An introduction of Plant Tissue Culture

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A Text Book of

Tissue Culture

For
B. Sc., Students

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A Text Book of
Tissue Culture

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Dedicated to
My Parents

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Preface

Though the genetics goes forward advancing to the molecular level the importance of classical genetics remained as it is or became more important than ever before. There are many practical books on the cell and genetics but there are very few which deals with the problems in genetics. This is our humble attempt to put forth the concise book on the practical aspects on cell biology and problems related to genetical phenomenon. We are grateful to our teacher Shri. S. N. Shinde for inculcating the principles of genetics in us at the graduate studies

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Author

Chapter... 1

INTRODUCTION

Introduction

Plant tissue culture is broadly refers to the in vitro cultivation of plants, seeds and various parts of the plants. The cultivation process is invariably carried out in a nutrient culture medium under aseptic condition.

Or

Plant tissue culture is the propagation of plants through "cloning" an asexual method of reproduction. A portion (explant) of a desired plant is cultured *in vitro* on a defined medium, which promotes rapid multiplication of cells. The new plants are removed from the culture and transferred to a standard potting medium.

Tissue culture is based on the theory of Totipotency;

Totipotency

Totipotency is the ability of plant cell to perform all the functions of development, which are characteristics of zygote, i.e., ability to develop into a complete plant. Morgan (1901) coined the term "Totipotency" to denote this capacity of cell to develop into an organism by regeneration. This unique and important property of plant cell known as Totipotency is mainly used in tissue culture techniques.

The potential of a cell to grow and develop a multicellular or multiorganed higher organism is termed as totipotency.

A multicellular explants are made of different types of cells. Some are dividing cells and some are non dividing. But all the cells are derived from a single celled zygote through the process of cell division and cellular differentiation. So the cells of the explants are present in differentiated state. When such explant is brought into callus culture, most of the cells including the non dividing mature cells within the explants start to divide and form mass of differentiated callus tissue. This phenomenon is termed as dedifferentiation and the cells of the callus tissue are termed as dedifferentiated cells. When such dedifferentiated cell mass is placed in a medium which is suitable for plant regeneration, the cells again form the whole plant organ. This phenomenon is known as redifferentiation and inherent capacity of the plant cell to regenerate the whole plant is known as totipotency.

During the redifferentiation, few dedifferentiated cells of particular culture system undergo cytoquiscence and cytosenescence. These changes mainly associated with the process of cytodifferentiation

History

About 250 years ago (1756), **Duharmel du Monceau H. L** discovered callus formation from the decorticated elm tree. This very old

work was foreword for the discovery of plant tissue culture. In 1839 **Schwann, T.H** expressed the view that each living cell of a multicellular organism would be capable of developing independently if provided with proper external conditions. In 1853, **Trecul. A** performed experiment on callus formation by decorticated trees such as *Robinia, Pawlonia* and *Ulmus* and published the pictures of callus formation. In 1901 **Morgan, T.H** coined the term totipotency to describe the capability of a cell to form an individual plant.

German botanis**t Gottlieb Haberlandt** (1902) regarded as the Father of plant tissue culture, published a paper on "Experiments on the culture of isolated plant cells: In that he says "I should like to point out the fact that, in my cultures, despite the conspicuous growth of the cells which frequently occurred, cell division was never observed. It will be the problem of future culture experiments to discover the condition under which isolated cells undergo division". He clearly set forth the purposes and potentialities of cell culture after having attempted and failed in the culture of isolated plant cells. The reasons for his failure may be

(i) Use of three monocotyledonous genera for much of his work,
(ii) Culture of mature differentiated green mesophyll and pallisade tissues,
(iii) Contamination during culture growth.

Gautheret, R. J. (1934) made preliminary attempts with liquid medium for cultivating plant issues but failed completely. Later he cultured the explants on medium solidified with agar, and got healthy calli from the explants **White, P.R.** (1934) obtained indefinite survival of cultured tomato roots on sub culturing in liquid medium.

White, P.R., Gautheret, R.J. and Nobecourt, P (1939) simultaneously announced the possibility of cultivating plant tissues for unlimited periods. **Van Overbeek, J., Conklin**, **M.E. and Blackeslee, A.F** (1941) established importance of coconut milk for growth and development of very young *Datura* embryos. **White, P.R. and Braun, A.C** (1942) initiated studies on crown gall and tumour formation in plants. **Skoog, F** (1944) started his work on organogenesis in tobacco callus. **Guha, S. and Maheshwari, S.C** (1964) cultured mature anthers of *Datura innoxia* to study the physiology of meiosis and accidentally noticed the development of embryoids from the anthers plated on basal medium supplemented with kinetin and coconut milk. **Murashige, T** (1974) developed the concept of developmental stages in cultures *in vitro*:

Stage I: Explant establishment; Stage II: Multiplication of propagule and Stage III: Rooting
and hardening for planting into soil.

Types of culture

Plant tissue culture includes two major methods:
(A) Type of in vitro growth-callus and suspension cultures.

(B) Type of explant— shoot and root cultures, meristem culture, anther culture and haploid production, protoplast culture and somatic hybridization, embryo culture, ovule culture, ovary culture, etc

I. Callus culture

Callus refers to an unorganized mass of cells generally parenchymatous in nature.

The unique feature of callus is that the abnormal growth has biological potential to develop normal root, shoots, and embryoids, ultimately forming a plant. Naturally, the callus is formed due to the infection of microorganisms from wounds due to stimulation by endogenous growth hormones, the auxins and cytokinins. However, it has been possible to artificially develop callus by using tissue culture techniques.

Auxins are added to culture medium for callus induction but the nature and quantity of auxin added, depends on the nature and source of explant and its genotype besides other factors. 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.

II. Organ culture

The culture of different plant organs is referred as organ culture. These organs may be shoots, root, embryo, seed, ovary, or anther.

Types of organ culture

a) Shoot culture
b) Root culture
c) Anther and pollen culture
d) Embryo culture
e) Ovary culture

a) Shoot culture

The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro*, producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation. Shoot meristem cultures are potential alternatives to the more commonly used methods for cereal regeneration (see the Case study below) as they are less genotype-dependent and more efficient (seedlings can be used as donor material).

b) Root culture

Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on fairly simple media. The growth of roots *in vitro* is potentially unlimited, as roots are indeterminate organs. Although the establishment of root cultures was

one of the first achievements of modern plant tissue culture, they are not widely used in plant transformation studies.
According to the Oxford University Press, root tissue cultures were one of the first plant tissues used in developing plant cultures. Plant roots can be grown virtually indefinitely as the cells aren't specifically assigned. While these cells were one of the first steps in the plant culture process, they are not widely used in modern cell transformation studies.

c) Anther 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.

d) Ovary Culture

Ovary culture is also used to produce haploid plants. The unpollinated ovaries used for this purpose. It is possible to trigger female gametophyte of angiosperm to develop into a sporophyte. The plants produced by this culture are called gynogenic haploid.

e) Embryo culture

Embryos can be used as explants to generate callus cultures or somatic embryos. Both immature and mature embryos can be used as explants. Immature, embryo-derived embryogenic callus is the most popular method of monocot plant regeneration.

III. Cell culture

Cell suspension is prepared by transferring a fragment of callus to the liquid medium and agitating them aseptically to make the cells free. Single cells can be isolated from either callus or any other part of the plant and cultured in liquid medium using both mechanical and enzymatic methods. Usually, the medium contains the auxin 2,4-D. Suspension cultures must be constantly agitated at 100-250 rpm (revolutions per minute). Suspension cultures grow much faster than callus culture.

IV. Protoplast culture

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions, although other sources can be used to advantage. Two general approaches to removing the cell wall can be taken—mechanical or enzymatic isolation.

Protoplasts are fragile and easily damaged, and therefore must be cultured carefully. Liquid medium is not agitated and a high osmotic potential is maintained, at least in the initial stages. The liquid medium

must be shallow enough to allow aeration in the absence of agitation. Protoplasts can be plated out on to solid medium and callus produced. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus. Protoplasts are ideal targets for transformation by a variety of means.

Application of plant tissue culture

1. Commercial production of secondary metabolites

The compounds/ biochemical are 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, 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 biochemical's 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 propagule as don't need hardening and can be sown directly in field.

3. Raising somaclonal variant:

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 plant:

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.

5. Micropropagation Methods:

The ability of mature cell to dedifferentiate into callus tissue and the technique of cloning isolated single cell in vitro discussed earlier in this chapter have demonstrated that the somatic cells can differentiate to a whole plant under particular conditions. This potential of cell to divide and develop into multicellular plant is termed as cellular totipotency. To

express totipotency, after dedifferentiation, the cell has to undergo redifferentiation or regeneration which is the ability of dedifferentiated cell to form plant or plant organs.

6. Germplasm conservation

Germplasm refers to the sum total of all the genes present in a crop and its related species. The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock for its use at any time in future. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the existing and primitive plants will be lost. A global organization-International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation and provides necessary support for collection, conservation and utilization of plant genetic resources throughout the world. The germplasm is preserved by following two ways.

(a) ***In-situ*** **conservation**-

The germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in a near natural habitat along with the several wild types.

(b) ***Ex-situ*** **conservation**-

This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or in vitro cultures are preserved and stored as gene banks for long term use.

Cryopreservation plays a vital role in the long-term in vitro conservation of essential biological material and genetic resources. It involves the storage of in vitro cells or tissues in liquid nitrogen that results in cryo-injury on the exposure of tissues to physical and chemical stresses.

7. Genetic transformation

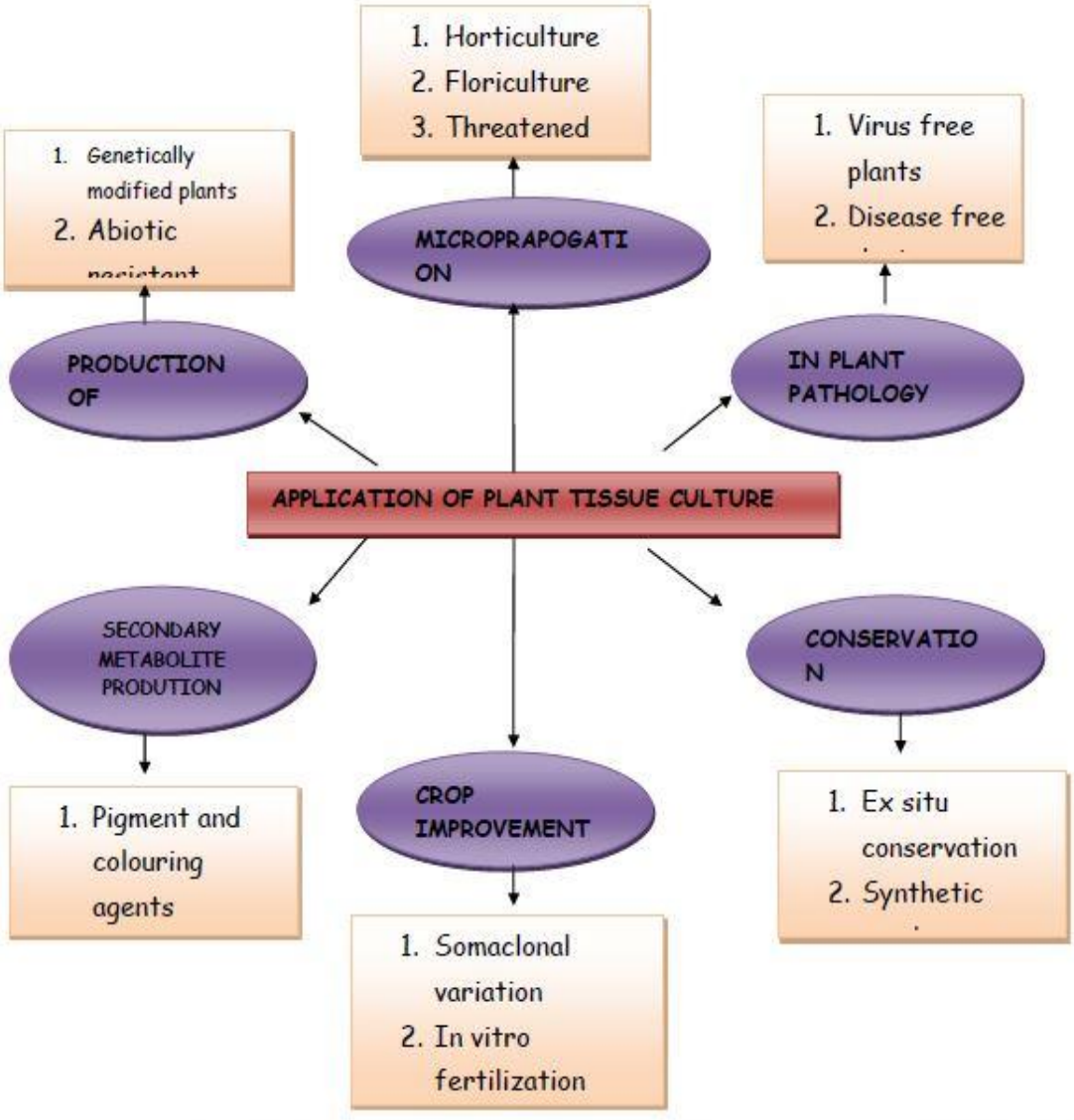
Genetic transformation is the most recent aspect of plant cell and tissue culture that provides the mean of transfer of genes with desirable trait into host plants and recovery of

transgenic plants. The technique has a great potential of genetic improvement of various crop plants by integrating in plant biotechnology and breeding programmes. It has a promising role for the introduction of agronomically important traits such as increased yield, better quality and enhanced resistance to pests and diseases.

8. Protoplast technology

Somatic hybridization is an important tool of plant breeding and crop improvement by the production of interspecific and intergeneric hybrids. The technique involves the fusion of protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and

regeneration of hybrid plants. Protoplast fusion provides an efficient mean of gene transfer with desired trait from one species to another and has an increasing impact on crop improvement. Somatic hybrids were produced by fusion of protoplasts from rice and ditch reed using electrofusion treatment for salt tolerance.



9. Haploid production

The tissue culture techniques enable to produce homozygous plants in relatively short time period through the protoplast, anther and microspore cultures instead of conventional breeding .

10. Production of transgenic plants with beneficial traits such as herbicide resistance, stress resistance, insect resistance, etc

- **Herbicide resistance**

Weeds are unwanted plants which decrease the crop yields and by competing with crop plants for light, water and nutrients. Several biotechnological strategies for weed control are being used.

The biological manipulations using genetic engineering to develop herbicide resistant plants are: (a) over-expression of the target protein by integrating multiple copies of the gene or by using a strong promoter., (b) enhancing the plant detoxification system which helps in reducing the effect of herbicide., (c) detoxifying the herbicide by using a foreign gene., and (d) modification of the target protein by mutation.

e.g. Glyphosate resistance

- **Stress resistance**

Resistance to abiotic stresses includes stress induced by herbicides, temperature (heat, chilling, and freezing), drought, salinity, ozone and intense light. These environmental stresses result in the destruction, deterioration of crop plants which leads to low crop productivity. Several strategies have been used and developed to build resistance in the plants against these stresses.

- **Insect resistance**

A variety of insects, mites and nematodes significantly reduce the yield and quality of the crop plants. The conventional method is to use synthetic pesticides, which also have severe effects on human health and environment. The transgenic technology uses an innovative and eco-friendly method to improve pest control management.

Chapter...2

PLANT TISSUE CULTURE LABORATORY

Tissue culture is rapidly becoming a commercial method for propagating new cultivars, rare species, and difficult-to-propagate plants. From a few research laboratories several years ago, a whole new industry is emerging. Currently, the demand for micropropagated plants is greater than the supply with some plants. Some growers specialize in only the micropropagation of plantlets, leaving the growing-on to others; many growers are integrating a tissue culture laboratory into their overall operation.

In designing any laboratory, big or small, certain elements are essential for a successful operation. The correct design of a laboratory will not only help maintain asepsis, but it will also achieve a high standard of work.

Any laboratory, in which tissue culture techniques are performed, regardless of the specific purpose, must contain a number of basic facilities. These usually include the following:

- A general washing area
- A media preparation, sterilization, and storage area
- An aseptic transfer area
- culture rooms

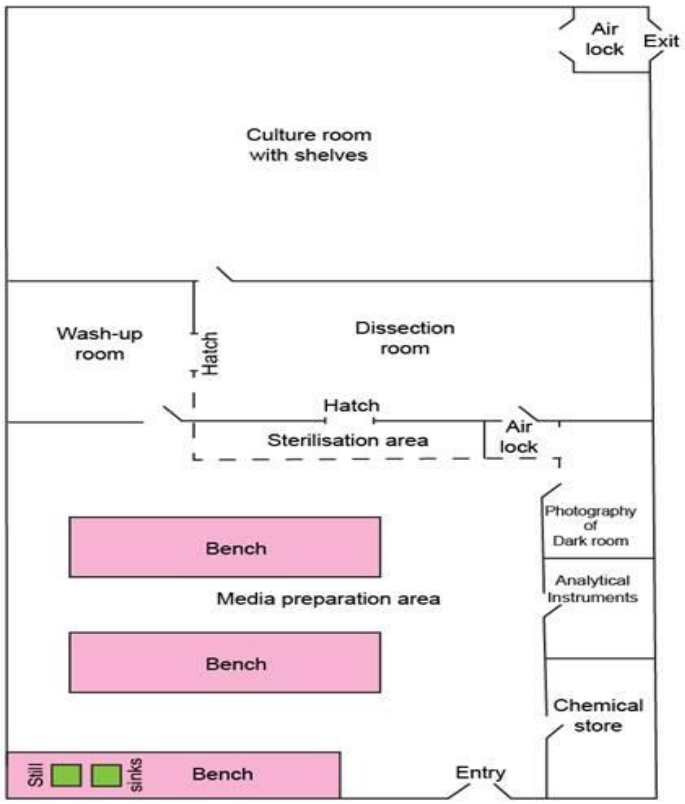


Fig: 2.1. Layout of plant tissue culture laboratory

1. Washing Area

The washing area should contain large sinks,drainage racks, demineralized, distilled and double distilled water. The glassware washing area should be located near the sterilization and media preparation areas. The room needs moisture tolerant floors and walls finished to withstand steam and cleaning. When culture vessels are removed from the growth area, they are often autoclaved to kill contaminants or to soften semi-solid media. The vessels can be easily moved to the washing area if the autoclave or pressure cooker is nearby. Locate the glassware storage area close to the wash area to expedite storage; these areas also need to be accessible to the media preparation area. The glassware area should be equipped with at least one large sink; two sinks are preferable. Adequate work space is required on both sides of the sink; this space will be used for glassware soaking tubs and drainage trays.

2. Media Preparation Area

This is the central section where most of the activities are preferred including media preparation as well as other activities that relate to handling of tissue culture matter. This area is provided with floors and walls with smooth surface to minimize dust accumulation and to facilate cleaning.

- Benches at a height suitable to work while standing
- pH meter is used to determine the pH of various media used for tissue culture. pH indicator paper can also be used for the purpose but it is less accurate. The standard media pH is maintained at 5.8.
- Hot-plate-cum-magnetic stirrer for dissolving chemicals and during media preparation
- An autoclave or domestic pressure cooker is crucial instrument for a tissue culture laboratory. High pressure heat is needed to sterilize media, water, labware, forceps, needles etc. Certain spores from fungi and bacteria can only be killed at a temperature of 121°C and 15 pounds per square inch (psi) for 15-20 min. A caution should be taken while opening the door of autoclave and it should be open when the pressure drops to zero. Opening the door immediately can lead to a rapid change in the temperature, resulting in breakage of glassware and steam burning of operator.
- Plastic carboys for storing distilled water required for media preparation and final washing of labware.
- Balances near dry corner of the media room. High quality microbalance are required to weigh smallest of the quantities. Additionally a top pan balance is required for less sensitive quantities.
- Hot-air oven to keep autoclaved medium warm before pouring into vessels. It is also used for the dry heat sterilization of clean glassware like, Petridishes, culture tubes, pipettes etc. Typical sterilizing conditions are 160-170 °C/1hr.

- Dishwasher for cleaning glass pipettes in running water

Weighing balance

Hot plate

Oven

Double distillation

3. Transfer Area

Under very clean and dry conditions, tissue culture techniques can be successfully performed on an open laboratory bench. However, it is advisable that a laminar flow hood or sterile transfer room be utilized for making transfers. Within the transfer area there should be a source of electricity, gas, compressed air, and vacuum.

4. Culture Room

All types of tissue cultures should be incubated under conditions of well-controlled temperature, humidity, air circulation, and light quality and duration. These environmental factors may influence the growth and differentiation process directly during culture or indirectly by affecting their response in subsequent generations. Protoplast cultures, low-density cell suspension cultures, and anther cultures are particularly sensitive to environmental cultural condition.

Typically, the culture room for growth of plant tissue cultures should have a temperature between 15° and 30° C, with a temperature fluctuation of less than ±0.5°C; however, a wider range in temperature may be required for specific experiments. It is also recommended that the room have an alarm system to indicate when the temperature has reached preset high or low temperature limits, as well as continuous temperature recorder to monitor temperature fluctuations. The temperature should be constant throughout the entire culture room (i.e., no hot or cold spots). The culture room should have enough fluorescent lighting to reach the 10,000 lux; the lighting should be adjustable in terms of quantity and photoperiod duration. Both light and temperature should be programmable for a 24-hr period. The culture room should have fairly uniform forced-air ventilation, and a humidity range of 20-98% controllable to ±3 percent. Many incubators, large growth chambers, and walk-in environmental chambers meet these specifications.

Storage area

- A deep freezer (-20°C to -80°C) / refrigerator for storage of enzyme solutions, stock solutions plant materials and all temperature-sensitive chemicals.
- Microwave oven to melt agar solidified media
- Upright and inverted light microscope with camera attachment for recording the morphogenic responses from various explants, calli, cells and protoplasts. Inverted microscope gives the clear views of cultures settled at the bottom of Petridishes

Greenhouse

The greenhouse facility is required to grow parent pants and to acclimatize in vitro raised plantlets. The size and facility inside the green house vary with the requirement and depends on the funds available with the laboratory. However, minimum facilities for maintaining humidity by fogging, misting or a fan and pad system, reduced light, cooling system for summers and heating system for winters must be provided. It would be desirable to have a potting room adjacent to this facility.

Chapter...3

PLANT TISSUE CULTURE MEDIA

In plant tissue culture, the growth medium or culture medium is a liquid or gel designed to support the growth and maturation of excised plant tissue. To date many researchers has been developed the different medium composition for the optimal growth of plant tissue, such as MS medium, Whites medium, Gamborg B5 medium etc. Culture media are largely responsible for the invitro growth & morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium with required concentration of macro and micronutrient for growth and maturation of plant source/tissue. As the nutrient requirement of plant tissues are different so no single medium is suitable for growth of plant tissues, consequently the suitable medium for particular plant may be prepared by trial and error method.
The basic nutritional requirements of cultured plant cells are very similar to those of whole plants.

Basic requirement of plant tissue culture medium

Culture media used for Successful *in vitro* cultivation of plant cells are consists of Complex mixture of inorganic salts such as Essential elements or mineral ions, Organic supplements such as vitamins and amino acids, Carbon source, Gelling agents, Plant Growth Regulators and Antibiotics.

A. Complex Inorganic salts:

It consists of microelement and microelement

1. Macro element

These are the inorganic salts which are required in more amounts. It includes nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S)-required for plant cell or tissue growth.

Nitrogen: Necessary for formation of amino acids, the building blocks of proteins and also Essential for plant cell division, vital for plant growth

- Directly involved in photosynthesis
- Necessary component of vitamins
- Affects energy reactions in the plant

Calcium:

- Utilized for Continuous cell division and formation
- Involved in nitrogen metabolism
- Reduces plant respiration
- Aids translocation of photosynthesis from leaves to fruiting organs

Phosphorus

- Involved in photosynthesis, respiration, energy storage and transfer and cell division.
- Promotes early root formation and growth

- Improves quality of fruits, vegetables, and grains
- Vital to seed formation
- Helps plants survive harsh winter conditions
- Increases water-use efficiency

Potassium:

- Carbohydrate metabolism and the break down and translocation of starches
- Increases photosynthesis
- Increases water-use efficiency
- Important in fruit formation
- Activates enzymes and controls their reaction rates
- Increases disease resistance

Magnesium:

- Key element of chlorophyll production
- Improves utilization and mobility of phosphorus
- Activator and component of many plant enzymes
- Increases iron utilization in plants

Sulphur:

- Integral part of amino acids
- Helps develop enzymes and vitamins
- Promotes nodule formation on legumes
- Aids in seed production

2. Microelement:

These are the essential inorganic salts which is required in very small quantity, These includes Manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc are regarded as microelements, although other elements like aluminium and nickel are frequently found in some formulations. These plant micro elements are used in very small amounts; they are generally acts as a cofactor for variety of enzymes and are just as important to plant development and profitable crop production as the major nutrients. Especially, they work "behind the scene" as activators of many plant functions.

Iron is usually added in the medium as iron sulphate, although iron citrate can also be used. Ethylenediaminetetraacetic acid (EDTA) is usually used in conjunction with the iron sulphate. The EDTA complexes with the iron so as to allow the slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide.

B. Organic supplements:

These include vitamins and amino acids.

Vitamins

A normal plant synthesizes vitamins required for their growth and development. Vitamins are required by plants as catalysts in various metabolic processes (mostly work as coenzymes). When plant cells and tissues are grown in vitro, some vitamins may become limiting factors for

cell growth. The vitamins most frequently used in cell and tissue culture media include thiamin (B_1), nicotinic acid, pyridoxine (B_6), and myo-inositol.

Amino acids

Although cultured cells are normally capable of synthesizing all of the required amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures and protoplast cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen.

The most common sources of organic nitrogen used in culture media are amino acid mixtures (e.g., casein hydrolysate), L-glutamine, L-asparagine etc.

C. Carbon source

The plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon source for energy. Among the energy sources, sucrose is the most preferred. During autoclaving of the medium sucrose gets hydrolyzed to glucose and fructose. The plant cells in culture first utilize glucose and then fructose as a sole source of carbon.

D. Gelling agents

As the liquid media in plant tissue culture brings some problems with subcultures and handling with plants and for the formation of developed roots and shoots, a some kind of support is required in liquid media, this can be done by solidifying the media by using gelling agents, commonly used gelling agents are,

Agar:

Agar is the most commonly used gelling agent for preparing semisolid and solid plant tissue culture media. Agar is actually the resulting mixture of two components, the linear polysaccharide agarose and a heterogeneous mixture of smaller molecules called agaropectin. Agar is obtained from red algae- *Gelidium amansii*. Agar has several advantages over other gelling agents. First, when agar is mixed with water, it forms a gel that melts at approximately 60°-100°C and solidifies at approximately 45°C thus, agar gels are stable at all feasible incubation temperature. Additionally, agar gel does not react with media constituents and is not digested by plant enzymes.

Agarose

It is obtained by purifying agar to remove the agaropectins. This is required where high gel strength is needed, such as in single cell or protoplast cultures.

Gelrite

It is produced by bacterium *Pseudomonas elodea* . It can be readily prepared in cold solution at room temperature. It sets as a clear gel which assists easy observation of cultures and their possible contamination. Unlike agar, the gel strength of gelrite is unaffected over a wide range of

pH. However, few plants show hyperhydricity on gelrite due to freely availability of water.

Gelatin

It is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C) and as a result the gelling property is lost.

E. Plant growth regulators

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. There are currently five recognized groups of plant hormones: auxins, gibberellins, cytokinins, abscisic acid (ABA) and ethylene.

Auxins

Auxins stimulate cell elongation and influence other developmental responses in host, such as root initiation, vascular differentiation, tropic responses, apical dominance and the development of auxiliary buds, flowers and fruits. Auxins are synthesized from amino acid tryptophan in the stem and root apices and transported through the plant axis by polar transport. The principal auxin in plants is indole-3-acetic acid (IAA). Auxins in plant tissue culture are used to induce callus from explants , and cause root and shoot morphogenesis

Table:4.2 Commonly used auxin, their abbreviation and chemical name.

Abbreviation/name	Chemical name
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
Dicamba	2-methoxy-3,6-dichlorobenzoic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MCPA	2-methyl-4-chlorophenoxyacetic acid
NAA	1-naphthylacetic acid
NOA	2-naphthyloxyacetic acid
Picloram	4-amino-2,5,6-trichloropicolinic acid

Cytokinin

Cytokinin is named because of their discovered role in cell division (cytokinesis), the cytokinins have a molecular structure similar to adenine. Naturally occurring **zeatin**, isolated first from corn (*Zea mays)*, is the most active form of the cytokinins. Cytokinins are found in sites of active cell division in plants—for example, in root tips, seeds, fruits, and leaves. They are transported in the xylem and work in the presence of auxin to promote cell division

Table-4.3: Commonly used cytokinins, their abbreviation and chemical name.

Abbreviation/name	Chemical name
BAP	6-benzylaminopurine
2iP (IPA)	[N6-(2-isopentyl)adenine]
Kinetin	6-furfurylaminopurine
Zeatin	4-hydroxy-3-methyl-trans-2-butenylaminopurine
Thidiazuron	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea

Ratio of auxin and cytokinin:

Differing cytokinin: auxin ratio changes the nature of organogenesis. If cytokinin is high and auxin low, shoots are formed; if cytokinin is low and auxin high, roots are formed. For all practical purposes, it is considered that the formation and maintenance of callus culture require both auxin and cytokinin. The actual concentration of the growth regulators in culture media is variable depending on the type of tissue explants and the plant species.

