CHRISTIAN EMINENT COLLEGE, INDORE

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E-Content

On

"CRYOPRESERVATION"

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CONTENT

1. Introduction

In recent years, with the enormous increase in the population, pressure on the forest and the land resources have increased. This results in depletion of population of medicinal and aromatic plant species. Even some of the plant species are at the verge of vanishing from the forest. The list of endangered species is growing day by day. The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

- Attack by pathogen and pests.
- Climatic disorders
- Natural disasters and
- Political and economic causes

In addition, the seeds of many important medicinal plants lose their viability in a short time under conventional storage system.

The conservation of germplasm can be done by two methods:

1. **In-situ preservation:** Preservation of the germplasm in their natural environment by establishing biospheres, national parks etc.
2. **Ex-situ preservation:** In the form of seeds or by *in vitro* cultures.

Seeds form the most common material to conserve plant germplasm, however, the method has the following disadvantages:

- Discrete clones cannot be maintained in the form of seeds.
- Some plants do not produce fertile seeds.
- Loss of seed viability.
- Seed destruction by pests, etc.
- Poor germination rate.
- This is useful for seed propagating plants and is not applicable to vegetatively propagated crops, like potato, ginger etc.

In vitro preservation by tissue culture has several advantages over seed preservation:

- Large amount of materials can be stored in a small area.
- The material could serve as an excellent form of nucleus stock to propagate large number of plants rapidly, when required.
- Under special storage conditions the plants do not require frequent splitting and pruning.
- Being free from known viruses and pathogens, the clonal plant material could be sent from country to country, thus, minimizing the obstructions imposed by quarantine systems on the movement of live plants across national boundaries.
- Protection from natural hazards.
- The plants are not exposed to the threat of changing government policies and urban development.

There are few disadvantages of in vitro system to be used for conservation of plant material:

- It is a costly process.
- In cultures, plants can be maintained by serial subcultures at frequent intervals for virtually unlimited periods.

However, the storage of germplasm by serial subcultures risks the loss of plant material by microbial contamination due to human error and also, is uneconomical. Moreover, in long-term callus and suspension cultures, the regeneration potential, biosynthetic properties and genetic make-up of the cells suffer. The basic requirement of a plant tissue culture method is the preservation of genetic resources, therefore, is to reduce the frequency of subcultures to a bare minimum.

Cryopreservation Introduction

Cryopreservation means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to zero metabolism or non-dividing state by mean of storage of germplasm at a very low temperatures, (i) Over solid CO_2 (-79°C), (ii) Deep freezers (-80°C), (iii) in vapor phase nitrogen (-150°C), (iv) in liquid nitrogen (-196°C).

Among these, the most commonly used non-lethal storage of biological material at ultra-low temperature is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

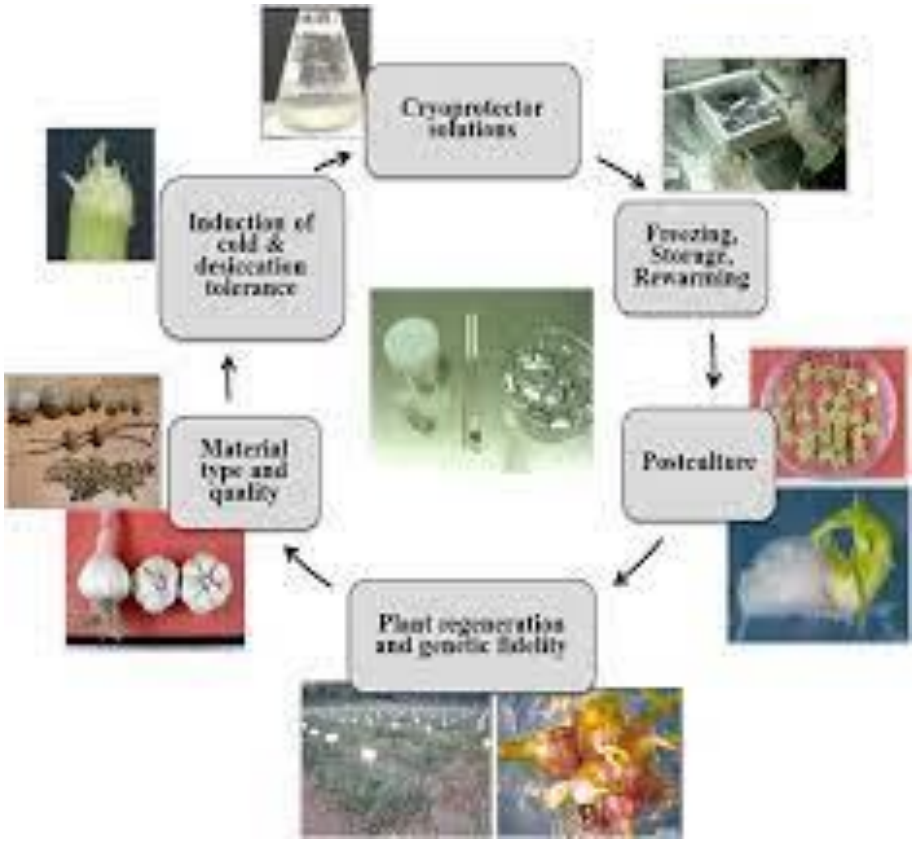
Steps involved in cryopreservation

The technique of cryopreservation involves the following steps:

