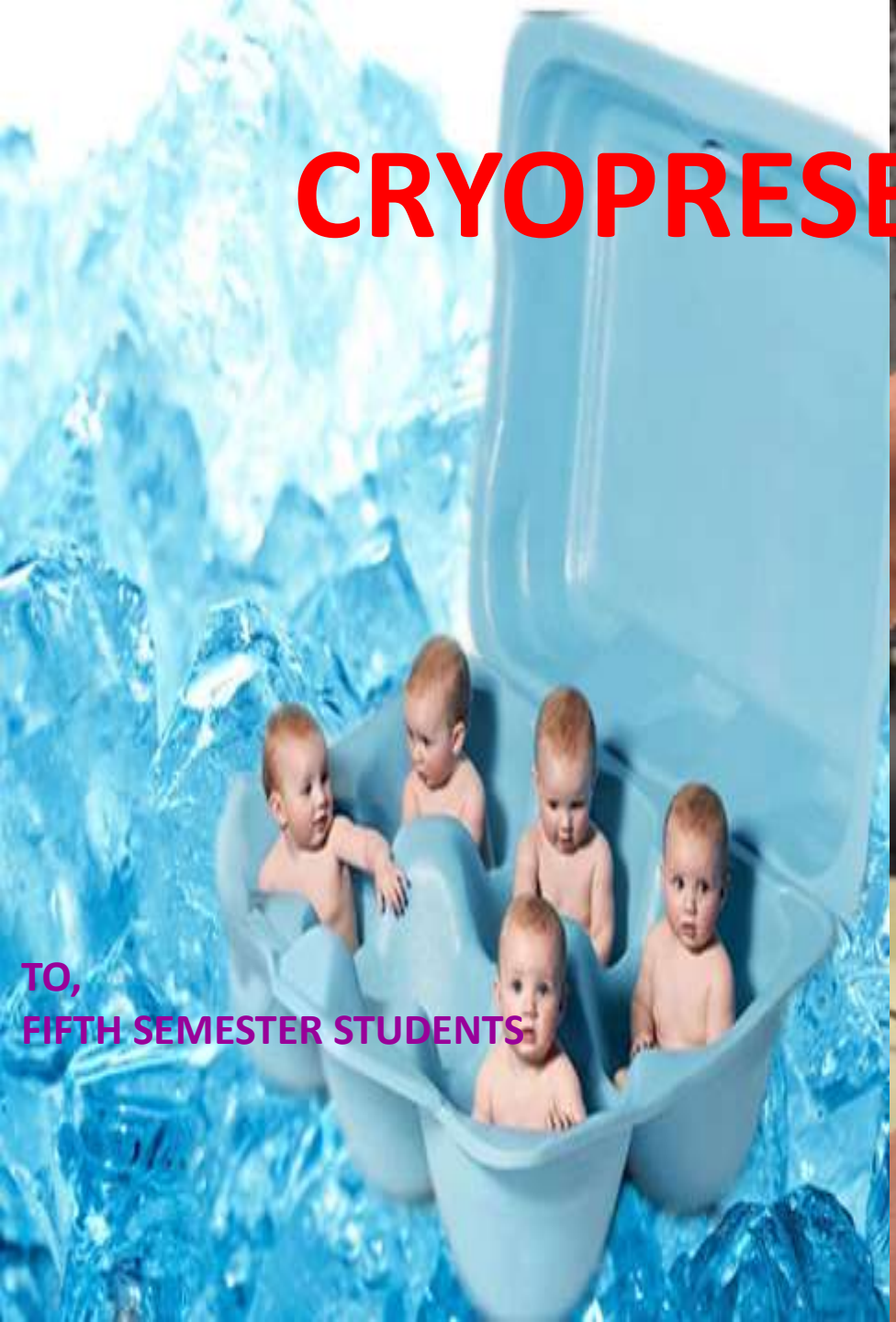
CRYOPRESERVATION



TO,
FIFTH SEMESTER STUDENTS

PRESENTED BY,
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ASST PROFESSOR ON CONTRACT BASIS
L F COLLEGE GURUVAYOOR