Gibberellins

The gibberellins are widespread throughout the plant kingdom, and more than 75 have been isolated to date. Rather than giving each a specific name, the compounds are numbered—for example, **GA1, GA2**, and so on. **Gibberellic acid three (GA3**) is the most widespread and most thoroughly studied. The gibberellins are especially abundant in seeds and young shoots where they control stem elongation by stimulating both cell division and elongation (auxin stimulates only cell elongation). Gibberellins are transported by the xylem and phloem. Numerous effects have been cataloged that involve about 15 or fewer of the gibberellic acids. The greater numbers with no known effects apparently are precursors to the active ones.

Experimentation with GA3 sprayed on genetically dwarf plants stimulates elongation of the dwarf plants to normal heights. Normal-height plants sprayed with GA3 become giants.

Ethylene

Ethylene is a simple gaseous hydrocarbon produced from an amino acid methionine and appears in most plant tissues in large amounts when they are stressed. It diffuses from its site of origin into the air and affects surrounding plants as well. Large amounts ordinarily are produced by roots, senescing flowers, ripening fruits, and the apical meristem of shoots. Auxin increases ethylene production, as does ethylene itself—small amounts of ethylene initiate copious production of still more. Ethylene stimulates the ripening of fruit and initiates abscission of fruits and leaves. In monoecious plants (those with separate male and female flowers borne on the same plant), gibberellins and ethylene concentrations determine the sex of the flowers: Flower buds exposed to high concentrations of ethylene produce carpellate flowers, while gibberellins induce staminate ones.

Abscisic acid

Abscisic acid (ABA), despite its name, does not initiate abscission, although in the 1960s when it was named botanists thought that it did. It is synthesized in plastids from carotenoids and diffuses in all directions through vascular tissues and parenchyma. Its principal effect is inhibition of cell growth. ABA increases in developing seeds and promotes dormancy. If leaves experience water stress, ABA amounts increase immediately, causing the stomata to close.

F. Antibiotics

Antibiotics are substances produced by certain microorganisms that suppress the growth of other microorganisms and eventually destroy them. Their applications include:

a. Suppresses bacterial infections in plant cell and tissue culture.

b. Suppresses mould and yeast infections in cell cultures.

c. Eliminates Agro bacterium species after the transformation of plant tissue.

d. As a selective agent in plant transformation experiments.

G. pH of medium

The optimal pH range for the most tissue cultures is in the range of 5.0 to 6.0.The pH generally falls by 0.3 to 0.5 units after autoclaving. Before sterilization pH can be adjusted to the required optima level while preparing the medium. At pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. In general, pH above 6.0 gives the medium hard appearance, while pH below 5.0 does not allow gelling of the medium.

Major Types of media

Several media formulations are commonly used for the majority of all cell and tissue culture work. The most commonly used tissue culture media as follows

- **Whites medium:**

This is one of the earliest plant tissue culture media developed for root culture.

- **MS medium:**

Murashige & Skoog Medium (MS) is established by Murashige & Skoog (1962) for in vitro callus culture of *Nicotiana tobacum* (family-Solanaceae). This medium was modified by incorporating plant growth regulators for the development of micropropagated explants in vitro. This medium used to induce organogenesis & regeneration of plants in cultured tissues. These days this medium is widely used for many types of culture systems.

- **B5 medium:**

Gamborg B5 Medium is established by Gamborg O.L. (1968) for callus and cell suspension culture of *Glycine max* (Family- Fabaceae). This medium is widely used for in vitro plant cell, tissue and organ culture.

- **N6 medium:**

Chu C.C. *et al.* (1975) has developed CHU (N) Medium for in vitro anther culture of *Oryza sativa* (Family-Graminae). It is useful to generate new useful genetic varieties in gramineous plants by the initiation, growth, and differentiation of callus from rice pollen culture.

- **Nitsch's medium:**

Nitsch Medium is established by Nitsch J. P. (1969) for in vitro anther culture of Nicotiana (Family-Solanaceae).

Among the media referred above, MS medium is most frequently used in plant tissue culture work because of its success with several plant species.

Formulation of MS Medium (Murashige and Skoog, 1962) for the Preparation of Stock Solutions

TABLE -Ingredients Stocks Final concentration in MS Medium

Ingredients	**Stocks**	**Final concentration in MS Medium**
A) Macronutrients	**mg/l (20x)**	**mg/l**
(NH4) NO_3	20 x 1650=33000	1650
KNO_3	38000	1900
$CaCl_3.2H_2O$	8800	440
$MgSO_4.7H_2O$	7400	370
KH_2PO_4	3400	170
b) Micronutrients mg /l (100x)		

$MnSO_4.4H_2O$	22.3 x 100=2230	22.3
$ZnSO_4.7H_2O$	860	8.6
$H_3BO_3.7H_2O$	620	6.2
KI	83	0.83
$Na_2MoO_4.2H_2O$	25	0.25
$CuSO_2.5H_2O$	2.50	0.025
$CoCl_2.6H_2O$	2.50	0.025
c) Vitamins mg/l (200x)		
Glycine	2 x 200=400	2.0
Nicotinic acid	100	0.5
Pyridoxine HCl	100	0.5
Thiamine HCl	20	0.1
d) Iron mg/l (200x)		
$Na_2EDTA.2H_2O$	33.6 x 200=6720	36.2
$FeSO_4.7H_2O$	5560	27.8
e**) Myo-inositol mg/l (100x)**		
Myo-inositol	100 x 100=10000	100

Stock preparation

I. Macro stock (MS-I) in 1000 ml

Take 500 ml double distilled water in a 2.0 liter beaker, weight, add and keep on dissolving each salt sequentially in a descending order (dissolve by magnetic stirring) and finally make up the volume to 1000 ml by double distilled water. Store at 4º C.

II. Calcium stock (MS-II) in 1000 ml

Take 500 ml double distilled water in a 2.0 liter beaker, weight, add $CaCl_2$ .2 H_2O and keep on dissolving (dissolve by magnetic stirring)

and finally make up the volume to 1000 ml by double distilled water. Store at 4º C.

III. Micro stock (MS-III) in 1000 ml.

Take 500 ml double distilled water in a 2.0 liter beaker, weight, add and keep on dissolving each salt sequentially in a descending order (dissolve by magnetic stirring) and finally make up the volume to 1000 ml by double distilled water. Store at 4º C. **Notes** that Na2MoO4. 2H2O should be dissolved separately in approximately 200 ml of double distilled water and added to the stock after dissolving $ZnSO_4$. 7 H_2O. Also note that $MnSO_4$. H_2O takes time to dissolve.

IV. MS Iron EDTA stock (MS-IV) in 1000 ml

Take 1000 ml double distilled water in 1500 ml amber colored bottle and warm the water near boiling. Now weight and add Na_2 EDTA. 2 H_2O while stirring under magnetic stirrer; after Na2 EDTA. 2H2O has been dissolved add gradually $FeSO_4$. 7 H_2O under mild magnetic stirring. Immediately after adding $FeSO_4$. 7 H_2O close the bottle and keep on stirring at least for an hour store at 4º C.

V. Vitamin stock (MS-V) in 1000 ml.

Take 500 ml double distilled water in a 2.0 liter beaker, weight, add and keep on dissolving each salt sequentially in a descending order (dissolve by magnetic stirring) and finally make up the volume to 1000 ml by double distilled water. Store at 4º C. Vitamin stock is very prone to microbial contamination. Therefore always check the stock before use.

Preparation of hormone stock solution

- **Gibberellic acid (GA3): [1mg/ml stock]**

1. Weigh 0. 2 g of gibberellic acid and dissolve well with some alcohol drops. Add 200 ml of distilled water
2. Keep in a conveniently labeled vial at 0°C. The gibberellic acid may be sterilized together with the culture medium: however, the loss of some activity is also possible. One ml of concentrate solution (1,000 ppm) contains 1 mg of gibberellic acid.

- **1-naphthylacetic acid (NAA) : [1mg/ml stock]**

1. Weigh 0.2 g of NAA and dissolve well with some NaOH 1N drops.
2. Add 200 ml of distilled water. Keep it in a conveniently labeled vial at 0^0C. One ml of stock solution (1,000 ppm) contains 1 mg of NAA.

- **Indole-3-acetic acid (IAA): [1mg/ml stock]**

1. Weigh 0.2 mg of IAA and dissolve well with some alcohol drops. Add 200 ml of distilled water.
2. Keep it in a conveniently labeled vial at 0°C. Sterilization by filtration is recommended. One ml stock solution (1,000 ppm) contains 1 mg of IAA.

- **Indole-3-butyric acid (IBA) [1mg/ml stock]**

1. Weigh 0.2 mg of IBA and dissolve well with some alcohol drops. Add 200 ml of distilled water.
2. Keep it in a conveniently labeled vial at 0°C. Sterilization by filtration is recommended. One ml stock solution (1,000 ppm) contains 1 mg of IBA.

- **2, 4-D (2,4-dichlorophenoxyacetic acid): [1mg/ml stock]**

1. Weigh 0.2 g of 2,4-D and dissolve well with some alcohol drops. Add 200 ml of distilled water.
2. Keep in a vial conveniently labeled at 0°C. 2,4-D may be sterilized together with the culture medium; however, a loss of its activity is also possible. One ml of the stock solution (1 000 ppm) contains 1 mg of 2, 4-D.

- **6- Benzyl amino purine (BAP] [1mg/ml stock]**

1. Weigh 0. 2 g BAP and dissolve well with some drops of NaOH 1N. Add 200 ml distilled water.
2. Keep in a conveniently labeled vial at 0°C.BAP may be sterilized together with the culture medium; however, the loss of some activity is also possible. One ml of stock solution (1,000 ppm) contains 1 mg of BAP.

- **Kinetin (KIN) [1mg/ml stock]**

1. Weigh 0.2 g KIN and dissolve well with some drops of NaOH 1N. Add 200 ml of distilled water.
2. Keep in a conveniently labeled vial at 0°C. KIN may be sterilized together with the culture medium; however, the loss of its activity is also possible. One ml of the stock solution (1,000 ppm) contains 1 mg of KIN.

Maintenance of stock solution

- ✓ All salts stock should be stored in the refrigerator and are stable for several months.
- ✓ Always prepare stocks with glass distilled or dimineralized water
- ✓ Label the stock solutions clearly with date.
- ✓ Reagent grades of chemical should be maximum purity.
- ✓ Any stock showing cloudy or has bacterial or fungal growth should be discarded.

Preparation of 1 L of MS basal medium

- ✓ For preparation of 1.0 liter of MS Basal medium, the above stocks solutions should be added sequentially in about 500 ml of doubled distilled water.
- ✓ Weigh and add required quantities of sucrose (20 or 30 g) and dissolve by magnetic stirring. According to the purpose of the medium growth regulator and other medium conjugates/ additives are added, and the volume of the medium is made up to 1000 ml by distilled water. **Note** that thermolabile growth regulators and additives should be added by filter sterilization only after autoclaving.

- ✓ Adjust pH of the medium to 5.8 using 0.1 NaOH or 0.1 N HCL before autoclaving. **Note** that the pH meter should be calibrated by standard buffers (4.0 and 7.0) immediately before adjusting the medium.
- ✓ For preparing semisolid medium, add agar at the rate of 6.0-8.0 gm, and heat until near boiling in a microwave oven or gas oven with intermittent stirring. Measured volume of semisolid media is dispense into culture tubes, containers. For preparing liquid medium, pH adjusted media are directly poured in suitable containers. For plating experiments, semisolid medium is poured in sterile petridishes under a LFCA workstation.
- ✓ Plant tissue culture media are usually autoclaved at 121º C For 20 min (15 lb in or 1.05 kg cm2). Autoclaving is generally done in a horizontal or vertical autoclave. Minimum time necessary for steam sterilization of media is dependent on volume of medium per vessel and is described separately.
- ✓ Autoclaved media are kept in ambient temperature for a day and then transferred in a dust-free closed cabinet for subsequent use. Semisolid medium starts drying up, and therefore should be used within a fortnight after its preparation.

Sterilization of media

The culture medium is usually sterilized in an autoclave at 121^0C and 15 psi for 20 min. Hormones and other heat sensitive organic compounds are filter sterilized and added to autoclaved medium.

Chapter...4

STERILIZATION AND ASEPTIC TECHNIQUES

The sterilization and aseptic condition are more essential to maintain a completely aseptic environment and for successful tissue culture procedure. There are several sources of contamination. These are the plant tissue culture media which contain sucrose and other growth material which support the growth of microorganism. As microorganism grow faster than the cultured tissue it make nutrient unavailable for plant tissue. Secondly the initial explants are the major source of contamination, then the environment of culture room and transfer room.

Sterilization

Sterilization is the killing or removal of all microorganisms, including bacterial spores which are highly resistant. Sterilization is an absolute term, i.e. the article must be sterile meaning the absence of all microorganisms.

Disinfection

Disinfection is the killing of many, but not all microorganisms. It is a process of reduction of number of contaminating organisms to a level that cannot cause infection, i.e. pathogens must be killed some organisms and bacterial spores may survive.

Disinfectants are chemicals that are used for disinfection. Disinfectants should be used only on inanimate objects.

Antiseptics

Antiseptic are the mild form of disinfectants that are used externally on living tissues to kill microorganism. e.g. on the surface of skin and mucus membrane.

Four categories of sterilization

1. Sterilization of vessels and instruments
2. Sterilization of nutrient media
3. Sterilization of explants
4. Sterilization of culture room

Table 3. 1:- Sterilization techniques used in Plant Tissue Culture

Techniques	Materials sterilized
Steam sterilization/Autoclaving (121°C at 15 psi for 20-40 min)	Nutrient media, culture vessels, glasswares and plasticwares
Dry heat (160-180°C for 3h)	Instruments (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares (heat resistance)
Flame sterilization	Instruments (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	Worker's hands, laminar flow cabinet
Surface sterilization (Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	Explants

1. Sterilization of vessels and instruments

Reusable glasswares for tissue culture should be emptied immediately after use and soaked. Glasswares are soaked in the detergent solution for suitable period and then thoroughly rinsed under tap water and then with distilled water.

To reuse the glasswares, that have contaminated tissue or media, it is extremely important to autoclave them without opening the caps so that all the microbial contamination are destroyed. The washed glasswares and plasticwares are then subjected to sterilization.

a. Dry heat sterilization

Vegetative bacteria are killed by dry heat at 100^0 C for 60 min. fungal spores are killed at 115^0 within 60 min and bacterial spores at 120^0-160^0C within 60 min. Glass, culture vessels, metal instrument and aluminum foil can be sterilized by exposure to dry air (160-180^0 C) for 2-4 hour in a hot air oven.

Mechanisms of dry heat sterilization

- ✓ Protein denaturation
- ✓ Oxidative damage
- ✓ Toxic effect of elevated electrolyte (in absence of water).

Dry heat at 160°C (holding temperature for one hour is required to kill the most resistant spores). The articles remain dry. It is unsuitable for clothing which may be spoiled.

Hot Air Oven

It is one of the most common method used for sterilization. Glass wares, swab sticks, all-glass syringes, powder and oily substances are sterilized in hot air oven. For sterilization, a temperature of 160°C is maintained (holding) for one hour. Spores are killed at this temperature. It leads to sterilization.

Hot Air Oven is an apparatus with double metallic walls and a door. There is an air space between these walls. The apparatus is heated by electricity or gas at the bottom. On heating, the air at the bottom becomes hot and passes between the two walls from below upwards, and then passes in the inner chamber through the holes on me top of the apparatus. A thermostat is fitted to maintain a constant temperature of 160°C.

Flaming

Burning the article in methylated spirit or exposing it to a spirit of gas flame. The instrument such as foreceps, scalpels, needles, and spatulas are sterilized by dipping in 95 % ethanol, followed by flaming and cooling. This technique is called flame sterilization.

b. Moist heat:-

This can be at temperature below 100^0C, at 100^0C (boiling water) and above 100^0C under increased pressure at saturated steam, e.g. autoclaves. The first two are used as disinfection methods and only the third one is suitable for sterilization and killing of spores. Moist heat at temperature below 100^0C is used for milk, vaccines and eating utensils.

Boiling

This is sufficient to kill all non sporing organism within 5-10 minutes. It is used to disinfect blades, syringes, etc. when the article is removed from the boiler it should be allowed to dry before handled to prevent contamination by bacteria from the skin of the handless finger.

Autoclave

Autoclaving is one of the most common method of sterilization.

Principle: In this method sterilization is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. The temperature of boiling depends on the surrounding atmospheric pressure. A higher temperature of steaming is obtained by employing a higher pressure. When the autoclave is closed and made air-tight, and water starts boiling, the inside pressures increases and now the water boils above 100°C. At 15 Lb per sq. inch pressure, 121°C temperature is obtained. This is kept for 15 minutes for sterilization to kill spores. It works like a pressure cooker. Autoclave is a metallic cylindrical vessel. On the lid, there are:

- A gauge for indicating the pressure,
- A safety valve, which can be set to blow off at any desired pressure,

and

- A stopcock to release the pressure. It is provided with a perforated diaphragm. Water is placed below the diaphragm and heated from below by electricity, gas or stove.

Operator instructions

I. Prepare and package material suitably

- Loose dry materials must be wrapped or bagged in steam-penetrable paper or loosely covered with aluminum foil. Wrapping too tightly will impede steam penetration, decreasing efficiency of the process.
- All containers must be covered by a loosened lid or steam-penetrable bung.
- Containers of liquid must be a maximum of 2/3 full, with lids loosened.
- Glassware must be heat-resistant borosilicate.
- Plastics must be heat-resistant eg: polycarbonate (PC), PTFE ("Teflon") and most polypropylene (PP) items.
- Sharps must be in a designated 'Sharps' container.
- Items or baskets must be tagged with autoclave tape to verify sterilization.
- Loosen all lids to prevent pressure buildup.
- Add water to containers as appropriate.

II. Operating Autoclave

- Check to be sure that the water reservoir is filled (approximately 1" from top), and if not, fill with distilled water.
- Choose appropriate cycle (e.g. fluid, dry etc) for the material.
- Set appropriate temperature for the cycle.
- Turn the autoclave to fill and wait until the water in the bottom of the autoclave covers the fill plate.
- Turn the autoclave to sterilize and load autoclave. Close and lock door.
- A steam cycle is approximately 40 minutes if the autoclave is cold and approximately 20 minutes if it is already warmed from a previous steam cycle.
- When cycle is complete, turn autoclave to vent and remain there until pressure drops. The heat stays on during the venting and without the steam plastics will melt.
- Turn the autoclave to power off.
- Do not attempt to open the door while autoclave is operating.
- The manuals for operation of the autoclave are located on the wall behind the autoclave.

2. Sterilization of nutrient medium

a. Autoclaving

Tissue culture media are generally sterilized by autoclaving at 121^0C and 1.05 kg/cm^2 (15-20 psi). The time required for sterilization depends upon the volume of medium in the vessel. Dispense medium in small aliquots whenever possible as many media components are broken down on prolonged exposure to heat. Medium exposed to temperatures more than 121^0C may not properly get or may result in poor cell growth. Minimum autoclaving time includes the time required for the liquid volume to reach the sterilizing temperature (121 ^{0}C) and 16 min. at 121 ^{0}C.

Several medium components are considered thermolabile and should not be autoclaved. Stock solutions of the heat labile components are filter sterilized. The filtered solution is aseptically added to the culture medium, which has been autoclaved and allowed to cool, approximately 35-40^{0}C. The medium is then dispensed under sterile conditions.

b. Filter sterilization

Filtration is an excellent way to reduce microbial population in heat sensitive solutions. It can be used to sterilize solutions. Rather than directly destroying contaminating microorganisms, the filter simply removes them. Bacteria can be stopped by using filter of pore size less than 0.75µm. The following types of filters are known.

- ✓ Asbestos filters
- ✓ Sintered glass filters
- ✓ Micro filters
- ✓ Depth filters
- ✓ Cellulose membrane filters

3. Sterilization of explants

The initial explant is the major source of contamination. To avoid the contamination, it is necessary to sterilize the explant before using it for tissue culture process.

During sterilization it is very essential to remove dirt and debris from the plant tissue and should be washed in a detergent solution and rinsed several times with distilled water. In case where the explant carries heavy load microorganism, it is better to wash under running tap water for an hour or more. The sterilization procedure that is needed for plant materials, which are to be directly used as explant source, can also cause damage to the tissue. Therefore, the concentration of the sterilizing agent and the duration of treatment should be adjusted to minimize tissue death. After surface sterilization treatment, the plant material must be rinsed 3-4 times in sterile distilled water to remove all traces of sterilizing agent.

Explants have to be treated with suitable chemosterilant to make them free from contamination. For each type of explants and plant species, a procedure of surface sterilization in order to obtain high percentage of aseptic but most proliferating cultures. While standardizing the procedure the TCDC formula is applied.

Where,

T- Selection of the type of chemical to be used.

C- Effective concentration of the selected chemical.

A- The required duration of the treatment.

C- Combination of the treatment if required.

While standardizing the procedure, it is ensured that the treatment is effective enough to kill the contaminants while its concentration should not be toxic to the proliferating explants.

Table: Common explant sterilizing agents

Sterilizing agent	Concentration %	Time of sterilization (Minutes)
Mercuric chloride	0.01-01	0.5-10
Calcium hypochlorite	9-10	5-30
Sodium hypochlorite	2	5-30
Hydrogen peroxide	10-12	5-15
Bromine water	1-2	2-10
Silver nitrate	1	5-30
Silver chloride	0.1-1	2-30

4. Sterilization of culture room

The air is full of contaminants, molds or bacteria in a medium; therefore, great care is required during transfer of media. Cleanliness is of primary importance in the culture room, especially in the laminar flow hood where cultures are shortly outside of their sterile protective container. This is a time when they have the greatest danger of becoming contaminated.

Sterilization of air

Air is also sterilized by filtration. Laminar flow biological safety cabinet employing high efficiency particulate air (HEPA) filters, which removes 99.97% of 0.3µm particles. Laminar flow biological safety cabinets force air through HEPA filters, then project a vertical curtain of sterile air across the cabinet opening. This protects a worker from microorganism being handled within the cabinet and prevents room contamination.

Except for the HEPA filter, the hood should be wiped down daily with a disinfectant such as 10% bleach, 70% alcohol, or Lysol or some other disinfectant.

Guidelines to use laminar air flow hood

- Close the hood and turn on its germicidal ultraviolet (UV) light. Let it run for several minutes. This disinfects the surfaces inside the hood.
- Turn off the UV light. Turn on the blower and the regular light.
- Raise the hood slowly until you feel resistance, and then stop. Raising the hood beyond this point reduces its effectiveness; many hoods have a warning system that will buzz if you raise the hood too high.
- Wear laboratory gloves, and wipe them down with alcohol every time you insert your hands under the hood.
- Wipe the surfaces inside the hood with alcohol on a clean paper towel, to provide further disinfection. Wipe the surfaces of all materials you intend to use before placing them into the hood.
- Move the supplies for your procedure inside the hood. Keep them a few inches back from the front of the hood to ensure that they remain uncontaminated. Do not place them too far back, as this may interfere with the airflow.
- Remove all of your supplies when you are done using the hood. You should never store anything in a laminar flow hood.
- Wipe down the surfaces inside the hood with alcohol once again, and close the hood.
- Turn on the UV light again. Let it run for several minutes, and then turn it off.

Chapter...5

CALLUS CULTURE

Callus on wounded parts or on a culture medium is made of an amorphous aggregate of loose parenchyma cells which proliferate from the mother plant. Or Callus is the undifferentiated and unorganized mass of plant cells. It is basically a tumor tissue which usually forms on wound of differentiated tissues or organs Or Callus formation in vivo is frequently observed as a result of wound at the cut edges of stem or root. Callus formation has been found in angiosperm.

Callus tissue is not of one single kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single explant. Sometimes the type of callus obtained its degree of cellular differentiation and its capacity to regenerate new plants, depend upon the origin and age of the tissue chosen as an explant. Loosely packed or 'friable' callus is usually selected for initiating suspension cultures.

Callus culture

The callus culture is based on a basic principle, a cell from any part of the plant like shoot apex, bud, leaf, mesophyll cells, epidermis, cambium, pollen, fruits, etc. when inoculated suitable medium results into the formation of an amorphous mass of cells known as callus, which can be induced to redifferentiated on appropriate medium to develop embryoids which directly developed into the plantlets, eventually giving rise to a whole viable plant.

During callus formation there is some degree of dedifferentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology (callus is usually composed of unspecialized parenchyma cells) and metabolism. One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesize. This has important consequences for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant. This necessitates the addition of other components such as vitamins and, most importantly, a carbon source to the culture medium, in addition to the usual mineral nutrients.

Development stages of callus

A typical unorganized plant callus initiated from a new explant or piece of previously initiated calli has three stages of development

- ✓ The induction of cell division.\
- ✓ A period of active cell division during which differentiated cells lose specialized features, they may have acquired and became dedifferentiated.

- ✓ A period when cell division slows down or ceases there is increasing cellular differentiation.

Callus culture techniques

- **Preparation and sterilization of explants**

A] Choice of explant

The starting material for callus culture may be the differentiated tissue from any part of the plant such as root, stem, leaf, anther, flowers, etc.

B] Preparation

The desirable portion of the plant is removed by cutting with the help of sharp knife. This portion is transferred for sterilization and then for inoculation. The dried, damaged and mature part/portion is removed. Then that explants are used for callus culture.

C] Surface sterilization

The explants are washed under running tap water for 15 min to remove the surface contaminants and soil particles and immersed in detergent (laboline) for 5 min and rinsed with distilled water for four times. The explant was deeped in 70% ethanol for 1 min. Then the explants were soaked in 0.1% (w/v) mercuric chloride solution for 1-2 min and thoroughly rinsed with sterile distilled water for four times. The explants are cultured on Murashige and Skoog basal medium supplemented with different concentrations of plant growth regulators.

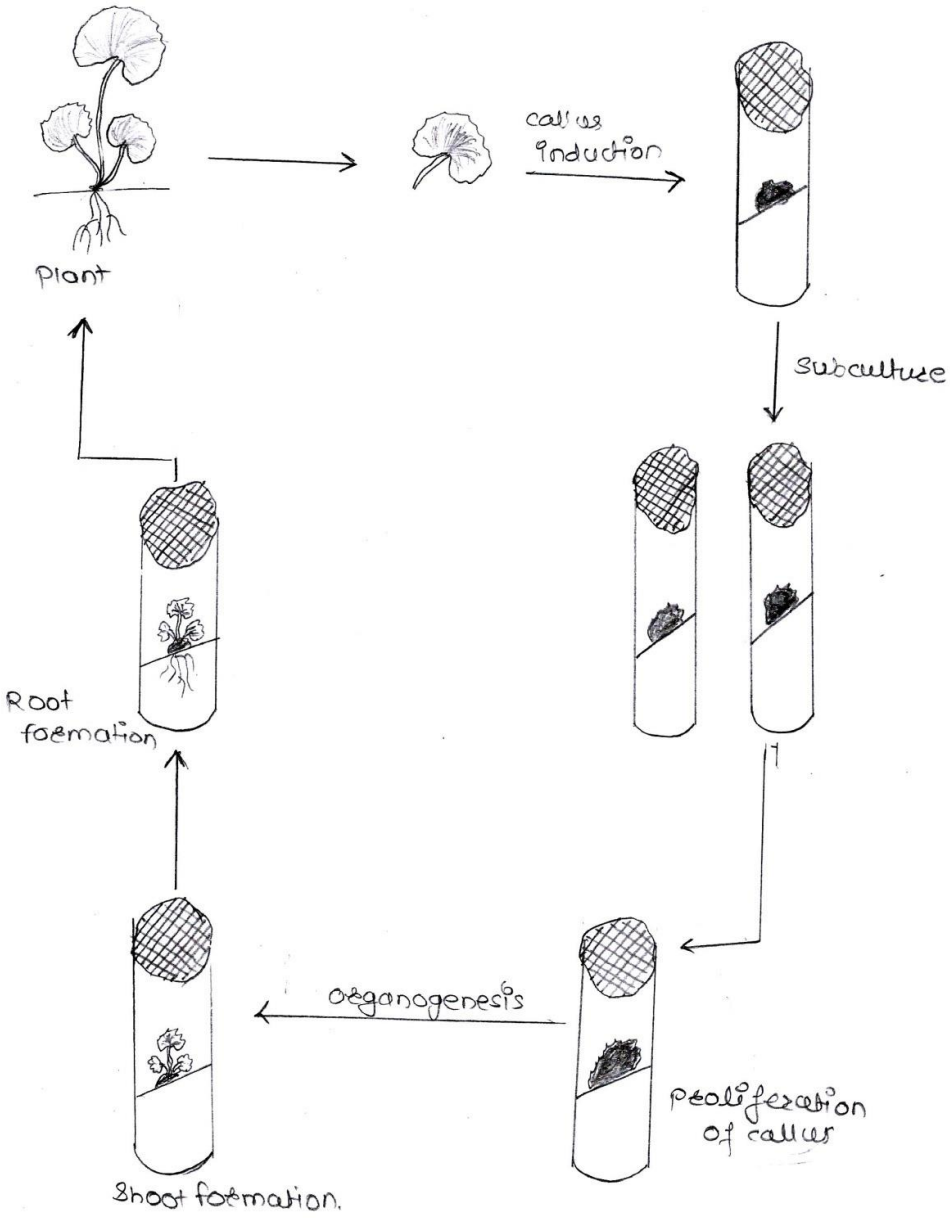
D] Inoculation of explants

All the glassware's are sterilized by autoclaving under steam pressure. The inoculation chamber is sterilized by wiping with ethyl alcohol and UV radiation for 15 minutes before use.