1. Selection of plant material
2. Pre-culture
3. Cryoprotective treatment
4. Freezing and storage
5. Thawing
6. Reculture

Selection of plant material

The morphological and physiological conditions of the plant material, prior to freezing, considerably influence its ability to survive freezing at -196°C. Generally, small, richly cytoplasmic and meristematic cells survive better than the larger, highly vacuolated cells. Therefore, cell suspensions should be frequently subcultured and frozen in the late lag phase or exponential phase when the majority of the cells are in the preferred condition. While preservation of cell lines remains useful with respect to *in vitro* production of secondary metabolites, cultured cells are not the ideal system for germplasm storage. Instead, organized structures, such as shoot apices, embryos or young plantlets are preferred. The reasons to shift from cell cultures to organized cultures are as follows:



- The genetic instability of cells in long term callus and cell suspension cultures is a very common phenomenon and there is no effective measure to control it so far. Moreover, most of the callus cultures are initiated from non-meristematic cells of the plant body which might exhibit polysomaty. Hence, the cultured cells may exhibit genetic heterogeneity from the very beginning. In contrast, plants raised from shoot apices have generally proved to be true- to-type.
- ii. Cultured cells of several important plants do not exhibit totipotency. Moreover, in few cases these cells initially form organs/ embryos and whole plants but this potentiality is often lost after some time in culture. Besides, shoot apices possess a high regeneration ability which is retained in prolonged cultures. Shoot apices are mostly preferred to develop a virus free plants and also for the rapid clonal multiplication.

- Haploidy, which is highly unstable in callus and suspension cultures can be maintained through shoot tip culture and axillary-bud proliferation.
- The cells of shoot-tip and young embryos are small and meristematic. They ppear to be better suited than larger cells to survive liquid nitrogen (LN) freezing and thawing.

Pre-culture

In several cases, a brief culture of shoot apices for at least 48h at 4°C before freezing has proved beneficial for consistently high frequency of survival of shoot apices after freezing in liquid nitrogen. The other treatments include the application of additives that known to enhance plant stress tolerance, for example ABA, proline, osmoticum (sucrose, mannitol), dimethylsulfoide (DMSO, 1-5%). Sugars acts as osmotically effective agents, although they do not penetrate inside the cells. Dehydration of cells/tissues occurs in the presence of sugars during the preculture, which prevents lethal ice crystal formation during freezing. Proline may act by reducing the level of latent injury to the cells or it may actively participate in recovery metabolism.

Cryo-protective treatments

There are two potential sources of cell damage during cryopreservation (1) Formation of large ice crystals, inside the cells, leading to rupture of organelle and the cell itself, (2) intracellular concentration of solutes increases to toxic levels before or during freezing as a result of dehydration.

Addition of cryoprotectants controls the appearance of ice crystals in cells and protects these cells from the toxic solution effect. Cryoprotectants are categorized as: (a) Penetrating, which exert their protective colligative action, (b) Non-penetrating, which affect through osmotic dehydration. A large number of heterogeneous groups of compounds have been shown to possess cryoprotective properties with different efficiencies, e.g. glycerol, DMSO etc. Cryoprotectant depress both the freezing and super-cooling point of water, i.e. the temperature at which the homogeneous nucleation of ice occurs, thus, retarding the growth of ice crystal formation in cells and protect cells from toxic effect. The cryoprotectants used in cryopreservation are:

- **Alcohols:** Ethylene glycol, glycerol, propylene glycol, sorbitol, mannitol
- **Sulphur containing compounds:** Amino acids, dimethyl sulphoxide (DMSO), sugar (glucose, saccharose)
- **Polymers:** Hydroxyethyl amidon, polyethylene glycol, polyvinyl pyrrolidine

At a sufficiently low temperature, highly concentrated aqueous solutions of cryoprotective agents become so viscous that they solidify into an amorphous "glassy" state, without ice crystal formation (crystallization) at practical cooling rates, this phenomenon is called vitrification. The significance of vitrification in cryopreservation of biological materials is that the cells applied with highly concentrated solution of osmotially active compounds, are protected from internal damage from ice crystal formation during freezing. This pretreatment also causes dehydration of cells. The commonly used cryoprotectants are employed for vitrification like DMSO.