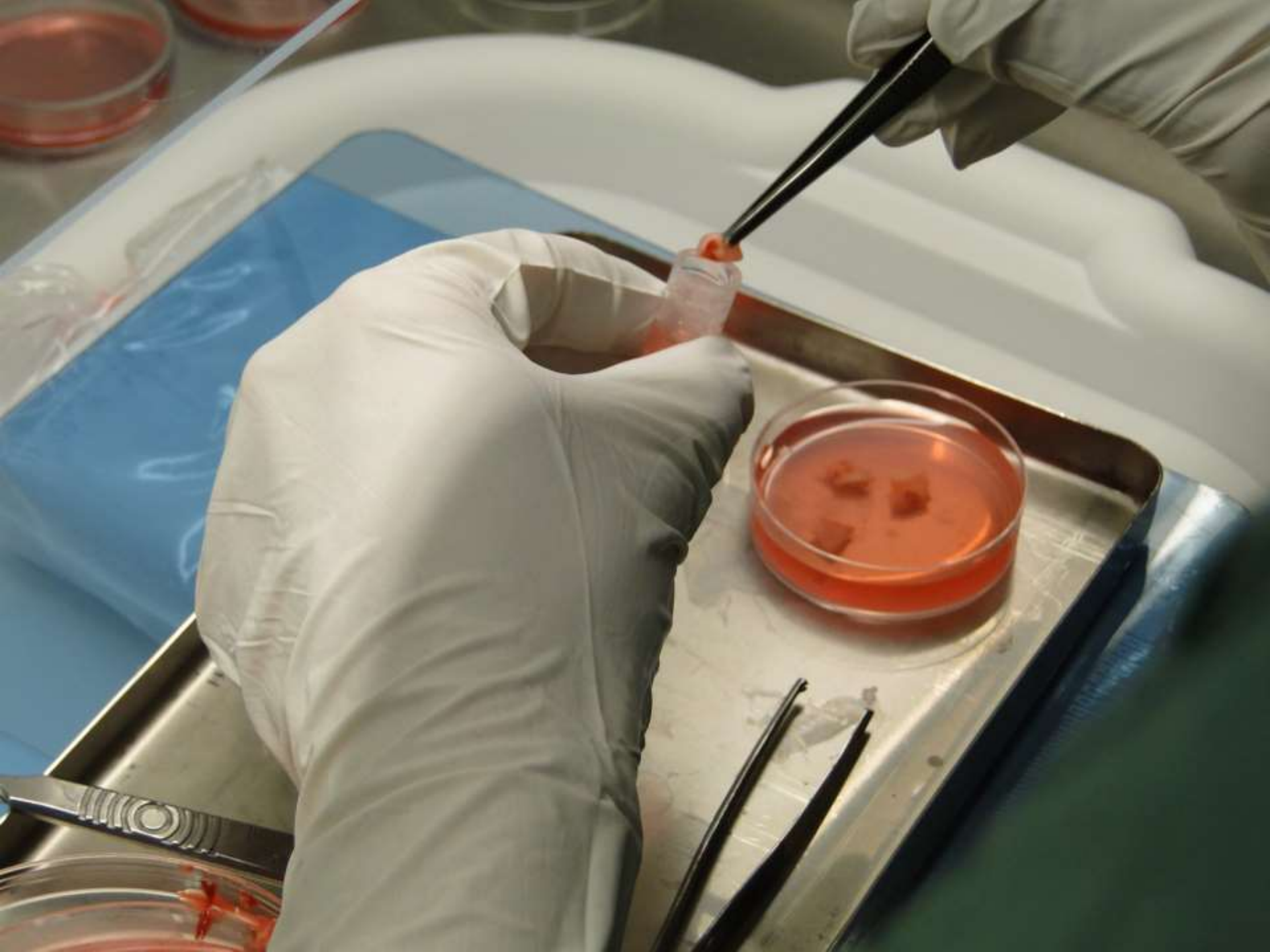
- Also called freeze preservation.
- Long term storage of living tissues or organs in the frozen state for future use.
- In liquid nitrogen (-196^{o} C)
- Temperatures at sub-zero level.
- Plant cells remain completely inactive state.
- Zero metabolism – principle
- Cryoprotectants are used.
- Potato, pea, chickpea, rice, sugarcane etc…

FACTORS INFLUENCING CRYOPRESERVATION

1. Age, nature & density of cells.
2. Cryoprotective agent.
3. Rate of freezing.
4. Storage temperature.
5. Method of thawing & culture conditions.

MAJOR STEPS OF CRYOPRESERVATION

1. Selection of source material
2. Vitrification
3. Exposure of culture to super-low temperatures.
4. Storage of the frozen cultures in liquid nitrogen (-196^{o}C)
5. Thawing
6. Removal of cryoprotectants by washing.
7. Determination of viability.
8. Re – culture
9. Induction of growth & regeneration of plantlets.



