PLANT BIOTECHNOLOGY

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Paper-VIII : PLANT BIOTECHNOLOGY

Unit-I

Lesson 1

Concept and Scope of Biotechnology – Techniques of Plant Tissue Culture

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1.0 OBJECTIVES

The concept of Biotechnology, its scope and one of the important techniques of tissue culture are explained are explained in this lesson.

1.1 INTRODUCTION

The term Biotechnology was coined by Karl Ereky a Hungarian Engineer in 1919. The origin of biotechnology can be traced back to prehistoric times when micro-organisms were used for processes like fermentation, formation of yoghurt and cheese from milk and acetone from starch by *Closteridium acetobutylicum* or the production of antibiotics like peneillin from *Pencillium notatum*. Thus the origin of Biotechnology can be traced back to the origin of human civilization.

With the discovery of restriction enzymes in 1970, that cut DNA, there is development of variety of gene technologies for genetic manipulation, which can't be carried out in conventional breeding. Programmes because in conventional breeding, the transfer of genes is severely limited by species barriers. Hence, these technologies provide better approach for manipulation of targets of our choice.

1.2 SCOPE AND INTERDISCIPLINARY NATURE OF BIOTECHNOLOGY

Attempts have been made to define biotechnology and it has been interpreted in different ways by different groups of workers. Biotechnology is broadly defined as any technique that uses living organisms or parts of organisms to make or modify products to improve plants or animals to develop microorganisms for specific use. Biotechnology is the controlled use of biological agents, such as microorganisms or cellular components for beneficial use (US National Science Foundation). Among many definitions, the following definition seems to be most appropriate and simple.

Biotechnology is a multi-disciplinary applied science which is based on cell biology, genetics, microbiology, biochemistry, Molecular Biology, Physiology, Chemical Engineering, Material Science etc.

What constitutes the core of biotechnology? It is the gene technology followed by cell technology. The gene technology, also called recombinant DNA technology constitutes precise molecular biotechnologies. These include:

gene isolation
gene cloning
gene sequencing
gene expression and characterization of expressed product
gene manipulation
gene transfer etc.

For instance, the common bacterial cells can't consume oil or carry human insulin genes in them. Similarly, plants were not meant to harbor genes from bacteria, rats, human etc. But these organisms were re-programmed to produce chemicals, enzymes, drugs and biopesticides etc. Hence, in Biotechnology some of the important methods that can bring biological revolution are Recombinant DNA technology, Genetic Engineering, Tissue Culture, immunotechnology, protein engineering processing technology, DNA chip technology etc.

Biotechnology in India

Though Biotechnology progressed since 1970s in other countries, its growth in India is from 1987. In view of the significance, a separate Department of Biotechnology in the Ministry of Science and Technology was created in 1986. Among these disciplines, time culture is International Centre for Genetic Engineering and Biotechnology (ICGEB) for developing countries has been established. This Centre has two locations one at Delhi and at Italy Biotechnology Centres in India have established nearly two decades times. Some of them are Indian Institute of Science, Bangalore; Madurai Kamaraj University, Madurai; Bose Institute, Calcutta; Jawaharlal Nehru University, New Delhi; University of Poona, Pune; Indian Agricultural Research Institute, New

Delhi; Centre for Cellular and Molecular Biology, Hyderabad; National Institute of Immunology, New Delhi; Institute of Microbial Technology, Chandigarh; Department of Biotechnology, New Delhi and several subcentres have come in various Institutes and Universities.

The European Federation of Biotechnology was started in 1978, and people working in various disciplines of Science like microbiology, biochemistry, molecular biology, medicine, virology, agriculture etc. have put all their efforts for unraveling the Biotechnological solutions which lead to the mass production of growth hormones, vaccines, insulin, immuno-proteins, polypeptides, biofertilizers, biopesticides, production of plants resistant to diseases, stress, antibiotics, enzymes, biofuels etc. Production of majority of these have been achieved using recombinant DNA technology, in countries like USA, Sweden, Switzerland etc.

Human health care is the important area which came under focus and all the Countries over the globe have been doing collaborative work in this direction. Scientists are receiving training through famous centers and institutes like the International Centre for Co-operative research and training on Microbial engineering at Japan, Institute of Biotechnological studies in UK, Regional Microbiology net work for South East Asia supported by Japan & UNESCO; Microbiological Resource Centers (MICRENS) supported by UNESCO; United National Environmental Programs (UNEP), Inter National Cell Research Organization (ICRO) etc.

The increasing world's population demands a consequential increase in (Agricultural produce) food grain production. The achieved green revolution may not be sufficient to meet with the food demand for the ever increasing human populations. The solution could be production of varieties with the gene manipulation and through tissue culture technology which may improve the productivity.

Thus emergence of plant Biotechnology in the plant science fore front has been a mile stone in the manipulation of biological systems. The most exciting method being gene cloning for generation of transgenics, micro propagation of plants through tissue culture & recombinant DNA technologies etc.

Genetic engineering has contributed significantly in the genetic modification of plants either through the manipulation of their own genome or through introduction of genes from other organisms like those of microbes and animals etc.

Technical developments in science occur which enable an increment in the knowledge through enhancement in innovative potentialities. Such changes have occurred in the Molecular biology and biomedical research fields resulting in gene manipulation studies which permitted isolation of DNA stretches from their host organism and its propagation in the same or a different host.

Biotechnology can produce great impact on several aspects of our life. There are rapidly increasing opportunities for the application of biotechnology to the manufacture of medical and

veterinary products, chemicals, foods, energy, pollution control and waste management etc. (see Lesson 4).

1.3 TISSUE CULTURE TECHNOLOGY

Tissue culture Technology is based on the toti potency concept whereby a cell has the genetic potential to develop into a complete organism and it involves a number of experimental methods for the mass production of large number of plants from cells, tissues or organs under controlled and sterile conditions. The experimental materials are grown on nutrient medium containing mineral nutrients, Vitamins, phyto hormones etc. This technology is highly useful for mass production of plants under *in vitro* conditions in the laboratory, which cannot be propagated by the conventional methods available. Tissue culture work needs different types of instruments, glassware, chemicals for the preparation of nutrient medium, and a sterile chamber in which inoculation operations are carried on.

