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INSTANT NOTES ON
PLANT CELL TISSUE AND ORGAN CULTURE

(***Private circulation only***)

Compiled
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Chapter 1: Introduction

Background

Plant tissue culture is one of the most rapidly growing areas of biotechnology because of its high potential to develop crop plants with improved characters. Due to the advancement made in the tissue culture technology; it is now possible to regenerate species of any plant in the laboratory.

With the advances made in genetic engineering, it became possible to introduce foreign genes into cell and tissue culture systems. This led to the development of genetically modified (GMO) or genetically engineered organisms (GEO) transgenic crops which had improved traits and characteristics. An important aspect of all biotechnology processes is the culture of either the plant cells or animal cells or microorganisms. The cells in culture can be used for recombinant DNA technology, genetic manipulations etc.

The knowledge obtained from plant tissue cultures has contributed to our understanding of metabolism, growth, differentiation and morphogenesis of plants cells. Further, developments in tissue culture have helped to produce several pathogen-free plants, synthesis of many biologically important photochemical and secondary metabolites. Thus, plant tissue culture attracts the attention of molecular biologists, plant breeders and industrialists.

Definition

The aseptic culture of plant protoplasts, cells, tissues or organs under conditions which lead to cell multiplication or regeneration of organs or whole plants

(Or) "It is a scientific technique of growing plant cells, tissues and organs in an artificially prepared static or liquid nutrient medium maintained under sterile/aseptic conditions.

Other terms

In vitro culture

In vitro propagation

Micropropagation or Clonal propagation

Pioneers in plant tissue culture

Gotlieb Haberlandt
Father of plant tissue culture

Panchanan Maheshwari
First to develop haploid plants through anther culture

Basic principles in plant Tissue culture:

1. **Totipotency**

It is the potential or inherent capacity of a plant cell or tissue to develop into an entire plant if suitably stimulated. Totipotency implies that all the information necessary for growth and reproduction of the organism is contained in the cell. Although theoretically all plant cells are totipotent the meristematic cells are best able to express it.

2. Dedifferentiation

It is the capacity of mature cells to return to meristematic condition and development of a new growing organ

3. Competency

Describes the endogenous potential of a given cell or tissue to develop in a particular way. For example, as embryogenically competent cells are capable of developing into fully functional embryos. The opposite is non competent or morphogenetically incapable.

Chapter: 2
Historical achievements in Plant Tissue Culture

1902 **Gottlieb Haberlandt**, a German plant physiologist, called as the father of plant tissue culture. Haberlandt proposed concept of *in vitro* cell culture

1904 **Hannig** cultured embryos from several cruciferous species

1922 **Kolte and Robbins** successfully cultured root and stem tips respectively

1926 **Went** discovered first plant growth hormone –Indole acetic acid

1934 **White** introduced vitamin B as growth supplement in tissue culture media for tomota root tip

1939 Gau**theret, White and Nobecourt** established endless proliferation of callus cultures

1941 **Overbeek** was first to add coconut milk for cell division in Datura

1946 **Ball** raised whole plants of Lupinus by shoot tip culture

1954 **Muir** was first to break callus tissues into single cells

1955 **Skoog and Miller** discovered kinetin as cell division hormone

1957 **Skoog and Miller** gave concept of hormonal control (auxin: cytokinin) of organ formation

1959 **Reinert and Steward** regenerated embryos from callus clumps and cell suspension of *Daucus carota*

1960 **Cocking** was first to isolate protoplast by enzymatic degradation of cell wall

1960 **Bergmann** filtered cell suspension and isolated single cells by plating

1962 **Murashige and Skoog** developed MS medium with higher salt concentration

1962 **Kanta and Maheshwari** developed test tube fertilization technique

1966 **Steward** demonstrated totipotency by regenerating carrot plants from single cells of tomato

1966 **Guha and Maheshwari** produced first haploid plants from pollen grains

of Datura

1970 **Smith and Nathans** discovered first restriction enzyme from *Haemophilus influenza* (*HindIII*)

1970 **Baltimore** isolated Reverse transcriptase from RNA tumour virus

1972 **Carlson** produced first interspecific hybrid of *Nicotiana* by protoplast fusion

1972 **Berg** produced first recombinant DNA , combining SV40 virus and λ virus

1974 **Zaenen** *et al* discovered Ti plasmid is tumour inducing principle of agrobacterium

1975 **O'Farrel** developed high resolution two dimensional gel electrophoresis system

1977 **Chilton** *et al* successfully integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants

1977 **Sanger, Maxam-Gilbert** gave technologies for DNA sequencing

1980 **Zambryski** detailed structure of T-DNA and border sequences

1983 **Kary Mullis** invented Polymerase chain reaction (PCR), for amplification of DNA.

1984 **Horsh** *et al* developed transgenic tobacco by transformation with *Agrobacterium*

1987 **Klien** *et al* developed biolistic gene transfer method for plant transformation

1995 **Fleischmann** *et al* sequenced *Haemophilus influenzae*

1997 **Blattner** *et al* sequenced *E coli* genome

2001 Human genome Project consortium and **Venter** *et al* sequenced human genome successfully

2005 Rice genome sequenced under International Rice Genome Sequencing Project

Chapter 3: Terminology

Culture: Growing of cells tissues plant organs (or) whole plants in nutrient medium under aseptic conditions. Depending upon the explant source it can be named as follows:

Anther	anther culture
Pollen	Pollen culture
Embryo	Embryo culture
Cell	Cell culture
Protoplast	Protoplast culture
Callus	callus culture

Adventitious: adventitious: developing from unusual points of origin, such as shoots or roots from callus or from leaf explants, or embryos from other sources than a zygote

Amplification: Creation of many copies of a segment of DNA by PCR / Duplication of genes within a chromosomal segment.

Aseptic culture: Arising of culture from a tissue (or) an organ after elimination of bacterium, fungi and micro organism

Auxins : A class of growth hormone which cause cell elongation, apical, dominance, root initiation Eg : NAA, IAA, 2.4-D

Batch culture: Cell suspension grown in fixed volume of liquid Medium

callogenesis is a formation of undifferentiated mass of plant cell called "calli" form from the source of wounded viable plant tissue

Callus: A tissue arising from disorganized proliferation of cells either in culture (or) in nature

Caulogenesis or Stem organogenesis: induction of shoot development either directly from the explants or from the callus or from somatic embryo.

Cell culture: culturing of single cell (or) a small group of similar cells.

Clonal propagation: Asexual multiplication starting from single individual

Clone-Group of plants genetically identical in which all are derived from one selected individual by vegetative or in-vitro propagation, without the sexual process.

Continuous culture: A suspension culture continuously supplied with nutrients by continuous flow of fresh medium. The volume of culture medium is normally constant

Cryopreservation: ultra low temperature storage of cells, tissues, organs or seeds; it is usually carried out at temperatures below -1000C, as in liquid nitrogen (-1960C)

Cybrid: Plant (or) a cell which is a cytoplasmic hybrid produced by fusion of protoplast cytoplast

Cytokinens : A class of growth hormone which cause cell division, shoot differentiation, breaking of apical dominance etc. kinetin, zeatin etc.

Cytoplast: Protoplast – nucleous enucleated protoplast

De-Differentiation: loss of specialised function, usually characterised by small isodiametric cells

Differentiation: acquisition of specialised cell functions; the differentiated status is usually stable and in plant tissue culture the term is often taken to indicate the formation of shoots, roots or even other organs

Embryogenesis The process of embryo initiation origin of plantlet in a developmental pattern that closely resembles the normal embryo development from fertilized egg or ovum.

Embryoid / Somatic embryos: Non zygotic embryo's formed in culture.

Explant : A piece of tissue used to initiate tissue culture or removing shoots from callus separating individual shoots from proliferating mass of shoots.

Heterokaryon: A cell in which two or more nuclei of unlike genetic make up are present

Hetroplas t: Cell containing foreign organells

Homokaryon: A cell with two or more nuclei of similar genetic makes up Synkaryon: Hybrid cell produced by fusion of nuclei in heterokaryon

Hyperhydricity: physiological abnormality occuring in tissue culture; formerly the word vitrification was used

In vitro: A latin word literally means in glass / living in test tube applied to any process carried out in sterile culture under controlled condition in the laboratory

In vivo : a latin word literally means in living applied to any process occur in a whole organism under field condition where there is no control over the environmental conditions

Meristem culture: culture of a shoot apical meristem in view of avoiding contaminations, especially specific viruses and bacteria

Meristem: A group of actively dividing cells from which permanent tissue systems such as

root, shoot, leaf, flower etc are derived

Meristemoid: A gp of meristematic cells with in a callus with a potential to form primordial

Micro propagation: Production of miniature planting material (somatic embryos (or) plantlets) in large number by vegetative multiplication through growth hormone regeneration

Morphogenesis: (a) the evolution of a structure from an undifferentiated to a differentiated state; (b) the process of development and of growth of differentiated structures

Mother plant/source plant: plant from which a tissue culture is initiated

Nutrient medium : A solid (or) liquid combination of nutrients and water usuallyincluding several salts, carbohydrates in the form of sugar and vitamins such a medium is called basal medium. The basal medium may be supplemented with growth harmone occasionally with other defined and undefined substances.

Organ culture: Culture of isolated plant organs such as root tips, shoot tips, leaf

Organogenesis: Type of morphogenesis which results in the formation of organs and / or origin of shoots roots. The floral organs from tissue culture (or) suspension culture

Parasexual hybridization: Hybridization by non-sexual methods. Eg:- protoplast fusion

Passage time: The time interval between two successive sub cultures Primordial, immature parts of flower etc.

Primary metabolite: essential metabolites (e.g. carbohydrates, proteins, lipids), components of the essential metabolic pathways

Protoplast: A single cell with their cell walls stripped off a cell without a cell wall

Regeneration In tissue culture it is used for development of new organs (or) plantlets from a tissue, callus culture (or) from a bud.

Rhizogenesis: Formation of roots from the shoots

Secondary metabolite: a metabolite that is not part of the primary metabolic network of the cells. Primary metabolites are components of fundamental biochemical

pathways (e.g. glycolysis) that are present in all cells. Secondary metabolites only appear in cells that are specialised in some way e.g. in defence

Shoot apical meristem: undifferentiated tissue, located within the shoot tip, generally appearing as a shiny dome-like structure distal from the youngest leaf primordia and measuring less than 0.1 mm in length when excised

Somaclonal variation: genotypic or phenotypic variations occuring in tissue culture, the latter being either genetic or epigenetic in origin

Sub culture: Aseptic transfer of a part of a culture to a fresh medium

Suspension culture : Culturing of cells (or) cell aggregates in liquid medium

Suspension Culture: a type of culture in which cells, or aggregates of cells, multiply while suspended in liquid medium

Totipotency: The ability inherent property of a cell (or) tissue to give rise to whole plant irrespective of their ploidy level and the form of specialization

Sterilization is a procedure used for elimination of micro organisms and maintaining aseptic (or) sterile conditions for successful culture of plant tissues (or) organs.

Chapter 4
Sterilization or aseptic culturing technique

Sterilization

The media used for plant tissue culture contain sugar as a carbon source there by attracting a variety of micro organisms including bacteria and fungi. These organisms grow much faster than the cultured tissues and produce metabolic substances which are toxic to plant tissues. There are a number of sources through which the media may get contaminated which include culture vessels, instruments, media, explant, transfer area and culture room. There fore sterilization is absolutely essential to provide and maintain a completely aseptic environment during in vitro cultivation of plant cells (or) organs.

Sterilization is a procedure used for elimination of micro organisms and maintaining aseptic (or) sterile conditions for successful culture of plant tissues (or) organs.

The different techniques used for sterilization of plant tissue culture growth room chambers and instruments are

1. Dry sterilization

2. Wet heat / autoclaving / steam sterilization

3. Ultra filtration (or) Filter sterilization

4. Ultra violet sterilization

5. Flame sterilization

6. Surface sterilization (or) chemical sterilization

7. Wiping with 70% alcohol

Techniques in brief

1. Dry heat sterilization

Empty glass ware (culture vessels, petriplates etc) certain plastic ware (Teflon, FFp), Metallic instruments (scalpels, foreceps, needles etc) aluminium foils, paper products can be sterilized by exposure to hot dry air at 160O- 180OC for 2-4hr in hot air oven. All items should be properly sealed before sterilization.

2. Wet heat sterilization (or) autoclaving steam sterilization

It is a method of sterilization with water vapour under high pressure to kill all microbes by exposing to the super heated steam of an autoclave. Normally the tissue culture media in glass containers sealed with cotton plugs (or) Aluminium, foils, plastic caps are autoclaved

With a pressure of 15psi at 121^0C for 15-20 minutes. From the time the medium reaches the required temperature some types of plastic glassware can also be repeatedly sterilized by autoclaving (Good sterilization relies on time, pressure, temperature and volume of the object to be sterilized). The advantages of an autoclave are speed, simplicit y and distruction of viruses, while disadvantages are change in pH by 0.3 – 0.5 units.

3. Ultra filteration / Filter sterilization:

Vitamins, amino acids, plant extracts, harmones, growth Regulators are thermolabile and get destroyed during autoclaving. Such chemicals are filter sterilised by passing through a bacterial proof membrane filter under positive pressure. A millipore (or) seitiz filter with a pore size of not more than

O,2µ is generally used in filter sterilization. This procedure has to be carried out only in aseptic working space created by laminar air flow cabinet. Filter sterilised thermolabile compounds are added to an autoclaved media after cooling at about 40OC temperature.

Laminar air flow cabinets are used to create an aseptic working space blowing filter sterilized air through an enclosed space. The air is first filtered through a coarse free filter to remove large particles. It is then passed through HEPA filters which filters out all particles larger than 0.3 lm. This sterilized air blows through the working area in a cabinet at a constant speed of 1.8km/hr-1. These filters not only eliminate dust and other particles but also fungal and bacterial spores. Thus an aseptic environment is maintained at the time of tissue inoculation.

4. Ultra violet sterilization:

UV light sterilizes the interior portion of the inoculation chamber and eliminates atmospheric contamination. Materials like nutrient media, disposable plastic ware used for tissue culture and other similar materials are sterilized using UV rays to remove the contaminates.

5. Flame sterilization:

Metalic instruments like foreceps, scalpels, needle, and spatula are sterilised by dipping in 95%. ethanal followed by flaming and cooling. This technique is called flame sterilization. Autoclaving of metalic instrument is generally avoided as they rust and become blunt. These instruments are repeatedly sterilized during their use and time of inoculation to avoid contamination. The mouths of culture vessels are need to be expose to flame prior to inoculation (or) sub culture

6. Chemical sterilization / Surface:

The explant before their transfers to the nutrient medium contain in the culture vessels is treated with an appropriate sterilizing agent to inactivate the microbes present on their surfaces. This is known as surface sterilization.

The most commonly used sterilization for surface disinfection are

Mercuric chloride 0.1% for 3-10min

Calcium hypochlorite 5% for 20 min

Sodium hypochlorite 0.5-1% for 15 min

Bromine water 1% for 2-10min

Chloramines 10-20% for 20-30min

Other H2O2 AgNO3 Antibiotic etc. are also used

The plant material to be sterilized is dipped in sterilant solution for prescribed period and then the explant is taken out and washed with sterile distilled water for 2-3 times thoroughly so as to remove all the traces of sterilant adhere to the plant material before its transfer to nutrient media.

7. Wiping with 70% ethanol:

The surfaces that can not be sterilized by other techniques example plot form of laminar air flow cabinet, hands of operator etc are sterilized by wiping them thoroughly with 70% alcohol and the alcohol is allowed to dry.

Table 1 Sterilization techniques used in Plant Tissue Culture

Technique	Material	Materials sterilized
Steam sterilization/Autoclaving	Autoclave (121°C at 15 psi for 20-40 min)	Nutrient media, culture vessels, glassware and solvents such as water etc
Dry heat/hot air sterilization	Hot air oven (160-180°C for 3h)	Only glassware
Red hot/Flame sterilization	Spirit lamb	scalpel, forceps, needles etc, mouth of culture vessel
Filter sterilization	(membrane filter made of cellulose nitrate or cellulose acetate of 0.45-0.22µm pore size)	Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
Alcohol sterilization	70% ethanol	Worker's hands, laminar flow cabinet
Surface sterilization	(70%ethanol, Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	Explants
Radiations	Ionizing UV lights	Sterilize the laminar inner cabinet
Fumigations	Fumigants-HCHO and KMNO4	Sterilize the entire laboratory

Chapter 5: Plant nutrient Medium

Definition

Substrate for plant growth and refers to the mixture of certain chemicals to form a nutrient rich gel or liquid for growing cultures.

The nutritional requirement for optimum growth of plant organ, tissue and oplast in vitro generally vary from species to species even tissues from different parts of plant may have different requirement for satisfactory growth. Therefore no single media as such can be suggested as being entirely satisfactory for all types of in vitro culture. In order to formulate a suitable medium for a new system a well known basal medium such as Ms (Murashigel and skoog) B5 (gamborg et al) etc.

Commonly used tissue culture media

MS (Murashige and Skoog, 1962) and **LS** (Linsmaier and Skoog, 1972) for plant regeneration of both monocots and dicots

B5 (Gamborg *et al*., 1969) developed for culture of soybean cell suspensions but also has been effectively used for methods of plant regeneration. **B5** and its various derivatives have been valuable for cell and protoplast cultures.

SH: Schenk and Hildebrandt (1972) introduced this for culture of monocots and dicots, . Widely used especially for legumes.

WPM: Lloyd and McCown (1980, 1981). This is post MS media.. WPM is increasingly used for propagation of Ornamental shrubs and trees in commercial labs.

N6 (Chu) was developed for cereal anther culture and used in the success in other cereal anther culture. In special cases, NN (Nitsch and Nitsch) was also used.

Types of media – Solid and liquid media – Advantages and limitations

Culture medium is a general term used for the liquid (or) solidified formulations upon which plant cells, tissues (or) organs develop in the plant tissue culture. Thus normally the explants are grown in two different types of media

1) Solid Medium

2) Semi solid medium

3) Liquid Medium

1) Solid Medium:-

A solidifying or a gelling agent is commonly used for preparing semisolid (or) solid tissue culture medium. The plant material is placed on the surface of the medium. The tissue remains intact and the cell multiplication is comparatively slow.

Advantages

1) Solid medium is most widely used in plant tissue culture because of its simplicity and easy handling nature.

2) Acquires sufficient aeration without a special device since the plant material is placed on the surface of the medium.

Disadvantages

1) Only a part of the explant is incontact with the surface of the medium. Hence there may be inequality in growth response of tissues and there may be a nutrient gradient between callus and medium

2) There will be a gradiation in the gaseous exchange

3) Solid medium represent a static system. Hence there will be polarity of the tissues due to gravity and there will be variation in the availability of light to the tissues

4) Considerable damage to the tissues may occur during subculturing

5) Some physiological experiments which requires the immersion of tissues in the culture medium can not be conducted by using the solid medium

2) Semi solid medium:-

Those medium which contains only half of the solidifying agent recommended for a solid medium

3) Liquid medium:-

All the disadvantage of solid medium can be overcome by use of liquid medium. It does not contain a gelling or solidifying agent. So the plant material is immersed in the medium either partially or completely. Liquid medium is used for suspension cultures and for a wide range of research purposes.

