**PLANT TISSUE CULTURE**

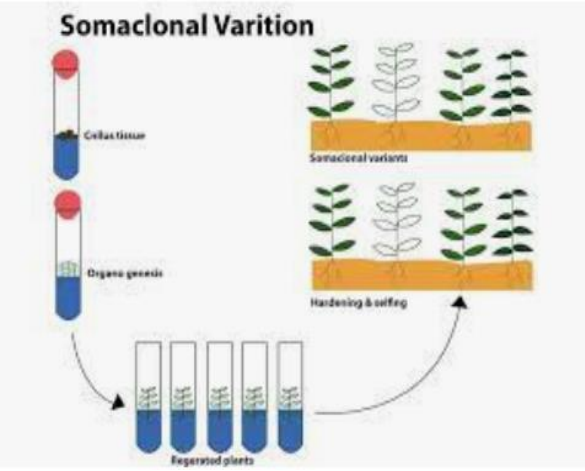
**UNIT II**

## SOMOCLONAL VARIATION: -

**Somaclones are the genetically identical plants produced by using the somatic cells or the vegetative parts of the plants through the tissue culture technique. Somoclonal variation is defined as genetic variation observed among progeny plants obtained after somatic tissue culture in vitro.**

**The word somoclonal variation is derived from Greek, Soma-vegetative cells, clonal-to make copy, variations-to make changes.**

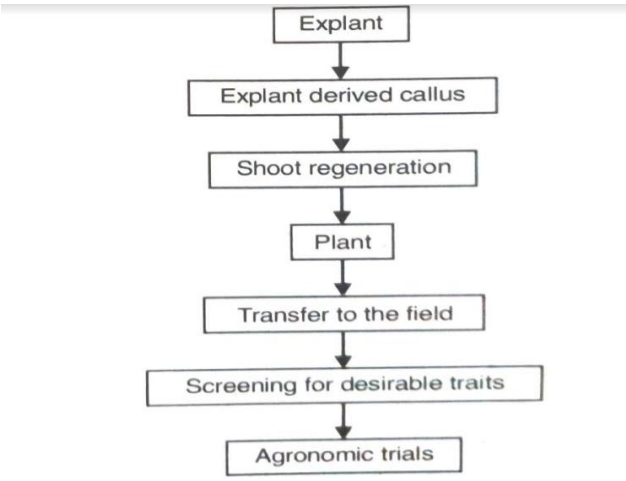
The term somoclonal variation by **Larkin** and **Scowcroft (1981**) was given for the variability generated by the use of a tissue culture cycle. While most tissues of the clone retain uniformity and identity to parent, genetic changes occur in some tissues and these changes are transmitted to regenerated plants. Such plants that differ from their parents in one or few traits are called somatic variants. The formation of somatic variants among the tissues in cultures is called somoclonal variation. It may occur spontaneously during repeated subcultures or due to induced mutations. Now a days, production of somoclonal variants is one of the objectives of tissue culture. Plants differ from their parents in or free traits are called **somatic variants.** The formation of somatic variants among the tissue cultures are called **somatic variation.** Somoclonal variants are obtained from the cultures of embryos, meristem, anther, leaf callus, tip of inflorescence, microspores, ovaries and protoplasts. Before regenerating plantlets, the Calli are tested for the desired trait (variation) expected in them. Somoclonal variants have been selected for the agricultural traits. This variation includes aneuploids, sterile plants and morphological variants, sometimes involving traits of economic importance in case of crop plants. The usefulness of variation was first demonstrated through the recovery of disease resistant plants in potato (resistance against late blight and early blight) and sugarcane (resistance against eye-spot disease, Fiji disease and downy mildew)




**Diagrammatic representation of somoclonal variation**

**Without in vitro Selection for agronomic traits**

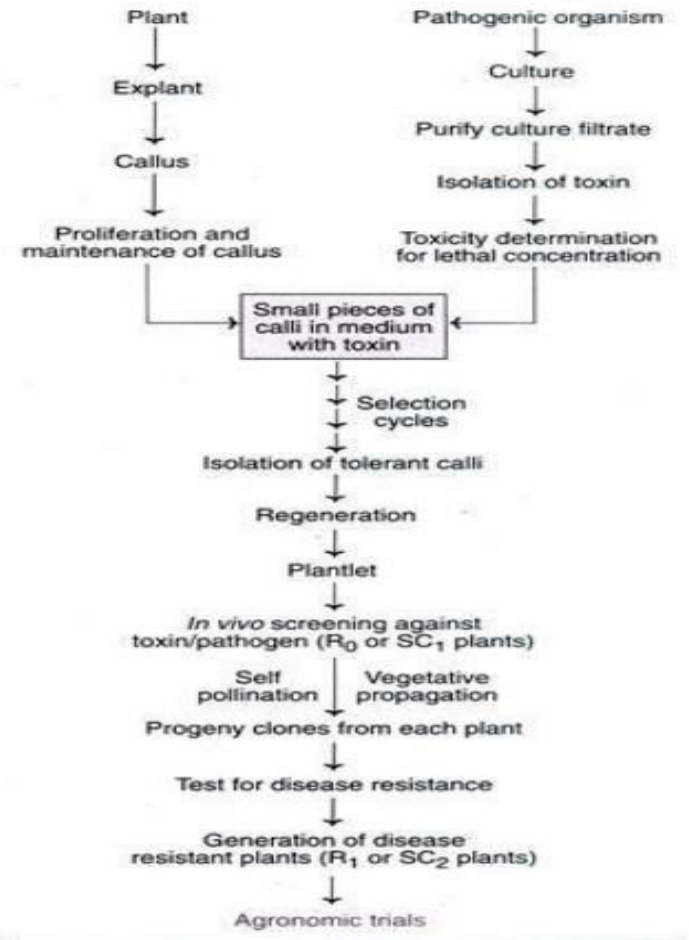
An explant is cultured on a suitable medium, e.g., small shoot segments (1-2 cm) of sugarcane, cotyledons, hypocotyls, protoplasts, leaves, embryos, etc. The basal medium supplemented with growth regulators which support dedifferentiation stage, i.e., callus. Normally these cultures are subculture and then transferred to shoot induction medium for plant regeneration. The plants so regenerated are transferred to pots, grown to maturity and analysed for variants. The approach is to find somoclonal variants among the regenerated plants for various characters. Here no directed approach is used and appearance of desirable variant is a chance event. In such a method, both dominant and homozygous recessive traits can be directly selected. If the regenerants are heterozygous, then recessive traits can be selected in the progenies of regenerants. Epigenetic variation will also be avoided when progenies are used. Thus, in case of self-fertilizing crops it is recommended to screen the progenies of the regenerants. Unfortunately, a disadvantage of this approach is that it is time consuming due to the fertilization step and requires screening of many plants.




**A flow diagram for generation of somoclonal variation without in vitro selection.**

**Within vitro Selection for disease resistance: -**

Isolation of somaclones with in vitro selection method basically involves handling of plant cells in cultures (protoplast, callus) like microorganisms and selection of biochemical mutants. It is now well known that in vitro culture of higher plants can be used for selection of mutants. Protoplast, cell suspension and callus cultures are handled like microorganisms to search for biochemical mutants. Selection for resistance is the most straightforward method for mutant selection, whereby resistant cells in a large population can be selected by their ability to grow in the presence of an inhibitor while the sensitive cells do not. The protocol has been described in Fig. Here the dedifferentiated culture (callus) is subjected to selection against inhibitors like antibiotics, amino acid, pathotoxins, etc. These compounds are put in the medium at a concentration such that some cell population survives and can be further grown on a selective medium. Different selection cycles are performed to get tolerant cells/callus cultures that are subsequently regenerated into plants. These plants are then in vivo screened against the inhibitor. If the plants are resistant to the inhibitor, then stable transmission of that character is analyzed in subsequent generations. In this approach, variants for a particular character are selected rather than the general variation obtained in first case where selection is done at the plant level.




