Plant tissue culture

Historical perspective

CC-14
UNIT-1

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Introduction

- Tissue culture involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile conditions.
- Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots, and, with the addition of suitable hormones new roots.
- These plantlets can also be divided, usually at the shoot stage, to produce large numbers of new plantlets.
- The new plants can then be placed in soil and grown in the normal manner.

History of Plant tissue culture

1838- Cell theory, indicating towards totipotentiality of cells by Schleiden and Schwann.

1902- First but unsuccessful attempt of tissue culture using monocots by Haberlandt. He also explained the concept of cell totipotency.

1904- First attempt in embryo culture of selected Crucifers by Hannig.

1924- Callus formation on carrot root explants by use of lactic acid by Meyer.

1941- Coconut Milk used for growth and development of very young Datura embryos by Overbeek.

History of Plant tissue culture

1944- First *In vitro culture of tobacco used to study adventitious shoot* formation by Skoog.

1948- Formation of adventitious shoots and roots in tobacco by Skoog.

1957- Discovery that root or shoot formation in culture depends on auxin: cytokinins ratio by Skoog and Miller.

1958- *In vitro culture of excised ovules of Papaver somniferum by* Maheshwari.

1958- Regeneration of somatic embryos from nucleus of Citrus ovules by Maheshwari and Rangaswamy.

1962- Development of MS medium by Murashige and Skoog.

1964- First haploid plants from Datura androgenesis by Guha and Maheshwari.

History of Plant tissue culture

1978- Somatic hybridization of tomato and potato resulting pomato by Melchers.

1978- Industrial scale fermentation of plant cells for production of shikonin.

(Selection of cell lines with higher yield of secondary products) by Tabata.

1981- Introduction of the term somaclonal variation by Larkin.

1985- Infection and transformation of leaf discs with *Agrobacterium tumefaciens* and regeneration of transformed plants by Horsch.

History of Plant tissue culture

1985- Development of binary vector system for plant transformation.

1985- Gene transfer in protoplasts of Dicot and Monocot plants by electroporation.

1993- *In vitro fertilization with isolated single gametes resulting in zygotic* embryogenesis and recovery of fertile maize plants by Kranz.

1996- Development of method of plant transformation by Hansen.

Significance of Plant tissue culture

- Plant tissue culture technology is being widely used for large scale production of specific plant type .
- Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites.
- Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in the relatively short time period and space under controlled conditions, irrespective of the season and weather on a year-round basis.

Significance of Plant tissue culture

- Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on the number of initial plants and space.
- In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants.
- The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities

Significance of Plant tissue culture

- Alongwith, plant tissue culture has become of great interest to the molecular biologists, plant breeders and even to the industrialists, as it helps in improving the plants of economic importance.
- In addition to all this, the tissue culture contributes immensely for understanding the patterns and responsible factors of growth, metabolism, morphogenesis and differentiation of plants.

Status of tissue culture technology in India

- India is reported to have one of the largest groups of tissue culture scientists in the world. Most of the research is directed towards the development of improved plants for agriculture, horticulture and forestry using tissue culture methods.
- The Department of Biotechnology (DBT), Government of India, New Delhi is playing a vital role in promoting research in the area of plant tissue culture. Several laboratories are being supported by providing funds for development of tissue culture technology for the improvement of crop plants.
- The important Biotechnology centres are

1. Indian Agricultural Research Institute (IARI), New Delhi.
2. Bhaba Atomic Research Centre (BARC), Mumbai.
3. Central Institute of Medicinal and Aromatic plants (CIMAP), Lucknow.
4. Dr.M.S. Swaminathan Research Institute (MSSRI), Chennai.

General Techniques of Tissue Culture

1. Choice of explant

- Explants could be shoot tips (meristem), nodal buds, sections from internodes, leaves, roots, centres of bulbs, corms or rhizomes or other organs.
- The choice depends on the species to be multiplied and the method of shoot multiplication to be followed.
- Actively growing (shoot tips), juvenile (seedlings) or rejuvenated tissues (suckers) are preferred.

General Techniques of Tissue Culture

2. Establishment of Germfree (aseptic/sterile) culture

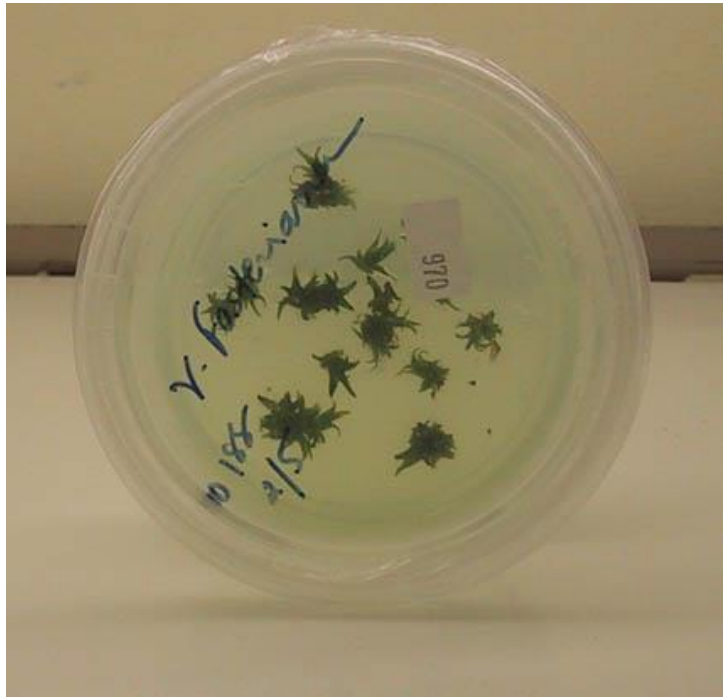
Excised part of plant is surface sterilized and transferred to sterile nutrient medium contained in glass vessel.

The cultures are maintained in growth rooms.

General Techniques of Tissue Culture

3. Production of shoots/propagules

Once growth is initiated by induction of meristematic centres, buds develop into shoots by multiplication of cells. There are three types of multiplication systems for production of shoots-



i) Multiplication by axillary shoots

ii) ii) Multiplication by adventitious shoots

iii) iii) Multiplication by somatic embryos (embryoids)

General Techniques of Tissue Culture

4. Preparation of micro-cuttings for establishment in the natural environment.

Young axillary or adventitious shoots are finally separated from clusters (micro cutting) for initiation and development of roots.

After separation, they are transferred individually to a medium containing rooting hormone (auxin) and continued to be maintained in the growth rooms until the roots are formed.

General Techniques of Tissue Culture

5. Establishment in the natural environment(Hardening)

The most critical stage in propagation by tissue culture is the establishment of the plantlets in the soil. The steps involved are as under

- washing of media from plantlets,
- transfer of plantlets to compost/soil in high humid green house,
- gradual decrease in humidity from 100% to ambient levels over 3-4 weeks,
- and gradual increase in light intensity.

General Techniques of Tissue Culture

Plantlets during their growth in laboratory do not photo synthesize and their control of water balance is very weak. They use sugar contained in medium as source of energy. They exist as heterotrophs. They need to be converted to autotrophs. This acclimatization on the harsh real environment, outside artificial laboratory milieu takes place gradually.

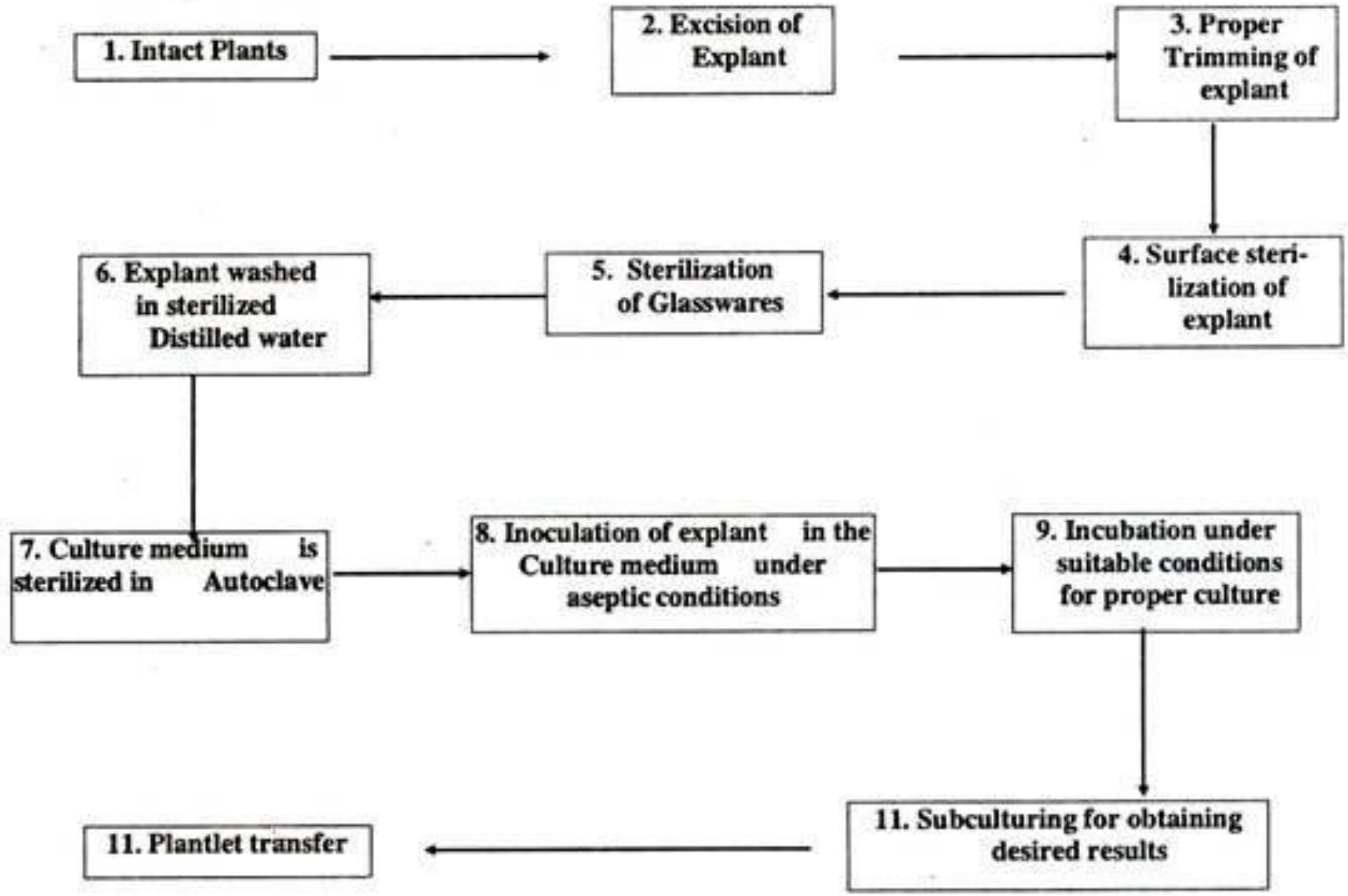


Fig. 1. Steps in general technique of Plant tissue culture.