Advantages

1) The tissue is more easily supplied with nutrients.

2) The culture of plant tissue in an agitated liquid medium facilitates

a) Gaseous exchange

b) Removes any polarity of the tissue due to gravity

c) Eliminates nutrient gradient within the medium and at the surface of the cells

3) Toxic waste products can be easily removed

4) Growth and Multiplication of cells tissues occur at a faster rate

5) There will be less damage to the tissues while sub-culturing

6) Isolation of secondary metabolites is easy

7) Liquid media are suitable for studies on the effect of any selective agent on individual's cells.

8) Therefore screening can be done at the cellular level for resistance to biotic and abiotic stresses.

9) Liquid medium can be easily changed without re-culturing and are preferred for some plant species whose explants exude phenols from their cut surfaces

Disadvantages

1. The explant gets submerged in liquid medium hence it requires some special devices for proper aeration. Usually Filter Paper Bridge may be used to keep the explant raised above the level of the medium.

2. The cultures may be regularly aerated either by bubbling sterile air / gentle agitation on a gyratory shaker

3. Needs to be sub-cultured frequently

Table shows the content of solidifying agent in medium

Medium	**% of Solidifying agent**	
	Agar agar	**phytagel**
Solid	15	4
Semi solid	7.5	2
Liquid/suspension culture	-	-

Some of the elements important for plant nutrition and their physiological function. These elements have to supplied by the culture medium in order to support the growth of healthy cultures *in vitro*

Element	Function
Nitrogen	Component of proteins, nucleic acids and some coenzymes Element required in greatest amount
Potassium	Regulates osmotic potential, principal inorganic cation
Calcium	Cell wall synthesis, membrane function, cell signalling
Magnesium	Enzyme cofactor, component of chlorophyll
Phosphorus	Component of nucleic acids, energy transfer, component of intermediates in respiration and photosynthesis
Sulphur	Component of some amino acids (methionine, cysteine) and some cofactors
Chlorine	Required for photosynthesis
Iron	Electron transfer as a component of cytochromes
Manganese	Enzyme cofactor
Cobalt	Component of some vitamins
Copper	Enzyme cofactor, electron-transfer reactions
Zinc	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum	Enzyme cofactor, component of nitrate reductase

Chapter 6: Plant Growth Regulators

Definition

Plant growth regulators (also called plant hormones) are numerous chemical substances that profoundly influence the growth and differentiation of plant cells, tissues and organs. Plant growth regulators function as chemical messengers for intercellular communication. In plants, only five substances are classically defined as plant hormones. However, some other substances are being studied that may eventually be classified also as hormones.

The five classical hormones:

1. Auxin
2. Gibberellins
3. Cytokinin
4. Abscisic acid
5. Ethylene

Additional substances gaining recognition as hormones:

Polyamines

Jasmonates

Salicylic acid

Brassinosteroid

Brief

1. Auxins

Auxins stimulate new cell division, cell enlargement, the formation of shoot apices or buds, the induction of somatic embryogenesis, and may promote rooting.

e.g. Indole-3-acetic acid (IAA),

Indole-3-butyric acid,

1-Naphtalene acetic acid (NAA),

2,4-Dichlorophenoxyacetic acid (2,4-D),

2,4,5-Trichlorophenoxy acetic acid (2,4,5-T) and

picloram.

2. Cytokinins

Cytokinins stimulate cell division, cell and/or shoot differentiation, lateral bud break, *etc*

natural : zeatin (ZEA),

Isopentenyl Adenine (2iP or IPA), (adenine)

synthetic: benzylaminopurine (BAP = benzyladenine BA),

kinetin (KIN)

Auxin - Cytokinin Interaction

In case of high auxin-cytokinin ratio, root formation, embryogenesis and callus formation is initialised. In case of low auxin-cytokinin ratio, formation of adventitive or axillary shoots is induced. The auxin-cytokinin ratio is also essential for chloroplast formation (and for most of another process).

3. **Gibberellins**

The main effect of gibberellins in plants is to cause stem elongation and flowering. They are also prominently involved in mobilization of endosperm reserves during early embryo growth and seed germination. Gibberellins are an extensive chemical family based on the ent-gibberellane structure. There exit over 80 different gibberellin compounds in plants but only giberrellic acid (GA3) and GA4+7 are often used in plant tissue culture. In tissue culture, gibberellins are used to induce organogenesis, particularly adventitious root formation.

4. Abscisic Acid

Abscisic Acid: Abscisic acid (ABA) in plants is a terpenoid involved primarily in regulating seed germination, inducing storage protein synthesis and modulating water stress. In plant tissue culture, it is used to help somatic embryogenesis, particularly during maturation and germination.

5. Ethylene: Ethylene is a simple gaseous hydrocarbon with the chemical structure H2C=CH2. Ethylene is apparently not required for normal vegetative growth. However, it can have a significant impact on development of root and shoots. Usually, ethylene is not used in plant tissue culture.

Others

6. Brassinosteroids

Brassinosteroids (BRs) are a class of polyhydroxysteroids that have been recognized as a sixth class of plant hormones. These were isolated from *Brassica napus* reported promotion in stem elongation and cell division

Promote shoot elongation at low concentrations

Strongly inhibit root growth and development

Promote ethylene biosynthesis and epinasty

Jasmonates

Jasmonates are represented by jasmonic acid and its methyl ester. Jasmonic acid is considered by some to be a new class of plant growth substance. Inhibition of many processes such as tissue culture growth, embryogenesis, seed germination, pollen germination, flower bud formation, chlorophyll formation, Differentiation in plant tissue culture, adventitious root formation, breaking of seed dormancy, pollen germination,

Polyamines

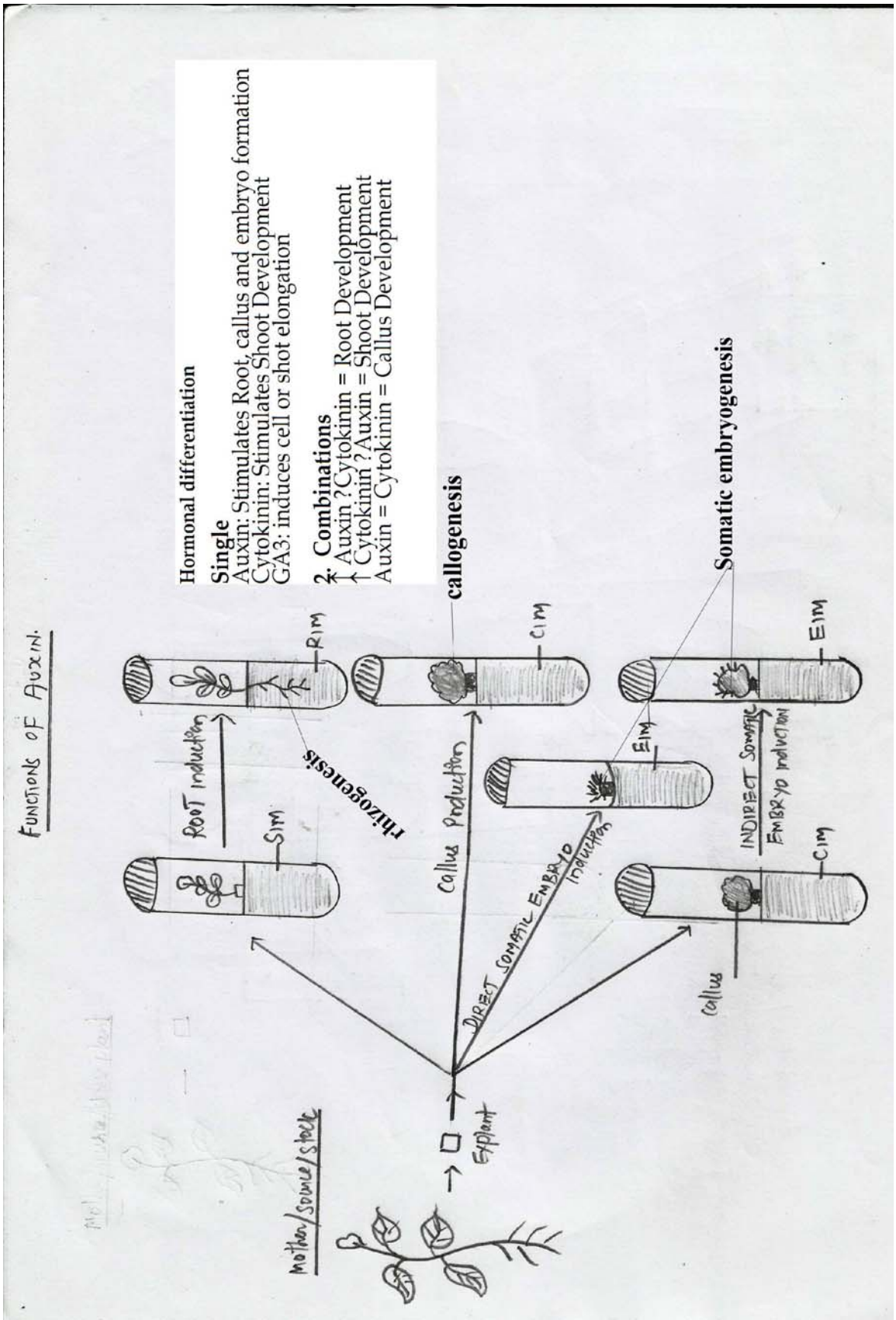
There is some controversy as to whether these compounds should be classified with hormones. They appear to be essential in growth and cell division.

Salicylic Acid

SA is thought by some to be a new class of plant growth substances. It promotes flowering, inhibits ethylene biosynthesis and reverses the effects of ABA .

List of growth hormones and their functions

Hormone	Categories	Function in Plant Tissue Culture
Auxins	Indole-3-Acetic Acid Indole-3-Butyric Acid Indole-3-Butyric Acid, Potassium Salt α-Naphthaleneacetic Acid 2,4-Dichlorophenoxyaceti	Adventitous root formation (high concen) Adventitious shoot formation (low concen) Induction of somatic embryos Cell Division Callus formation and growth Inhibition of axillary buds Inhibition of root elongation
Cytokinins	6-Benzylaminopurine 6-Y,Y-Dimethylallylaminopurine (2iP) Kinetin Thidiazuron (TDZ) N-(2-chloro-4-pyridyl)-	Adventitious shoot formation Inhibition of root formation Promotes cell division Modulates callus initiation and growth Stimulation of axillary's bud breaking and growth Inhibition of shoot elongation Inhibition of leaf senescence
Gibberellins	Gibberellic Acid	Stimulates shoot elongation Release seeds, embryos, and apical buds from dormancy Inhibits adventitious root formation Paclobutrazol and ancymidol inhibit gibberellin
Abscisic Acid	Abscisic Acid	Stimulates bulb and tuber formation Stimulates the maturation of embryos
Polyamines	Putrscine Spermidine	Promotes adventitious root formation Promotes somatic embryogenesis Promotes shoot


FUNCTIONS OF AUXIN.
Hormonal differentiation
Single
Auxin: Stimulates Root, callus and embryo formation
Cytokinin: Stimulates Shoot Development
GA3: induces cell or shot elongation
2. Combinations
↑ Auxin ?Cytokinin = Root Development
↑ Cytokinin ?Auxin = Shoot Development
Auxin = Cytokinin = Callus Development
Somatic embryogenesis
callogenesis
Rhizogenesis
ROOT induction
SIM
RIM
CIM
EIM
Callus Production
DIRECT SOMATIC EMBRYO Induction
INDIRECT Somatic EMBRYO induction
Explant
Callus
Mother/source/stock

Chapter 7:

Nutritional requirements of a medium
or
Composistion or Components of a Plant Tissue Culture Medium

1. Inorganic Nutrients: In vitro growth of plants also requires combination of macro and micronutrients like in vivo growth.

- **Macronutrients** are classified as those elements which are required in concentration greater than 0.5 mM/l. They include nitrogen, potassium, phosphorus, calcium, magnesium and sulphur in form of salts in media. Nitrogen is usually supplied in form of ammonium (NH_4^+) and nitrate (NO_3^-) ions. Nitrate is superior to ammonium as the sole N source but use of NH_4^+ checks the increase of pH towards alkalinity. Culture media should contain atleast 25mM/l nitrogen and potassium. Other major elements are adequate in concentration range of 1-3mM/l.
- **Micronutrients** are those elements which are required at a concentration less than 0.05mM/l. These include iron, manganese, zinc, boron, copper and molybdenum. These inorganic elements although required in small quantity are essential for plant growth, most critical of them being iron which is not available at low pH. Therefore, it is provided as iron EDTA complex to make it available at wide range of pH.

2. Carbon Source: Sugar is very important part of nutrient medium as energy source, since most plant cultures are unable to photosynthesize effectively owing to inadequately developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels etc. Hence they lack auxotrophic ability and need external carbon for energy. The most preferred carbon or energy source is sucrose at a concentration of 20-60g/l. While autoclaving the medium, sucrose is hydrolysed to glucose and fructose which are then used up for growth. Fructose, if autoclaved is toxic. Other mono or

disaccharide and sugar alcohols like glucose, sorbitol, raffinose etc may be used depending upon plant species. Carbohydrates also provide osmoticum and hence in anther culture higher concentration of sucrose (6-12%) is used.

3. Organic Supplements:

- **Vitamins:** are organic substances required for metabolic processes as cofactors or parts of enzymes. Hence for optimum growth, medium should be supplemented with vitamins. Thiamine (B1), nicotinic acid (B3), pyridoxine(B6), pantothenic acid(B5) are commonly used vitamins of which thiamine (0.1 to 5mg/l) is essentially added to medium as it is involved in carbohydrate metabolism. Rest vitamins are promontory.

- **Amino acids:** Addition of amino acids to media is important for stimulating cell growth in protoplast cultures and also in inducing and maintaining somatic embryogenesis. This reduced organic nitrogen is more readily taken up by plants than the inorganic nitrogen. L-glutamine, L-asparagine, L-cystein, L-glycine are commonly used aminoacids which are added to the culture medium in form of mixtures as individually they inhibit cell growth.
- **Complex organics/ Other organic supplements** are group of undefined supplements such as casein hydrolysate, coconut milk, yeast extract, orange juice, tomato juice etc. These compounds are often used when no other combination of known defined components produce the desired growth. Casein hydrolysate has given significant success in tissue culture and potato extract also has been found useful for anther culture. However, these natural extracts are avoided as their
- **Antibiotics** Some plant cells have systematic infection of micro organisms. To prevent the growth of these microbes it is essential to enrich the media with antibiotics**.** Eg:- Streptomycin or Kanamycin at low concentration effectively controls systemic infection and do inhibit the growth of cell cultures

composition is unknown and vary from lot to lot and also vary with age affecting reproducibility of results.

- **Activated charcoal:** acts both in promotion and inhibition of culture growth depending upon plant species being cultured. It is reported to stimulate growth and differentiation in orchids, carrot, ivy and tomato whereas inhibits tobacco, soybean etc. It absorbs brown-black pigments and oxidized phenolics produced during culture and thus reduce toxicity. It also absorbs other organic compounds like PGRs, vitamins etc which may cause the inhibition of growth. Another feature of activated charcoal is that it causes darkening of medium and so helps root formation and growth.

4. PGRs: stimulate cell division and hence regulate the growth and differentiation of shoot and roots on explants and embryos in semisolid or in liquid medium cultures. The four major PGRs used are auxins, cytokinin, gibberellins and abscissic acid and their addition is must to the culture medium.

- **Auxins:** induce cell division, cell elongation, apical dominance, adventitious root formation, somatic embryogenesis. When used in low concentration, auxins induce root initiation and in high, callus formation occurs. Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA). 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3 acetic acid (IAA), indolebutyric acid (IBA) etc. Both IBA and IAA are photosensitive so the stock solutions must be stored in the dark. 2,4-D is used to induce and regulate somatic embryogenesis.

- **Cytokinins:** promote cell division and stimulate initiation and growth of shoots in vitro. Zeatin, 6- benzylaminopurine (BAP), kinetin, 2-iP are the frequently used cytokinins. They modify apical dominance by promoting axillary shoot formation. When used in high concentration, CK inhibits root formation and induces adventitious shoot formation. The ratio of auxin and cytokinin in the culture decides morphogenesis. If this ratio is high, leads to

embryogenesis, callus initiation and root initiation whereas if ck/auxin is high, it gives rise to axillary and shoot proliferation.

- **Gibbrellins and abscissic acid:** are lesser used PGRs. Gibbrellic acid (GA3) is mostly used for internode elongation and meristem growth. Abscissic acid (ABA) is used only for somatic embryogenesis and for culturing woody species.

5.Solidifying agents: are used for preparing semisolid tissue culture media to enable explant to be placed in right contact with nutrient media (not submerged but on surface or slightly embedded) to provide aeration. Agar is high molecular weight polysaccharide obtained from sea weeds and can bind water. It is added to the medium in concentration ranging from 0.5% to 1 %(w/v). Agar is preferred over other gelling agents because it is inert, neither does it react with media constituents nor digested by plant enzymes. Agarose, a purified extract of agar is used for protoplast culture. Alternative gelling compounds like gelrite etc form clear gels (unlike agar which is translucent) and hence easier to detect contamination which might develop during culture growth. Mechanical support for cell or tissue growth can also be provided without using any gelling agent by Filter Paper Bridge, perforated cellophane and polyurethane foam etc.

6. pH: pH affects absorption of ions and also solidification of gelling agent. Optimum pH for culture media is 5.8 before sterilization. Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development in vitro. The pH of culture media generally drops by 0.3 to 0.5 units after autoclaving and keeps changing through the period of culture due to oxidation and also differential uptake and secretion of substances by growing tissue.

Chapter 8: Basic technique of Plant tissue culture

Plant tissue culture is the aseptic method of growing cells and organs such as meristems, leaves, roots etc either in solid or liquid medium under controlled condition. In this technique small pieces of viable tissues called ex- plant are isolated from parent plants and grown in a defined nutritional medium and maintained in controlled environment for prolonged period under aseptic condition.

The general techniques of plant tissue culture involve four main stages. They are

Initiation of culture

Multiplication (or) sub culture

Development and differentiation

Hardening

1. Intiation of culture

The most important factor in tissue culture technique is the maintenance of aseptic condition. For this purpose the culture medium generally, a hormone-free medium is used Immediately after preparation the culture vessel has to be plugged and autoclaved at $121^{O}C$ at 15 psi (pounds per sq. inch) for an about 15-20min. The plant material has to be surface sterilized with a suitable sterilent. The transfer area should also maintain free of micro organisms. Strict precautions are to be taken to prevent the entry of micro organisms. The plug of a culture vessel is removed carefully to transfer plant material to the nutrient medium during sub culturing. After inoculation the cultures are incubated in culture room under controlled condition at $25 \pm 12^{O}C$ temperature and 1000 lux light intensity generated by florescent tube and at a constant photoperiod regulated.