1.4 TECHNIQUES

The general techniques involve: (i) medium preparation, (ii) sterilization, (iii) aseptic manipulation and maintenance of cultures.

1.4.1 Preparation of culture medium

For well organized growth and development of the plant, nutrients and other growth requirements are necessary. The cells, tissues and organs of an intact plant (*in vivo*) receive the nutrients and other growth promoters are necessary for their organized growth and development directly from the plant body ,while isolated cells and tissues of the explant material need nutrients for their growth and development *in vitro* which are supplied in the form of synthetic nutrient media.

Composition of the nutrient medium

Among different culture media, Murashige and Skoog's (1962, MS) medium is of wider use in tissue culture experiments. In addition to the necessary nutrients, these media also contain various inorganic constituents. The specific purpose of the different media varies - for e.g. Gamborg's B_5 medium is specifically used for cell and protoplast culture, while N_6 medium is used for androgenic haploid production in rice.

The success of any medium for callus induction depends on the hormonal concentration while the process of regeneration mainly depends on the ratio of auxin to cytokinin.

The inorganic nutrients, carbon, energy source, vitamins organic supplements that include nitrogen, acids and complex substances are required in addition to growth regulators (detailed information on composition of different media is presented in table -1).

Table 1 Nutritional components of some plant tissue culture media[a]

Components	Amount (mg l^{-1})[b]					
	White's	MS	B5	Nitsch's	N6	E1
Macronutrients						
$MgSO_4 \cdot 7H_2O$	750	370	250	185	185	400
KH_2PO_4	—	170	—	68	400	250
$NaH_2PO_4 \cdot H_2O$	19	—	150	—	—	—
KNO_3	80	1900	2500	950	2830	2100
NH_4NO_3	—	1650	—	720	—	600
$CaCl_2 \cdot 2H_2O$	—	440	150	—	166	450
$(NH_4)_2 \cdot SO_4$	—	—	134	—	463	—
Micronutrients						
H_3BO_3	1.5	6.2	3	—	1.6	3
$MnSO_4 \cdot 4H_2O$	5	22.3	—	25	4.4	—
$MnSO_4 \cdot H_2O$	—	—	10	—	3.3	10
$ZnSO_4 \cdot 7H_2O$	3	8.6	2	10	1.5	2
$Na_2MoO_4 \cdot 2H_2$	—	0.25	0.25	0.25	—	0.25
$CuSO_4 \cdot 5H_2O$	0.01	0.025	0.025	0.025	—	0.025
$CoCl_2 \cdot 6H_2O$	—	0.025	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	—	0.8	0.8
$FeSO_4 \cdot 7H_2O$	—	27.8	—	27.8	27.8	—
$Na_2EDTA \cdot 2H_2O$	—	37.3	—	37.3	37.3	—
EDTA Na ferric salt	—	—	43	—	—	43
Sucrose (g)	20	30	20	20	50	25
Organic supplements						
Vitamins						
Thiamine HCl	0.01	0.5	10	0.5	1	10
Pyridoxine HCl	0.01	0.5	1	0.5	0.5	1
Nicotinic acid	0.05	0.5	1	5	0.5	1
Myoinositol	—	100	100	100	—	250
Others						
Glycine	3	2	—	2	—	—
Folic acid	—	—	—	0.5	—	—
Biotin	—	—	—	0.05	—	—
pH	5.8	5.8	5.5	5.8	5.8	5.5

[a] Growth regulators and complex nutrient mixtures used by various workers are not included here as the quantity and constituents of these compounds vary for specific tissue and organ.

[b] Abbreviations and references:

White's (1953; *Am. J. Bot.* 40: 517–524, or 1963; The Cultivation of Plant and Animal Cells, 2nd Edition, The Ronald Press, NY, 228 pp.).

MS (Murashige and Skoog 1962; *Physiol. Plant.* 15: 473).

B5 (Gamborg et al. 1968; *Exp. Cell Res.* 50: 151).

Nitsch's (Nitsch and Nitsch 1969; *Science,* N.Y., 163: 85).

N6 (Chu 1978; *Proc. Symp. Plant Tissue Culture,* Science Press, Peking, p. 43).

E1 (Gamborg et al. 1983; *Plant Cell Rep.* 2: 209).

Note: LS (Linsmaier and Skoog 1965; *Physiol. Plant.* 18: 100-127) medium has same composition as MS, but differs in concentration of thiamine (6.4 mg l^{-1}); inositol (100 mg l^{-1}) is used instead of other vitamins and glycine.

Inorganic nutrients

Among the inorganic nutrients, calcium forms a component of the cell wall; nitrogen- a part of amino acids, proteins, nucleic acids and vitamins, while, magnesium is the main component of chlorophyll. Zinc, molybdenum and iron are constituents of some enzymes. Apart from carbon, nitrogen, hydrogen and oxygen, about 12 more elements are necessary for plant growth.

Some of the elements like N, K, P, Ca ,S and Mg required in concentrations of 0.5mM/l are known as macros while those necessary in very low concentrations form the microelements and these include Fe, Cu, Mn, B, Zn, I, Mo, and Co.

In general, the nutrient medium should contain from 25 to 60 mM inorganic nitrogen. In order to check the difference in the pH of the medium caused by addition of nitrogen in the form of nitrates, an ammonium compound is added to the medium.

Carbon Source

Sucrose or glucose form the sources of carbon in the medium. Rarely fructose is used, but it is less efficient compared to either sucrose or glucose. Generally, 2.5% concentration of glucose is used. In some cases, lactose, maltose, galactose and starch also have been used as carbon sources which seem to be inferior to the other carbohydrates used. In many media preparations, 100 mg/l myo insitol is used for cell growth.

In comparison to the intact plants that synthesize vitamins, cultured cells require the supply of vitamins for their growth and developments.

Hormones

Plants synthesize Hormones, which regulate their growth and development .They are produced at one site in the plant but become effective at another site. The hormones are categorized into auxins and cytokinins The former are commonly endowed with induction of cell division, cell elongation and swelling of tissues and cause adventitious root formation, while, the latter are adenine derivatives and induce shoots.

