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**Introduction to Plant Tissue Culture
and Propagation**

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1.1.What is Plant Tissue culture?

Definition: Plant tissue culture is a technique with which plant cells, tissues or organs are grown on artificial nutrient medium, either static (solid) or liquid, under aseptic and controlled conditions. Plant tissue culture is widely used to produce clones of a plant in a method known as **micropropagation**. The production of exact copies of plants that produces particularly good flowers fruits or has other desirable traits. The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.

1.2.Applications of Cell and Tissue Culture:

Plant tissue cultures are associated with a wide range of applications the most important being the production of pharmaceutical, medicinal and other industrially important compounds. In addition, tissue culture is useful for several other purposes.

- ✓ Micropropagation
- ✓ Somaclonal variations
- ✓ Production of virus-free plants (virus eradication).
- ✓ Production of synthetic seeds
- ✓ Production of secondary metabolites (useful biochemicals)
- ✓ Organogenesis
- ✓ Germplasm preservation (*in vitro*)
- ✓ Protoplast culture and fusion
- ✓ Production of somatic hybrids and cybrids
- ✓ Plant breeding (Plant improvement)
- ✓ Application of plant tissue culture in forestry.

1.2.1. Micropropagation /Clonal Propagation:

Clonal propagation refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. The tissue culture methods of plant propagation, known as 'micropropagation' utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium. The micropropagation is rapid and has been adopted for commercialization of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. *Orchids*) and other plants. Only a small amount of tissue is required to regenerate millions of clonal plants in a year.

1.2.2.Somaclonal variation:

The genetic variations found in the *in vitro* cultured cells are collectively referred to as somaclonal variation and the plants derived from such cells are called as 'somaclones'. Larkin and Scowcroft have proposed a general term "somaclones" for plant variants achieved from tissue cultures. It has been observed that the long-term callus and cell suspension culture and plants regenerated from such cultures are often associated with chromosomal variations. In some callus culture there is a major tendency of the callus tissues towards the numerical variation of the chromosomes in the cells that occurs after a number of serial subcultures. Thus tissue culture is providing to be rich and novel sources of variability with a great potential in crop improvement without resorting to mutation or hybridization. Variant selected through tissue culture has been variously termed to as calliclones (from callus culture) or protoclones (from protoplast culture). Such variant plants show some useful characters such as resistant to a particular disease, herbicide resistant, stress tolerance etc.

1.2.3.Production of virus free plants:

The viral diseases in plants transfer easily and lower the quality and yield of the plants. It is very difficult to treat and cure the virus infected plants therefore tea plant breeders are always interested in developing and growing virus free plants. In some crops like ornamental plants, it has become possible to produce virus free plants through tissue culture at the commercial level. This is done by regenerating plants from cultured tissues derived from-

- A) Virus free plants and
- B) Meristems which are generally free of infection.
- C) Chemical treatment of the media- attempts have been made to eradicate the viruses from infected plants by treating the culture medium with chemicals e.g. addition of **cytokinins** suppressed the multiplication of certain viruses.

1.2.4.Production of synthetic seeds:

The somatic embryos are encapsulated in a suitable matrix (e.g. **sodium alginate**), along with substances like *mycorrhizae*, insecticides, fungicides and herbicides. These artificial seeds can be utilized for the rapid and mass propagation of desired plant species.

Benefits: They can be stored up to a year without loss of viability and they can be directly sown in the soil like natural seeds and do not need acclimatization in green house.

1.2.5.Production of secondary metabolites: Man depends on plants for many compounds other than food such as medicines, pigments, vitamins, hormones, flavouring agents, latex,

alkaloids, glycosides (steroids and phenolics), terpenoids, phytoalexins, tannins etc. The most important chemicals (phytochemicals) produced using cell culture are secondary metabolites, which are defined as 'those cell constituents which are not essential for survival & metabolism'. When cells undergo morphological differentiation and maturation during plant growth, some of the cells specialize to produce secondary metabolites.

Table showing plant species and secondary metabolites obtained from them using tissue culture techniques:

Product	Plant source	Uses
Artemisin	<i>Artemisia spp.</i>	Antimalarial
Azadirachtin	<i>Azadirachta indica</i>	Insecticidal
Berberine	<i>Coptis japonica</i>	Antibacterial, anti inflammatory
Capsaicin	<i>Capsicum annum</i>	Cures Rheumatic pain
Codeine	<i>Papaver spp.</i>	Analgesic
Camptothecin	<i>Camptotheca accuminata</i>	Anticancer
Cephalotaxine	<i>Cephalotaxus harringtonia</i>	Antitumour
Digoxin	<i>Digitalis lanata</i>	Cardiac tonic
Pyrethrin	<i>Chrysanthemum cinerariaefolium</i>	Insecticide (for grain storage)
Morphine	<i>Papaver somniferum</i>	Analgesic, sedative
Quinine	<i>Cinchona officinalis</i>	Antimalarial
Vincristine	<i>Catharanthus roseus</i>	Anticarcinogenic
Scopolamine	<i>Datura stramonium</i>	Antihypertensive
Atropine	<i>Atropa belladonna</i>	Muscle relaxant
Reserpine	<i>Rauwolfia serpentina</i>	Hypotension
Jasmine	<i>Jasmiun sp.</i>	Perfume
Ajmalicin	<i>Catharanthus roseus</i>	Anticancer
Pyrethrins	<i>Tagetes erecta</i>	Insecticide
Nicotine	<i>Nicotiana tabacum</i>	Insecticide
Furoquinoline, furocoumarin Rutanensin, rutamarin, rutacultin, chalepin	<i>Ruta chalepensis</i>	Antispasmodic, antipyretic, anti-inflammatory. analgesic

1.2.6.Organogenesis: Refers to differentiation of organs, such as shoot and root from an undifferentiated mass of cells. When an explant is placed in an artificially enriched nutrient

medium, its differentiated cells first de-differentiate and form a mass of unorganized cells known as callus.

Cytokinins and Auxins: Cytokinins such as kinetin and adenine promote shoot differentiation, while auxins, such as indole acetic acid (IAA) and naphthalene acetic acid (NAA) promote root differentiation.

The production of roots shoots or leaves.

These organs may arise out of pre-existing-meristems or out of differentiated cells.



1.2.7. Germplasm Conservation (*In vitro*): Germplasm refers to the sum total of all the genes present in a crop and its related species.

- (a) ***In-situ* conservation-** The germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries.
- (b) ***Ex-situ* conservation-** This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or *in vitro* cultures are preserved and stored as gene banks for long term use.
- (c) The method involved in the *in vitro* conservation of germplasm is:

Cryopreservation- In cryopreservation (Greek-krayos-frost), the cells are preserved in the frozen state. The germplasm is stored at a very low temperature using solid carbon dioxide (at -79°C), using low temperature deep freezers (at -80°C), using vapour nitrogen (at -150°C) and liquid nitrogen (at -196°C). The cells stay in completely inactive state and thus can be conserved for long periods. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Certain compounds like- DMSO (dimethyl sulfoxide), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline, acetamide etc are added during the cryopreservation. These are called cryoprotectants and prevent the damage caused to cells (by freezing or thawing) by reducing the freezing point and super cooling point of water.

1.2.8. Protoplast culture and fusion: Protoplasts are plant cells, whose cell wall is digested. Two different species of plants have been successfully fused to produce a single protoplast containing the genetic material and the cytoplasm of both the fusing protoplasts.

The process of fusion is not straightforward. It is facilitated by some agents, known as fusogens e.g. use of polyethylene glycol (PEG).

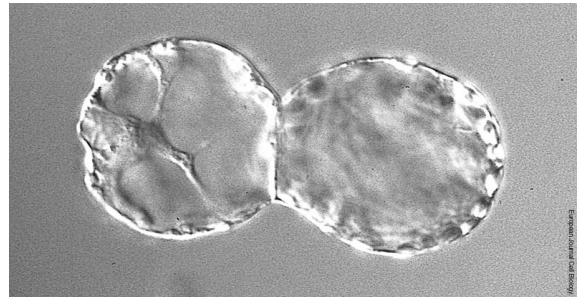
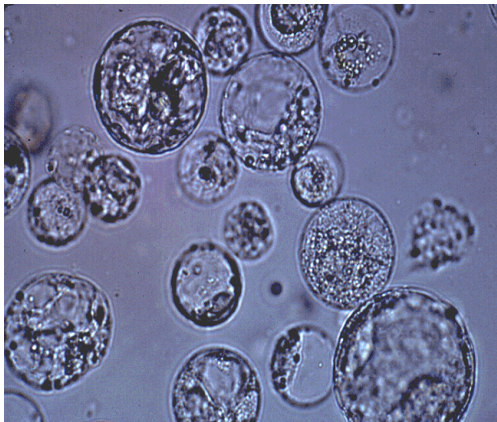
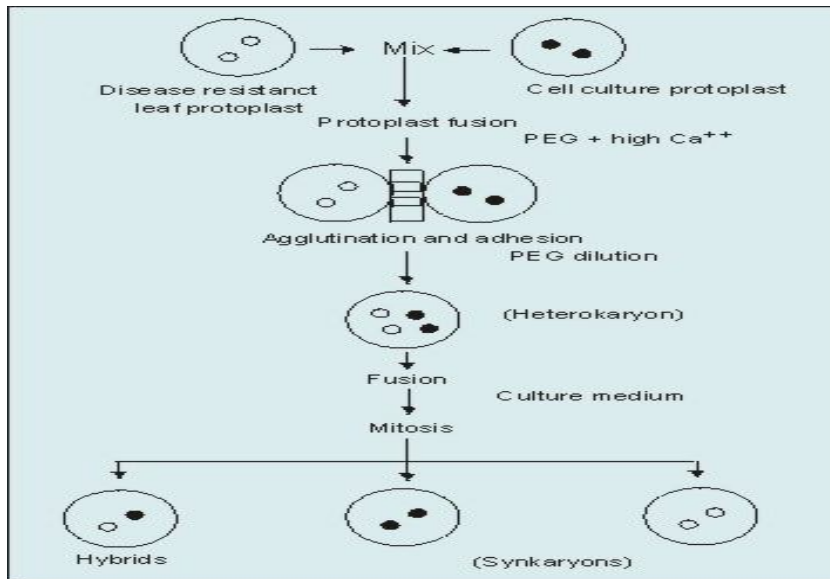


FIGURE 6.6a Two tobacco plant protoplasts, obtained by digesting away the cell wall, are fused (6a and 6b) to produce a cell that acquires some of the characteristics of both genetic backgrounds and can be regenerated into a plant with some traits from both parental plants.

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- Protoplasts are made from two species that you want to cross
- The membranes are made to fuse
- Regenerate the hybrid fusion product
- Contain genome from both organisms
- This process is very-2 difficult.

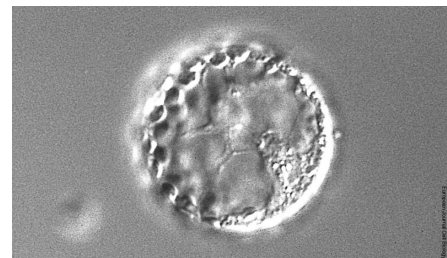


FIGURE 6.6b Two tobacco plant protoplasts, obtained by digesting away the cell wall, are fused (6a and 6b) to produce a cell that acquires some of the characteristics of both genetic backgrounds and can be regenerated into a plant with some traits from both parental plants.

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1.2.9. Production of Somatic hybrids and cybrids:

The Somatic cell hybridization/ parasexual hybridization or Protoplast fusion offers an alternative method for obtaining distant hybrids with desirable traits significantly between species or genera. **Somatic hybridization:**

Somatic hybridization broadly involves *in vitro* fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant. These treatments include the use of fusogens like NaNO_3 , high pH with high Ca^{2++} ion concentration, use of polyethylene glycol (PEG).

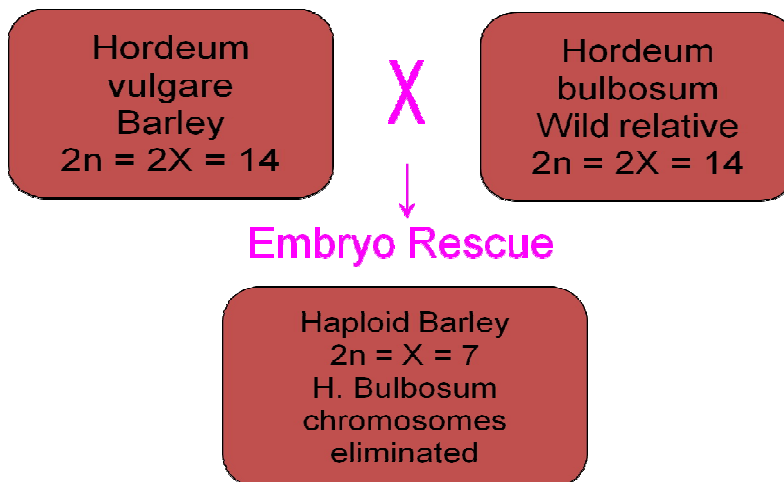
Carlson *et al.* (1972) produced the first inter-specific somatic hybrid between *Nicotiana glauca* and *N. langsdorffii*.

Melchers *et al.* (1978) developed the first inter-generic somatic hybrids between *Solanum tuberosum* and *Lycopersicon esculentum*. The hybrids are known as '**Pomatoes or Topatoes**'. However, this hybrid is of little commercial value.

Cybrids:

The cytoplasmic hybrids where the nucleus is derived from only one parent and the cytoplasm is derived from both the parents are referred to as cybrids. The process of formation of cybrids is called cybridization.

1.2.10. Plant breeding (Plant improvement): *Hordeum vulgare* or *Triticum aestivum* (used as male) is crossed with *H. bulbosum* (used as female) the chromosome complement of *H. bulbosum* is eliminated from the developing embryo. Most of the seedlings obtained from such crosses are haploid, having only one set of chromosomes either from *H. vulgare* or *T. aestivum* parent.