The sterilized medium is distributed in culture tubes or bottles and their plugged with sterile cotton plug under the aseptic condition After solidification of medium, the surface sterilized explants are inoculated on semi solid medium in culture tubes and gently pressed in the medium so that there is a good contact between the explants and the medium.

E] Incubation

After inoculation the cultured tubes/ bottles are transferred for incubation. For incubation the temperature is maintained at 25-28^{0}C which is helpful for callus production. The room must have small amount of illumination to ensure the better growth of callus tissues. The Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus.


Plant
callus induction
subculture
proliferation of callus
organogenesis
Shoot formation.
Root formation

- **Proliferation**
  After 3- 4 weeks of incubation the callus developed. The well developed callus is cut into small pieces with the help of sterile knife. These pieces are then transferred to another medium containing an altered concentration of hormones.
  This medium induces the growth of more callus tissues called callus proliferation. In the proliferation medium, the callus tissues multiply more rapidly by their fast growth and cell division.
  2, 4- D is a synthetic plant growth hormone which induces the callus formation. Once callus is formed 2, 4-D is avoided in proliferation medium.
- **Subculture**
  After proper growth of callus tissues, they are frequently transferred to a fresh medium at regular intervals. This transfer of callus helps to maintain the cells in a viable condition.

Example- Callus culture of *Centella asiatica* .L
Culture Media and Culture Conditions:-
Leaf explants were inoculated on MS media supplemented with different concentration of phyto- hormones like, BA, KIN, IBA and IAA. The medium was further supplemented with KIN & BA 1.8, 2.0 mg/L. with combination of 1 mg/L each IBA and IAA gives maximum growth. The cultures were incubated under at 25 ± 2ºC & light (16 hours light and 8 hours dark). Details regarding quantity of callus color, type and number of days to callus formation were observed and results were recorded.
Result and discussion: Callus development was observed from the leaf explants of *C. asiatica* within a week of inoculation and the highest frequency of callus was observed after 3 weeks on MS medium supplemented with different concentration of KIN & BA 1.8, 2.0 mg/l, with combination of 1 mg/l each of IBA and IAA. Higher concentration of 2.0 mg/l BA in combination of 1 mg/l IBA and IAA.showed 95% callusing response with yellowish green and more friable callus.

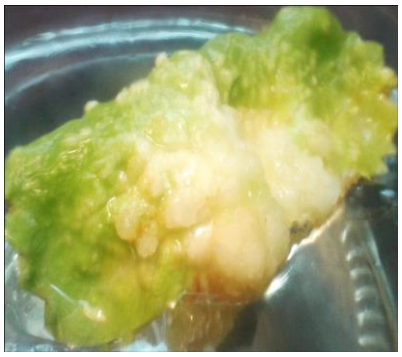


Fig:- A

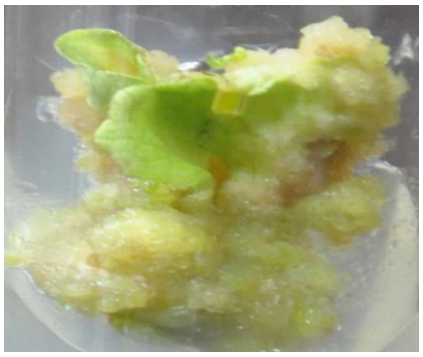


Fig:-B

Fig:-C

Fig:-D

Fig: Shows Stages of callus proliferation from leaf explants of *Centella asiatica*. (A) Callus induction after two weeks of culture on KIN & BA 1.8 mg/L with combination of 1mg/L IAA & IBA; (B & C)Callus induction after three weeks of culture on KIN & BA 2.0 mg/L with combination of 1mg/L IAA &IBA; (D) More friable and brownish callus formation after 6 weeks of culture.

Application of callus culture

Callus culture offer a unique system for the study of

- Nutrition of plants
- Cell and organ differentiation and morphogenesis.
- Somaclonal variation and its exploitation.
- Developing cell suspension culture and protoplast cultures.
- Genetic transformation using ballistic particle gun technology
- In the production of secondary metabolite and their regulation.

Chapter...6

CELL SUSPENSION CULTURE

The callus proliferates as an unorganized mass of cell so it is very difficult to follow many cellular events during its growth and development of phases. To overcome such limitations of callus culture cultivation of free cells or as well as cell aggregate in a chemically defined medium [liquid] as a suspension.

Cell suspension cultures are initiated by transferring friable callus to liquid nutrient medium. Plant tissues remains submerged which leads to anaerobic conditions and ultimately death of the cells. Therefore such cultures are agitated on rotary shaker.

Agitation serves both to aerates the cultures and to disease the cells. Cell from the inoculums are separated during this process and a suspension of cells is produced. The division rate of suspension culture cells at the exponential phase is typically higher than the callus but doubling times are slower. Cell suspensions are maintained by routine sub culturing in a fresh medium when cells are at their stationary phase.
As the cells are incubated in suspension cultures, the cell divides and enlarges. The incubation period is depend on

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

Among these, cell density is very crucial the initial cell density used in the subculture is very critical and largely depend on the type of suspension culture being maintained with low initial cell densities, the lag phase and log phase of growth get prolonged. Whenever a new suspension culture is started, it is necessary to determine the optical cell density in relation the volume of culture medium, so that the maximum cell growth can be achieved with the low cell densities, the culture will not grow properly.
The normal incubation time for the suspension cultures is in the range of 21-28 days.

Types of Suspension Culture
The suspension cultures are grouped as

1. Batch culture
2. Continuous culture

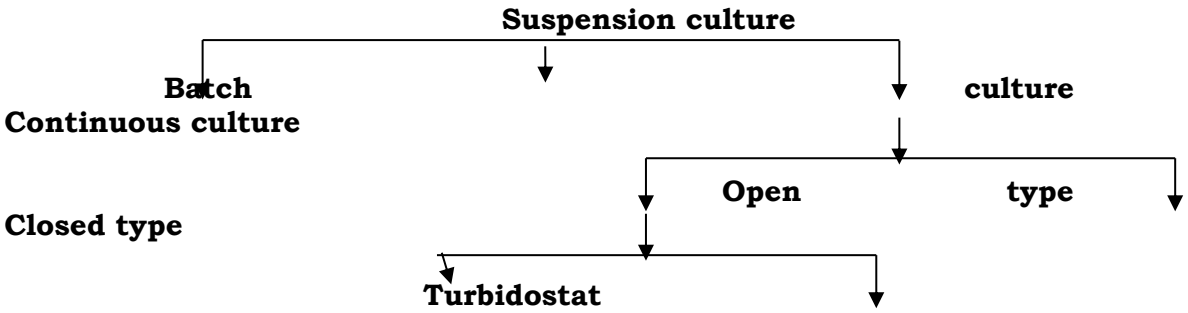


Fig: Types of suspension culture

1. Batch culture

A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. In batch culture, the same medium and all the cells are produced are retained in the culture vessel. In batch culture, cell division and cell growth coupled with increase in biomass occur until one of the factors in the culture environment becomes limiting . the cell number or biomass of batch culture exhibit a typical sigmoidal curve (include 5 phases) when the cell number in suspension culture is plotted against the time of incubation.

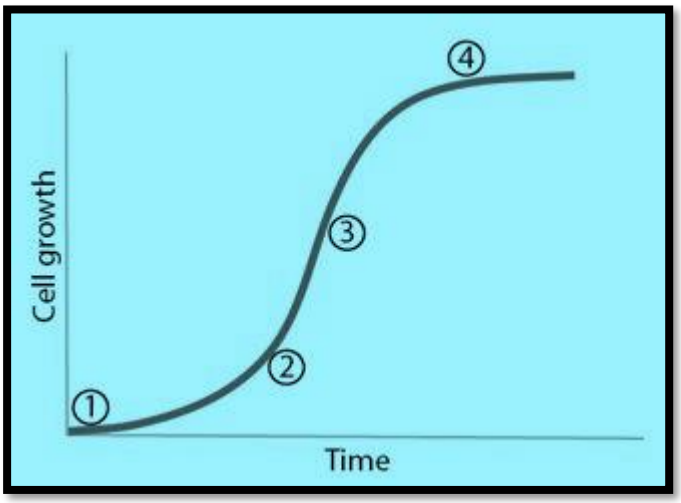


Figure: Growth curve for plant cell suspension grown in closed system. The four different growth phases are labeled: (1) Lag phase, (2) Exponential phase, (3) Linear phase, (4) Stationary phase.

a. Lag phase:-

Characterized by preparation of cells to divide. The lag phase duration depends mainly on inoculums size and growth phase of culture.

b. Log phase/ exponential phase:-

When the rate of cell multiplication is highest. The log phase lasts about 3-4 cell generations, and the duration of a cell generation may vary from 22-48 hours mainly depending on the plant species.

c. Linear phase: -
This phase is represented by slowness in cell division and increase in cell expansion.

d. Deceleration phase: -
Characterized by decrease in cell division and cell expansion.

e. Stationary phase:-
The depletion of nutrients and accumulation of cellular wastes causes the stationary phase. This phase is represented by a constant number of cells and their size.

Batch culture is used for initiation of cell suspension, which may be used for cell cloning and scaling up or for continuous culture. Batch cultures are more convenient than continuous cultures and thus are routinely used.

2. Continuous culture

In continuous cultures there is a regular addition of fresh nutrient medium and draining out the used medium so that culture volume is normally constant. These cultures are carried in specially designed culture vessels [bioreactor]. Continuous culture is carried out under defined and controlled condition i.e. cell density, nutrients, oxygen, pH etc. Cells in these cultures are mostly at an exponential phase of growth. The cell population is maintained in a steady state by regularly replacing a portion of the used or spent medium by fresh medium. Such culture system is either open type or closed type.

a. Open type: -

In these cultures, the inflow of fresh medium is balanced with the outflow of the volume of spent medium along with the cells. The addition of fresh medium and culture harvest are so adjusted that the cultures are maintained indefinitely at a constant growth rate. At a steady state, the rate of cells removed from the cultures equals to the rate of formation of new cells. The open type cultures are of either chemostat or turbidostat types.

Chemostat: -

In this type the cellular growth rate and density are kept constant by limiting a nutrient (glucose, nitrogen, phosphorus) in the medium. Except limiting nutrient, all other nutrients are kept at higher concentration. As a result any increase or decrease in limiting factor results in increase or decrease in growth rate.

Turbidostat: -

In this addition of fresh medium is done whenever there is an increase in turbidity so that the suspension culture system is maintained at a fixed optical density. Thus, in these culture systems, turbidity is preselected on

the basis of biomass density in cultures, and they are maintained by intermittent addition of medium and washout of cells.

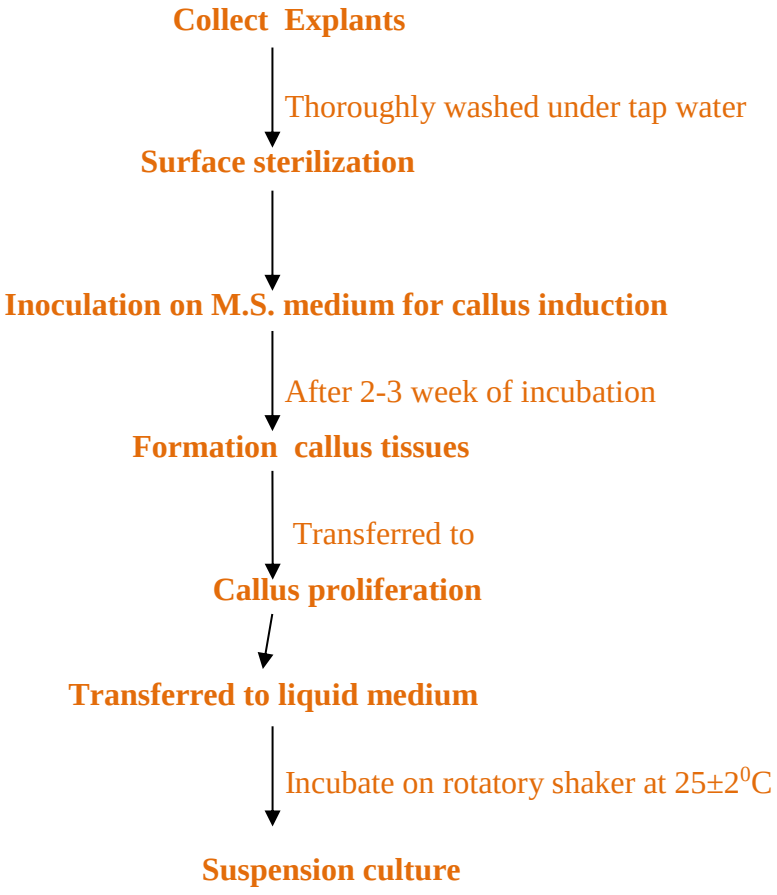
b. Closed type:-

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

Establishment of Culture

The essential stages of establishment of the suspension culture are stated below

- A well developed callus tissue is transferred aseptically to liquid medium. The composition of this liquid medium is same as that of callus proliferation medium.
- These flasks agitated by using rotary shaker system, the medium is agitated with 30-150 rpm..
- All the production of sufficient number of cells in the suspension, a small volume of the suspension is transferred to a fresh medium to establish subculture.



Synchronization of Suspension Culture

Normally in the suspension culture, cells vary greatly in size, shape, DNA and nuclear content therefore cell cultures are mostly referred as asynchronous. This variation is not suitable for genetic, biochemical and physiological studies. Hence it is essential to manipulate the synchronization of cells.

Synchronization of cultured cells broadly refers to the organized existence of majority of cells in the same cell cycle phase simultaneously a synchronous culture is one in which the majority of cells proceed through each cell cycle phase simultaneously. Synchronization is expressed as percentage synchrony of cells in suspension cultures. There are two methods by which synchronization achieved.

A. Physical method
B. Chemical method

A. Physical methods

The environmental growth condition such as light, temperature etc. and the physical properties of cells can be monitored to achieve high degree of synchronization. Some of them are as follows.

✓ **Cold treatment:-**

In the cold treatment method, the cells are subjected to low temperature shock i.e. around 4^0C.low temperature shocks with nutrient starvation gives the better result.

✓ **Selection by volume:-**

The cells in suspension culture can be selected based on the size of the aggregates.

B. Chemical method

In the chemical method of synchronization of cell, the cells are first arrested at particular stage of the cell cycle and subsequently allowed to undergo simultaneous division. The chemical method includes use of chemical inhibitor and deprivation of essential nutrient.

✓ **Inhibition**

The cells are arrested at G1 phase of cell cycle by using DNA synthesis inhibitors e.g. 5-aminouracil, hydroxyurea, 5-flurodeoxypurine, etc. when these inhibitors added to the cultures results in accumulation of cells at G1 phase. And on removal of inhibitors, synchronization of cells occurs.

Colchicine is a strong inhibitor to arrest the growth of cells at metaphase stage of cell cycle.

✓ **Starvation**

Starvation is achieved on the basis of depriving the suspension cultures of an essential growth compounds leading to a stationary growth phase. When the missing nutrient is added to culture cell resumption occurs synchronously.

Measurement of Growth

It is necessary to assess the growth of cells in suspension culture. The growth can be measured by using following parameters. These are

1. Cell counting
2. Packed cell volume
3. Cell fresh weight

1. Cell counting

The number of cells found in the cell suspension can be counted directly by using hemocytometer. The number of cells found in a unit volume of sample is counted and calculated with reference to the total volume of suspension. This method has some disadvantages such as

- it is tedious and time consuming method
- cells in suspension culture exist as colonies in varying size.

To overcome this problem, the cells have to be disrupted by treating with chromic acid or pectinase.

Chromic acid treatment

- The 1 volume of cell suspension culture is treated with 2 volume of 8% chromic acid solution.
- This mixture is heated at 70^0 C for 2-15 minutes.
- The mixture is allowed to cooled and the agitated vigorously for 10 min. on shaking machine to separate the individual cells from the mass of cells.
- The suspension is now centrifuged, the chromic acid poured off and the pellet resuspended in 8% NaCl solution.
- After 10- 15 min. cells are counted on a hemocytometer.

Pectinase enzyme treatment

- The suspension is treated with 0.25% pectinase enzyme which dissolves middle lamella present in between the two adjacent cells.
- This results in separation of cells from cell mass.
- Then the suspension is used to count the cell number.

2. Packed cell volume

This is the easiest method for determination of cell growth in suspension cultures. Packed cell volume (PCV) is expressed as ml of pellet per ml of culture.

- 10 ml of sample is transferred to graduate measuring tube aseptically.
- This sample is centrifuged for the deposition of cells at the bottom of the tube.
- After the settlement of all cells , the volume of packed cells can be measured against the reading of graduated measuring tube.

3. Cell fresh weight

The weight of cells in the sample can be measured by simple procedure.

- The required volume of culture is filter through pre weighed nylon fabric filter with the help of funnel.
- Collect the cells remain on filter paper, drain off excess water.

- The filter paper containing cells is weighed properly by using following formula.

Cell fresh weight = weight of filter paper containing cells – weight of filter paper

4. Cell dry weight

The procedure similar to that fresh weight except that the filter paper are dried in an oven for 12 hr at 60^0 C. After cooling, the dried paper weighed and cell weight is expressed as gm/ml.

Measurement of Viability of Cultured Cells

The viability of cells is the most important for the growth of cells. Viability of the cells can be assessed by microscopic examination of cells directly or after staining them.

- **Phase contrast microscopy**

Cytoplasmic streaming and the presence of a healthy nucleus indicate that the cells are viable. For this purpose phase contrast microscope is used.

- **Reduction of Tetrazolium salts**

This test is used to measure respiratory efficiency of cell. By reduction of 2,3,5-triphenytetrazolium chloride to red dye formazon. Formazon can be extracted and measured by using spectroscopic method.

- **Fluorescein Diacetate (FDA) method**

The cell suspension is incubated with 0.01% of FDA solution. FDA is cleaved by esterase enzyme of viable cells which results in emission of green fluorescence under UV light. The viable cells can be detected by the fluorescence.

- **Evan's blue staining**

In this method, a dilute solution of Evan's blue dye is used for staining. Evan's blue stains the dead or damaged cells while the viable cell remain unstained.

Importance of Cell Suspension Culture

Plant cell suspension cultures have found wide application both for research and for commercial exploitation.

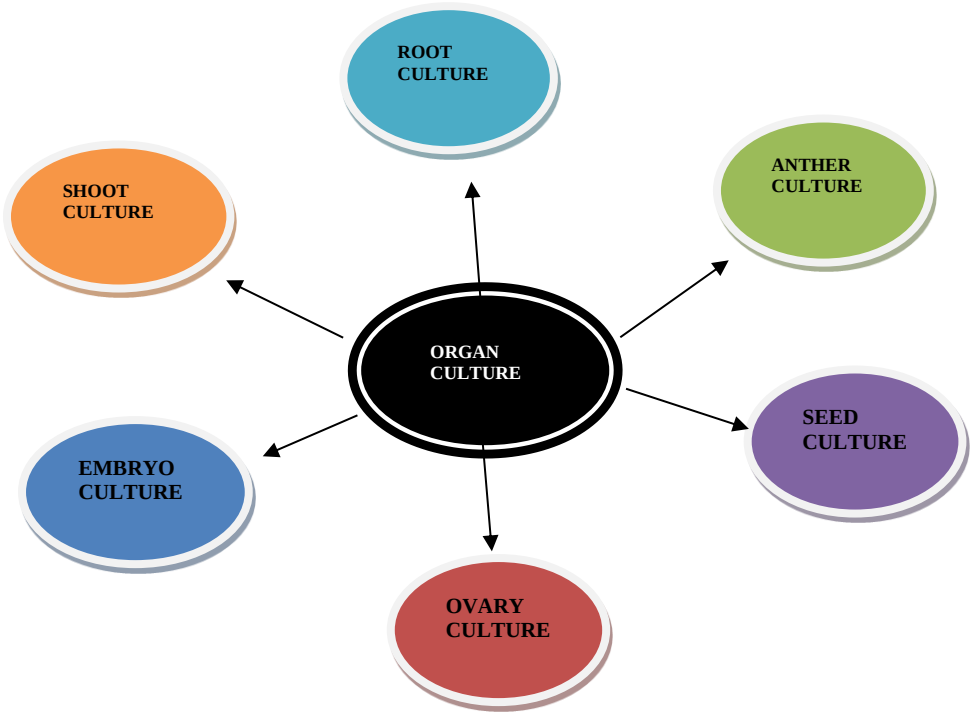
- It helps in understanding in clonal studies.
- Cell suspension cultures are easy to maintain and allow great flexibility of experimental approach.
- Selection of variant cell lines, mutants and production of secondary metabolites.
- The formation of somatic embryos in suspension cultures is ideal for the large scale production of commercial plants

Chapter...7

ORGAN CULTURE

Organ culture is used as a general term for those types of culture in which an organized form of growth can be continuously maintained.
It includes aseptic isolation of plant organ such as embryo, seed, root, endosperm, anther pollen, ovary, shoot, etc and their growth in vitro.
Differentiated plant organs can usually be grown in culture without loss of integrity.

Types of organ culture



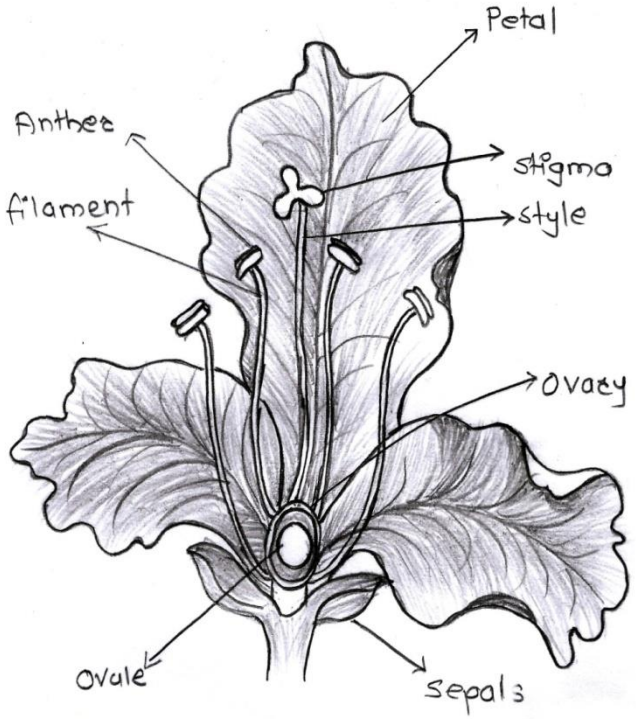


Fig: Different parts of typical flower

1. SHOOT CULTURE

The growing points of shoots can be cultured in such a way that they continue uninterrupted and organized growth. As these shoot initials ultimately give rise to small organized shoots which can then be rooted, their culture has great practical significance for plant propagation. Culture of larger stem apices or lateral buds (ranging from 5 or 10 mm in length to undisected buds) is used as a very successful method of propagating plants.

The term shoot culture is now preferred for cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by the repeated formation of axillary branches. In this technique, newly formed shoots or shoot bases serve as explants for repeated proliferation; severed shoots (or shoot clumps) are finally rooted to form plantlets which can be grown *in vivo*. This is the most widely used method of micropropagation

Explant size. Shoot cultures are conventionally started from the apices of lateral or main shoots, up to 20 mm in length, dissected from actively-growing shoots or dormant buds. Larger explants are also sometimes used with advantage: they may consist of a larger part of the shoot apex or be stem segments bearing one or more lateral buds; sometimes shoots

from other *in vitro* cultures are employed. When apical or lateral buds were used almost exclusively as explants, the name 'shoot tip culture'

Regulating shoot proliferation

The growth and proliferation of axillary shoots in shoot cultures is usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Robbins (1922) seems to have been the first person to have successfully cultured excised shoot tips on a medium containing sugar.

A notable feature of shoot cultures of most plant species is the need for high cytokinin levels at Stage II to promote the growth of multiple axillary shoots.

Cytokinin growth regulators are usually extremely effective in removing the apical dominance of shoots. A cytokinin treatment can not only promote the formation of multiple shoots (axillary and/or adventitious), but also (if the compound used is unsuitable, or the concentration used is too high), cause the shoots formed to be too short for rooting and transfer.

The cytokinin growth regulators added to shoot culture media at Stage II to promote axillary shoot growth, usually inhibit root formation. Single shoots or shoot clusters must therefore be moved to a different medium for rooting *in vitro* before being transferred as plantlets to the external environment.

2. ROOT CULTURE

Root cultures can be established from root tips taken from primary or lateral roots of many plants.

3. ANTHER AND POLLEN CULTURE

Anther culture [called androgenesis] is a technique by which the developing anther from unopened flower bud are cultured on a nutrient medium where the microspores within the cultured anther develop into callus tissue or embryoids that gives rise to haploid plantlets either through organogenesis or embryogenesis.

The basic principle of anther and pollen culture is the production of haploid plants exploiting the Totipotency of microspores and the occurrence of single set of chromosome in microspore.

Anther is a male reproductive organ, diploid in chromosome number. The aim of anther and pollen culture is to get haploid plants by induction of embryogenesis. Haploid plants are characterized by possessing only a single set of chromosome. Haploid plants are of great significance for the production of homozygous lines and for the improvement of plants in plant breeding programme.

Guha and Maheshwari (1964) reported the direct development of haploid embryo and plantlets from microspores of *Datura innoxia* by the anther culture.

- Anther culture

The flowers are collected from young plants and surface sterilized. The anther are removed from flower with their filaments. Anther are excised under aseptic condition in their proper developmental stages.

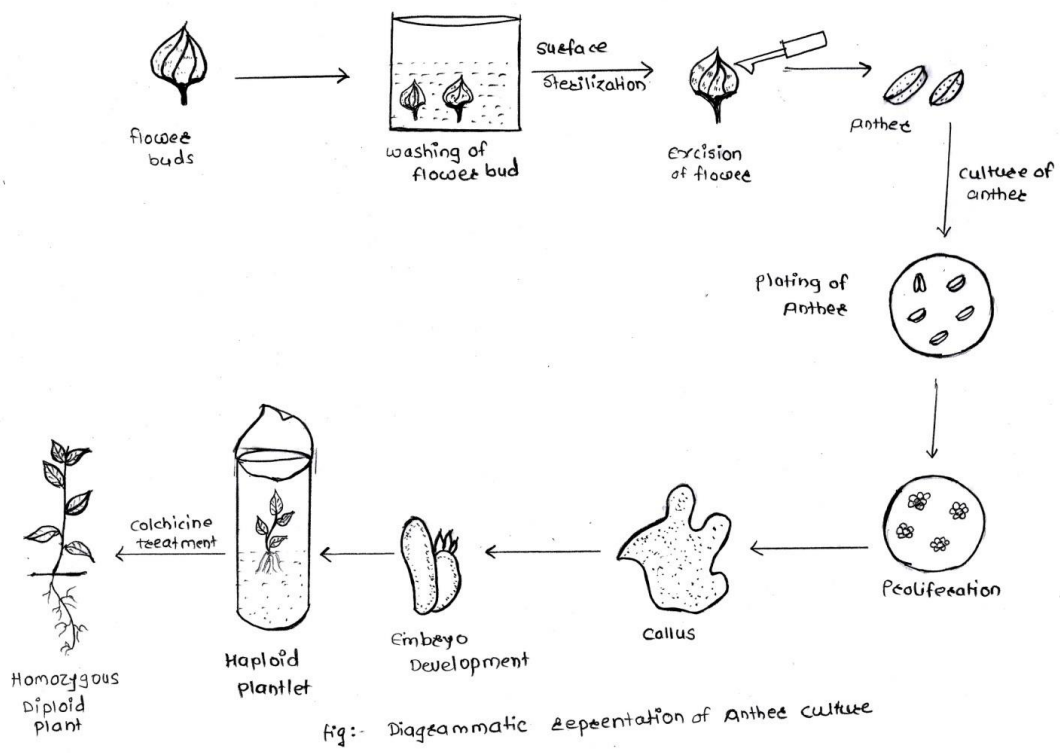


fig:- Diagrammatic representation of Anther culture

Pollen culture

Pollen grains are male reproductive cells they are produced by the stamens of flowers and participate in fertilization.

Pollengrain also called microspores. Microspores culture may b e preferred over anther culture,even though the degree of success is low in this case.

Microspores collected first using the following steps.

1. Collect the flowers from young plants and washed thoroughly under tap water.
2. Anthers are excised and surface sterilized.
   Pollen is isolated either by squeezing or floats culturing of anthers.

Squeezing method

Anthers are placed in 20ml of medium. Squeezed gently with a glass rod to separate pollens from anther walls. The prepared solution is filtered through a nylon mesh of suitable pore size. Filtrate is centrifuged for 5 min. at 500-800 rpm. The resulting pollen dense pellet is collected, washed twice.

Float culture

Anthers are excised. Those are placed in petridishes containing liquid medium. Anthers float in liquid medium. The anthers release their pollen grains into the medium in a few days

1. Transfer the pollen pellet liquid medium.
2. The microspore is then inoculated on a solid or in a liquid medium.
3. It is maintained at 25^0C and 16hr light and hr dark for 15 days
4. Subculture of cells in a fresh medium for callus induction.
5. Regenerate the plantlets from callus tissue.
6. Transfer to green house for acclimatization
7. Transfer them to field.