**A flow diagram for generation of somoclonal variation with in vitro selection for disease resistance.**

**Factors/Causes for somoclonal variation**: -Somoclonal variations occur as a result of genetic and non-genetic factors in plant tissue cultures.

**Genetic factors: -**

1. Chromosome structure
2. Chromosome number
3. Mitotic crossing over
4. Cytoplasmic genetic changes.
5. DNA-methylation.
6. Transposable elements.
7. Nuclear changes.

**Non-genetic factors: -**

1. **Nature of explant**: -The nature of genotype of the plants influences the frequency of regeneration and frequency of production of somaclones. Explants can be taken from any part of plant — leaves, roots, internodes, ovaries etc. The source of explant is very critical for somoclonal variations.
2. **Duration of cell culture: -** In general, for many plant cultures, somoclonal variations are higher with increased duration of cultures. For example, it was reported that genetic variability increased in tobacco protoplasts from 1.5 to 6% by doubling the duration of cultures.
3. **Growth hormone effects: -**The plant growth regulators in the medium will influence the karyotypic alterations in cultured cells, and therefore development of somaclones. Growth hormones such as 2, 4-dichlorophenoxy acetic acid (2, 4-D) and naphthalene acetic acid (NAA) are frequently used to achieve chromosomal variability.

**4.Culture Condition:** - It has been known that growth regulator composition of the culture medium can influence frequently of karyotypic attention in cultured cells.

**Nomenclature of somoclonal variation:**

- Though different letters and symbols have been used, two symbols are generally used.
- Chaleff (1981) has labelled the plants regenerated from tissue culture as R or R0 plants and the self-fertilized progeny of R0 plants as R1. The somaclones that are regenerated from tissue cultures directly are regarded as R0 or R plants. The self-fertilized progeny of R0 plants represent R1 plants. R2, R3, R4 etc. plants are the subsequent generations. Some workers use other nomenclature — somaclones (SC1 = R0), SC2, SC3, SC4 etc. for subsequent generations.
- Subsequent generations produced by self-fertilization are termed R2, R3, R4, etc. Larkin and Scowcroft (1981) have referred regenerated plants as SC1 (=R0) and subsequent self-fertilized generations as SC2, SC3, SC4, etc

**Somoclonal variation in agronomic traits: -**

Agronomic traits mean improving the plant height, total biomass, productivity, good, photosynthetic capacity, increased crop yield, improved nutritional content, change in maturity time.

**Examples**

1. Rice-Number of tillers per plant, fertile tillers per plant, panicle length, plant height, early maturity, seed fertility, disease resistance, drought tolerance and cold tolerance.
2. Wheat-Plant height, awns, tiller number. grain colour, spike shape, gliadin protein, maturity, leaf wax, a-amylase, temperature tolerance and disease resistance.
3. Maize-Plant height, node number, ear arrangement, stalk number, toxin resistance, mitochondrial pattern, etc.
4. Brassica-Flowering time, plant height, leaf wax and disease resistance.
5. Tobacco-Plant height, leaf size, alkaloid content
6. Tomato-Disease resistant and early maturity.
7. Sugarcane-Sugar content, auricle length, disease resistance and early maturity.
8. Potato-High protein content, early maturity, resistance to viruses and Phytophthora
9. Legumes-Resistance to pod borer, Fusarium wilt.
10. Groundnut-Shallow depth of pod development, induction of dormancy, resistance to Fusarium and Aspergillus
11. Sunflower-Self compatibility, resistance to Alternaria blight, Rhizoctonia and Fusarium.

Somoclonal variants of sugarcane with resistance to Fiji disease virus, downy mildew and eye-spot disease were isolated from tissue cultures. **In India, the Sugarcane Breeding Institute (Coimbatore) has released sugarcane varieties obtained through somoclonal variation. Such varieties are resistant to red rot, higher in sugar yield and cane yield.** somoclonal variants of potato from leaf protoplast cultures of Russet Burbank variety. These somoclonal variants have high growth rate, early maturity, tuber uniformity and tuber skin colour, onset of tuberization. Potatoes with altered

**Applications of Somoclonal Variations**

i. **Production of Novel variants**: An implication of somoclonal variation in breeding is that novel variants can arise and these can be agronomically used. A number of breeding lines have been developed by somoclonal variation. **Example**: An example of heritable somoclonal variation is the development of pure thornless blackberries

**ii. Distinctive mutations** may sometimes give rise to elite characters in the regenerants which cannot be achieved by conventional methods of breeding.

**iii. Disease resistant genotypes** of various plants can be attained. Resistance was first reported in sugarcane for eye spot disease (*Heliminthosporium sacchari*) and Fiji virus disease by regenerating plants from callus of susceptible clones.

**iv.** Plants with characteristic resistance to abiotic stress (cold, draught, acidic or alkaline soil) can be obtained as somaclones.

**v. Seed quality improvement:** - Recently, a variety Bio L 212 of lathyrus (*Lathyrus sativa*) has been identified for cultivation in central India which has been developed through somoclonal variation and has low ODAP (β-N-oxalyl -2-α, β diamino propionic acid), a neurotoxin indicating the potential of somoclonal variations for the development of varieties with improved seed quality.

**vii.Production of Drought tolerance:** - Drought tolerant rice lines were obtained by in vitro selection of seed induced callus on a media containing polyethylene glycol as a selective agent which simulated the effect of drought in tissue culture conditions.

**vii.Production of Salt tolerance:** - Plant tissue culture techniques have been successfully used to obtain salt tolerant cell lines or variants in several plant species, like. tobacco, alfalfa, rice, maize, Solanum *nigrum*, sorghum, etc. In most cases, the development of cellular salt tolerance has been a barrier for successful plant regeneration, or if plants have been obtained, they did not inherit the salt tolerance.

**vii**.**Production of Aluminium tolerance:** -Plant species or cultivars greatly differ in their resistance to aluminium stress. In recent years, considerable research has been focused on the understanding of physiological, genetic and molecular processes that lead to aluminium tolerance.

**IX Production of Herbicide resistance:** Through in vitro selection several cell lines resistant to herbicides have been isolated and a few have been regenerated into complete plants.
Among the important achievements are tobacco, soybean, wheat, maize, etc. resistant to various herbicides such as glyphosate, sulfonylurea, imidazolines, etc

**X)Production of Cold tolerance:** Azar et al. (1988) developed somoclonal variants for freezing tolerance in Norstar winter wheat. A significant positive correlation between proline level and frost tolerance has been found in a broad spectrum of genotypes. In vitro selection and regeneration of hydroxyproline resistant lines of winter wheat with increased frost tolerance and increased proline content has been reported. The results showed strong correlation of increased frost tolerance with increased proline content.

**Limitations of Somoclonal variations**

i. Poor plant regeneration from long-term cultures of various cell lines.
ii. Regeneration being limited to specific genotypes which may not be of much interest to breeders.
iii. Some somaclones have undesirable features, such as aneuploidy, sterility etc.
iv. Unpredictable variations that are often generated are of no use.
v. Variations attained may not always be stably integrated.
vi. Variants attained may not always be novel. In majority of cases improved variants are not even selected for breeding programs.
vii.Uncontrollable and unpredictable nature of variation and most of the variations are of no apparent use.
it. The variation is cultivar dependent.
ixThe variation obtained is not always stable and heritable. The changes occur at variable frequencies.

**Somatic embryogenesis: -**

**Somatic embryogenesis is the development of embryos form vegetative cells with in vitro systems. Specific tissues have a capacity for somatic embryogenesis in cultural systems. This allows the clonal propagation of normally seed-propagated crops analogous to the production of apomictic seedlings** Somatic embryogenesis is the process wherein somatic cells differentiate into somatic embryos. It is not a naturally occurring process, an artificial one wherein an embryo or plant is obtained from one somatic cell. Somatic embryos take form from the cells of the plants, which usually do not take part in embryo development. Neither a seed coat nor endosperm is formed around the somatic embryo. In the process, one cell or a cluster of cells initiates the developmental route, which results in reproducible regeneration of non-zygotic embryos, which can germinate for the formation of an entire plant. The cells which are derived from potential source tissues are subject to a culture medium for the formation of an undifferentiated cluster of cells referred to as the callus. In the tissue culture medium, the plant growth regulators can be formed for the induction of the formation of calluses and hence modified to induce the embryos for the formation of calluses.