Cryoprotective dehydration

If cells are sufficiently dehydrated they may be able to withstand immersion in liquid nitrogen without further application of traditional cryoprotectant mixtures. Dehydration can be achieved by growing the cultures in the presence of high concentration of osmotically active compounds (sugars) and /or air desiccations in laminar-air-flow cabinet or over silica gel. Dehydration reduces the amount of water available for the ice formation.

Encapsulation and dehydration

This involves the encapsulation of tissues in calcium alginate beads which are pre-grown in liquid culture media containing high concentrations of sucrose. The beads are transferred to sterile airflow in a laminar cabinet and desiccated further. After these treatments, the cells are able to withstand exposure to liquid nitrogen without application of chemical cryoprotectants.

Freezing and storage

The type of crystal water within stored cells is very important for survival of the tissue. Different tissues have different sensitivities for cooling rates. In general, there are three different types of freezing procedures:

Rapid freezing

The plant material is placed in vials, liquid nitrogen is poured directly in the vial and dipping the vial into an open flask filled with liquid nitrogen. In this procedure, cooling between -10°C and -70°C occurred at the rate of >1000°C/min. The quicker the freezing is done, smaller the intracellular ice crystals are formed. In combination with desiccation or vitrification pre-treatments, ultra rapid cooling is proved to be the most attractive method for cryopreservation of plant materials. This method has been successfully used for the cryopreservation of shoot-tips, somatic embryos and embryonal axes from zygotic embryos of a number of plant species. The survival rate of cryopreserved tissues by this method is high and when the desiccation pretreatment is applied even the cryoprotectants are not required.

Slow freezing

The tissue is slowly frozen at a slow cooling rate of 0.5-4°C/min from 0 to -100°C, and then transferred to liquid nitrogen. Survival of cells frozen at slow freezing rates may involve some beneficial effects of dehydration, which minimizes the amount of water that freezes intracellularly. Slow cooling permits the flow of water from the cells to the outside, thereby promoting extracellular ice formation instead of intracellular freezing. It is generally agreed that upon extracellular freezing the cytoplasm will be effectively concentrated and plant cells will survive better when adequately dehydrated. This has been successfully employed for cryopreservation of meristems of few plants and has proved especially successful with cells from suspension cultures.

Stepwise freezing

Firstly, the material is cooled gradually (ca 1°C/min) or step-wise (5°C/min) to an optimum intermediate temperature (-30°C to -50°C) for about 30 min, and then rapidly cooled by dipping into liquid nitrogen. The method is highly favorable for freeze preservation of shoot apices and buds. It is equally successful to cells from suspension cultures.

The initial slow freezing reduces the amount of intracellular freezable water by dehydrating the cells. Early in the freezing process ice is formed first outside the cells, and the unfrozen protoplasm of cells loses water due to the vapor pressure deficit between the supercooled

protoplasm and the external ice. This initial cooling, thus, acts as another pre-treatment for dehydration of the cells.

Storage

Maintaining the frozen material at the correct temperature is as important as proper freezing itself. Temperatures above -130°C may allow ice-crystal growth inside the cells and, as a result reduce their viability. Long-term storage of the material frozen at -196°C, therefore, requires a liquid nitrogen refrigerator.

Generally, the frozen cells or tissues are immediately kept for storage at temperature ranging from -70°C to -196°C. The storage is ideally done in liquid nitrogen refrigerator at -150°C in the vapor phase or -196°C in the liquid phase. The temperature should be sufficiently low for long term storage of cells to arrest all metabolic activities and to prevent biochemical injury.

Thawing

Rapid thawing of the material frozen at -196°C is achieved by plunging it into water at 37 to 40°C which gives thawing rate of 500-750°C/min. After about 90s, the material is transferred to an ice bath and maintained there until recultured or its viability is tested. The transfer is necessary because the cells might get damage if it is left long in the water bath 37-45°C. Rapid thawing protects the cells from the damaging effects of ice crystal formation, which may occur during slow warming.

Re-culturing

The material after thawing should be washed several times to remove the cryoprotectant which may otherwise be toxic to the cells. A gradual dilution of the cryoprotectant is desirable in-order to avoid any deplasmolysis injury to the cells. The plant material frozen at -196°C may need some special requirements for better survival when re-cultured. Shoot-tips from frozen seedlings of tomato directly developed into plantlets only if the medium was supplemented with GA_3. In its absence, apices callused, followed by the differentiation of adventitious shoots.

Applications of Cryopreservation:

- This method of preservation is widely used in different sectors including cryosurgery, molecular biology, ecology, food science, plant physiology, and in many medical applications.
- Traditional cryopreservation is a highly effective method for storing cells and tissues, which works by keeping cells in "suspended animation"
- Sanger Institute explains. "By freezing cells, it stops the metabolic activity and preserves the compounds within cells such as enzymes."
- Without cryopreservation you must keep cells and tissues alive in continuous culture – which means growing and splitting them to generate more cells.
- But cells change as they multiply over time, and this can cause them to lose important characteristics. By freezing them, it reduces the heterogeneity which would otherwise