Application

- Development of homozygous lines
- Generation of exclusive male plants
- Production of disease resistant plants.
- Cytogenetic research
- Evolutionary studies
- Classical plant genetics and cytogenetic
- Modern molecular genetics including
- Induced mutagenesis,
- site-directed mutagenesis,
- Genetic transformation research,
- Genome mapping
- Assessing distant genome relationships,
- Gene dosage effects,
- Analysis of linkages,
- Mechanisms of the genetic control of chromosomal pairing and
- In conventional plant breeding studies

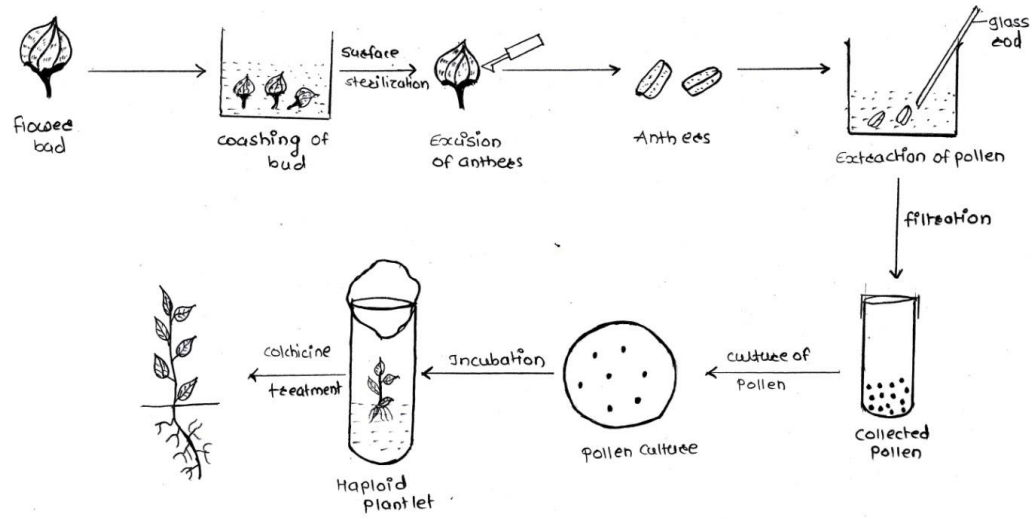


Fig: Diagrammatic representation of Pollen culture for production of Haploid and Diploid plants

HAPLOID PRODUCTION

Haploid plants are characterized by possessing only sinle ste of chromosome. In nature, haploid arie as result of parthenogenesis. Haploid plants are of great significance for the production of homozygous line and in plant breeding programme.

HISTORY

In 1921,A.D. Bergner discovered haploid plants in *Datura stramonium.* The Indian scientists Guha and Maheshwari (1964) reported the direct development of haploid embryo and plantlets from microspore of *Datura innoxia* by the cultures of excised anthers.

In 1967, Bougin and Hitsch obtained the first full pledged haploid plants from *Nicotiana tobacum.*

Approaches

Haploids are produced by two approaches. These are as follows.

A. In vivo
B. In vitro

A. In vivo

There are several methods to induce haploid production in vivo. There are

i. Gynogenesis:-
   Production of a haploid individual by the development of an unfertilized egg cell (by delayed pollination).
ii. Androgenesis:-
   Production of a haploid individual by development of an egg cell containing the male nucleus. For successful in vitro androgenesis

Diploidisation of haploid plants: Haploid plants produced from anther culture maintained *invitro* 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 haploids, 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.
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

4. EMBRYO CULTURE

Embryo culture deals with the sterile isolation and in vitro growth of a mature or an immature embryo with an ultimate objective of obtaining a viable plant.

The embryo of different developmental stages, formed within the female gametophyte through sexual process, Can be isolated aseptically and cultured in vitro under aseptic condition in order to grow into plantlets. In 1904, Hanning published a paper describing the first systematic attempt to culture isolated mature embryos of angiosperms Cochleria and Raphanus aseptically.Laibach cultured excised embryos from seeds of an interspecific cross Linum perenne × L. austrianum and succeeded in raising hybrid plants. The technique of zygotic embryo culture is also useful for exploring the nutritional and physical conditions required for embryonic development, skipping seed dormancy in order to shorten the breeding cycle, determination of seed viability and microcloning of the source material.

Growth factors

Sucrose is the most commonly used source of energy for embryo culture. Maltose, lactose, raffinose, or mannitol may also be used for embryo culture. Mature embryos grow fairly well at low sucrose concentrations but younger embryos require higher levels of carbohydrate various concentrations of sucrose used in embryo cultures depend on the species and size/age of the embryo.

Embryos which have an excellent enzyme system can reduce nitrates to ammonium. Ammonium nitrate is significantly superior to KNO_3, $NaNO_3$ and $(NH_4)_2HPO_4$.especially, the presence of NH^+_4 in the medium has been found essential for proper growth and differentiation of embryos.

Type of embryo culture

a. Mature embryo culture
b. Immature embryo culture

a. Mature embryo culture

Mature embryo culture are carried out in the following condition.

- When the embryo remain dormant for long periods.
- Low survival of embryos in vivo.
- To avoid inhibition in the seed for germination.
- For converting sterile seed to viable seedling.

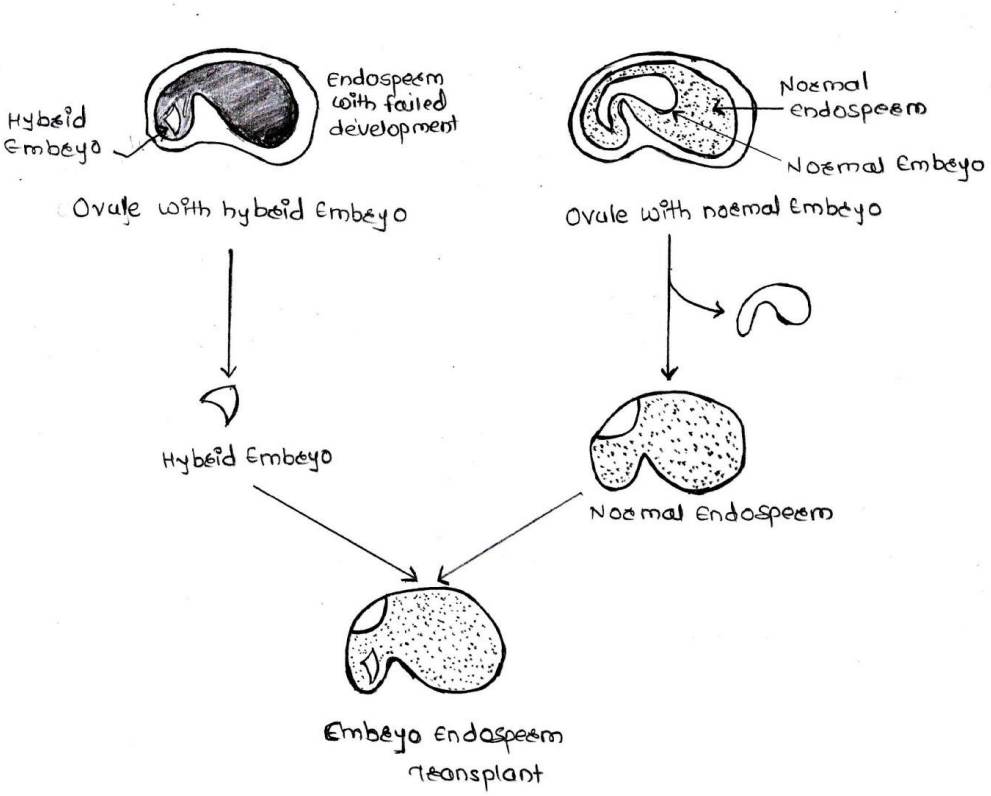
Embryo culture is a simple technique. It requires simple nutrient medium. Embryo of seed plants normally develop inside the ovule which , in turn , are covered by ovaries. As embryos already exist in a sterile environment, so disinfection of the embryo surface is not necessary. The mature seeds or ovule or fruits are surface sterilized and then embryos remove aseptically.

The general method of embryo culture is as follows:

1. Collect healthy and mature fruits from the field and wash thoroughly under running tap water for about an hour
2. Surface sterilize with 0.01% Tween-20 for15 min. rinse seeds several times with distilled water and finally treated with 0.1 % mercuric

chloride for 10- 15 min. finally rinse it for six times with sterile distilled water.

3. Break seed aseptically and isolate embryo. The embryo can be isolated by usin needles, scalpels, blades etc. if seeds has hard seed coat, soak it in water for few hour before sterilization.
4. Culture embryo on callus proliferation medium.
5. Incubate the cultures at 22-25^{0}C under a 16 hour photoperiod of 2000 lux luminous intensity.
6. After 2 weeks of incubation the embryo begins to swell on callus proliferation medium. Distinct callus growth is observed after weeks.
7. After 8 weeks of incubation transfer the callus on shoot regeneration medium. Within 4 weeks of transfer into second medium the callus turns green and produces soft spongy tissue. Some of these tissues are differentiated into embryoids/
8. The embryoids produce cluster. When sub cultured onto shoot regeneration medium which grow into shoots within 12 weeks.



OVARY CULTURE

Ovary culture is also used to produce haploid plants. The unpollinated ovaries used for this purpose. It is possible to trigger female gametophyte f angiosperm to develop into a sporophyte. The plant produced by this culture ar called gynogenic haploid. In 1976, San Noeum firstly demonstrated the gynogenesis b ovary culture of *Hordeum vulgare*. Late, in 1979, Zhu and Wu obtained haploid plants from cultured unpollinated ovaries of *Triticum aestivum* and *Nicotina tobacum.*

Protocol

Collect flower buds (24-48 r prior to anthesis from unpollinated ovaries)
Remove calyx, corolla and stamens of flower
The ovaries are subjected to surface sterilization.
After surface sterilization, the ovaries inserted in solid culture medium. If liquid culture medium is used, the ovaries are placed on filter paper or allowed to float over the medium with pedical inserted through filter paper.

Chapter...8

MICROPROPAGATION

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.

Plants can be propagated through their two developmental life cycles; the sexual, or the asexual. In the sexual cycle new plants arise after fusion of the parental gametes, and develop from zygotic embryos contained within seeds or fruits.

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.

The in vivo clonal propagation of plants is tedious, expensive & frequently unsuccessful. In vitro clonal propagation through tissue culture is referred as micropropagation. Use of tissue culture technique for micropropagation was first started by Morel (1960).This is the only process of plant biotechnology, which is being utilized by industries in India for commercial production of mainly ornamental plants and fruit trees.

Micropropagation methods:

The ability of mature cell to dedifferentiate into callus tissue and the technique of cloning isolated single cell in vitro discussed earlier in this chapter have demonstrated that the somatic cells can differentiate to a whole plant under particular conditions. This potential of cell to divide and develop into multicellular plant is termed as cellular totipotency. To express totipotency, after dedifferentiation, the cell has to undergo redifferentiation or regeneration which is the ability of dedifferentiated cell to form plant or plant organs. This may occur through either of two processes:

1. Organogenesis
2. Embryogenesis

1. Organogenesis

Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds. These shoot buds are monopolar structures which in turn give rise to leaf primordial and the apical meristem. Or

Organogenesis is the process of morphogenesis involving the formation of plant organs.

A high level of auxin to cytokinin favoured root formation and the reverse favoured shoot formation . in organogenesis, the shoot or root may form first depending upon the nature of growth hormone in the basal medium.

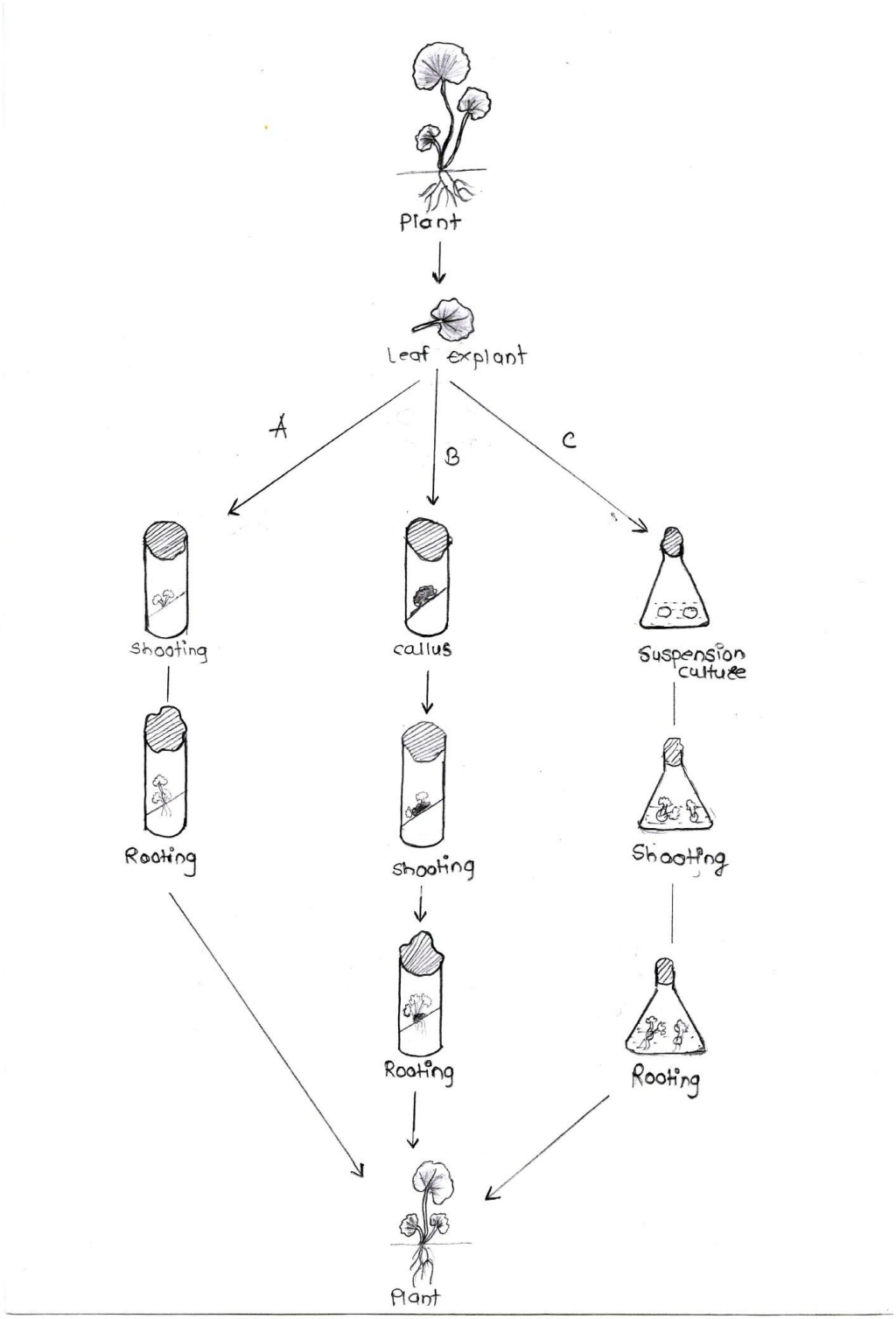


Figure: Direct and indirect organogenesis in plant

A- Direct organogenesis

B- Indirect organogenesis

C- Indirect organogenesis through suspension culture

The genesis of shoot or root from the explants or calli is termed a caulogenesis or rhizogenesis respectively.
There are two types of organogenesis
a. Direct organogenesis
b. Indirect organogenesis

a. Direct organogenesis
In direct organogenesis, the tissue undergoes morphogenesis without going through callus formation stage.

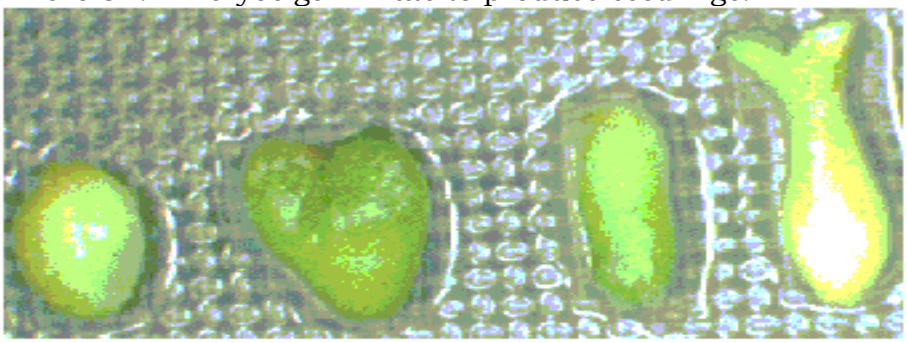
b. Indirect organogenesis

2. **Somatic embryogenesis:**
Somatic embryogenesis is a process involving redifferentiation of meristematic cells into nonzygotic somatic embryo which are capable of germinating to form complete plants.
Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non zygotic embryos capable germinating to form complete plants. Under the natural conditions this pathway is not normally followed but from tissue cultures somatic embryogenesis occurs most frequently and as an alternative to organogenesis for regeneration of whole plants. Somatic embryogenesis was first described in carrot (Daucus carota) by Reinert in 1958 and Steward et.al. in 1958.
Kohlenbach (1978) has proposed the following classification of embryos.
- **Zygotic embryo:** those formed from fertilized egg or the zygote.
- **Non zygotic embryo:** those formed by cells other than zygote.
- **Somatic embryo:** those those formed by sporophytic cells either in vitro or in vivo.
- **Parthenogenetic embryo:** those formed by unfertilized egg.
- **Androgenic embryo:** those formed by male gametophyte.

Somatic embryo are structurally similar to zygotic embryos and they can be excised from parental tissue. Somatic embryos are bipolar structures with radical and plumule is contrast to monopolar shoot bud with only plumular end in organogenesis. While developing into somatic embryo, the mersitematic cells break any cytoplasmic or vascular connections with other cells around it and become isolated. Therefore, unlike shoot bud, the somatic embryos are easily separable from explants. Development of somatic embryos can be done in plant culture using somatic cells, particularly pidermis, parenchymatous cells of petioles or secondary root phloem.

Somatic embryogenesis involves three distinct steps which are absent in organogenesis:
• **Induction:** is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called embryogenic clumps (ECs). These ECs develop into initial stages of somatic embryo i.e. globular stage.

• **Maturation:** In this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped, torpedo to cotyledonary stages. The mature embryo here undergoes biochemical changes to acquire hardiness.
• **Conversion**: Embryos germinate to produce seedlings.



Somatic embryogenesis is influenced by following factors:
• **Growth regulators**: The presence of auxin (generally 2,4-D) in the medium is essential for induction phase. Tissues or calli maintained continuously in an auxin free medium generally do not form embryo, therefore, somatic embryogenesis is achieved in two steps

- First, the callus is initiated and multiplied on a medium rich in auxin (e.g. 2,4-D) which induces the differentiation of localized groups of meristematic cells called embryogenic clumps (EC).
- Second, the ECs develop into mature embryos when transferred to a medium with a very low level of auxin or no auxin at all.

Consequently, the medium with auxin is called a proliferation medium and without auxin an embryo development medium.
Maturation is achieved by culturing somatic embryos on high sucrose medium. Also, ABA is added as it gives hardening due to water loss which is important for embryo maturation.
Ethylene inhibits both somatic embryogenesis and organogenesis. Therefore, silver nitrate is added to the medium as inhibitor of ethylene for plant regeneration.
• **Nitrogen source:** Reduced form of nitrogen i.e. ammonium salts is required for somatic embryogenesis. $NH4^+$ form of nitrogen is essential for induction of somatic embryogenesis while $NO3^-$ form is required during maturation phase.
• **Other factors:** Like shoot bud differentiation, explant genotype has influence on somatic embryogenesis also. In cereals, use of maltose as carbohydrate source promotes both somatic embryo induction and maturation.
Two routes of somatic embryogenesis are known

I. Direct somatic embryogenesis

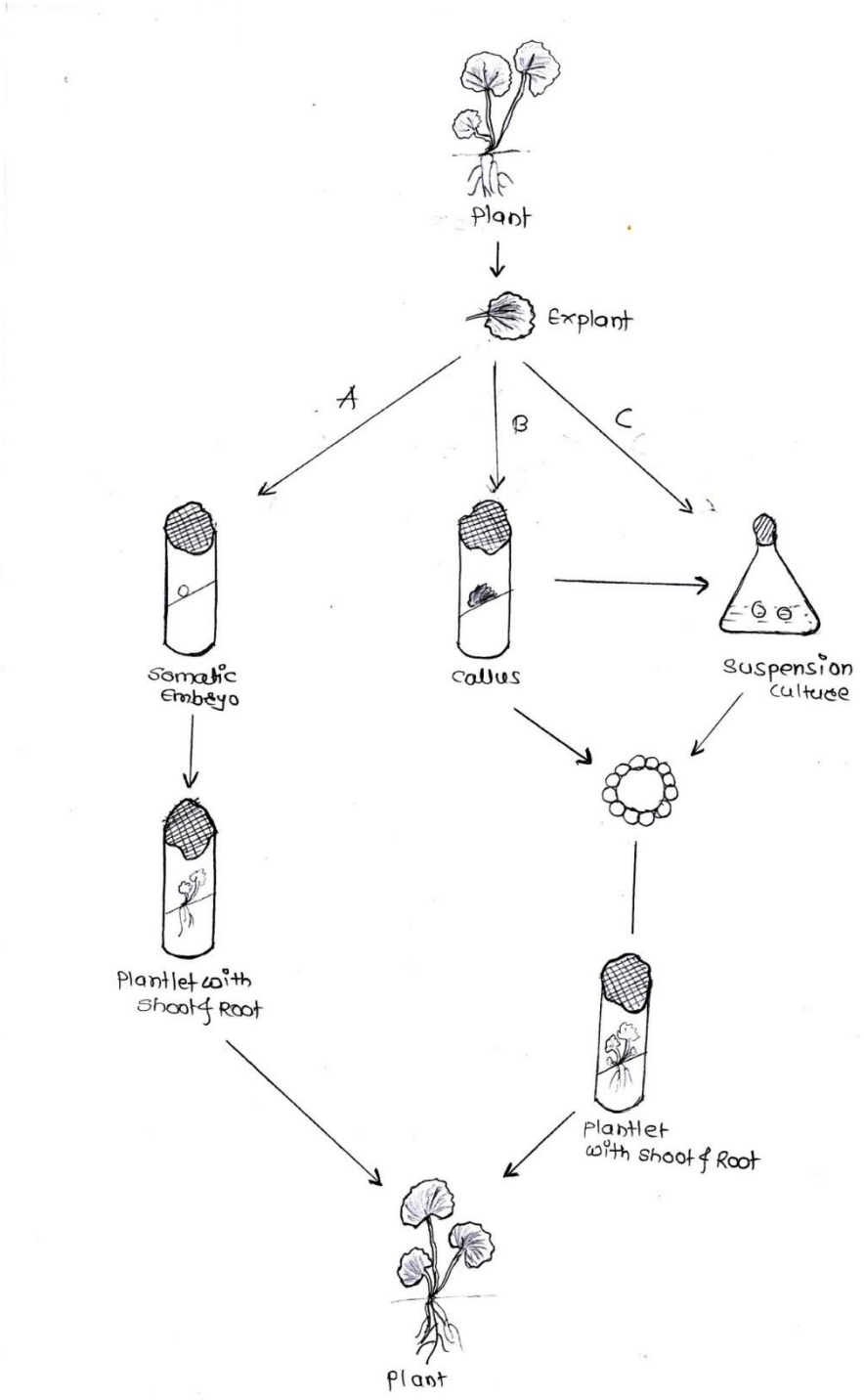


Figure: Somatic embryogenesis in plants
A- Direct Somatic embryo genesis
B- Indirect Somatic embryogenesis

In the direct somatic embryogenesis, the somatic embryo develops directly on excised plant without undergoing callus formation. This direct somatic embryogenesis is possible due to presence of pre-embryonic determined cells [PEDC]. In the pre embryonic determined cells , the embryogenic pathway is predetermined and cells appear to only wait for the synthesis of an inducer to resume independent mitotic divisions in order to express their potential. Once the embryogenic state has been reached cells proliferate as embryogenic determined cells[EDCs]. The cells may gives rise to embryoids and plantlets are then produced directly by following the full embryogenic pathway as a co-ordinated group of EDCs. By the direct somatic embryogenesis the possibility of somaclonal variations is reduced.

II. Indirect somatic embryogenesis

When the cells from explants are made to proliferate and form callus, from which cell suspension can be raised which forms somatic embryos. Induced embryogenic determined cells (IEDC) from the cell suspension forms the somatic embryos. IEDCs, require redetermination to the embryogenic state by exposure to specific growth regulators such as 2,4-D. Once the embryogenic state has been reached cells proliferate as embryogenic determined cells [EDCs]. The cells may gives rise to nodular embryogenic callus consisting of proembryoids. These embryo like structure are bipolar units and germinate into full plantlets under suitable cultures conditions.

C- Indirect Somatic embryogenesis through suspension culture

Applications of somatic embryogenesis

- Somatic embryogenesis offers immense potential to speed up the clonal propagation of plants being bipolar in nature.
- Being single cell in origin, there is a possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds.
- The bipolar nature of embryos allows their direct development into complete plantlet without the need of a rooting stage as required for plant regeneration via organogenesis.
- Epidermal single cell origins of embryos favor the use of this process for plant transformation.
- It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds.
- The production of artificial seeds using somatic embryos is an obvious choice for efficient transport and storage.
- The embryo culture technique is applied to overcome embryo abortion, seed dormancy and self-sterility in plants.

Limitations of somatic embryogenesis

- Complete conversion into plantlets or poor germination of embryos is a major limitation of somatic embryogenesis in many plants. Therefore,

the process of germination needs to be studied in detail for successful plantlet conversion.

- Compared to other plant species active research on somatic embryogenesis involving forest trees has been very slow.
- The paucity of knowledge controlling somatic embryogenesis, the synchrony of somatic embryo development and low frequency of true to type embryonic efficiency are responsible for its reduced commercial application
- To obtain a complete conversion into plantlets it is necessary to provide optimum nutritive and environmental conditions.

EXPERIMENT

Aim :-Micropropagation of Centella asiatica by using axillary bud

Equipment:-Autoclave, pH-meter, Magnetic stirrers, Magnetic beads, Weighing balance, Laminar-air-flow

Materials: Murashige and Skoog's (MS; 1962), sucrose, agar, conical flasks, measuring cylinders and beakers of various sizes. distilled water. Cotton plugs, aluminum foils, muslin cloth, scissor, media stocks, 1N NaOH, 1N HCl, myo-inositol. rubber bands, borosil glass test-tubes (150mm x 25mm without rim). Black markers, micropipette, micropipette-tips, test-tube stands, fresh of *Centella asiatica* L.

Methods

Collection of plant material

↓

Leaf explants are washed under running tap water for 15 min.

↓

Immersed in detergent (labolene) for 5 min and rinsed with distilled water for four times.

↓

The explants were deeped in 70% ethanol for 1 min.

↓

Explants were surface sterilized with 0.1% (w/v) mercuric chloride solution for 1-2 min.

↓

Thoroughly rinsed with sterile distilled water for four times.

↓

Induction of somatic embryo 2,4-D (0.5, 1.0,1.5, and 2.0 mg/L)

↓

Development of somatic embryo these calli were subcultured every 3 to 4 weeks to the same medium for the production of SEs and were kept in the dark. For further development and their germination, SEs were subcultured into hormone-free MS medium.

↓

Shoot formation KIN & BA 0.5, 1.0, 1.5, 2.0mg/L with combination of 0.2 mg/l IAA

Root formation IAA 1.5, 2.0, mg/L & NAA 2.0 mg/L

↓

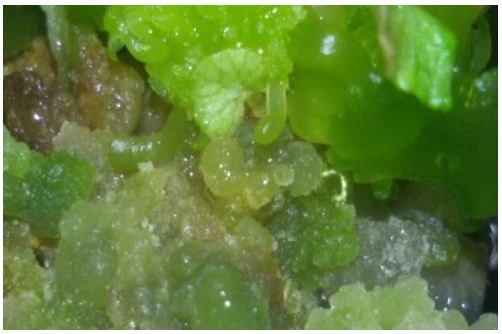
The cultures were incubated at 25 ± 2°C & light period (16 hours light and 8 hours dark).

Results

Somatic embryos were induced on leaf explants when cultured on MS basal medium supplemented with 2,4-D. Onset of nodular callus and

development of SEs was observed on explants after 3 to 4 weeks of culturing in the dark. Younger unfurled leaves showed

Photoplate 1 Callus formation



Photoplate 2 Induction of somatic embryogenesis

Photoplate3 & 4 Regeneration from somatic embryoides

Photoplate5 & 6 Regeneration of plant

Techniques of Micropropagation

The following processes are employed for micropropagation.

Stage 0- Source of explants-

This is the initial step of micropropagation, & involves the selection& growth of stocks plants for about 3 months under controlled condition.

Stage I- Establishment of aseptic cultures:-

The process of micropropagation starts with the establishment of aseptic cultures. The choice of explants depends upon the pathway of micropropagation and objective to be achieved. In case of stimulatory shoot bud proliferation, nodal segment from sexually mature or seedling material are to be used. To induce adventitious shoot buds, somatic embryogenesis or callus, the explants such as a stem or hypocotyls segments cotyledon or leaves etc. are used.

Explants have to be treated with suitable chemosterilant to make them free from contamination. For each type of explants and plant species, a procedure of surface sterilization in order to obtain high percentage of aseptic but most proliferating cultures. While standardizing the procedure the TCDC formula is applied.

Where,

T- Selection of the type of chemical to be used.

C- Effective concentration of the selected chemical.

D- The required duration of the treatment.

C- Combination of the treatment if required

While standardizing the procedure, it is ensured that the treatment is effective enough to kill the contaminants while its concentration should not be toxic to the proliferating explants.