**Somatic Embryos:- Somatic embryos are bipolar structure with both apical and basal meristematic regions which are capable of forming shoot and root.**Somatic embryos develop through stages similar to zygotic embryos, however, the final size for the cotyledons are usually reduced and there is no development of endosperm or seed coat

**Somatic Embryogenesis formation of embryos from somatic cells while culturing them in vitro is called somatic embryogenesis**. **The embryos thus formed from somatic cells are called embryoids/somatic embryoids. s**omatic embryo (SE) is an embryo derived from somatic cell other than zygote, usually on culture in vitro. Somatic embryogenesis may be defined as process of development of a bipolar structure like zygotic somatic cell.

Somatic embryogenesis can be induced both in suspension cultures and in callus cultures.

**Ammirato (1983) has reported that at present somatic embryogenesis is observed in tissue culture of plants belonging to about 30 families.**

**Somatic cells are induced to become embryogenic by auxins in the culture medium. Among various auxins being used for tissue culture, 2,4-D (2,4-Dichlorophenoxy acetic acid) and 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) are powerful to initiate embryogenic cells, but**

**IAA(Indole Acetic Acid), IBA (Indole-3-butyric acid)and NAA (Naphthaleneacetic acid)are ineffective.** After the formation of embryogenic cells, the callus or cell suspension is transferred to a medium lacking auxin for the development of embryoids. Reduced amount of nitrogen and high level of potassium and proline favour for embryogenesis from the embryogenic cells. Embryoid is a mass of tissue produced during culture. Formation of embryoid from pollen in tissue culture medium is due to cellular totipotency. Cell potency is a cell's ability to differentiate into other cell types. The more cell types a cell can differentiate into, the greater its potency. Embryoid is a small, well-organised structure comparable to the sexual embryo, which is produced in tissue culture of dividing embryo genic potential somatic cells.

**Somatic embryos show the following salient features:**

- Somatic embryos are structurally similar to zygotic embryo in the seed.
- As in the development of zygotic embryo, they also pass through globular, heart-shaped and torpedo stages.
- They have enough totipotency to grow into complete plants.
- They take their development from somatic cells.
- In contrast to zygotic embryos, all embryoids produced from a single cell or tissue are genetically identical.
- Somatic embryos offer an efficient method of clonal propagation.
- Somatic embryos do not become dormant.
- No vascular connection in somatic embryo.
- Zygotic embryos are usually diploid (sometimes may be polyploidy) but somatic embryos may be haploid, diploid or triploid

**Process of Somatic Embryogenesis**

The somatic embryogenesis procedure is a three-step procedure, which causes the induction of embryogenesis, development of the embryo and its maturation. The principle of somatic embryogenesis finds its basis on the topic of totipotency of the plant cells.

**Figure showing the embryoid formation**

Somatic Embryogenesis Stages – Steps of Somatic Embryogenesis

**1)Induction**

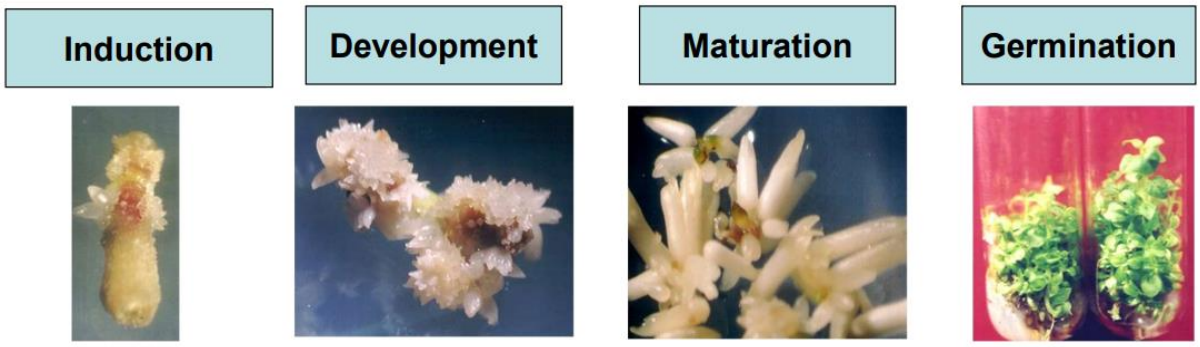
For the process of induction, auxins, specifically 2, 4-D are typically essential. The necessity of exogenous auxin to induce somatic embryogenesis is based on the nature of the explants, which are made use of with a proportional concentration of the auxins.

**2)Development**

Once reinitiation of the process of cell division and a stage of cell proliferation occurs in the presence of auxins, embryogenic cells are liberated in the auxin-free medium. Such cells are in groups of cytoplasmic cells referred to as the PEMs (Pro Embryonic Mass of Cells).

**3)Maturation**

The standard of the somatic embryos in aspects of their conversion into plants or germinability is degraded as a result of usually normal-seeming somatic embryos, which in actuality are incomplete in their development. The somatic embryos, as opposed to seed embryos, do not experience the last stage of embryogenesis referred to as embryo maturation that is distinguished by the collection of embryo-specific reserve food substances and proteins imparting desiccation tolerance to the embryos. The size of the embryos does not increase at this stage.




**Types of Somatic Embryogenesis**

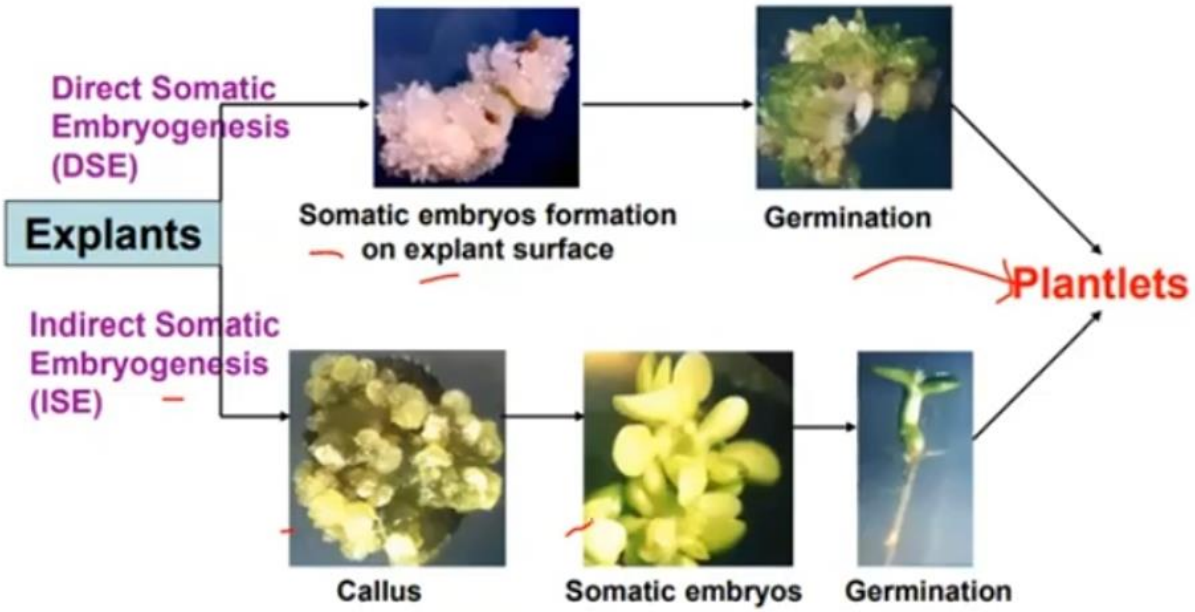
**Direct somatic embryogenesis**

It involves the development of the embryos in a direct way from the cells of the explants, such as the cells of the immature embryos. Here, there is no intermediary stage (like the formation of the callus). The explants of the somatic embryogenesis are seen to entail PEDCs (pre-embryogenic determined cells). **Direct embryogenesis involves the development of embryos directly from the cells of explants, like from the cells of immature embryos. It doesn't involve any intermediary stages, such as callus formation.** The explants of somatic embryogenesis are observed to carry pre-embryogenic determined cells (PEDCs). The embryo initiates directly from the explant tissue in the absence of callus proliferation. This occurs through pre-embryonic determined cells are the cells are committed to embryonic its development and need only to be released. Such cells are found in embryonic development and need only to be released. Such cells are found in embryonic tissues certain tissues of young in-vitro grown plantlets like hypocotyl in Daucus Corota.