Among the auxins, Indole Acetic Acid (IAA) and among the Cytokinins- Zeatin are of natural occurrence while hormones like Indole Butyric acid (IBA), Naphthyl Acetic acid (NAA) ; Kinetin(kn) Benzyl adenine (BA) etc belong to the synthetic category. Other hormones are important for specific purposes like meristem culture (GA3), Gibberellins for inter node elongation, meristem growth and in general to inhibit adventitious root as well as shoot formation.

Aminoacids

Even though cultured cells synthesize amino acids, the nutrient medium includes amino acids like glutamine, aspargine and nucleotides like adenine as sources of organic nitrogen.

Organic acids

Organic acids cannot be used by plant cells as sources of carbon, but citrate, malate, succinate or fumarate when added to the medium having ammonia as nitrogen source, cause cell growth.

Other Complex substances

In some cases complex substances like yeast extract, coconut milk, fruit juices etc. are added to the medium but these in higher concentrations cause adverse effects on cell growth.

Phenolic substances produced by the plant cells into the medium disturb the pH of the medium and this can be regulated by the addition of activated charcoal or polyvinyl pyrollidin (0.2% -3%)

Gelling agent or Agar

Agar, a polysacharide derived from sea weed is used for solidification of the medium . Higher concentrations of agar show negative effects on *in vitro* growth. In place of agar sometimes alginate (for protoplast culture), Gelrite or biogel P20 can also be used.

pH

Different aspects of structure and action of biological molecules are determined by the hydrogen ion concentration or pH of the medium. For a better *in vitro* growth, the medium pH should lie between 5 & 6. The pH of the medium changes during autoclaving. The growth and development *in vitro* are effected by the alkaline pH (>7) as well as acidic < 4.5) pH.

5 to 8% Agar or any other gelling agent is to be added to the liquid medium (for solidification) and heated to 60^0C with stirring until agar is dissolved. The medium is autoclaved for 20mts. at 121^0 C and 15 lbs pressure. Filter sterilized hormones and other compounds are to be added to the autoclaved medium only after cooling it to 50^oC 60^o C in the Laminar Flow Unit while using 0.22 mm filter. Then the medium is to be poured in to tubes, flasks or petriplates.

The tubes are to be fitted with non absorbent cotton plugs wrapped in gauze cloth and covered with brown paper and a rubber band is tied. The tubes, flasks or petriplates containing the medium should be marked with the date and concentration of the hormone. Infection occurring in the medium indicates improper sterilization of the medium.

1.4.2 Sterilization

The culture medium promotes growth of micro organisms, bacteria and fungi, when it is supplemented with sugars, these organisms destroy the explant growth due to their faster growth and short life cycle. Hence, procedure used for elimination of microorganisms, which hinder the growth of the explant is called sterilization.

Sterilization through Autoclaving

One of the most commonly used sterilization procedures is autoclaving under steam. The instruments, filter papers, glass rods, and distillated water used for inoculation of the explant, as well as the explant it self may cause infection. Thus, the medium, as well as the non-living particles mentioned above as well as the explant used should be maintained under sterile conditions.

While autoclaving, screw capped glass vials, the screw caps should not be set too tightly, expansion of gases result in explosion.

After autoclaving, the glass vials are taken out and kept in the Laminar air flow cabinet and the caps are tightened after they attain the room temperature.

Even though sterilization process in an autoclave is speedy and the operation is simple, pH of the medium changes by 0.3 to 0.5 units resulting in occurrence of chemical reactions due to separation of the components of the chemicals as a result of which the activity of the medium is likely to be reduced.

While using an autoclave care should be taken to maintain the correct duration, since higher temperatures, caramelize sugars, which become toxic. Autoclaving for longer periods precipitates salts and de polymerizes agar.

Dry sterilization

Glassware and metallic instruments wrapped in brown paper or aluminum foil, can be sterilized by heating them in an oven at $160^{o} – 180^{0}$C for 3 hrs. The autoclaves available presently have progress for both steam as well as dry sterilization. For sterilizing liquid materials like media or water, wet or steam sterilization using autoclave or pressure cooker is to be employed.

Filter sterilization

Heat labile growth factors (hormones) like amino acids, vitamins get destroyed during autoclaving and these are filtered through sieve or filtration assembly using 0.45 to 0.22 mm filters available from Millipore, sartorius, or Nalgone companies. These filters are made of cellulose acetate/cellulose nitrate and are available in pre-sterilized, plastic disposable units. If a thermo

labile substance is to be added to the medium, this substance is first autoclaved in a flask, while the medium is still in liquid state, it is injected with hypothermic syringe.

UV-Sterilization

Use of radiation (Gamma rays) for sterilization of nutrient media in tissue culture is very rarely used due to its expensive nature as well as the risk involved in its use.

Sterilized plastic ware (disposable) are exposed to ultra violet (UV) radiation, the sterilized media are transferred in to the sterilized plastic containers in the Laminar Flow chamber.

Aseptic conditions: In order to create aspect condition, the walls and floor of the Laminar Flow Cabinet should be sprayed with ethanol and the workers should wash their hands with an antibacterial detergent and ethanol is to be applied to the hands.

Flame sterilization

The instruments have to be dipped in 70% alcohol and from time to time heated on a Bunsen burner flame kept in the Laminar Air Flow unit. The culture vessels (tubes/flasks/Petriplates) should be closed soon after transfer of explant material.

While carrying on the sterilization and the inoculation operations, the worker should remain silent and his/her hands should remain inside the cabinet. All the materials, containers and instruments have to be kept aside in the hood of Laminar Air Flow cabinet such that the sterile air flow will not be disturbed.

Explant sterilization

In order to eradicate the micro organisms present on the explant, it is to be surface sterilized with mercuric chloride, Sodium hypochlorite, etc., in the hood of the Laminar Flow chamber. The concentration of the chemicals should be such that, it can remove the micro organisms but should not be lethal to the explant tissues. Hence, the concentration of the surface sterilizing agent should be standardized for the different explants.

The explant material should be thoroughly washed in tap water and then immersed in 5% solution of a liquid detergent like “Teepol” for 10-15 minutes; thoroughly washed with tap water for 5 minutes and then with distilled water out side the Laminar Flow for 5 minutes; the explant is to be immersed in 70% ethanol for 60-75 seconds, then transferred to any one of the following chemicals used for surface sterilization like , 0.01 aqueous solution of Mercuric chloride for 2 to 10 mts or 1 to 1.4% sodium hypochlorite for 5 to 30 mts or 9-10% calcium hypo chlorite for 5-30 mts or 10-12% hydrogen peroxide for 5 – 15 mts, 1-2% bromine water for 2 – 10 mts, or 1% silver nitrate for 5-30 mts or 4-50 mg of antibiotic solution for 30-60 mts.