2. Multiplication / Subculture

After 2-3 weeks the explants show visible growth by forming either callus (or) differentiated organs like shoots, roots (or) complete plantlets,

depending upon the composition of the medium. Periodically sub-culturing of callus (or) organs (or) plantlets to the fresh medium is done to multiply the callus (or) organs (or) to obtain large number of plantlets from the callus

3. Development and Diffentiation / organogenesis

The conce ntration of phytoharmones in the medium are altered to induce differentiation in callus. A high cytokinins to auxin ratio induces shoot formation (caulogenesis) (basal medium + low cytokinins / GA_3 medium is used before they can be rooted. Higher concentration (>2 mg/l BAP) of cytokinins induce adventitious shoot buds and retard shoot growth. Very high auxins to cytokinin ratio induces root formation (Rhizogenesis). The development of organ structures like shoot, roots etc. from the cultured cells (or) tissues is known as organogenesis. Alternatively media composition can also be altered to induce the development of somatic embryos and the process is known as somatic embryogenesis. Further, an entire plantlet can be induced to grow on culture media by manipulating the phytoharmone balance correctly a nd the process is called Regeneration. The regeneration may be either direct or callus mediated. The *in vitro* induced shoots must be transferred to the culture media that supports root induction.

4) Hardening:

The *in vitro* cultured rooted pla nts are first subjected to acclimatization before transferring to the field. The gradual acclimatization of *in vitro* grown plant to *in vivo* conditions is called hardening. The plantlet is taken out from the rooting medium and is washed thoroughly to remove entire agar from the surface of plantlet as agar may attract microbes to grow and destroy the plantlets. The plantlet is now kept in a low minimal salt medium for 24-48hrs and transferred to a pot that contains autoclaved sterilized mixture of clay soil, coarse sand and leaf moulds in 1 : 1 : 1 ratio proportion. The pot containing plantlet is covered generally with the transparent polythene cover having holes for aeration to maintain the humidity. The plantlets are maintained for about 15-

30 days in this condition. The plantlets are then transferred to the soil and are ready for transfer either to the green house or main field.

Applications of plant tissue culture in crop improvement

1. Micro propagation helps in mass multiplication of plants which are difficult to propagate through conventional methods.

2. Some perennial crop plants like ornamental and fruit crops can not be propagated through seeds. The vegetative propagation like grafting, budding are tedious and time consuming. In such crops micro propagation helps in rapid multiplication.

3. Rapid multiplication of rare and elite genotypes such as Aromatic and Medicinal plants. Isolation of *in vitro* mutants for a large number of desirable character Eg:- Isolation of biochemical mutants and mutants resistant to biotic (pest and disease) abiotic (salt and drought, cold, herbicide etc) stresses through the use of somaclonal variation

4. Screening of large number of cells in small space.

5. Cross pollinated crops like cordamum, Eucalyptus, coconut, oil palm do not give true to type plants, when multiplied through seed. Development of genetically uniform plants in cross pollinated crops is possible through tissue culture

6. In case of certain horticultural crops orchids etc seed will not germinate under natural conditions, such seed can be made to germinate *in vitro* by providing suitable environment.

7. Induction of flowering in some trees that do not flower or delay in flowering. Eg:- Bamboo flowers only once in its life time of 50 years

8. Virus free plants can be produced through meristem culture

9. Large amount of germplasm can be stored within a small space and less cost for prolonged periods under *in vitro* condition at low temperature. The preservation of cells tissues, organs in liquid Nitrogen at – 196OC is called cryopreservation

10. Production of secondary metabolites. Eg:- Caffine from *coffea arabica,* Nicotine from *Nicotiana rustica.*

11. Plant tissue culture can also be used for studying the biochemical pathways and gene regulation.

12. Anther and pollen culture can be used for production of halploids and by doubling the chromosome number of haploids using cholchicine homogygous diploids can be produced. They are called dihaploids.

13. In case of certain fruit crops and vegetative propagated plants where seed is not of much economic important, triploids can be produced through endosperm culture.

14. Inter specific and inter generic hybrids can be produced through embryo rescue technique which is not possible through conventional method. In such crosses *in vitro* fertilization helps to overcome pre-fertilization barrier while the embryo rescue technique helps to over come post fertilization barrier.

15. Somatic hybrids and cybrids can be produced through protoplast fusion (or) somatic hybridization

16. Ovary culture is helpful to know the physiology of fruit development.

17. Development of transgenic plants.

Advantages of tissue culture

- ❖ Rapid multiplication within a limited space
- ❖ It is not time bound and not season bound
- ❖ Free from pests and diseases

Limitations (or) Disadvantages

- ❖ Laborious, costly, special risk is required.

Chapter 8a

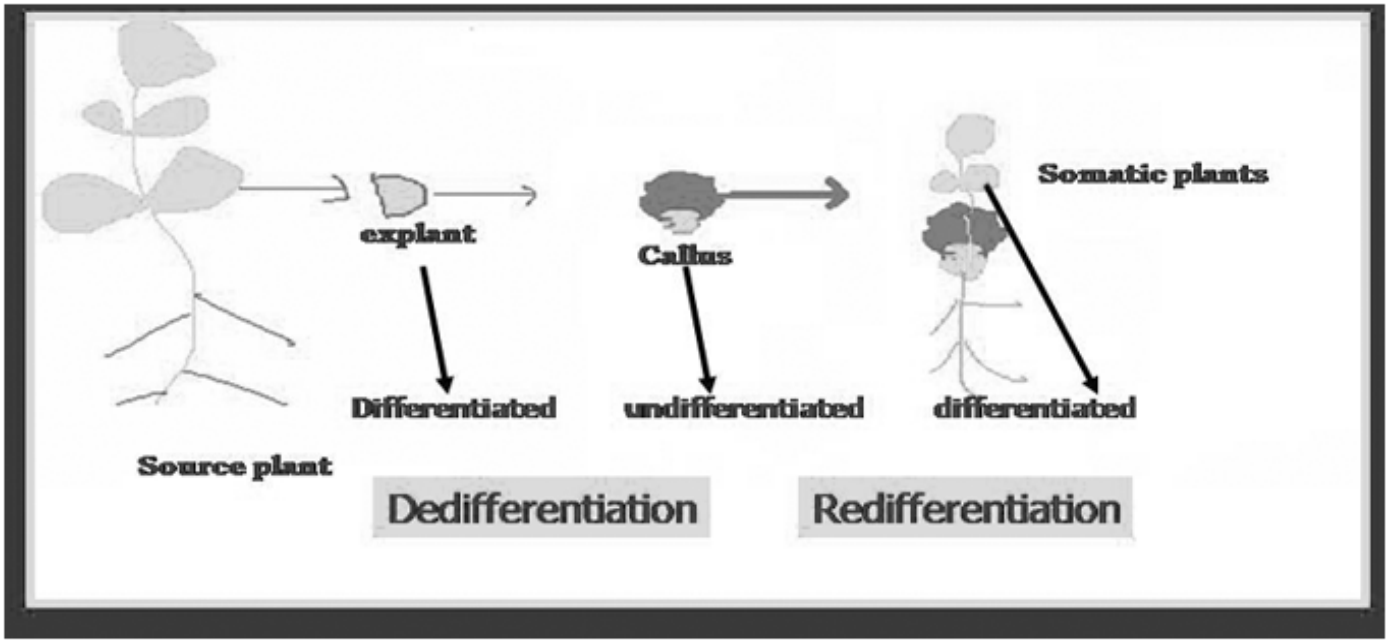
Vascular or cytodifferentiation

Dedifferentiated tissue made of meristematic cells

Undifferentiatied tissue made of parenchymatous cells

Dedifferentiation: The transformation of differentiated tissues such as explants into a state of undifferentiated tissue like callus.

Redifferentiation: The transformation of undifferentiated tissues such as callus into a state of differentiated tissue like shoots and roots form the callus.



Chapter 9:

Types of cell or tissue culture in plants

or

Types of in vitro culture in plants

There are different types of tissue culture techniques, mainly based on the explants used.

1. Callus culture
2. Suspension culture
3. Single cell culture
4. Protoplast culture
5. Organ culture- {anther culture, pollen culture,
Culture of embryo, ovary, root,
Shoot, floral buds, Meristem, fruit,
Seed, leaf primordia etc}

1) Callus culture:-

Callus refers to an unorganized mass of cells generally parenchymatous in nature. Callus culture may be derived from a wide variety of plant organs roots, shoots, leaves (or) specific cell types. Eg:- Endosperm, pollen. Thus when any tissue (or) cell cultured on an agar gel medium forms an unorganized growing and dividing mass of cells called callus culture. In culture, this proliferation can be maintained more (or) less indefinitely by subculturing at every 4-6 weeks, in view of cell growth, nutrient depletion and medium drying. Callus cultures are easy to maintain and most widely used in Biotechnology. Manipulation of auxin to cytokinin ratio in medium can lead to development of shoots or somatic embryos from which whole plants can be produced subsequently. Callus culture can be used to initiate cell suspensions which are used in a variety of ways in plant transformation studies. Callus cultures broadly speaking fall into one of the two categories.

1) Compact 2), Friable callus.

In compact callus the cells are densly aggregated. Where as in friable callus the cells are only loosly associated with each other and callus becomes soft and break a part easily. It provides inoculum to form cell suspension culture.Callus cultures can be maintained for prolonged periods by repeated sub-culturing. Callus cultures are

used for a) plant regeneration, b) preparation of single cell suspensions and protoplasts, and, c) genetic transformation studies.

Factors affecting Callus culture

- The source and the genotype of the explant
- Composition of the medium (most commonly used-MS medium)
- Temperature (22-280C suitable for callus formation)
- Growth regulators e.g. auxins, cytokinins alone or combination of these.
- Age of the plant
- Physiology and growth condition of the plant

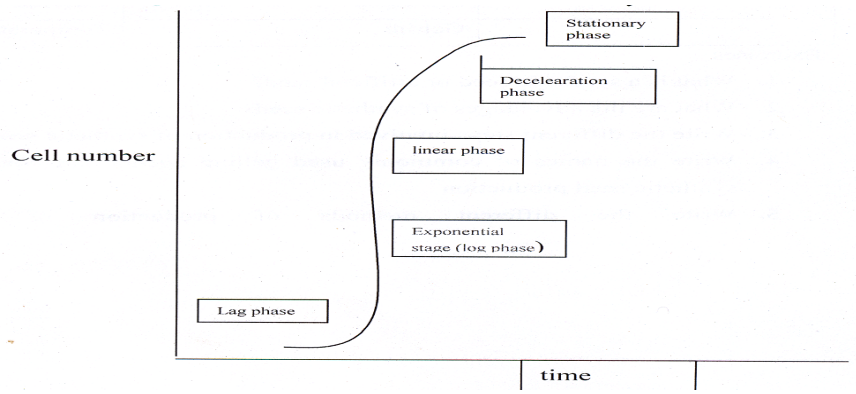
2) Suspension culture

The culture of tissues and cells cultured in a liquid nutrient medium produce a suspension of single cells and cell clumps, this is called **suspension culture**. A callus mass friable in texture is transferred to liquid medium and vessel is incubated on shaker to facilitate aeration and dissociation of cell clumps into smaller pieces. Gradually, over several weeks by subculturing, cells of callus dissociate and a liquid suspension culture is obtained. Cell suspensions are also maintained by subculturing of cells in early stationary phase to a fresh medium. Their growth is much faster than callus cultures and hence need to be subcultured more frequently (3-14days). Cell suspension cultures when fully established consist of a nearly homogeneous population. This system has an advantage that the nutrients can be continually adjusted and hence it is the only systems which can be scaled up for large scale production of cells and even somatic embryos.

The suspension cultures are broadly classified as:

a) Batch culture

A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. Cell suspension increases in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting and the growth ceases. The cells in culture exhibit the following five phases of a growth cycle.



1. Lag phase, where cells prepare to divide
2. Exponential phase, where the rate of cell division is highest.
3. Linear phase, where cell division shows but the rate of cells expansion increases.
4. Deceleration phase, where the rates of cell division and elongation decreases.
5. Stationary phase, where the number and size of cells remain constant.

When cells are subculture into fresh medium there is a lag phase. It is the initial period of a batch culture when no cell division is apparent. It may also be used with reference to the synthesis of a specific metabolite or the rate of a physiological activity. Then follows a period of cell division (exponential phase). It is a finite period of time early in a batch culture during which the rate of increase of biomass per unit of biomass concentration (specific growth rate) is constant and measurable. Biomass is usually referred to in terms of the number of cells per ml of culture. After 3 to 4 cell generations the growth declines. Finally, the cell population reaches a stationary phase during which cell dry weight declines. It is the terminal phase of batch culture growth cycle where no net synthesis of biomass or increase in cell number is apparent.

In batch culture, the same medium and all the cells produced are retained in the culture vessel (Eg. culture flask 100-250 ml). The cell number or biomass of a batch culture exhibits a typical sigmoidal curve. Batch cultures are maintained by sub-culturing and are used for initiation of cull suspensions.

b) Continuous culture: Here steady state of cell density is maintained by regularly replacing a portion of the used up medium with fresh medium. Continuous culture is further classified into two types:

1) **Closed**

2) **Open**

1) In **closed** type, the used medium is replaced with fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

2) In **open** type, both cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate. There are further two types of open continuous suspension culture: **turbidostat** and **chemostat.** In **turbidostat**, cells are allowed to grow upto a certain turbidity (decided on the basis of optical density) when a predetermined volume of the culture is replaced by fresh culture. On the other hand, in **chemostat**, the fresh culture medium to be added has one nutrient kept at a concentration so that it is depleted rapidly and becomes growth limiting while other nutrients are still in concentration higher than required. Increase or decrease in the concentration of growth limiting factor is correspondingly expressed by increase or decrease in growth rate of cells. Thus, the desired rate of cell growth can be maintained by adjusting the level of concentration of growth limiting factor with respect to that of other constituents. Chemostats are useful for the determination of effects of individual nutrients on cell growth and metabolism.

(3) Single cell culture

Free cells isolated from plant organs or cell suspensions when grown as single cells under *in vitro* conditions thus producing a clone of identical cells is called single cell culture.

Isolation of single cell from plant organs*:* Leaf tissue in particular is utilized as it has homogeneous population of cells using either of the following two methods:

1. **Mechanical:** Small pieces of leaves are cut and macerated in mortar and pestle in a grinding buffer. This homogenate is filtered through muslin cloth followed by centrifugation to finally pellet down the cells.
2. **Enzymatic:** Leaves are cut into moderate pieces after peeling the lower epidermis off. Cut pieces are then incubated with macerozyme or pectinase which degrade the middlelamella and cell wall of parenchymatous tissue. A suitable osmoticum like 0.3M mannitol is added to the culture which provides protection to cell wall from any damage to cells by enzymatic action.

Isolation of Single Cells from Cell Suspension: Suspension cultures are prepared from friable calli as described earlier from which isolation is carried out by filtering and harvesting the cells by centrifugation.

Culture of single cells: Isolated single cells are unable to divide in normal tissue media, therefore, they are cultured on nurse tissue where well grown callus cultures are made to diffuse their exudates through filter paper placed on them. The single cells placed on the filter paper derive their nutrition from these exudates and thus called nurse tissue. This technique of culturing single cell is known as Filter paper-Raft nurse tissue technique. Besides this, there are other techniques for single cell culture like microchamber, microdrop, Bergmann's plating technique, thin layer liquid medium etc., out of which Bergmann's plating technique is widely used. In this technique, free cells are suspended in a liquid medium. Culture medium with agar (1%) is cooled and maintained at 35°C in a water bath. Equal volumes of liquid and agar medium are mixed and rapidly spread in a petridish. The cells remain embedded in the soft agar medium. These embedded cells in the soft agar are observed under inverted microscope. When cell colonies develop, they are isolated and cultured separately.

Other point to be taken care of here is that since light has detrimental effect on cell proliferation, single cells should be cultured in dark.

Synchronisation of suspension cultures*:* Cell suspension is mostly asynchronous, different cells of different size, shape, DNA, nuclear content and also in different

stages of cell cycle (G1, S, G2, M). This is not desirable in cell metabolism studies. Hence, it is essential to obtain synchrony in suspension cultures and can be achieved by following methods:

- **Starvation:** Cells are starved of a nutrient like a growth regulator which is necessary for cell division resulting in arrest of cell growth during G1 or G2. After some time, when the nutrient is supplied, all arrested cells enter divisions synchronously.
- **Inhibition:** Using a biochemical inhibitor of DNA synthesis like 5 aminouracil, cells are arrested at G1 so that removal of inhibitor leads to synchreonous division of cells.
- **Mitotic arrest:** Colchicine is widely used to arrest cells at metaphase but only for short duration as longer colchicine treatment may induce anormal mitosis and chromosome stickiness.

Cell viability test: The objective of cell suspension culture is to achieve rapid growth rates and uniform cells with all cells being viable. The viability of cells can be determined by following approaches:

- **Phase contrast microscopy**: Live cells having a well defined healthy nucleus and streaming cytoplasm are easily observed under phase contrast microscope.
- **Reduction of tetrazolium salts**: When cell masses are stained with 1-2% solution of 2, 3,5-triphenyl teterazolium chloride (TTC). The living cells reduce TTC to red coloured formazon which can be extracted and measured spectrophotometrically for quantification of viability. This approach is not used for single cells.
- **Fluorescein diacetate (FDA)**: Esterase present in live cells cleaves FDA to produce fluorescein which fluoresces under UV so that live cells appear green under UV.
- **Evan's blue staining**: This is the only dye which is taken up by dead cells. Therefore, evan's blue is used usually to complement FDA.

Applications of Cell Culture

- **Mutant screening and selection:** Induced mutagens produce more frequency of mutants than spontaneous ones and screening them at cellular level also inhibits chimeric formation which is a drawback in mutation breeding.
- **Production of secondary metabolites:** Plants being important source of variety of chemicals used in pharmacy, medicine and industry, cell cultures are effectively utilized for production of these chemicals on a commercial scale for enhanced yield and better production control.

4. Protoplasts- isolation, culture and regeneration

Protoplasts are naked cells that can be obtained through mechanical/enzymatic degradation of cell walls. They are plant cells with a plasma membrane but without cell wall.

Sources: Young leaves, roots stems, petals, reproductive organs, friable callus, tissues/cells, fast growing cell suspensions. Coleoptiles, aleuronic layers, Plant cell tumors/galls.

Commercial enzyme preparations

Onozuka R10 Cellulase (Japan)
Cellulase R 10 (Japan)
Macerozyme R 10, Pectinase (USA)
Hemicellulase (USA)
Driselase, Cellulase (Japan)
Celulysin, Cellulase (USA)
Pectinase (USA, Sigma)
Macerase (USA)
Rhozyme HP 150, Hemicellulase (USA).

Enzyme mixture is normally dissolved in culture media together with an osmotic stabilizer. The osmotic agents may be sugar, alcohols, sorbitol or mannitol (13% w/v). Sucrose can be used for this purpose.