SELECTION OF SOURCE MATERIAL

- Young , small & thin walled meristematic cells.
- These cells taken from actively growing & periodically transferred suspension cultures.
- Explant ability to survive at -196^{0}C.
- Apical meristem, ovules, anthers, seeds etc..
- Culture cells are not ideal for cryopreservation.
- Shoot pieces, young plantlets are desirable for cryopreservation.

VITRIFICATION BY ADDITION OF CRYOPROTECTANTS.

- **Conversion of plant material into a less desirable state (free of crystal formation).**
- **Partial dehydration of the material either in vacuum or by treating them with a specially concentrated solution, called vitrification solution.**
- **Inorder to avoid cryodestruction, cryogens are used.**
- **Cryoprotectant are the compound that can prevent the damage caused to cells by freezing(antifreezing agents)**

What You Need For Cryopreservation?

Liquid nitrogen (liquid phase or vapor phase)

- Characteristics of liquid nitrogen:
  - Chemically inert
  - Relatively low cost
  - Non toxic
  - Non flammable
  - Readily available

Cryofreezer

Cryoprotectant: organic or inorganic additive which will protect the cell from freezing injuries during cryopreservation.

- Characteristics of cryoprotectants:
  - Should easily penetrate into cell
  - Non- electrolyte
  - Easily misible with water
  - E.g: Glycerol, DMSO, PVP, PEG etc.

CRYOPROTECTANTS

- Sugars
- Glycols
- Sugar alcohols (sorbitol, mannitol)
- Polyvinyl pyrrollidone (PVP)
- PEG
- Dimethyl sulphoxide (DMSO)
- Dextrans
- Glycerine
- Aminoacids (Proline)

CRYOPROTECTANTS

Permeating cryoprotectants

- DMSO
- Methanol
- Glycerol

Non-permeating cryoprotectants

- Sugar
- Sugar alcohols
- dextrans

CRYOPROTECTANTS- chemicals that minimise injuries to the cell due to ice formation or it suppresses ice formation.

CRITERIA FOR CHOOSING A CRYOPROTECTANT

1. Least toxic to cells
2. Should be permeable to cells
3. Should be soluble in water during freezing

TWO categories

A. Permeating cryoprotectants – permeable to cell memb.

Function by

1. Reducing the rate of diffusion of water from cell to extracellular ice crystal.
2. Reducing the cell volume change
3. Reducing the rate of ice crystal growth.

Common **Permeating cryoprotectants** –

1. **DMSO (Dimethyl Sulfoxide)√**
2. Glycerol
3. Methanol
4. Propylene glycol

B. Non permeating cryoprotectants

1. Not permeable to cell memb.
2. Act by depressing the freezing point and raising the ice formation temperature of extracellular solution.

Commonly used chemicals- sucrose, glucose, dextran, egg yolk serum, skim milk Antifreeze protein .

Extenders – Solution of balanced salts.
Used to dilute the milt as undiluted milt is not suitable for freezing.
Also inhibits the activation of spermatozoa and functions as a medium for cryoprotectant.

The **diluent** used for milt - **combination of extender and cryoprotectant.**
Kept in the fridge, as it is an exothermic reaction.
Three categories of diluents-

I. Extender A 90ml, DMSO- 5 ml, Glycerol- 10ml.
II. Extender B 90ml, DMSO- 8 ml, Glycerol- 10ml.
III. Extender C 65ml, DMSO- 15 ml.

Any one of the diluents – mixed with milt in a proportion- 4:1(diluent : milt)

CRYOPROTECTANTS

- **Low toxicity & high water-solubility are the desirable qualities of cryoprotectants.**
- **DMSO excellent cryoprotectant.**
- **Low molecular weight substance , easily miscible with solvent, non-toxic at low concentration, easily permeable into cells, easily washable.**


99.9% PURE
DMSO
DIMETHYL SULFOXIDE
GEL 90%
DISTRIBUTED BY
FWI, TULSA, OK 74116

CRYOPROTECTANTS

- **Materials to be preserved is put in the culture medium & treated with cryoprotectant.**
- **Transferred to sterile cryovials or ampules, made up of polypropylene.**
- **5-10% cryoprotectant is added into ampules & the ampules are tightly closed with screw cap.**


1.0
1.0

FREEZING

- **Without causing intracellular crystal formation.**
- **Test tubes are sealed and kept in freezer.**
- **If long storage in liquid nitrogen, pre-freezing is needed.**
- **Mini freezers, programmed users are used for this.**
- **Uniform freezing**
- **Differential freezing of different parts by programmed cell, freezing equipment.**