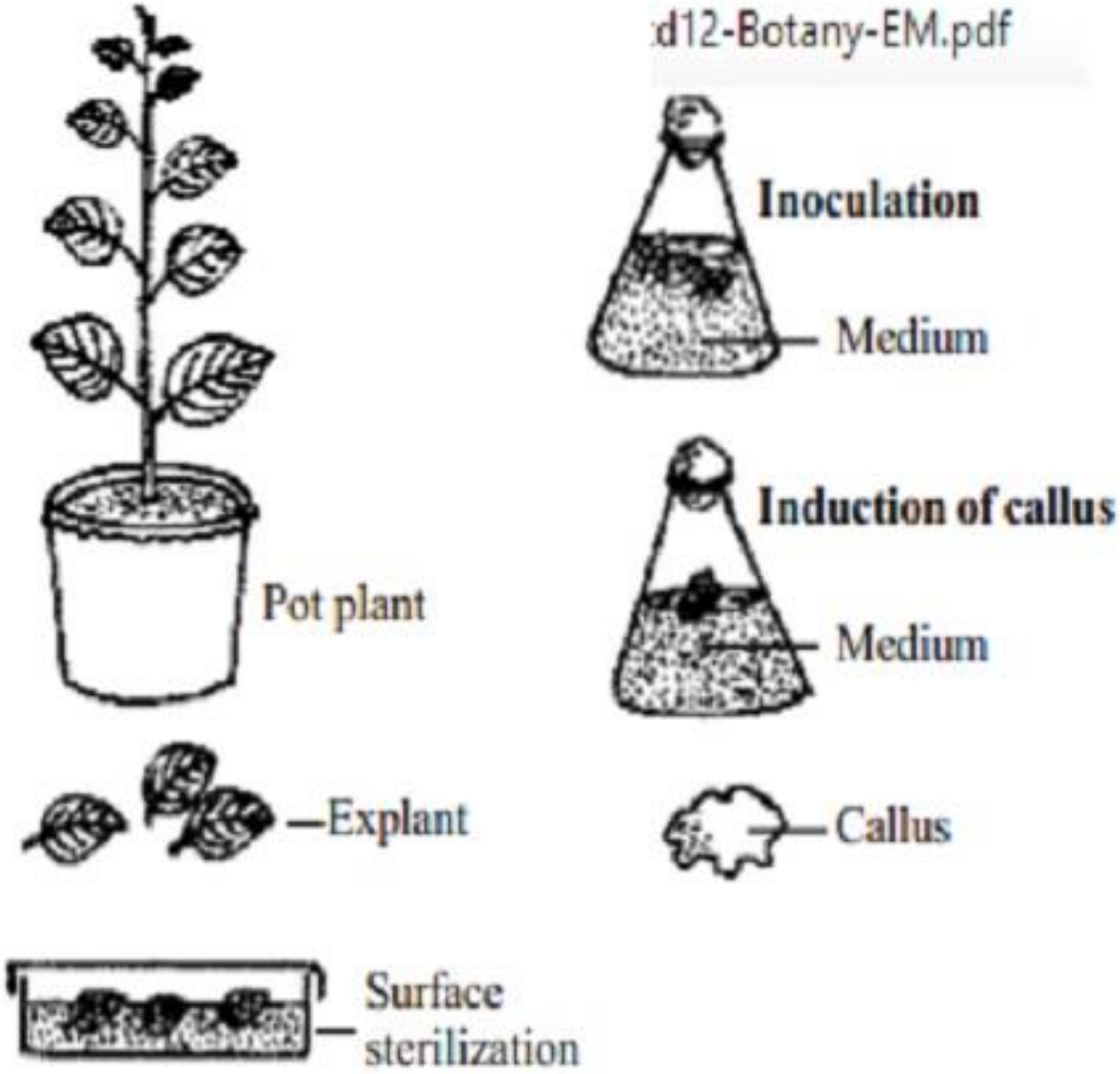


Fig. Techniques of tissue culture

Tissue culture media

Generally all culture media are made up of:

- **Macronutrients**
- **Micronutrients**
- **Vitamins**
- **Growth regulations**
- **Carbohydrates (Sucrose)**

Formulation designed by Murashige and Skoog (1962), revised by Linsmairand Skoog (1965) can be regarded as standard.

Inorganic Nutrients

Macro elements:-

- **C- Carbon forms the backbone of many plants Bio-molecules,** including starches and cellulose.
- **H- Hydrogen also is necessary for building the plant and it is obtained** almost entirely from water.
- **O- Oxygen is necessary for cellular respiration.**
- **N- Nitrogen is an essential component of all proteins.**
- **P- Phosphorus is important in plant bioenergetics as a component of** ATP. Phosphorus can also be used to modify the activity of various enzymes by phosphorylation and can be used for cell signaling.

Inorganic Nutrients

Microelements: -

- These are essential as catalysts for many biochemical reactions;
- Microelement deficiency symptoms include Leaf chlorosis (Fe, Zn, and Mn) Shoot tip necrosis (B, Co, Ni) inhibits ethylene synthesis.

Organic Nutrients

Vitamins:

Plants can produce their requirements of vitamins.

However, plant cell cultures need to be supplemented with certain vitamins like Thiamine (vit B1), Niacin (vit B3), Pyridoxine (vit B6), and Myo-inositol (Member of the vit. B complex).

Thiamine – Involved in the direct biosynthesis of certain amino acids and essential co-factor of carbohydrates metabolism.

Vit E – Antioxidants.

Vit C- Prevent blacking during explant isolation.

Vit D- Growth regulatory effect

Amino Acids – Glycine- has little benefit in the growth of plant. They may be directly utilized by plant own be provided as N2 source.

Carbon Sources

Sucrose (is most commonly used carbon source) at a concentration of 3%, glucose and fructose also known to support plant growth. Sucrose in the medium is necessary for various metabolic activities.

Growth Regulators

Auxin – Auxin are involved in cell division and elongation and in cell wall synthesis. IAA, IBA, NAA, 2, 4-D are the most frequently used auxin in plant tissue culture.

Cytokinin –These hormones, are concerned with cell division, modification of apical dominance, shoot differentiation etc. Most commonly used cytokinins are BAP, BA, Kinetin, 2 ip and Zeatin. They usually promote cell division if added together with an auxin. Of these, BAP is the most effective cytokinins for stimulating axillary shoot proliferation.

Gibberellins – There are over 20 known gibberellins. Of these, generally, GA3 is used. They are rarely used and reported to stimulate normal development of plantlets from *in vitro formed adventives embryos*.

Others – Abscisic acid is most often required for normal growth and development of somatic embryos **and only in its presence they resemble zygotic embryos.**

Gelling Agent

In static cultures if liquid medium is used the tissue would get submerged and die due to lack of oxygen. A gelling agent is generally used to circumvent this problem.

Agar – This is obtained from red algae, especially *Gelidium amansii.* Complex mixture of related polysaccharides built up from the sugar, galactose.

Agarose- Is commonly preferred over agar for protoplast culture.

Gelrite- Is a good alternative to agar not only because of its lower cost per liter of medium (0.1-0.2% is sufficient) but also for the many advantage it offers.

Major types of media

1. White's medium - is one of the earliest plant tissue culture media
2. MS medium - The most extensively used nutrient medium is MS medium

(developed by Murashige and Skoog in 1962)

3. B5 medium - developed by Gamborg for cell suspension and callus culture and at present its modified form used for protoplast culture.
4. N6 medium - formulated by Chu and used for cereal anther culture.
5. Nitsch's medium- developed by Nitsch and used for anther culture.

Cellular Totipotency, Organogenesis and Embryogenesis

Cellular Totipotency

The potential of a plant cell to grow and develop into a whole new multicellular plant is described as cellular totipotency.

Or

The property of a single cell for differentiating into many other cell types is called as totipotency.

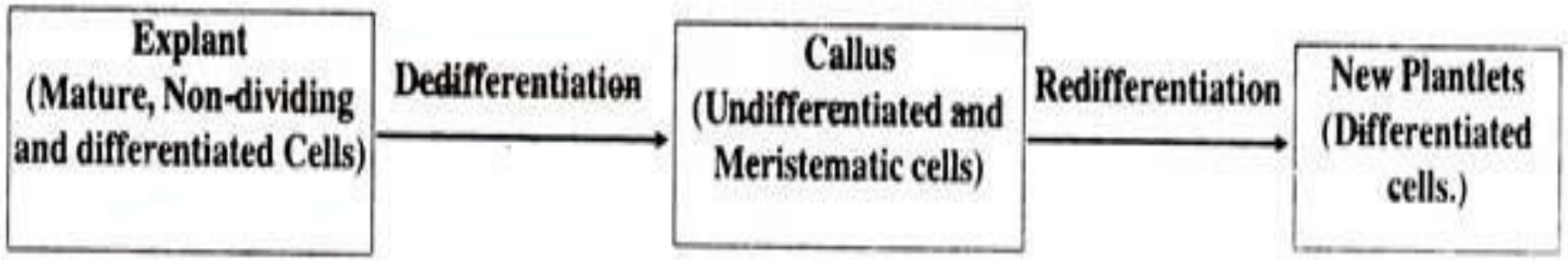
The term totipotency was coined in 1901 by Morgan.

Cellular Totipotency

To show totipotency, such mature, non-dividing cells undergo changes which revert them into a meristematic state (callus state). This phenomenon of reverting back of mature tells to dividing state is called dedifferentiation.

Now, these dedifferentiated cells have the ability to form a whole plant or plant organ. This phenomenon is termed as re-differentiation.

Dedifferentiation and re-differentiation are the two inherent phenomena involved in the cellular totipotency.



Differentiation

In broad sense, it is defined as the process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other.

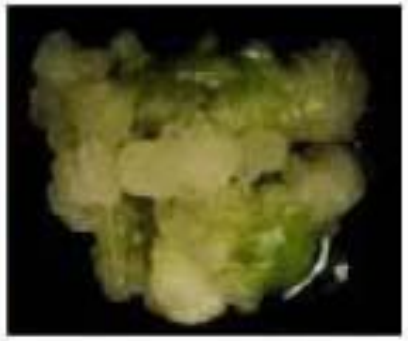
De-differentiation

The term is used to denote the process of formation of unorganised tissues from the highly organized tissues.

Re-differentiation

The process of differentiation occurring in an undifferentiated tissue.

A newly wounded leaf disc



Callus tissue forming on leaf disc

A mass of undifferentiated callus

Cellular Totipotency

Usually the dedifferentiation of the explant leads to the formation of a callus. However, the embryonic explants, sometimes, result in the differentiation of roots or shoots without an intermediary callus state.

all the genes responsible for dedifferentiation or re-differentiation are present within the individual cells and they become active for expression under adequate culture conditions. As totipotent cells are the basis of whole plant tissue culture techniques, so, by the exploitation of this potential of plant cells, biotechnologists are trying to improve the crop plants and other commercially important plants.

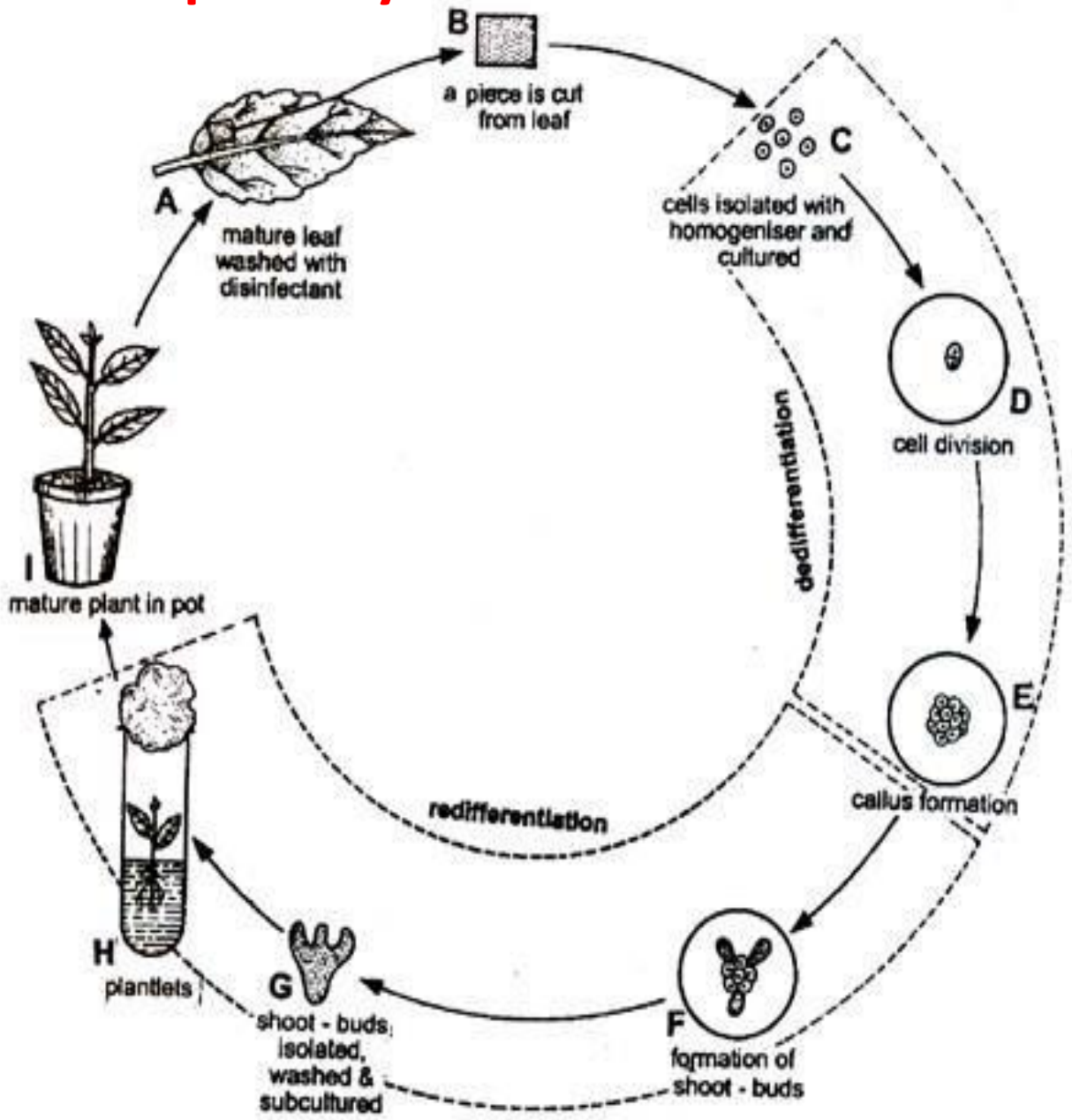


Fig. 3. Scheme depicting the totipotency of plant cells.