Stage II-Multiplication stage:

The shoot produced via micropropagation pathway can be multiplied for an indefinite period by repeated subculturing. In case of somatic

embryogenesis both shoot and root develop simultaneously and therefore, there is no separate phase of shoot multiplication and rooting.
Cytokinins are generally incorporated individually or in combination with small quantity of auxin to stimulate shoot multiplication. BAP (6- Benzyl Amino Purine) has been considered to be a better cytokinin as compare to other.
The period of subculture depends upon the plant species and pace of the growth of shoots. The rate of shoot multiplication depends upon how readily the axillary meristem respond to an available cytokinin.
The prolonged use of cytokinin in the medium though induce, large number of shoots may become hyperhydric (glassy in appearance) and remain stunted and are not suitable for rooting. In order to obtain longer shoots, the multiplying shoots are sub cultured on low concentration of cytokinin for at least one passage at a terminal phase prior to rooting.

Stage III-Rooting or preplant stage:

In this phase, shoots of suitable size are excised from multiplying shoot culture and inoculated on a medium containing root inducing hormone. Auxin like Indole-3- butyric acid [IAA], Indole-3- acetic acid [IAA], Naphthalene acetic acid [NAA] are generally used in their various concentration and combination to induce roots in vitro developed shoots.

Stage IV- Hardening and Acclimatization:

Hardening is a step in which a tissue culture plant is made to harden with respect to physiological disorders which it develops in cultures. Hardening and Acclimatization are the, most important and critical step to achieve success in micropropagation. The plants grow in tissue culture have became adapted to laboratory environment. In culture room, the light intensity is very low as compared to external environment while the temperature is maintained around 25-28^0 C for 24 hours and throughout 3the time period. Moreover, the plants in culture, because of aseptic conditions are not in direct conflict with microbes and therefore face no competition. All these factors together, make tissue culture plants adapted to laboratory conditions. Such plants when transferred ex vitro cannot withstand the transplantation and die. In order to solve this problem, the culture plants are subjected to gradual and systematic. Hardening and acclimatization procedures enabling them to establish in soil successfully. In the beginning, the plants after their removal from the rooting medium are transferred in culture bottles containing soil rite which is moistened with nutrient solution of reduced salt concentration and kept closed in culture room in aseptic environment. By doing so, the tissue culture plants become normal. Such bottles are subsequently shifted to greenhouse environment, which maintains a reducing humidity and temperature 28-30^{0}C and light intensity.

Micropropagation mostly in vitro clonal propagation by two approaches.

1. Multiplication by axillary buds/ apical shoots.
2. Multiplication by adventitious shoots.

1. Multiplication by axillary buds/ apical shoots:-

Axillary and apical shoots contain quiescent or active meristem depending on the physiological state of the plant. The axillary buds located in the axils of leaves are capable of develop in into shoots. In the in vivo state, however only in limited number of axillary meristems an form shoots. By means of induced in vitro multiplication in micropropagation, it is possible to develop plants from meristem and shoot tip culture.

Apical meristem is a dome of tissue located at the extreme tip of shoot. The apical meristem along with the young leaf primordial constitutes the shoot apex. Meristem tips should be cultured.

Meristem or shoot tip is located from a stem by a V shaped cut. The size of explant is critical for culture. In general, the larger the explant the better are the chances of survival.

The most widely used media for meristem culture are MS medium or Whites medium. Addition of growth regulators namely cytokinin and auxin will support the growth and development. High level of cytokinin requires for the axillary shoot proliferation and for root formation low cytokinin and high level of auxin is required.

Apical bud culture

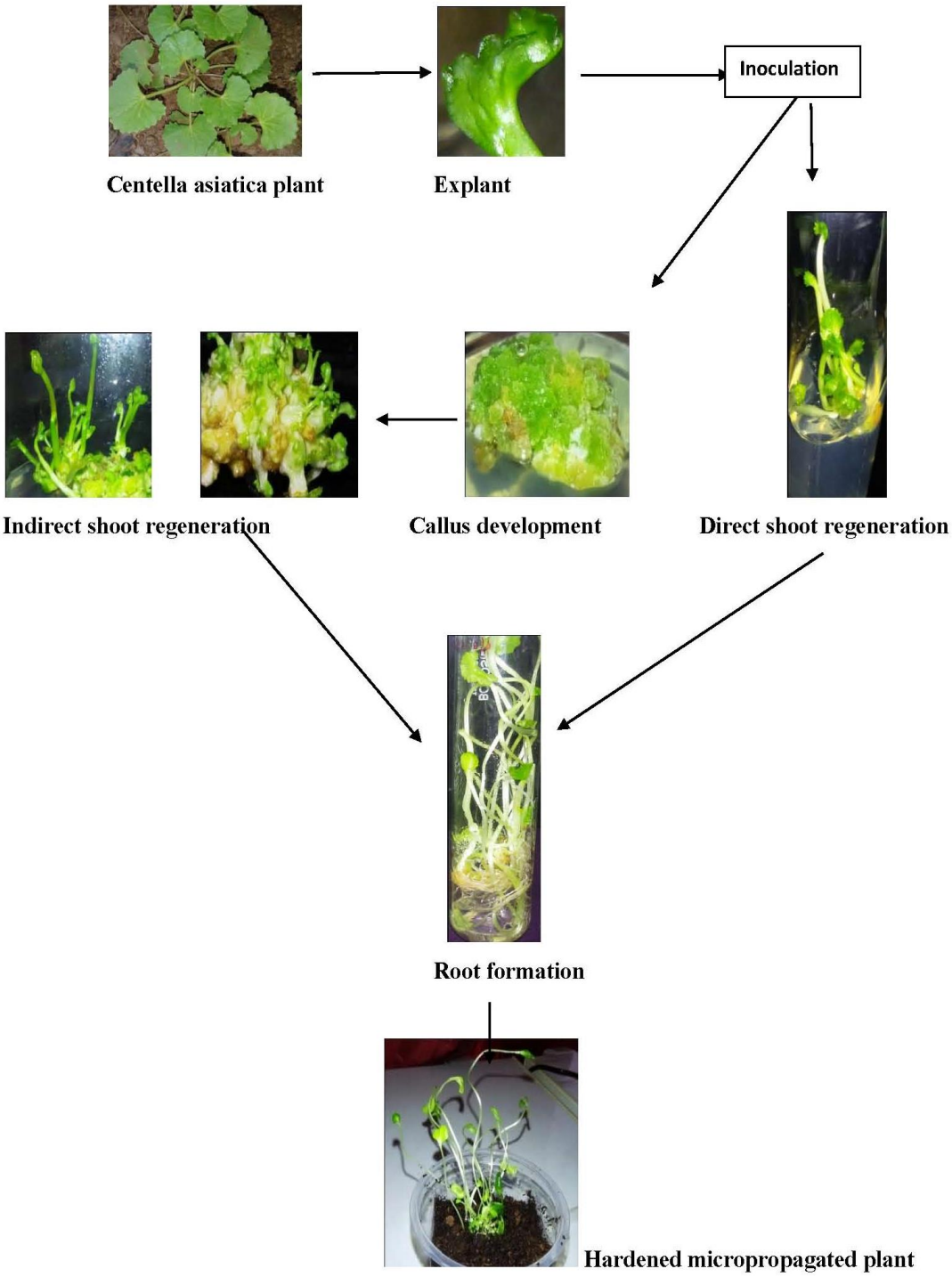
Axillary buds are usually present in the axil of each leaf and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods. The species with strong apical dominance show the growth of axillary buds into shoot only if the terminal bud is removed or injured. Two types of bud culture are used

- Single node culture
- Axillary bud culture
- **Single node culture**

This is a natural method for vegetative propagation of plants both in vivo and in vitro conditions. The bud found in the axil of leaf is comparable to the stem tip, for its ability in micropropagation. A bud along with the piece of stem is isolated and cultured to develop into a plantlet. Closed buds are used to reduce the chances of infections.

- **Axillary bud culture**

In this method, a shoot tip along with axillary bud is isolated. The cultures are carried out with high concentration. In plant tissue culture, the rate of shoot multiplication can be determined by enhanced axillary branching. As medium is supplemented with cytokinin, the axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explants transformed into a mass of branches. There is a limit to which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, if shoots are excised and planted on a fresh medium of same composition, the shoot multiplication cycle can be repeated. This process can go on indefinitely, and can be maintained throughout the year independent of the season and the region.


Inoculation
Centella asiatica plant
Explant
Indirect shoot regeneration
Callus development
Direct shoot regeneration
Root formation
Hardened micropropagated plant

2. Multiplication by adventitious shoots.

Adventitious shoot proliferation in plant cell and tissue culture, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognizable organs. Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase. Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication (Marcotrigiano and Jagannathan 1988; Thorpe et al. 1991). Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation. Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

Methods of Micropropagation	**Stage of culture**		
	I. Initiating a culture Growth of excited tissues/organs *in vitro* free from algae, bacteria, fungi and other contaminants	**II. Increasing propagules** Growth of excited tissues/organs *in vitro* free from algae, bacteria, fungi and other contaminants	**III. Preparation for soil transfer** Separating and preparing propagules to have a high rate of survival as individual plants in the external environment
Shoot Cultures	Transfer of disinfected shoot tips or lateral buds to solid or liquid media and the commencement of shoot growth to *ca.* 10mm.	Induce multiple (axillary) shoot formation and growth of the shoots to a sufficient size for separation, either as new Stage II explants or for passage to III.	Elongation of buds formed at Stage II to uniform shoots. Rooting the shoots in vitro or outside the culture vessel.

Meristem culture	Transfer of very small shoot tips (length 0.2-0.5mm) to culture. Longer shoot tips (1-2mm) can be used as explants if obtained from heat treated plants.	Growth of shoots to *ca.* 10mm, then as shoot tip culture, or as shoot multiplication omitted and shoots transferred to Stage III.	As for shoot tip cultures.
Direct shoot regeneration from explants	Establishing suitable explants of mother plant tissue (e.g. leaf or stem segments) in culture without contamination.	The induction of shoots directly on the explant with no prior formation of callus. Shoots so formed can usually be divided and used as explants for new Stage II subcultures or shoot tip culture.	As for shoot tip cultures.
Indirect shoot regeneration from morphogenic callus	Initiation and isolation of callus with superficial shoot meristems	Repeated subculture of small callus pieces followed by transfer to a shoot-inducing medium. The growth of shoots *ca.* 10mm in length.	Individual shoots are grown and rooted.
Indirect embryogenesis from embryogenetic callus or suspension cultures	Initiation and isolation of callus with the capacity to form somatic embryos, OR obtaining embryogenic suspension cultures from embryogenic callus or by *de*	Subculture of the embryogenic callus or suspension culture followed by transfer to a medium favoring embryo development.	Growth of the somatic embryos into "Seedlings".

	novo induction.		
Direct embryogenesis	Establishing suitable embryogenic tissue explants or previously-formed somatic embryos.	The direct induction of somatic embryos on the explants without prior formation of callus.	Growth of the embryos into plantlets which can be transferred to the outside environment.

APPLICATION OF MICROPROPAGATION

1. Production of disease free plant

Meristem cultures are generally employed to develop pathogen free plant. Eg. Micropropagation is successful used for the production of virus free plants of sweet potato, cassava, etc.

2. High rate of plant propagation

A large number of plant can be grown from a piece of plant tissue within a short period. It can be carried out throughout the year, i.e. irrespective of seasonal. Through micropropagation propagule can be stored (germ plasm storage) and transported across international boundries.

3. Production of seeds in some crops

Micropropagation through axillary bud proliferation method is suitable for seed production in some plants.

4. Micropropagation is cost effective process.

Trouble shooting

- Few explants exude dark colored compounds, like phenols, pigments etc which leach into the medium from the cut ends of the explant. It results in the browning of tissues and the medium as well. The browning of medium is associated with poor culture establishment and low regeneration capacity of the explants. This can be overcome by:

1. Minimizing the wounding of explants during isolation and surface disinfection to reduce this browning response.
2. Washing or incubation of explants for 3-5 hrs in sterile distilled water to remove phenolics responsible for browning of medium or explants.
3. Frequent subculture of explants with excision to fresh medium at regular intervals.
4. Initial establishment of cultures in liquid medium and later transfer to the semi-solid medium.
5. Culture of explants on porous substrate or paper bridges.
6. Addition of activated charcoal (AC) or polyvinylpyrrolidone (PVP) for adsorbtion of phenolics.
7. Antioxidants like ascorbic acid, citric acid etc. can also be used to prevent browning of tissues in culture.

- Appearance of vitrified tissues (hyperhydricity), a physiological disorder occurring in the *in vitro* cultures due to which the tissues look transparent and fluffy resulting from excessive intake of water. Hyperhydricity can be caused by a high concentration of cytokinin or low concentration of gelling agent or high water retention capacity of explants if the container is tightly closed.
- Loss of regeneration ability in long-term cultures due to epigenetic variations (temporary variations) and culture aging, including transition from juvenile to mature stage. Epigenetic variation are phenotypic temporary variations which disappear as soon as the culture conditions are removed.
- Genotypic variations are also seen in the cultures, therefore, cytological, biochemical and molecular analyses are required to confirm clonal fidelity of *in vitro* regenerants. Besides, morphological and physiological testing is also required to remove undesired genetic variability.

EXPERIMENT

Aim :-Micropropagation of Centella asiatica by using axillary bud

Equipment:-Autoclave, pH-meter, Magnetic stirrers, Magnetic beads, Weighing balance, Laminar-air-flow

Materials: Murashige and Skoog's (MS; 1962), sucrose, agar, conical flasks, measuring cylinders and beakers of various sizes. distilled water. Cotton plugs, aluminum foils, muslin cloth, scissor, media stocks, 1N NaOH, 1N HCl, myo-inositol. rubber bands, borosil glass test-tubes (150mm x 25mm without rim). Black markers, micropipette, micropipette-tips, test-tube stands, fresh of *Centella asiatica* L.

Methods

Collection of plant material

↓

Leaf explants are washed under running tap water for 15 min.

↓

Immersed in detergent (labolene) for 5 min and rinsed with distilled water for four times.

↓

The explants were deeped in 70% ethanol for 1 min.

↓

Explants were surface sterilized with 0.1% (w/v) mercuric chloride solution for 1-2 min.

↓

Thoroughly rinsed with sterile distilled water for four times.

⬇

Nodal explants were inoculated on MS medium supplemented with various concentrations of **KIN [0.5, 1.0, 1.5, 2.0 mg/L]**or **BA [0.5, 1.0, 1.5, 2.0 mg/L]** individually or in combination **[BA 0.5, 1.0, 1.5, 2.0 mg/L & KIN 0.5 mg/L each]** & **IAA [0.5, 1.5, 2.5 mg/L] or NAA [0.5, 1.5, 2.5 mg/L]** individually or in combination **[IAA 0.5, 1.5, 2.5 mg/L & NAA 0.5mg/L each]**

⬇

The cultures were incubated at 25 ± 2°C & light period (16 hours light and 8 hours dark).

Results

The 1st treatment of BA (0.5-2.0 mg/l) along with IAA resulted in shoot as well as root induction. Maximum shoot numbers per explant (11.66±0.56) was recorded, when MS media supplemented with 2 mg/L BA + 0.2 mg/L IAA, followed by 1.5 mg/L BA + 0.2 mg/L IAA (6.66±0.88), which were significantly higher than others, while maximum shoot length (5.76±0.42) was observed on MS media supplemented with 2.0 mg/LBA + 0.2 mg/L of IAA, which was significantly higher than others (Table I). The 2nd treatment of KIN (0.5-2.0 mg/l) in combination with IAA (0.2mg/L) results in maximum number of shoots than BA.

Fig.A

Fig.B

Fig. C

Fig. D

Fig. E

Fig. F

Figs A-F A. initiation of shoot from the explant. B. multiplication of shoots. C&D. multiplication of shoot and basal callus formation. E. initiation of roots from regenerated shoots. F. induction of rooting and basal callus formation

EXPERIMENT -2

Aim :-Micropropagation of Centella asiatica by using Leaf explant

Equipment:-Autoclave, pH-meter, Magnetic stirrers, Magnetic beads, Weighing balance, Laminar-air-flow

Materials: Murashige and Skoog's (MS; 1962), sucrose, agar, conical flasks, measuring cylinders and beakers of various sizes. distilled water. Cotton plugs, aluminum foils, muslin cloth, scissor, media stocks, 1N NaOH, 1N HCl, myo-inositol. rubber bands, borosil glass test-tubes (150mm x 25mm without rim). Black markers, micropipette, micropipette-tips, test-tube stands, fresh of *Centella asiatica* L.

Methods

Collection of plant material

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Leaf explants are washed under running tap water for 15 min.

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Immersed in detergent (labolene) for 5 min and rinsed with distilled water for four times.

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The explants were deeped in 70% ethanol for 1 min.

↓

Explants were surface sterilized with 0.1% (w/v) mercuric chloride solution for 1-2 min.

↓

Thoroughly rinsed with sterile distilled water for four times.

↓

Leaf explants inoculated on MS medium supplemented with various concentrations of KIN & BA 1.8, 2.0 mg/l, with combination of 1 mg/l each IBA and IAA gives maximum growth.

↓

Incubate the cultures at 25 ± 2°C temperature and 50-60% relative humidity under a 16/8 hour (light/dark) photoperiod with diffuse light (1000-2000 lux).

↓

Observe the cultures at regular intervals for adventitious shoot proliferation directly or indirectly via callus proliferation, and remove the

contaminated culture, if any. Record the time of shoot proliferation, number of shoots per culture and the size of each shoot in single culture.

Results

Highest shoot multiplication is observed 2.0 mg/L BA in combination of 1 mg/L IBA & IAA. However, root initiation was achieved from the bases of excised shoots in the presence of IAA or NAA. The 1.5 mg/L IAA produced maximum number of roots after 2-3 weeks incubation with basal callusing

Fig: A

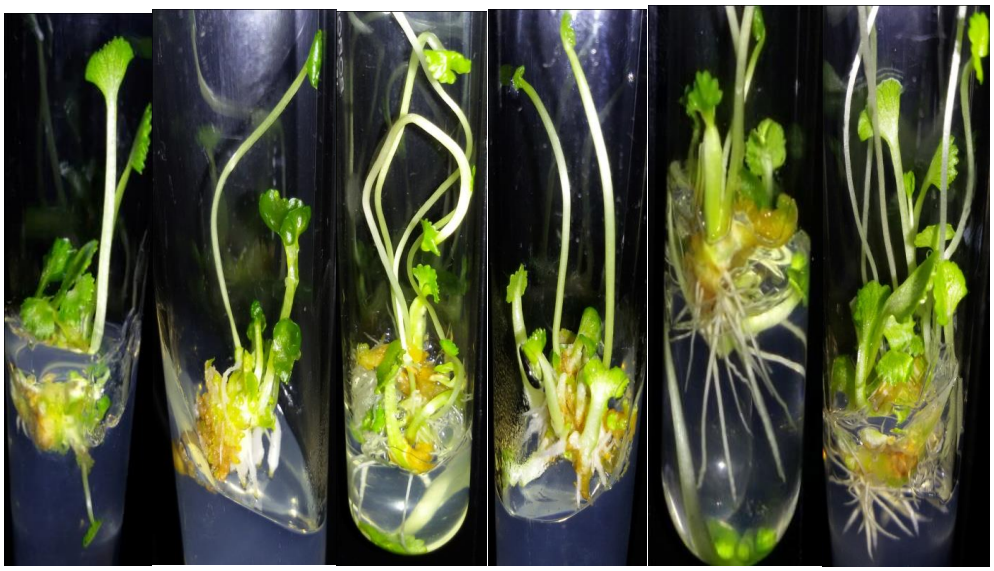


Fig: B Fig:C Fig:D Fig:E Fig:F Fig:G

Figs A-G A.Callus formation B. initiation of shoot from the explant. B. multiplication of shoots. C&D. multiplication of shoot and basal callus formation. And intiation of roots E. initiation of roots from regenerated shoots. F. induction of rooting and basal callus formation G. multiplication of roots.

Chapter...9

PROTOPLAST TECHNOLOGY

Protoplast is naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components. The absence of cellulosic cell wall permits advantages to fuse protoplast of similar or on different species and the fused product can regenerate into whole plant. This process is called somatic hybridization.

Method of isolating plant protoplasts have made significant advantage during recent years and protoplasts have now been obtained from whole plant organ or tissue culture of many different species. Techniques have also been developed to enable isolated protoplast to be cultured so that by growth of a cell wall followed by cell division, they may give rise to callus and then to whole plant

HISTORY

The term protoplast was introduced in 1880 by Hanstein. The first isolation of protoplast was achieved by Klercker (1892) employing mechanical method.

In 1960, Coking used the enzyamatic method for isolation of protoplast.

Takabe and co worker in 1971 were successful to achieve the regeneration of whole tobacco plant from protoplast.

IMPORTANCE OF PROTOPLAST

The isolation, culture and fusion of protoplast is fascinating field in plant research. Protoplasts have a wide range of application. These are as follows

1. Hybrid can be produced from protoplast fusion.
2. Protoplast helps in crop improvement by somatic hybridization.
3. Protoplast can regenerate in whole plant when cultured on suitable medium.
4. Protoplast is used in the establishment of cybrids through fusion.
5. The isolated protoplasts are also having the tendency to uptake the foreign gene from the culture.
6. It is easy to perform single cell cloning with protoplasts.
7. Genetic transformation can be achieved through genetic engineering of protoplast DNA.
8. Isolation of cell organelles and chromosomes is easy from protoplasts.

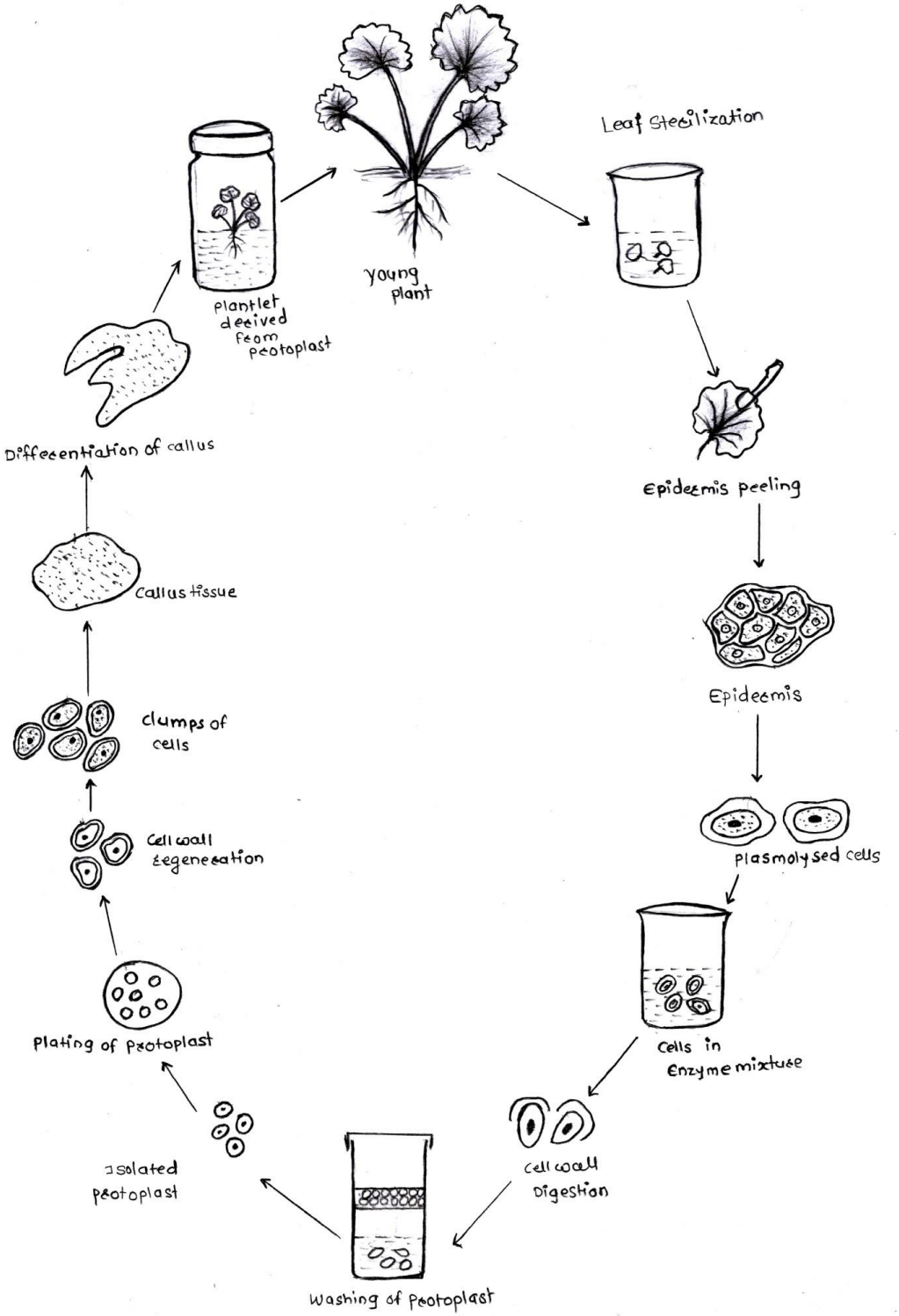


Fig: Steps involved in Protoplast

ISOLATION OF PROTOPLAST

Source:- Protoplast can be isolated from almost all plant parts such as roots, leaves, fruits, tubers, root nodule, endosperm, pollen mother cell, etc. but the most convenient and popular source of plant protoplasts is the leaf, as the mesophyll cells are loosely arranged and the chemical used for isolation have easy access to the cells.

Protoplast are isolated by two techniques-
A] Mechanical method
B] Enzymatic method

A] MECHANICAL METHOD

In this method the cell walls are mechanically removed from the cell with the help of scissors, needles, and forceps, etc.

Protoplast isolation by mechanical method is crude and tedious procedure. This method yields small number of protoplast as compare to enzymatic method.

The isolation technique involves following steps,

1. A small piece of epidermis from plant s selected.
2. It must be kept in a hypertonic solution for a few minutes which causes the plamolysis. Plasmolysis results protoplasts to shrink away from the cell walls.
3. The cell wall of plasmolysed cells are broken with the help of micro scalpel and needles.
4. The resulting protoplasts are separated from the cell wall material.

This method has some disadvantages over enzymatic isolation method. These are

- It yields small number of protoplast.
- This method is laborious and tedious

B] ENZYMATIC METHOD

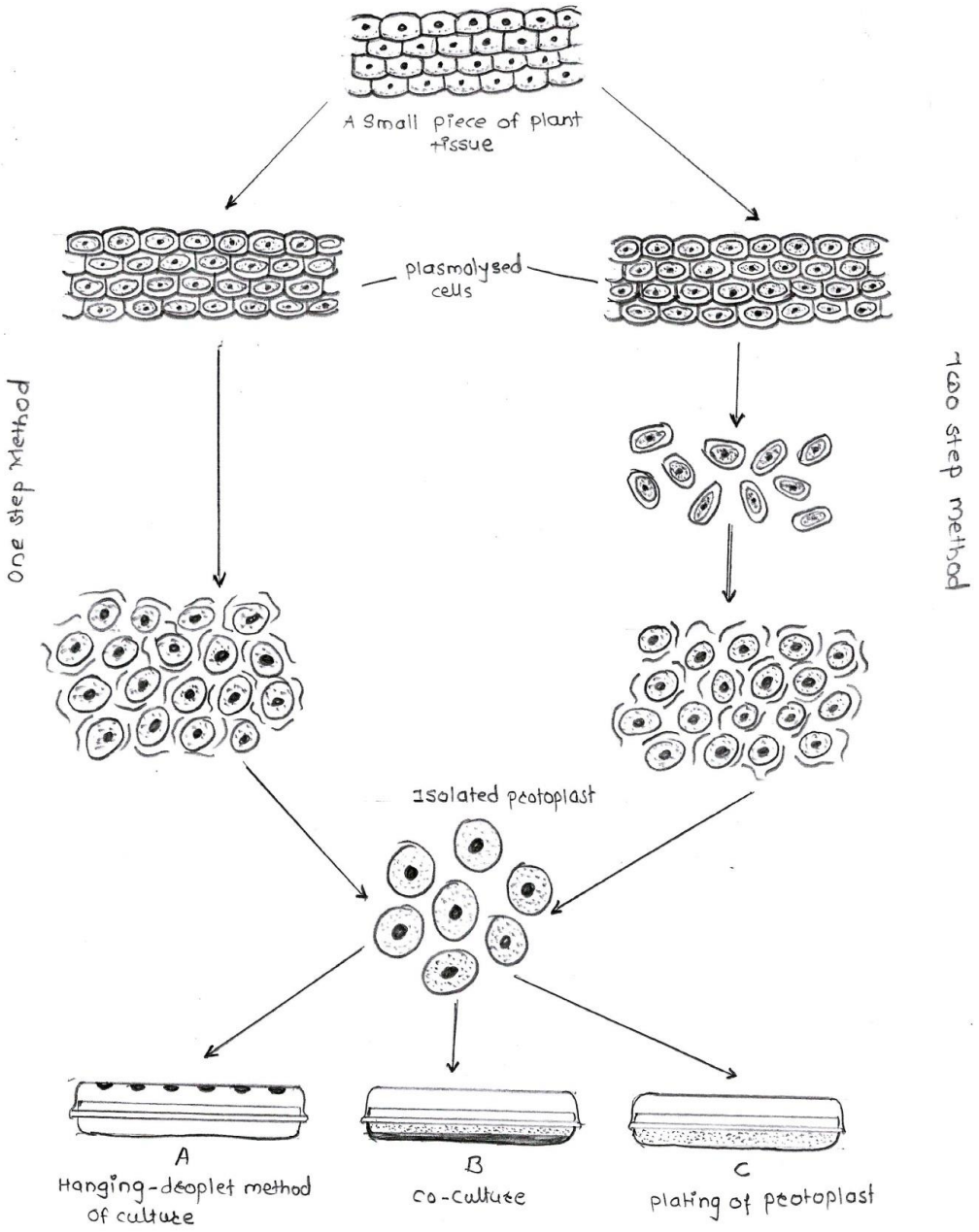
In this method protoplasts are produced by treating the cells with an enzyme mixture which digest the cell wall of plant cells. Enzymatic method is a very widely used technique for isolation of protoplast. Enzymatic method generate very large number of protoplasts in comparison to mechanical methods with very less damage to the cells and lesser shrinkage to the protoplast.