**Indirect somatic embryogenesis**

It includes the formation of somatic embryos by reiterating numerous cycles of cell divisions. It includes intermediary steps of growth of the callus, and hence the process includes multiple steps.The cells which do not carry the pre-embryogenic determined cells are caused to

differentiate for the formation of the embryo by revealing different treatments. The cells modify into IEDs (induced embryogenic pre-determined cells).




**Indirect embryogenesis:** -The embryogenesis can be best explained by following method: -

1. Explant (petiole or stem or leaf) is grown on MS medium containing **(auxin)** to induce the formation of callus and embryogenic cells. Along with auxin sucrose (2-5%) is added which induces the production of callus.
2. The callus is dispersed in liquid B5 medium containing **do not have auxin for 7 days**. During this period, embryogenic cells get separated and remain in the suspension.
3. The cell suspension is sieved to get 220500mm fraction containing small masses of embryogenic cells.
4. The fraction containing embryogenic clumps is transferred to solid BOi2Y medium lacking 2,4-D and incubated for 7-10 days at 25°C. The embryoids undergo morphological changes like globular, heart-shaped and torpedo stages.
5. After attaining the torpedo stage, the embryoids are collected and transferred to enriched BOi2Y medium containing high level of sucrose, nitrogen and sulphur. During this step, the embryoids accumulate food reserves and increase in size and dry weight. This is often called first maturation phase.

6. After reaching 1-2 mg dry weight, the embryoids are transferred to modified BOi2Y medium containing abscisic acid (ABA) for 3 days. Now, the embryoids grow in size and become resistant to desiccation. This is called second maturation phase.

7. The embryoids are collected from the culture and washed with sterile water to remove nutrients and sugars.

8. The washed embryoids are dried by keeping them in a sealed chamber till the moisture content of embryoids becomes approximately 15%.

9. These embryoids can be stored for 1 year with good viability. Thus, as many as 900 embryoids can be obtained from a single Petri plate.

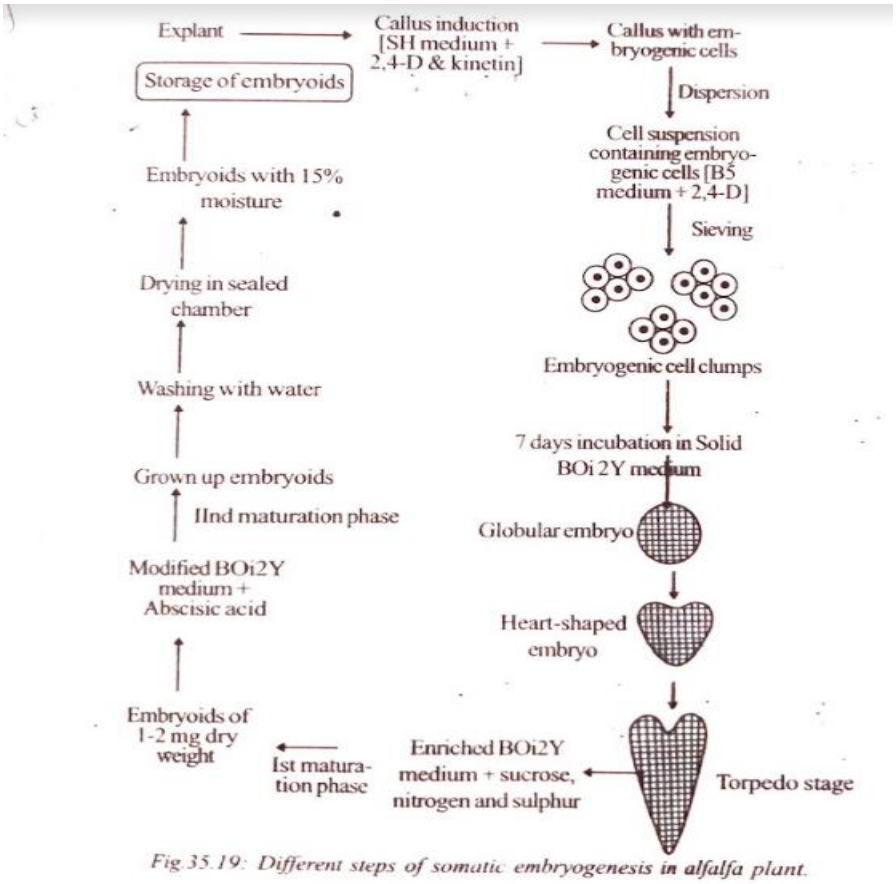
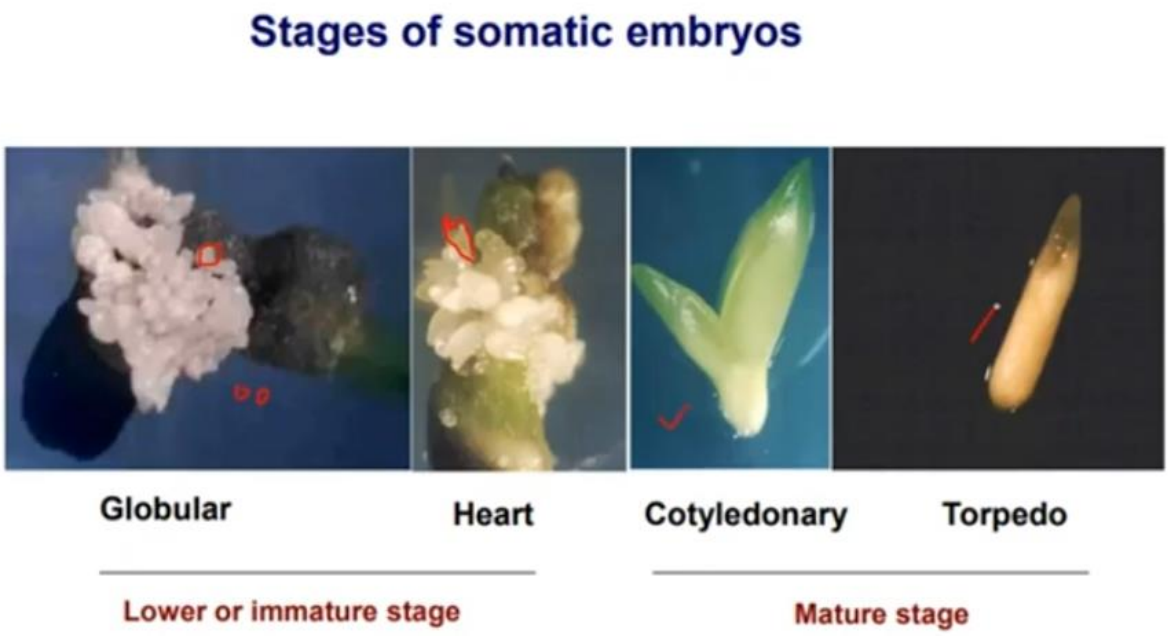



*Fig 35.19: Different steps of somatic embryogenesis in alfalfa plant.*

**Diagrammatic representation of indirect somatic embryogenesis**




**Factors effecting the process of somatic embryogenesis:**

**1)Traits of explant**

Despite the fact that variations of explants can be used, the apt stage of development of explants is vital too to initiate the embryogenic callus; whereas juvenile explants tend to give rise to more somatic embryos compared to older explants. Also, different explant explants tissues from the same mother plant generated embryogenic callus at varying frequencies.