After surface sterilization with any one of the above mentioned chemicals, the explant is to be thoroughly washed with sterile distilled water to remove any traces of the sterilizing agents, since the traces of chemicals will effect the establishment of cultures.

1.4.3 Aseptic manipulation

The sterilized explant material should be cut into discs and these are to be transferred to the tubes or petriplates containing the sterilized medium using the sterile forceps and the culture tubes or petriplates should be fitted with cotton plugs or closed with the tape respectively. These are to be kept in the racks and exposed to16 hours of light (2000 lux) and 8 hours of dark periods.

1.5 SUMMARY

The advancements in the field of various disciplines of biology and technology have led to the development of biotechnology is described. Biotechnology has helped in solving many problems in the fields like medicine, agriculture, environment etc.

The totipotency concept has led to the development of tissue culture technology which is mainly used for developing the total organism from any part of the original organism. This technology is based on growing any explant under *in vitro* conditions on a nutrient medium. The medium contains organic and inorganic nutrients, sugars as carbon source, growth hormones, gelling agent etc. Sterilization is an important process used in tissue culture technology for production of plantlets in aseptic conditions. A brief outlines of the techniques are described.

1.6 MODEL QUESTIONS

1. Explain the concept of Biotechnology? How is it important in 21st century.
2. Explain the recent advancements achieved in Biotechnology.
3. Write an account of the applications of Biotechnology.
4. Write short notes on:
   a) Culture media
   b) Laminar air flow chamber
   c) Explant sterilization
5. Give an account of the different methods of sterilization.
6. Give an out line of the main constituents of a culture medium.
7. Give an account of the general techniques used for culturing the plant tissues *in vitro*.

1.7 REFERENCE BOOKS

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Unit-II

Paper-VIII : PLANT BIOTECHNOLOGY

Lesson 2

CELL SUSPENSION CULTURES AND SECONDARY METABOLITES

2.0 OBJECTIVE:
In this lesson, Isolation of single cells and establishment of cell suspension cultures, types of cultures are given. Applications of cell suspension cultures, such as production of secondary metabolites and biotransformations have been discussed.
2.1 INTRODUCTION
2.2 ISOLATION OF SINGLE CELLS AND ESTABLISHMENT OF CELL SUSPENSION CULTURES
2.3 GROWTH AND STRUCTURE OF CELL SUSPENSION CULTURES
2.4 TYPES OF CELL SUSPENSION CULTURES
2.5 CULTURE MEDIUM
2.6 MEASUREMENT OF GROWTH IN CELL SUSPENSION CULTURES
2.7 SYNCHRONISATION OF CELL SUSPENSION CULTURES
2.8 VIABILITY OF CULTURED CELLS
2.9 CULTURE OF ISOLATED SINGLE CELLS
2.10 APPLICATIONS OF CELL CULTURES
2.11 BIOTRANSFORMATION
2.12 MUTANT SELECTION
2.13 SUMMARY
2.14 MODEL QUESTIONS
2.15 REFERENCE BOOKS

2.1 INTRODUCTION

Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Haberlandt (1902) made the attempt to culture isolated single cells from leaves of flowering plants. Although he failed to achieve divisions in isolated single cell, but his work subsequently stimulated several workers to study further on this line. To date, a spectacular progress has been achieved in inducing divisions in isolated single cells and regenerating complete mature plant *in vitro*. This generated much interest among plant biotechnologists and they realized the potentiality of cell cultures to synthesize natural products.

Besides, cell cultures have great potential in crop improvement and these are useful to describe the pathways of cellular metabolism.

2.2 ISOLATION OF SINGLE CELLS AND ESTABLISHMENT OF CELL SUSPENSION CULTURES

The leaf tissue is suitable source for the isolation of cells since it gives more or less homogeneous population of cells. From the leaf tissue, single cells can be isolated by mechanical or enzymatic methods.

(i) Mechanical method: Gnanam and Kulandavelu (1969) developed the procedure in which involves mild maceration of 10 g leaves in 40 ml of the grinding medium (20 μ mol sucrose, 10 μ mol $MgCl_2$, 20 μ mol tris-HCl buffer, pH 7.8) with a mortar and pestle. The homogenate is passed through two layers of muslin cloth and the cells thus released are washed by centrifugation at low speed using the same medium. Leaf mesophyll cells and bundle sheath cells can be isolated mechanically by using this method.

(ii) Enzymatic method: Cells are isolated enzymatically by using pectinase (macerozyme) which degrades the middle lamella and cell wall of the parenchymatous tissue. Tissue is incubated in the enzyme mixture consisting of potassium dextran which improves the yield of free cells. Isolated cells can be achieved with minimum damage by providing osmotic protection while incubation.

(iii) Isolation of cells from cultured tissues: Cultured tissues have been widely used to isolate single cells. Freshly cut pieces from surface-sterilized plant organs are simply placed on a solidified nutrient medium consisting of a suitable proportion of auxins and cytokinins to initiate cultures. Explant produces callus on the medium. Now this callus is separated and transferred to fresh medium of the same composition to enable it to build up a mass of tissue. Repeated subculturing of this on an agar medium improves the fragility of callus, a prerequisite for establishing a cell suspension cultures. The pieces of undifferentiated and friable callus are transferred to the liquid medium dispensed in autoclaved flasks. Agitation is done by placing the culture flasks on an orbital-platform shaker. Due to continuous agitation, mild pressure exerted on small pieces of tissue, breaking them into free cells and small cell aggregates. Further, it facilitates the gaseous exchange between the culture medium and the culture air, and also ensures uniform distribution of cells as well as cell clumps in the liquid medium.

2.3 GROWTH AND SUBCULTURE OF CELL SUSPENSION CULTURES

Cell suspensions are clonally maintained by subculture (routine transfer) of cells in the early stationary phase to a fresh medium. During incubation period, the biomass increases due to cell divisions and cell enlargement. This continues for a limited period since the viability of cells in

suspension after the stationary phase decreases due to exhaustion of nutrients or accumulation of toxic substances in the medium. When they are in active state, an aliquot of cell suspension with uniformly dispersed free cells is transferred to fresh medium.