1.2.11.Application of plant tissue culture in forestry:

R. J. Gautheret (1934) first time cultured cambium tissues of some trees (*Salix caprea*, *Populus nigra*) on knop's solution containing glucose and cysteine hydrochloride.

R. J. Gautheret (1940) organogenesis in cambial tissues of *Ulnus campestris* in culture.

E. A. Ball (1950) first obtained buds from callus tissues of *Sequoia semipervirens*.

L. L. Winton (1968) found the regeneration of true triploid plant from the callus tissues of *Populus tremuloide*.

1.3.History of Plant Tissue Culture:

1. Schleiden and Schwann (1838-1839) proposed cell theory (cell is autonom and totipotent).
2. Henri-Louis Duhamel du Monceau's (1756) has done experiment on wound healing by spontaneous callus formation in elm plants.
3. Gottlieb Haberlandt (1902) developed the concept of *in vitro* cell cultured on glucose, peptone and knop's salt solution.
4. Sinnon (1908) achieved success in the regeneration of callus, buds, and roots from stem segments.
5. Kotte and Robbins (1922) observed meristematic cells in root tip.
6. Gautheret (1934) cultured cambium cell of some trees on glucose, cysteine hydrochloride, proliferation of cells stops after 6 months.
7. Van Overveek *et al.* (1941) used coconut milk for embryo development and callus formation.
8. Caplan and Steward (1948) reported callus explants by using coconut milk enhanced more proliferation of callus than auxin.
9. Muir (1953) transfer callus into liquid medium and used shaking machine. It's breakdown the callus into single cells.
10. Cocking (1960) isolated protoplast and cultured them.
11. Vasil and Hildebrandt (1965) raised whole plantlets from a single cell.
12. Guha and Maheswari (1966) cultured pollen immature anthers of *Datura innoxia* and raised haploid embryods and plants.
13. Power (1970) reported the fusion of protoplasts.
14. Takebe (1971) raised plants from protoplasts.
15. Sundberg and Glemelius (1986) produced somatic hybrid in Brassicaceae.

2.1. Organization of Plant Tissue Culture Laboratory (*In Vitro* Methods):

Objective

This lesson deals with the basic laboratory set-up that is required to initiate plant tissue culture work. The laboratory setup for tissue culture depends on the nature of the research undertaken and the availability of funds. For a standard tissue culture laboratory, following minimum facilities are necessary:

1. Washing & storage facilities
2. Media preparation, sterilization and storage room
3. Transfer area for aseptic manipulations
4. Culture rooms or incubators for maintenance of cultures under controlled conditions of temperature, light & humidity
5. Observation or data collection area

2.1.1. Washing & Storage Facilities:

New glassware is always washed using detergents especially designed for the purpose to remove all traces of acids, etc. Finally, the glassware is rinsed in tap water and then in distilled water. For this, sufficient area is required to accommodate large sinks (acid-lined), hot and cold running water, draining boards/ racks and sufficient distilled/demineralised water. Space should also be available to set up drying ovens; for storage of washed and dried labware; the washing area should have dustproof cupboards, etc.

In tissue culture, glassware should be resistant to heat. Cleaning of glassware is done by soaking it in sodium dichromate/sulphuric acid (conc.) for 4 hrs and then washing under tap water. The glassware is then soaked in a detergent solution for 16 hrs, and then rinsed first in tap water followed by a second rinse in distilled water. Glassware is then dried in oven at high temperature.

Instead of glasswares, a wide range of presterilised polystyrene culture containers (plastic labwares) are now available which can be disposed off after use. Reusable plastic labware is also available now. These can be washed with a mild detergent and rinsed with tap and distilled water. Washed and dried labware is finally stored in a closed cupboard (dust free).

2.1.2. Media Preparation, Sterilization and Storage Room:

This room should have sufficient space for storage of chemicals, labware, culture vessels, pH meters, balances, water baths, burners etc. A microwave oven, autoclave, or domestic pressure cooker for sterilizing media, culture vessels and instruments are appliances most needed for media preparation.

2.1.3. Transfer Area for Aseptic Manipulations:

The simplest type of transfer area is an enclosed plastic box which can be sterilized with a UV light and by cleaning the floor surface with 70% ethyl alcohol. A small wooden hood may also be used for tissue culture work.

When a large number of cultures or transfers are being manipulated, large equipment is required and the most desirable arrangement is a dust-free room equipped with an overhead UV and positive pressure ventilation unit. The ventilation should possess a high-efficiency particulate air (HEPA) filter. It has been observed that a 0.3 μ m HEPA filter shows almost 100 percent efficiency in not allowing the bacteria to pass through. All the surfaces in the room can be thoroughly cleansed and disinfected regularly. The room should be air-conditioned.

Another type of transfer area used currently in most laboratories is a 'laminar airflow cabinet'. A small motor blows air into the unit first through a coarse filter, where large dust particles are separated, and then, passes through a 0.3 μ m HEPA filter. The air is directed either downward (vertical flow unit) or outward (horizontal flow unit) over the working surface. The air coming out of the fine filter is ultraclean (free from fungal or bacterial contaminant) and its velocity (27+ 3m/min) adequately prevents the microcontamination of the working area by a worker sitting in front of the cabinet. Various other contaminants (e.g. hair, salts, etc.) are also blown away by the ultraclean airflow. In this way, an aseptic environment is maintained when the cabinet is switched on. Laminar airflow cabinets are commercially available in various sizes and shapes. The advantage of using such cabinets is that the flow of air does not hamper the use of a spirit lamp or Bunsen burner and each cabinet occupies a relatively small space within an ordinary lab. At places where atmospheric dust is very high, it is advisable to keep the airflow cabinet in a culture room fitted with double doors to prolong the life of filter. An important precaution is that a laminar airflow cabinet should never face a window or door that is frequently used.

2.1.4.Culture Room:

All types of plant tissues are incubated under conditions of well-controlled temperature, humidity, illumination and air circulation. Usually, air-conditioners and heaters are used to maintain the temperature around 25 ± 2 °C. Cultures are generally grown in diffused light. Other requirements are a humidity range of 20-98%, controllable to $\pm 3\%$ and uniform forced-air ventilation. Culture room should have enough shelves illuminated by a set of fluorescent tubes for storing cultures. All the cultures should be labelled, giving details of the experiment (name of the plant, explants, medium, date of culture and other information) to ensure identity and for recording monitored results. Shakers with controlled temperature and light are also installed in a culture room. Incubators, large plant growth chambers, are readily available in the market. They occupy less space and have the range of control and flexibility desirable for growth of tissues under *in vitro* conditions.

2.1.5.Observation or Data Collection Area:

The growth and development of tissues cultured *in vitro* is generally monitored by observing cultures at regular intervals, in the culture room. Plants regenerated from *in vitro* tissue cultures are transplanted to soil in pots. The potted plants are ultimately transferred to green houses or growth cabinets and maintained for further observations under controlled conditions of light, temperature and humidity.

2.2.Equipment and supplies:

Equipments commonly used in a Laboratory for many tissue culture techniques

2.2.1.Preparation of Media:

1. Gas, water, and electricity supplies.
2. Water heater or small stove.
3. Hot plate with magnetic stirrer.
4. Glass or stainless steel containers for heating and dissolving media.
5. Autoclave or pressure-steam steriliser.
6. pH meter.
7. Centigram balances.
8. Graduated measuring cylinders, flasks, beakers, Petri dishes and pipettes with teats.
9. Culture tubes, bottles and other glassware with suitable closures (such as cotton plugs, aluminium foil, plastic film, or metal caps).
10. Small transfer instruments such as spatulas, scalpels, forceps, or dissecting needles.

11. Hot-air oven for rapid heating of media and agar mixtures (microwave oven will also do rapid thawing of frozen items).
12. Distilled or double-distilled water units.
13. Chemicals for preparing culture media or commercially available powdered culture media, growth hormones and other organic constituents.
14. Detergents, disinfectants.
15. Pipette washers (acid proof).
16. Drying and draining racks.
17. Storage space for chemicals, glassware, nutrient media, sterile water and other items.

2.2.2.Isolation of Cultures:

1. Laminar airflow cabinet.
2. Spirit lamp or bunsen burner in the inoculation cabinet.
3. Stereo-microscope.
4. Ethyl alcohol (70%) for sterilisation and flaming of small metal instruments.
5. Tiles or glass plates for use during sterile cutting.
6. Hypochlorite solution for sterilisation of plant material.

2.2.3.General Equipment:

1. Refrigerator, deep-freeze.
2. Automatic dish-washer.
3. Dispensing devices (e.g., wire-mesh baskets, trolleys with trays and metal racks for holding test-tubes or culture vials in the autoclave).
4. Acid proof baths for cleansing glassware.
5. Microscopes (e.g., compound, inverted) with microphotographic equipment.
6. Markers, labels and vitafilm (or similar material for wrapping culture vessels, glassware, tiles and other labware).

2.2.4.Culture Room:

1. Temperature control (17-27°C).
2. Electricity supply essential for lighting, cooling and heating.
3. Shelves for culture racks.
4. Fluorescent tubes for lighting.
5. Timer for regulating day-length.
6. Racks for culture vials.
7. Rotary shaker.
8. Observations table.

2.2.5. Transplantation Facility:

A small area where high humidity, light and temperature can be controlled in the form of a greenhouse or a small room.

2.3. Basic Techniques of Plant Tissue Culture:

1. The required explants (buds, stems and seeds) are trimmed subjected to sterilization in a detergent solution.
2. Washing with distilled water.
3. Explants are placed in suitable culture medium (liquid and semisolid) and incubated (22-28 °C).
4. This results in the establishment of culture.
5. The mother cultures can be divided, as frequently as needed, to give daughter cultures.

Explants→trimming→surface sterilization→washing with distilled water→establishment of culture→incubation→subculture.

The most important aspect of *in vitro* culture technique is to carry out all the operations under aseptic conditions. Bacteria and fungi are the most common contaminants in plant tissue culture. They grow much faster in culture and kill the plant tissues. The contaminants produce toxic compounds to the plant tissues. Therefore, it is absolutely essential that aseptic conditions are maintained throughout the tissue culture operations.

Sterilising Plant Material:

To obtain sterile plant material is difficult because in the process of sterilisation living materials should not lose their biological activity, only bacteria or fungal contaminants should be eliminated. Plant organs or tissues are surface sterilised by treatment with a disinfectant solution at suitable concentrations for a specified period. Some disinfectants are following-

Disinfectant	Concentration (%)	Duration of treatment (min.)
Benzalkonium chloride	0.01-0.1	5-20
Bromine water	1-2	2-10
Calcium hypochlorite	9-10	5-30
Ethyl alcohol	75-95	0.1-5
Hydrogen peroxide	3-12	5-15
Mercuric chloride	0.1-1.0	2-10
Silver nitrate	1	5-30
Sodium hypochlorite	0.5-5	5-30

Chapter-3 Plant Tissue Culture Media:

Culture media are largely responsible for the *in vitro* growth and morphogenesis of plant tissues. The success of plant tissue culture depends on the choice of the nutrient medium. The basic requirements of cultured plants are similar to those of whole plants in practice. Some tissues show better response on solid medium while others prefer a liquid medium. Plant tissue culture media that influence the growth of various types of cells or explants and further help to achieve the regeneration of plants. It may be noted that plants in nature can synthesize their own food material. However, plants growing *in vitro* are mainly **heterotrophic** i.e. they can not synthesize their own food.

3.1. Media Composition:

The composition of the culture media is primarily dependent on two parameters-

1. The particular species of the plants.
2. The type of material used may be for culture i.e. cells, tissues, organs, protoplasts.

Thus, the composition of the medium is formulated considering the specific requirements of given culture system. The media used may be solid (solid medium) or liquid (liquid medium) in nature. The selection of solid or liquid medium is dependent on better response of a culture.

Major types of Media:

White's medium – developed for root culture

MS-medium - induce organogenesis and regeneration of plants in cultured tissues.

B₅ medium - developed by Gamborg, cell suspension and callus culture, used for protoplast culture

N₆ medium- formulated by Chu, cereal anther culture

Nitsch medium - anther culture

Constituents of media: Many elements are needed for plant nutrition and their physiological functions.

Thus, these elements have to be supplied in the culture medium to support adequate growth of callus *in vitro*. The culture media usually contain the following constituents-

1. Inorganic nutrients
2. Carbon and energy sources
3. Organic supplements

4. Growth regulators
5. Solidifying agents
6. pH medium.

3.1. Inorganic nutrients: The inorganic nutrients consists of-

1. Macronutrients –C, H, O, N, P, K, Ca, Mg, S
2. Micronutrients – Fe, Mn, Zn, Bo, Cu, Mo

A wide range of mineral salts (elements) supply the macro and micronutrients. The inorganic salts in water undergo dissociation and ionization. Consequently, one type of ion may be contributed by more than one salt e.g. in MS-medium, K^+ ions are contributed by KNO_3 and KH_2PO_4 while nitrate ion comes from KNO_3 and $NH_4 NO_3$.

Macroelements

NH_4NO_3 , KNO_3 , $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4

Microelements

KI, H_3BO_3 , $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$ $CoCl_2 \cdot 6H_2O$

3.1.1. Carbon and energy sources:

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy e.g. glucose, sucrose, fructose, galactose, maltose raffinose.

3.1.2.Organic supplement:

The organic supplements include vitamins, amino acids, organic acids, organic extracts, activated charcoal and antibiotics.

Vitamins: Plants synthesize vitamins endogenously and are used as catalyst in various metabolic processes. When plants and tissues are grown *in vitro*, some essential vitamins are synthesized but in suboptimal quantities, inadequate to support growth. Therefore, the medium should be supplemented with vitamin to achieve good growth of cells e.g. thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid, myoinositol, para-aminobenzoic acid and vitamin E.