The desired species of plants to be induced for embryogenesis decides the choice of explants. For the majority of plant species, explants of immature zygotic embryos are apt for somatic embryogenesis.

**2)Growth regulators**

Cytokinins: These have been in use in the primary medium consistently at the time of embryogenesis of the crop plants. They are vital in speeding up the process of maturation of somatic embryos, the cotyledon development, precisely.

Auxins: These alone or in combination with cytokinin seemingly are vital for the start of growth and the induction of the embryogenesis of all the auxins. Auxins find immense importance in the

first step of this process – the step of induction. High levels of auxins can lead to the inhibition of embryogenesis in the explants of the citrus plants.

Abscisic acid: These are supplied at the inhibitory levels. It facilitates the development and maturation of the somatic embryos, while also inhibiting the unusual proliferation and the initiation of the accessory embryos.

**3)Genotype**

The process of embryogenesis is also affected by the genotypic variation seen in different plants; as per research, it can also be as a result of the endogenous levels of the hormones.

**4)Sources of nitrogen**

Nitrogen forms that are utilised in the media have an influence on the process of embryogenesis in plants. Forms of nitrogen have a marked influence on somatic embryogenesis. Somatic embryo development takes place on a medium that contains $NO^{-}_{3}$ as the only source of nitrogen.

**5)Polyamines**

The concentration of polyamines in media or explants is said to have an effect on the process. Experts observe the concentration of polyamines to be seen in higher concentrations in the polyembryonates compared to monoembryonates.

**6)Electrical stimulation**

Electrical stimuli apparently facilitate the differentiation of the structured embryo by influencing the cell polarity via modifications in the structure of the microtubules and the induction of first asymmetric division

7) Charcoal media shows lower levels of phenylacetic acid and benzoic acid compounds which inhibit somatic embryogenesis.

8)Environmental condition of light, temperature, density of embryogenic cell in medium are important.

**Practical application of somatic embryogenesis**:

**1)Clonal propagation**:

Somatic embryogenesis has the potential application in plant improvement. Since both the growth of embryogenic cell and subsequent development of somatic embryo can be carried out in a liquid medium it is possible to combine somatic embryogenesis with engineering technology to create large scale mechanised or automated culture system.

**2)Genetic transformation**: In seed embryogenesis zygotic embryo are seated deep inside the nuclear tissues. They live in a protected environment besides being genetically heterogeneous. Somatic embryos remain virtually unprotected and more or less give rise to genetically uniform plants

**3)Conservation of genetic resources**: Embryogenic culture as well as embryos remain viable upon storage and ambient temperature , cold storage or cryostases. Therefore, somatic embryogenesis has a great importance in plant germplasm conservation.

**4) Synthetic of Metabolites:**The repetitive embryogenesis system is of potential use in the synthesis of metabolites such as pharmaceuticals and oils. Borage contains high level of Y-Linoleic acid, used as precursor of post-glandins or in the treatment of atopic eczema. Somatic embryos of borage also produce this metabolite but through repetitive somatic embryogenesis a continuous supply of Y-lenolenic acid is ensured. Which otherwise would be limited to the growing season in the zygotic embryos. The same principle can be applied for production in vitro of industrial lubricant from jojoba and leo-palmitostearin from cacao.

**5) Source of Regenerable Protoplast System:**Embryogenic callus, suspension cultures, and somatic embryos have been employed as source of protoplast isolation for a range of species. Cells or tissues in these system have demonstrated the potentiality to regeneration in culture and therefore, yield protoplast that are capable to forming whole plants.

**6)** Since the process of somatic embryogenesis does not entail the procedure of fertilization, it promotes the large scale propagation of plants at a faster rate. In addition, it also assists in the

genetic transformation of plants, serving as a promising resource for the cryo-storage of the embryo and germplasm.

7)**Embryo Cloning:**

Repetitive embryogenesis often provides innumerable number of somatic embryos, which in turn is useful in the mass production of plant propagules. Several embryo specific metabolites like seed storage proteins and lipids of industrial value can be recovered

**8)Micro-Propagation Industries:**

One of the most promising applications of somatic embryogenesis is large scale propagation of somatic embryos, which shows several advantages such as innumerable number of embryo production (60,000-70,000 embryos per litre of media), presence of both root and shoots meristems, easy to scale up and convert them into seedlings efficiently as far as commercial significance

9)It is a preferred in-vitro propagation method for woody plants. Here, it plays a critical role in clonal propagation, synthetic seed production, germplasm conservation, and cryopreservation.

10)It is used for rapid large-scale propagation of plants for the production of secondary compounds and drugs.

**Advantages of Somatic Embryogenesis**

- A huge number of embryos are obtained
- The development and environmental stage of somatic embryos can be regulated
- This process of embryogenesis can be monitored easily
- Production of artificial seeds
- Higher rate of propagation
- Apt in suspension culture
- Labour savings
- Large number of embryos can be obtained easily.
- The environment and the development phase of somatic embryos can be controlled.
- It is an ideal system for the basic studies of plant cell biology and embryo development.
- It also provides a better system for the understanding of the differentiation and mechanism of totipotency expression in plant cells

**Limitations/Disadvantages of somatic embryogenesis: -**

- Response tissue specific (explants)
- Confined to few species
- Inability to generate large numbers of normal, free living plantlets
- Barriers to high frequency plantlets production may occur at any of a number of points between induction and the production of a plantlets capable of surviving transfer to ex-vitro condition
- May also include, low frequency embryo production, production of malformed embryos, incomplete embryo maturation, unbreakable embryo dormancy or low plantlet.
- May create unwanted genetic variation (Somoclonal variation)

**Artificial Seeds/synthetic seeds: -**The immediate benefit of somatic embryogenesis is the production of synthetic seeds or artificial seeds which have many diverse applications in agriculture. An artificial seed is a synthetic seed consisting of a somatic embryo surrounded by nutrient medium protected by a thin chemical membrane. Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under in vitro or ex vitro conditions and that retain this potential also after storage. In simple words synthetic seed contains an embryo produced by somatic embryogenesis enclosed within an artificial medium that supplies nutrients and is encased in an artificial seed covering. The technology designed to combine the advantages of clonal propagation with those of seed propagation and storage. The first synthetic seeds were produced by Kitto and Janick in 1982 using carrot Artificial seeds are prepared by encapsulating somatic embryos in a nutrient mix and chemical membrane. Murashige first attempted to produce artificial seeds in 1977. **Artificial seeds are also called encapsulated seeds**. Artificial seeds produced for Avocado, chestnut, coffee, cocoa, coconut, cardamom.

**Why Synthetic Seeds?**

In some of the horticultural crops seeds propagation is not successful due to;

- Heterozygosity of seeds particularly in cross pollinated crops
- Minute seed size eg; orchids
- Presence of reduced endosperm
- Some seeds require mycorrhizal fungi association for germination eg: orchids
- No seeds are formed

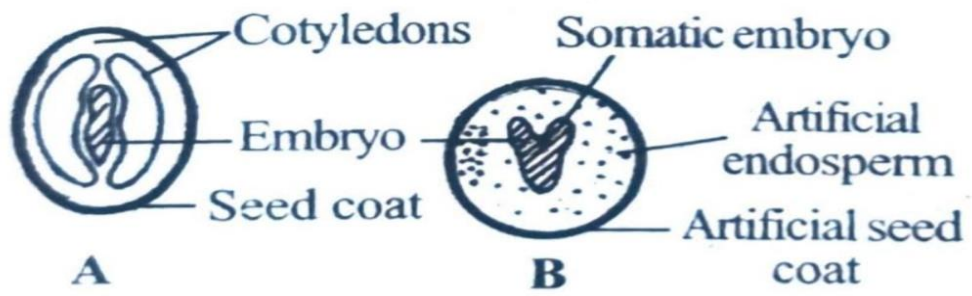



*Fig.35.20: Structure of seeds: A. Natural seed; B. Artificial seed.*

**Characteristics of Synthetic Seeds**

1. Large scale propagation method
2. Maintains genetic uniformity of plants
3. Direct delivery of propagules to the field, thus eliminating transplants
4. Lower cost per plantlet
5. Rapid multiplication of plants.

