Course : Plant Genetic Resources: Conservation & Sustainable Use
Chapter **:** 06
Topic : Techniques for conservations
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TECHNIQUES FOR PLANT GERMPLASM CONSERVATION

Plant Genetic Resources

The sum totals of hereditary material i.e. all the alleles of various genes, present in a crop species and its wild relatives is referred to as germplasm. This is also known as genetic resources or gene pool or genetic stock.

Important features of plant genetic resources are given below.

- Genetic pool represents the entire genetic variability or diversity available in a crop species.
- Germplasm consists of land races, modern cultivars, obsolete cultivars, breeding stocks, wild forms and wild species of cultivated crops.
- Germplasm includes both cultivated and wild species and relatives of crop plants.
- Germplasm is collected from centres of diversity, gene banks, gene sanctuaries, farmer's fields, markers and seed companies.
- Germplams is the basic material for launching a crop improvement programme.
- Germplasm may be indigenous (collected within country) or exotic (collected from foreign countries)

GERMPLASM CONSERVATION

Conservation refers to protection of genetic diversity of crop plants from genetic erosion. There are two important methods of germpalsm conservation or preservation.

i) In-situ conservation and

ii) ex situ conservation.

These are described below.

i) In - situ conservation:

Conservation of germplasm under natural conditions is referred to as in situ conservation. This is achieved by protecting the area from – human interference, such an area is often called natural park, biosphere reserve or gene sanctuary. NBPGR, New Delhi, established gene sanctuaries in Meghalaya for citrus, north Eastern regions for musa, citrus, oryza and *saccharum*. **Or**

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. 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.

Merits:

- In this method of conservation, the wild species and the compete natural or seminatural ecosystems are preserved together.

Demerits:

- Each protected area will cover only very small portion of total diversity of a crop species, hence several areas will have to be conserved for a single species.
- The risk of losing germplasm due to environmental hazards
- The cost of maintenance of a large number of genotypes is very high.

iii) Ex - situ conservation:

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or in form of in- vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions. This is the most practical method of germplasm conservation.

Advantages

- It is possible to preserve entire genetic diversity of a crop species at one place.

- Handling of germplasm is also easy.
- This is a cheap method of germplams conservation.

Limitations

- Viability of seeds is reduced or lost with passage of time.
- Seeds are susceptible to insect or pathogen attack, often leading to their destruction.
- This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.
- It is difficult to maintain clones through seed conservation

Forms/ classification of Ex - situ conservation

1) Seed banks:

Germplam is stored as seeds of various genotypes. Seed conservation is quite easy, relatively safe and needs minimum space. Seeds are classified, on the basis of their storability into two major groups. 1) Orthodox and 2) Recalcitrant

Orthodox seeds: Seeds which can be dried to low moisture content and stored at low temperature without losing their viability for long periods of time is known as orthodox seeds. (eg.) Seeds of corn, wheat, rice, carrot, papaya, pepper, chickpea, cotton, sunflower.

Recalcitrant: Seeds which show very drastic loss in viability with a decrease in moisture content below 12 to 13% are known as recalcitrant seeds. (e.g) citrus, cocoa, coffee, rubber, oilpalm, mango, jack fruit etc.

Seed storage:

Based on duration of storage, seed bank collects are classified into three groups.

(1) Base collections. (2) Active collections and (3) Working collection.

Base collections: Seeds can be conserved under long term (50 to 100 years), at about -20 ^{O}C with 5% moisture content. They are disturbed only for regeneration.

Active collection: Seeds are stored at 0 ^{O}C temperature and the seed moisture is between 5 and 8%. The storage is for medium duration, i.e., 10-15 years. These collections are used for evaluation, multiplication, and distribution of the accessions.

Working collections: Seeds are stored for 3-5 years at 5-10 ^{O}C and the usually contain about 10% moisture. Such materials are regularly used in crop improvement programmes.