Amino Acids: Cultured tissues can synthesize amino acids to a certain extent, but addition of amino acids to media stimulate cell growth in protoplast cultured and help in establishment of cells lines. Amino acids are taken up more rapidly by plant cells as

compared to inorganic nitrogen. Organic nitrogen- casein hydrolysate, L-glutamine, L-asparagine, L-arginine and L- cysteine.

Organic Acids: Addition of kreb's cycle intermediates such as citrate, malate, succinate, or fumarate allow the growth of plant cells. Pyruvic acid also enhances the growth of cultured cells.

Organic extracts: Culture media are often supplemented with a variety of organic extract such as-protein (casein) hydrolysates, coconut milk, yeast and malt extract, ground banana, orange juice, tomato juice and potato extract.

It is avoidable the use of natural extracts due to high variations in the quality and quantity of growth promoting factors in them. Natural extracts have been replaced by specific organic compounds e.g. Yeast by L- asparagines, fruit extracts by L-glutamine.

Activated charcoal: Supplementation of the media with activated charcoal stimulates growth and differentiation of certain plant cells. Some toxic/inhibitory (phenols) produced by cultured plants are removed (absorbed) by activated charcoal and this facilitates efficient cell growth in cultures. It is found inhibitory for some plants due to absorption of growth stimulants such as phytohormones.

Antibiotics: It is sometimes necessary to add antibiotics to the medium to prevent the growth of micro-organisms e.g. Streptomycin or kanamycin is used at low concentration. As far as possible, avoid the use of antibiotics because they have inhibitory influence on the cell growth.

3.1.3.Growth regulators:

Plant hormones or phytohormones are a group of natural organic compounds that promote growth, development and differentiation of plants. Four broad classes of growth regulators are used for culture of plant cells- Auxins, cytokinin, gibberellins and abscisic acid. They promote growth, differentiation and organogenesis of plant tissue in cultures.

Auxins: Auxins induce cell division, cell elongation, and formation of callus in cultures. At low concentration, auxins promote root formation while at high concentration callus formation occurs e.g.

(2, 4-D) 2, 4-dichlorophenoxy acetic acid,

(IAA) Indole-3-acetic acid,

(IBA) Indole-3-butyric acid,

(NAA) Naphthaline acetic acids are most effective and are widely used in culture media.

Cytokinin: Cytokinins are adenine (purine) derivatives. These adenine derivatives are involved in cell division, shoot differentiation and somatic embryo formation. Cytokinins promote RNA synthesis and stimulate protein and enzyme activities in tissues e.g.

BAP – 6-benzylaminopurine

BA- benzyl adenine

2iP (IPA) - N⁶-(2-isopentyl) adenine

Kinetin – 6- furfuryl aminopurine

Zeatin – 4-hydroxy 3 methyl Trans 2-butanyl aminopurine

Thidiazuron – 1 phenyl 3- (1, 2, 3- thiadizol 5- yl) urea.

Ratio of Auxin and Cytokinin: The concentrations of the growth factors namely auxins and cytokinins are crucial for the morphogenesis of culture systems. When the **ratio of auxins to cytokinins is high, embryogenesis, callus initiation and root initiation occur. On the other hand, when the ratio of auxins to cytokinins is low axillary and shoot proliferation occur.** Auxin is needed for root culture and cytokinin for shoot culture.

Gibberellins: About 20 different gibberellins have been identified as growth regulators. Gibberellins A₃ (GA₃) is most commonly used for tissue culture. It promotes the growth of culture cells, enhance callus growth and induce dwarf plantlets to elongate. Gibberellins are capable of promoting or inhibiting cultures, depending on plant sps.

Abscisic acid: The callus growth of cultures may be stimulated or inhibited by ABA. Abscisic acid is an important growth regulator for induction of embryogenesis.

3.1.4.Solidifying agents: For preparation of solid or semi solid tissue culture media gelling agents are required. Agar is a polysaccharides obtained from seaweeds (red algae e.g. *Gelidium*, *Gracilaria* and *Pterocladia*).

1. It does not react with media constituents. Agar at a concentration of 0.5 to 1% in the medium can form a gel.
2. It does not digested by plant enzymes and is stable at culture temperature.

Gelatin used at high concentration (10%) and melt at low temperature (25°C) and gelling property is lost. Other gelling agents are – biogel (polyacrylamide pellets) phytigel, alginate, methacel, and gelrite. They can form gels at a low concentration (1.0 to 2.5 g /L).

3.1.5. pH: Plant cells and tissues require optimum pH for growth and development in cultures. The optimal pH for most tissue culture is *in vitro* in the range of 5.0-6.0. The pH generally falls by 0.3 – 0.5 units after autoclaving. At a pH higher than 7 and lower than 4.5, the plant cells stops growing in cultures.

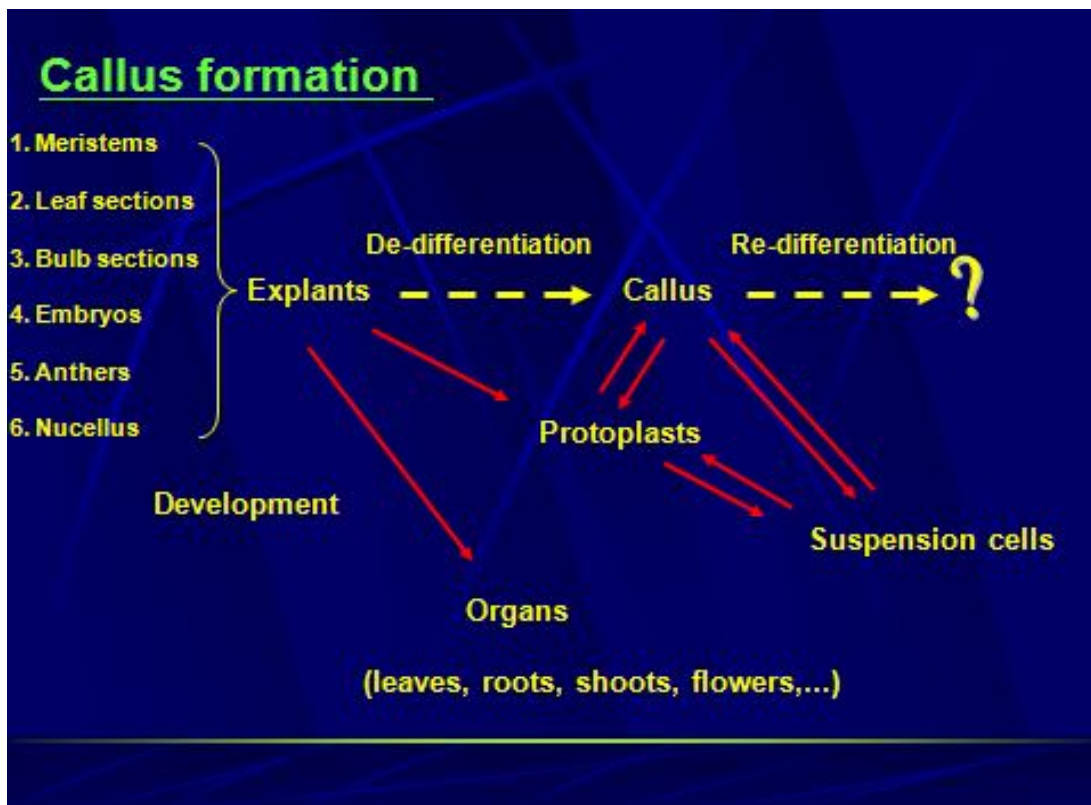
3.2. Media Preparation: Media preparation is time consuming process. The simplest method of preparing media is -

1. Weigh the required amount of inorganic and organic nutrients.
2. Dissolve these nutrients in separate flask containing some quantity of distilled water.
3. Thoroughly mix contents in distilled water then mix sugar and melted agar.
4. It is desirable to weigh and dissolve each ingredient separately before mixing them together.
5. The volume is made up to one litre.
6. The pH is adjusted by using 0.1 NaOH or 0.1 HCl.
7. Pour the medium into the desired vessels (250-500 ml flasks).
8. The vessels containing medium are then covered with aluminium foil and autoclaved at 121 °C (15 lb/in² or 15 psi or 1.06 kg/cm²) for 15-20 min.
9. The autoclaved medium is allowed to cool to approximately 60 °C and finally dispensed into sterilized vials aseptically.
10. Vitamin and auxins may be added after autoclaving.
11. Most of the growth regulators are not soluble in water. They have to be dissolved in NaOH or alcohol.

4.1.Callus Induction:

To induce callus formation, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Callus induction medium consists of agar and a mixture of macronutrients and micronutrients for the given cell type. Plant hormones, such as auxins, cytokinins, gibberellins, and vitamins (Gamborg B₅ vit.) are supplemented into the medium to initiate callus formation or somatic embryogenesis. There are several types of basal salt mixtures used in plant tissue culture, but most notably modified Murashige and Skoog medium.

Auxin is an essential factor for callus induction from differentiated tissues of plants and, in fact, many kinds of calli have been induced by auxin. Generally, monocotyledonous plants require relatively high concentrations of auxin, while callus tissues are easily induced from dicotyledonous plants.



4.2.Callus Culture:

This involves the culture of differentiated tissues from explants which dedifferentiate to form callus. **Callus is the undifferentiated and unorganized mass of plant cells.** It is basically a tumour tissue which usually forms on wounds of differentiated tissue or organs. Callus cells are parenchymatous in nature.

Callus formation *in vivo* is frequently observed as a result of wounds at the cut edges of stems or roots and forms a protective layer of cells to cover an injury. Invasion of microorganisms or damage by insect feeding usually occurs through callus. An outline of callus culture is following-

Explants for callus culture: The starting materials (explants) for callus culture may be the differentiated tissue from any part of the plant (root, stem, leaf anther, flower etc). The selected explants tissues may be at different stages of cell division, cell proliferation and organization into different distinct specialized structures. If the explant used possesses meristematic cells, then cell division and multiplication will be rapid.

4.2.1.Factors affecting callus culture:

Many factors are known to influence callus formation in *in vitro* culture. These include the sources of the explants and its genotype, composition of the medium (MS medium most commonly used), physical factors (temperature, light etc.) and growth factors. Other important factors affecting callus culture are- age of the plant, location of explants, physiology and growth conditions of plant.

Physical factors: A temperature in the range of 22-28 °C is suitable for adequate callus formation. Light is largely dependent on the plant species, light may be essential for some plants while darkness is required by others.

Growth Regulators: The growth regulators to the medium strongly influence callus formation. Based on the nature of the explant and its genotype, and the endogenous content of the hormone, the requirements of growth regulators may be categorized into 3 groups-

1. Auxin alone
2. Cytokinin alone
3. Both auxin and cytokinin.

4.2.2.Applications of callus cultures: Callus cultures are slow growth plant culture systems in static medium. This enables to conduct several studies related to many aspects of plants (growth, differentiation and metabolism) as listed below-

- Nutritional requirements of plants
- Cell and organ differentiation
- Development of suspension and protoplast cultures.
- Somaclonal variations
- Genetic transformations
- Production of secondary metabolites.

4.3.Cell Culture:

The first attempt to culture single cells (obtained from leaves of flowering plants) was made in as early as by Haberlandt (1902). Although he was unsuccessful to achieve cell division *in vitro*, his work gave a stimulus to several researchers. In later years, good success was achieved not only for cell division but also to raise complete plants from single cell cultures. The culture of individual cells, obtained from an explant tissue or callus is regarded as cell culture. These cultures are carried out in dispersion medium and are referred to as **cell suspension cultures**.

4.3.1.Applications of cell cultures: Cultured cells have a wide range of applications in biology.

1. Serve as good targets for mutation and selection of desirable mutants
2. Production of secondary metabolites of commercial interest.
3. Good potential for crop improvement.

Cell Culture Technique: The *in vitro* cell culture technique broadly involves the following aspects:

1. Isolation of single cells.
2. Suspension cultures growth and subculturing.
3. Types of suspension cultures.
4. Synchronization of suspension cultures.
5. Measurement of growth of cultures.

Isolation of single cells: The cells employed for *in vitro* culture may be obtained from plant organs and from cultured tissues.

A. From Plant Organs: Plant leaves with homogenous population of cells are the ideal sources for cell culture. Single cell can be isolated from leaves by mechanical and enzymatic methods.

i) Mechanical Methods: Surface sterilized leaves are cut into small pieces ($> 1\text{cm}^2$), suspended in a medium and subjected to grinding in a glass homogeniser tube. The homogenate is filtered through filters or muslin cloths and then centrifuged at low speed to remove the cellular debris. The supernatant is removed and diluted to achieve the required cell density.

ii) Enzymatic Method: The enzyme **macerozyme** can release the individual cells from the leaf tissues. Macerozyme degrades middle lamella and cell walls of parenchymatous tissues.

B. From Cultured Tissues: Single cells can be isolated from callus cultures (grown from cut pieces of surface sterilized plant parts). Repeated subculturing of callus on agar medium improves the friability of callus so that fine cell suspensions are obtained.

4.3.2. Suspension cultures growth and subculturing:

The isolated cells are grown in suspension cultures. Cell suspensions are maintained by routine subculturing in fresh medium.