Cellular Totipotency

Applications of Totipotency:

i. It has potential applications in the crop plant improvement.

ii. Micro-propagation of commercially important plants.

iii. Production of artificial or synthetic seeds.

iv. It helps in conservation of germplasm (genetic resources).

v. This ability is utilized for haploid productions.

Cellular Totipotency

Applications of Totipotency:

vi. Applied in producing somatic hybrids and cybrids.

vii. Helps in cultivation of those plants whose seeds are very minute and difficult to germinate.

viii. Also helps to study the cytological and histological differentiations.

ix. For high scale and efficient production of secondary metabolites.

x. The genotypic modifications can also be possible.

Plant regeneration categories

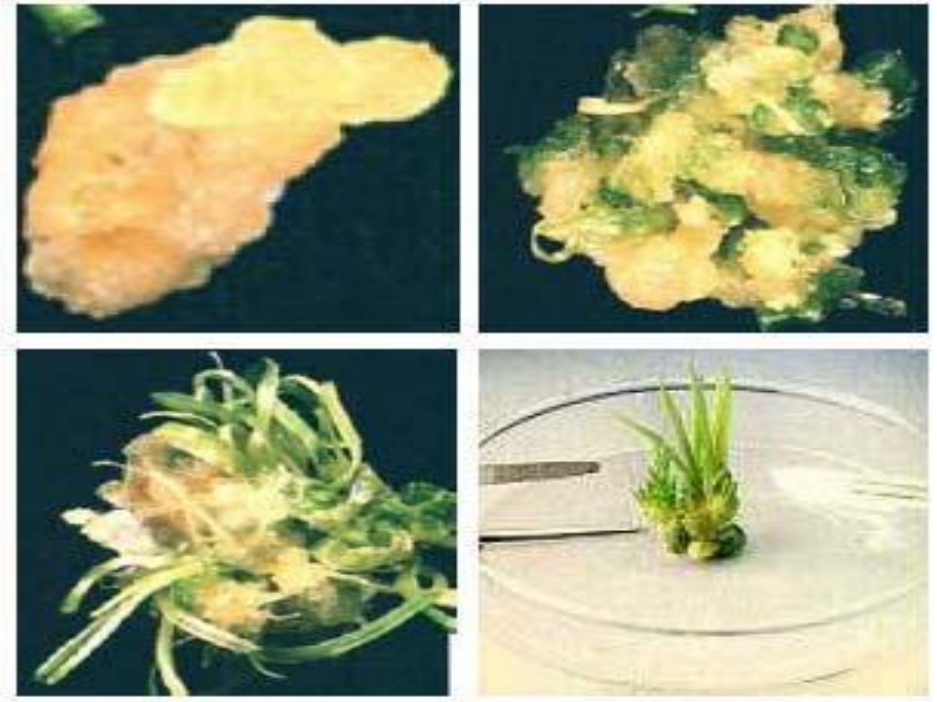
1. Enhanced release of axillary bud proliferation, multiplication through growth and proliferation of existing meristem.
2. Organogenesis - formation of individual organs (shoots, roots, flower ….) either directly on the explants where a preformed meristem is lacking or de novo origin from callus and cell culture induced from the explants.
3. Somatic embryogenesis -formation of a bipolar structure containing both shoot and root meristem either directly from the explants (adventitive origin) or de novo origin from callus and cell culture induced from the explants.

Organogenesis

Process of differentiation by which plant organs are formed (roots, shoot, buds, stem etc.).

It refers to the development or regeneration of a complete organised structure (or whole plant) from the cultured cells/tissues.

Plant development through organogenesis is the formation of organs either de novo (from callus) or adventitious (from the explants) in origin.



Organogenesis

- Organogenesis commences with the stimulus produced by the components of culture medium, the substances initially present in the original explants and also by the compounds produced during culturing.
- Among different organs, which can b induced in plant tissue culture are included the roots, shoots, flower buds and leaves. Regenerations into flower buds and leaves occur in a ver low frequency.

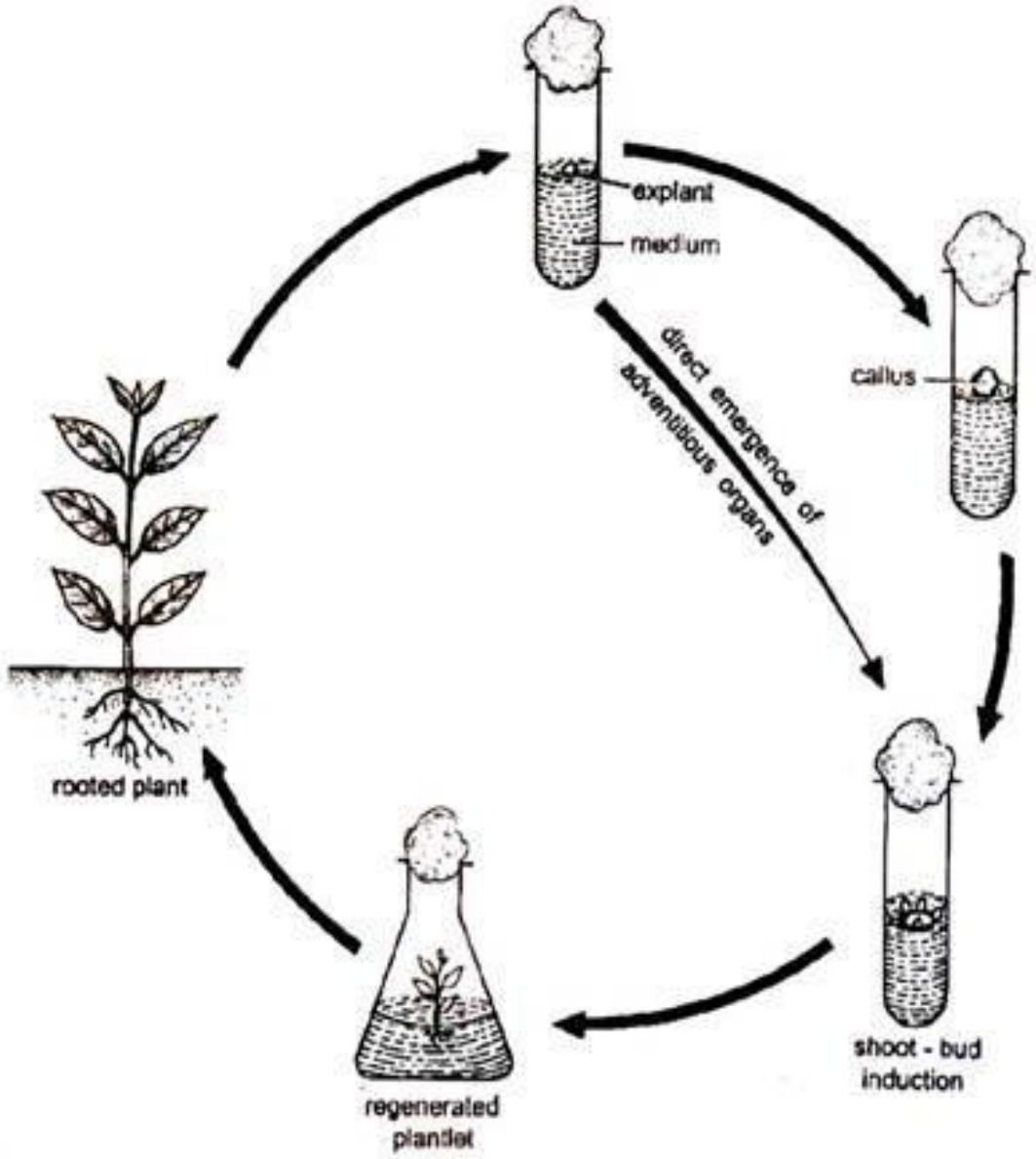


Fig. 4. Organogenic Differentiation.

Organogenesis

- The initiation of roots is termed as rhizogenesis while the initiation of shoots is called as caulogenesis and these two phenomena are affected by alterations in the auxin : cytokinin ratio in the nutrient medium. A group of meristematic cells called as meristemoids is the site of organogenesis in callus. Such meristemoids are capable of producing either a root or a shoot.
- Shoot bud differentiation was first of all demonstrated by White (1939). Shoot bud differentiation refers to the formation of shoot buds from the cultured cells by providing appropriate culture conditions and nutrient medium.
- Further, in 1944, Skoog indicated that organogenesis could be chemically controlled.

Organogenesis

Factors affecting organogenesis:

(i) Auxin: Cytokinin ratio in medium is an important factor affecting root/shoot bud differentiation in most plants.

(ii) Usually Gibberellic acid inhibits organogenesis.

(iii) Physiological state and size of explant play important role in organ differentiation.

(iv) Genotype of the donor plant plays a crucial role.

(iv) Physical factors like light, temperature, moisture, etc., play effective role in organogenesis.

Somatic embryogenesis

Somatic embryogenesis

Somatic embryogenesis is the process in which a single cell or a small group of cells follow a developmental pathway that leads to reproducible regeneration of non-zygotic embryos which are capable of producing a complete plant.

These non-zygotic embryos may originate directly from other organs or parthenogenetic embryos (without fertilization) or androgenetic embryos (from the male gametophyte).

In general somatic embryos are those which are formed from the somatic tissue in cultural i.e., in vitro condition. Embryos formed in cultures have been variously designated as accessory embryos, adventive embryos, embryoids and supernumerary embryos.

Somatic embryogenesis

Types of Embryo

I. Zygotic Embryos: These are formed by fertilized egg or the zygote.

II. Non-zygotic Embryos: These are formed by cells other than zygote.

(a) Somatic Embryos: These are formed by sporophytic cells (except zygote), directly arising from other embryos or organs which are termed as adventive embryos.

(b) Parthenogenetic Embryos: These are formed by unfertilized egg.

(c) Androgenetic Embryos: These are formed by the male gametophyte i.e., microspore or pollen grains.

Somatic embryogenesis

- Due to the totipotent nature of plant cell, the somatic cell having the complete set of genome, may be induced in cultures to form the organised bipolar structures bearing cotyledons resembling the zygotic embryo.
- This phenomenon is called in vitro somatic embryogenesis.
- The term 'embryoids' are used to distinguish these somatic embryos from the zygotic embryos.
- Any sporophytic cell or gametophytic cell may undergo somatic embryogenesis due to some changes occurring at molecular level i.e., due to induction of some genes, protein production and high metabolic activity.

