CRYOPRESERVATION AND IT'S APPLICATION IN PLANT GERMPLASM CONSERVATION

Present Applications

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1. Introduction

1.1 Germplasm Conservation

Germplasm broadly refers to the hereditary component transmitted to the offspring through germ cells. The germplasm provides the breeder with raw material for the development of various crops. So, conservation of germplasm assumes significance in all breeding programs.

As people of old age learnt about the usefulness of plants for food and shelter, they developed the habit of preserving selected seeds or vegetative propagules from one season to the next. In other words, it can be considered primitive but conventional germplasm preservation and management, which is extremely valuable in the breeding program.

The main objective of germplasm conservation or storage is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in the future. In recent years, many new plant species with desirable and improved characteristics have begun to replace primitive and commonly used agricultural plants. Preserving endangered plants is important or otherwise losing some of the valuable genetic characteristics present in primitive plants.

1.2 Approaches for germplasm conservation of plant genetic materials

There are two approaches for germplasm conservation of plant genetic materials:

1. In-situ conservation
2. Ex-situ conservation

In-situ conservation

The germplasm conservation is regarded as In-situ conservation by setting up biosphere reserves or national parks / gene sanctuaries in their natural environment. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

The major limitations of the in-situ conservation

i. The cost of maintenance of a large number of genotypes is very high.
ii. The risk of losing germplasm due to environmental disaster.

Ex-situ conservation

Ex-situ conservation is the main method of preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures can be stored as gene banks for long-term storage under appropriate circumstances. For successful establishment of gene banks, sufficient knowledge of the genetic structure of plant populations and the techniques involved in sampling, regeneration, maintenance of gene pools are essential.

Germplasm conservation in the form of seeds

Generally, seeds are the most common and useful materials for the conservation of plant germplasm.

The certain limitations in the conservation of seeds

There are however, certain limitations in the conservation of seeds, there are:

i. This method is restricted to seed propagating plants. Therefore it is of no use for vegetatively propagated plants e.g. Potato, Ipomoea, Dioscorea.
ii. Over time the validity of the seed is reduced or lost.
iii. Seeds are susceptible to insect or pathogen attack and often lead to their destruction.
iv. It is difficult to maintain clones through seed conservation.

In vitro methods for germplasm conservation

In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

The advantages associated with in vitro germplasm conservation

There are several advantages associated with in vitro germplasm conservation, there are:

i. The germplasm preserved can be maintained in an environment, free from pathogens.
ii. Large quantities of materials can be preserved in small space.
iii. From the germplasm stock, large number of plants can be obtained whenever needed.
iv. Obstacles for their transport through national and international borders are minimal.
v. It can be protected against the nature’s disasters.

1.3 Approaches for the in vitro conservation of germplasm

There are mainly three approaches for the in vitro conservation of germplasm, these are:

1. Cryopreservation
2. Cold storage
3. Low-pressure and low-oxygen storage

Cryopreservation

Cryopreservation (Greek, *krayos-frost*) literally means preservation in the frozen state. Cryopreservation is a process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. The principle involved with cryopreservation is to bring plant cells and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants. For example, the technology of cooling and storing cells at a temperature below the freezing point ('196' C) permits high rates of survivability of the cells upon thawing.

Cryopreservation broadly means the storage of germplasm at very low temperatures

i. Over solid carbon dioxide (at -79°C)
ii. Low temperature deep freezers (at -80°C)
iii. In vapour phase nitrogen (at -150°C)
iv. In liquid nitrogen (at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

Mechanism of Cryopreservation

The technique of cryopreservation or freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C).

When stored at low temperatures, the metabolic processes and biological degradation of cells / tissues become almost stagnant.

Precautions and limitations for successful cryopreservation

Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

Other precautions (the limitations that should be overcome) for successful cryopreservation are listed below:

i. High intracellular concentration of solutes may also damage cells.
ii. Cryoprotectants also affect the viability of cells.
iii. Formation of ice crystals inside the cells should be prevented due to injury to the organelles and the cell.
iv. Sometimes, certain solutes from the cell may leak out during freezing.
v. The physiological status of the plant material is also important.

Technique of Cryopreservation

An outline of the protocol for cryopreservation of shoot tip is illustrated in ***Fig. 1***.