Somatic embryos obtained from well-known strain of plant are used to make artificial seeds by encapsulation. To nourish the embryo, the minimum essential amount of medium is allowed to wrap around that embryo. The nutrient medium may be one that is required to grow explants of the plant or any other nutrient formulations used for tissue culture. **The gelling agents such as agar, alginate, polyco2133, carboxy methyl cellulose, sodium pectate, tragacanth gum, polyoxy-ethylene oxide and polyacrylamide are found to be suitable for seed encapsulation.** Among these gels, **alginate is found to be most suitable for the production of artificial seeds. Alginate is chosen because it enhances capsule formation and rigidity of the capsules is also high enough to protect the seeds from mechanical injury**.

## Types of Artificial Seeds

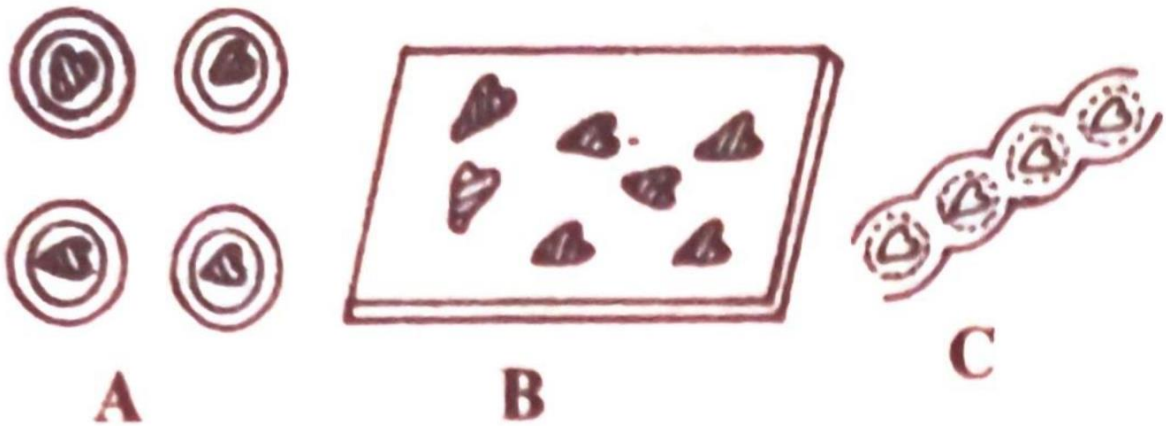
Based on the method of production, artificial seeds are classified into three types:

A. **Encapsulated Seeds:** Somatic embryo in a nutrient suspension are encapsulated in a gel to form individual seeds.

B. **Seed Gel Suspension**: Somatic embryos are kept suspended in a semi-solid gel of nutrient medium with agar. This method produces straps of artificial seeds.

C. **Seed Tapes**: Somatic embryos are placed in one or two rows between two tapes of semi-solid medium.

**The following are the major steps in the production of artificial seeds:**




**Two types of artificial seeds (encapsulated somatic embryos) are commonly produced: desiccated and hydrated artificial seeds**

1) **Desiccated artificial seeds:-** Desiccation tolerance (DT) refers to the capacity of an organism to dehydrate to below 10% water on a fresh weight basis (or 0.1 g $H_2O$/g dry weight) without accumulation of lethal damage. This mechanism was essential for the colonization of terrestrial habitats by the early land plants. Vegetative DT is very common in basal plants and is mostly confined to seeds of spermatophytes (Oliver et  However, seed DT has been lost in some plants adapted to environments where conditions are conducive to immediate germination  Desiccated artificial seeds are achieved from somatic embryos either naked or encapsulated in polyoxyethylene glycol followed by their desiccation. Desiccation can be applied either

rapidly by leaving artificial seeds in unsealed petri dishes on the bench overnight to dry, or slowly over a more controlled period of reducing relative humidity These types of artificial seeds can be only made in plants whose somatic embryos are desiccation-tolerant. The desiccation tolerance of somatic embryos can be induced using a high osmotic potential of the maturation medium. The osmotic potential could be increased by using a high gel strength or by the addition of permeating osmoticants such as mannitol, sucrose, etc. Desiccation can also be induced by applying sub-lethal stresses such as nutrient deprivation or low temperature, since these treatments have been reported to have similar effects on desiccation tolerance.

**2) Hydrated artificial seeds**

1. Somatic embryos are mixed with a suspension of nutrient medium containing 3% sodium alginate. Inoculum density of 0.8 gram of embryo per 25ml of medium is suitable for seed preparation and storage.

2. The alginate gel containing embryos is dropped into the $CaCl_2.2H_2O$ solution to form round firm beads. These beads are retained in that solution for 30 minutes to form a rigid capsule by ion exchange between Na in sodium with Ca in $CaCl_2.2H_2O$ solution. Therefore, the hardness and rigidity of capsule depends on the concentration of calcium and sodium ions.

3. The beads are sieved through a nylon mesh to collect the artificial seeds. The remaining $CaCl_2.2H_2O$ solution is reused to drop fresh somatic embryos.

4. Viability of the artificial seeds is tested by placing them on moist filter paper in a petridish or sowing them on soil treated with fungicides and pesticides.

5. Artificial seeds are then stored at 4°C in a dark room to sow in the field.

The survivability of the encapsulated seeds can be increased by adopting some pre-encapsulation procedures. Artificial endosperm with 12% sucrose instead of 2% increases the duration of survival of somatic embryo in the seed Cold storage of embryo at 4°C before seed production provides some tolerance to the seeds during storage.

**Advantages of synthetic seeds**

- This method is for large scale productions.
- It maintains genetic uniformity for a high number of generations. Most plant tissue culture methods fail to maintain genetic uniformity for longer durations.
- Artificial seeds have no dormancy period.
- The viability of artificial seeds is good.
- Artificial seeds can be made in large numbers when we are in need.
- Storage and transportation are easier than natural seeds.
- The germination of artificial seeds is uniform.
- All the clones are identical.
- As compared to plantlets, it is easy to handle and transport synthetic seeds.
- Ease of handling while in storage
- Easy to transport
- Has potential for long term storage without losing viability
- Maintains the clonal nature of the resulting plants
- Serves as a channel for new plant lines produced through biotechnological advances to be delivered directly to the green house or field
- Allows economical mass propagation of elite plant varieties

**Disadvantages/Limitations of synthetic seeds**

1. Limited production of viable micro-propagules that are useful in synthetic seed producer
2. Asynchrous development of somatic embryos
3. Improper maturation of somatic embryos that makes them inefficient for germination and conversion in to normal plants
4. Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds
5. Somo clonal variations which may alter the genetic constituent of the embryos
6.Somatic embryos have low survival rates for most plant species, which also limits the value of synthetic seeds.
7.There are not many protocols available to produce propagules from different plant parts using plant tissue culture methods. Hence less useful material available for producing synthetic seeds.

In some cases, inefficient maturation of somatic embryos leads to poor germination and hence poor growth and development.

9.According to scientists, somatic embryos from some plants species are not capable of germinating out of the capsule or coating. Hence, they are not able to form normal plants rapidly.

10.The concentration of coating material is also a limiting factor for producing synthetic seeds. It should have nutrient supplementing materials for facilitating germination and growth.

11.When the shape of synthetic seeds is not matching the farm machinery then it is hard to use them for transplantation. Hence, seeds should be transplantable.

**Applications/Uses of Artificial Seeds**

1. They are used for the propagation of non-seed producing plants and polyploids which have high economic value.

2. They are used to propagate male or female sterile plants for breeding programs.

3. Cryopreserved artificial seeds are used for germplasm storage. Eg. Mango, Coconut, Cocoa, etc.

4. Transgenic plants with original genotype can be stored in the form of somatic embryos in artificial seeds.

5. Somatic embryos are potential tools for genetic engineering in plants.

6. Artificial seeds germinate and give rise to pure plants that do not require continuous selection during conventional breeding programmes.