During the incubation period the biomass of the suspension cultures increases due to cell division and cell enlargement. Cell division and cell enlargements continues for a limited period since the viability of cells in suspension after the stationary phase decreases due to accumulation of toxic substance in the medium. The timing of subcultures is very important. The incubation period is dependent on:

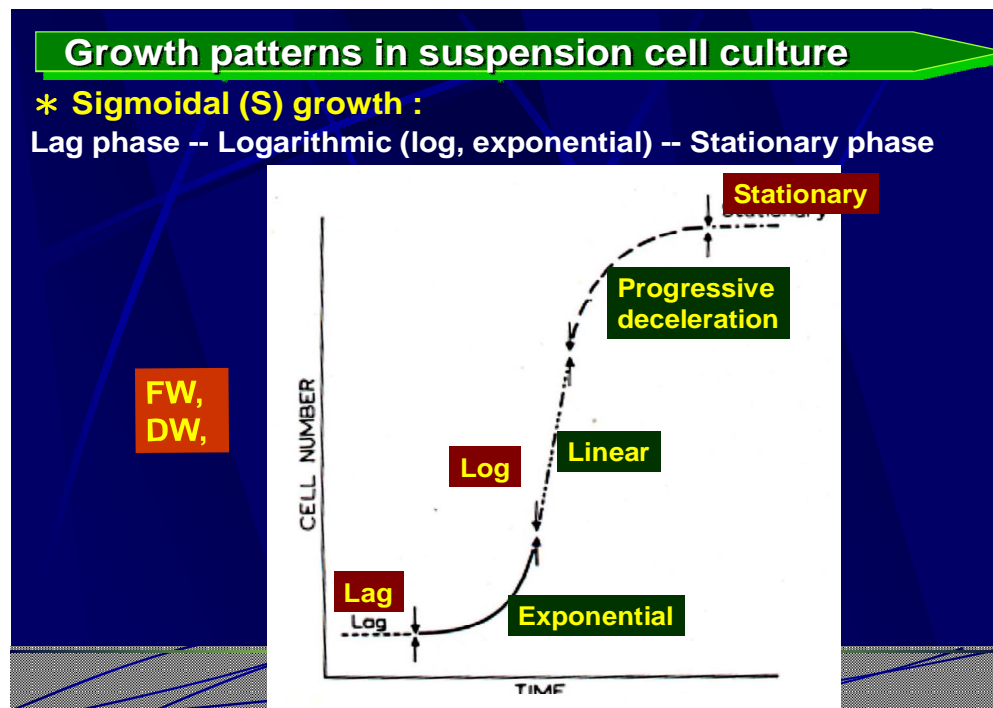
- Initial cell density
- Duration of lag phase
- Growth rate of cells.

With low initial cell densities, the lag phase and log phases of growth get prolonged. With low cell densities, the culture will not grow well and requires additional supplementation of metabolites to the medium. The normal incubation time for the suspension cultures is in the range of 21-28 days.

4.3.3. Types of suspension cultures: There are mainly two types of suspension culture- Batch cultures and continuous cultures.

4.3.3.1. Batch Cultures: A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. In batch culture, cell division and cell growth coupled with increase in biomass occur until one of the factors in the culture environment becomes limiting. The cells exhibit the following five phases of the growth when the cell number in suspension culture is plotted against the time of incubation.

1. **Lag phase:** characterized by preparation of cell to divide
2. **Log phase:** the rate of cell multiplication is highest
3. **Linear phase:** represented by slowness in cell division and increase in cell size expansion.
4. **Deceleration phase:** characterized by decrease in cell division and cell expansion
5. **Stationary phase:** represented by a constant number of cells and their size.



The batch cultures can be maintained continuously by transferring small amounts of the suspension medium to fresh medium at regular intervals (2-3 days).

Batch cultures are characterized by a constant change in the pattern of cell growth and metabolism.

4.3.3.2. Continuous cultures: There is a regular addition of fresh nutrient medium and draining out the used medium so that the culture volume is normally constant. Continuous cultures are carried out under defined and controlled conditions. Continuous cultures are two types- **open** and **closed**.

Open continuous cultures: The inflow of fresh medium is balanced with the outflow of the volume of spent medium along with the cells. The rate of cells removed from the cultures equals to the rate of formation of new cells.

Closed Continuous cultures: In these cultures, the cells are retained while the inflow of fresh medium is balanced with the outflow of corresponding spent medium. The cells present in the out flowing medium are separated and added back to the culture system. As a result there is a continuous increase in the biomass in closed continuous cultures. These cultures are useful for the production of certain secondary metabolites.

4.3.4. Synchronization of suspension cultures:

A synchronous culture is one in which the majority of cells proceed through each cell cycle phase (G_1 , S, G_2 and M) simultaneously. Suspension cultures may be grouped under two categories- physical and chemical.

4.3.5. Measurement of growth of cultures:

It is necessary to assess the growth of cells in cultures. Assessment of the growth in suspension cultures can be accomplished by following selected parameters at regular intervals. These include:

- (a) Cell counting
- (b) Packed cell volume
- (c) Cell fresh weight
- (d) Cell dry weight.

Cell counting: Although cell counting to assess culture growth is reasonably accurate, it is tedious and time consuming. This is because cells in suspension culture mostly exist as colonies in varying sizes. These cells have to be first disrupted (by treating with pectinase or chromic acid), separated, and then counted using a haemocytometer.

Packed cell volume: Packed cell volume (PCV) is expressed as ml of pellet per ml of culture. To determine PCV, a measured volume of suspension culture is centrifuged (usually at 2000 rpm for 5 min) and the volume of the pellet or packed cell volume is recorded. After centrifugation the supernatant can be discarded, the pellet washed, dried overnight and weighed. This gives **cell dry weight**.

Cell fresh weight: The wet cells are collected on a preweighed nylon fabric filter (supported in funnel). They are washed to remove the medium, drained under vacuum and weighed. This gives the fresh weight of cells. However, large samples have to be used for accurate weights.

Micropropagation is the growing of plants from meristematic tissue or somatic cells of superior plants on nutrient suitable media under controlled aseptic physical conditions.

5.1. Stages of Micropropagation(Steps of Micropropagation:)

Micropropagation procedure is divided in stages for the sake of understanding. Murashige proposed three (I to III) stages, Debergh and Maene added stage '0'. Currently we have accepted five stages procedure (0 to IV).

- ❖ Stage 0
- ❖ Stage I
- ❖ Stage II
- ❖ Stage III
- ❖ Stage IV

Stage 0: Selection of mother plants and its maintenance or Selection and maintenance of stock plants for culture initiation:

This stage was basically introduced to overcome the problem of contamination. Stock plants are grown under more hygienic conditions to reduce the risk of contamination.

Stage I: 1. Explant isolation(Initiation and establishment of culture) We can take virtually any part of the plant.

Vegetative portions: Shoot tip, meristem, leaves, stems, roots.

Reproductive portions: Anthers, pollen, ovules, embryo, seed, spores.

Shoot tip and auxiliary buds are most often used. Size of explant, age of the stock plant, physiological age of explant, developmental age of explant these are some of the factors which decide the success rate of stage I.

2. Disinfestation or Surface sterilization – Explants are surface sterilized by treating it with disinfectant solution of suitable concentration for a specific period. Ethyl alcohol, bromine water, mercuric chloride, silver nitrate, sodium hypochlorite, calcium hypochlorite etc. can be used as disinfectant.

3. Washing – Washed with distilled water.

4. Establishment of explant on appropriate medium: – There is no one universal culture medium; however modifications of Murashige and Skoog basal medium (Murashige and Skoog, 1962) are most frequently used. Medium must contain all components necessary to nourish explants.

- a. Inorganics:** Macronutrients(N,P,K,Ca,Mg) & Micronutrients(B,Co,Cu,Mn,Fe,Zn)
- b. Organics** - carbon source: needed since plants do not seem to photosynthesize well in culture
- c. Vitamins:** Thiamine (essential), Myoinositol, B vitamins, folic acid, & biotin
- d. Growth regulators:** Cytokinins, Auxins, GA, ABA rarely used
- e. Complex organics:** natural orange juice, coconut milk, bananas
- f. Inert supports:** Agar, foam rubber, filter paper bridge, liquid
- 5. Growth regulators:** Basic research findings of Miller and Skoog have been born out by many investigators.

In general: Cytokinins induce shoot bud formation and **Auxins** induce root formation.

6. Environmental conditions:

Light: light intensity, photoperiod, quality important.

Temperature - there are usually high and low cut-offs. In some cases a specific temperature is needed.

Stage II: Multiplication of shoots or somatic embryo formation (rapid) using a defined culture medium:

In this stage, rapid multiplication of the regenerative system is carried out for obtaining large number of shoots. Cultures obtained from stage I are placed on a suitable medium. Normally, medium for stage I and II is same, but cytokinin proportion is increased for stage II to produce numerous shoots. This stage can be repeated a few cycles until a desired number of shoots are developed to carry out for rooting.

Stage III: Rooting of regenerated shoots *in vitro* (Pretreatment for transfer to soil):

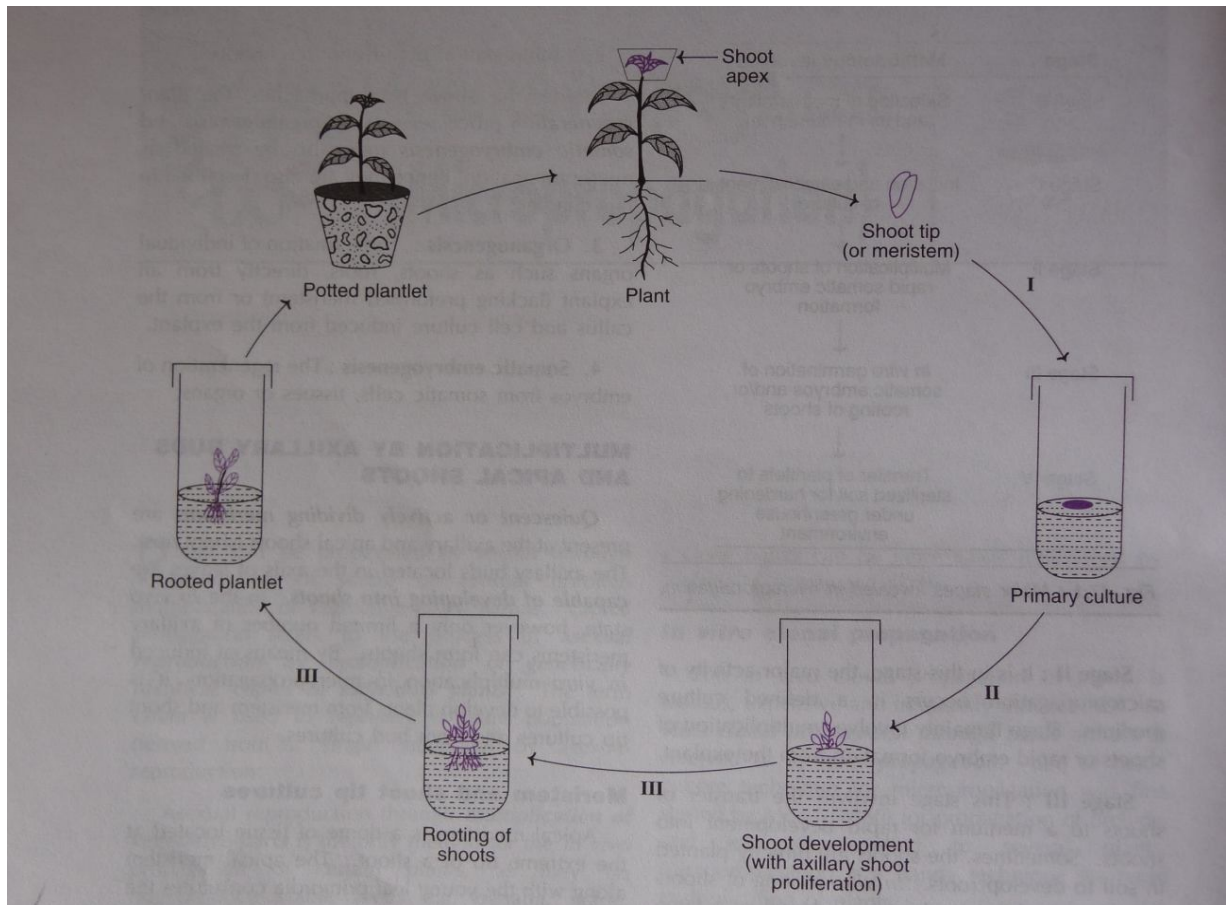
In this stage, shoots or shoot clusters from stage II are prepared to transfer to soil. Shoots are separated manually from clusters and transferred on a rooting medium containing an auxin. Elongation of shoots prior to rooting, rooting of shoots and prehardening cultures to improve survival are some of the activities carried under this stage. NAA used for herbaceous plants and IBA for woody plants. Lower or in darkness for 1 week for root formation.

Stage IV:

Transfer of plantlets to sterilized soil for Hardening under Green House environment:

"Hardening" refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in "ideal" conditions; designed to encourage rapid growth. This process is done for acclimatization of plantlets to the soil as they were

previously grown in plant media. It is performed *in vitro*, or in a sterile "test tube" environment. After growing for some days the plantlets are transferred to the field. When taken out of culture, the plantlets need time to adjust to more natural environmental conditions. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to a normal growth environment for the species in question.



Stages of Micropropagation.

5.2.Types or Processes or Methods of Micropropagation: Types of micropropagation are following-

- Micropropagation by axillary and apical buds
- Micropropagation by axillary shoots (buds, bulbs and protocorms)
- Micropropagation through callus culture
- Artificial seeds
- Somaclonal variations

5.3.Applications of Micropropagation:

Plant tissue culture is used widely in plant science; it also has a number of commercial applications. Applications include:

- Micropropagation is widely used in forestry and in floriculture.
- Micropropagation can also be used to conserve rare or endangered plant species.
- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.
- In the production of plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- For production of doubled monoploid (dihaploid) plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes, usually by treatment with colchicine which causes doubling of the chromosome number.
- As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
- Certain techniques such as meristem tip culture can be used to produce clean plant material from virused stock, such as potatoes and many species of soft fruit.
- Micropropagation using meristem and shoot culture to produce large numbers of identical individuals.