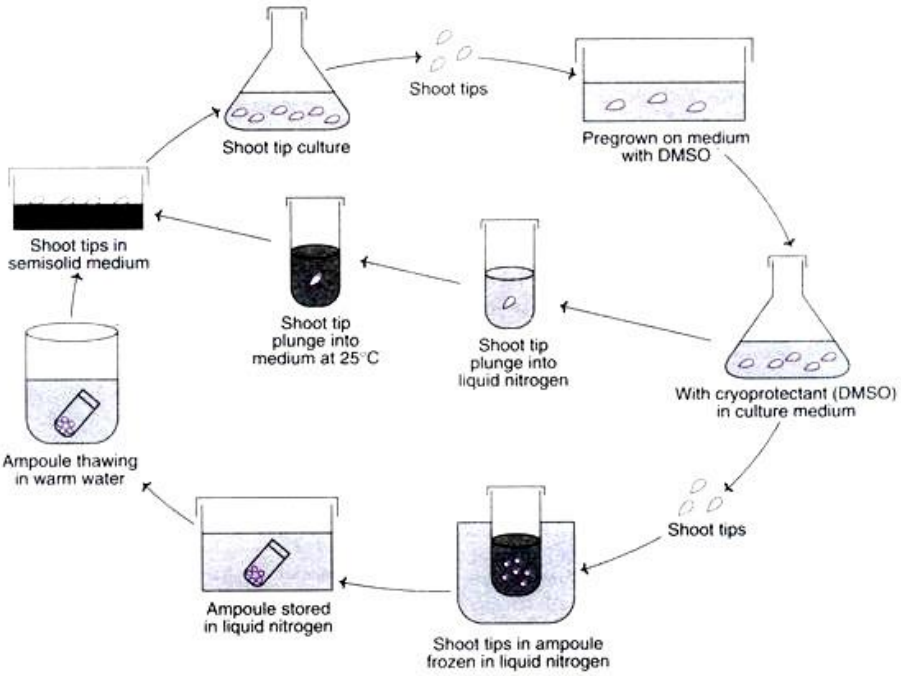

Fig. 1 : *An outline of the protocol for cryopreservation of shoot tip (DMSO – Dimethy Sulfoxide)*

The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages.

1. Development of sterile tissue cultures
2. Addition of cryoprotectants and pretreatment
3. Freezing
4. Storage
5. Thawing
6. Re-culture
7. Measurement of survival/viability
8. Plant regeneration.

The main features of the above stages are briefly described.

1. DEVELOPMENT OF STERILE TISSUE CULTURE

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

2. ADDITION OF CRYOPROTECTANTS 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. There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide.

3. FREEZING

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Different types of freezing methods

There are four different types of freezing methods are used. These are:

1. Slow-freezing method

The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows out of the cell. This promotes the formation of extracellular ice rather than intercellular freezing. As a result of this, the plant cells are partially dehydrated and survive better.

2. Rapid freezing method

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000° C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells.

3. Stepwise freezing method

This is a combination of the slow and rapid freezing procedures, and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen.

4. Dry freezing method

It has reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries.

4. STORAGE

Maintaining a freezing culture at certain temperatures such as freezing is just as important. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduce viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase. The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. Proper documentation of the germplasm storage has to be done.

The documented information must be comprehensive with the following particulars:

i. Taxonomic classification of the material
ii. History of culture
iii. Genetic manipulations done
iv. Morphogenic potential
v. Somaclonal variations
vi. Culture medium
vii. Growth kinetics

5. THAWING

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water with a temperature of 37-45°C bath with vigorous swirling. By this approach, rapid thawing at the rate of 500- 750°C min-1 occurs, and this protects the cells from the damaging effects ice crystal formation.

6. RE-CULTURE

Usually thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

7. MEASUREMENT OF SURVIVAL / VIABILITY

The viability/survival of the frozen cells can be measured at any stage of cryopreservation. It also can be measured after thawing or re-culture. The techniques employed to determine the viability of cryopreserved cells are the same as used for cell cultures. The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture.

$$\frac{No.of\ cell/organs\ growing}{No.of\ cell/organs\ thawed}\ X\ 100$$

8. PLANT REGENERATION

The final purpose of cryopreservation of germplasm is to reproduce the desired plant. The cryopreserved cells or tissues have to be carefully nursed and grown for appropriate plant growth and regeneration. A selected list of plants in various forms that have been successfully used for cryopreservation is given in ***Table 1***.