The incubation period from culture initiation to the stationary phase is determined primarily by (a) initial cell density, (b) duration of lag phase, and (c) growth rate of cell line. The cell density used to subculture is critical and depends largely on the type of suspension culture to be maintained. While initiating new suspension culture, it is necessary to determine optimal cell density, proportionate to the volume of the culture medium, in order to achieve maximum growth. At an initial cell density of 9-15 x 10^3 ml^{-1}, the cells will generally undergo an eight fold increase in cell number before entering the stationary phase. Cell cultures initiated at very low cell densities will not grow unless the medium is supplemented with metabolites to grow single cells.

The normal incubation time of stock cultures is 21-28 days between subcultures although cloning may occur within 18-25 days.

2.4 TYPES OF CELL SUSPENSION CULTURES

(i) Batch Cultures

Batch cultures are maintained continuously by propagating a small aliquote of the inoculum and transferring it to a fresh medium at regular intervals. The biomass growth in batch cultures follows a fixed pattern (Fig. 2.1). The growth curve shows that initially the culture passes through a lag phase, followed by a brief exponential growth phase which is the most fertile period for active cell divisions. The growth declines after 3 to 4 generations, indicating that the culture has entered the stationary phase.

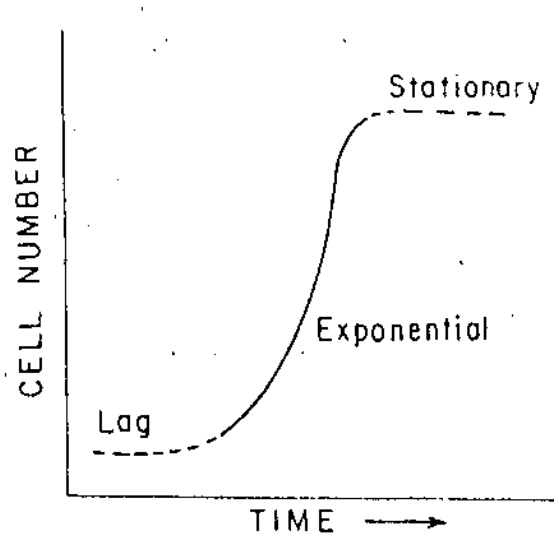

Fig. 2.1 Model curve showing different growth phases in batch cultures

Cultures can be maintained continuously in exponential phase by frequent (every 2-3 days) subculture of the suspensions.

Batch cultures are characterized by a constant change in the pattern of cell growth and metabolism. In these cultures, exponential growth with constancy of cell doubling time may be achieved, but there is no period of steady-state growth in which relative concentrations of metabolites and enzymes are constant. So batch cultures are not ideal systems to study various aspects of cellular behaviour.

(ii) Continuous cultures (mass cultures)

The large-scale cultures grown under steady state for longer periods by adding fresh medium and draining out the used medium in a number of specially designed vessels are known as continuous cultures (mass cultures). These are following two types:

A. Open Continuous Culture - A continuous culture in which the influx of fresh medium is equal to the efflux of old medium together with cells. In this culture, cells are constantly washed out with the old out-flowing medium. A steady state is achieved when the loss of cells is equal to new cells formed. A situation of balanced growth is achieved, i.e. majority of cells in the culture are in a similar metabolic state. The growth rate and cell density are held constant by a fixed rate of input of growth limiting-nutrients and, removal of cells and old medium.

The open continuous systems may be two types: **(a) Chemostat cultures** in which growth rate and cell density maintain steadily by a constant inflow of fresh medium consisting of growth limiting nutrients, such as nitrogen, phosphorous, glucose etc.; all other constituents are present at concentrations higher than the required. Increase or decrease in the concentration of growth-limiting factor is correspondingly expressed by increase or decrease in the growth rate of cells. Thus, the desired rate of cell growth can be maintained by adjusting the level of concentrations with respect to the growth-limiting factors and other constituents; (b) **Turbidostat cultures** in which fresh medium flows in response to increase in the turbidity so as to maintain the culture at a fixed optical density of suspension cultures. A pre-selected biomass density is maintained by the washout of cells.

B. Closed continuous culture – In this culture system, cells are retained from out-flowing medium and reintroduced into the cultures. An amount of inflow of fresh medium is balanced by the equal amount of out-flow of old medium (spent medium only). Cells are mechanically separated from the old medium. Thus in closed continuous culture, cell biomass continues to increase as the growth proceeds. These cultures are useful for studying the cytodifferentiation and harvesting the secondary metabolites (coumarins, lignins, polysaccharides, monoterpene derivatives produced by ell suspension cultures) continuously.

A wide range of bioreactor configurations and sizes have been designed for continuous cultures depending on the variety of plant cells.

2.5 CULTURE MEDIUM

Friable callus is essential to initiate the cell suspension cultures. This can be obtained through media manipulations and repeated subculturing of callus. Medium supplemented with 2,4-D (dichlorophenoxy acetic acid), small amount of hydrolytic enzymes (cellulase and pectinase), or substances such as yeast extract, appears to be have promotory effect on cell dispersion.

ER (Erikson) and B5 media are specially recommended for suspension cultures of higher plants. These and other synthetic media have been used for initial population density of 5×10^4 cells ml^{-1} or more.

Conditioning medium: To initiate cell cultures at very low cell density, the medium requires to be conditioned or enriched with various other compounds. A simple method is to filter out cells growing at high density from 4-6 week-old liquid cultures and to use this medium in drops to culture single cell/cells at low population density. Another method involves the separation of a high-density cell culture from a low-density culture medium by a barrier that permits the diffusion of solutes and air. A high-density cell suspension (the nurse culture) kept inside the dialysis tube (Fig. 2.2) is suspended into the flask containing the culture medium with low cell density. Metabolites produced by the nurse medium are diffused into the low cell density medium, thereby increasing the latter's growth-promoting activity. In this way, it meets the conditions of growth for low cell density populations.