This method has some advantages over mechanical method.

- Cell is not damaged
- Yield large amount of undamaged protoplasts
- Osmotic shrinkage of protoplast is much less.

Enzyme for protoplast isolation

The physiological state of the leaves is extremely important for the successful isolation of protoplasts from the leaves. The leaves normally should be fully expanded and from young plants. The lower epidermis is removed and is used to isolate epidermal and quard cell protoplasts.



The remaining peeled leaf is used for isolation of mesophyll protoplast. Plant cell wall mainly composed of cellulose, pectin & hemicelluloses. These can be degraded by enzyme cellulose, pectinase & hemicellulase respectively. Enzymes are usually used at a pH 4.5 to 6.0, temperature 25 to 30^0 C.

An enzymatic isolation of protoplasts can be carried out by two apparatus.

1. Two step or sequential method.
2. One step or simultaneous method.

1. Two step method

In this method, the cells are treated with two enzyme mixture for isolation of protoplasts. The tissue is firstly treated with macerozyme (pectinase) which break cell aggregates into individual cells. Then these free cells are exposed to cellulose enzyme which removes cell wall.

2. One step method

This is the preferred method for protoplast isolation. It involves the simultaneous use of both the enzymes- macerozyme and cellulose.

PROTOCOL FOR ISOLATION OF PROTOPLAST

The process of isolating the protoplast from leaves involves number of steps. These includes

a. Surface sterilization of leaves
b. Removal of epidermis
c. Enzymatic treatment

Surface sterilization of leaves

- The leaves selected for experiment should be from young plants.
- The selected young leaves are surface sterilized. The leaves are dipped into 70% ethanol for 1 minute and then in 2 % solution of sodium hypochlorite for 20- 30 min.
- Then it is washed with sterile distilled water for 3-4 times to remove traces of sodium hypochlorite.

Removal of epidermis

- After surface sterilization the lower epidermal layer of the leaf is carefully peeled off and the mesophyll tissue is cut into small pieces

Enzymatic treatment

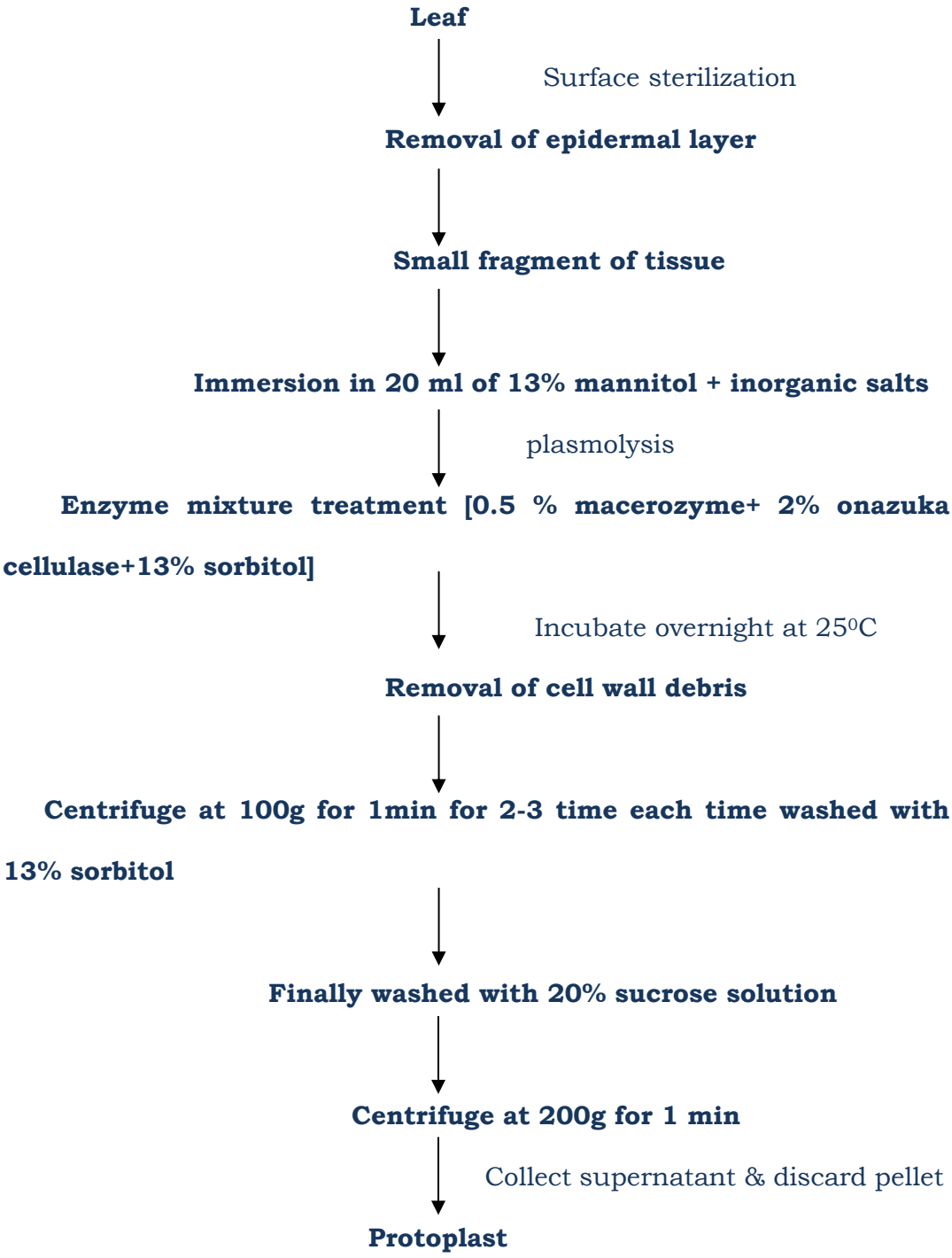
The protoplast is generally isolated by enzymatic treatment by two approaches.

A] One step / sequential method

In the sequential method only one type of enzyme mixture is used for isolation of protoplast so it is called one step method.

Steps

- The small leaf segments are kept immersed in 20 ml solution of 13% mannitol and inorganic salts for one hour.
- The plasmolysed leaf segments are dipped in an enzyme mixture which contains 0.5 % macerozyme, 2 % onazuka cellulase and 13% sorbitol and mannitol. The pH of enzyme mixture adjusted to pH 5.4. This enzyme mixture and leaf segment incubated overnight at 25^0C.
- After incubation, leaf segments gently teased to liberate the protoplasts. This mixture is filtered through fine wire gauze to remove cell wall debris from the enzyme mixture.
- Centrifuge the solution at 100g for 1 min this is repeated for 2-3 times and protoplasts are washed with 13% sorbitol solution.
- For final washing, 20% sucrose solution is used in place of sorbitol and centrifuge at 200g for 1 min.
- The protoplast is present in supernatant.
- The protoplasts are carefully separated from the tube and in suitable place for further use.


Leaf
Surface sterilization
Removal of epidermal layer
Small fragment of tissue
Immersion in 20 ml of 13% mannitol + inorganic salts
plasmolysis
Enzyme mixture treatment [0.5 % macerozyme+ 2% onazuka cellulase+13% sorbitol]
Incubate overnight at 25⁰C
Removal of cell wall debris
Centrifuge at 100g for 1min for 2-3 time each time washed with 13% sorbitol
Finally washed with 20% sucrose solution
Centrifuge at 200g for 1 min
Collect supernatant & discard pellet
Protoplast

B] Two step/ simultaneous method

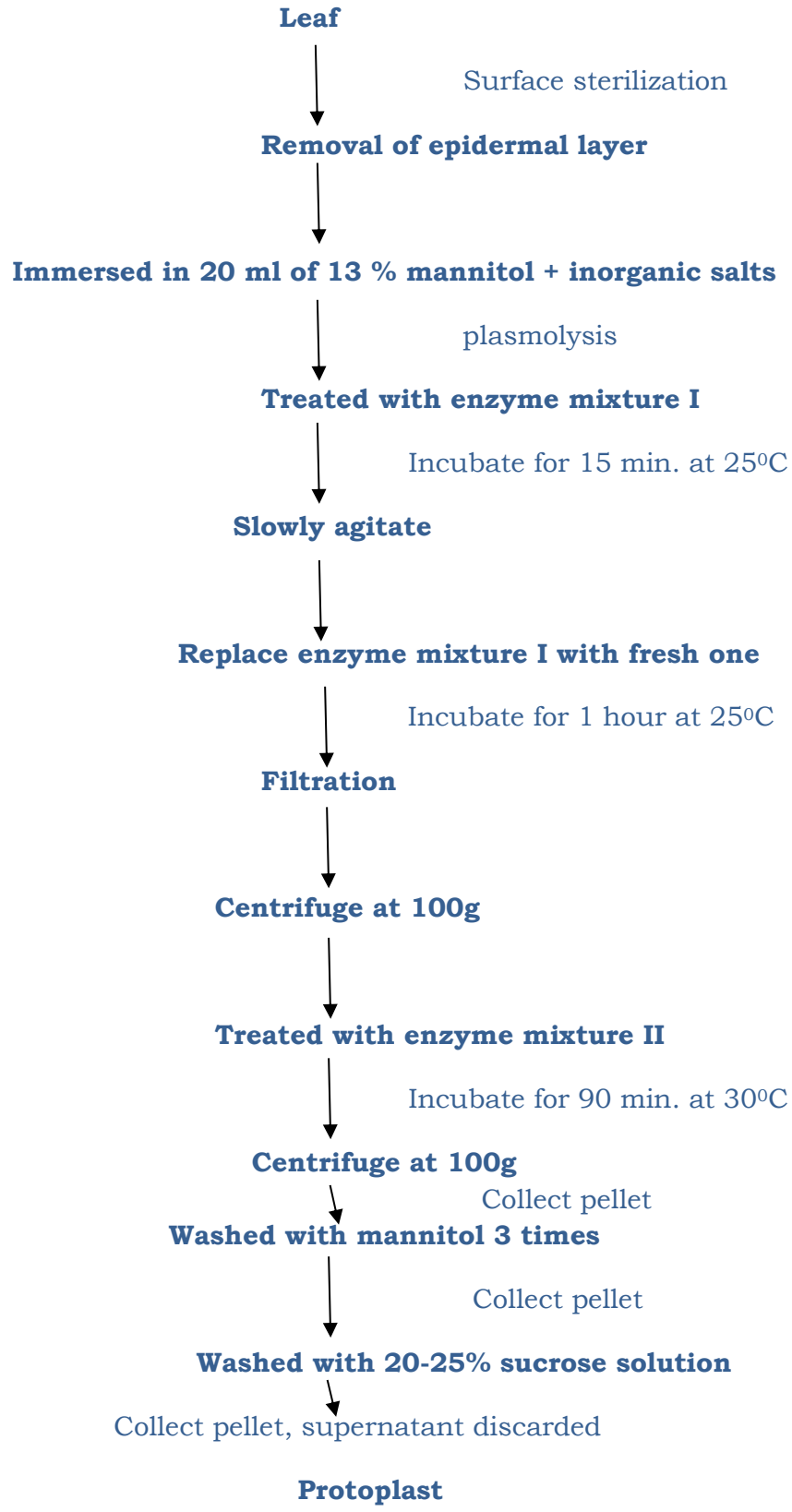
In this method, the cells are treated with two enzyme mixture for isolation of protoplast.

Enzyme mixture I- 0.5 % macerozyme+ 0.3 potassium dextran sulphate+13% mannitol (pH 5.8)

Enzyme mixture II- 2% cellulase+13% mannitol (pH 5.4)

Steps

- Collect the leaves and surface sterilized.
- Removal of epidermal cells from the leaves and leaves cut into small pieces.
- Pieces kept in hypertonic solution for plasmolysis.
- The leaf segment with plasmolysed ells treated with enzyme mixture I for 15 min. at 25^0 C.
- Agitate slowly.
- After sometime, the enzyme mixture is replaced by fresh one and is again incubated for one hour. By this process cells are completely separated.
- The isolated cells are filtered, then centrifuge at 100g for 1 min.
- Isolated cells are now treated with an enzyme mixture II for 90 min. at 30^0C and then centrifuge at 100g for 1 min.
- Supernatant is discarded and the protoplast are washed 3 times with mannitol
- The protoplasts are finally cleaned with 20-25 % sucrose solution


Leaf
Surface sterilization
Removal of epidermal layer
Immersed in 20 ml of 13 % mannitol + inorganic salts
plasmolysis
Treated with enzyme mixture I
Incubate for 15 min. at 25⁰C
Slowly agitate
Replace enzyme mixture I with fresh one
Incubate for 1 hour at 25⁰C
Filtration
Centrifuge at 100g
Treated with enzyme mixture II
Incubate for 90 min. at 30⁰C
Centrifuge at 100g
Collect pellet
Washed with mannitol 3 times
Collect pellet
Washed with 20-25% sucrose solution
Collect pellet, supernatant discarded
Protoplast

REGENERATION AND CULTURE OF PROTOPLAST

The first step of protoplast culture is the development of cell wall around the cell membrane. Protoplasts are cultured either in semisolid agar or liquid medium.

1. Agar culture

In this method the plating of protoplast is carried out by Bergmann's cell plating method. In agar culture, protoplasts remain in a fixed position, divide and form clones.

Bergmann's cell plating method:-

The Bergmann's cell plating method developed by Bergmann in 1960 for cloning of single cells.

The cell suspension is filtered through a sieve to obtain a single cells in the filtrate. The free cells are suspended in a liquid medium, at a density twice than the required density for cell plating. Mow equal volume of melted agar and medium containing cells are mixed. The agar medium with single cells is poured and spread out in apetridishes are sealed with parafilm and incubated at 25^0C in dark or diffused light. The single cells divide and develop into clones.

2. Liquid culture

It is preferred method for protoplast culture. Osmotic pressure of liquid medium can be altered as desired.

It is easy to dilute and transfer.

CULTURE MEDIA

In general the nutritional requirement of protoplast is similar to those of cultured plant cells. Some variation in protoplast culture media are

- The medium should be devoid of ammonium and the quantities of iron and zinc should be less.
- Calcium concentration should be higher than standard tissue culture media.
- To induce cell division high auxin/kinetin ratio is used. For regeneration high kinetin /auxin ratio.

Osmoticum and osmotic pressure

During the isolation process, there is the possibility of bursting of isolated protoplast due to their osmotic fragility. To prevent bursting the isolation and culture of protoplast require osmotic protection until they develop a strong cell wall without osmotic protection they will burst.

To induce the osmotic pressure some reagents or chemicals are added which is referred as osmoticum. The osmoticum are of two types

1. **Non ionic osmoticum**: e.g. mannitol, sorbitol, glucose, fructose, galactose and sucrose.
2. **Ionic osmoticum**: e.g. potassium chloride, calcium chloride and magnesium phosphate.

   Use of both type of osmoticum gives advantage. As growth of protoplasts, cell wall generation occur.

Culture methods

- ✓ **Microdrop culture**

For Microdrop culture, specially designed dishes namely cuprak dishes with outer and inner chambers are used. The inner chamber carries several wells wherein the individual protoplast in outer chamber is filled with water to maintain humidity. This method allows the culture of fewer protoplasts for droplet of the medium.

- ✓ **Embedded in Agar/ Agarose :** Agarose is a preferred choice in place of agar and this has improved the culture response. This method of agar culture keeps protoplast in fixed position, thus, prevents it from forming clumps. Immobilized protoplasts give rise to clones which can then be transferred to other media. In practice, the protoplasts suspended in molten (40°C) agarose medium (1.2% w/v agarose) are dispensed (4ml) into small (3.5-5cm diameter) plates and allowed to solidify. The agarose layer is then cut into 4 equal sized blocks and transferred to larger dishes (9 cm diameter) containing liquid medium of otherwise the same composition. Alternatively, protoplasts in molten agarose medium are dispensed as droplets (50-100 µl) on the bottom of petri plates and after solidification the droplets are submerged in the same liquid medium.
- ✓ **Feeder layer technique**

  Fast growing protoplasts are sometimes made mitotically blocked protoplast by low doses of X- ray treatment. Such irradiated protoplasts are plated with agar medium. Upon this thin solidified layer of irradiated protoplast, desirable protoplasts are again plated at a low density with agar medium. As result, it makes two agar layers containing irradiated protoplast in lower layer and desirable protoplast in lower in upper layer. The lower layer irradiated protoplast is known as feeder layer which improves the growth and development of normal protoplasts even at lower density.

- ✓ **Co-culture of protoplasts**

  Protoplast from two different species haval so been co- cultured to promote their growth or that of the hybrid cells. Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together so that there is cross-feeding between the two types. This enables the protoplasts or cells at low density to undergo sustained divisions. The co-culture method is generally used where calli arising from two types of protoplasts can be morphologically distinguished.

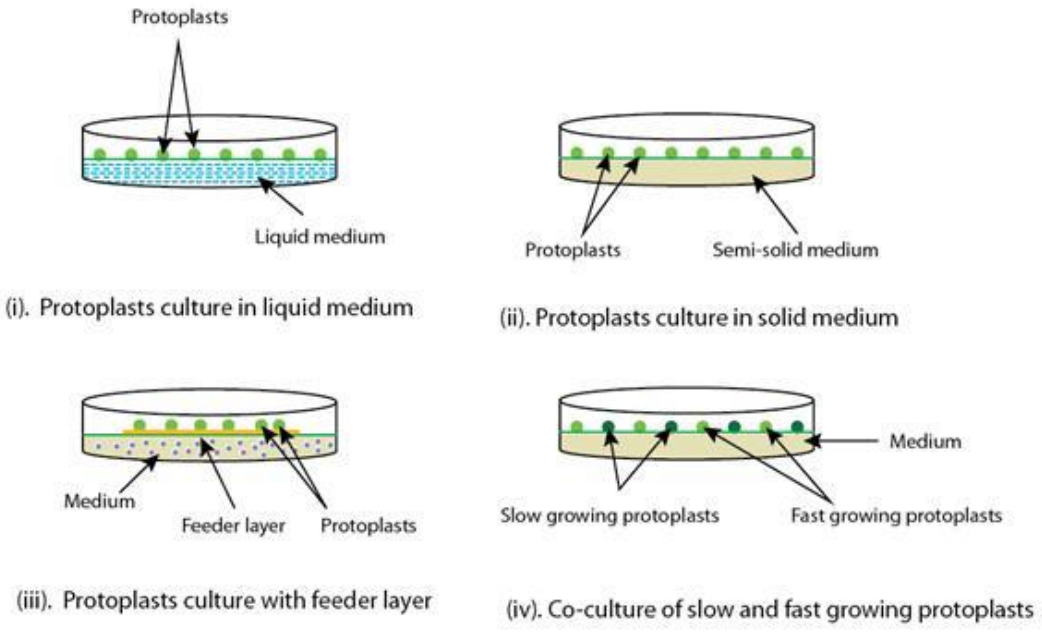


Figure : protoplast culture techniques

Regeneration of protoplast

Protoplast development occurs in two stages.

A. Formation of cell wall
B. Development of callus/ whole plant

A. Formation of cell wall

In culture, protoplasts starts developing a wall around it within few hours and it takes only few days to complete the process. Wall materials are progressively deposited at the surface of plasmalemma. The cellulose is deposited either between the plasmalemma and the multilamellar wall material on the plasmalemma.

As the cell wall development occurs the protoplast lose their characteristics spherical shape. The newly developed cell wall by protoplast can be identified by using calcofluor white fluorescent stain.

The process of cell wall development requires continuous supply of nutrient. The protoplasts with proper cell wall development undergo normal cell division. On the other hand, protoplast with poorly regenerated cell wall show budding and fail to undergo normal mitosis.

B. Development of callus/ whole plant

As the cell wall formation around protoplasts is complete, the cells increases in size, and the first divisions generally occurs within 7 days. After 3rd week small visible colonies are formed.

These colonize are then transferred to an osmotic free medium for further development to callus. With appropriate manipulation of nutrient media the callus can undergo organogenesis or embryogenesis and finally forms whole plant.

PROTOPLAST FUSION

The isolated protoplasts are used in the study of biochemistry, physiology and gene expression in plants. The fact that isolated protoplasts are devoid o cell walls makes them easy tool for undergoing fusion in vitro.

In the conventional method to improve the characteristics of cultivated plants, sexual hybridization method used. The major limitation of this method is that it can be performed within a plant species or very closely related species. This species barrier encounter in sexual hybridization can be overcome by somatic hybridization.

Thus the plant protoplasts, would offer exciting possibilities in the field of somatic cell genetics and crop improvement.

The in vitro fusion of protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant, this is known as somatic hybridization.

Somatic hybridization is an effective method used to breed sexually imcompactible plants i.e. interspecific, intergenic or even interkingdom levels.

Somatic hybridization involves the following aspects

- Fusion of protoplast
- Selection of hybrid cells
- Identification of hybrid plants

Methods of somatic hybridization

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

1. Spontaneous fusion:

Cell fusion is a natural process as is observed in case of egg fertilization. During the course of enzymatic degradation of cell walls, some of the adjoining protoplasts may fuse to form homokaryons. These fused cells may sometimes contain high number of nuclei. This is mainly because of expansion and subsequent coalescence of plasmodermal connections between cells. The frequency of homokaryon formation was found to be high in protoplasts isolated from dividing cultured cells.spontaneously fused protoplasts, however, cannot regenerate into whole plants except undergoing a few cell divisions.

2. Mechanical fusion

In this process, the isolated protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulator and perfusion micropipette. This micropipette is partially blocked within 1mm of the tip by a sealed glass rod. In this way the protoplasts are retained and compressed by the flow of liquid.

3. Induced method

This is the chemically method of protoplast fusion. Freshly isolated protoplasts can be fused by induction. The chemical compounds which are called fusogens. Fusogens brings the two protoplast together and induce them to fuse with one another.

The fusogens are as follows,

Sodium nitrate, High pH/Ca^{2+}, polyethylene glycol, polyvinyl alcohol, lysozyme, concavalin A, fatty acids, electrofusion, dextran, etc.

a. Treatment with sodium nitrate:

The isolated protoplasts are exposed to a mixture of 5.5% sodium nitrate in 10% sucrose solution. This mixture is called aggregation mixture. The step involved in the protoplast fusion re as follows.

- ✓ The protoplasts of two different strains are isolated separately.
- ✓ These two strain of protoplast treated with aggregation mixture is then incubated at 35^0C for about 5 minutes.
- ✓ At the time of incubation, sodium nitrate brings about plasmolysis in protoplast.
- ✓ After incubation, centrifugation is carried out at 200g for 5 minutes.
- ✓ The protoplast pellet is kept in a water both at 30^0C for about 30 minutes. During this period majority of protoplasts fuse together.
- ✓ Then the protoplasts suspension is transferred to fresh medium containing 0.1% $NaNO_3$ and then the protoplasts are washed with distilled water and used to demonstrate further cultures.

This method results in a low frequency of heterokaryon formation, particularly when mesophyll protoplasts are fused.

b. High pH / Ca^{2+} ion treatment:

Keller and Melchels (1973) studied the method effect of Ca^{2+} ions in protoplast fusion in tobacco. In this method, the protoplasts are incubated with solution containing 0.4M mannitol, 0.05M CaCl2 at pH 10.5 at 37^0C for 30- 40 minutes. The protoplasts form aggregates, and fusion usually occurs within 10 minutes. High pH and high Ca^{2+} ion neutralize the surface charges on the protoplasts. This allows closer contact and membrane fusion between agglutinated protoplasts.

c. Polyethylene glycol

Kao and Michayluk in 1974 discovered another fusion inducing chemical i.e. polyethylene glycol high is most effective agent discovered so far. Many fusion experiment are performed by a polyethylene glycol due to its high success rate, for the fusion of protoplasts from many plant species. PEG induces protoplast aggregation and subsequent fusion. PEG causes rapid agglutination and formation of clumps of protoplasts. This results in the formation of tight adhesion of membranes and consequently their fusion.

The isolated protoplasts in culture medium (1 ml) are mixed with equal volume (1 ml) of 28-59% PEG in a tube. This tube is shaken and then allowed to settle. The setteled protoplasts are washed several times with culture medium.

PEG treatment method is widely used for protoplast fusion as it has several advantages.

- ✓ It results in a reproducible hih frequency of heterokaryon formation.
- ✓ Reduced formation of binucleate heterokaryons.
- ✓ Low toxicity to cells.
- ✓ PEG induced fusion is nonspecific and therefore can be used for a wide range of plants.

For better results PEG is used with other chemical agent. These are

- ➢ PEG is more effective when it is mixed with 10-15% dimethyl sulfoxide (DMSO).
- ➢ Addition of concanavalin A to PEG increases protoplast fusion frequency.

d. Electrofusion:-

In this method , electrical field is used for protoplast fusion. Two glass capillary microelectrdes are placed in contact with the protoplasts. An electric field of low strength gives rise to dielectrophoretic dipole generation within the protoplast suspension. This leads to pearl chain arrangement of protoplasts. Electrofusion technique is simple, quick and efficient and hence preferred by many workers.

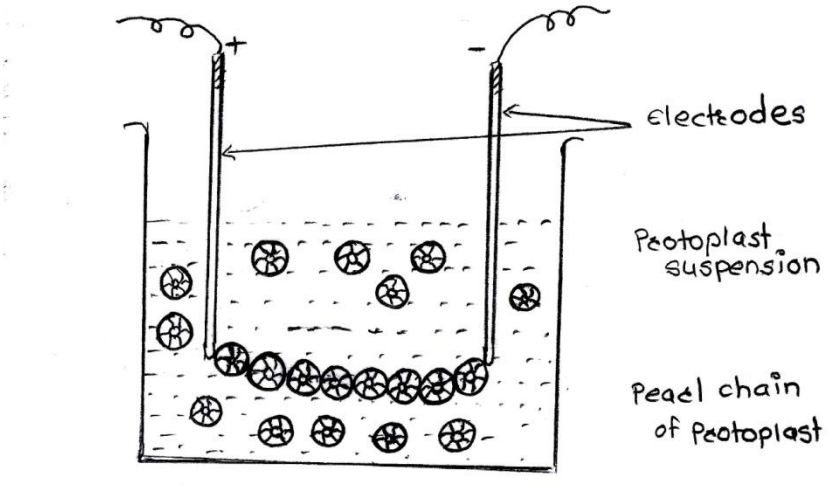


Fig: Electrofusion of protplasts

SELECTION OF HYBRID CELLS

In the protoplast fusion process about 20 – 25% of protoplasts are actually involved after the fusion process , the protoplast population consists of a heterogenous mixture of of unfused chloroplasts, homakaryons and heterokaryons. It is therefore necessary to select the hybrid cells.to select the hybrid cells following methods are employed.

Biochemical method

The biochemical methods for selection of hybrid cells are based on the use of biochemical compound in the medium. These compounds helps t sort out the hybrid and parental cells based on their differences in the expression of characters. Drug sensitivity and auxotrophic mutant selection methods are used.

1. Drug sensitivity

This method is useful for the selction hybrids of two plant species, if one of them is sensitive to drug. Power et.al in 1976, utilized the differential sensitivity of protoplasts isolated from *Petunia parodii* and *Petunia hybride* to the drug actinomycin D.