7. Artificial seeds produce pathogen free plants.

8. Since they are free from pathogens, artificial seeds can be transported easily without much risk of quarantine and bulkiness.

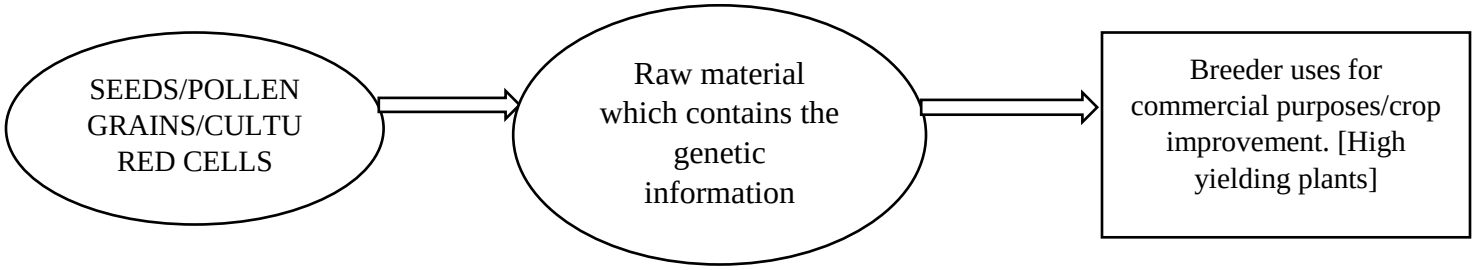
9. Artificial seeds are good analytical tools for the comparison with zygotic embryos.

10. Artificial seeds can be handled as seeds being used in agriculture and horticulture.

11. Artificial seeds reduce the cost of transplants.

**GERMPLASM CONSERVATION AND PRESERVATION: -**

**Germplasm is the sum of total genes and their alleles present in the crop and their related species. Germplasm in a broad way can be defined as the hereditary material i.e., total content of genes which is inherited by the off springs through germ cells Germplasm material is stored in the form of seeds, cultured cells, callus culture and .pollens. The in-situ/ex-situ preservation of theses materials is called Germplasm conservation.**




**Need for Germplasm conservation.**

- To save the endangered species, rare species, economically important and medicinal important species.
- The main objective of germplasm conservation is to preserve the genetic diversity of selected plants or genetic stock for its utilization at any time in future.
- germplasm preservation and management, which is highly precious in breeding programmes.
- In recent years, the primitive and conventionally used agricultural plants are being replaced by many new plant species with desired and improved characteristics.
- It is very crucial to conserve the endangered plants otherwise some of the important genetic traits possessed by the primitive plants may be lost.
- Germplasm serves as the raw material for the breeder to produce various crops.

Types of germplasm conservation:

There are mainly two types of germplasm conservation which are

- **In-situ conservation**
- **Ex-situ conservation**

**In-situ conservation:**

- The conservation of germplasm in their natural habitat by constructing national parks/gene sanctuaries is termed as in-situ conservation.
- It is regarded as a high priority germplasm preservation programme.
- It helps in the continuation of plant life in the ecological community.
- It aims in conservation of great number of cultivated and wild species in less area.
- One of the advantages of in-situ conservation is that it continues the evolution of the species along with the allowance of appearance of new recombinant form.

**Limitations of in-situ conservation**

- Security is very low in absence of controlled monitoring.
- Various environmental hazards can degrade the germplasm.
- Expensive maintenance cost for large number of genotypes.

**Ex-situ conservation:**

- The genetic materials can be conserved either by collecting plants and kept in normal growing conditions or in the form of seeds in seed banks, through tissue culture and low temp maintained by liquid $N_2$. This type of conservation is termed as ex-situ conservation.
- It is the major method for the preservation of germplasm obtained from both wild and cultivated plant materials.
- Genetic resources either in form of seeds or plants cells, tissues or organs can be preserved as gene banks for long term storage under favourable conditions.
- Proper knowledge of plant diversity, their genetic structure and the methods involved in sampling, regeneration, maintenance of gene pools is important for the successful accomplishment of gene banks.
- Ex-situ conservation cannot allow the plants to continue its evolutionary process but it ensures the availability of stored genetic materials in need and its safety.
- Sugarcane, cocoa and rubber are stored in this way.

Technique of Germplasm conservation

Seeds conservation:

- Usually, seeds are the most efficient, simple, economic and convenient resources to conserve plant germplasm.

- This is because many plants are regenerated through seeds, and seeds cover relatively small space. In addition, seeds can be easily moved to various places.
- Seed conservation is the most broadly used method of ex-situ conservation.
- It is performed by drying the seeds at 10-25°C, 10-15% relative humidity followed by storage at -18°C.

**CRYOPRESERVATION: -**

The preservation of biological materials in the frozen state is called cryopreservation. The basic principle of cryopreservation is that, when biological materials are maintained at zero metabolism, their cells remain undivided without losing the viability for a long time) Low temperature storage helps to keep the cells at zero metabolism at which there is no enzyme activity. Plant tissues, Calli, cell suspensions, pollen grains, seeds, protoplasts, animal cell lines, animal organs, hybridoma, etc. are preserved for a long time by using cryopreservation techniques. During cryopreservation, the biological materials are kept on solid carbon dioxide (-79°C) or in deep freezers (-80°C) or in nitrogen vapour (-150°C) or in liquid nitrogen (196ººC). These super -low temperatures preserve the shelf-life of the biological materials.

Cryopreservation provides opportunities for long-time storage of biological materials in vitro and reuse of the materials when we are in need. The low temperature storage and reuse of biological materials is called cryopreservation technology.

Plant tissues, calli, cell suspensions, embryos and protoplasts can be preserved for a long time by keeping them at -196°C in liquid nitrogen. This is called cryopreservation. This freeze-preservation has several applications in plant tissue culture for maintaining genetic stocks (germplasms) of agricultural, horticultural and forest crops. During cryopreservation, there is no loss of specimens due to insect and pest attack; the specimens never suffer from worst climatic changes and political and economic causes do not interfere with the preserved material. Above all, it saves the originality of genetic stocks as such for carrying out breeding programs. No one in conventional storage method provides all these facilities while preserving the plant materials. Liquid nitrogen maintains low temperature for the long-term storage. The low temperature reduces the growth rate of the stored cells and delays ageing. Therefore, the plant materials do not lose their viability for a long time. Plants can be regenerated from the cryopreserved plant materials after a long period of in vitro storage.

Callus tissues of potato, pea, coconut, rice, wheat, oil palm, strawberry, sugarcane, etc. are stored by cryopreservation.

**It is useful for**

- Preservation of germplasm of plants.
- Long-time storage of microbial cultures in microbial culture collection centres.
- Storage of animal stocks cell lines, semen and hybridoma.
- Protection of animal from genetic drift.
- Preservation of recalcitrant seeds in the form of embryos.
- Preservation of human organs for transplantation.

**1. Selection of Plant Materials:-**Wide range of plant materials have been preserved by employing cryopreservation method. Suitable plant materials are selected from different plant species for cryopreservation.

**Examples**

- Meristem-Potato, Cassava, sugarcane, chickpea, peanuts, etc.
- Root tip-Potato
- Pollen-Peanut, mustard, rape, cotton, wheat, rice, tobacco, Primula, etc.
- Pollen embryo - Peanut, mustard, rape, cotton, wheat, rice, tobacco, etc.
- Zygotic embryo-Wheat, rice, Triticale, coconut, Citrus, coffee, Avocado, cocoa, cardamom, oil palm, rubber, tea, walnut, etc.
- Fused protoplasts- Pea X wheat, rice X pea, etc.
- Protoplasts- Datura, Belladonna, rice, wheat, etc.
- Callus-Potato, sugarcane, black gram, green gram, cotton, wheat, rice Triticale, Petunia, Primula, etc.
- Cell suspension- Datura, tobacco, Belladonna, Acer pseudoplatanus, Chrysanthemum, carrot, soybeans, potato etc.
- Meristem, root tips, embryos and Calli are carried in small vessels containing little amount of nutrient medium. This low amount of medium, as it is a limiting factor, retards the growth rate during preservation. The cell suspension contains a large proportion of nutrient medium. The excess medium is removed after sedimentation of cells by keeping the culture in an ice-bath.