Isolation Methods

1. Mechanical

The cells are kept in a suitable plasmalyticum and protoplasts are isolated by cutting the plasmolysed tissues with a sharp blade so that protoplasts are released from cells through the cell wall when the tissue is again deplasmalysed. This method is suitable for isolation of protoplast from highly vacuolated cells of storage tissues, Example:- onion bulb, scales, raddish roots etc.

However this method has certain limitations

1) Only a small number of protoplasts can be isolated

2) Method is tedious and time consuming

3) Not useful for isolating protoplast from meristematic tissues, mature and less vacuolated cells

4) Viability of protoplasts is low because of the presence of substances released by damaged cells.

Enzymatic method

Preplasmolysis –Enzyme digestion-Washing- Purification-Culture

Direct (one step) method

Treatment with Macrozyme or Pectinase and Cellulase is done simultaneously. The enzyme mixture in direct method consists of 0.5% Macerozyme + 2% cellulase in 13% sorbitol or mannitol at pH 5.4.

Sequential (two step) method: In two step method, leaf segments with mixture A (0.5% macerozyme+ 0.3% potassium dextran sulphate in 13% Mannitol at pH 5.8) are vacuum infiltrated (in a desiccators) for about 5 min and washed cells are then, incubated with enzyme mixture B (2% Cellulase in a 13% solution of Mannitol at pH 5.4) for above 90 min at 30°C. After incubation, the mixture is centrifuged at 100g for 1 minute, so that protoplasts form a pellet, which is cleaned three times as in one step method.

Protoplast culture and Regeneration of plants

Like the totipotency of plant cells, isolated protoplasts regenerate a cell wall around themselves to reconstitute a cell and undergo repeated division to form callus. Protoplasts can be cultured following 'Bergman's cell plating technique', hanging

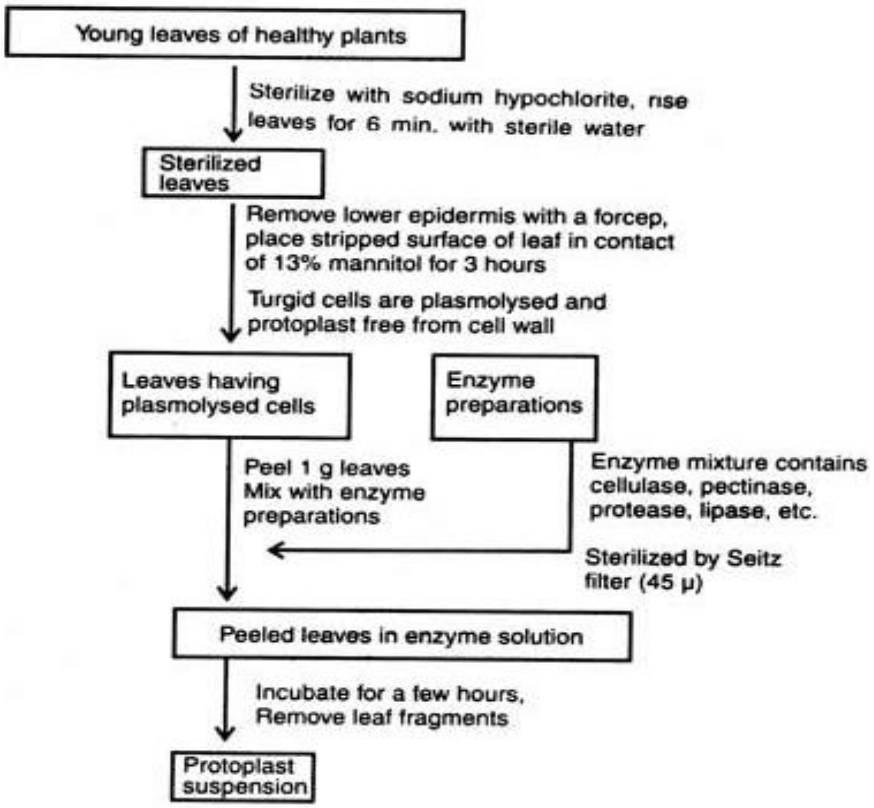
drops or in micro chambers or following multiple drop array method Isolated protoplasts is mixed with 1.0% agar culture medium and maintained at 40-45°C. Small amount of agar (liquid) protoplasts mixture is then poured into sterile plates. The parameters to be calculated before culture are:

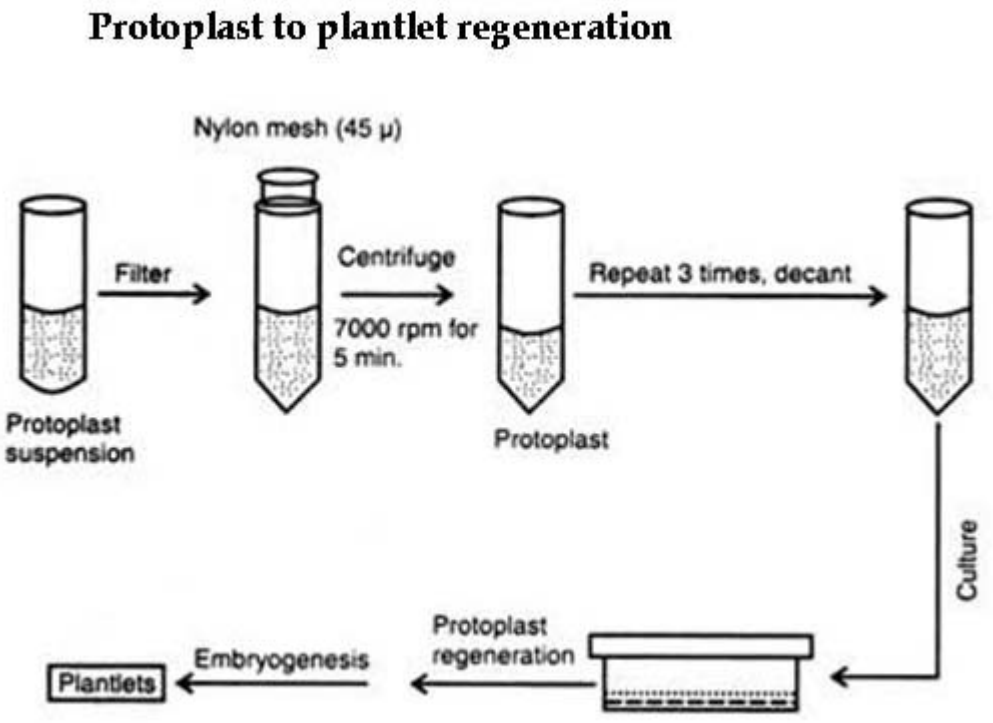
1. Viability and Plating density of protoplasts

2. Minimum Plating density (MPd) :In general, protoplast density within a range of 5 x 104 to 105/ml seems to be suitable. Normally, for the induction of division in the protoplasts, they have to be plated at final densities higher than 104/ml of the medium.

Protoplasts in culture start to regenerate a wall within a few hours and may take two to several days to complete. After three weeks, colonies are visible. Once small colonies are formed, their further growth is slowed down/inhibited altogether if they are allowed to remain on original high osmotic medium. The colonies therefore should be transferred to Mannitol free medium.

Steps in Protoplast isolation





Protoplast Fusion And Somatic Hybridization

Protoplast fusion is especially important in sexually incompatible plants and in cases where conventional methods of breeding fails to operated.

Protoplast fusion can be classified into three categories.

1. Spontaneous fusion,
2. Mechanical Fusion
3. Induced fusion.

Spontaneous fusion

Young leaves are more likely to produce such spontaneously induced multi nucelate protoplasts. It is strictly inter specific.

Mechanical fusion

This is a mechanical method to bring isolated protoplasts into intimate contact through micro manipulators and perfusion micropipette. By this method, occasional

fusions of protoplasts from soybean. *Arachis hypogea* and *Vinca rosea* were observed. In this procedure, protoplasts are likely to get injury.

Induced fusion

Induced fusion of protoplasts does not necessarily involve fusion of the same plant species which requires an inducing agent.

Use of fusogens

1. Treatment with NaNO3: Isolated protoplasts are suspended in an aggregation mixture of 5.5% Sodium nitrate in 10% sucrose solution

2. Effect of proteins: Gelatin and early products of its degradation at a concentration of 2.5% induced aggregation at high frequency within one hour E.g. Vicia, Glycine and Allium.

3. Immunological Method: Soybean and brome grass antibody cross reacted with and agglutinated *Vicia* protoplasts..

4. Calcium ions at High pH: This involves spinning the protoplasts in a fusion inducing solution (0.05M CaCl2 2H2O in 0.4 M mannitol at pH 10.5)

5. Polyethylene Glycol (PEG)

When protoplasts are available in sufficient quantities, 1 ml of the protoplasts suspended in a culture medium is added to 1 ml of 56% solution of PEG and the tube shaken for 5 Seconds. When micro quantities of protoplasts are available drop cultures can be used. Two types of protoplasts are mixed in equal quantities, 4-6 micro drops (100µl each) are placed in small plastic petridishes and allowed to settle for 5-10 minutes at Room temperature.

6. Miscellaneous: Additives such as Poly L-ornithine, poly D lysine, cocanavalin DM so, lysozyme, cytocholasin B and protamine sulphate have been employed.

7.Electric fusion: There are two steps:

Protoplasts are exposed to high frequency alternating electric field (0.5-1.5 NH2) that generates dipoles through dielectricphores. So, protoplasts find together to form, pearl chains.

Application of one/more short (10-100μs) direct current (DC) (1.3 k/cm-1). Cause reversible membrane breakdown resulting in pores in the aligned membranes. The contact membranes can be fused and fusion opens the way for hybrid cell formation. To insure maintenance of close membrane contact during fusion, AC field is reapplied.

Selection of fused protoplasts

After fusion, the protoplast population consists of a mixture of parental types, homokaryons and heterokaryons, of which heterokaryons (potential source of future hybrids) often make only 0.5%-10%.

1. Visual selection: This utilizes protoplasts for fusion studies that are visually distinct at the light microscope level. This is laborious. Only limited fusion products can only be selected.

2. Biochemical basis of selection/Nutrional selection: This is based on nutritional requirements of parents and hybrids

3.Complementation Selection is based upon the ability of the two genomes present in the heterokayon/hybrid to complement E.g. *N. tabaccum Petunia* Datura.

4. Drug resistance/sensitivity: Parental types are sensitive to actinomycin. (*Petunia parodic, P. hybrida*). Somatic hybrids (*P. hybrida* + *P. parodri*) are resistant to actinomycin.

5. Labelling: Protoplasts of two parents may be labeled by different fluorescent agents, which will then enable the selection of hybrids

(1) Octadeconyl amino fluorscein.

(2) Octadecyl palamine.

Culture of hybrid cells

The isolated hybrid cells may be cultured on a suitable medium and can be induced to develop a new cell wall, divide and regenerate either through embryoid formation (or) production of calli.

Regeneration of plants from hybrid tissue:-

Once the hybrid calli (or) embryoids are obtained, the plants are induced to regenerate from them since this is a pre requisite for exploitation in crop improvement. Further, the hybrid plants must be at least partially furtile, in addition to having some useful property to be of any use in breeding schemes. The hybrid plants thus obtained through somatic hybridization may be

1) Symmetric hybrids
2) Asymmetric hybrids
3) Cybrids

The regenerated plants are characterized using morphological, cytological.
Biochemical and Molecular biology techniques.

a) Symmetric hybrids:-

When normal protoplasts of two species are fused, the resulting somatic hybrid plants may retain the somatic chromosome complements of both the fusion parents. Such somatic hybrids called **symmetric hybrids**, immediately give rise to a new species Example:- pomato obtained by the fusion of potato and tomato mesophyl protoplast. Some of the symmetric hybrids may be superior to their parents in some traits of economic value, and could be ultimately develop into useful crops, but they may more often serve as useful sources of valuable genes. Many somatic hybrids have been produced between sexually incompatible species. Some of these hybrids possess and express useful genes, and are fertile. Some of these hybrids possess and express useful genes, and are fertile.

Symmetric hybrids provide the following opportunities in crop improvment programmes

1) Production of hybrids between non flowering or sterile lines
2) Widening of the genetic base of an allopolyploid species
3) Creation of a superior somatic hybrids
4) Gene transfer from related species into cultivated species
5) Generation of novel materials for scientific studies.

6) Symmetric hybrids can also be used in a backcross programme for cytoplasm transfors

b) Asymmetric hybrids

Many somatic hybrids exhibits the full somatic complement of one parental species while all or nearly all chromosomes of other parental species are lost during proceding mitotic divisions. Such hybrids are referred to as asymmetric hybrids. Such hybrids are likely to show a limited introgression of chromosome segments from the eleminated genomes due to drastically enhanced chromosome aberrations and / or mitotic crossing over *in vitro.* Asymemtric hybrids are produced due to spontaneous chromosome elimenation in certain fusion combinations. Asymmetric hybrids are essentially cytoplasmic hybrids (or) cybrids except for the introgressed genes (or) chromosomes, intensive somatic hybrids is being carried out in Brassica and potato either to generate new sources of cytoplasmic male sterility (or) to transfer genes for disease resistant (or) other treatments.

c) Cytoplasmic hybrid (or) cybrid

Cybrid or cytoplasmic hybrid may be defined as cell (or) plant which is produced by fusion of protoplast of one parent and cytoplast of another parent. Cybr id posses nuclear genes from only one parent and cytoplasm from both parents. In cultures cybrids may be produced by one of the following means.

1) Fusion of a normal protoplast of one species withan enucleated protoplast (cytoplast) (or) a protoplast having an inactivated nucleus of other species

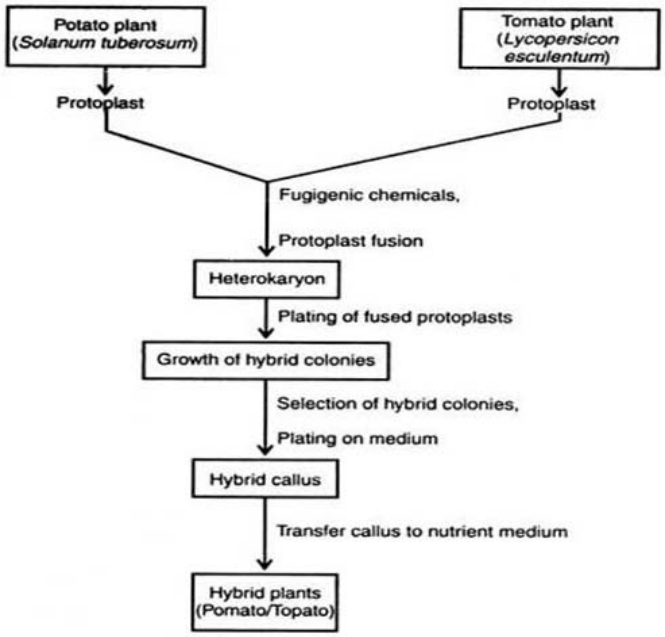
2) Elimination of nucleus of one species from a normal heterkokaryon or gradual elimination of chromosome of one species from a hybrid cell during the subsequent mitotic divisions.

3) The objective of cybrid production is to be combine cytoplasmic genes of one species with nuclear and cytoplasm of genes of another species. This provides a unique opportunity 1) for transfer of plasmagenes from one species into nuclear background of another species in a single generation. 2) The cybrid approach has been used for the transfer of cytoplasmic male sterility from *N. tabacum* to *N.*

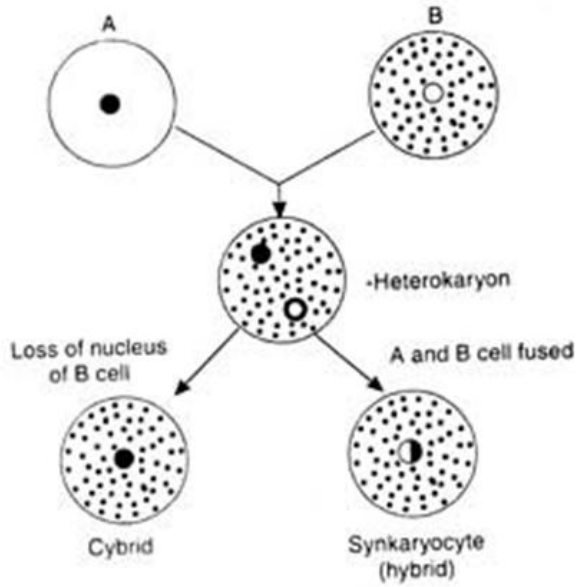
sylvestris from *petunia* hybrid to *petunia axillaries* etc. and even in sexually incompatible combination.

3) Recovery of recombinants between the parental mitochondrial or chloroplast DNAs (genomes).

Protoplast fusion for hybrid productions



Protoplast fusion for cybrid production



Applications of somatic hybridization

1. Production of novel interspecific and intergeneric crosses between the plants that are difficult (or) impossible to hybridise conventionally

Eg:- Tomato x Potato = Pomato

2. **Transfer of desirable genes for disease resistance**. Eg:- potato protoplast have been fused with those of *Solanum brevidens* and *Solanum phuraja* and *lyco persicon* the resulting somatic hybrids are resistant to potato leaf roll virus, potato virus and *Erwinia* soft rot. Somatic hybrids resistant to Phomalingam (Black log disease) were produced by the fusion of protoplast of *Brassica napus* with those of *Brassica nigra* (resistant to pomalingam)

3. **Transfer of desirable genes for Abiotic stress resistance,** Atrazine (resistance) has been transferred from wild species of tomato *lycopersicon peruvianum* into cultivated species (*Lycopersicon esculentum*)

4. **Transfer of desirable genes for quality characters**: somatic hybrids produced

between *Brassica napus* and *Erucasativa* were fertile and had low concentration of Erucic acid.

5. **Transfer of cytoplasmic male sterility:** Male sterility can be induced by alloplasmic association. It results in interaction between nucleus of onespecies and cytoplasm of another species. Male sterile lines were developed by fusing protoplast of *N. tabacum* with X-ray irradiated protoplasm of *N. africana*

6. **Overcoming sub in compatable barriers:** Hybrids between two sexually incompatable species posses some desirable features that may make them commercially useful. Eg:- somatic hybrids between *Datura innoxa* x *D. discolor* and *D. innoxa x D. stramonium* show heterosis for alkaloid content (20-25%) higher then their parents.

7. Production of auto tetraploids

8. Protoplast of sexually sterile plants (haploid, triploid, anueploid) can be fused to produce fertile diploids and polyploids.

9. Hybridization become possible between plants that are still in Juvenile phase

10. Production of unique nuclear cytoplasmic combinations

11. Production of heterozygous lines with in a single species that normally could only be propagated by vegetative means Eg:- potato, other tubers root crops

12. Photosynthetic efficiency of plants can be enhanced through the transfer of efficient foreign chloroplast into the plants having less photosynthetic systems.