Protoplasts of *Petunia hybride* can form macroscopic callus on MS medium , but are ensitive to actinomycin D. *Petunia parodii* protoplasts form small colonies, but are resistant to actinomycin D. when these two species are fused, the fused protoplasts derive both the characters i.e. formation of macroscopic colonies and resistance to actinomycin D. The parental protoplast of both the species fail to grow. A similar procedure is in use for the selection of other somatic hybrids e.g. hybrids between *Nicotiana syivestris* and *Nicotiana knightiana.*

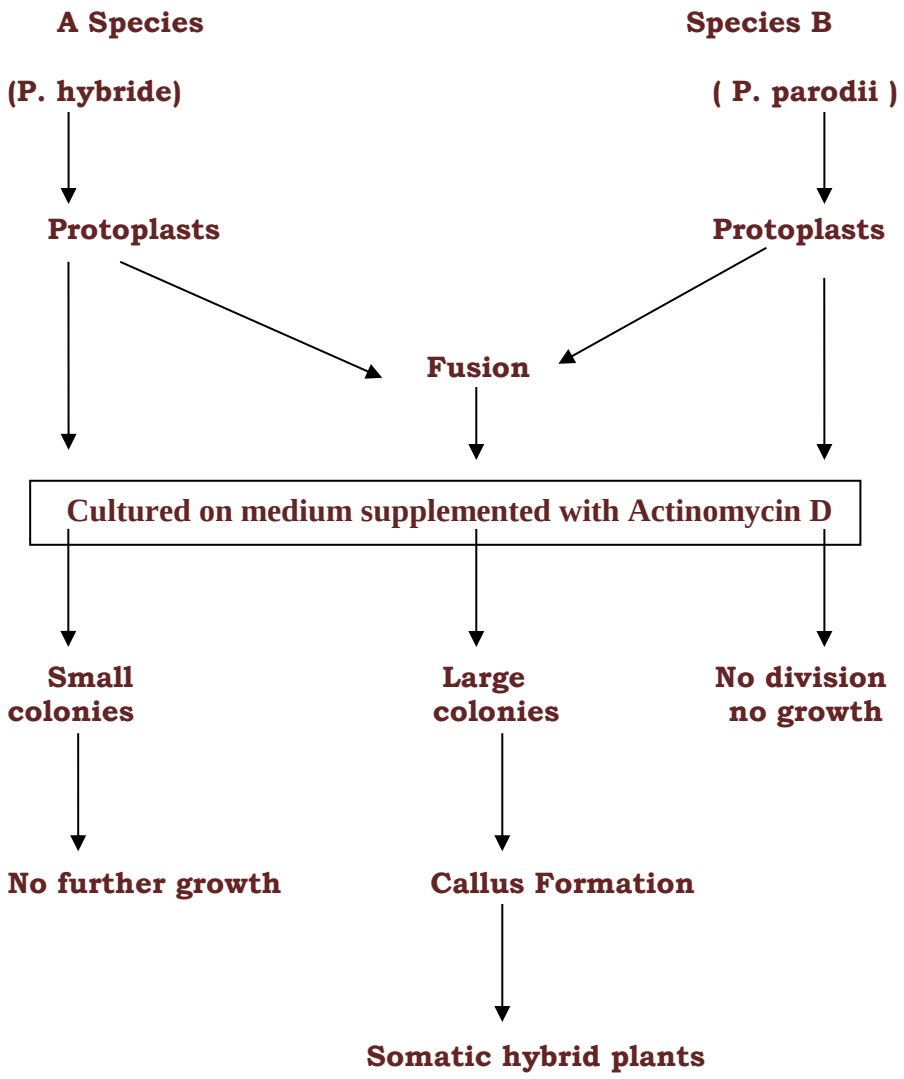


Figure: Drug sensitivity method for the selction of hybrid cells

2. Auxotrophic mutants

Auxotrophs are mutants that can not grow on a minimal medium and therefore require specific compounds to be added to the medium. The selection of somatic hybrids as a result of complementation by auxotrophic mutants may be useful as only the hybrid lines are expected to survive in the minimal medium. Glimelius et. al. in 1978 succeeded in selection of numerous somatic hybrids by utilizing protoplasts of nitrate reductase deficient and chlorate resistant mutants lines of tobacco.

The parental protoplasts of tobacco cannot grow with nitrate as the sole source of nitrogen while hybrid can grow.

The selection of auxotrophic mutants is possible only if the hybrid cells can grow on a minimal medium.

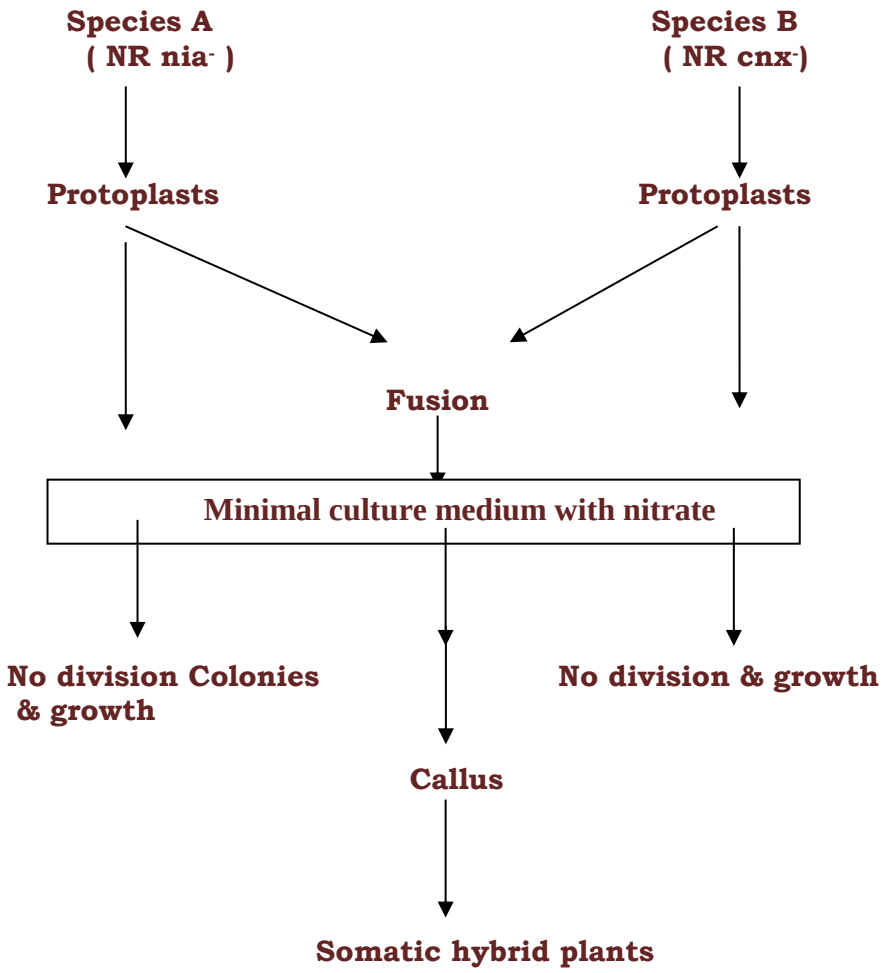


Figure : Auxotrophic mutant selection method of hybrid selection. [NR nia- -Nitrate reductase apoenzyme deficient, NR cnx- - Nitrate reductase lacking molybdenum cofactor

Visual Method

In most of the somatic hybridization experiments , chloroplast deficient protoplasts of one parent are fused with green protoplasts of another parent.this facilates the visual selection of heterokaryons under the light microscope. The heterokaryons are bigger and green in color whle the parental protoplasts are either small or colorless. There are two approaches of this methods.

1. Visual selection coupled with differential media growth

Visual selection procedure is coupled with complementary natural differences in the sensitivity of parental protoplasts to media constituents

which enable only the hybrid cells to develop in cultures and regenerate plants. A diagrammatic representation of visual selection coupled with differential media growth of heterokaryons on a selection medium is as follows.

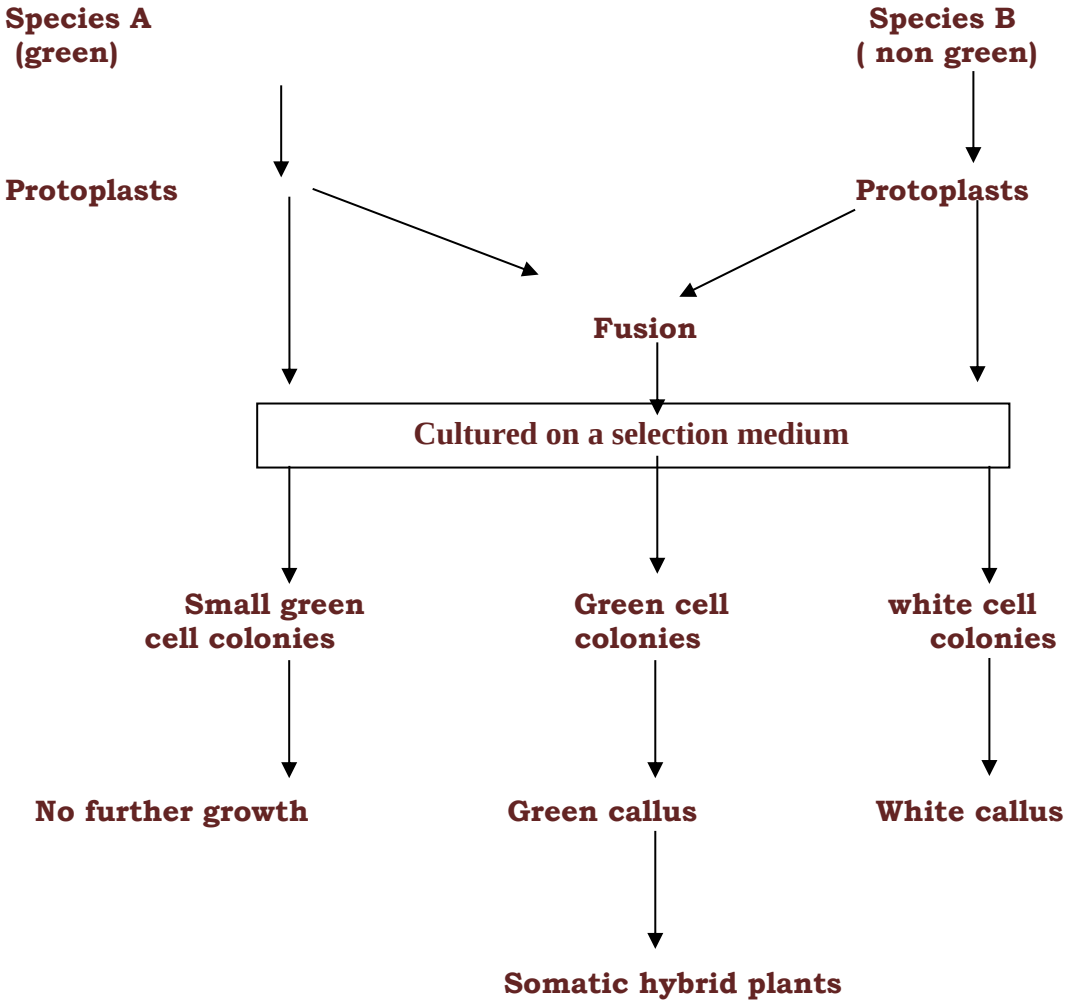


Figure : A diagrammatic representation of visual selection coupled with differential media growth of heterokaryons on a selection medium

2. Mechanical isolation

This method involve the mechanical isolation of hybrid cells. The use of special pipette namely Drummond pipette. The so isolated heterokaryons can be cloned to finally produce somatic hybrid plants.

This method has a limitation i.e. it requires a special culture medium for its growth. This limitation can be overcome by using Microdrop culture of single cells using feeder layers.

Cytometric methods

The cytometric technique involves the use of flow cytometry and fluorescent activated cell sorting techniques.

IDENTIFICATION OF HYBRID PLANTS

The development of hybrid cells followed by the generation of hybrid plants requires a clear proof of genetic contribution form both the parental protoplasts.

- ✓ **Morphology of hybrid plants**
  Morphological features of hybrid plants which usually are intermediate between two parents can be identified. For this, the vegetative and floral characters are considered.

- ✓ **Isoenzyme analysis**
  Isoenzymes are the multiple form of enzyme catalyzing the same reaction. Somatic hybrids posses specific isoenzymes of one or the other parent or both the parents simultaneously. There are many enzymes possessing unique isoenzymes that can be used for the identification of somatic hybrids. e.g. Esterase, Aspartate aminotransferase , Amylase, Isoperoxidase.

- ✓ **Chromosome constitution**
  Chromosome counting of the hybrid is an easier and reliable method to ensure hybridity as it also provides the information of ploidy level. Cytologically the chromosome count of the hybrid should be sum of number of chromosomes from both the parents. Besides number of chromosomes, the size and structure of chromosomes can also be monitored. However, the approach is not applicable to all species, particularly where fusion involves closely related species or where the chromosomes are very small. Moreover, sometimes the somaclonal variations may also give rise to different chromosome number.

- ✓ **Molecular techniques**
  Many molecular techniques are used to identify hybrid plants. These are PCR, AFLP, RFLP, RAPD, etc.

CYBRIDS

Cybrids are defined as the Cytoplasmic inclusion where the nucleus derived from one species and cytoplasm derived from both the parental species. The phenomenon of formation of cybrids regarded as cybridization.

Normally, cybrids are produced when protoplasts from two phytogenetically distinct species are fused.

Methods of cybridization

During the hybridization, the nuclei can be stimulated to segregate so that one protoplast contributes to the cytoplasm while other contributes nucleus alone. By this way cybridization can achieved. Following are some approaches of cybridization.

- To ensure an effective Cytoplasmic gene transfer, the cytoplasm donor species are irradiated. The protoplast of cytoplasm donor species are irradiated with X-rays or γ rays. Due to this protoplast inactivated. This treatment completely arrests division of the nonfused cells and serves as a selection factor for screening cybrids. When this protoplast fused with other, it donate the Cytoplasmic constituents to recipient protoplasts.
- Normal protoplast can directly fused with enucleated protoplasts.
- Protoplasts are inactivated by metabolic inhibitors.
- Suppression of nuclear division of donar protoplast and fuse them with normal protoplasts.

Cybrids provide the following unique opportunities:

(i) Transfer of plasmogenes of one species into the nuclear background of another species in a single generation, and even in
(ii) Sexually incompatible combinations,
(iii) Recovery of recombinants between the parental mitochondrial or chloroplast DNAs (genomes), and
(iv) Production of a wide variety of combinations of the parental and recombinant chloroplasts with the parental or recombinant mitochondria.

Applications of somatic hybridization

1. Novel interspecific and intergeneric crosses which are difficult to produce by conventional methods can be easily obtained.
2. Important characters, such as resistance to diseases, ability to undergo abiotic stress and other quality characters, can be obtained in hybrid plant by the fusion of protoplasts of plant bearing particular character to the other plant which may be susceptible to diseases.
3. Protoplasts of sexually sterile haploid, triploid, aneuploid plants can be fused to obtain fertile diploids and polyploids.
4. Studying cytoplasmic genes may be helpful to carry out plant breeding.
5. Most of the agronomically important traits, such as cytoplasmic male sterility, antibiotic resistance and herbicide resistance, are cytoplasmically encoded, hence can be easily transferred to other plant.
6. Plants in juvenile stage can also be hybridized by means of somatic hybridization.
7. Somatic hybridization can be used as a method for the production of autotetraploids.

Limitations of somatic hybridization

1. Application of protoplast methodology requires efficient plant regeneration system from isolated protoplasts. Protoplasts from two species can be fused, however, production of somatic hybrids is not easy.
2. Lack of a proper selection method for fused products (hybrids) poses a problem.
3. The end product of somatic hybridization are often unbalanced (sterile, misformed and unstable)
4. Somatic hybridization of two diploids leads to formation of amphidiploids which is unfavorable.
5. It is not sure for a character to completely express after somatic hybridization.
6. The regeneration products of somatic hybridization are often variable due to somaclonal variation, chromosome elimination, organelle segregation.
7. All diverse intergeneric somatic hybrids are sterile and, therefore, have limited chances of development of new varieties.
8. To transfer useful genes from wild species to cultivated crop, it is necessary to achieve intergeneric recombination or chromosome substitution between parental genomes.

Chapter...10

APPLICATION OF PLANT TISSUE CULTURE

Germplasm conservation

Germplasm conservation broadly refers to the hereditary material transmitted to the offspring through germ cells. Germplasm provides the raw material for the breeder to develop various crops. Thus conservation of germplasm assumes significance in all breeding programme.

The principle of germplasm conservation is to preserve the maximum possible genetic diversity of a particular plant or genetic stock future use.

A global body namely International Board of Plant Genetic resource (IBPGR) has been established for germplasm conservation. The objective of IBPGR is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

There are two approaches of germplasm conservation are

1. In situ conservation
2. Ex situ conservation

1. In situ conservation

The conservation of germplasm in their natural environment by establishing biosphere reserves is regarded as in situ conservation.

This approach is used to preserve landplants in a near natural habitat along with several wild relatives with genetic diversity.

In-situ conservation has some limitations, these are as follows

- There is a risk of the material being lost due to environmental hazards.
- Cost of maintenance.

2. Ex situ conservation

This is the chief method of conservation of germplasm which may include cultivated and wild material. In this seeds or in vitro maintained plant cells, tissue and organs are preserved under appropriate conditions for long term storage as gene bank.

This requires considerable knowledge of the genetic structure of population, sampling techniques, method of regeneration and maintenance of varietal gene pools particularly in cross pollinated plants.

Seeds are more convenient for conservation because they occupy less space and easily transported to various places.

But there are some limitations of seed conservation. These are

- ✓ Viability of seeds is susceptible to insect or pathogen attack, often leading to their destruction.
- ✓ Difficult to maintain clones through seed conservation.
- ✓ Not applicability to vegetatively propagated crops.
- ✓ In vitro methods of employing shoots meristem and embryos are ideally suited for the conservation of germplasm of vegetatively

propagated plants. The plant with recalcitrant seeds and genetically engineered plant can also be preserved by this method.

- ✓ The potential advantages of conservation include
- ✓ Requirement of less space for preservation of a large number of clonally multiplied plants.
- ✓ Maintenance of the material in an environment free of pest for pathogens.
- ✓ Protection against dangers of natural environment hazards.
- ✓ Availability f nucleus stock to propagule a large number plants rapidly whenever necessary.
- ✓ Obstacles for their transport through National and International borders are minimal.

Method of in vitro conservation

There are mainly three approaches for in vitro conservation.

A. Cryopreservation
B. Cold storage
C. Low pressure and low oxygen storage.

A. Cryopreservation

Cryopreservation literally means preservation in the frozen state. The principle underlying cryopreservation basically involves bringing the plant cell and tissue cultures to a non subjecting them to super low temperature in the presence or absence of cryoprotectants. At this stage plant cell become to a zero metabolism or non dividing state. Cryopreservation broadly means the stage of germplasm at very low temperature.

- ➢ Over solid carbon dioxide (at -79^{0}C)
- ➢ Low temperature deep freezers (at -80^{0}C)
- ➢ In vapour phase nitrogen (at -150^{0}C)
- ➢ In liquid nitrogen (at -196^{0}C)

Liquid nitrogen is widely used method of cryopreservation. At the temperature of liquid nitrogen (-196^{0}C), the cells stay in a completely inactive state and thus can be conserved for long periods.

Cryopreservation is most useful for long term storage of plant germplasm since cells at ultralow temperature do not divide and remain genetically stable. In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat peanut cassava, sugarcane , strawberry, coconut. Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

Precaution:

- ➢ It requires technical skill.
- ➢ It essential that cells during freeze preservation be protected against cryogenic injuries.
- ➢ Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
- ➢ High intracellular concentration of solutes may also damage cells.

- Sometimes, certain solutes from the cell may leak out during freezing.
- Cryoprotectants also affect the viability of cells.
- The physiology status of the plant Material is also important.

Mechanism of cryopreservation

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze compared to the freezing point of pure water. When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

Technique of cryopreservation

The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages

a. Development of sterile tissue culture
b. Addition of cryoprotectants
c. Freezing
d. Storage
e. Thawing
f. Reculture
g. Survival measurement
h. Plant regeneration

a. Development of sterile tissue culture

The morphological and physiological characters of explants are considered for cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristem, embryos, endosperms, ovule, seeds, cultured plant cells, protoplast, callus, etc. the meristematic cells and cell suspension cultures are more suitable.

b. Addition of cryoprotectants

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing.

During the cryopreservation, ice crystals are formed due to super cooling. The freezing point and super cooling point of water are reduced by the presence of cryoprotectants. The cryoprotectants include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, glucose, etc. from which DMSO, sucrose, glycerol are widely used. Mixtures of cryoprotectants are also used for effective results.

c. Freezing

There are six different type of freezing method.

1. Slow- freezing method
2. Rapid freezing method
3. Step wise freezing method
4. Dry freezing method
5. Vetrification

6. Encapsulation/ dehydration

1. Slow freezing method

The tissue or the requisite plant material is slowly frozen at low cooling rates of 0.5-5^{0}C / min from 0^{0}C to -100^{0}C, and then transferred to liquid nitrogen. The advantage of slow freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. This method is successfully used suspension culture.

2. Rapid freezing method

This is simple technique. It involves plunging of the vial containing plant material into liquid nitrogen. The temperature decrease rapidly, at the rate -300^{0}C to -1000^{0}C / min occurs. Seibert in 1976 used this rapid freezing procedure. This method is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method

In this method both slow and rapid freezing procedures combinly used and carried in stepwise manner. The plant material is cooled stepwise to an intermediate temperature, maintained at that temperature for 30 min, and then rapidly cooled by plunging it into liquid nitrogen. In the initial slow freezing, ice is formed outside the cells and the unfrozen protoplasm of the cells loses water due to the vapour deficit pressure between the supercoiled protoplasm and the external ice.

The stepwise freezing method can be used wide range of plant materials which include shoot apices, buds and suspension culture.

4. Dry freezing method

Material dehydrated by drying in an oven or under vacuums demonstrates remarkable resistance to cryogenic. The basic idea for te dry freezing method originate from the fact that non-germinated dry seeds are able to survive freezing at superlow temperatures in contrast to water imbibing seeds which show susceptibility to cryogenic injuries.

5. Vitrification

Cell material contains water content. During the freezing process it is more important to maintain viability of cells with more water content after cooling to the temperature of liquid nitrogen. So it is essential to avoid intracellular freezing. This can be accomplished either by drying or vitrification.

Vitrification is an effective mechanism against freezing wherein a highly concentrated cryoprotective solution supercools to very low temperature and solidifies into metastable glass without undergoing crystallization. In this method cells or meristems are sufficiently dehydrated with a highly concentrated vitrification solution (e.g. PVS3 and PVS2) at 25^{0}C or 0^{0}C without causing injury prior to immersion in liquid nitrogen. The

vitrification method has been successful in cryopreservation of species and cultivars of herbaceous and woody plants.
Steps:

- Cells or meristem are subjected cryoprotectants.
- Cells are dehydrated by exposure to highly concentrated vitrification.
- Transfer of cells and meristem to minicryotubes followed by proper sealing and immersion in liquid nitrogen.
- Rapid thawing in water bath at 40^0C
- Removal of vitrification solution and reculture for shoot or plantlet regeneration.

6. Encapsulation:

Vitrification method requires careful control of the procedure for dehydration and cryoprotectants permeation in order to prevent injury by chemical toxicity or excess osmotic stress. In the encapsulation technique, the cells or tissue are trapped into calcium alginate beads followed by incubation in 0.85 M sucrose for 4-16 hr and rapid freezing in liquid nitrogen.
Before immersion of beads in liquid nitrogen, they are air dried for 3-4 hr in a laminar flow chamber. This technique has found application in cryopreservation of somatic embryo and meristems.

d. Storage

In general, the frozen cell/tissues are kept for storage at temperatures in the range of -70 to -196^0C. The main objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196^0C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential.

Proper documentation of the germplasm storage has to be done. The documented information must be comprehensive with the following particulars.

- ✓ Taxonomic classification of the material
- ✓ History of culture
- ✓ Morphogenic potential
- ✓ Genetic manipulation done
- ✓ Somaclonal variations
- ✓ Culture medium
- ✓ Growth kinetics

e. Thawing

In thawing process, plunging the frozen samples in ampoules into a warm water at 37-40^0C which gives a rapid thawing rate of 500-750^0C/min. After the thawing the material is transferred to either an ice bath or water bath or at room temperature and maintained.
Rapid thawing protects cells from the damaging effects of ice crystal formation.

f. Reculture

Thawed materials are washed several times to remove the cryoprotectants so as to avoid any deplasmolytic injury to cells. The washed material is then recultured in a fresh medium.

g. Survival measurement

The viability or survival of frozen cells can be measured at any stage of cryopreservation. The measurements of survival determine same as cell culture. The cryopreserved materials are stained by triphenyl Tetrazolium chloride (TTC), Evans blue and Fluorescein diacetate.

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

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

h. Plant regeneration

For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of some growth promoting substances is necessary for successful plant regeneration.

B. Cold storage

Germplasm conservation by storing materials in cultures at low and nonfreezing temperatures i.e. 1-9^{0}C has also been carried out. In this the aging of the plant material is slowed down but not completely stopped as in cryopreservation.

Advantages: -

- ✓ Major advantage of this is that plant material not subjected to cryogenic injuries.
- ✓ Simple, cost effective

Many in-vitro developed shoots/plants of fruit tree species have been successfully stored by this approach.

e.g. grape plants, strawberry plants.

C. **Low- pressure and low-oxygen storage**

As alternative to cryopreservation and cold storage, low-pressure storage (LPS) and low-oxygen storage (LOS) have been developed for germplasm conservation.

Low-pressure storage(LPS)

In LPS, the atmospheric pressure surroundings the plant material is reduced. This results in a partial decrease of the pressure exerted by the gases around the germplasm. The lowered partial pressure reduces the in vitro growth of plants. This method is useful in short-term (e.g. fruits,

vegetables, cut flower, plant cuttings) and long-term storage (germplasm grown in culture).

LPS has an advantage of reducing the microbial activity in cultures in which the cells or tissue carry them systemically.

Low-oxygen storage (LOS)

In the LOS, the oxygen concentration is reduced, but the atmospheric pressure is maintained by addition of inert gases (such as nitrogen).

The partial pressure of oxygen below 50mm Hg reduces plant tissue growth. This is due to fact that with low concentration of oxygen, the production of CO_2 is low. As a consequence, the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

This method has a limitation, that the long term conservation of plant material by low oxygen storage is likely to inhibit the plant growth after certain dimensions.

Application of germplasm storage

1. Maintenance of stock cultures- plant material of several species can be cryopreserved and maintained for several years, and used as and when needed.
2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolite.
3. Disease free plant material can be frozen and propagated whenever required.
4. Conservation of pollen for enhancing longevity.
5. Plant material from endangered species can be conserved.
6. Conservation of somaclonal and gameclonal variations in cultures.

Chapter...11

PRODUCTION OF DISEASE FREE PLANTS

The horticultural and forest crops are infected by systemic diseases caused by fungi, bacteria , viruses, mycoplasma and nematode. Due to this , it reduces the yield and quality of crops. Eradication of pathogen s is highly desirable to optimize the yields. The plant infected with bacteria and fungi frequently respond to chemical treatment by bactericides and fungicides. However it is very difficult to cure the virus infected plants. Further, viral disease are easily transferred in seed propagated plant species. Plant breeder are always interested to develop disease free plants. This is obtain through tissue culture.

In general it is suggested that the apical meristem of infected plant generally either free or carry low concentration of viruses, for the following reasons

- Meristem lacks the vascular system, as viruses move readily through vascular system.
- High metabolic activity in the actively dividing meristematic cells dose not allow virus replication
- Virus replication is initiated by high concentration of endogenous auxin in shoot apices.

Tissue culture techniques employing meristem tips are successfully used production of disease free plants.

Methods of virus elimination

In general, plant are infected with many viruses, the nature of some of them may be unknown. The commonly used methods for virus elimination in plants are as follows

1. Heat treatment
2. Meristem tip culture
3. Chemical treatment of media
4. Other Invitro methods

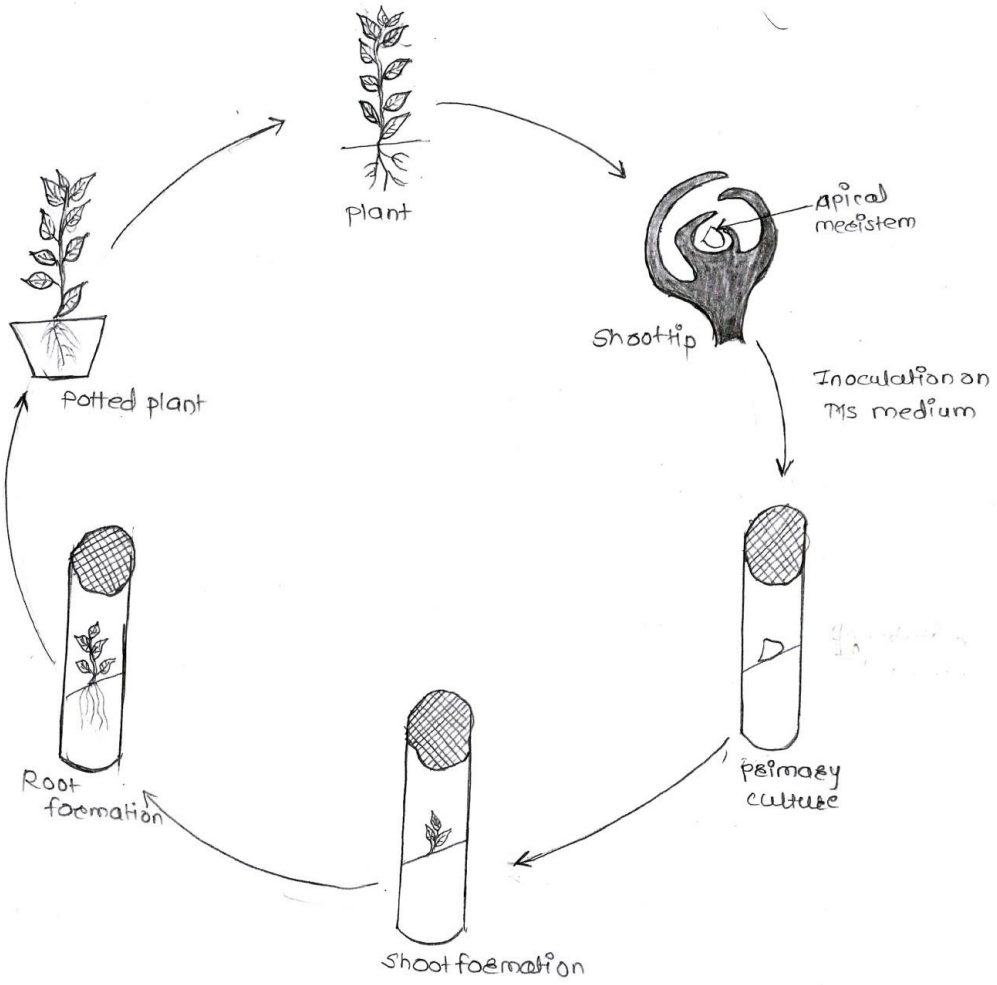
1. Heat treatment

The heat treatment is also called as thermotherapy.