Somatic embryogenesis

Two routes for somatic embryogenesis:

1. Direct Embryogenesis:

The embryos initiate directly from the explant without callus formation and here some cells which are called as 'Pre-embryonic determined cells' (PEDC) initiates embryonic development, only those cells need to be released.

2. Indirect Embryogenesis:

Here, the embryos are developed through cell proliferation i.e., callus formation. The cells from which embryos arise are called as 'Induced embryogenic determined cells' (IEDC). Here growth regulators with specific cultural conditions are required for initiation of callus and then redetermination of those cells into the embryo development.

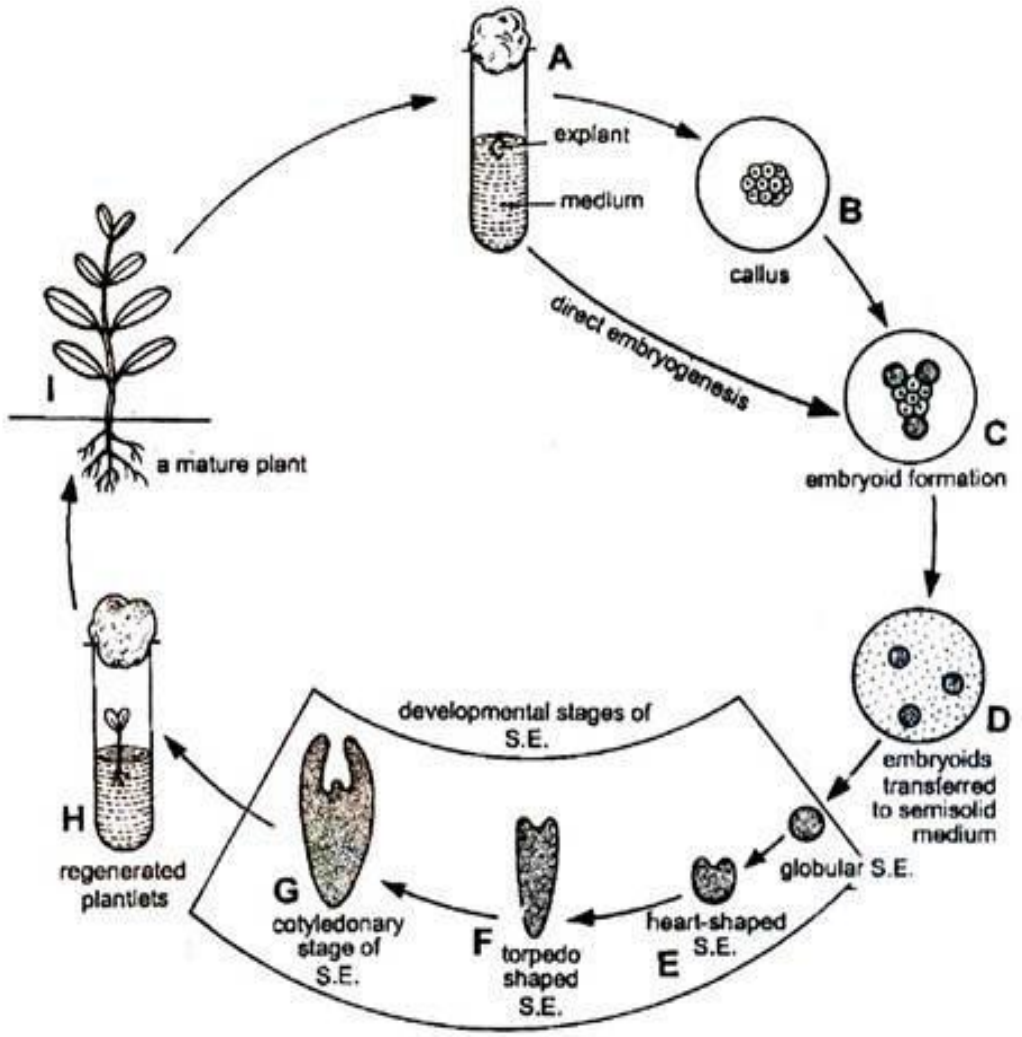


Fig. 5. Somatic Embryo (S.E.) Differentiation

Somatic embryogenesis

Somatic embryoids may be formed from:

(a) Vegetative cells of a mature plant,

(b) Reproductive cells other than zygote or

(c) Cotyledons, hypocotyl or young plantlets.

According to Sharp (’80) embryoids are formed in two ways:

(a) Directly, without callus formation, from pre-embryonic cells, i.e., the cells that are destined to form the embryo,

(b) After callus formation from induced embryo genic cells, as in carrot.

Somatic embryogenesis

- In carrot root culture it has been observed that single cell should produce a cell aggregate (pro-embryo) first, before embryo initiation.
- During initiation embryogenic cells have protoplasmic connections with adjacent cells.
- Embryogenic cells have certain characteristics. These cells have dense cytoplasm, prominent nucleolus, large nucleus, conspicuous starch grains, high concentration of protein and RNA and have dehydrogenase activity.

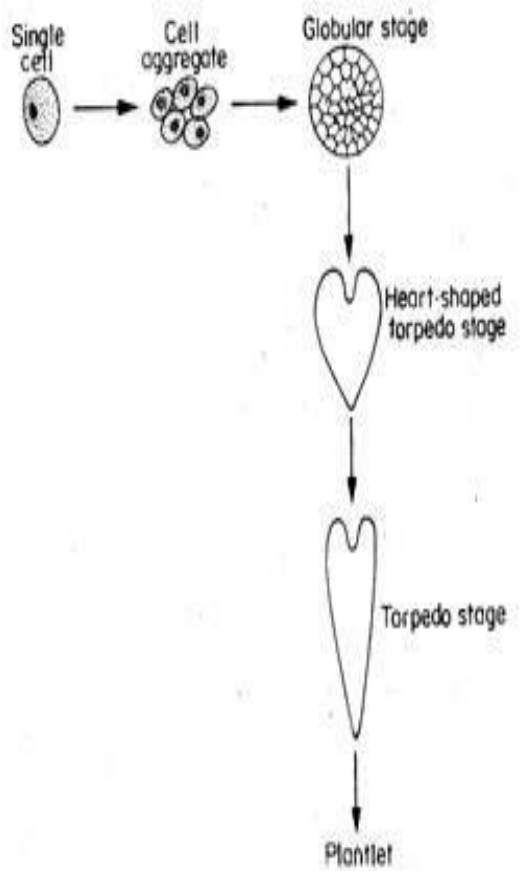


Fig. 20. Embryoid development in tissue culture passes through various stages, namely, globular stage, heart-shaped stage and torpedo stage before plantlet formation.

Somatic embryogenesis

Embryoid development in tissue culture passes through three stages, namely,

1. Globular stage,
2. Heart-shaped stage and
3. Torpedo stage.

During embryoid formation there is first cyto-differentiation of proembryoid cells followed by occurrence of various developmental stages.

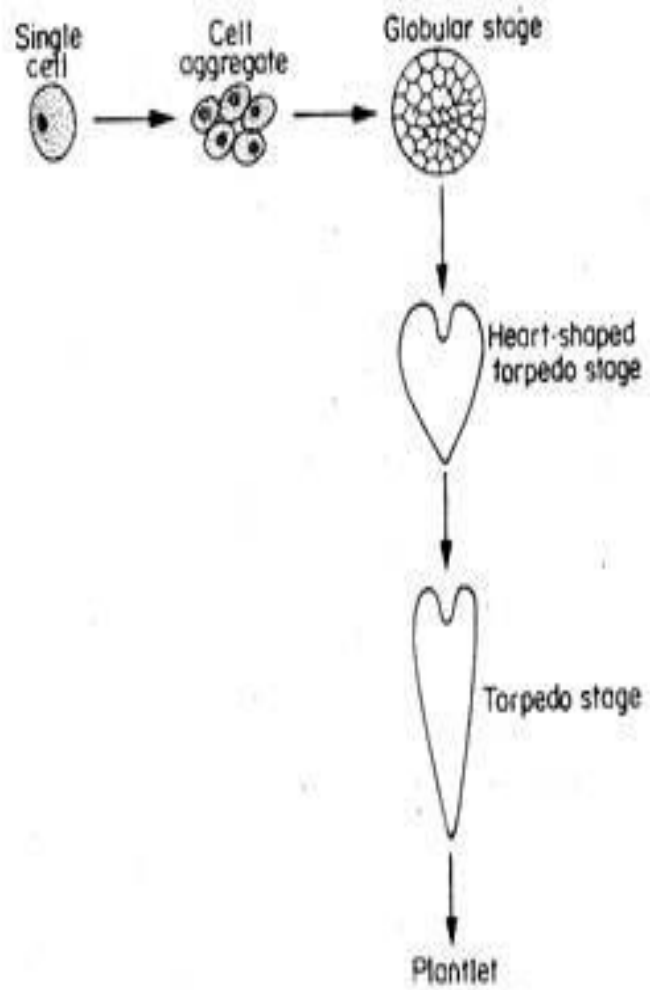


Fig. 20. Embryoid development in tissue culture passes through various stages, namely, globular stage, heart-shaped stage and torpedo stage before plantlet formation.

Types of Somatic Embryogenesis

1. Primary somatic embryogenesis: In this type, the embryogenesis can only be induced by using the explants.
2. Secondary somatic embryogenesis: In this phenomenon, the development of the embryo is induced through existing somatic embryos.

- The process of somatic embryogenesis involves four key steps which are, induction, maintenance, development, and regeneration.
- However, the two ways of inducing somatic embryogenesis include:

A. Direct somatic embryogenesis: In this process, the embryo is developed without any intermediate callus stage. The embryo can be developed by directly inducing the explant for the genesis.

B. Indirect somatic embryogenesis: In this process, the development of an embryo occurs with an intermediate callus stage. So, it is a multistep process.

Characteristics differ from Zygotic embryos

1. Somatic embryogenesis is an inductive process and embryogenesis can takeplace without the involvement of fertilization or gametic fusion.
2. Early divisions do not follow sequence in somatic embryos
3. Suspensor is absent in somatic embryos
4. Somatic embryos do not become dormant
5. Lack of endosperm differentiation in somatic embryos
6. No vascular connection in somatic embryo
7. Unlike the zygotic embryos, SEs, often show secondary embryogenesis andtheir development is asynchronous.
8. Zygotic embryos are usually diploid (sometimes may be polyploidy) but somatic embryos may be haploid, diploid or triploid

Somatic embryogenesis

Factors influencing somatic embryogenesis:

(1) Auxin:

- In medium having relatively high concentration of auxin embryonal budding or embryonal clumps have been observed. For cell differentiation the medium should contain auxin and reduced nitrogen.
- Subsequent development takes place in medium with no auxin or low concentration of auxin and reduced nitrogen.

(2) Nitrogen:

- The ratio of nitrogen to auxin is an important factor controlling embryogenesis.
- Embryo development can be initiated on White's medium with low nitrogen content only in absence of auxin.

Somatic embryogenesis

(3) Cytokinin:

Embyogenesis in carrot cell suspension is stimulated by addition of zeatin in medium lacking auxin but inhibited by the addition of kinetin.

(4) Activated charcoal:

Presence of activated charcoal in the medium helps embryogenesis in several cases, as in Daucas carota, Hedera helix etc. Activated charcoal may adsorb the inhibitory substances present in the medium.

(5) Age of the culture:

Embryogenesis usually occurs in short-term cultures. With older cultures this ability decreases and ultimately it is completely lost. This may be due to either the inability to synthesise some embryogenetic substances or changes in the ploidy level which may lead to loss of morphogenetic potential.

