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Cryopreservation: A Tool of Germplasm Conservation

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Cryopreservation is the technique of freezing cells and tissues at very low temperatures at which the biological material remains genetically stable and metabolically inert, while minimizing ice crystal formation. In general, when a tissue is subjected to low temperatures, ice crystals will eventually form. These crystals may disrupt the cell membrane leading to the death of the cell. Cryopreservation (Greek.,Kryos = frost) means " preservation in the frozen state". In practice, this is generally meant to be storage at very low temperature, e.g. over solid carbon dioxide, in low temperature deep freezers (-80^0 C or above) ,in vapour phase of nitrogen (-150^0 C) or in liquid nitrogen (-196^0 C).Generally the plant material is frozen and maintained at the temperature of liquid nitrogen (-196^0 C).Storage can be done in,

1. Over solid carbon dioxide (at -79 degree)
2. Low temperature deep freezer (at -80 degree)
3. Invapour phase nitrogen (at -150 degree)
4. In liquid nitrogen (at -196 degree) Cryopreservation

Theoretical basis of cryopreservation

At this temperature, the cells stay in a completely inactive state. The theoretical basis of freeze preservation is the transfer of water present in the cells to the solid state.

Advantages

- Once the material is successfully conserved to particular temperature it can be preserved indefinitely.
- Once in storage no chance of new contamination of fungus or bacteria.
- Minimal space required.
- Minimal labour required
- Protected against the nature's hazards

Steps of cryopreservation

1. Raising sterile tissue cultures.
2. Addition of cryoprotectants and pretreatment.
3. Freezing
4. Storage
5. Thawing
6. Determination of survival / viability.
7. Plant growth and regeneration. (Engelmann, 2004)

Raising sterile tissue cultures.

The morphological and physiological conditions of the plant material influence the ability of an explant to survive freezing at -196 0 C. Different types of tissues can be used for freezing such as the apical and lateral meristems , plant organs (embryos, endosperm, ovules, anther/pollen),seeds,

cultured plant cells, somatic embyros, seeds, cultured plant cells, protoplast, calluses etc. In general, small, richly cytoplasmic, meristematic cells survive better than the larger, highly vacuolated cells.

Addition of Cryopreservation and pretreatment

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation. Mainly pretreatment is done in two ways

Encapsulation- Dehydration: In this method, explants are suspended in 3-5% sodium alginate solution and picked up to dispense individually into 100Mm CaCl2 followed by shaking.Well-formed beads, formed within in 10-20 min are dehydrated in sucrose – enriched media for a minimum period of 17 hr. Beads are later desiccated in laminar airflow cabinet before freezing in liquid nitrogen.

Vitrification: It involves the treatment of tissues with cryoprotectants in vitrification solution followed by fast freezing. Most commomly used vitrification solution is PVS2(Plant Vitrification Solution2) which contains glycerol 30%, dimethyl sulphoxide (DMSO)15% and ethylene glycol 15%.Initially this procedure was developed for shoot apices, cell suspensions and somatic embryos but recently this technique has been applied to zygotic embryos and embryonic axes also.

Freezing

Freezing temperature depends on the sensitivity of species. There are,

1. **Slow freezing method -** the tissue or plant material is slowly frozen at slow cooling rate (0.5-5°C/min from 0°C to -100°C). The advantage is the plant cells are partially dehydrated and survive better. There will be extracellular ice formation mainly used for suspension culture.
2. **Rapid freezing method** - it involves plunging the vials in liquid nitrogen. The temperature decreases from -300 to -1000 degree rapidly. Mainly done to embryo culture and suspension.
3. **Stepwise freezing method** - this is combination of both slow and rapid freezing method. The process is carried out in step wise manner. Cooled to intermediate temperature for 30 minutes and rapid cooling is done. Mainly done to embryo culture, shoot tip and suspension.
4. **Dry freezing method** - in this method dehydrated cells and seeds are stored. Mainly done for recalcitrant seeds.

Storage

The maintenance of the frozen cells or material at specific temperature is very important. In general the temperature is kept -70 to -196 degree. Prolong storage is done at temperature of -196 degree in liquid nitrogen. To prevent damage, continuous supply of nitrogen is done.

Thawing

For the reuse of frozen cell thawing should be done to maintain the cell to normal condition.Usually carried out by plunging the vials into warm water bath with vigorous swirling. As thawing occurs the vials are transferred to another bath at 0 degree. It is important for survival of the tissue that the tubes should not be left in the warm bath after the ice melts.

Determination of survival / viability

There is possibility of death of cells due to storage stress. Thus viability can be found at any stage. It is calculated by formula: No of cells growing / no of cells thawed * 100. Staining techniques using triphenyltetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA).

Plant growth and regeneration

Mainly biophysical changes occur in the cell during freezing and thawing. The freshly thawed cell is strongly prone to further damage and requires appropriate nursing.Washing away the cryoprotectant and allow to grow in a regrowth medium with special additives

Applications

I. It is ideal method for long term conservation of material.
II. Disease free plants can be conserved and propagated.
III. Recalcitrant seeds can be maintained for long time.
IV. Endangered species can be maintained.
V. Pollens can be maintained to increase longevity.
VI. Rare germplasm and other genetic manipulations can be stored. (Sakai, 2000)

Limitations

I. Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
II. High intracellular concentration of solutes may also damage cells.
III. Sometimes, certain solutes from the cell may leak out during freezing.
IV. Cryoprotectants also affect the viability of cells.
V. The physiological status of the plant material is also important.

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