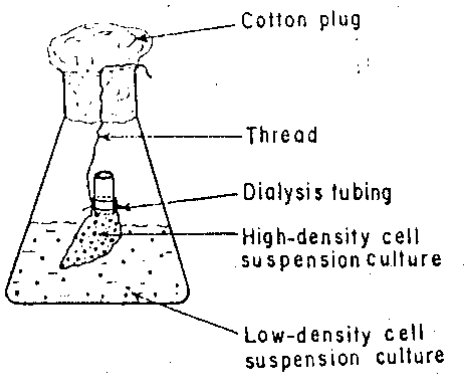

Fig. 2.2 Apparatus designed for conditioning of a low-density cell culture medium (adopted from Razdan, 1994)

2.6 MEASUREMENT OF GROWTH IN CELL SUSPENSION CULTURES

The following methods are adopted to measure the growth of cell suspension cultures at regular intervals.

(i) Cell Counting – Increase in cell number depends on the rate of mitotic index of cells in suspension cultures. So the cell count is a relatively more accurate method adopted to measure the growth of cultures. In this method, it is essential to disrupt cell aggregates by treating them with 5-15% chromic acid and heat at 70^{o}C for few min. Later, the mixture is cooled down, and agitated vigorously for 10 min. on rotary shaker. The suspension is centrifuged and, pellet is taken out and resuspended in 8% saline (NaCl) solution. After 10-15 min. free cells are counted on haemocytometer. Heating is avoided when the enzyme is used to disrupt cell aggregates.

(ii) Cell fresh weight – The cells are collected on a preweighed (wet) circular filter of nylon fabric and these filter disc are reweighed along with cells.

(iii) Cell dry weight – Procedure is similar to that of fresh weight except that the filter discs are dried in an oven for 12 hr at 60^{o}C. After cooling in a desiccator, the dried filter is reweighed and cell weight is expressed as g ml^{-} of culture.

(iv) Packed Cell Volume (PCV) – A small aliquote of sample (10 ml) is taken from the uniformly dispersed suspension culture and centrifuged in 15 ml graduated conical centrifuge tubes at 1000 g for 15 min. The packed ell volume is expressed as ml pellet ml^{-1} culture.

2.7 SYNCHRONISATION OF CELL SUSPENSION CULTURES

Cell cultures are mostly asynchronous because of presence of varied shape, size and nuclear content of cells; moreover, cell cycle time is also varied considerably among individual cells. This situation complicates the studies of biochemical, genetic, physiological and other aspects of cell metabolism. So it is essential to achieve a high degree of synchronisation of cell suspension cultures. In synchronous cultures, the majority of cells are in similar cell cycle phase.

(i) **Physical methods:** Physical properties of cells, such as size of individual cell/small cell aggregates (or) environment factors (light, temperature) can be monitored successfully to achieve a high degree of synchronisation.

(A) Selection by volume – Synchronisation is achieved on the basis of selecting the size of cell aggregates present even in the finest possible suspension cultures. This can be achieved by cell fractionation technique. This approach proved successful for carrot suspension cultures since 90% cell aggregates isolated were in early embryogenic stages.

(B) Temperature shock – To achieve the synchronous cultures, temperature shock is given at 4^{o}C for few days.

(ii) Chemical methods: Various chemical methods have been adopted to achieve synchronisation.

(A) Starvation method – It is based on the deprivation of an essential growth compound from suspension cultures that leads to a stationary phase. Resupplying the missing compound is

expected to induce resumption of cell growth synchronously. It has been reported that sycamore (*Acer pseudoplatanus*) cells are arrested in G1 and G2 by deprivation of phosphorus and carbohydrate respectively. Nitrogen-starved cells accumulate at G1 stage.

(B) Use of inhibitors – Synchronisation is achieved by using some biochemical inhibitors that temporarily block the progression of events in the life cycle and accumulate cells in a specific stage. On removing the inhibitor, these blocked cells will synchronously enter the next stage. Generally, 5-fluorodeoxyuridine (FudR), excess thymidine (TdR) and hydroxyurea (HU) are used that accumulate cells at G1/S interface stage.

(C) Mitotic arrest by colchicine method – Colchicine is the most effective spindle fibre inhibitor, which arrest the cells at metaphase stage of cell division. Suspension cultures in exponential growth are treated with 0.02% (w/v) colchicine for 4-8 hr. This synchronisation is measured in terms of mitotic index (the percentage of cells in the mitotic phase of the cell cycle).

2.8 VIABILITY OF CULTURED CELLS

The growth of the cultures is largely dependent on the viability of cells. This can be assessed by using dyes and observe under microscope.

(i) Phase contrast microscope: Cytoplasmic streaming and healthy nucleus observed under phase contrast microscope indicate that the cells are viable.

(ii) Reduction of tetrazolium salts: This test is performed to measure the respiratory efficiency of cells by reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to the red dye formazon. Formazon is extracted and measured spectrophotometrically.

(iii) Fluroscein diacetate (FDA) method: Stock solution of FDA (0.5% w/v) is prepared in acetone and stored at 0^{o}C. Viability is tested by adding this solution to cell/protoplast suspension. After 5 min. incubation, cells are examined under a microscope with a suitable excitation or suppression filter. FDA, with non-fluorescing and non-polar, is cleaved by esterase activity inside the living cell and releasing the polar portion of fluorescein which fluoresces green under UV. It accumulates only in viable cells. In a dead cell, the fluorescein is lost and remains invisible.

2.9 CULTURE OF ISOLATED SINGLE CELLS

Isolated single cells can be cloned by Bergmann plating technique. Success in the culture of single cells depends on the technique adopted and various factors affecting cell plating.

(i) Bergmann's Plating Technique: This technique is the most popular one for plating of single cells. Suspension cultures are filtered aseptically through filter which allows only the single cells to pass through (Fig. 2.3); the cell aggregates are discarded.