5. Organ culture

A body of higher plants has complex inter-relationships between different organs like root, shoot, meristem, leaf primordial, floral buds, ovary, ovule anther lobs, pollen grains, and fruit, seed etc. In this method a particular organ is isolated and cultured under laboratory conditions in a chemically defined medium where they retain their characteristic structures and other features and continue to grow as usual. In organ culture, organ are not induced to form callus, therefore, it differs from the callus culture where the organization of the intact tissues is lost.

Organ culture may be grouped into two major categories: vegetative organs (root culture, leaf culture, and shoot tip culture) and reproductive organs (complete flower culture, isolated ovary culture, isolated ovule and embryo culture, pollen mother cell culture, seed and fruit culture).

1. Embryo culture

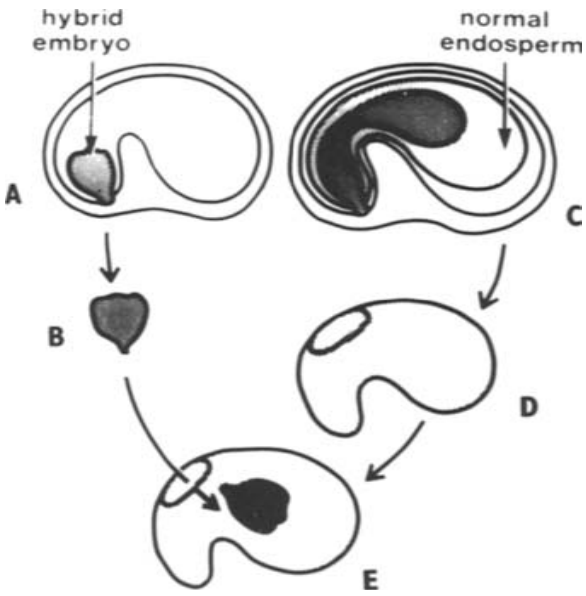
Besides, roots, shoots, and pollen, embryos can also be cultured to produce haploid plants. The embryo culture is very useful in conditions where embryo fails to develop due to degeneration of embryonic tissues. It has been used as a routine technique in orchid propagation, in breeding of species showing dormancy.

2. Embryo rescue

It has been observed that sometimes, inspite of successful pollination and fertilization, the embryos do not develop. The incompatibility between the embryo and the endosperm or some inherent deficiency also results in the under development of embryo. These immature embryos can be dissected out from the seeds and can be grown artificially on culture medium. These embryos differentiate into shoot, root and plantlets under culture conditions. This technique of growing immature embryo is termed as 'embryo rescue'. This technique is very useful in hybridization, breaking dormancy of certain seeds, and to achieve complete growth of embryo into a plant.

3**. Embryo-nurse endosperm transplant**

They inserted an excised hybrid embryo into a cellular endosperm dissected out from a normally developing ovule of one of the parents or a third species and cultured the nurse endosperm with the transplanted embryo on an artificial medium. Surface-sterilized pods carrying hybrid embryos or normally developing embryos (for nurse endosperm) were placed in sterile petri-dishes lined with moist filter paper until dissection. Two ovules, one carrying a hybrid embryo and the other with a normal embryo were removed from their pods and placed on a small (2 x 2 mm) pre-sterilized 'carrier' square of moist filter paper on the sterilized microscope stage.



Endosperm transplant for the culture of hybrid embryos in Trifolium, Lotus or Ornithopus. (A,B) The hybrid embryo is removed from the ovule in which endosperm development has failed. (C) To provide transplant endosperm a normally developing intraspecifically pollinated ovule is dissected at a stage when it contains cellular endosperm enclosing a heart-shaped to torpedo- shaped embryo. (D) The normal embryo is pressed out of the sac of endosperm leaving an exit hole. (E) The hybrid embryo is inserted into the normal endosperm through the exit hole (after Williams and De Lautour, 1980).

4. Micropropagation of pre-existing meristem

Meristem tip Culture (For virus elimination) **See chapter 11**

5. Anther Culture/pollen/microspore culture

Culturing anther on a suitable media to regenerate into haploid plants is called anther culture. First time, haploid plants were discovered in Datura stramonium by A.D. Bergner in 1921. Guha and Maheshwari (1964) pioneered the formation of embryos from anthers of Datura innoxia grown in vitro. After this, haploid plants have been produced via anther culture in more than 170 species. The anther culture technique is useful in haploid production. Haploid production: Haploid plant is defined as a sporophyte with gametophytic chromosome number. The in vitro production of haploid plants can be achieved by many techniques like:

- **Delayed pollination** which may not result in fertilization and hence only female genome grows up to form a haploid plant.
- **Temperature shock –** Extremes of temperature (both high and low) are used to suppress syngamy or make pollen inactive, thus leading to induction of haploidy.
- **Irradiation effect** - X rays, UV rays induce chromosomal breakage in pollen cells thus making them sterile which in turn results in haploid production.
- **Chemical treatment –** Treatment with colchicines, maleic hydrazide and toluene blue etc also induces chromosomal elimination.
- **Genome elimination by distant hybridization –** In case of distant crosses like inter-generic and inter-specific crosses where during the developmental process; one of the parental genomes is selectively eliminated subsequently leading to formation of haploid plants.

Therefore, production of a haploid plant where egg cell is inactivated and only male genome is present is called androgenesis. Similarly, production of haploid by development of unfertilized egg cell due to inactivation of pollen is called gynogenesis. Among all the methods illustrated above, anther culture is the most popular and successful for haploid production.

Anther culture procedure:

Step1

Experimental material: Young healthy plants grown under controlled conditions are used as experimental material from which flower bud of right stage (varies with species) is excised.

Step2

Disinfestation, excision and culture of anther: Flower buds are surface sterilized in laminar flow chamber followed by excision of anther from the bud. Stage of pollen development is determined by squashing an anther in acetocarmine and observing it under microscope. While excising anthers from flower buds, care is taken that

anthers are not injured as injury leads to callusing hence giving mixture of diploids, haploids and aneuploids.

Step3

Culture medium conditions: The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period. Medium should have sucrose for induction of embryogenesis.

Step 4

Haploid plants: In species following direct **androgenesis** i.e. which develop through embryoid formation, small plants emerge in 3-8 weeks after culturing which are then transferred on to a rooting medium with low salt and small amount of auxin. Those species undergoing indirect androgenesis involving callus formation, callus is removed from the anther and placed onto a regeneration medium with suitable ratio of cytokinin to auxin. The haploid plants thus produced in both cases are transplanted to soil in small pots and maintained under controlled conditions in greenhouse.

Diploidisation of haploid plants: Haploid plants produced from anther culture maintained *in vitro* can grow till the flowering stage but cannot be perpetuated. Since these plants are haploid and have only one set of homologous chromosomes of the diploid species, they cannot form viable gametes and hence no seed setting takes place for further perpetuation. Therefore, it is necessary to double the chromosome number of haploids to obtain homozygous diploids or dihaploid plants followed by their transfer to culture medium for further growth.

Application of haploid production:

Diploidisation of haploid plants result in rapid achievement of homozygous traits in doubled haplouds, hence these anther derived haploid plants have been used in breeding and improvement of crop species.

1. **Production of homozygous lines:**

The most important use of haploids is the production of homozygous lines which may be used directly as cultivars or may be used in breeding programme. For e.g. doubled haploids have been used for rapid development of inbred lines in hybrid maize programme.

The anthers from F1 hybrids of selected or desirable cross are excellent breeding material for raising anther derived homozygous plants or doubled haploids in which complementary parental characteristics are combined in one generation. The doubled haploid plants are subjected to selection for superior plants **(F**ig.2**)**. This approach is described as **hybrid sorting** where recombinant superior gametes are virtually being selected since the heterozygous gene combination in the F1 hybrid is transformed into homozygous combinations. Hybrid sorting reduces the time required for haploid breeding by 4-5 years as in conventional breeding by pedigree/ bulk method, the same requires ten years. Also, selection among DH lines reduces the size of breeding population.

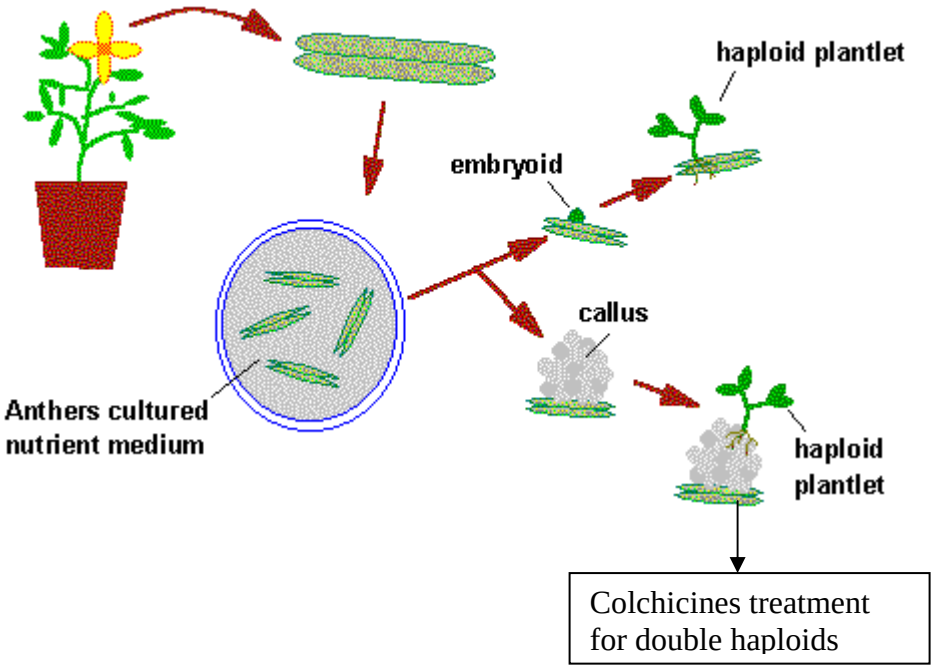
2. **Gametoclonal variation:** The variation observed among haploid plants having gametic chromosome number developing from anther culture is called gametoclonal variation. Such variations resulting in desirable traits are subjected to selection at haploid level followed by diploidisation to get homozygous plants which can be released as new varieties.

3. **Selection of desirable mutants:** Haploids offer a system where even recessive mutations are expressed unlike diploids where they express only in segregrating single plant progeny in M2 generation. Therefore, in several crops desirable mutants including traits like resistance to diseases, antibiotics, salts etc have been isolated from haploids derived from anther culture. For e.g. tobacco mutants resistant to black shank disease and wheat lines resistant to scab (*Fusarium graminearum*) have been selected and used as improved cultivars.

Problems associated with haploid plants:

- • Many species are not yet amenable for haploid production

- • Deleterious mutations may be induced during *in vitro* phase.
- • Plants having more or lesser than gametic chromosome number is also obtained which necessitates cytological analysis first.
- • Occurrence of gametoclonal variation limits the use of anther derived embryos for genetic transformation



Regeneration of anther for haploid productions

Chapter 10:
Micropropagation/Clonal propagation

In vitro propagation of plants vegetatively by tissue culture to produce genetically similar copies of a cultivar is referred to as **micropropagation** or **clonal propagation**. Sexually propagated plants (through generation of seeds) demonstrate a high amount of heterogeneity since their seed progenies are not true to type whereas asexual reproduction (by multiplication of vegetative parts) gives rise to genetically identical copies of parent plant. Thus, it permits perpetuation of the parental characters of the cultivars among the plants resulting from micropropagation.

Micropropagation proves useful for propagation of:

- Sexually sterile species like triploids, aneuploids which cannot be perpetuated by seeds.
- Seedless plants like banana
- Cross bred perennials where heterozygosity is to be maintained
- Mutant lines like auxotrophs which cannot be propagated in vivo
- Disease free planting material of fruit trees and ornamentals

General techniques of Micropropagation

Micropropagation generally involves four stages:

Stage 0, Selection of mother plant and its maintenance

Stage 1, initiation of aseptic cultures;

Stage 2, multiplication of shoots or somatic embryo formation

Stage 3, in vitro germination of somatic embryos or rooting of shoots

Stage 4, transfer of plants to greenhouse or field conditions (transplantation).

Debergh and Maene (1981) introduced the Stage 0, making micropropagation a five stage process. Each stage has its special requirements.

Stage 0

This is the initial step in micropropagation, and involves the selection and growth of Stock/mother/source plants for about three months under controlled conditions.

Stage I

In this stage, the initiation and establishment of culture in a suitable medium is achieved. Selection of appropriate explant is important. The commoniest used explants are organs, shoot tips and axillary buds. The chosen explant is surface sterilized and washed before use

Stage II

In this stage, the major activity of micro propagation occurs in a defined culture medium. Stage II involves the multiplication of shoots or rapid embryo formation from the explants.

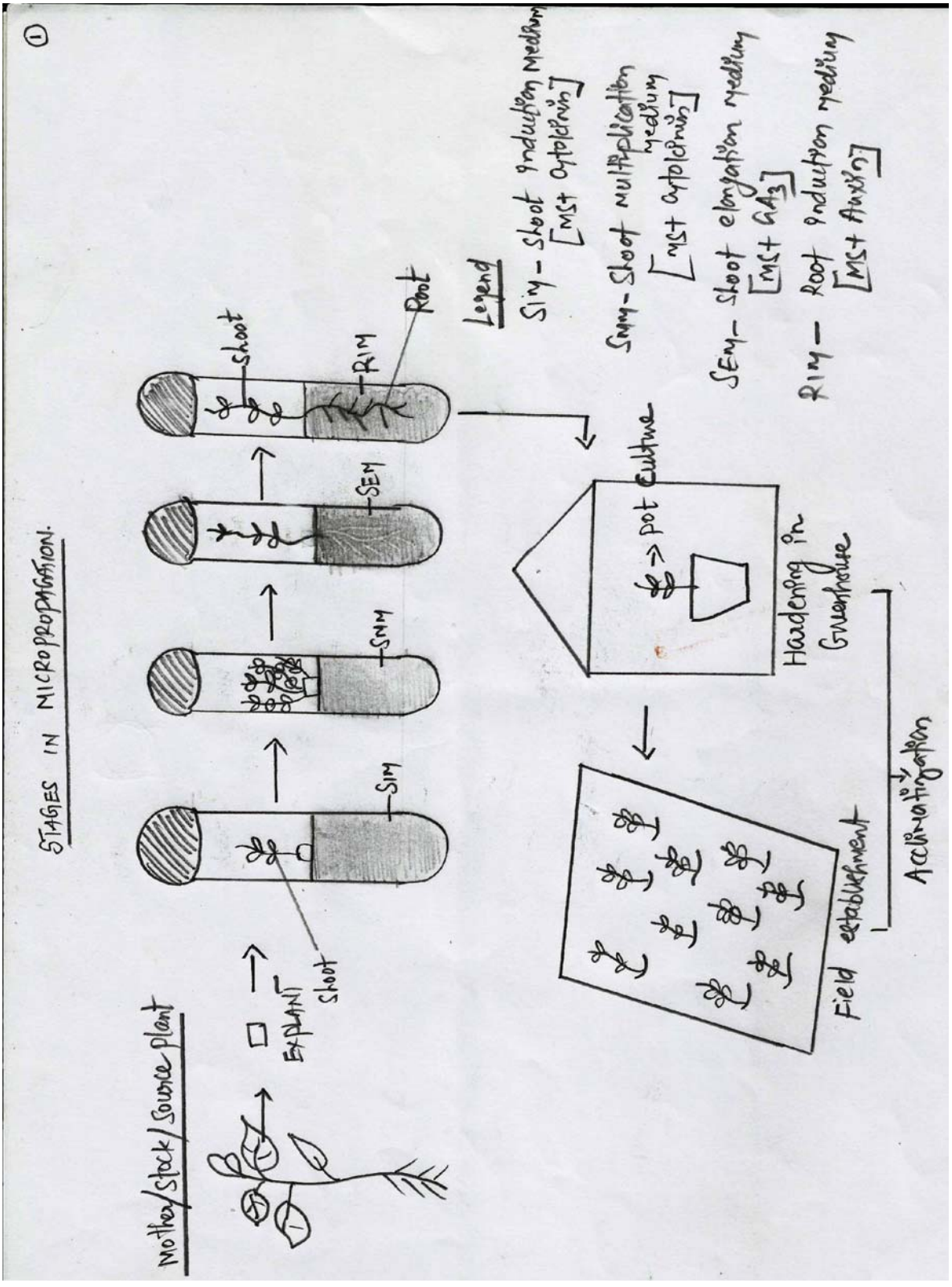
Stage III

In this stage, shoots are separated manually from clusters and transferred on a rooting medium containing an auxin. Elongation of shoots prior to rooting, rooting of shoots (individual or clumps), and prehardening cultures to improve survival are some of the activities carried under this stage. Sometimes, shoots are directly established in soil as micro-cuttings to develop roots.

Stage IV

Hardening

This stage involves the establishment of plantlets in soil. This is done by transferring the pantlets in stage III from the laboratory to the environment of greenhouse. For some plant species, stage III is skipped and unrooted stage II shoots are planted in soil or in a suitable compost mixture.


①
STAGES IN MICROPROPAGATION.
Mother/Stock/Source plant
EXPLANT
shoot
SIM
SMM
SEM
RIM
shoot
Root
Legend
SIM - shoot induction Medium [MS+ cytokinin]
SMM - Shoot multiplication medium [MS+ cytokinin]
SEM - Shoot elongation medium [MS+ GA3]
RIM - Root induction medium [MS+ Auxin]
Pot Culture
Hardening in Greenhouse
Field establishment
Acclimatization

Advantages of Micropropagation

- High multiplication rates (10raise to 6 plants /year from a single explant).
- Very small size explants can be used for micropropagation. This is impossible with conventional technique. Important when limited explant is available.
- Material multiplied by micropropagation can be maintained in small place, packing and transport is also easy due to small size.
- Micropropagation is the only viable method of multiplying genetically modified cells or cells after protoplast fusion.
- In case of dioecious species, where one of the sex is more desirable then under such circumstances plants of desired sex can be selectively multiplied by this technique.
- The output is clean, healthy and pathogen free, as during micropropagation, fungi and bacteria are usually eliminated.
- Easy export, no quarantine problem, as plants obtained is pathogen / virus / disease free.
- Independent of the season; can be carried out through out the year.

Technical problems in Micropropagation

1) Microbial contamination

Bacterial and fungal contaminations in culture do not allow propagules to grow and contaminated cultures have to be usually discarded. Such a problem can be overcome by growing the donar plant in growth chamber, by effective sterilization of explants, by performing inoculation in the laminar air flow cabinets and by using sterlised surgical instrument. Fumigation of inoculation with dilute formaldehyde solution helps to minimise this problem.