2. Plant Bank: (Field or plant bank) is an orchard or a field in which accessions of fruit trees or vegetative propagated crops are grown and maintained-

Limitations:

1. Require large areas
2. Expensive to establish and maintain
3. Prone to damage from disease and insect attacks
4. Man – made
5. Natural disasters
6. Human errors in handling

3. Shoot tip banks: Germplasm is conserved as slow growth cultures of shoot-tips and node segments. Conservation of genetic stocks by meristem cultures has several advantages as given below.

- Each genotype can be conserved indefinitely free from virus or other pathogens.
- It is advantageous for vegetatively propagated crops like potato, sweet potato, cassava etc., because seed production in these crops is poor
- Vegetatively propagated material can be saved from natural disasters or pathogen attack.
- Long regeneration cycle can be envisaged from meristem cultures.
- Regeneration of meristerms is extremely easy.
- Plant species having recalcitrant seeds can be easily conserved by meristem cultures.

4. Cell and organ banks: A germplasm collection based on cryopreserved (at – 196 ^{O}C in liquid nitrogen) embryogenic cell cultures, somatic/ zygotic embryos they be called cell and organ bank.

5. DNA banks: In these banks, DNA segments from the genomes of germplasm accessions are maintained and conserved.

SPECIFIC METHODS GERMPLASM CONSERVATION (ex-situ /In-vitro)

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.

There are several advantages associated with in vitro germplasm conservation:

i. Large quantities of materials can be preserved in small space.

ii. The germplasm preserved can be maintained in an environment, free from pathogens.

iii. It can be protected against the nature's hazards.

iv. From the germplasm stock, large number of plants can be obtained whenever needed.

v. Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

Approaches conservation

A. Cryobank and Cryopreservation (freeze-preservation)

B. Cold storage

C. Low-pressure and low-oxygen storage

A.CRYOBANK AND CRYOPRESERVATION (FREEZE-PRESERVATION)

Cryobank

A cryobank is a facility that is capable of storing germplasm (normally in cell/ tissue form) under a such low temperatures that the cells are prevented from damaging themselves or evolving from their initial state.

1. Cryopreservation:

Cryopreservation (***Greek word- krayos-frost***) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a

zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

Cryopreservation techniques are probably the most promising approach for preserving *ex situ* plant genetic resources and are of extreme importance when applied to plant species in danger of extinction. The ultralow temperatures of liquid nitrogen (-196ºC) allow the conservation of germplasm for a long period of time without deterioration, because at these temperatures all metabolic processes are drastically reduced. Different plant materials, such as seeds, shoot tips, nodal explants, cell suspensions, dormant buds and others, can be cryo-preserved.

Plant germplasm stored in liquid nitrogen does not undergo cellular divisions. In addition, metabolic and most physical processes are stopped. Therefore, plant germplasm preserved under cryogenic storage can be maintained for very long periods of time and problems that are typical for storage in the active growth state, like genetic instability and the loss of accessions due to contamination, loss of vigour and toti-potency and human error during continual sub-culturing, are overcome.

Forms of Cryopreservation

- Over solid carbon dioxide (at -79°C)
- Low temperature deep freezers (at -80°C)
- In vapour phase nitrogen (at -150°C)
- 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 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 temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

***Precautions for successful cryopreservation* :**

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.

***Technique of Cryopreservation*:**

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 salient 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 cryo-protectants and pre-treatment:

Cryo-protectants 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. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

3. Freezing:

Freezing methods -

3.1. 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 from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures

3.2. 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. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. 3. Stepwise freezing method:

This is a combination of slow and rapid freezing procedures (with the advantages of both), 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. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

3.4. Dry freezing method:

Some workers have 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. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

4. Storage:

Maintenance of the frozen cultures at the specific temperature is as important as freezing. 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 reduces 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. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

5. Thawing:

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 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.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

6. Re-culture:

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a 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 or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{No.of\ cells/organs\ growing}{No.of\ cells/organs\ thawed} \times 100$$

8. Plant regeneration:

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

B. COLD STORAGE:

Cold storage basically involves germplasm conservation at a 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. Hence, cold storage is regarded as a slow growth germplasm conservation method. The major advantage of this approach is that the plant material (cells/tissues) is not subjected to cryogenic injuries.