Chapter-6 **Organogenesis:**

Organogenesis is the process of morphogenesis involving the **formation of plant organs** i.e. shoots, roots flowers, and buds from explants or cultured plant tissues. Organogenesis in plant tissue culture involves two distinct phases: dedifferentiation and re-differentiation. Dedifferentiation begins shortly after the isolation of the explants tissues with an acceleration of cell division and a consequent formation of callus. Re-differentiation starts from the callus into bud formation. Organogenesis is of two types-

1. Direct organogenesis
2. Indirect organogenesis

1. Direct Organogenesis: Tissues from leaves, stems roots and inflorescences can be **directly cultured to produce plant organs**. In direct organogenesis tissues undergoes morphogenesis without going through a callus or suspension cell culture stage. The term **direct adventitious organ formation** is also used for direct organogenesis.

Induction of adventitious shoot formation directly on roots, leaves and various other organs of intact plants is a widely used method for plant propagation. This approach is particularly useful for herbaceous species.

For appropriate organogenesis in culture system, exogenous addition of growth regulators- auxin and cytokinin is required. The concentration of the growth promoting substance depends on the age and nature of the explants, besides the growth conditions.

2. Indirect Organogenesis:

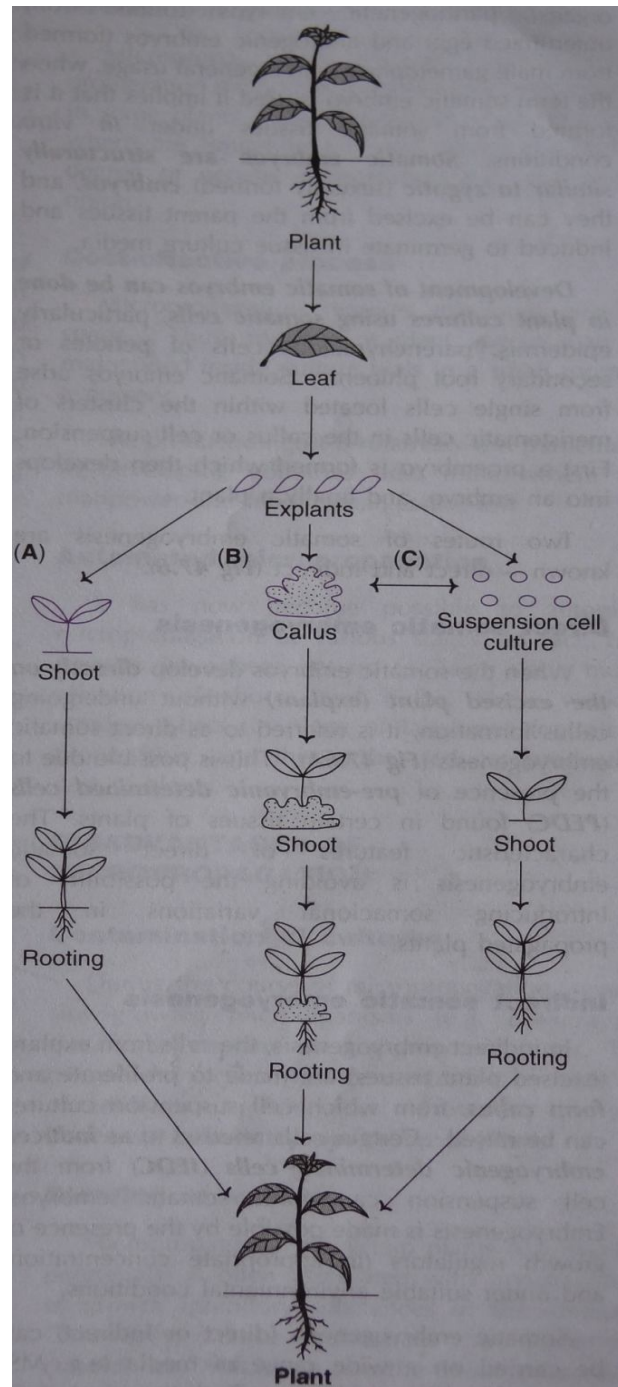
When the organogenesis occurs through callus or suspension cell culture formation is called indirect organogenesis. Callus growth can be established from many explants (leaves, roots, stem, flower petals etc.) for subsequent organogenesis.

The explants for good organogenesis should be mitotically active immature tissues. It is advantageous to select meristematic tissues for efficient indirect organogenesis. This is because their growth rate and survival rate are much better.

For indirect organogenesis, the cultures may be grown in liquid medium or solid medium. Many culture media (MS, B₅ White's etc) can be used in organogenesis. The concentration of growth regulators in the medium is critical for organogenesis. By varying the concentration of auxins and cytokinins, *in vitro* organogenesis can be manipulated.

- Low auxin and low cytokinin concentration will induce callus formation.
- Low auxin and high cytokinin concentration will promote shoot organogenesis from callus.
- High auxin and low cytokinin concentrations will induce root formation.

Fig.1 Micropropagation of plants by organogenesis. (A) Direct organogenesis (B) Indirect organogenesis through callus (C) Indirect organogenesis through callus suspension cell culture



The process of **regeneration of embryo from somatic cells, tissues or organs** *in vitro* conditions is regarded as somatic (or asexual) embryogenesis. Somatic embryogenesis may result in non-zygotic embryos or somatic embryos (directly formed from somatic organs) and parthogenetic embryos (formed from unfertilized eggs). **Somatic embryos are structurally similar to zygotic embryos**, and they can be excised from the parent tissues and induced to germinate in tissue culture media.

Development of somatic embryos can be done in plant tissue culture using somatic cells, particularly epidermis, parenchymatous cell of petioles or secondary root phloem. Somatic embryos arise from single cells located within the clusters of meristematic cells in the callus or cell suspension. First proembryo is formed which then develops into an embryo, and finally a plant.

It is of two types-

1. Direct somatic embryogenesis
2. Indirect somatic embryogenesis

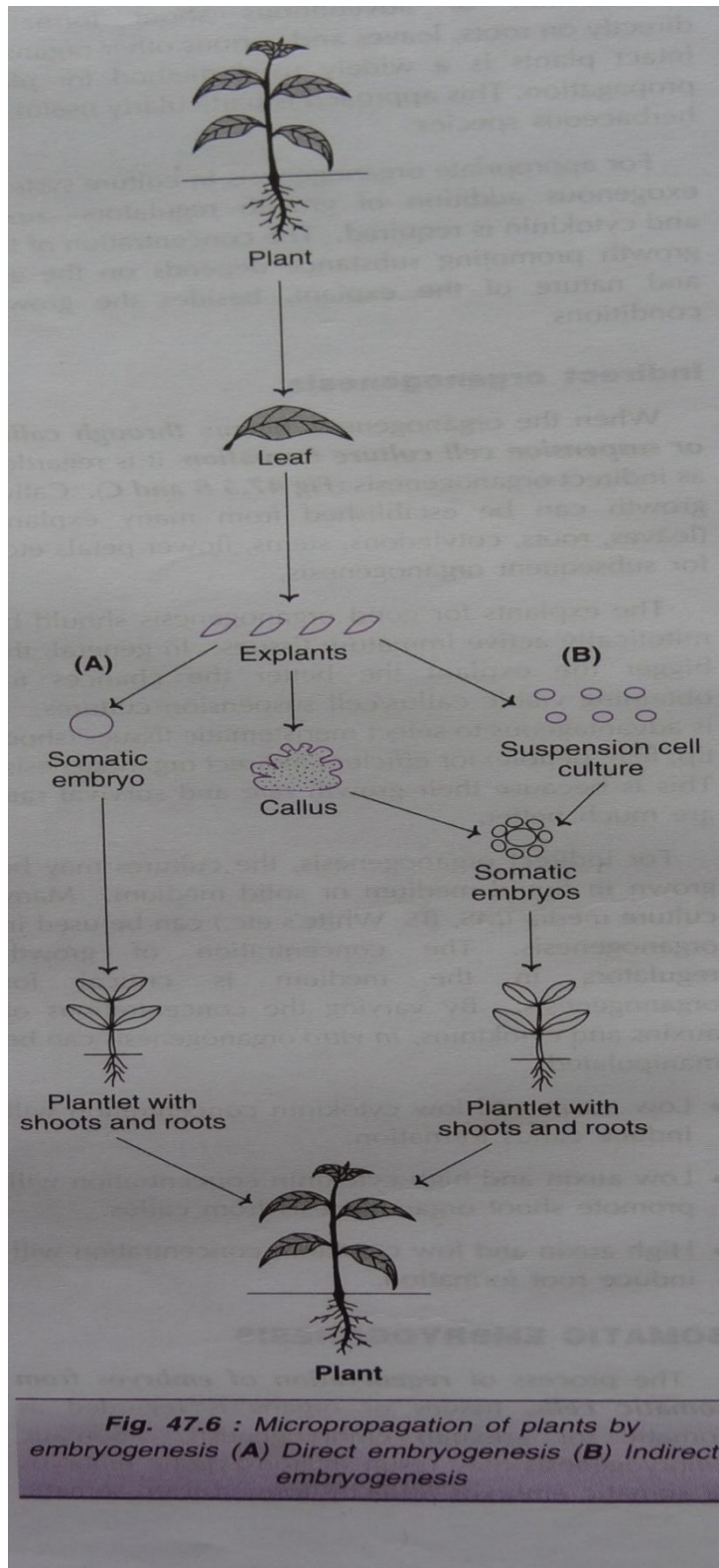
1. Direct somatic embryogenesis: When the somatic embryos develop directly on the excised plant (explant) without undergoing callus formation is called direct somatic embryogenesis. This is possible due to the presence of **pre-embryonic determined cells (PEDC)** found in certain tissues of plants. Direct somatic embryogenesis is avoiding the possibility of introducing somaclonal variations in the propagated plants.

2. Indirect somatic embryogenesis: The cells from explants are made to proliferate and form callus, from which cell suspension cultures can be raised. Certain cells referred to as **induced embryonic determined cell (IEDC)** from the cell suspension can form somatic embryos.

Somatic embryogenesis can be carried on MS and White's media. The addition of the amino acid **L-glutamine** promotes embryogenesis. **Auxin such as 2, 4-dichlorophenoxy acetic acid** is essential for embryo initiation. Indirect somatic embryogenesis is commercially very attractive since a large number of embryos can be generated in a small volume of culture medium.

Artificial seeds from somatic embryos:

Artificial seeds can be made by encapsulation of somatic embryos. The embryos coated with sodium alginate and nutrient solution, are dipped in calcium chloride solution. The calcium ions induce rapid cross linking of sodium alginate to produce small gel beads, each containing an encapsulated embryo.

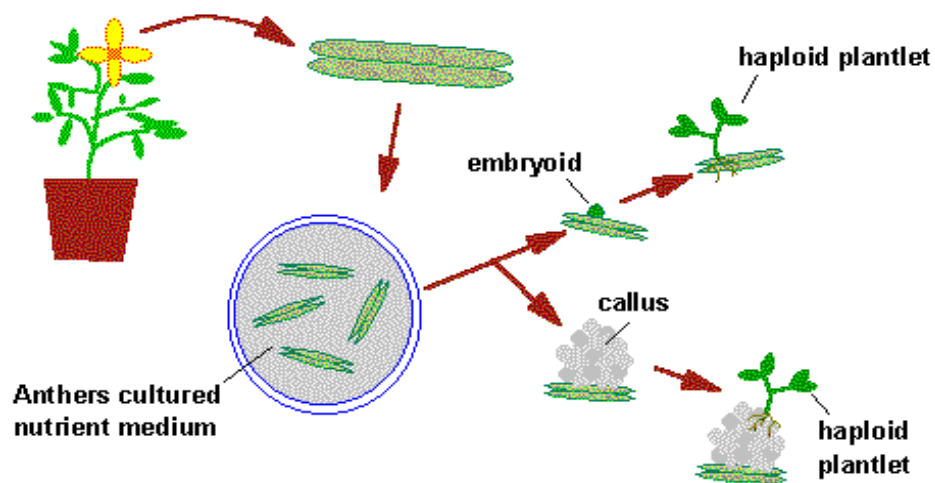


Chapter-8 Anther/ Microspore Culture:

Anther culture is the process of using anthers to culture haploid plantlets on a suitable, synthetic culture medium (*in vitro*). Progeny developed by this technique contains a single set of chromosomes ($n=1$). The technique was discovered by **Guha and Maheshwari(1964)**. More than one thousand plantlets or calluses have occurred under optimal conditions from one anther.