METHOD OF FREEZING

1. Slow freezing or equilibrium freezing
2. Rapid freezing or non-equilibrium freezing
3. Droplet freezing
4. Stepwise freezing
5. Storage in liquid nitrogen
6. Thawing
7. Washing and reculturing
8. Determination of viability
9. Regeneration of plantlets

SLOW FREEZING

- **Freezing is very low**
- **Flow of water from inside to outside**
- **Prevents intracellular ice formation & freezing injury.**
- **Cellular dehydration occur**
- **Dehydrated cells survive for a longer duration**
- **Done by using computer-controlled freezers.**

RAPID FREEZING

- **Simple**
- **Preservation of shoot tips**
- **Direct immersion of the cryoprotectant – treated material into liquid nitrogen**
- **Freezing occurs quickly.**
- **Only least chance for the formation of intracellular ice crystals.**
- **Ultra cooling prevents ice crystal formation.**

DROPLET FREEZING

- **Cryoprotectant treated material is dispended as droplets in a petridish.**
- **Cooled slowed to a sub-zero temperature.**

STEPWISE FREEZING

- **Include both rapid freezing & slow freezing.**
- **Temperature is lowered by -20^0 C to -40^0 C.**
- **Allows protective freezing of cells.**
- **Further freezing stopped for 30 minutes.**
- **Good results.**

STORAGE IN LIQUID NITROGEN

- **Prevents biochemical injury**
- **Preserves the viability of cells.**
- **Liquid nitrogen help in this process.**

THAWING

- **Also called warming.**
- **Bringing the frozen material back to normal temperature between 35 to 45 degree celsius for timely use.**
- **Removes extracellular ice crystals.**
- **Re-establishes the normal water balance of cells.**
- **Restores their normal metabolic activities.**

- **Place container in cold water bath 25-25 degree celsius.**
- **Gentle agitation accelerates thawing process.**
- **37^0 C effective in stabilizing the cells.**
- **It depends on cells used & cryoprotectants involved.**

WASHING & RECULTURING

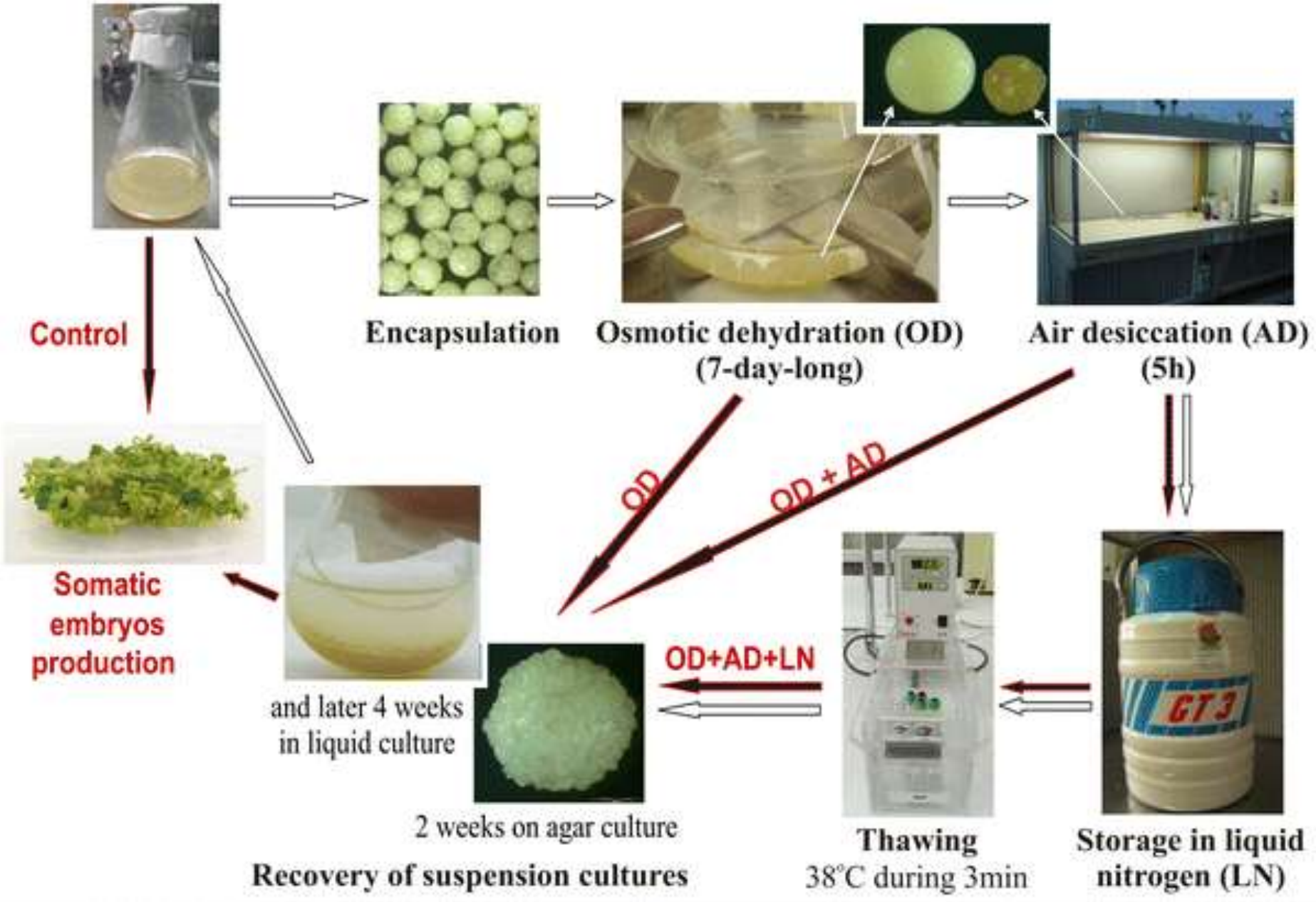
- If non-toxic cryoprotectants are used no need of washing.
- Washing done with distilled water.
- Dilution
- Resuspension
- Centrifugation
- Removal of cells