Plant material	Plant species
Cell suspensions	Oryza sativa Glycine max Zea mays Nicotiana tabacum Capsicum annum
Callus	Oryza sativa Capsicum annum Saccharum sp
Protoplast	Zea mays Nicotina tabacum
Meristems	Solanum tuberosum Cicer arientinum
Zygotic embryos	Zea mays Hordeum vulgare Manihot esculenta
Somatic embryos	Citrus sinensis Daucus carola Coffea arabica
Pollen embryos	Nicotiana tabacum Citrus sp Atropa belladonna

Table 1 : *A selected list of plants in various forms that are successfully cryopreserved*

Cold Storage

Cold storages basically involve germplasm conservation at low and non-freezing temperatures (1-9°C). The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation. Therefore, cold storage is considered as a slow-growth germplasm conservation method. The main advantage of this approach is that the plant material (cells or tissues) does not suffer cryogenic injury.

Virus free strawberry plants could be preserved at the temperature of 10°C for about 6 years with the addition of a few drops of medium periodically once in 2-3 months.

Low-Pressure and Low-Oxygen Storage

As alternatives to cryopreservation and cold storage, Low-Pressure Storage (LPS) and Low-Oxygen Storage (LOS) have been developed for germplasm conservation. A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure and low-oxygen is illustrated in ***Fig. 2***.

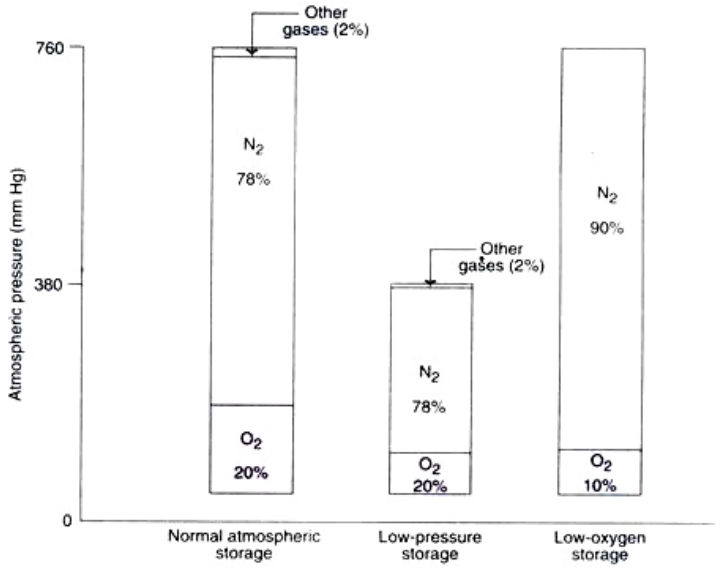

Fig. 2 : *A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure, and low-oxygen*

Low-Pressure Storage (LPS)

In Low-Pressure Storage (LPS), atmospheric pressure is reduced around the plant material. This results in a partial decrease of the pressure exerted by the

gases around the germplasm. The in-vitro growth of plants of organized or unorganized tissues is reduced when partial pressure is reduced. The short-term storage is particularly useful to increase the shelf life of many plant materials e.g. plant cuttings fruits, cut flowers, vegetables.

Low-Oxygen Storage (LOS)

In Low-Oxygen Storage (LOS), the oxygen concentration is reduced; however the atmospheric pressure is maintained by adding inert-gases. The partial pressure of oxygen below 50 mm Hg reduces growth of plant tissues. As a consequence, the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

Applications of Germplasm Storage:

The germplasm storage has become a boon to plant breeders and biotechnologists.

Some of the applications are briefly described below-

1. Maintenance of stock cultures: Plant materials of several species can be cryopreserved and maintained for several years, and used as and when needed.
2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce medicines.
3. Plant materials from endangered species can be conserved.
4. Recalcitrant seeds can be maintained for long.
5. Disease free plant materials can be frozen and propagated whenever required.
6. Conservation of pollen for enhancing longevity.
7. Cryopreservation is a good method for selection of cold resistant mutant cell lines which could develop into frost resistant plants.
8. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:

The major limitations of germplasm storage are the expensive equipment and the trained personnel. It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.