>ST</td>
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<tr>
<td>D. deltoidea</td>
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<td>V</td>
<td>ST</td>
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<tr>
<td>Ensete glaucam</td>
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<td>AD</td>
<td>ZE</td>
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<tr>
<td>Musa spp.</td>
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<td>DV, V, AD</td>
<td>SM, ECS, ZE</td>
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<td>M. acuminata</td>
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<td>SM, ZE</td>
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<td>M. balbisiana</td>
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<td>AD, DV</td>
<td>SM, ZE</td>
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<tr>
<td>Vaccinimum ovatum</td>
<td>7</td>
<td>ED</td>
<td>ST</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>191</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPLICATION OF CRYOPRESERVATION IN CONSERVATION

App. for 150-200 species cryopreservation procedures are available

Most cryopreservation procedures - academic research work

Germplasm conserved in the liquid nitrogen is ~10,000 accessions

>80% potato cassava, banana, mulberry and Garlic

Cryopreservation protocols vary from one species to other and between accessions

No real standardised methods are available
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Practice and Patience is the key to Success
THANK YOU!

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