DETERMINATION OF VIABILITY

- **Cell viability done by using FDA (fluorescein diacetate) staining & growth measurements.**
- **Cell number, cell volume, dry & fresh weight, plating efficiency etc…**

REGENERATION OF PLANTLETS

- **Viable cells are cultured on non specific growth media under controlled aseptic conditions to promote regeneration of new plantlets.**
- **Plantlets are transferred to fields after proper hardening & acclimatization.**


Control
Encapsulation
Osmotic dehydration (OD)
(7-day-long)
Air desiccation (AD)
(5h)
OD
OD + AD
Somatic
embryos
production
and later 4 weeks
in liquid culture
OD+AD+LN
2 weeks on agar culture
Recovery of suspension cultures
Thawing
38°C during 3min
Storage in liquid
nitrogen (LN)

POTENTIALS AND PROSPECTS OF CRYOPRESERVATION OF GERMPLASM

- Stores disease free stocks.
- International exchange of materials.
- Prolonged pollen longevity
- Cold acclimatization
- Preservation of haploids
- Conservation of important & rare germplasm
- Maintains genetic stability.
- Long term storage
- Saves man power
- Saves space
- Saves medium
- Avoids subculture
- Prevents ageing

PLANT CELL BANK OR CELL CRYOBANK

- Also called germplasm bank
- First suggested by Bajaj (1977) & Popov(1985).
- Stock stored in large-sized cylinders(30-50L capacity).
- Not require refilling of liquid nitrogen for a period of 6 to 8 months.
- Facilitates for the cryopresevation of the genetic resources of a variety of plants & also for the national & international supply of germplasm.

POLLEN BANK

- **In-vitro collection of pollens.**
- **Enhances the longevity & storage life of pollens so that they can be stored for more than one year.**

ADVANTAGES

- Growth of plants at different places.
- Hybridization between plants which flower at different times.
- Reduction in the spreading of diseases by pollination vectors.
- Maintenance of germplasm & enhancement of longevity.

ACHIEVEMENTS

1. Cryopreservation of cell lines
2. Cryopreservation of pollen & pollen embryos
3. Cryopreservation of excised meristems
4. Cryopreservation of vegetatively propagated crops.
5. Cryopreservation of recalcitrant seeds & embryos.

ADVANTAGES OF CRYOPRESERVATION

- Conservation of genetic uniformity.
- Storage potential of original germplasm
- Preservation of rare genomes of somaclonal & gametoclonal variations.
- Storage of cell cultures
- Acclimatization & freeze resistance
- Slows down metabolism & ageing.

SLOW GROWTH METHOD

- Storage of germplasm at very low temperature, under non-freezing conditions & slow growth rate.
- Prevents cold injuries & damages.
- Similar to bonsai technique
- Growth reduced to minimum by a combination of limiting factors, such as nutrients, medium, temperature, light, hormones, etc..
- Biochemical and physiological processes are brought down.