Procedure for *in vitro* Anther culture:

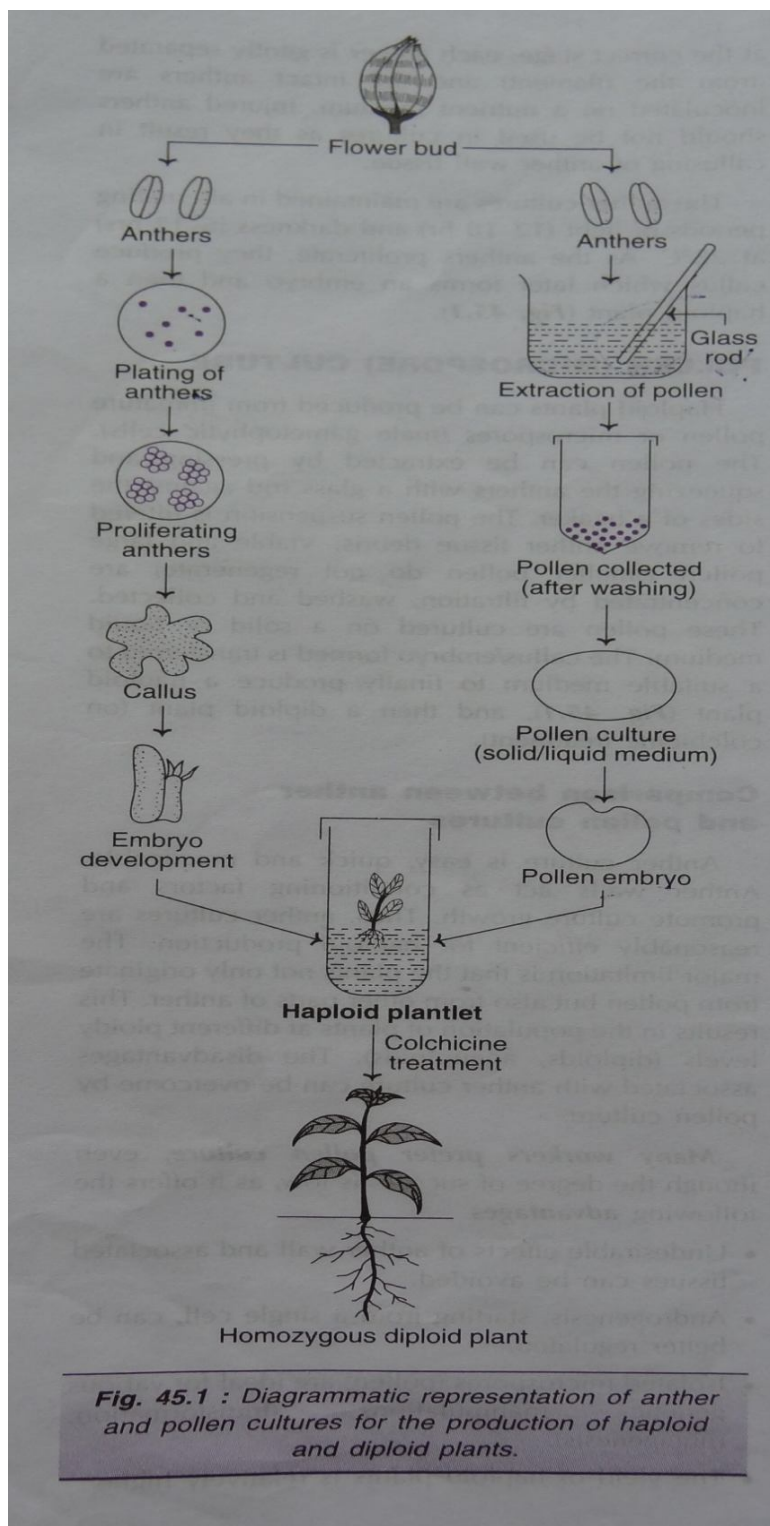
The selected flower buds of young plants are surface sterilized and anthers removed along with their filaments. Anthers are excised under aseptic conditions. Each anther is gently separated (from the filament) and the intact anthers are inoculated on a nutrient medium. The anther cultures are maintained in alternating periods of light (12-18 hr) and darkness (6-12 hr) at 28 °C. Anthers proliferate and they produce callus which later forms embryo and then a haploid plant.



Pollen (Microspore) Culture:

Pollen culture (microspore culture) is a technique in which haploid plants are obtained from isolated pollen grains. Immature pollen or microspores produce haploid plants. The pollen can be extracted by pressing and squeezing the anthers with a glass rod against the sides of a beaker. The pollen suspension is filtered to remove anther tissue debris. Viable and large pollen are concentrated by filtration, washed and collected. These pollens are cultured on solid or liquid medium. Pollen produces callus/embryo. The callus /embryo is transferred to a

suitable medium to finally produce **haploid plant** and then a **diploid plant** (on colchicine treatment).



Chapter-9 Embryo Culture:

In vitro cultures of sexually produced mature or immature embryos are called embryo culture. There are two types of embryo culture-

1. Mature embryo culture and
2. Immature embryo culture

Mature Embryo culture: Mature embryos are isolated and cultured *in vitro*. Mature embryo cultures are carried out in the following conditions.

- When the embryos remain dormant for long period
- Low survival of embryos *in vivo*.
- For converting sterile seeds to viable seedlings.

Seed dormancy in plant species is a common occurrence. This may be due to chemical inhibitors or mechanical resistance exerted by the structures covering the embryo. Seed dormancy can be successfully bypassed by culturing the embryo *in vitro*.

Embryo Rescue: Embryo rescue involves the **culture of immature embryos** to rescue them from unripe or hybrid seeds which fail to germinate. This approach is very useful to avoid embryo abortion and produce a viable plant.

In the normal circumstances, endosperm first develops and supports embryo development nutritionally. Thus, majority of the embryo abortions are due to failure in endosperm development. Embryo abortion can be avoided by isolating and culturing the hybrid embryos prior to abortion. Hybrid endosperm fails to develop leading the abortion of hybrid embryo. The endosperm may also produce toxins that ultimately kill the embryo.

The most important application of embryo rescue is **the production of interspecific and intergeneric hybrids from wild plant species.**

9.1.Culture technique for embryo rescue:

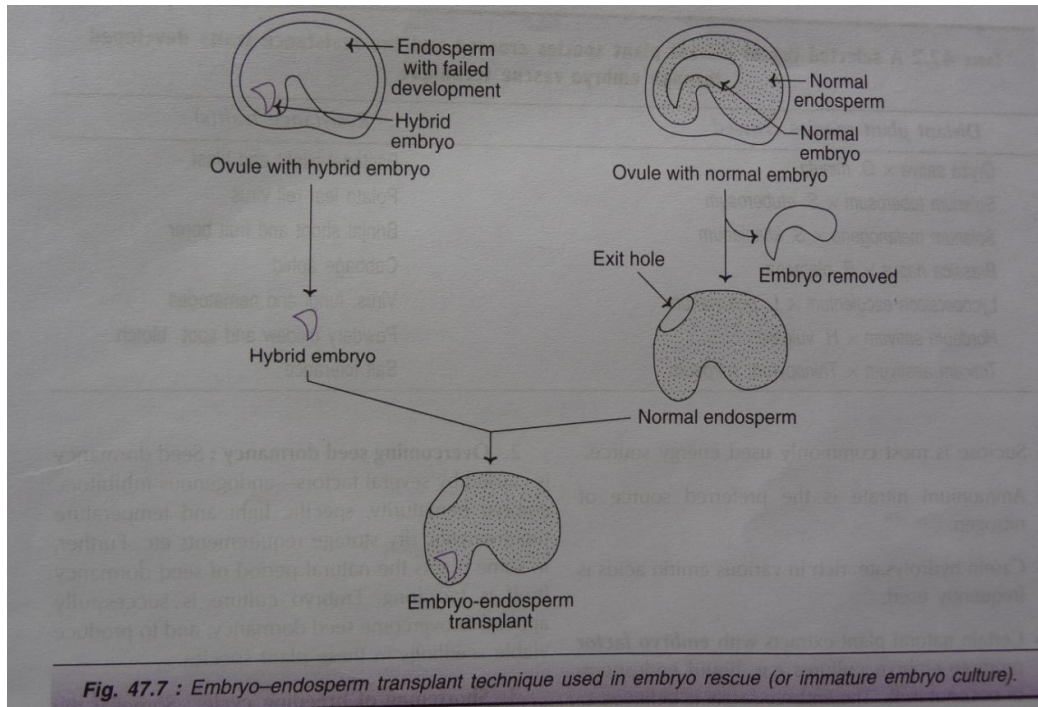
The aseptically isolated embryos can be grown in a suitable medium under optimal conditions. Complex nutrient medium is required for culture methods involving embryo rescue. For adequate nutritional support of immature embryos, embryos-endosperm transplant is used.

Embryo-endosperm transplant:

The hybrid embryo from the ovule in which endosperm development has failed is taken out by excision. Another normally developed ovule with endosperm enclosing an embryo is

chosen. This ovule is dissected and the normal embryo is pressed out. This leaves a normal endosperm with an exit hole. Now the hybrid embryo can be inserted through exit hole. This result in embryo-endosperm transplant which can be cultured in a suitable medium.

By using embryo-endosperm transplant, many interspecific and intergeneric plants have been raised e.g. hybrid plants of legumes.



9.2.Nutritional requirements of embryo culture:

There are two phases in the embryo development and the nutritional requirement-

- 1. Heterotrophic phase:** This is an early phase and the embryo is mostly dependent on the endosperm and maternal tissues for nutrient supplies.
- 2. Autotrophic phase:** This phase is characterized by the metabolic capability of the embryo to synthesize substances required for its growth which slowly makes it independent.

9.3.Composition of the Medium:

- Inorganic constituents of MS, B₅ or White's media are adequate.
- Ammonium nitrate is the preferred source of nitrogen.
- Certain natural plant extracts with embryo factor promote embryo cultures e.g. liquid endosperm of coconut milk.

- Embryo grow well in the pH range of 5 to 7.5.
- An incubation temperature of 24-26 °C is ideal.
- Better growth of embryo is observed in darkness which is then transferred to light for germination.

9.4.Applications of embryo culture:

1. **Prevention of embryo abortion:** Interspecific and intergeneric hybridization programmes leading to embryo abortion can be successfully overcome by embryo rescue.
2. **Overcoming seed dormancy:** Seed dormancy is caused by endogenous inhibitor, specific light and temperature requirements.
3. **Shortening of breeding cycle:** Some of the plants in their natural state have long breeding cycles.
4. **Production of haploid plants:** embryo culture has been successfully used for the production of the haploid plants e.g. barley.
5. **Overcoming seed sterility:** Certain plant species produce sterile seeds that do not germinate. Using embryo cultures, it is possible to raise seedlings from sterile seeds.

Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane. Protoplast of different plant species can be fused to generate a hybrid and this process is known as somatic hybridization (protoplast fusion).

Klercker (1892) first time isolated the protoplast by mechanical method.

Cocking (1960) removed the cell wall by enzymatic methods.

The main aim of protoplast fusion is to improve plant genetic material and the development of transgenic plants.

Step 1: Development of cell wall around the plasma membrane followed by cell division and form small colony.

Step2: The cell colony may be grown continuously as cultures (callus) or regenerated to whole plants.

Isolated protoplasts are cultured in semisolid agar or liquid medium. Sometimes protoplast are allowed to develop cell wall in liquid medium and then transferred to agar medium.

Agar culture: Agar solidify the culture media. The concentration of the agar should be such that it forms a soft agar gel when mixed with the protoplast suspension. The plating of protoplast is carried out by Bergmann's cell plating technique. In agar cultures, the protoplasts remain in a fixed position, protoplast divide and form cell clones. The advantage with agar culture is that clumping of protoplast is avoided.

Liquid culture: Liquid culture is the preferred method for protoplast cultivation for the following reasons-

1. It is easy to dilute and transfer.
2. Density of cells can be manipulated as desired.
3. The cells of some plant species cannot divide in agar medium; therefore liquid medium is the only choice.
4. Osmotic pressure of liquid medium can be altered as desired.

10.1.Culture Media: Mostly, MS and B₅ media with suitable modifications are used. Some of the special features of protoplast culture media are following-

1. Medium should be devoid of ammonium, and the quantities of iron and zinc should be less.

2. The concentration of calcium should be 2-4 times higher than used for cell culture. This is needed for membrane stability.
3. High auxin/kinetin ratio is suitable to induce cell divisions while high kinetin/auxin ratio is required for regeneration.
4. Glucose is preferred as source of carbon by protoplast.
5. The vitamins used for protoplast cultures are the same as used in standard tissue culture media.

Osmoticum and Osmotic Pressure: Osmoticum broadly refers to the reagents/chemicals that are added to increase the osmotic pressure of a liquid. The isolation and culture of protoplast require osmotic protection until they develop a strong cell wall. If the freshly isolated protoplasts are transferred to the normal culture medium, protoplasts will burst. So, Addition of an osmotic is indispensable for both isolation and culture media of protoplast to prevent their rupture. The osmotic are of two types- non-ionic and ionic.

i) Non-ionic osmotica: The non-ionic osmotica are soluble carbohydrates such as mannitol, sorbitol, glucose fructose, sucrose and galactose. Mannitol, being metabolically inert, is most frequently used.

ii) Ionic osmotica: Calcium chloride, potassium chloride and magnesium phosphate are the ionic substances in use to maintain osmotic pressure.

When protoplasts are transferred to a culture medium, the use of metabolically active osmotic stabilizers (glucose, sucrose) along with metabolically inert osmotic stabilizers (mannitol) is advantageous.

10.2.Culture Methods: The culture techniques of protoplasts are almost the same that are used for cell culture with suitable modifications.

Feeder Layer Technique: This method is also important for selection of specific mutant or hybrid cells on plates. This technique consists of exposing protoplast cell suspension to X-ray (to inhibit cell division with good metabolic activity) and then plating them on agar plates.

Co-culture of Protoplast: Protoplasts of two different plant species (one slow growing and another fast growing) can be co-cultured. This type of culture is advantageous since the growing species provide the growth factors and other chemicals which help in the generation of cell wall and cell division.