2) Callusing

Callus formation is highly undersirable as it often effects the normal development of shoots and roots and may lead to variability among the regenerated plants. Additon of tri-iodo-benzoic acid, flurogauicinol and flurorizin into the culture medium (or) reduction of inorganic salt concentration helps in overcoming this problem

3) Tissue culture induced variation

The Micro propagation plants exhibit genetic (or) epigenetic variations which may be a major problem in getting true to type plants. It can be controlled by careful selection of initial explant, tha t is selecting meristems and controlling the cultural environment favouring slow multiplication rates

4) Browning of medium

In many species, phenolic substances leach into the medium from the cut surfaces of explant. These phenolics turn brown on oxidation and lead to browning (or) blackening of medium and or explants. These oxidation products are detrimental to the cultures as they cause necrosis and eventually death of the cultures. This problem is very common incase of woody species particularly when explants are taken from mature trees. This problem can be overcome by one of the following ways.

- Frequent subculturing of explant (about every 15 days)
- A brief period of culture in liquid medium (about 3 to 7 days), it is helpful in removing phenolics and other inhibitory substances.
- Use of antioxidants (like ascorbic acid or citric acid (150 mg / lit)) may check oxidation of polyphenols.
- Adsorbents like activated charcoal (0.5- 2g/lit) (or) PVP (poly vinyl pyrrolidon) may be used to adsorb the poly phenols secreted in to the medium
- Culture incubation in dark may be helpful since light enhances polyphenol oxidation as well as polyphenol bio -synthesis.

5) Vitrification

Some shoots developed in vitro appear brittle glassy and water soaked. This is called vitrification (or) hyper hydration. The plants appear abnormal because of abnormal leaf morphology. Poor photosynthetic efficiency malfunctioning of stomata reduced epicuticular waxes. It can be reduced by reducing the relative humidity in culture vessels. Reducing the cytokinins level (or) NH4 levels (or) salt concentration in the medium, addition of flurorizin, flurorogluc inol (or) Cacl2 in medium etc

6) Vulnerability of micro propagation plants to transplantation shocks

High mortality rates upon transferring the tissue cult ure derived plants to soil continuously to be a major bottle neck in micro propagation of many plants species. Conservation of moisture by creating high humidity around the plant, partial defoliation, application of antitransperants have met with good suc cess.

Limitations of Micropropagation

- Requirement of sophisticated facilities
- High production cost
- Requirement of skill in handling and maintenance.
- Somaclonal variations may arise during in vitro culture when a callus phase is involved.
- For many valuable species suitable micropropagation techniques are not available (e.g. mango).
- Vitrification can be a problem in some species.

Application of micropropagation

1. **Commercial production of secondary metabolites:** The compounds/ biochemicals which are not directly involved in primary metabolic processes like respiration, photosynthesis etc are secondary metabolites. These include a variety of compounds like alkaloids, terpenoids, phenyl propanoids etc with various biological activities like antimicrobial, antibiotic, insecticidal, valuable pharmacological and pharmaceutical activities. Therefore, micropropagation allows their commercial scale production from cell cultures viz. shikonin derivatives used in dyes, pharmaceuticals are produced from cell cultures of Lithospermum erythrorhizon. Also, cultured cells of many plant species produce novel biochemicals which have otherwise not been detected in whole plants.

2. **Production of synthetic seeds:** Synthetic seed is a bead of gel containing somatic embryo or shoot bud with growth regulator, nutrients, fungicides, pesticides etc needed for development of complete plantlet. These are better propagules as donot need hardening and can be sown directly in field.

3. **Raising somaclonal variants:** The genetic variability occurring in somatic cells, plants produced in vitro by tissue culture are referred to as somaclonal. When these

variations involve traits of economic importance, these are raised and maintained by micropropagation.

4. **Production of disease free plants:** Most of the horticultural fruit and ornamental crops are infected by fungal, viral, bacterial diseases. Micropropagation provides a rapid method for production of pathogen free plants. In case of viral diseases especially, the apical meristems of infected plants are free or carry very low concentrations of viruses. Thus culturing meristem tips provides disease free plants.

Chapter 11. Micropropagation of pre-existing meristem

(Meristem tip Culture for virus elimination)

Meristem:

- A localized group of actively dividing cells, from which permanent tissue system, i.e., root, shoot, leaf, and flower, are derived.

Meristem culture:

- In vitro culture of a generally shiny, dome-like structure measuring less than 0.1 mm in length when excised, most often excised from the shoot apex.

Meristem tip culture

Only the meristematic dome and 1 pair of subtending leaves should be excised. If larger pieces are taken, it is likely that the virus will be transmitted. The size of a meristem plus the subtending leaves ranges from 0.1-0.5 mm. The apical dome itself measures from 0.1-0.25 mm depending on the species.

Meristem tip culture is used successfully to remove viruses, bacteria, and fungi from plants. In a majority of cases heat therapy is combined with meristem tip culture in order to produce the greatest number of plants that are "virus free".

The term meristem, shoot tip, meristem tip are often interchanged. The term shoot tip to refer to an apical tip ranging from 1-3 cm. The meristem is strictly the meristematic dome without any primordial leaves. The term meristem tip will be used to denote the meristem together with 1-2 primordial leaves and measuring between 0.1 and 0.5 cm in height. After successful heat therapy and meristem tip culture one can only say that a plant is free from the actual viruses for which one tested.

Thermotherapy /Heat treatment: Heat treatment is used in those plants in which viruses cannot be eradicated just by meristem tip culture alone. In plant tissue cultures viruses can also be eliminated with incidence of higher temperatures (heat treatment.).In such case explants are exposed to the incidence of higher temperatures, which are not lethal for plant cells, but they are lethal for viruses. Mostly used temperature range is 50-52oC with exposition about 10-30 minutes. In

case this method is applied on whole plants, lower temperatures have to be used (32-40oC) with exposition about 4 - 30 days (depends on plant species and virus type).

Chemotherapy : Virus-free plants can be also obtained when antivirus matters are added into nutrient solution (Ribavirin or 2-thiouracil). Mostly combination of thermo-therapy and meristem culture is used for virus-free plants production (e.g., cassava, bananas, citruses, strawberry, Irish potato, apples, chrysanthemas, garlic). Heat therapy combined with meristem tip culture is able to eradicate viruses, bacteria, and fungi but does not remove viroids. Unlike viruses, viroids are RNA without a protein coat – thus they are known as 'naked' RNA and are very difficult to eradicate. Usually the infected plant must be destroyed.

Principle of virus elimination

Several hypotheses exist to explain why heat therapy and meristem tip culture when used together are effective in eradicating viruses.

1. Virus distribution is uneven in a plant and is much less in a meristem.
2. Viruses cannot travel quickly enough through plasmodesmata to keep up with actively growing tip.

Plants in which virus eradication is commonly used

Garlic, pineapple, cymbidium orchid, carnation, hyacinth, sweet potato, lily, apple, banana, narcissus, gooseberry, raspberry, potato, grape, dahlia, strawberry, cassava, perlagonium (geranium) sugarcane. Ginger.

Procedure of Meristem culture

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water. This sterile explant can be cultured in ms basal medium supplemeted with cytokinin for-shoots initiation.

2. Multiplication of shoot initials: Rapid multiplication of the induced shoots can be attained in a regeneration medium comprises of MS medium containing MS + BAP or kinetin.

3. Elongation of shoots: The individual shoots can be elongated on a MS Medium containing GA3.

4. Rooting initiation: The elongated multiple shoots were split into individual shoots and separated shoots were rooted on rooting medium containing MS medium supplemented with IBA or IAA.

5. Acclimatization (Hardening and field establishment):

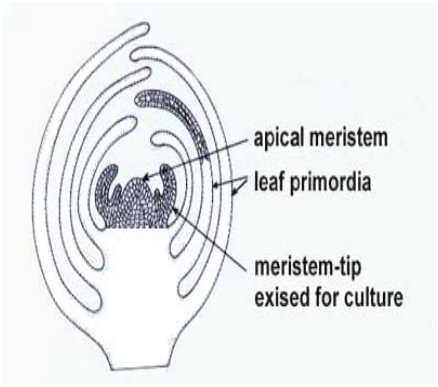
The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.

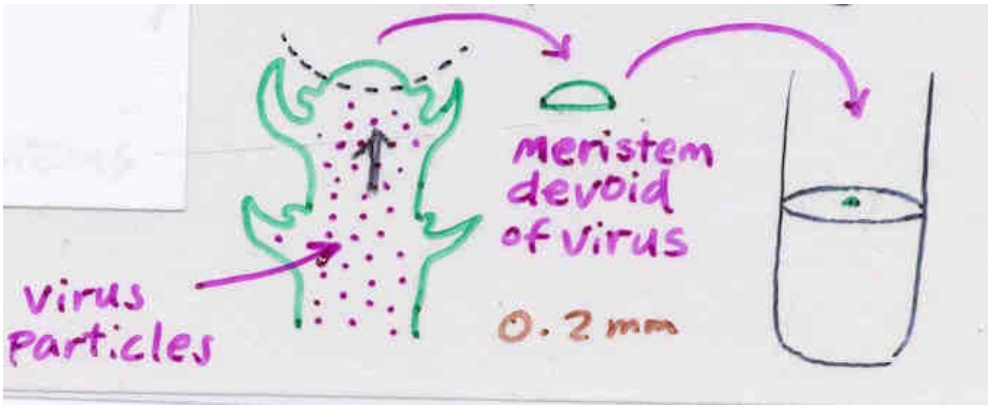
Applications and limitations

1. Virus elimination generally improves the yield by 20-90% over infected controls.
2. Virus-free plants serve as excellent experimental materials for evaluating the detrimental effects of infections by various viruses.
3. The virus free bulbs grew more rapidly, plants were more vigorous, and they produced a greater number of larger flowers that had richer colour than the virus infected stock.
4. The virus -free plants are deliberately infected by known viruses, and effects of the infection on performance of the host are assayed.
5. Meristem culture can also help eliminate other pathogens, e.g., mycoplasmas, bacteria and fungi. Bacteria and fungi present in explants show up when they are cultured in vitro since tissue culture media provide excellent nutrition for the microbes.

6. Meristem culture has been used to eliminate systemic bacteria form Diffenbachia and Pelargonium, and Fusareum roseus from carnations.

Structure of meristem





Chapter 12:
Methods of Micropropagation in plants
or
Methods of in vitro cultures in plants
or
Regeneration pathways of plants in plant cell tissue and organ culture
or
Regeneration pathways of plants

The plants can be regenerated by a) organogenesis and b) somatic embryogenesis.

A. Organogenesis refers to the formation of organs from the cultured explants. The shoot buds or monopolar structures are formed by manipulating the ratio of cytokinin to auxin in the cultures.

They are categorized into direct and indirect organogenesis

Direct

- Organ formed directly from a cell or a group of cells
- Common explants are reproductive tissues (nucellus, styles or pollen)

Indirect

- Explants produce calli
- Organ formed from the callus

B. Somatic embryogenesis (SE)

In Somatic embryogenesis, the totipotent cells may undergo embryogenic pathway to form somatic embryos which are grown to regenerate into complete plants. It was demonstrated for the first time in carrots (***Daucus carota***), where bipolar embryos developed from single cells. The somatic embryogenesis is influenced by plant extracts, growth regulators, and by the physiological state of calli.

They are categorized into direct and indirect Somatic embryogenesis

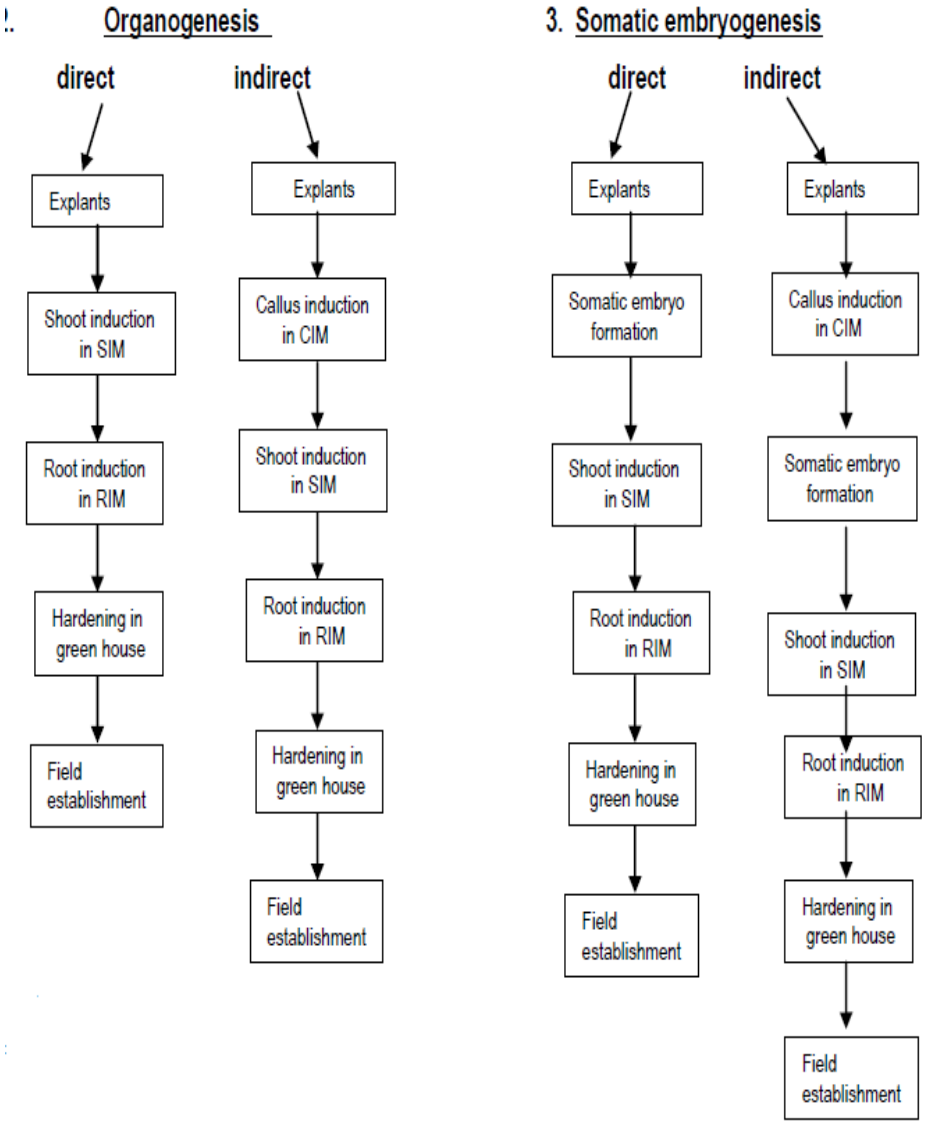
Direct SE

- Embryo formed directly from a cell or a group of cells (pre-embryogenic determined cells " (PEDC)
- Common explants are reproductive tissues (nucellus, styles or pollen)

Indirect SE

- Callus produced from explants
- Embryos produced from callus (induced embryogenic determined cells " (IEDC)

Stages in Organogenesis and somatic embryogenesis



A. Organogenesis pathway

1. Direct organogenesis: Organ either shoot/root formed directly from explants

Procedure of Direct organogeneis

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation for shoot initiations.

2. Shoot initiation: The sterilized explants were inoculated in the shoot induction medium (SIM) which comprises of MS basal medium supplemented with different concentrations of cytokinin for shoot initiations.

3. Rooting initiation: The shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with different concentrations of auxins like NAA or IBA or IAA for root initiations.

4. Acclimatization (Hardening and field establishment):

The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.

2. Indirect organogenesis pathways: Organs either shoot/root formed the callus

Procedure of indirect organogeneis

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation in medium for callus initiations.

2. Callus initiation: The sterilized explants were inoculated in the Callus induction medium (CIM) which comprises of MS basal medium supplemented with different concentrations of any auxins for callus formation.

3. Shoot initiation: The calli were transferred to shoot induction medium (SIM) which comprises of MS basal medium supplemented with different concentrations of cytokinin for the formation of shoots.

4. Rooting initiation: The shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with different concentrations of auxins like NAA or IBA or IAA for root formations

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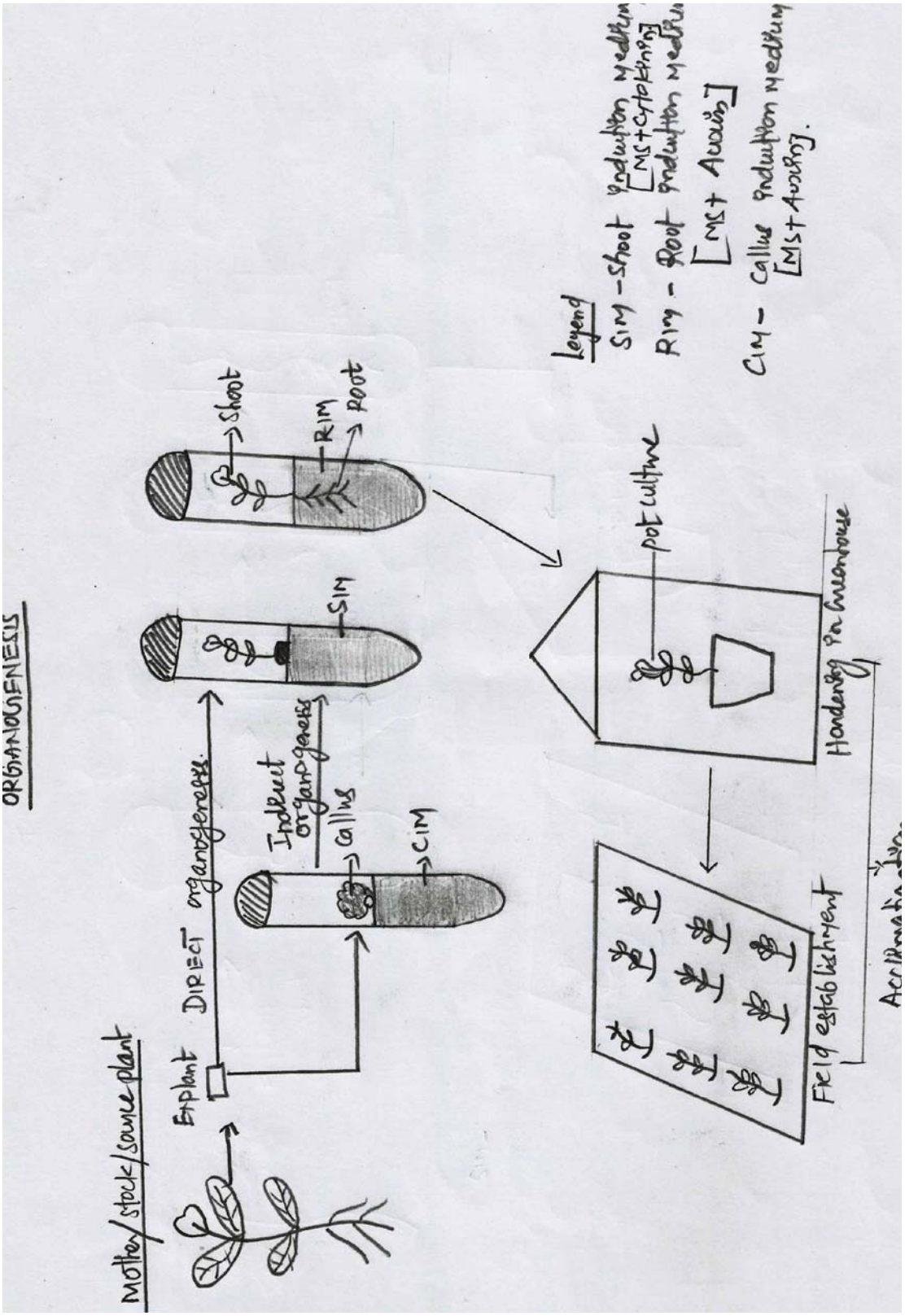

ORGANOGENESIS
Mother/stock/source plant
Explant
Direct organogenesis
Indirect organogenesis
Callus
SIM
CIM
shoot
RIM
Root
pot culture
Hardening in greenhouse
Field establishment
Legend
SIM - shoot production medium [MS+Cytokinin]
RIM - Root production medium [MS+Auxin]
CIM - Callus production medium [MS+Auxin].