Somatic embryogenesis

Applications of Somatic Embryogenesis:

(i) Large Scale Propagation Compared to Zygotic Embryos:

(ii) More Useful than Organogenesis: **The adventitious embryo is a bipolar structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture.**

(iii) Useful for Mutagenic Studies and Mutant Production: **The somatic embryos generally arise from single cells, so it may be advantageous for mutagenic studies.**

(iv) Useful for Genetic Manipulation Technique: **In plant biotechnological application, if the transformed cell gives rise to plantlet via somatic embryogenesis then this method of multiplication system is very much useful.**

Somatic embryogenesis

Applications of Somatic Embryogenesis:

(v) Useful for Pathogen-Free Plant Production:

Plants derived from this kind of somatic embryos may be free from viral or other pathogens. So it may be an alternative approach of disease free plant production.

(vi) A Good Source of Protoplast Culture:

Embryogenic cultures are specially valuable in providing a source of regenerable protoplasts in the graminaceous and coniferous plants.

(vii) Conservation of Genetic Resources:

Somatic embryos which originate from single cells and subsequently regenerate mostly genetically uniform plants are good materials for genetic resource conservation.

Zygotic Embryo Culture

Growth of embryo on an artificial nutrient medium is known as embryo culture. In many cases, development of embryo has been found to fail even after gametic fusion. Seeds formed in them were non-viable. When embryo is taken out of such ovule and grown on artificial nutrient medium, it grows normally.

Recent research has shown that in such cases it is frequently possible to excise the young embryos from the ovules and culture them in artificial media.

Embryo culture was first done successfully by Hanning (1902) in certain crucifers e.g. *Raphanus sp*. He used sugars, mineral salts, plant decoctions, certain amino acids and gelatin as different media.

The cultured embryos tended to skip the stages of development and grew directly into seedling.

Zygotic Embryo Culture

- The excised embryos are transferred into culture bottles containing a nutrient medium.
- All the steps are made in sterilized condition.
- Laminar flow chamber and various chemicals are available for sterilization purposes.
- Some embryologists observed that increased Osmotic value of the culture medium is essential for favouring the growth of the embryo. Some suggested that growth factors like IAA control the growth and differentiation of excised embryo on culture medium.

Importance of Embryo Culture

1. Embryo culture experiments has also helped in finding out the nature of factors which are involved in dormancy of seeds after ripening and also vernalization of seeds to some extent.

2. Embryo culture experiments has also made it clear that the physiological differences between different species are due to genetic differences.

3. Embryo culture technique has given very clear understanding about the environment inside the seed.

4. Crosses between plants of different ploidy within the same species have been found to be possible. e.g. Iris and Zea.

Importance of Embryo Culture

5. Inter specific hybrids have been obtained in many cases, e.g. *Gossypium, Datura, Lycopersicum*.

6. Some inter-generic hybrids have been obtained e.g. *Hordeum* and *Secale*, between *Datura* and *Brugmansia*, between *Triticum* and *Elymus* etc.

7. The culture of embryos from normal seeds has been used to shorten breeding cycles. In roses also, which requires a whole year to come into flowering, embryo culture has made it possible to shorten the breeding cycle and produce two generations in a year.

8. Embryo culture has served as a tool in studies of the influence of specific substance on the morphology of embryos.

Micropropagation

Micropropagation

Definition-

Micropropagation can be defined as a technique in which any vegetative (meristmatic) part of plant such as shoot tip, shoot bud etc is excised aseptically and cultured on sterile media under controlled conditions to give rise to plantlet which is exact copy of its donor plant.

In Simple words, it can be defined as clonal propagation *invitro*.

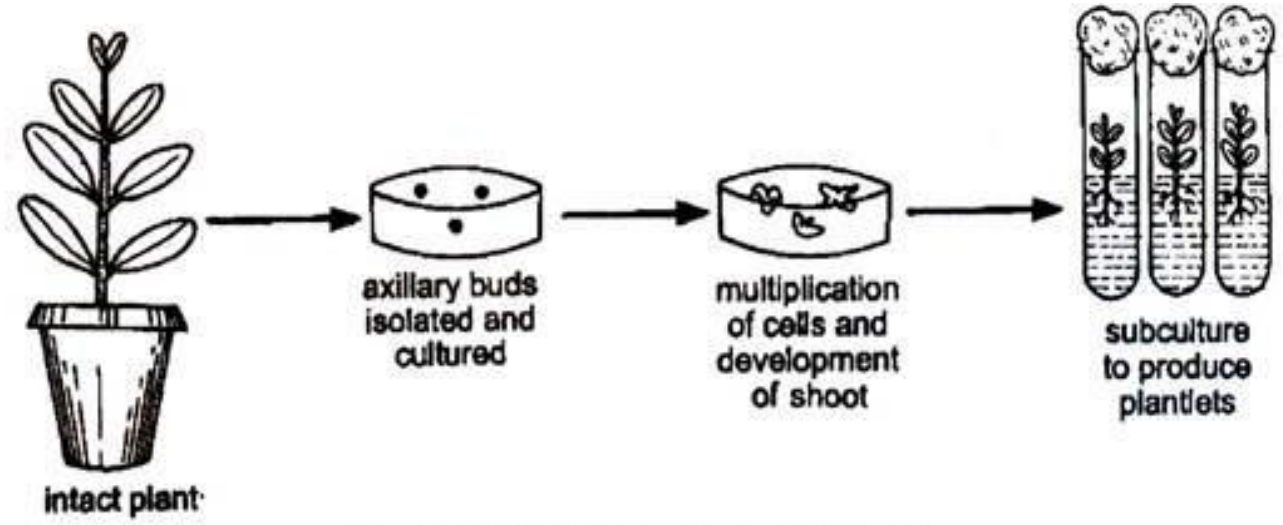


Fig. 12. Micropropagation of plants.

Micropropagation

- It is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods.
- It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

General Technique of Micropropagation

Stage 0: Pre-propagation Stage

The pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels and net covered tunnels, provide high quality explant source plants with minimal infection.

Collection of explants for clonal propagation should be done after appropriate pre-treatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro cultures.*

General Technique of Micropropagation

Stage 1: Initiation of Aseptic Culture:

- In this stage sterilization of explants and establishment of explants were done. The plant organ used to initiate a culture is called explant.The choice of explant depends on the method of shoot multiplication to be followed.
- For micropropagation work the explant of choice is nodes
- For callus culture work the explant of choice is internodes and leaves.
- For somatic embryogenesis the explant is internodes and leaves.

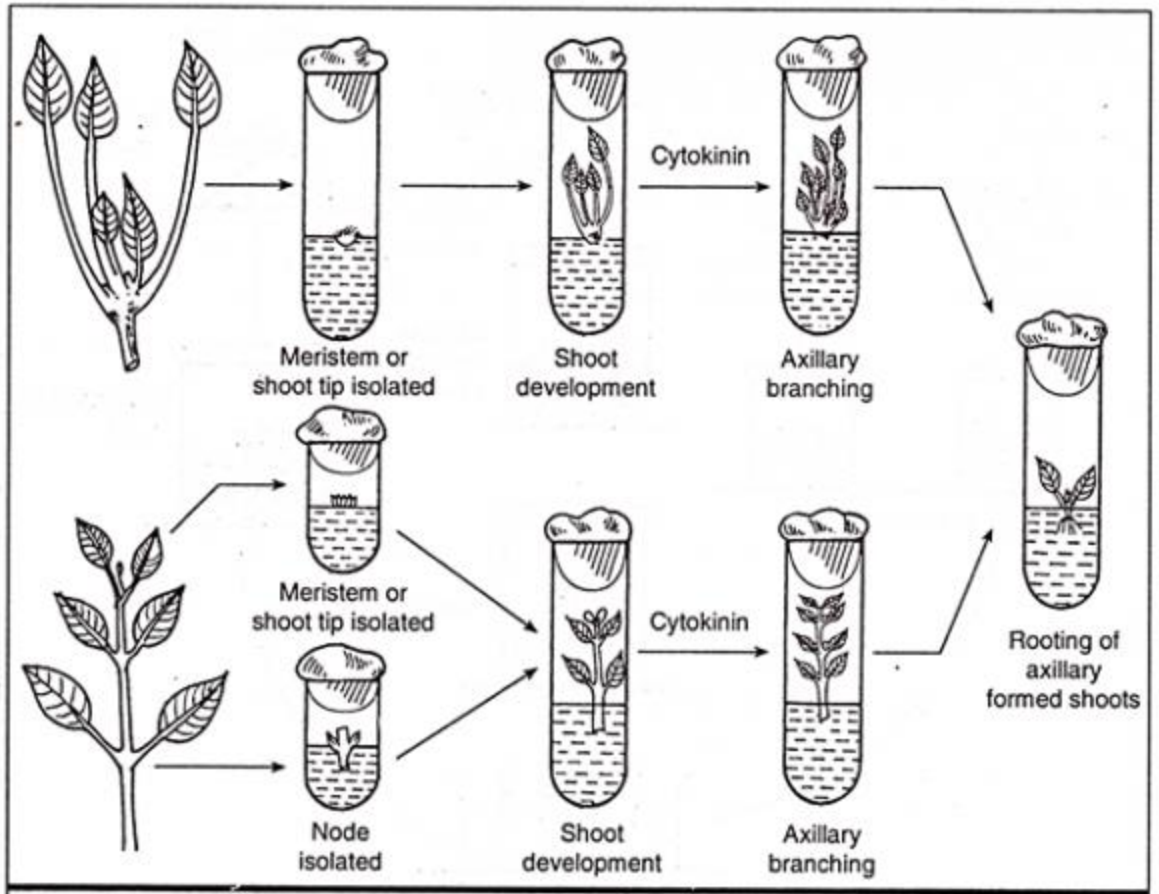


Fig. 18.9A: Scheme showing the *in vitro* micropropagation of plants by the axillary bud method. Upper row: as applied to rosette plants; bottom row: applied to plants which elongate

General Technique of Micropropagation

Stage 2: Multiplication of Culture:

This is the most important stage and the rate of multiplication determines the

largely success of micropropagation system this can be achieved by-

A. Enhanced axillary branching-The axillary bud present in the axil of each leaf either develops into a single shoot or form a cluster of shoots in the presence of cytokinins (BAP 1.0mg/l)in the medium.

B. Adventitious bud formation-Buds arising from any part other than the leaf axils or shoot apex are called adventitious buds. It is a standard horticulture practice.

C. Through callusing-This either gives rise to shoot bud or bipolar structure resembling embryo (somatic embryo).

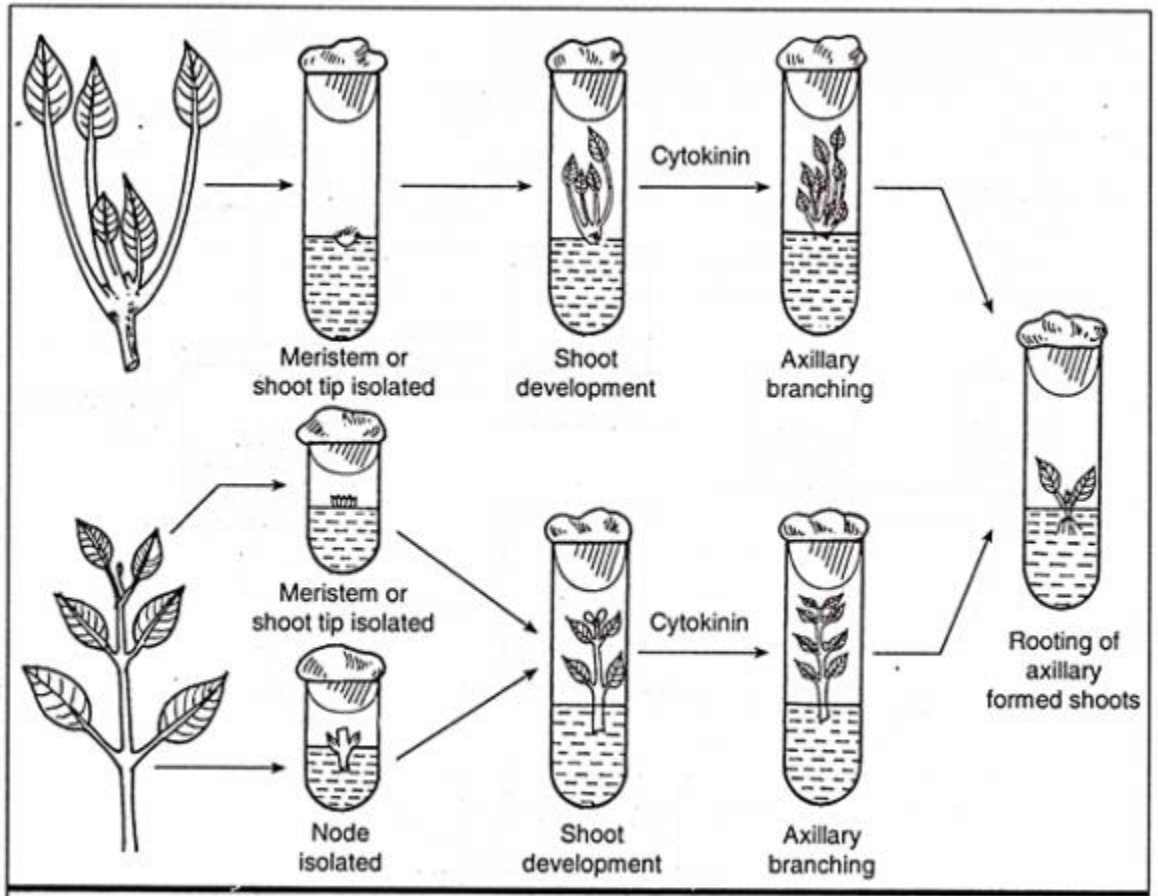


Fig. 18.9A: Scheme showing the *in vitro* micropropagation of plants by the axillary bud method. Upper row: as applied to rosette plants; bottom row: applied to plants which elongate

General Technique of Micropropagation

Stage 3: *In Vitro* Rooting of Shoots

In-vitro grown shoots lack root system. For induction of roots they were transferred to rooting medium.