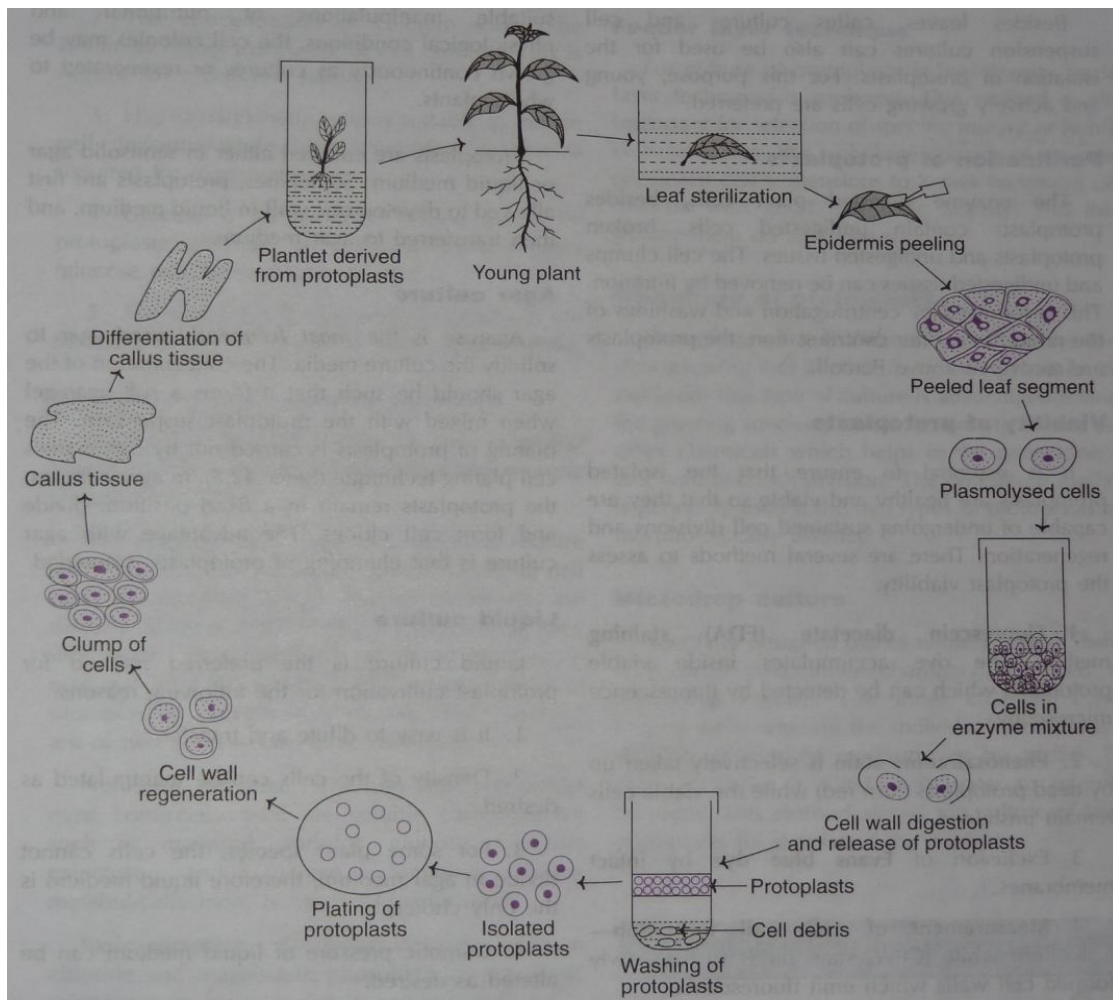
10.3.Regeneration of Protoplasts (Protoplast Development):

Protoplast development occurs in two stages-

1. Formation of cell wall.
2. Development of callus/whole plant.

Formation of cell wall: The process of cell wall formation in cultured protoplasts starts within a few hours after isolation that may take two to several days under suitable conditions. As the cell wall development occurs, the protoplasts lose their characteristic spherical shape.

Development of callus/whole plant: As the cell wall formation around protoplasts is complete, the cell increase in size and start to divide within 2-7 days. After 3 week visible colonies are formed. These colonies are transferred to osmotic free (mannitol, sorbitol free) medium for callus formation. The callus undergoes organogenic or embryogenic differentiation to finally form the whole plant.



Major steps involved in protoplast isolation, culture and regeneration of plants.

The genetic variations found in the *in vitro* cultured cells are collectively referred to as somaclonal variations. The plants derived from such cells are referred to somaclones. Some authors use the terms **calliclones** and **protoclones** to represent cultures obtained from callus and protoplasts respectively.

The term somaclonal variation was first used by **Larkin and Scowcraft (1981)** for variation arising due to culture of cells i.e. variability generated by a tissue culture. Somaclonal variations are reported in all types of plant tissue culture.

The term **gametoclonal variation** is used for the variations observed in the regenerated plants from gametic cells e.g. anther cultures. For the plants obtained from protoplast cultures, **protoclonal variation** is used.

11.1.Basis of somaclonal variations: Somaclonal variations occur as a result of genetic heterogeneity (change in chromosome number and structure) in plant tissue cultures. This may due to:

1. Expression of genetic disorder
2. Spontaneous mutations due to culture conditions.

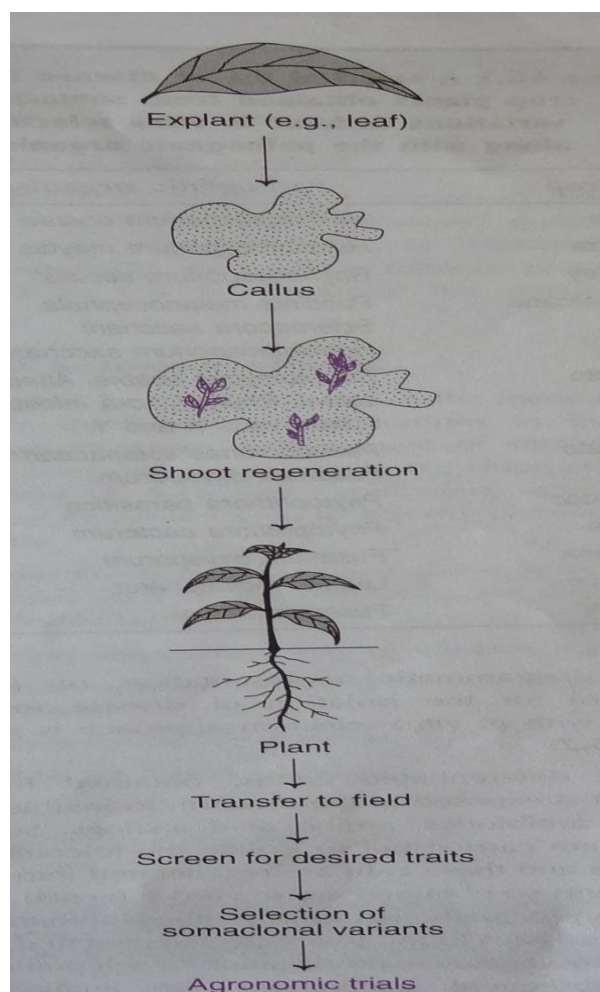
Mutations may be due to varied nutrients, culture conditions and mutagenic effects of metabolic products that accumulate in the medium. Somaclonal variations due to mitotic crossing over and changes in the cytoplasmic genome have been reported.

11.2.Isolation of Somaclonal variants:

There are two procedures to obtain the somaclonal variation-

1. Without *in vitro* selection
2. With *in vitro* selection.

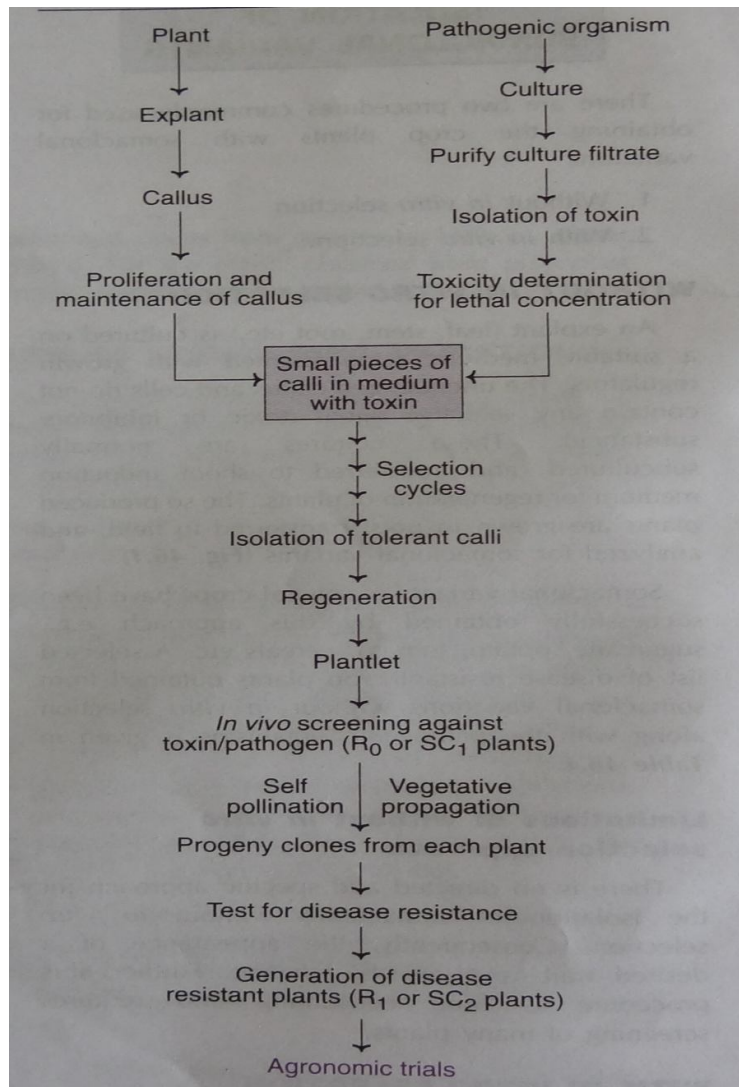
1. Without *in vitro* selection: An explant is cultured on a suitable medium, supplemented with growth regulators. The unorganised callus and cells do not contain any selective agent (toxic or inhibitory substance). These cultures are normally subcultured, and transferred to shoot induction medium for regeneration of plants. Produced plants are grown in pots, transferred to field and analysed for somaclonal variants. Somaclonal variations of several crops have been obtained by this method e.g. potato, tomato, sugarcane and cereals. Appearance of desired trait is by chance.



Isolation of somaclone without *in vitro* selection

2. With *in vitro* selection:

This method involves handling of plant cells in cultures (protoplast, callus). Cells are screened from plant cultures for their ability to survive in the presence of toxins/inhibitory substances in the medium or environmental stress conditions. The callus is exposed in the medium to inhibitors (toxins). Isolate the tolerant callus cultures and these calli are regenerated into plants. Obtained plants are screened *in vitro* against the toxin or pathogen. The plants resistant to the toxin are selected and grown further by self pollination or vegetative propagation. Subsequent generations are analysed for disease resistant against the specific pathogenic organism.



Isolation of disease resistant plants *in vitro* selection.

Advantages of with *in vitro* selection approach:

The major advantage of with *in vitro* selection method is the specific selection of the desired trait rather than a general variation found at the plant level.

11.3.Factors affecting production of somaclonal variants: The nature of genotype of the plants influences the frequency of regeneration and frequency of production of somaclones.

Duration of cell culture: Somaclonal variations are higher with increased duration of cultures. Genetic variability increased in tobacco protoplasts from 1.5 to 6% by doubling the duration of cultures.

Growth hormone effects: growth hormone such as 2, 4-dichlorophenoxy acetic acid (2, 4-D) and naphthalene acetic acid (NAA) are frequently used to obtain chromosomal variability.

11.4.Application of Somaclonal Variations:

Somaclonal variations are highly useful in plant breeding programmes. The genetic variations with desirable characters can be introduced into the plants. The important applications of somaclonal variations are-

1. Production of agronomically usefull plants e.g. thornless blackberries, rice, wheat, potato, sugarcane and carrot etc.
2. Resistance to disease.
3. Resistance to abiotic stresses (freezing, salt and aluminium tolerance).
4. Resistance to herbicides
5. Improved seed quality

Chapter-12 Plant Transformation:

The transfer of desirable gene(s) from one plant species to another with subsequent integration and expression of the foreign gene(s) in the host genome. The term **transgene** is used to represent the transferred gene. The genetically transformed new plants are regarded as transgenic plants. The development of transgenic plants is the outcome of an integrated application of recombinant DNA (rDNA) technology, gene transfer method and tissue culture techniques.

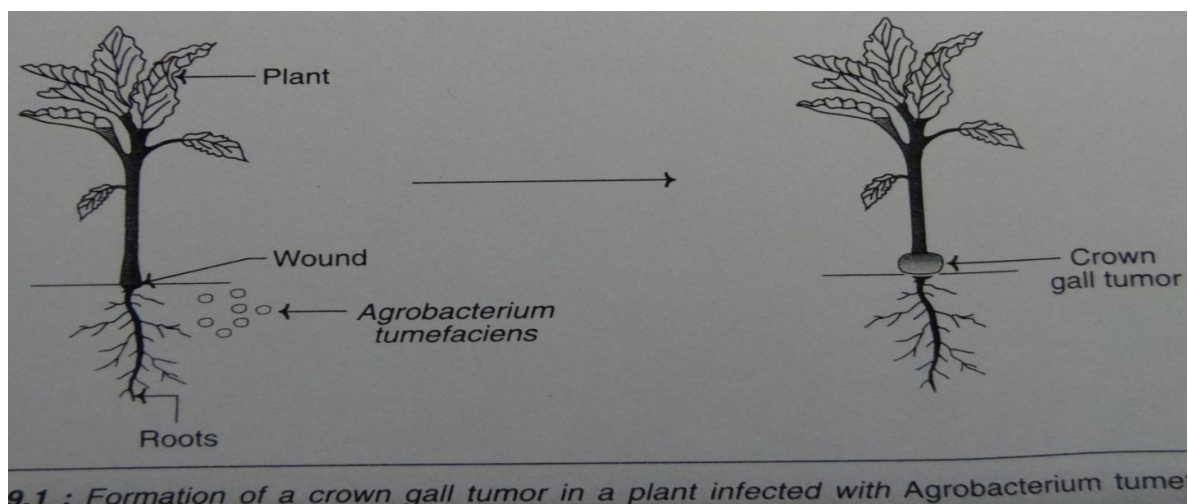
12.1. Gene transfer method:

There are two categories for gene transfer

1. Vector mediated gene transfer
2. Vectorless DNA transfer or Direct.

Vector mediated gene transfer: Vector mediated gene transfer is carried out either by *Agrobacterium*- mediated transformation or by use of plant viruses as vector.

***Agrobacterium* mediated gene transfer:** *Agrobacterium tumefaciens* is a soil-borne, pathogenic gram negative bacterium, produce tumour crown gall in wounded plants. The entry of the bacterium into the plant tissues is facilitated by the release of certain phenolic compounds (**acetosyringone, hydroxyacetosyringone**) by the wounded sites. Bacterium releases its **Ti plasmid** (Tumour inducing plasmid) into the cytoplasm of plant and produce crown gall. A fragment of **Ti plasmid (T-DNA)** is transferred from the bacterium into the host where it gets integrated into the plant cell chromosome. Crown gall is a naturally evolved genetic engineering process.