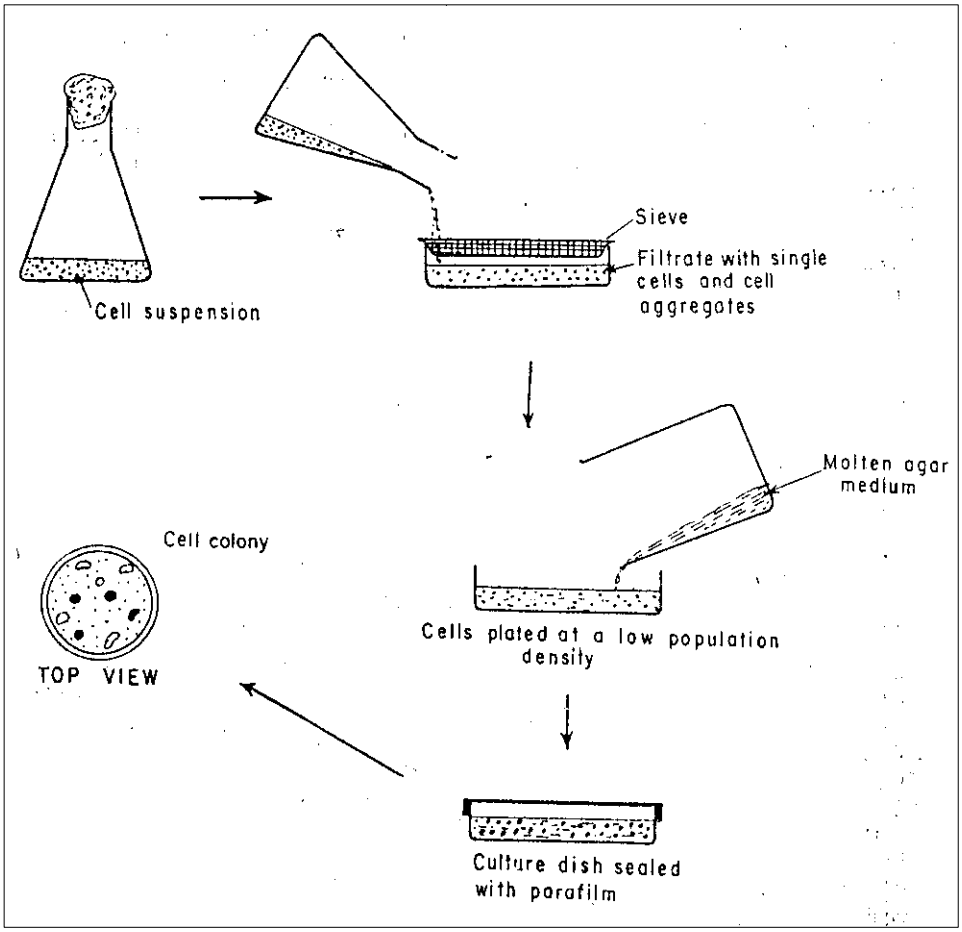

Fig. 2.3 Bergmann's technique of cell plating

About 2 ml aliquot of cell suspension of suitable density (10^3-10^5 cell ml^{-1}) is mixed with equal volume of agar nutrient medium and plated on petridish. On solidification, the culture plates are sealed and maintained in an inverted position and incubated in diffused light or dark at 25°C.

Plating efficiency – If known volume of suspension is transferred to each petridish, it is possible to assess the plating efficiency (PE) quantitatively by using the following formula.

$$PE = \frac{\text{Final number of colonies}}{\text{Initial number of cell units}} \times 100$$

Usually plating at cell densities of 10^3-10^5 cells in ml^{-1} or more yields a high plating efficiency. Other parameters to obtain high PE are: (a) using a conditioned medium or synthetic medium, (b) harvesting cells during exponential growth phase, and (c) incubating cells in diffused light or darkness.

(ii) The filter paper-raft nurse technique: In this technique, isolated single cells from suspension cultures are placed by means of micropipette on top of an actively growing callus but separated by a filter-paper raft (Fig. 2.4). After some days, cell is able to grow under the nurse effect of the callus.

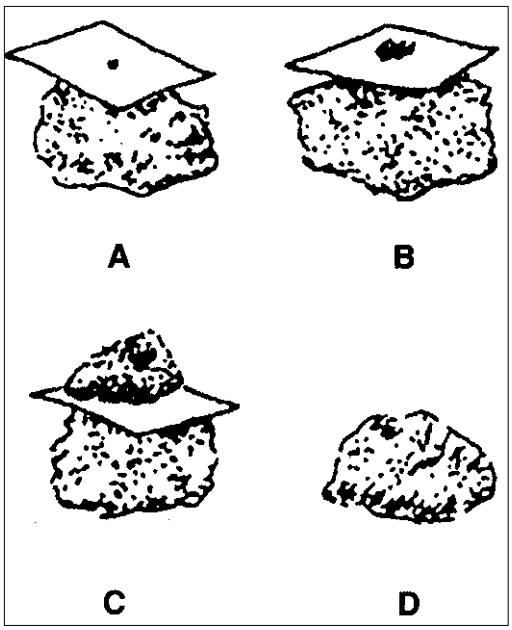

Fig. 2.4 Filter paper-raft nurse technique

Once, a macroscopic colony develops, it is transferred to an agar medium for further growth and maintenance under aseptic conditions. This method is now widely used to clone isolated single cells.

(iii) The microchamber technique: This technique is also known as **hanging-drop culture technique** which is widely used to initiate cell cultures at very low-density (see Section 3). The microchamber technique enables visual monitoring of the divisions in an isolated cell.

2.10 APPLICATIONS OF CELL CULTURES

Cell suspension cultures and immobilized cells are being used for the production of secondary metabolites on commercial scale due to the following advantages over the extraction from whole plants.

(i) Production of secondary metabolites: (a) The yield and quality of product is more consistent in cell cultures because it is not influenced by any internal and external environment. (b) The production scheduled can be predicted and controlled in the laboratory/industry.

Cell cultures have been exploited to produce secondary metabolites such as alkaloids, glycosides (steroids and phenolics), terpenoids, variety of flavors, perfumes, agrochemicals,

commercial insecticides of plant origin etc. These secondary metabolites useful to mankind are commercially extracted from cell cultures. The most important example is shikonin, a naphthaquinone used both as dye and as pharmaceutical, extracted from cell cultures of *Lithospermum erythrorhizon*. The tropical plant, *Catharanthus roseus* is a rich source of terpenoid indole alkaloids. Another, the most economically important natural plant insecticides are pyrethrins produced from the members of Compositae, such as *Chrysanthemum, Tagetus* etc. Cephalotaxine, an antitumour compound, extracted from *Cephalotaxus harringtonia*.