For rooting half strength MS medium supplemented with 1.0mg/l auxin was used.

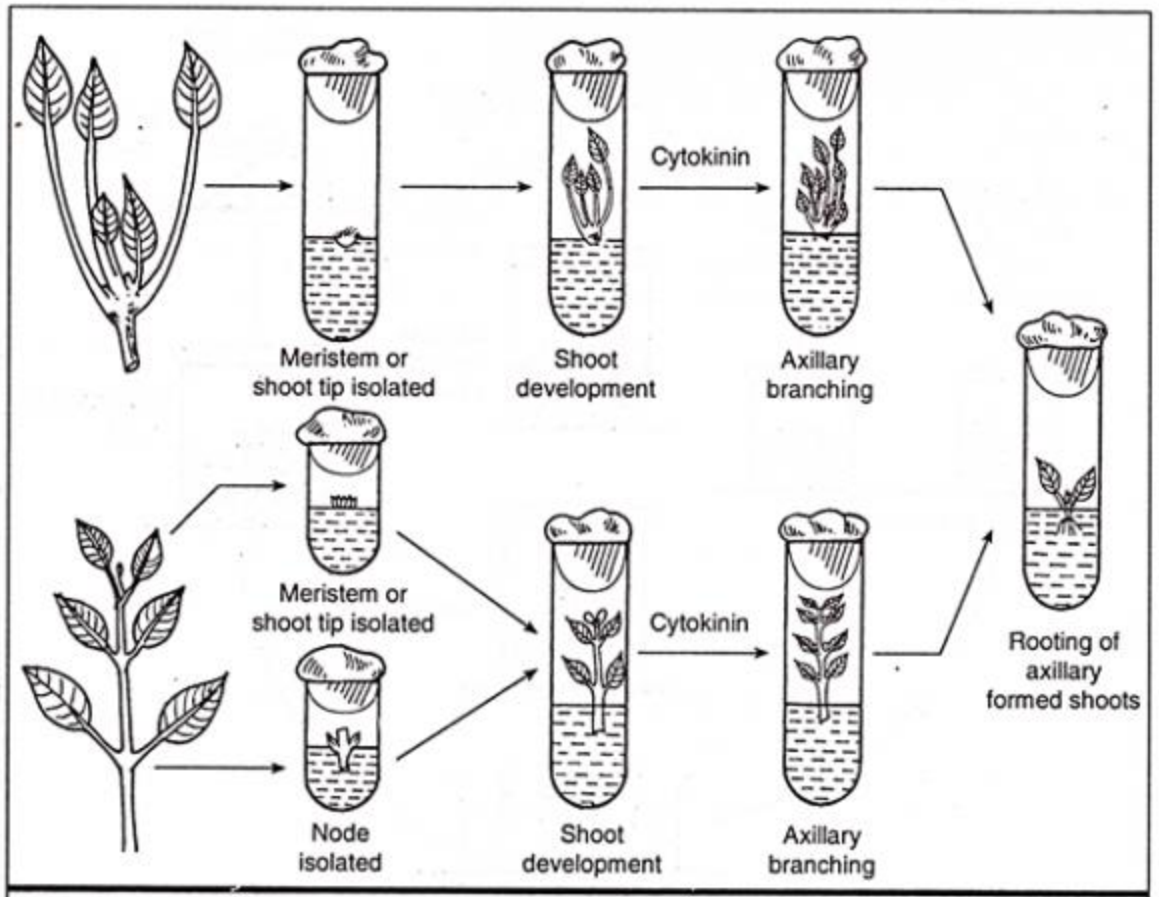


Fig. 18.9A: Scheme showing the *in vitro* micropropagation of plants by the axillary bud method. Upper row: as applied to rosette plants; bottom row: applied to plants which elongate

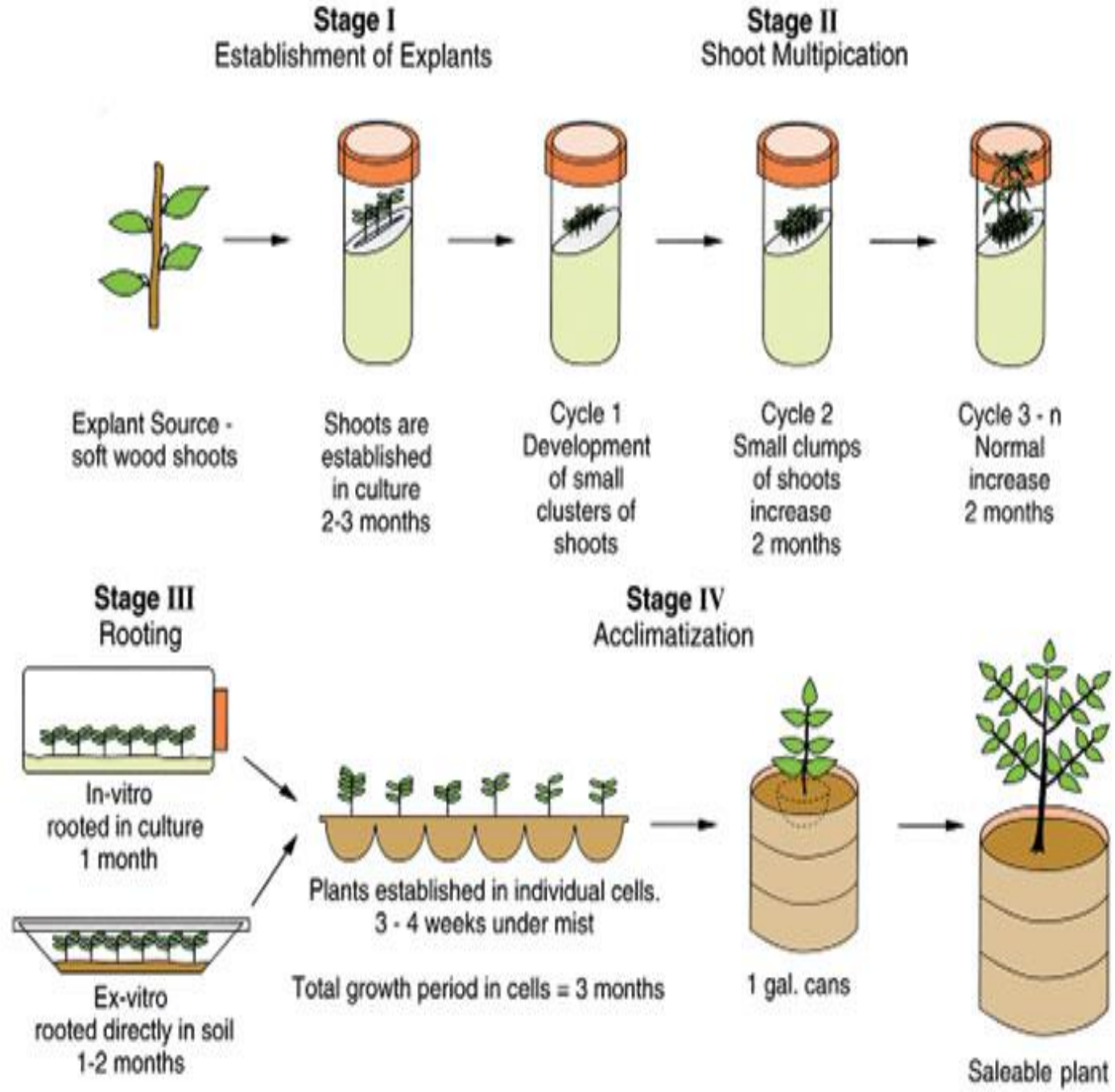
General Technique of Micropropagation

Stage 4: Hardening and Acclimatization of Tissue Culture Plantlets

- This is the final stage and requires careful handling of plants.
- The transplantation from completely controlled conditions should be gradual.
- This process of gradually preparing the plants to survive in the field conditions is called acclimatization.
- Optimum conditions were provided to plants in green house.

Stage	Methodology involved
Stage 0	Selection of mother plant and its maintenance
↓	
Stage I	Initiation and establishment of culture
↓	
Stage II	Multiplication of shoots or rapid somatic embryo formation
↓	
Stage III	*In vitro* germination of somatic embryos and/or rooting of shoots
↓	
Stage IV	Transfer of plantlets to sterilized soil for hardening under greenhouse environment

Fig. 47.1 : Major stages involved in micropropagation.



Advantages of Micropropagation

- The main advantage of micropropagation is the production of many plants that are clones of each other.
- Micropropagation can be used to produce disease-free plants.
- Micropropagation produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.
- It can have an extraordinarily high frequency rate, producing thousands of propagules while conventional techniques might only produce a fraction of this a number.
- It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.

Advantages of Micropropagation

- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored.
- Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
- Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
- A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

Disadvantages of Micropropagation

1. It is very expensive, and can have a labour cost of more than 70%.
2. A monoculture is produced after micro propagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same infections.
3. An infected plant sample can produce infected progeny. This is uncommon as the stock plants are carefully screened and vetted to prevent culturing plants infected with virus or fungus.
4. Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produce secondary metabolic chemicals that stunt or kill the explant.
5. Sometimes plants or cultivars do not come true to type after being tissue cultured. This is often dependent on the type of explant material utilized during the initiation phase or the result of the age of the cell or propagule line.
6. Some plants are very difficult to disinfect of fungal organisms.

Conventional propagation Vs Micropropagation

Conventional propagation	Micropropagation
Cuttings, budding, grafting, layering	Tissue culture using axillary buds and meristems
• Equipment costs minimal. • Little experience or technical practice • Inexpensive • Specialized techniques for growth control (e.g. grafting onto dwarfing rootstocks).	• From one to many propagules rapidly • Multiplication in controlled lab conditions & Reduce time and space • Expensive method • Require experienced and technical practice.

Micropropagation Vs Tissue culture

Micropropagation and tissue culture are both techniques used to produce large quantities of plants under aseptic and environmentally controlled conditions. While often used interchangeably, there are stark differences between the two techniques:

Micropropagation	Tissue Culture
1. Vegetative propagation of new plants in tissue culture.	1. A technique in which plant cells, tissues or organs is artificially grown under aseptic and controlled conditions
2. Uses tissue fragments derived from the meristem.	2. Uses cell, tissue or organ samples from any part of the plant
3. Takes place after tissue culture	3. It Is the first step in the process of micropropagation.