**2. Addition of Cryoprotective Agents (cryoprotectants): -**A cryoprotective agent is added to the concentrated culture to protect plant materials from damaging effects from cold storage. The cryoprotective agents remove crystallizable water in the cells so that the cell organelles and enzymes would be safe during cold storage. Further, they prevent the accumulation of toxic substances in the cells. **They are often named as cryogens**. Dimethyl sulfoxide (DMSO) (5-10%), propylene glycol, polyethylene glycol(PEG), polyethylene oxide (PEO), hexamethylene tetramine glucose, dimethyl acetamide, sucrose (10%), etc. are important cryogens being used in storage of plant and animal materials. Of these, DMSO is an ideal cryoprotectant. The cryogens maintain the original relationships of cell components during cold storage (Rowe, 1960). Meantime, they act as antifreezing agents (Lovelock, 1953). Therefore, the cells remain safe even at -196°C.In some cases, a combination of two or more substances is used as a cryoprotectant. Examples: -DMSO 24% +Glucose 10%, DMSO 2.5% + Glycerol 2.5% + Sucrose 6.5%

**3) Freezing Treatment:-**After adding the cryoprotectant, the ampoules containing plant tissue are closed tightly with screw caps and kept in a freezer. Plant cells require proper pre-cooling treatment before long-term cold storage. It prevents the formation of intra-cellular ice-crystals in the cells. During pre-freezing, temperature of cell or protoplast suspension is lowered gradually to the rate of 10°C for every 3 minutes. In such a way temperature is reduced upto -135°C because sudden reduction in temperature cause crystallization of intracellular water. At this temperature, almost all fractions of freezable water ooze out of the cell. So, the cells will not be affected during long-term cold storage. This is called stepwise freezing. To preserve meristem, embryo and other plant tissues, the ampoules are kept in liquid nitrogen to reduce the temperature rapidly. So this method is called rapid freezing. It is suitable for preserving the tissue at -79°C on dry ice.

Freezing units like LR-33 Biological freezer-6, Programmed freezer R201 and Mini-freezer R202 are being employed for the pre-freezing treatment.

**4. Long-term Cold Storage: -**After pre-freezing treatment, the cell culture is transferred to a small flask or beaker. The flask is insulated with a thick plastic cover to prevent damages by liquid nitrogen. The culture flask is then kept in liquid nitrogen in a container. The container is

kept in a refrigerator. The liquid nitrogen keeps the temperature -196°C inside the culture vessel. At this temperature, there is no metabolic activity in plant cells due to inactivation of cellular enzymes. So, the cells remain as such for a long time. any considerable change. By using this method, plant materials can be stored even for 5 years or more.

**5. Thawing**: -After a sufficient period of cold storage, the culture flask is taken out of the liquid nitrogen and heated gently. The heat is raised slowly but gradually, upto 35-40°C. This temperature treatment is called thawing. Quick heating cause dehydration of cells and it results in death of the cells. So sudden heat is avoided. Thawing melts extracellular ice crystals formed during the long-term storage. Immediately after melting of ice crystals, the culture flask is kept in a water-bath.

**6. Removal of Cryogen:** -The stored plant ceils have cryogen molecules on the surface of cell walls. The cryogens interfere with growth of the cells into callus and plant regeneration. So cryogens are washed out of the cells by rinsing the cells with distilled water. The water is then centrifuged out to get a pellet of plant cells.

**7. Viability Tests:** -Cryopreserved plant materials remain alive and capable of regenerating into entire plants after several months. Whether the specimen under cold storage is viable or not is determined by subjecting a sample of the specimen to invitro culture test. This culture test involves thawing, removal of cryogen, callus induction and plant regeneration. These steps are similar to those used for plant regeneration from the cryopreserved plant materials after storage. The survival of cell suspensions and protoplast cultures can be determined by fluorescein diacetate staining or Triphenyl tetrazolium chloride (TTC) staining or invitro growth measurements. The survival capacity of plant materials differs from part to part of a plant and from species to species. The survival of meristem is 7-57% depending upon species

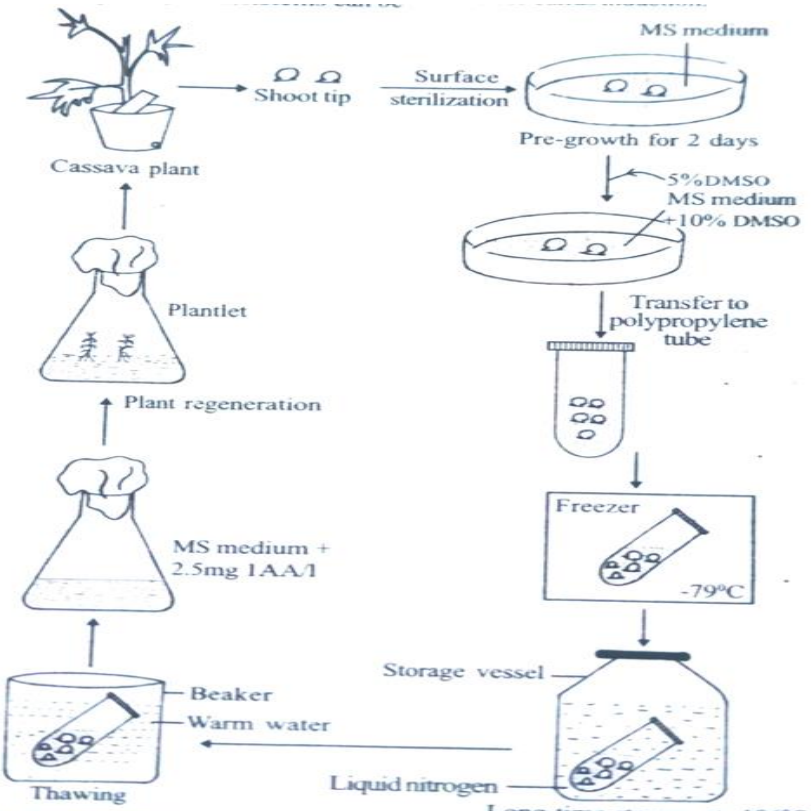



*Fig 37.4: Steps involving in cryopreservation of shoot tip of Cassava plant*

**Applications/Advantages of germplasm conservation: -.**

- Germplasm serves as the raw material for the breeder to produce various crops. Therefore, conservation of germplasm has importance in all breeding programmes.
- The possibility of life on earth is mainly due to the plants as it is the crucial component of the ecosystem, thus its preservation is our responsibility for the continuation of life.
- In other words, it may be regarded as the conventional germplasm preservation and management, which is highly precious in breeding programmes.
- In recent years, the primitive and conventionally used agricultural plants are being replaced by many new plant species with desired and improved characteristics.
- It is very crucial to conserve the endangered plants otherwise some of the important genetic traits possessed by the primitive plants may be lost.
- It has been estimated that up to 100,000 plants, depicting more than one third of all the world's plant species, are currently threatened or face extinction in the wild.

- Its main aim is to provide essential support for collection, conservation and utilization of plant genetic resources all over the world.

**Limitations:**

- Viability of seeds is decreased or lost along with time.
- Seeds are destructed by insect or pathogen attack.
- This approach is only confined to seed propagating plants, and thus it is useless for vegetatively propagated plants e.g. potato.
- Heterogenous seeds are not suitable for true genotype maintenance.
- Only orthodox seeds can be conserved by this method as the recalcitrant and intermediate seeds cannot stand the desiccation.