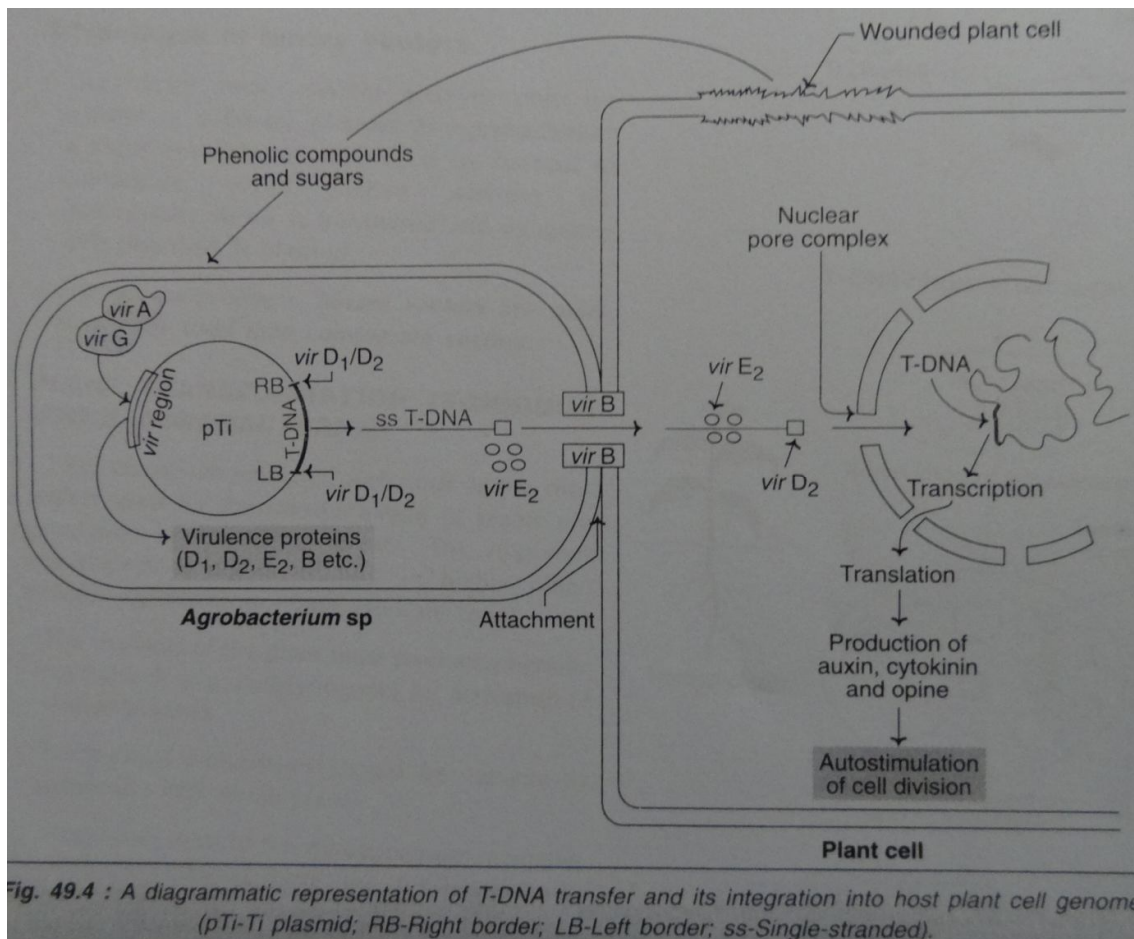
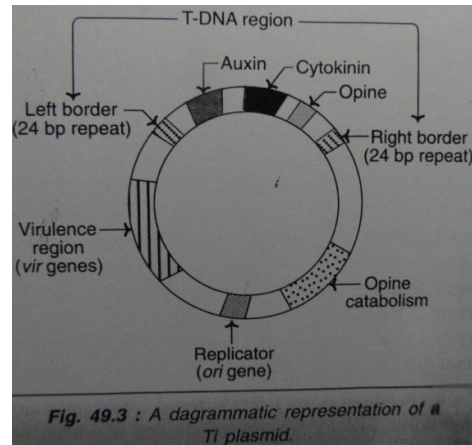
T-DNA transfer and integration:

The process of T-DNA transfer and its integration into the host plant genome is following-

1. **Signal induction to *Agrobacterium*:** The wounded plant cells release certain chemicals phenolic compounds and sugars which are recognized as signals by *Agrobacterium*. The signals induced result in sequence of biochemical events in *Agrobacterium* that ultimately helps in the transfer of T-DNA of T-plasmid.

2. **Attachment of *Agrobacterium* to plant cells:**

The *Agrobacterium* attach to plant cells through polysaccharides. Several chromosomal virulence (*chv*) genes responsible for the attachment of bacterial cells to the plant cells have been identified.



3. **Production of virulence proteins:** As the signal induction occurs in the *Agrobacterium* cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates *vir A* which in turn activates *vir G*. *Vir G* induces expression of virulence genes of Ti plasmid to produce the corresponding virulence proteins (**D₁, D₂, E₂, B** etc.). Sugars like glucose, galactose and xylose induce genes.
4. **Production of T-DNA strand:** The right and left borders of T-DNA are recognized by *vir D₁/ vir D₂* proteins. These proteins are involved in the production of single stranded T-DNA (ss T-DNA). The ss T-DNA gets attached to *vir D₂*.
5. **Transfer of T-DNA out of *Agrobacterium*:** The ss T-DNA- *vir D₂* complex in association with *vir G* is exported from the bacterial cells. *Vir B* products form the transport apparatus.
6. **Transfer of T-DNA into plant cells and integration:** The T-DNA *vir D₂* complex crosses the plasma membrane. In the plant cells, T-DNA gets covered with *vir E₂*. This covering protects the T-DNA from degradation by nucleases. *Vir D₂* and *vir E₂* interact with a variety of plant proteins which influences T-DNA transport and integration. The T-DNA *vir D₂ vir E₂* plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to illegitimate recombination.

12.2.Plant Transformation Techniques using *Agrobacterium*:

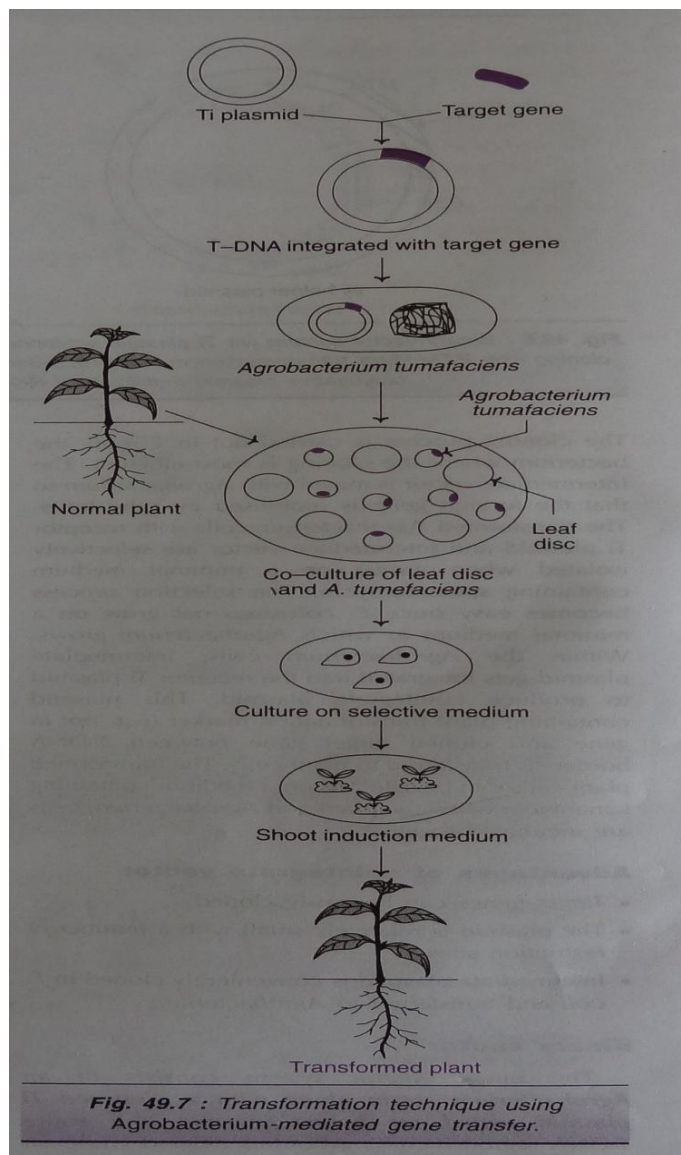
Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through *Agrobacterium* mediation are following-

- The explants of the plant must produce phenolic compounds for activation of virulence genes.
- Transformed cells/tissues should be capable to regenerate into whole plants.

In general, most of the *Agrobacterium* mediated plant transformations have been following basic protocol:

1. Development of *Agrobacterium* carrying the cointegrate or binary vector with the desired gene.

2. Identification of a suitable explants
e.g. cells, protoplasts, calluses, organs.
3. Co-culture of explants with *Agrobacterium*.
4. Killing of *Agrobacterium* with a suitable antibiotic without harming the plant tissues.
5. Selection of transformed plant cells.
6. Regeneration of whole plants.



12.3. Advantages of *Agrobacterium* mediated transformation:

- This is a natural method of gene transfer.
- *Agrobacterium* can infect any explants.
- Even large fragments of DNA can be efficiently transferred.
- Stability of transferred DNA is reasonably good.
- Transformed plant can be regenerated effectively.

Chapter-13 **Germplasm Preservation**

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells. A global body namely **International Board of Plant Genetic Resources (IBPGR)** has been established for germplasm conservation. The main objective of this board is to provide necessary support for collection and utilization of plant genetic resources throughout the world. There are two types of germplasm conservation:

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

1. *In-situ* conservation: Conservation of germplasm in their natural environment by establishing national parks is known as *in situ* conservation.

2. *Ex-situ* conservation: Genetic materials in the form of seed or from *in vitro* cultures can be preserved as gene banks for long term storage under suitable environmental conditions.

Germplasm conservation in the form of seeds: germplasm can be stored in the form of seeds but-

- Viability of seeds reduced or lost with passage of time
- Seeds are susceptible to insect or pathogen attack and leading to their destruction.

***In vitro* method for germplasm conservation:** There are several advantages associated with *in vitro* germplasm conservation-

- Large quantities of materials can be preserved in small space
- The germplasm preserved can be maintained in an environment free from pathogens
- It can be protected against the nature's hazards
- Large number of plants can be obtained from the germplasm
- Germplasm is maintained under aseptic conditions.

There are three methods for the *in vitro* conservation of germplasm-

1. Cryopreservation
2. Cold storage
3. Low pressure storage

Cryopreservation:

Preservation in the frozen state is called cryopreservation. In cryopreservation, bringing the plants cell and tissue cultures to a zero metabolism or non dividing state by reducing the

temperature in the presence of cryoprotectants. Cryopreservation means storage of germplasm at very low temperatures.

- Low temperatures deep freezers at -80 °C
- In vapour phase nitrogen at -150 °C
- In **liquid nitrogen at -196 °C**
- Over solid carbon dioxide at -79 °C

The cells stay totally in inactive state at liquid nitrogen temperature (-196 °C).

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. Reculture
7. Measurement of survival/viability
8. Plant regeneration

1. Development of sterile tissue cultures: Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, seed, ovules, cultured plant cells, protoplasts and calluses.

2. Addition of cryoprotectants and pretreatment: Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. There are several cryoprotectants e.g. **dimethyl sulfoxide (DMSO)**, glycerol, ethylene, mannose, proline and acetamide.

3. Freezing: i) Slow freezing method: Plant material or tissues is slowly frozen at slow cooling rates of 0.5-5 °C/ min from 0 °C to -100 °C and then transferred to liquid nitrogen. This method is used for the cryopreservation of suspension culture.

ii) Rapid freezing method: During rapid freezing, a decrease in temperature -300 to -1000 °C/min occurs. This method is used for the cryopreservation of shoot tips and somatic embryos.

iii) Stepwise freezing: This is the combination of slow and rapid freezing method. The plant material is first cooled to an intermediate temperature and maintained for 30 min and then rapidly cooled into liquid nitrogen. This method is used for the cryopreservation of shoot tips, suspension cultures and buds.

4. Storage: The frozen cells/tissues are kept for storage at -70 to -196 °C. The temperature of liquid nitrogen (-196 °C) is ideal for long storage.

5. Thawing: Frozen samples are plunged into warm water at 37-45 °C which gives a rapid thawing rate of 500-750 °C/min.

6. Reculture: Thawed germplasm is washed several times to remove cryoprotectants. This material is then recultured in a fresh medium.

7. Measurement of viability: We can measure the viability of frozen cells by **Evan's blue** and **fluorescein diacetate (FDA)** method.

8. Plant regeneration: The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant.

Cold storage: Cold storage basically involves germplasm conservation at a low and non freezing temperatures (1-9 °C). Virus free strawberry plants could be preserved at 10 °C for about 6 years. Several grape plants have been stored for over 15 years by cold storage (at 9 °C) by transferring them to a fresh medium.

3. Low Pressure Storage (LPS): The lowered partial pressure reduces the *in vitro* growth of plants.

Application of Germplasm Storage: The germplasm storage is boon to plant breeders and biotechnologists. Some of the applications are following-

1. Maintenance of stock culture: Plant materials of several species can be cryopreserved and maintained for several years and used when needed.
2. Disease free plant materials can be frozen and propagated whenever required.
3. Plant materials from endangered species can be preserved.
4. Rare germplasms developed by somatic hybridization and other genetic manipulations can be stored.
5. Establishment of germplasm banks for exchange of information at the international level.