Factors Affecting Micro propagation

1. Genotype of the plant:

Selection of the right genotype of the plant species (by screening) is necessary for improved micro propagation. In general, plants with vigorous germination and branching capacity are more suitable for micro- propagation.

2. Physiological status of the explants:

Explants (plant materials) from more recently produced parts of plants are more effective than those from older regions. Good knowledge of donor plants' natural propagation process with special reference to growth stage and seasonal influence will be useful in selecting explants.

3. Culture media:

The standard plant tissue culture media are suitable for micro propagation during stage I and stage II. However, for stage III, certain modifications are required. Addition of growth regulators (auxins and cytokinins) and alterations in mineral composition are required. This is largely dependent on the type of culture (meristem, bud etc.).

Factors Affecting Micro propagation

4. Culture environment:

Light:

Photosynthetic pigment in cultured tissues does absorb light and thus influence micro- propagation. The quality of light is also known to influence in vitro growth of shoots, e.g blue light induced bud formation in tobacco shoots. Variations in diurnal illumination also influence micro propagation. In general, an illumination of 16 hours day and 8 hours night is satisfactory for shoot proliferation.

Temperature:

Majority of the culture for micro propagation requires an optimal temperature around 25°C. There are however, some exceptions e.g. Begonia X Cheimantha hybrid tissue grows at a low temperature (around 18°C).

Haploid Production

Androgenesis

The technique of production of haploids through anther or microspore culture is termed as androgenesis.

It is a excellence method for the large scale production of haploids through tissue culture.

Androgenesis technique for haploid production is based on the in-vitro culture of male gametophyte i.e., microspore of a plant resulting into the production of complete plant from it.

It is achieved either-

1. by anther culture or
2. by microspore (pollen) culture.

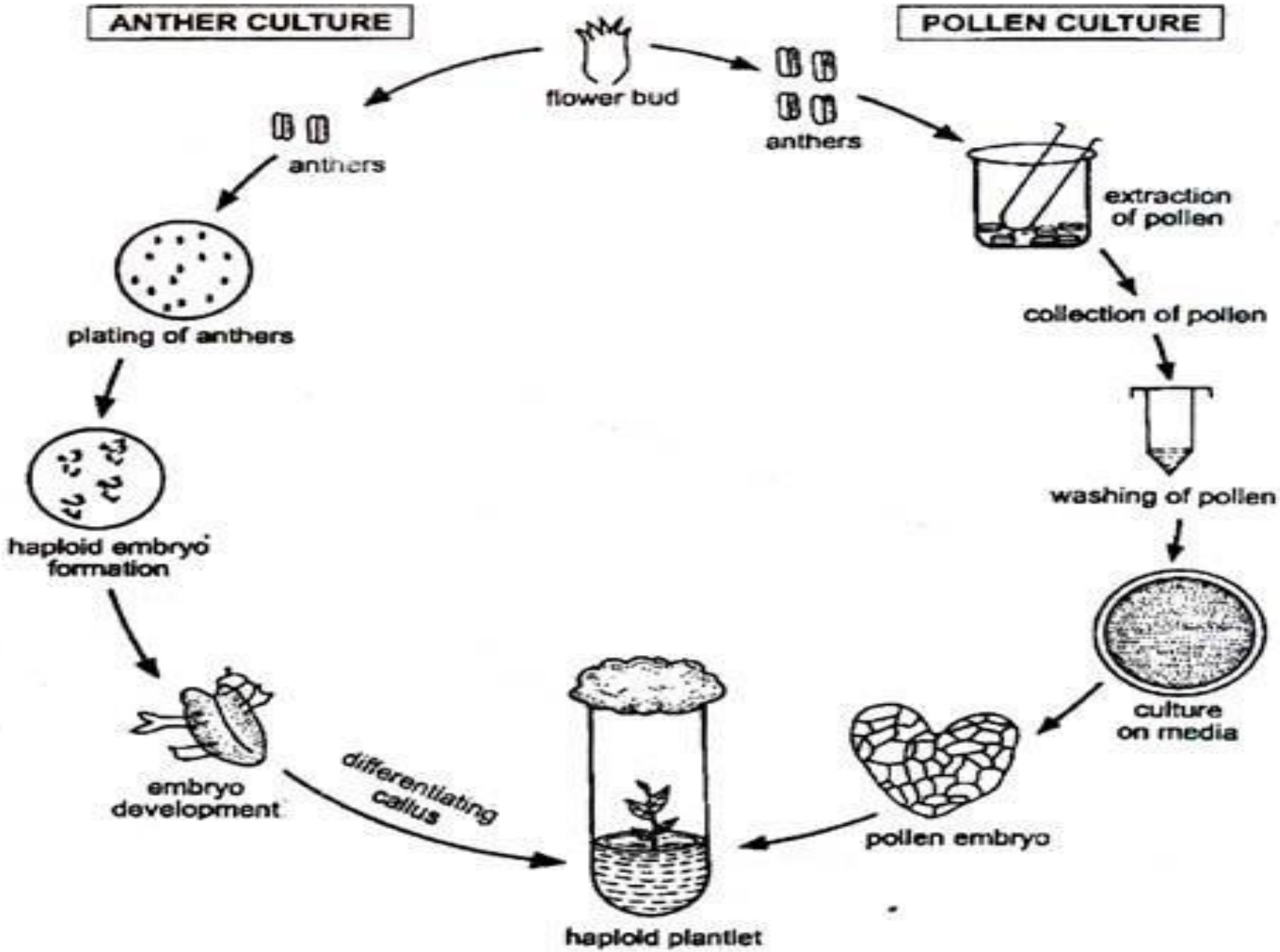


Fig. 9. Androgenesis for Haploid Production.

Androgenesis

Anther culture Vs Microspore (Pollen) culture

- The technique of another culture is quicker for practical purposes and is an efficient method for haploid production.
- But sometimes during another culture, the plantlets may originate from different other parts of anther also (along with from the pollens).
- On the other hand, microspore culture is free from any uncontrolled effects of the anther wall or other tissues.
- Microspore culture is ideal method for studying the mutagenic and transformation patterns.

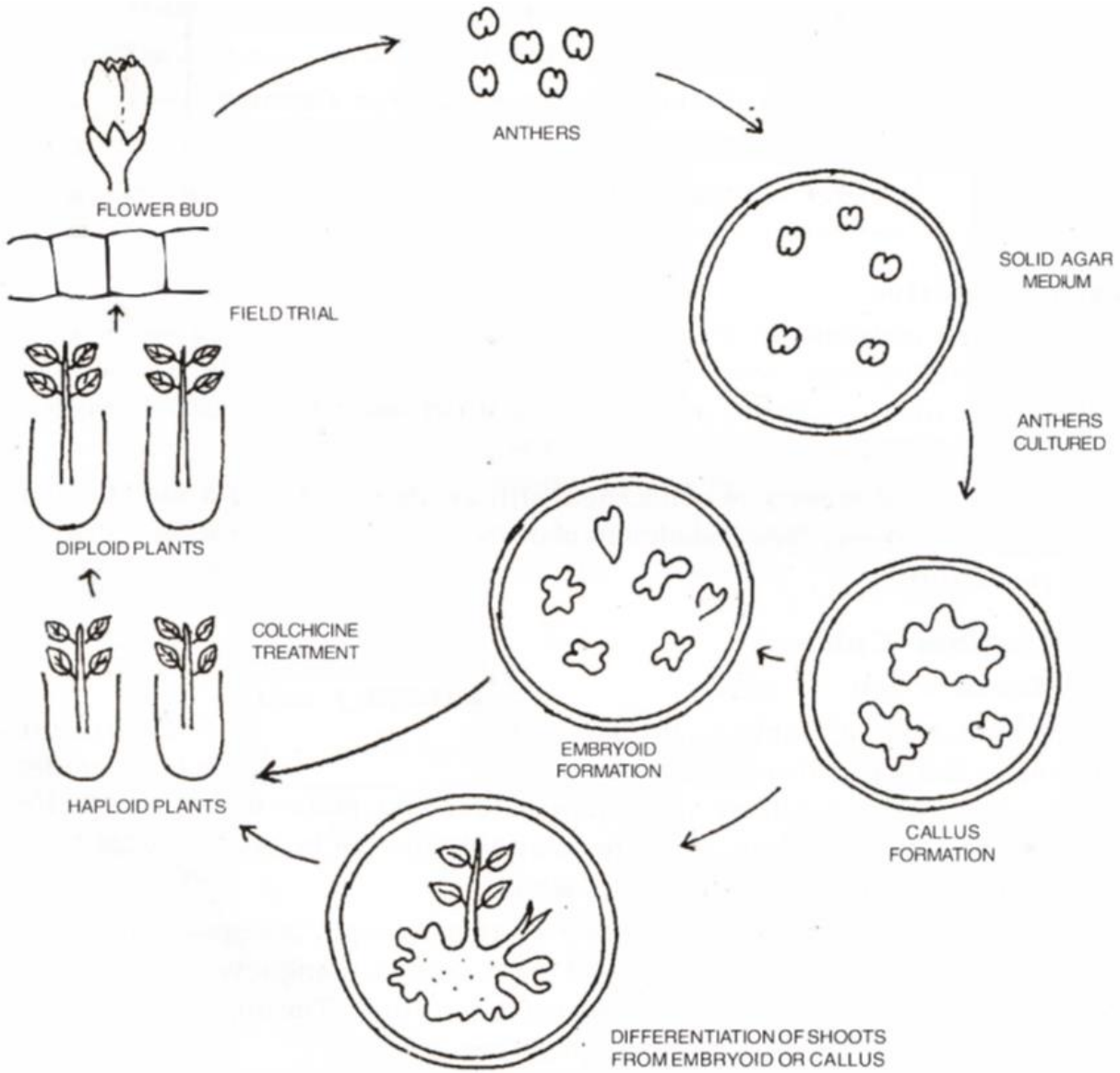
Anther Culture

Anther Culture is an ‘in vitro’ technique to produce haploid from normal diploid plant through culturing anthers on artificial nutrient medium.

Haploids are defined as sporophytes with gametophytic chromosome number and have been produced in a variety of plant species using a variety of methods.

Anther Culture

In this culture technique, the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and are cultured on a nutrient medium where the microspores within the cultured anther develop into callus tissue or embryoids that give rise to haploid plantlets either through organogenesis or embryogenesis.



Microspore (Pollen) culture

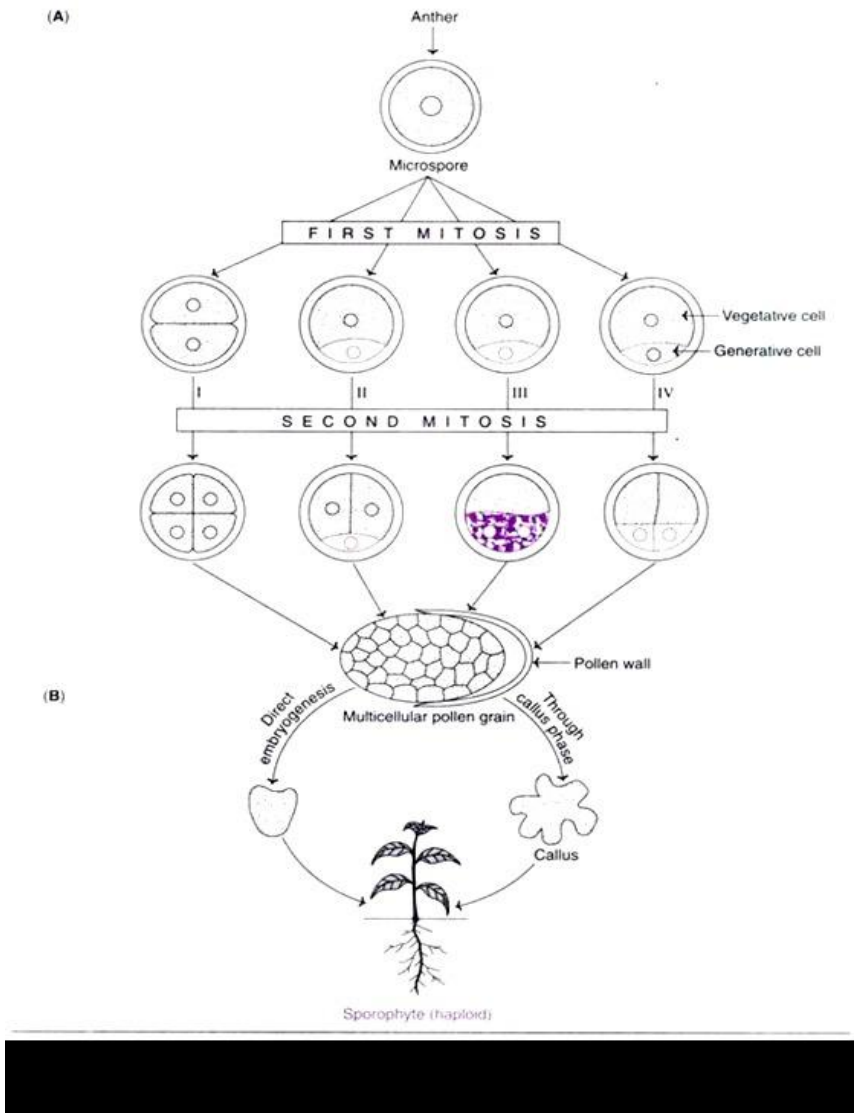
There are four different pathways to form the multicellular condition of pollen from the unicellular pollen.