B. Somatic embryogenesis (SE)

1. Direct SE: Somatic embryos formed directly from explants

Procedure for Direct SE

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation in medium for somatic embryo formation.

2. Somatic embryoid initiation: The sterilized explants were inoculated in embryo induction medium (EIM) which comprises of MS basal medium supplemented with different concentrations of auxins for somatic embryo formation.

3. Shoot initiation from embryos: The cotyledonary staged somatic embryos were cultured onto shoot induction medium (SIM) which comprises of MS basal medium supplemented with different concentrations of cytokinins for shoot conversion.

4. Rooting initiation: The somatic shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with different concentrations of auxins like NAA or IBA or IAA for root initiations.

5. Acclimatization (Hardening and field establishment):

The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.

2. Indirect (SE): Somatic embryos formed from the callus

Procedure of Indirect (SE):

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation in medium for callus initiations.

2. Callus initiation: The sterilized explants were inoculated in the Callus induction medium (CIM) which comprises of MS basal medium supplemented with different concentrations of any auxins for callus formation.

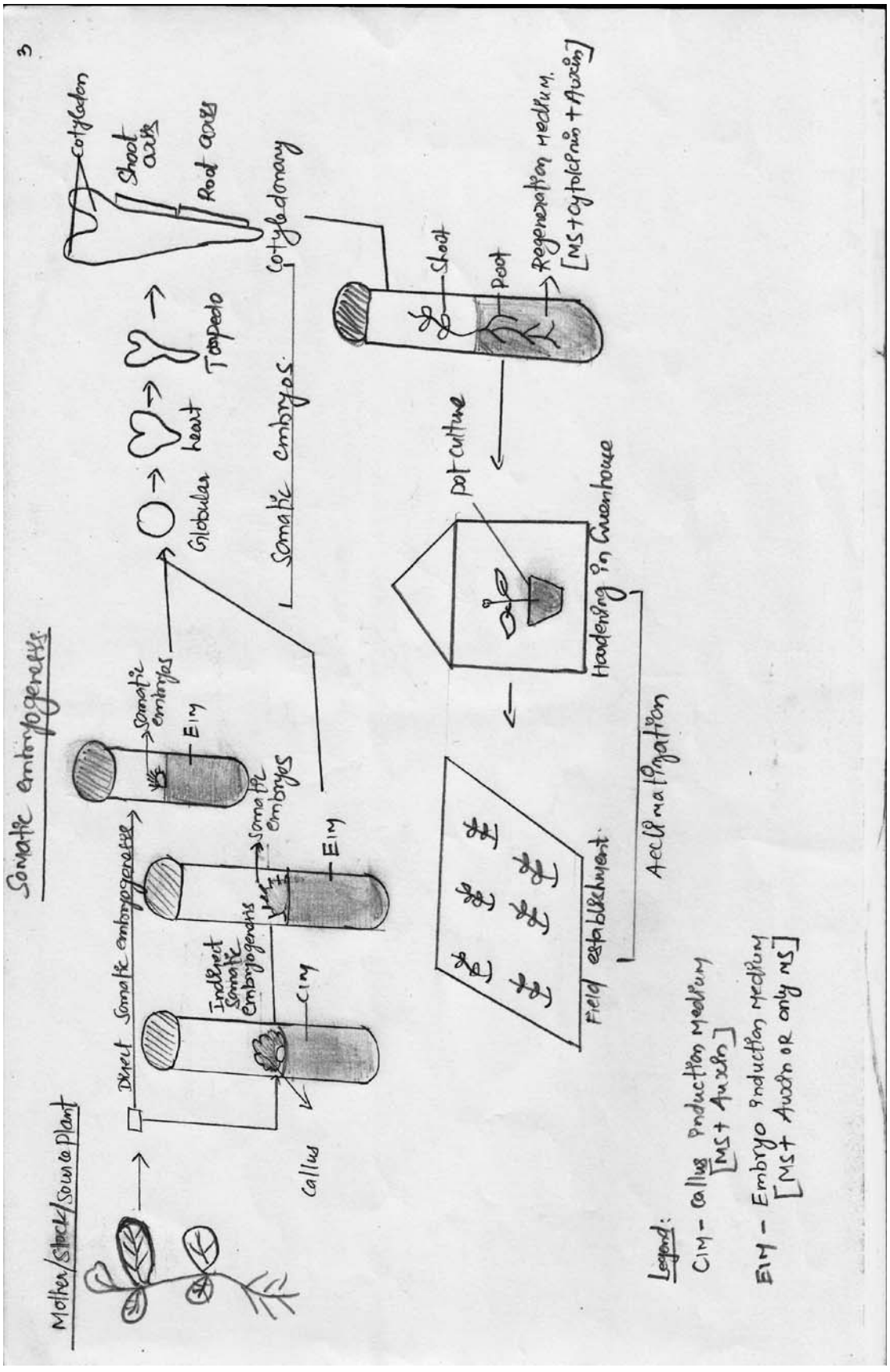
2. Somatic embryoid initiation: The embryogenic calli were inoculated in embryo induction medium (EIM) which comprises of MS basal medium supplemented with different concentrations of auxins for somatic embryo formation.

3. Shoot initiation from embryos: The cotyledonary staged somatic embryos were cultured onto shoot induction medium (SIM) which comprises of MS basal medium supplemented with different concentrations of cytokinins for shoot conversion.

4. Rooting initiation: The somatic shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with different concentrations of auxins like NAA or IBA or IAA for root initiations.

5. Acclimatization (Hardening and field establishment):

The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.


3
Somatic embryogenesis
Mother/Stock/Source plant
Direct Somatic embryogenesis
Indirect Somatic Embryogenesis
CIM
Callus
Somatic embryos
EIM
Somatic embryos
EIM
Globular
Heart
Torpedo
Somatic embryos
Cotyledonary
Cotyledon
Shoot axis
Root axis
Shoot
Root
Regeneration medium
[MS + cytokinin + Auxin]
pot culture
Hardening in Greenhouse
Field establishment
Acclimatization
Legend:
CIM - Calli Induction Medium
[MS + Auxin]
EIM - Embryo Induction Medium
[MS + Auxin or only MS]

Chapter 13: Somaclonal Variation and Crop Improvement

Larkin and Scowcroft (1981) proposed the term somaclone to describe the plants originating from any type of tissue culture. Genetic variation (Genotypic and Phenotypic Variability) found to occur between somaclones in plant tissue cultures was then called somaclonal variation.This variation includes aneuploids, sterile plants and morphological variants, sometimes involving traits of economic importance in case of crop plants. The usefulness of variation was first demonsrated through the recovery of disease resistant plants in potatao (resistance against late blight and early blight) and sugarcane (resistance against eye-spot disease, Fiji disease and downy mildew)

Types of somaclonal variation

Somaclonal variation can be divided into the following types

A. Based on the tissue from which variation originate

1) **Gametoclonal variation :** variation observed among the plants regenerated from gametic cultures

a) **Androclonal variation** observed among the plants regenerated from anther (or) pollen culture

b) **Gynoclonal variation:-** from ovule (or) ovary culture

2) **Protoclonal variation:-** variation observed among the plants regenerated from protoplast cultures

3) **Calliclonal variation:-** variation observed among the plants regenerated from callus cultures

B. Genetic variation - mutations or other changes in the DNA of the tissue those are heritable .This is only transmitted to the next generation and is thus important for crop improvement. Therfore it is necessary to study the transmission of variation to sexual progeny to facilitate the estimation of its utility for improvement of a sexually propagated crop. In several crops R0,R1 and R2 progeneies were analysed for genetic

analyses and 3:1 segregation leading to the isolation of true breeding variants was observed.

C. Epigenetic variation- non-heritable phenotypic variation. Epigenetic changes can be temporary and are ultimately reversible. However, they may also persist through the life of the regenerated plant.

D. Physiological variation- emporary in response to stimulus and disappear when it is removed.

Causes for variation/ Origin of Somaclonal variation

1. Changes of mother plant origin

Chimeral rearrangement of tissue layers Many horticultural plants are periclinal chimeras, that is, the genetic composition of each concentric cell layer (LI, LII, LIII) in the tunica of the meristematic tissues is different. These layers can be rearranged during rapid cellular proliferation. Therefore, regenerated plants may contain a different chimeral composition or may no longer be chimera at all. Cell variation also occurs if callus is initiated from explants containing differentiated and matured tissue s that have specialized function.

2. Explant derived variation

The most stable cultures are obtained from meristematic tissue of a mature plant or tissues of a very young organ of meristematic nature.Polyploid cells can give more variability than diploids

3. Genetic changes arising in culture

A. Changes of mother plant origin

Chimeral rearrangement of tissue layers many horticultural plants are periclinal chimeras, that is, the genetic composition of each concentric cell layer (LI, LII, LIII) in the tunica of the meristematic tissues is different. These layers can be rearranged during rapid cellular proliferation. Therefore, regenerated plants may contain a different chimeral composition or may no longer be chimera at all. Cell variation also occurs if callus is initiated from explants containing differentiated and matured tissue s that have specialized function.

B. Explant derived variation

The most stable cultures are obtained from meristematic tissue of a mature plant or tissues of a very young organ of meristematic nature.Polyploid cells can give more variability than diploids.

Ploidy Changes Three phenomena that occur during mitosis lead to most changes in ploidy: endomitosis (sister chromatids separate within the nuclear membrane, but there is no spindle formation nor cytoplasmic division) endoreduplication (chromosomes at interphase undergo extra duplications)

spindle fusion (giving binucleate or multinucleate cells).

Gross structural rearrangements appear to be a major cause of somaclonal variation. These involve large segments of chromosomes and so may affect several genes at a time.

Deletions (genes missing, for example 1,2,3,4 now 1,2,4)

Inversions (gene order altered, for example 1,2,3,4 now 1,3,2,4)

Duplications (1,2,3,4 now 1,2,2,3,4)

Translocations (whole chromosomal segments moved to a new location, for example 1,2,3,4 now 1,2,3,4,A,B,C).

Transposable elements are segments of DNA that are mobile and can insert into coding regions of genes, typically resulting in a lack of expression of the gene. The culture environment may make the transposable elements more likely to excise and move.

Point mutations (the change of a single DNA base), if they take place within a coding region of a gene and result in the alteration of an amino acid, can lead to somaclonal variation. Point mutations are often spontaneous and are more difficult to detect. Note that they result in single gene changes

Structural changes in the DNA sequence

Chromosomal rearrangements, point mutations, or transposition of transposable elements can occur during culture. These changes can occur spontaneously or can be induced with chemicals or radiation

4. DNA methylation: Most of the mutational events occasioned by tissue culture are directly or indirectly related to alterations in the state of DNA methylation. A decrease in methylation correlates with increased gene activity

5. Lack of nucleic acid precursors: Shortage of the precursor necessary for rapid nucleic acid biosynthesis, which occurs in many tissue cultures

6. Growth regulators: One of the triggers of polyploidy *in vitro* is growth regulators; both kinetin and 2,4-D have been implicated.

7. Composition of culture medium: The level of KNO3 influence the albino plants from wheat cultures. Level of organic N2, chelating agents and other micro nutrients are other factors.

8. Culture conditions: Temperature, Method of culture

9. Effect of the genotype

Effects of the culture process itself (lengthy culture periods, growth and other aspects of the culture medium may also affect the ploidy of the cultured cells. Medium that places cells under nutrient limitation will favor the development of "abnormal" cells. Chromosomal alterations, like ploidy changes, increase with increased lengths of culture. In mixed populations of cells with different ploidys, diploid cells retain their organogenic potential better than polyploid and aneuploid cells (probably due to an enhanced ability to form meristems).

One common alteration seen in plants produced through tissue culture is rejuvenation, especially in woody species. Rejuvenation may lead to changes in morphology, earlier flowering, improved adventitious root formation, and/or increased vigor.

Selection of somaclonal variants on subjecting the cells to selection pressure

Selection Selection of cells in the presence of

Resistance to herbicide - Herbicide

Resistance to salt - Sodium chloride / Aluminium

Resistance to drought - PEG / Mannitol

Resistance to frost - Hydroxy proline resistant lines

Resistance to pathogens - Pathotoxin / Culture filtrate

Crop improvement through somaclonal variation for desirable characters

Crop	Characters modified
Sugarcane	Diseases (eye spot, fiji virus,downy miledew, leaf scald)
Potato	Tuber shape, maturity date, plant morphology, photperiod, leaf colour, vigour, height, skin colour,Resistance to early and late blight
Rice	Plant height, heading date, seed fertility, grain number and weight
Wheat	Plant and ear morphology, awns,, grain weight and yield, gliadin proteins,amylase
Maize	T toxin resistance, male fertility, mt DNA
Medicago sativa	Multifoliate leaves, elongated petioles, growth, branch, no.of plants, dry matter yield.
Tomato	Leaf morphology, branching habit, fruit colour, pedicel, male fertility, growth
Avena sativa	Plant height, heading date,awns
Hordeum spp	Plant height and tillering
Lolium hybrids	Leaf size, flower,vigour, survival

Applications of somaclonal variations

1. Somaclonal variation has been described for a variety of both qualitative and quantitative traits

2. Isolation of regenerate resistant to diseases. Maize lines having Texas male sterile cytoplasm are susceptible to southern leaf blight caused by Helminth osporium maydis which produces a toxin that binds to mitochondria. Maize cells resistant to this toxin have been selected and plants regenerate from them were resistant to leaf blight caused by Helmithosporium maydis.

3. A tomato line resistant to bacterial wilt caused by psuedomonas solanacearum were isolated by screening of plants regenerated from un selected calli

4. A fiji disease resistant sugarcane line were isolated from the variety pindar is released as a new variety called "ono"

5. Variation may arise for useful morphological characters. An improved scented Geranium variety named velvet Rose has been developed from Rober's lemon rose

6. Isolation of variants resistant to abiotic stresses. Plant tissue culture techniques have been successfully used to obtain salt tolerant cell lines (or) variants in several plant species plant cells resistant to 4 to 5 times the normal toxic salt concentration (Nac1) have been isolated. In many cases, the plants regenerated from them were also tolerant to saline condition. Eg:- Tobacco plants regenerated from high salts (0.88%) tolerant cell lines were also salt tolerant.

7. Low temperature is another important environmental factor effecting survival and performance of crop plants. Cell lines resistant to chilling have been isolated in several cases. Eg:- Chillies, Nicotiana sylvestris.

8. Development of varieties with improved seed quality. A variety Ratan of Lathyrus which has low neurotoxin content has been develop through somoclonal variation

9. Isolation of mutants for efficient nutrient utilization. Tomato cell lines which are able to grow normally in phosphate deficient condition due to high secretion of enzyme. Acid phosphatage have been isolated through in vitro selection.

Chapter 14: Cryopreservation and Germplasm Storage

The genetic material especially its molecular and chemical constitution that is inherited and transmitted from one generation to other is referred to as germplasm. In other words, the sum total of all the genes present in a crop and its related species constitutes its germplasm. It is generally represented by a collection of various strains and species. Germplasm is valuable because it contains diversity of genotypes that is needed to develop new and improved genetic stocks, varieties and hybrids. Therefore, germplasm is the basic indispensable ingredient of all breeding programmes and great emphasis is placed on collection, evaluation and conservation of germplasm. The continuing search for high yielding varieties of crop plants with resistance to biotic and abiotic stresses necessitates the conservation of germplasm of different crops and their wild and weedy relatives.

In- situ conservation: In situ (on-site) conservation refers to the maintenance and use of wild plant populations in the habitats where they naturally occur and have evolved without the help of human beings. The wild populations regenerate naturally and are also dispersed naturally by wild animals, winds and in water courses. There exists an intricate relationship, often interdependence, between the different species and other components of the environment (such as their pests and diseases) in which they occur. The evolution is purely driven by environmental pressures and any changes in one component affect the other. Provided that changes are not too drastic, this dynamic co-evolution leads to greater diversity and better adapted germplasm. The conservation of the forests and other wild plant species is often carried out through protected areas such as national parks, gene sanctuary and nature reserves. However, this mode of conservation has certain limitations such as there is risk of loss of material due to environmental hazards.

Ex-situ conservation: Ex situ (off-site) conservation of germplasm takes place outside the natural habitat or outside the production system, in facilities specifically created for this purpose. This is the chief mode of preservation of genetic resources for both

cultivated and wild material. The most convenient method of ex-situ germplasm conservation is in the form of seeds. Thus, majority of field crops and vegetables which produce orthodox (dessication tolerant) seeds are conserved in gene banks by reducing their moisture content (3-7%) and storing under low humidity and low temperature.

In case of crops with dessication sensitive or recalcitrant seeds (which lose their viability after being dried below a critical limit) and also in vegetatively propagated crops, in vitro methods are the most useful for germplasm conservation. This tissue culture based method has been mainly utilized for conservation of somaclonal and gametoclonal variations in cultures, plant material from endangered sp., plants of medicinal value, storage of pollen, storage of meristem culture for production of disease free plants and genetically engineered materials.

In vitro Germplasm conservation: Germplasm can be stored in vitro in variety of forms including isolated protoplasts, cells from suspension or callus cultures, meristem tips, somatic embryos, shoot tips or propagules at various stages of development. Methods for in vitro germplasm conservation are classified into two groups based on culture growth:-

1. Slow growth cultures: where limited growth of culture is allowed. This is a simple, effective and economic method and can be used in all species where shoot tip/ nodal explant are available. In thses techniques, growth is suspended by either cold storage or lowering oxygen concentration. Such methods require serial subculturing for periodic renewal of cultures. The storage of germplasm by repeated cultures has some disadvantages like during subculturing there is risk of contamination by pathogen, genetic changes may also occur.

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2. Cryopreservation: Any growth in plant cell and tissue culture is brought to a halt still retaining its viability in this technique by storing at ultra low temperature (-196°C) using liquid nitrogen. This method, also called freezepreservation, is most popular and effective for indefinite storage. Cryopreservation for germplasm

purposes utilizes shoot tips and buds only but protoplasts, cells, tissues and somatic embryos are also cryopreserved for other tissue culture processes

Cryo preservation-Steps

1. Rising sterile tissue cultures and cell suspensions.
2. Addition of cryoprotective agent.
3. Subjecting cell cultures to super low temperature by regulated slow rate of cooling or after pre freezing.
4. Storage of frozen cells in liquid nitrogen.
5. Thawing or rapid re warming of cells.
6. Removal of cryoprotectant by repeated washing.
7. Determination of viability.
8. Re culture of the retrieved cells.
9. Induction of growth and regeneration of plants.