Long-term cold storage is simple, cost-effective and yields germplasm with good survival rate. Many in vitro developed shoots/plants of fruit tree species have been successfully stored by this approach e.g. grape plants, strawberry plants.

Virus- free strawberry plants could be preserved at 10°C for about 6 years, with the addition of a few drops of medium periodically (once in 2-3 months). Several grape plants have been stored for over 15 years by cold storage (at around 9°C) by transferring them yearly to a fresh medium.

C. 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 depicted in fig. below-

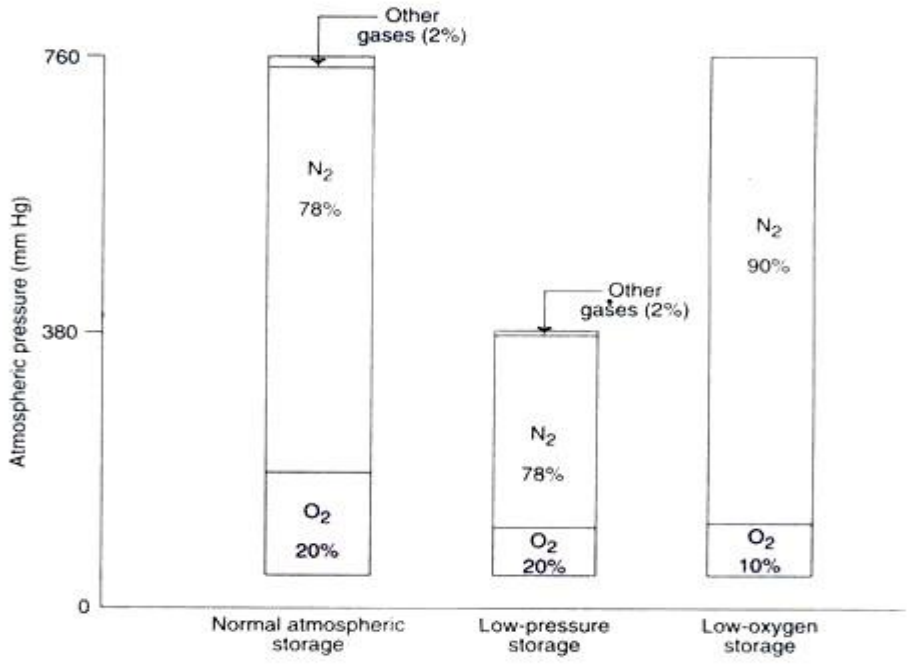

Fig. 48.2 : A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure, and low-oxygen.

C.1: Low-Pressure Storage (LPS):

In low-pressure storage, the atmospheric pressure surrounding the plant material is reduced. This results in a partial decrease of the pressure exerted by the gases around the germplasm. The lowered partial pressure reduces the in vitro growth of plants (of organized or

unorganized tissues). Low-pressure storage systems are useful for short-term and long-term storage of plant materials.

The short-term storage is particularly useful to increase the shelf life of many plant materials e.g. fruits, vegetables, cut flowers, plant cuttings. The germplasm grown in cultures can be stored for long term under low pressure. Besides germplasm preservation, LPS reduces the activity of pathogenic organisms and inhibits spore germination in the plant culture systems.

C.2: Low-Oxygen Storage (LOS):

In the low-oxygen storage, the oxygen concentration is reduced, but the atmospheric pressure (260 mm Hg) is maintained by the addition of inert gases (particularly nitrogen). The partial pressure of oxygen below 50 mm Hg reduces plant tissue growth (organized or unorganized tissue). This is due to the fact that with reduced availability of O_2, the production of CO_2 is low. As a consequence, the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

Source:

http://www.biologydiscussion.com/biotechnology/germplasm-conservation/germplasm-conservation-and-cryopreservation-with-diagram/10757
http://agritech.tnau.ac.in/crop_improvement/crop_imprv_plantgeni.html
http://www.plantbiotechnologylab.pt/index.php/en/research/24-germplasm-conservation
https://ivf.ilaya.com/what-is-a-cryobank/