Pathway I: The microspore divides by an equal division and two identical daughter cells contribute equally to the sporophyte development e.g., *Datura innoxia*.

Pathway II: The uninucleate microspores divide unequally forming vegetative and generative cell. The sporophyte arises through divisions of vegetative cell, the generative cell gets degenerated e.g., *Nicotiana tabacum*.

Pathway III: The uninucleate microspore undergoes a normal unequal division but the pollen embryos are formed from the generative cell alone. The vegetative cell does not divide, e.g., *Hyocyamus niger*.

Pathway IV: The division of microspore is asymmetrical as in pathway II, but both the cells take part in embryo formation and sporophyte development e.g., *Datura metal*.



Factor Affecting Androgenesis

(a) **Stage of Pollen:** Anthers with microspores ranging from tetrad to the bi-nucleate stage are responsive to culture. As soon as the starch deposition starts within the microspore there is no further development towards sporophyte.

(b) **Physiological Status of Donor Plant:** The anther should be collected from the flower buds of adult healthy plant and it is very important to use the healthy explant. The variation in response of anthers from plants grown under different environmental conditions may be due to the differences in endogenous level of growth regulators. Flowers from relatively younger plants, flowering at the beginning of flower-season are more responsive.

(c) **Genotype of Plants:** Success of anther culture is highly dependent on the genotype of the plant. It has been observed that various species and cultivars exhibit different growth responses in culture.

(d) **Pretreatment of Anthers:** The pathways towards androgenesis require to stop the development of pollen cell towards gamete formation and to force to develop the multicellular condition.

Importance of anther culture

1. Among the different importance of haploids, their use in crop improvement is considered to be the most significant and stands out as the most important reason for emphasis on haploid research.

2. Haploids have also been successfully utilized for barley, maize, sugarcane, oilseed etc.

3. In sugarcane selection among anther culture, derived haploids led to the development of superior lines with tall stem and higher sugar content.

4. Due to presence of only one set of chromosomes, even the recessive mutations are immediately expressed in haploids.

5. Anther can be plated on solid medium like single cells.

Importance of anther culture

6. Gametoclonal variation can be developed from culture derived from anthers. The gametoclonal variation is actually utilized in hybrid sorting during the use of haploids for plant breeding purposes.

7. In several crops, desirable mutants have been isolated among haploids derived in culture.

8. Haploids have been found to be useful in various areas of cytogenetics research, including the following:

(i) Production of aneuploids

(ii) determination of basic chromosome number, and

(iii) determination of the nature of polyploidy.

Germplasm Preservation

- Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.
- Germplasm provides the raw material for the breeder to develop various crops.
- Thus, conservation of germplasm assumes significance in all breeding programmes.

Objectives

- The very 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 future.
- In recent years, many new plant species with desired and improved characteristics have started replacing the primitive and conventionally used agricultural plants. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the primitive plants may be lost.
- A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

Methods of Conservation

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

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

In-Situ Conservation

- 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.
- Limitations:

i. The risk of losing germplasm due to environmental hazards

ii. The cost of maintenance of a large number of genotypes is very high.

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 from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions.

Germplasm conservation in the form of seeds:

Usually, seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space.

Ex-Situ Conservation

Limitations in the conservation of seeds:

i. Viability of seeds is reduced or lost with passage of time.

ii. Seeds are susceptible to insect or pathogen attack, often leading to their destruction.

iii. 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*.

iv. It is difficult to maintain clones through seed conservation.

Ex-Situ Conservation

Germplasm conservation by in vitro cultures (plant cells, tissues or organs):

In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants.

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.

Methods of Ex-Situ Conservation

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

1. **Cryopreservation (freeze-preservation)**
2. **Cold storage**
3. **Low-pressure and low-oxygen storage**

Cryopreservation

Cryopreservation 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 Methods:

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.

In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut.

Plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

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.

Many in vitro developed shoots/plants of fruit tree species have been successfully stored by this approach, e.g. grape plants, strawberry plants.

Advantages:

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.

Low-Pressure and Low-Oxygen Storage

As alternatives to cryopreservation and cold storage, for germplasm conservation.

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

Low-Pressure and Low-Oxygen Storage

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.

Limitations of LOS:

The long-term conservation of plant materials by low-oxygen storage is likely to inhibit the plant growth after certain dimensions.

Cryopreservation

It means preservation at ultralow temperature

- This technique is used mainly for long term storage of germplasm and thus helps in conservation of nature also.
- Plant tissues and organs are cryopreserved usually in liquid Nitrogen which has a temperature of 196°C.
- Cryopreservation technique has proved to be one of the most reliable methods for long term storage and preservation of plant germplasm in the form of pollens, shoot-tips, embryos, callus, protoplasts, etc.

Principles of Cryopreservation

Exposing cells to temperatures below 0°C without the aid of cryoprotectants is typically lethal. Since water constitutes approximately 80% of tissue mass, the freezing of water, both intra and extracellularly, imposes the largest influence over harmful biochemical, and structural changes that are thought to result in unprotected freezing injury.

Two independent theories exist that attempt to explain the harmful effects of freezing on cells:

(1) Ice crystals mechanically disrupt cellular membranes thus making it impossible to obtain structurally-intact cells after thawing; and

(2) Deadly increases in solute concentration occur to the remaining liquid phase as ice crystals form intracellularly during cooling.

Whether the mechanical or osmotic effects of freezing dominate, the end result is the same; unprotected cooling and thawing of cells is a process incompatible with life. To mitigate these effects, two protective actions must be carried out: use of a cryoprotectant, and selection of an appropriate cooling and thawing rate.

Principles of Cryopreservation

The method of preserving at low temperature varies depending upon the cells types among different mammalian species. It is generally be grouped into te following two types as described above which are slow freezing and fast freezing. Fast freezing of cryopreservation is also known as vitrification.

Whatever the method is, both the processes enable us to subzero storage of biological materials.

The major steps in cryopreservation are following;

- **The mixing of CPAs with cells or tissues before cooling;**
- **Cooling of the cells or tissue to a low temperature and its storage;**
- **Warming of the cells or tissues; and**
- **Removal of CPAs from the cells or tissues after thawing**

Therefore, appropriate use of CPAs is important to improve the viability of the sample to be cryopreserved.

Cryoprotective agents (CPAs)

- A cryoprotective agent is a substance that is used to prevent ice formation that causes freezing damage to the biological materials such as cells, tissues, or organs.
- It does so by reducing the ice formation at any temperature by increasing the total concentration of all the solutes present in the system.
- It is generally a fluid and able to penetrate the cells, and have low toxicity.
- Different cryoprotective agents are used in different cell types and therefore its use is also dependent upon cell type, cooling rate, and warming rate and its concentration are optimized accordingly.
- TheCPAs are divided into two categories, which are as follows;

1. Cell membrane-permeating cryoprotectants, such as dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol; and
2. Nonmembrane-permeating cryoprotectants, such as 2-methyl-2,4-pentanediol and polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, and various sugars.

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.**

Technique of Cryopreservation

Development of sterile tissue culture:

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.

Technique of Cryopreservation

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.

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.

Technique of Cryopreservation

Freezing:

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

Four different types of freezing methods are used:

1. **Slow-freezing method:**
2. **Rapid freezing method:**
3. **Stepwise freezing method:**
4. **Dry freezing method:**

Technique of Cryopreservation

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.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability.

Technique of Cryopreservation

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.

Technique of Cryopreservation

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.

Technique of Cryopreservation

Measurement of survival/viability:

- 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.

Technique of Cryopreservation

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.

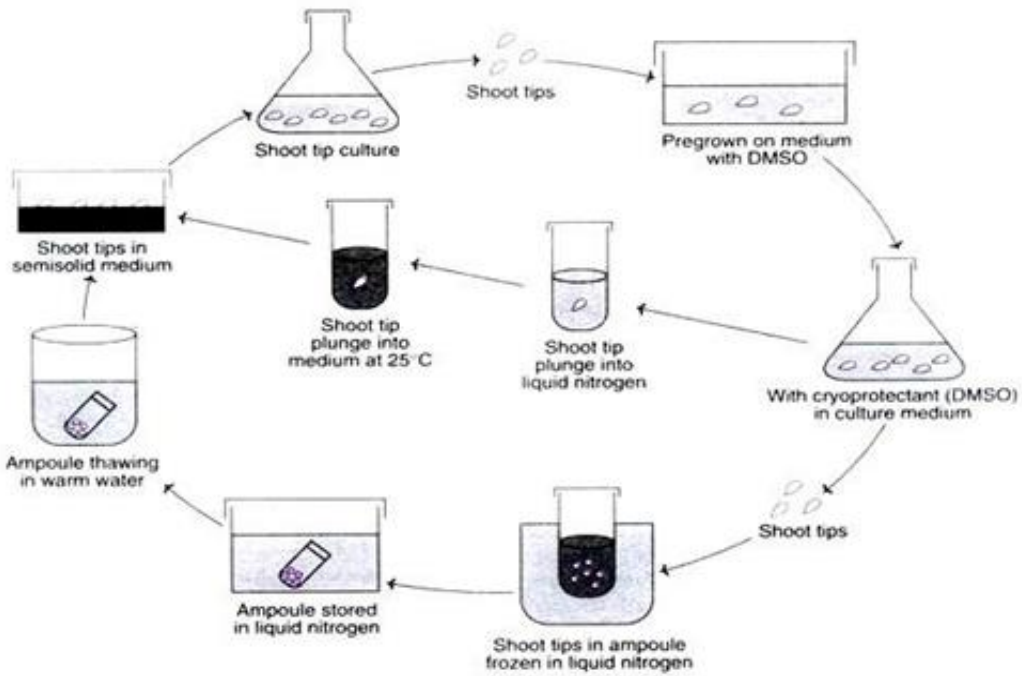


***Fig. 48.1** : An outline of the protocol for cryopreservation of shoot tip (DMSO–Dimethyl sulfoxide).*

Precautions/Limitations for Successful Cryopreservation

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

Other 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.

Application of Cryopreservation of plant

- Maintenance of stock cultures: Plant materials (cell/tissue cultures) of several species can be cryopreserved and maintained for several years, and used as and when needed.
- Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).
- Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.
- Cryopreservation can maintain recalcitrant seeds for long.
- Conservation of somaclonal and gametoclonal variations in cultures.
- Accordingly, it can preserve plant materials from endangered and rare species.
- Conservation of pollen for enhancing longevity.
- Also, it can store rare germplasms developed through somatic hybridization and other genetic manipulations.
- Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.
- Finally, establishment of germplasm banks for exchange of information at the international level.