Cryo protectants

Most of the experimental systems (meristem, shoot tips, cultured cells etc) contain high amount of cellular water and hence are extremely sensitive to freezing, injury protection from freezing and thawing injury has to be imposed artificially. A number of compounds such as glycerol, DMSO, ethylene glycol, polyethylene glycol, sugars and sugar alcohols either alone or in combination protect cells against damage during freezing and thawing. There are two types of cryo protectants.

Permeating: DMSO and Glycerol. DMSO permeates into cells more rapidly than glycerol and requires shorter treatment duration. Glycerol is less toxic to cells than DMSO while DMSO is superior in cryoprotection of plant cells and organs. **Non-permeating:** Sugars, sugar alcohols and high molecular additnes, polyvinyl pyrrolidone, PEG, dextran hyroxy ethyl starch etc.

Freezing Methods

1. Slow freezing: This is the most common method of freezing meristems, somatic embryos and cell cultures which is by regulated slow cooling at a rate of 0.5-1.0° C/min to either -30, -35 (or) -40°C followed by storage in liquid nitrogen.

2. Rapid Freezing: Meristems and somatic embryos for few plant species have been successfully cryo preserved by rapid freezing. Here, the temperature is rapidly lowered.

3. Droplet freezing: Mostly suitable for meristems E.g. Cassava. Here, the cryo protectant solution, (15% DMSO and 3% sucrose) is dispensed into droplets of 2-3µl in an aluminum foil contained in a petri dish. The advantage of droplet freezing on aluminum foil is homogeneous cooling due to efficient thermal conductivity.

4. Vitrification: This is based on the ability of highly concentrated solutions of cryoprotectants to super cool to very low temperature upon imposition of rapid cooling rates to become viscous at sufficiently low temperatures and solidity without the formation of ice. The advantage is being simple and doesn't require regulated cooling. The disadvantage is toxicity owing to high concentration of cryoprotectants.

e.g. Cell cultures of *Brassica*, naval orange, Somatic embryos of *Asparagus* and Daucus, mesophyll protoplasts of Secale and shoot tips of Mint, Potato, Papaya and Carnation.

Cryostorage and Thawing Long term preservation is accomplished at ultra low temperatures such as that of liquid nitrogen (-196°C). Thawing is generally carried out by immersing specimens rapidly for 1-2 minutes in 35-40°C water bath.

Viability assays: Viability can be assayed by fluorescent di acetate staining (FDA), Triphenyl tetrazolium chloride(TTC) reduction assay, and using other parameters like mitotic index, Cell number Cell volume, dry and fresh weight and plating deficiency.

Factors affecting viability of cells frozen for cryopreservation:

- **Physiological state of material**: Cells in the late lag or exponential phase are considered ideal for freeze preservation. After thawing, these cytoplasm rich cells are able to retain their viability and grow again from the actively dividing meristematic cell component. But in shoot tips, embryos etc, tissue

is large with highly vacuolated cells which get damaged by freezing and are unable to recover back.

- **Prefreezing treatment:** Conditioning treatment given to cells before freezing results in their hardening and increased survival rates. Such hardening treatments include growing culture in presence of cryoprotectant or growing at low temperature (4°C) (for cold dormant sp) or in presence of osmotic agents like sucrose. These treatments function by either changing the cell water content, metabolite content or membrane permeabilites.
- **Cryoprotectants:** are chemicals imparting protection to withstand low temperature. For plants, most frequently used cryoprotectant is Dimethyl sulphoxide (DMSO). About 5-10% of DMSO is prepared and added gradually to prevent plasmolysis of the cells. Other commonly used cryoprotectants include glycerol, polyvinyl pyrollidone, polyethylene glycol (PEG) etc.
- **Thawing rate and reculture:** For better survival of preserved samples, rapid thawing from -196°C to about 22°C is recommended. By thawing rapidly, the damaging effects of ice crystal formation (crystallization of cell water while freezing) are minimized. These thawed samples during reculturing require special growth conditions, for enhanced recovery rates like dim light, high osmoticum, gibbrellic acid, and activated charcoal in the medium.

Applications

1. In vegetatively propagated crops, to avoid high level of heterozygosity, they are clonally propagated through tubers or cuttings which have a limited life span, labor intensive and expensive and risks are associated with field maintenance. In seed crops problems on non-viability recalcitrance to storage, deterioration and heterozygosity exists. To circumvent the problems, Cryo preservation technology can be used.
2. Preservation of rare genomes.
3. Freeze storage of cell cultures.

4. Conservation of genetic uniformity.

5. Maintenance of Disease Free Material which is ideal for the international exchange.

6. Cold Acclimation and Frost Resistance.

7. Retention of Morphogenetic Potential.

8. Slow metabolism, which would prevent or virtually 'stop' the ageing process.

Chapter 15: Production of Secondary Metabolites

Plants are the source of a large variety of biochemicals which are produced as both primary and secondary metabolites. Primary metabolites include nucleic acids, proteins, carbohydrates and fats which along with their intermediates function for survival of cell and organism.. Compounds like alkaloids, non-protein aminoacids, terpenoids and phenolics are grouped under secondary metabolites which do not participate in vital metabolic function of cell. Primary metabolites essentially provide the basis for growth and reproduction, while secondary metabolites for adaptation and interaction with the environment. As secondary metabolites provide industrially important natural products like colour, insecticides, antimicrobials and fragrances, therapeutics etc, they are of great economic importance. Therefore, plant tissue culture is being potentially used as an alternative to plants for production of secondary metabolites. The first large scale production was successfully done for shikonin produced from *Lithospermum erythrorhizon*. It is used as antiseptic and as dye for cosmetics. Since then many valuable secondary metabolites like taxol, berberine etc. have been obtained using tissue culture.

Increasing productivity of secondary metabolites by cell cultures: In plants, most of the secondary metabolites are produced in differentiated cells or organized tissues. However, callus and cell suspension culture lack organ differentiation and hence produce low yields of these biochemicals. The yield of secondary metabolite by undifferentiated tissue or cell cultures can be increased by following techniques:-

- **Select proper cell line**: The heterogeneity within the cell population can be screened to select lines capable of accumulating higher level of metabolite.
- **Medium manipulation:** The constituents of culture medium like nutrients, phytohormones also the culture condition like temperature, light etc influence the production of metabolites.For e.g. if sucrose concentration is increased from 3% to 5%, the production of rosamarinic acid is increased by five times. In case of shikonin production, IAA enhances the yield whereas 2,4-D and NAA are inhibitory.

• **Elicitors:** Compounds that induce the production and accumulation of secondary metabolite in plants are known as elicitors. Elicitors produced within the plant cells include cell wall derived polysaccharides like pectin, pectic acid, cellulose etc. Product accumulation also occurs under stress caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, high salt concentration grouped under abiotic elicitors. These elicitors when added to medium in low concentration (50-250ng/l) enhance metabolite production.

• **Permeabilisation:** Secondary metabolites produced in cell are blocked in the vacuole. By manipulating the permeability of cell membrane, they can be elicited out to media. Permeabilisation can be achieved by electric pulse, UV, pressure, sonication, heat. Even charcoal is added to medium to absorb secondary metabolites.

• **Immobilisation:** Cell cultures encapsulated in agarose and Calcium alginate gels or entrapped in membranes are called immobilised plant cell culture. Here cell to cell contact is better while cells are also protected from high shear stresses. These immobilized systems effectively increase the productivity of secondary metabolites in number of species. Elicitors can also be added to these systems to stimulate secondary metabolism.

Limitations

- ❖ High production cost is involved.
- ❖ Lack of knowledge of biosynthetic pathways of many compounds is major bottleneck in improvement of their production.
- ❖ Cultured plant cells are often unable to produce high value compounds.

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

Product	**Plant source**	**Uses**
Artemisin	*Artemisia spp.*	Antimalarial
Azadirachtin	*Azadirachta indica*	Insecticidal
Berberine	*Coptis japonica*	Antibacterial, anti inflammatory
Capsaicin	*Capsicum annum*	Cures Rheumatic pain
Codeine	*Papaver spp.*	Analgesic
Camptothecin	*Campatotheca accuminata*	Anticancer
Cephalotaxine	*Cephalotaxus harringtonia*	Antitumour
Digoxin	*Digitalis lanata*	Cardiac tonic
Pyrethrin	*Chrysanthemum cinerariaefolium*	Insecticide (for grain storage)
Morphine	*Papaver somniferum*	Analgesic, sedative
Quinine	*Cinchona officinalis*	Antimalarial
Taxol	*Taxus spp.*	Anticarcinogenic
Vincristine	*Cathranthus roseus*	Anticarcinogenic
Scopolamine	*Datura stramonium*	Antihypertensive

Chapter 16: Artificial Seed

Definition

Artificial or synthetic or manufactured seed is encapsulated plant propagule (somatic embryo / shoot bud) in a suitable matrix, containing substances like nutrients, growth regulators, herbicides, insecticides, fungicides and mycorrhizae which will allow and help it to grow into a complete plantlet. The concept came into practical in 1970s.

The term artificial seed was coined by Murashige. It is also known by other names like manufactured seeds, synthetic seed. Though Murashige proposed encapsulation of somatic embryos to produce synthetic seed, the concept was further advanced by Redenbaugh et.al. (Plant Genetics Incorporation, California) and Kitoo and Janick (Purdue University). Redenbaugh et al. (1988) patented this artificial seed technology.

General procedure for the production of artificial seeds

- Somatic embryogenesis or callus culture
- Somatic embryo maturation or shoot bud formation
- Encapsulation of above with suitable matrix
- Evaluation of the resulted artificial seeds
- Mass production
- Open field / green house planting

Artificial seed production methods

Synthetic seed production methods can be of two types

Desiccated system

Hydrated system

Desiccated system for artificial seed production

Prior to encapsulation, somatic embryos are first hardened to withstand desiccation. Then these hardened embryos are encapsulated artificially with the use of appropriate growth medium.

Hydrated system for artificial seed production

The gel used to enclose somatic embryo remains hydrated. Various water soluble gels can be used for the purpose of encapsulation, like alginate, gel rite, locust bean gum, sodium alginate with gelatin. However, alginate is the most suitable gel.

Methods for artificial seed encapsulation

There are two methods for encapsulation listed as follows.

- **Dropping method**
- **Molding method**

Procedure for dropping method

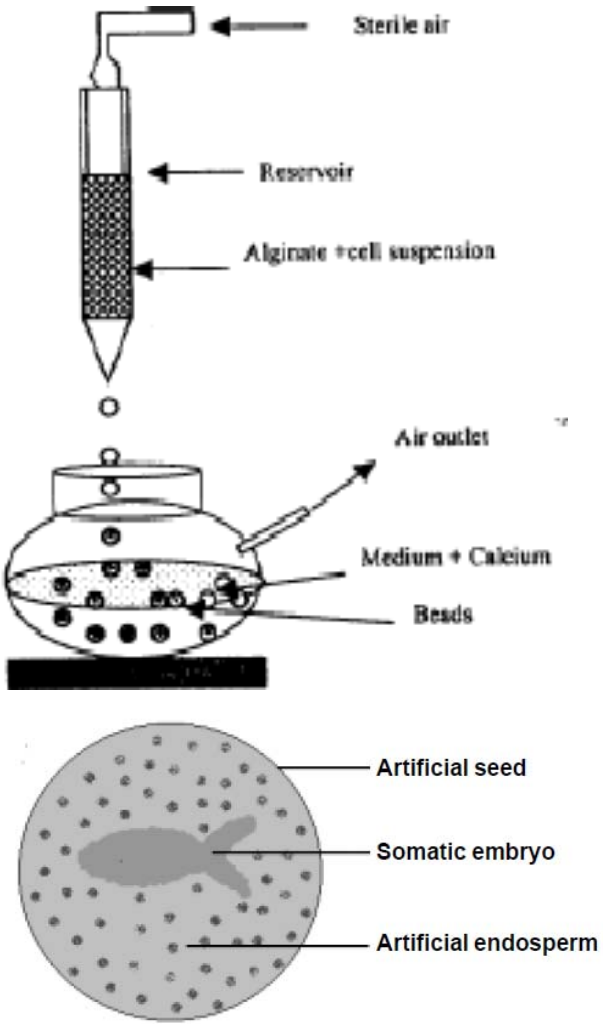
- Somatic embryos are dipped in hydrogel, this step encapsulate SEs.
- Hydrogel used may be any of the following.

  alginate – sodium alginate, agar from see weeds

  seed gums like guar gum, locust bean gum
- Here we have considered sodium alginate solution (1 – 5%), prepared in MS basal medium solution. SEs are dipped in this solution.
- These coated beads are added one by one with the help of pipette (5mm) into a complexation solution flask kept on magnetic stirrer and kept such for around 20-30 minutes. Here we considered calcium nitrate solution (100mM) as complexation solution.
- Embryos get covered by calcium alginate which is a stable complex due to ionic bond formation, become harder. Seeds become harder. Then gelled embryos are washed with water or MS basal medium.
- The synthetic seeds are ready.
- Alternatively, a burette is filled with sodium alginate solution (1 – 5%), dripped into a calcium nitrate solution (100mM) drop by drop. Somatic embryo is inserted into the drop formed at the burette tip. Sodium alginate drop along with SE falls into the solution of calcium nitrate.

- Useful adjuvants like growth regulators, herbicides, insecticides, fungicides and mycorrhizae such can be supplied to the SE while encapsulation along with the matrix.
- This method is applicable for embryo / auxiliary / apical / adventitious buds.

Steps in artificial seed productions



Molding method

•This method follows simple procedure of mixing of embryos with temperature dependent gel (e.g. gel rite, agar).

•Cells get coated with the gel at lowering of the temperature.

Applications of Synthetic Seeds:

1. Hybrid plants can be easily propagated using synthetic seed technology

2. Genetically modified plant or crops can be propagated using synthetic seed technology.

3. Endangered species can be propagated using synthetic seed technology.

4. Elite genotype can be preserved and propagated using artificial seed technology.

5. Synthetic seed production is cost effective when compared to traditional method.

6. Synthetic seeds can be directly used in fields

7. Genetic uniformity is maintained by using synthetic seed technology.

8. Synthetic seeds can be transported from one country to another without obligations from quarantine department.

9. Cereals, fruits and medicinal plants can be studied anywhere in the world using synthetic seeds.

10. Synthetic seed transportation is easy as these do not contain any disease causing agents. As synthetic seeds are produced using sterile plant materials produced using plant tissue culture techniques.

11. Synthetic seeds are small therefore they are easy to handle.

12. Synthetic seed encapsulation provides aseptic condition to the plant material or explant, which is present inside the capsule.

13. While producing the synthetic seed encapsulation herbicides can be added to the formulation, this herbicide will provide extra protection to the explants against pests and diseases.

14. Synthetic seed plantation can be done by using the sowing farm machinery.

15. Synthetic seed crops are easy to maintain because of uniform genetic constituent.

16. This technology improves the food production and also produces environment friendly plantation.

Limitations:

1) Limited production of viable micropropagules that are useful in synthetic seed production

2) Asynchrous development of somatic embryos

3) Improper maturation of somatic embryos that makes them inefficient for germination and conversion in to normal plants

4) Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds

5) Somo clonal variations which may alter the genetic constituent of the embryos

Chapter 17: Molecular detection or diagnosis/indexing of disease in crop plants

Molecular detection methods have been developed to determine the integrity of the transgene (introduced gene) into the plant cell.

1. Polymerase chain reaction or

PCR is a quick test to determine if the regenerated transgenic cells or plants contain the gene. It uses a set of primers (DNA fragments) – forward and backward primers, whose nucleotide sequences are based on the sequence of the inserted gene. The primers and single nucleotides are incubated with the single stranded genomic DNA and several cycles of DNA amplification is conducted in a PCR machine. Analysis of the PCR products in agarose gel will show if the plants are really transformed when DNA fragments equivalent in size with the inserted gene is present and amplified.

2. Southern blot analysis

Determines the integrity of the inserted gene: whether the gene is complete and not fragmented, at the correct orientation, and with one copy number. The DNA coding sequence is the probe binding to the single stranded genomic DNA of the transgenic plant which is implanted on a nitrocellulose paper. Autoradiography will reveal the transgenic status of the plant.

3. Northern blot analysis

Determines whether the transcript or the messenger RNA (mRNA) of the introduced DNA is present and is correctly transcribed in the transgenic plant. The messenger RNA of the transgenic plants are isolated and processed to bind to the nitrocellulose membrane. Labeled DNA is used to bind to the mRNA and can be visualized through autoradiography.

4. Western blot analysis or protein immuno blotting or ELISA

It is an analytical technique used to detect whether the transgenic plants produce the specific protein product of the introduced gene. Protein samples are extracted from the transgenic plants, processed into denatured proteins and

transferred to a nitrocellulose membrane. The protein is then probed or detected using the antibodies specific to the target protein.

Others

5. Insitu hybridization

The gene specific probes are radioactively labeled to detect the presence of disease causing pathogen in the plant system through a probe and plant genome hybridization. The detected positive signal shows the presence of disease.

Chapter 18. MS medium composition

Composition of a typical plant culture medium. The medium described here is that of Murashige and Skoog (MS)[a]

Essential element	Concentration in stock solution (mg/l)	Concentration in medium (mg/l)
Macroelements[b]		
NH_4NO_3	33 000	1 650
KNO_3	38 000	1 900
$CaCl_2.2H_2O$	8 800	440
$MgSO_4.7H_2O$	7 400	370
KH_2PO_4	3 400	170
Microelements[c]		
KI	166	0.83
H_3BO_3	1 240	6.2
$MnSO_4.4H_2O$	4 460	22.3
$ZnSO_4.7H_2O$	1 720	8.6
$Na_2MoO_4.2H_2O$	50	0.25
$CuSO_4.5H_2O$	5	0.025
$CoCl_2.6H_2O$	5	0.025
Iron source[c]		
$FeSO_4.7H_2O$	5 560	27.8
$Na_2EDTA.2H_2O$	7 460	37.3
Organic supplement[c]		
Myoinositol	20 000	100
Nicotinic acid	100	0.5
Pyridoxine-HCl	100	0.5
Thiamine-HCl	100	0.5
Glycine	400	2
Carbon source[d]		
Sucrose	Added as solid	30 000

[a] Many other commonly used plant culture media (such as Gamborg's B5 and Schenk and Hildebrandt (SH) medium) are similar in composition to MS medium and can be thought of as 'high-salt' media. MS is an extremely widely used medium and forms the basis for many other media formulations.
[b] 50 ml of stock solution used per litre of medium.
[c] 5 ml of stock solution used per litre of medium.
[d] Added as solid.