The principle of this method is that viruses in plant tissue are either partially or completely inactivated at higher temperature with minimal injury to the host plant.

The heat treatment (at 35-40^{0}C) was carried out by usinghot water or hot air for eliminationof viruses from shoot and buds.

For hot air treatment actively growing plants are placed in thermotherapy chamber. Temperature is adjusted to 35-40^{0}C for a period varying from a few minutes to several months.



An adequate humidity and light is should be maintained. The temperature of the air should be gradually raised during first few days until the desired temperature is reched. Small cutting are taken from shoot tips immediately after heat treatment and grafted onto healthy rootstocks.

Limitations:

1. Most of the viruses are not sensitive to heat treatment.
2. Many plant species do not survive after the thermotherapy.

2. Meristem tip culture

Apical meristem is dome of tissue located at the extreme tip of a shoot. For virus elimination the size of meristem used in culture is very critical since in most of the viruses exist by establishing a gradient in plant tissues. In general, the regeneration of virus free plant through cultures is inversely proportional to the size of meristem used.

The most widely used media for meristem culture are MS medium and whites medium. The optimal temperature for culture is in the range of 20-28^{0}C.

Stages of meristem culture

Stage I:- The culture of meristem is established. Addition of growth regulators namely cytokinins and auxin will support the growth and development.

Stage II:- In this stage , shoot are developed along with axillary shoot proliferation occurs.

Stage III:- In this stage developed shoots are transferred on MS medium supplemented with high auxin and low cytokinin concentration.

Stage IV:- Developed plantlet to sterilized soil for hardening under greenhouse environment.

Meristem culture influenced by following factors

- Physiological condition of the explant-actively growing buds are more effective.
- In some plants, heat treatment is first given and then meristem tips are isolated and cultured.
- Culture medium –MS medium with low auxin and cytokinin is ideal.

3. Chemical treatment of media:-

Some workers have attempted to eradicate viruses from infected plants by chemical treatment of the tissue culture media. The commonly used chemicals are growth substances (e.g. cytokinin) and antimetabolites (e.g. thiouracil acetyl salicylic acid). There are however, conflicting reports on the elimination viruses by chemical treatment of the media.

4. Other in vitro method:-

Besides meristem tip culture other in vitro methods are also be used. In this callus culture have been successful to some extent. The callus derived from the infected cells. In fact the uneven distribution of tobacco mosaic virus in tobacco leaves was exploited to develop virus free plants of tobacco. Somatic cell hybridization, gene transformation and somaclonal variation also useful to raise disease free plants.

Chapter...12

SOMACLONAL VARIATION

The genetic variation found in the in vitro cultured cells are known as somaclonal variations.

OR

The variations occurs as a result of genetic heterogeneity (i.e. change in chromosome number& / or structure) in plant tissue culture, known as Somaclonal variation.

The genetic variability present among cultured cells, plants derived from such cells or progeny of such plants is called as somaclones.

In 1981, **Larkin and Scowcroft** coined the term somaclonal variation for plant variant derived from any form of cell or tissue cultures. In 1984, **Evan's** prefer the term gamatoclonal variation for the variation observed in the regenerated plants from gametic cells.

Genetic variation occurs in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of regenerated plants. The genetically variability in culture express in the form of variant traits in regenerated plants which are then transmitted to the progeny through the sexual or vegetative propagation. The genetic heterogeneity in culture arrises mainly due to such factors as,

a) The expression of chromosomal mosaicism or genetic disorders in cells of the initial explants.
b) Spontaneous mutation due to culture condition
   The genetic changes associated with somaclonal variation include polyploidy, aneuploidy, chromosomal breakage, deletion, translocation and gene amplifications, besides several mutation.

Factors affecting production of somaclonal variants

Factors affecting production of somaclonal variants are as follows

1. Genotype & explant source
2. Duration of cell culture
3. Growth hormone effects

1. Genotype & explant source

The genotype influences both the frequency of regeneration and the frequence of somaclonal variation variation. Explant are generally taken from any tissue namely leaves, internodes, ovaries, roots and inflorescences. The source of the explants has often been considered a critical variable for somaclonal variation.

For instance, potato plants regenerated from callus of rachis and petiole are much higher (~50 %) comapared to those regenerated from callus of leave (~12 %).

2. Duration of culture

In general , for many cultures, somaclonal variation are higher with increased duration of culture. For example it was reported that genetic

variability increased in tobacco protoplasts from 1.5 to 6% by doubling the duration of cultures.

3. Growth harmone effects

The plant growth regulator in the medium will influence the karyotypic alteration in cultured cells, & therefore development of somaclone growth harmone such as 2,4D & NAA are frequently used to achieve chromosomal variability.

Isolation of somaclonal variants

The various approaches for isolation of somaclonal variants can be categorized as

a. Without in vitro selection
b. With Invitro selection

a. Without in vitro selection

- In this method , an explant is cultured on a suitable medium. This medium is supplemented with growth regulators and incubated at 25 ± 2^0C.
- After 1-2 week of incubation, the unorganized callus formed and cell do not contain any selective agent (toxic or inhibitory substance).
- For shoot initiation, the callus is subcultured on the suitable medium supplemented with high cytokinin to auxin ratio. The formed shoots transferred on to rooting medium for regeneration of plants. The so produced plants are grow in pots, transfer to field and analyzed for somaclonal variants.
- Somaclonal variants of several crop have been successfully obtained by this approaches. e.g. sugarcane, potato, cereals,etc.

Limitations:

- There is no directed and specific approach for the isolation of somaclones by without in vitro selection.
- It is time consuming method.
- Requires screening of many plants.

b. With in vitro selection

Isolation of somaclones with in vitro selection method basically involves handling of plant cells in cultures like microorganism and selection of biochemical mutants. The cell lines are screened from plant cultures for their ability to survive in the presence of a toxic/inhibitory substance in the medium or under conditions of environmental stress.

A diagrammatic representation of in vitro protocol for the isolation of disease resistance plants with in vitro selection approach is given in figure

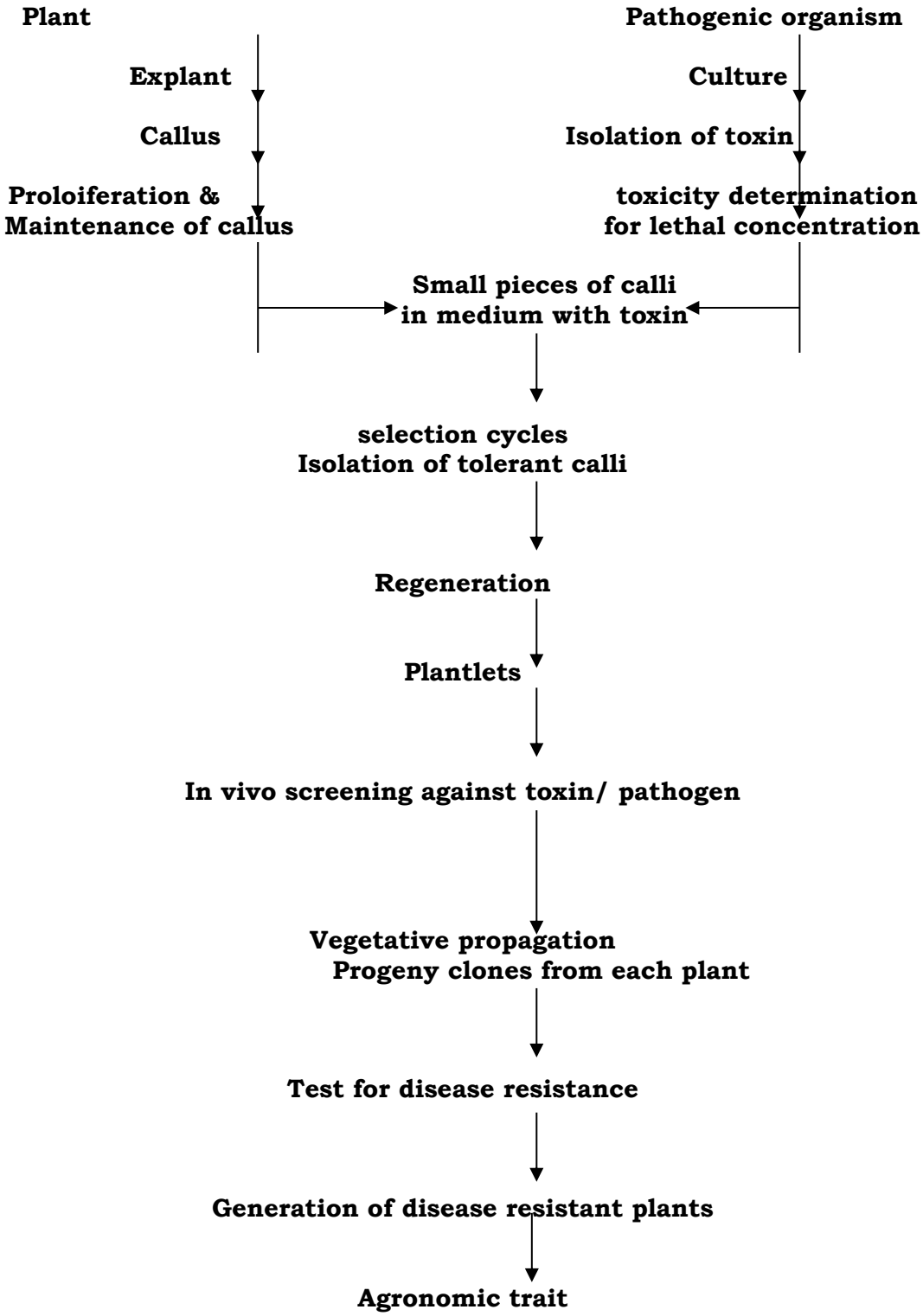


Figure: a diagrammatic representation of isolation of disease resistant plants with n vitro selection

The dedifferentiated callus, obtained from an explant is exposed in the medium to inhibitors like toxins, antibiotics, amino acid analogs. Selection cycles are carried out to isolate the tolerant callus cultures and these calli are regenerated into plants. The plants so obtained are in vitro screened against the toxins or any other inhibitors. The plant resistant to the toxin are selected and grown further by vegetative propagation of self pollination. The subsequent generations are analyzed for disease resistant plants against the specific pathogenic organism.

Major causes of Somaclonal Variation:

• Physiological:

Variations induced by physiological factors in culture medium for e.g. prolonged exposure to PGRs (2,4-D; 2,4,5-T) results in variability among the regenerants. Often such variations are epigenetic and hence donot follow Mendelian inheritance.

• Genetic:

All the alterations at chromosomal level are grouped under genetic cause of variation observed in regenerants. Chromosomal rearrangements such as deletion, duplication, translocation, inversion polyploidy, aneuploidy, have been reported to be the chief source of somaclonal variation. Meiotic crossing over involving symmetric and asymmetric recombination could also be responsible for variation observed among somaclones. Transposable elements like Ac-Ds in maize have been shown to get activated in *in vitro* culture. In maize (*Zea mays* L.) and broad beans (*Vicia faba* L), late replicating heterochromatin is the main cause of somaclonal variation. Single gene mutations in cultures also give rise to variations which are not detected in plants regenerated *in vitro* from any cell or tissue (R0 plants) but express in R1 plants (after selfing R0 plants).

• Biochemical:

The most common kind of biochemical variation is change in carbon metabolism leading to failure of photosynthesis *viz.* albinos is rice. Any variation in other cell processes like starch biosynthesis, carotenoid pathway, nitrogen metabolism, antibiotic resistance etc also lead to somaclonal varaiation.

Application of somaclonal variation

The important applications of somaclonal variations are briefly described

1. **Production of agronomically useful plants :-** As a result of somaclonal variations, several novel variants of existing crops have been developed.
   Example:- Rice, Wheat, Tobacco, Soybean, etc
2. **Resistance to disease:-** Somaclonal variations have largely contributed towords the development of disease resistance in many crops.
   Example:- Rice, Wheat, Maize, Sugarcane,etc.

3. **Resistance to abiotic stress:-**It has been possible to develop biochemical mutants with abiotic stress resistance.
- Freezing tolerance e.g. Wheat
- Salt tolerance e.g. Rice, Maize, Tobacco
- Aluminium tolerance e.g. Carrot, Sorghum

4. **Resistance to herbicide:-** certain somaclonal varisnts wih herbicide resistance have been developed .
- Resistant to Glyphosate, Sulfonlurea and Picloram. E.g. Tobacco

Limitation of somaclonal variations

Despite several applications of somaclonal variation, there certain limitation also.

1. Most of the somaclonal varistions may not be useful.
2. The variations occur in an unpredictable and uncontrolled manner.
3. Many time the genetic traits obtained by somaclonal variations are not stable and heritable.
4. Somaclones can be produced in only those species which regenerate to complete.

Gamatoclonal variations

The variations observed while culturingthe gametic cells are regarded as gamatoclonal variations.

The term gametoclone is used for the product of gamatoclonal variationsas the somatic cells divide by mitosis, the genetic material is equally distributed to the daughter cells. In contrast, the gamates, being the products of meiosis, possess only half of the parent cell genetic material.

The gamatoclonal variations differ from somaclonal variations by three distinctfeatures.

1. Mutants obtained from gamatoclonal variations give rise to haploid plants since a single set of chromosome are present.
2. Meiotic crossing over is the recombination process observed in gamatoclonal variations.

Chapter... 13

PRODUCTION OF SECONDARY METABOLITES

Plants are the source of a large variety of biochemical's 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 amino acids, terpenoids and phenolics are grouped under secondary metabolites which donot 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 color, 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.

Chapter... 14

GENETIC ENGINEERING

By the conventional plant breeding techniques, significant achievements have been made in the improvement of several food crops. These age-old classical methods, involving gene transfer through sexual and vegetative propagation, take very long time. For instance, about 6-8 years may be required to develop a new rice or a wheat by sexual propagation. Rapid advances in gene structure and function , coupled with the recent development made in the genetic engineering techniques have dramatically improved the plant breeding method to yield the desired results in a short period.

Plant genetic transformation technology basically deals with the transfer of desirable gene from one plant species to another with subsequent integration and expressionof the foreign genein the host genome.

The term transgene is used to represent the transferred gene, and the genetic transformation in plants is broadly referredto as plant transgenesis. The genetically transformed new plants are regarded as transgenic plants.

The development of of transgenic plants is the outcome of an integrated application of recombinant DNA (rDNA) technology, gene transfer – methods and tissue culture techniques.

Why transgenic plants?

The most important reason for developing transgenic plants are listed.

- To improve agricultural, horticultural or ornamental value of plants.
- To develop plant bioreactors for inexpensive manufacture of commercially important proctucts.
  E.g. Proteins, Medicines, Pharmaceutical compounds.
- To study the action of genes in plants during during development and various biological processes.

Gene transfer methods

The gene transfer techniques in plant genetic transformation are broadly grouped into two categories.

1. Vector-mediated gene transfer
2. Direct or vectorless DNA transfer
   The silent features of the commonly used gene transfer methods are given in table

Methods of gene transfer	Features
Vector-mediated DNA transfer Agrobacterium mediated gene transfer Plant viral vectors.	Very efficient, but limited to a selected group of plants Ineffective method, hence not widely used
Direct or vectorless DNA transfer **A] Physical method** Electroporation Particle bombardment Microinjection Liposome fusion Silicon carbide fibres **B] Chemical method** Polyethylene glycol (PEG) mediated Diethylaminoethyl dextran mediated	Mostly confined to protoplast that can be regenerated to viable plants. Very successful method used for a wide range of plants/tissues. Risk of gene arrangement high. Limited use since only one cell can be microinjected at time. Require skilled person. Confined to protoplasts that can be regenerated into viable whole plants. Require regenerable cell suspension. The fibres, however, require careful handling. Confined to protoplasts. Regeneration of fertile plants is frequently problematical. Dose not result in stable transformants.

Table:- Gene transfer methods in plants

1. Vector-mediated gene transfer

Vector mediated gene transfer is carried out either by *Agrobacterium* – mediated transformation or by use of plant viruses as vectors.

***Agrobacterium* – mediated gene transfer**

Among the various vectors used in plant transformation, the Ti plasmid of ***Agrobacterium tumefaciens*** has been widely used. This bacteria is known as "natural genetic engineer" of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti-plasmid in place of unwanted sequence.

Agrobacterium tumefaciens is a soil-borne, gram negative bacterium. It is rod shaped and motile, and belongs to the bacterial family of Rhizobiaceae.

There are mainly two species of *Agrobacterium.*

- A. tumefaciens that induces crown gall disease.
- A. rhizogenes that induces hairy root disease.

Virus-mediated gene transfer (Plant viruses as vectors)

Plant viruses are considered as efficient gene transfer agents as they can infect the plants and amplify the transferred genes through viral genome replication. They can introduce the desirable genes into almost all the plant cells since the viral infections are mostly systemic.

The plant viruses do not integrate into the host genome in contrast to the vectors based on T-DNA of A.tumifaciens which are integrative. The viral genomes are suitably modified by introducing desired foreign genes. These recombinant viruses are transferred, multiplied and expressed in plant cells. They spread systemically within the host plant where the new genetic material is expressed.

Criteria for a plant virus vector

An ideal plant virus fo its effective use in gene transfer is expected to posses the following characteristics.

- The virus must be capable of spreading from cell to cell hrough plasmodesmata.
- The viral genome should be able to replicate in the absence of viral coat protein and spread from cell to cell. This desirable since the insertion of foreign DNA will make the viral genome too big to be packed.
- The recombinant viral genome must elicit little or no disease symptoms in the infected plants.
- The virus should have a broad host range.
- The virus with DNA genome is preferred since the genetic manipulation involve plant DNA.

The three groups of viruses- Caulimovirus, Geminivirus and RNA viruses.

Caulimoviruses as Vectors

The caulimoviruses contain circular double-starnded DNA , and are spherical in shape. The caulimoviruses group has around 15 viruses and among these cauliflower mosaic virus (CaMV).

Cauliflower mosaic virus (CaMV)

CaMV infects many plants and can be easily transmitted, even mechanically. Another attractive feature of CaMV is that the infection is systemic, and large quantities of viruses are found in infected cells. The genome of CaMV consists of a 8kb relaxed but tightly packed circular DNA with six major and two minor coding regions. The genes II and VII are essential for viral infection.

For appropriate transmission of CaMV, the foreign DNA must be encapsulated in viral protein. Further, the newly inserted foreign DNA must not interferewith the native assembly of the virus.

CaMV genome does not contain any non coding regions wherein foreign DNA can be inserted. It is fortunate that two genes namely gene II and gene VII have no essential functions for the virus. It is therefore possible to replace one of them and insert the desired foreign gene.
Gene II of CaMV has been successfully replaced with a bacterial gene encoding dihydrofolate reductase that provides resistance to methotrexate. When the chimeric CaMV was transmitted to turnip plants, they were systemically infected and the plants developed resistance to methotrexate.

Limitation

- ✓ CaMV vector has a limitedfor insertion of foreign genes
- ✓ Infective capacity of CaMV is lost if more than a few hundred nucleotide are introduced.
- ✓ Helper viruses cannot be used since the foreign DNA gets expelled and wild-type viruses are produced.

Geminiviruses as vectors

The geminiviruses are so named because they have germinate morphological particles. These viruses are characterized by possessing one or two single- stranded circular DNA. The geminiviruses can infect a wide range of crop plants which attract plant biotechnologists to employ these viruses.
e.g. Curly top virus (CTV), Maize streak virus (MSV), Bean golden mosaic virus (BGMV)
It has been observed that a large number of replicate forms of a geminivirus genome accumulate inside the nuclei of infected cells. The single-stranded genomic DNA replicate in the nucleus to form a double-stranded intermediate.
Geminivirus vectors can be used to deliver, amplify and express foreign genes in several plants.

Limitation

- ✓ It is very difficult to introduce purified viral DNA into the plants.
- ✓ Require alternate arrangement is to take help of *Agrobacterium* and carry out gene transfer.

Direct or Vectorless DNA transfer

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The methods used for direct gene transfer in plants are

Chemical mediated gene transfer e.g. chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts. Calcium phosphate is also used to transfer DNA into cultured cells.
Microinjection where the DNA is directly injected into plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5 - 1.0 micrometer in diameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs, and the cells of early embryo.

Electroporation involves a pulse of high voltage applied to protoplasts/cells/ tissues to make transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleas.

Particle gun/Particle bombardment - In this method, the foreign DNA containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as gene gun/shot gun/ micro projectile gun).The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Two types of plant tissue are commonly used for particle bombardment- Primary explants and the proliferating embryonic tissues.

Transformation - This method is used for introducing foreign DNA into bacterial cells e.g. E. Coli. The transformation frequency (the fraction of cell population that can be transferred) is very good in this method. E.g. the uptake of plasmid DNA by E. coli is carried out in ice cold CaCl2 (0-50C) followed by heat shock treatment at 37-450C for about 90 sec. The transformation efficiency refers to the number of transformants per microgram of added DNA. The CaCl2 breaks the cell wall at certain regions and binds the DNA to the cell surface.

Conjuction - It is a natural microbial recombination process and is used as a method for gene transfer. In conjuction, two live bacteria come together and the single stranded DNA is transferred via cytoplasmic bridges from the donor bacteria to the recipient bacteria.

Liposome mediated gene transfer or Lipofection - Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adher to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells.

Selection of transformed cells from untransformed cells

The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For this, a marker gene (e.g. for antibiotic resistance) is introduced into the plant along with the transgene followed by the selection of an appropriate selection medium (containing the antibiotic). The segregation and stability of the transgene integration and expression in the subsequent generations can be studied by genetic and molecular analyses (Northern, Southern, Western blot, PCR).

Chapter...15

BIOCHEMICAL ANALYSIS

1. Qualitative analysis

Methods:-

Extracts were tested for the presence of active principles. Following standard procedures were used. [14] [15].

Test for Alkaloids:-

Ethanolic extract was warmed with 2% H2SO4 for two minutes. It is filtered and few drops of reagents were added and indicated the presence of alkaloids.

a. Mayer's reagent-A creamy- white colored precipitation positive.

b. Wagner's reagent-A reddish-brown precipitation positive.

c. Picric Acid (1%)-A yellow precipitation positive.

Test for Steroids Terpenoid and Triterpenoids:

a) Liebermann Burchard test: -

Crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids terpenoid and triterpenoids respectively.

b) Salkowski Test:-

The extract was mixed with 2ml of chloroform and concentrate H2SO4 (3ml) is carefully added to form a layer. A reddish brown coloration of the interface is formed to show positive result of the presence of steroids terpenoid and triterpenoids respectively.

Test for saponins:-

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for phenols and tannins:-

Crude extract was mixed with 2ml of 2% solution of FeCl3. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for Flavonoids:-

A small quantity of the extracts is heated with 10 ml of ethyl acetate in boiling water for 3 minutes. The mixture is filtered differently and the filtrates are used for the following test.

a) Ammonium Test:-

The filtrate was shaken with 1 ml of dilute ammonia solution (1%). The layers were allowed to separate. A yellow coloration was observed at ammonia layer. This indicates the presence of the flavonoid.

b) Aluminum Chloride Test:-

The filtrates were shaken with 1 ml of 1% aluminum chloride solution and observed for light yellow color. It indicated the presence of flavonoid and diluted NaOH and HCl was added. A yellow solution that turns colorless indicated positive.

Test for Carbohydrate:-

Benedict's test :-

Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath, observed for the formation of reddish brown precipitate to show a positive result for the presence of carbohydrate.

Test for Glycosides:-

Fehling's test:-

Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Test for proteins:-

Millon's test:-

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Test for Free Amino Acids:-[18]

Ninhydrin Test:-

Test solution when boiled with 0.2% solution of Ninhydrin, would result in the formation of purple color suggesting the presence of free amino acids.

Test for Vitamin C:-[17]

DNPH Test:-

Test solution was treated with Dinitrophenylhydrazine dissolved in concentrated sulphuric acid. The formation of yellow precipitate would suggest the presence of vitamin C.

For Carboxylic acid, test for NH_2, Nitrogen, Sulphur, Halogen, Amides, test for Unsaturation, test for Aromaticity [16].

Qualitative analysis by thin layer chromatography [15]

Extract was to begin with, checked by Thin Layer Chromatography (TLC) on analytical plates over silica gel. TLC was carried out to isolate the principle components that were present in most effective extracts of plant. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution.

Method

The above prepared plant extracts were applied on pre-coated TLC plates by using capillary tubes and developed in a TLC chamber using suitable mobile phase. The developed TLC plates were air dried and observed under ultra violet light UV at both 254 nm and 366 nm. They were later sprayed with different spraying reagents and some were placed in hot air oven for 1 min for the development of color in separated bands. The

movement of the analyze was expressed by its retention factor (Rf). Values were calculated for different sample.

$$R\,f = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}}$$

(Rf-Retention factor)

Detection

After drying the plates, they were exposed to Iodine vapours by placing in a chamber that was saturated with iodine vapours and also exposed to different spraying reagents. All plates were visualized directly after drying and with the help of UV at 254 nm and 366 nm in UV TLC viewer. The Rf value of the different pots that were observed was calculated.

Chemical Name	Solvent System	PP	R*f* values	Spray Reagent
Alkaloids	M:NH_4OH (17:3)	R	0.48, 0.55	Mayer's reagent
		S	0.64	
		L	0.71, 0.78	
Flavonoid	C:M (18:2)	R	0.26, 0.30, 0.45, 0.57, 0.82	UV light
		S	0.16, 0.20, 0.26, 0.33, 0.36, 0.48, 0.51, 0.57, 0.82	
		L	0.16, 0.30, 0.45, 0.60, 0.73, 0.82, 0.85	
Saponins	C:GA:M: W (6:2:1:1)	R	0.22	Iodine vapours
		S	0.10, 0.76	
		L	0.08	
Terpenoides	B:EA (1:1)	R	0.36, 0.37, 0.87	10% H2SO4
		S	0.36,0.39, 0.87	
		L	0.36, 0.40, 0.46, 0.73, 0.86	

14. Note : P P-Plant part., C-chloroform, M-methanol, B-Benzene, EA- Ethyl acetate, GA- Glacial acetate, W-water, R-root, S-stem, L-leaf

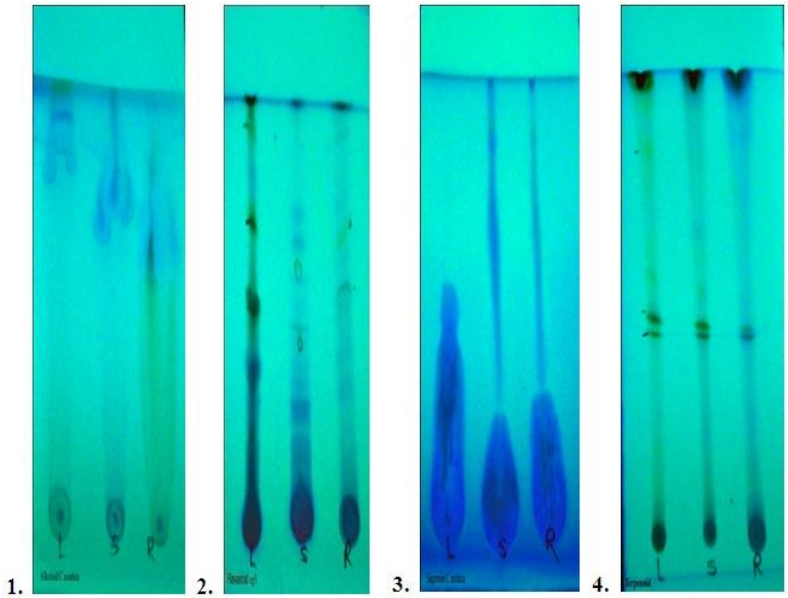


Photo plate 3: TLC of 1. Alkaloids 2. Flavonoid, 3. Terpenoides 4. Saponin of different parts of Centilla asiatica L

Quantitative analysis and Thin Layer Chromatography

Quantitative analysis:-

The phytochemicals which are present in the ethanol extracts of *Centella asiatica* were determined and quantified by standard procedures.

Alkaloid determination using Harborne (1973) method:-

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed [16].

Flavonoid determination using Boham and Kocipai- Abyazan (1994) method:-

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight [17].

Saponin determination using Obadoni and Ochuko (2001) method:-

10 g of samples powder was put into a conical flask and 50 ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 100 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at

about 90°C. The concentrate was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight and the Saponin content was calculated as percentage [18].

Total Terpenoides determination using Ferguson (1956) method:-

10g of plant powder were taken separately and soaked in alcohol for 24 hours. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids [19].

Plant part	Fresh wt. In gm	Dry weight	D.M. [%]	Alkaloid In gm Extraction. Each 5 gm	Alkaloid [%]	Flavonoid. In gm Extraction Each 10gm	Flavonoid [%]	Terpenoid In gm Extraction Each 10 gm	Terpenoid [%]	Saponin In gm Extraction Each 10 gm	Saponin [%]
Root	41.75	38.8	92.93	0.01	0.025	0.1	0.25	0.1	0.25	0.1	0.25
Stem	56.13	53.55	95.40	0.1	0.18	0.3	0.56	0.1	0.18	0.2	0.37
Leaf	48.43	45.34	93.61	0.2	0.44	1.4	3.0	0.7	1.55	0.2	0.44

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