Selection of cell lines producing high amounts of useful metabolites – There is a heterogeneity prevailed in cultured plant cells. It is essential to establish specific cell lines which can produce high amount of secondary metabolites.

Cell aggregate cloning is a suitable method for selecting specific cell line. It is simply a tissue culture method that permits the identification of cells with increased biosynthetic capacity for a particular secondary metabolite. Genetic variation is prevailed among cells in the cell aggregates. If this variation (somaclonal variation) is useful for high production of chemical compound and it can be exploited and establish the cell line.

A typical scheme for the selection of high secondary metabolite producing cell lines is shown in Fig. 2.5. The first step, in this direction is to initiate the growth of callus cultures on solid media. Cell suspension cultures with cell aggregates are initiated from calli. Now cell aggregates are plated on solid media and cultured. The resulting calli are then divided – one half for continued growth and other half for analysis of secondary metabolites. In this way, Yamada and Fujita (1973) selected high anthocyanin producing cell lines from cultured cells of *Euphorbia millii*.

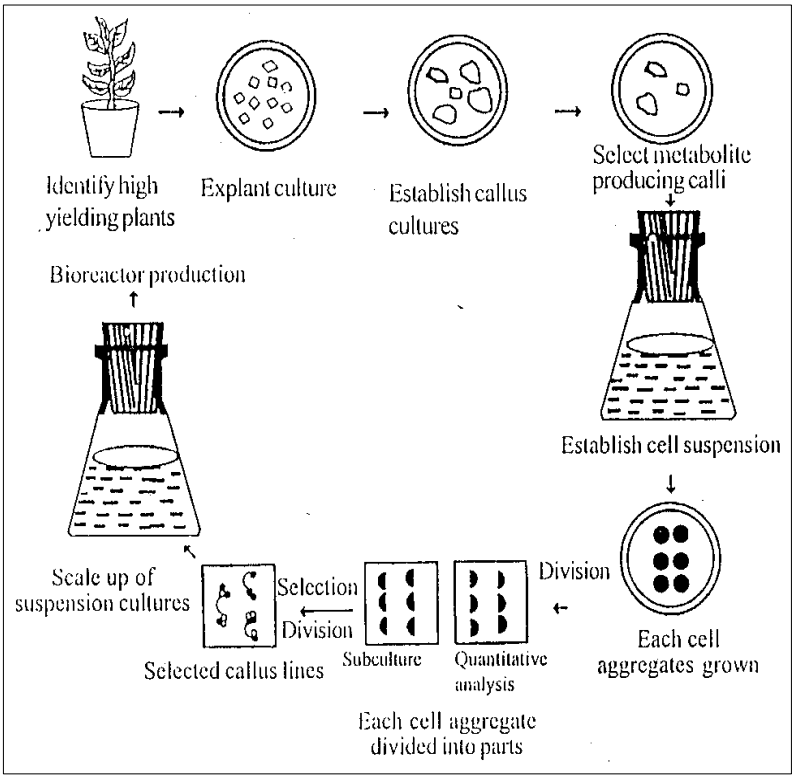

Fig. 2.5 Induction and selection of high secondary metabolite producing cell lines by cell aggregate cloning method

Hairy root cultures – Establishment of hairy root cultures by genetic transformation of plant tissues by the pathogenic bacterium, *Agrobacterium rhizogenes* is one of the recent organ culture systems employed for large scale production of secondary metabolites. These cultures are useful for the production of root-associated metabolites because of their high-growth rate and genetic stability.

Immobilized cell systems – Immobilized plant cells have been used to increase the efficiency of production system. The latest technique, known as **surface immobilized plant cell** (SIPC), is efficiently retains the inoculum and growing the biomass even at higher mixing rates. Methods of immobilization are widely based on gel or membrane entrapment. The cells are physically entrapped in a gel and substrate/product can diffuse to/from the immobilized cells. The cells are commonly immobilized by entrapment in calcium alginate, potassium, carrageenan or in agarose beads.

Immobilization in polyurethane matrix does not appear to affect cell viability. Immobilized cells of *Capsicum flutescens* produced more capsaicin than freely suspended cells cultured under similar conditions. Further reticulate polyurethane offers no barrier to the inward and outward diffusion of metabolites. SIPCs are protected from high-liquid shear stresses and better cell-to-cell contact is maintained.

The other substances which are being used for immobilization of cells include synthetic polyacrilamide, epoxy resin, and proteins – collagen, gelatin and fibrin. After the immobilization, tests may be conducted to study the viability of immobilized plant cells. The methods used for viability are same as used for cell suspension.

Use of ellicitors for secondary metabolite production – Antimicrobial compounds produced by plant in response to an attack by microbes are referred to as phytoalexins. It is defence mechanism adopted by plants against pathogens. While studying phytoalexins, compounds of pathogen origin were found to induce secondary metabolism in plants. Such pathogen-origin compounds which induce accumulation of secondary metabolites in plants are known as *illicitors*. e.g. endo-polygalacturonic acid lyase from *Erwinia carotovara* and *Rhizopus stolonifer*, chitasan from *Fusarium* cell walls, α-1,3-endoglucanase from *Phytophthora* cell walls.

2.11 BIOTRANSFORMATION

Plant cell cultures can also be used to accomplish certain changes in the structure and composition of industrially important chemical compounds. The conversion of a small part of chemical molecule by means of biological systems is referred to as biotransformation. It is a promising field in biotechnological application of plant cell cultures. In this technique, low-cost precursors are used as a substrate and are transformed into value-added high-cost product.

Digitoxin (or) methyldigitoxin → Suspension cultures of *Digitalis lanata* → Digoxin (or) methyldigoxin

The production of digoxin or methyldigoxin is achieved in airlift bioreactors using immobilized cells of *Digitalis*. Digoxin is useful in treatment of heart diseases.

Hydroquinone → *Datura* cell cultures → Arbutin (used as diuretic and urinary antiseptic)

Table 2.1. Some examples of biotransformations

Substrate biotransformed	**Plant cell culture**	**Product**
Codeinone	*Papaver somniferum*	Codeine
Ellipticine	*Choisya ternata*	5-formylellipticine
Solavetivone	*Solanum tuberosum*	Hydroxylated derivatives
2-succinylbenzoate	*Galium mollugo*	Anthraquinone
Valencene	*Citrus* spp.	Noothatone
Steviol	*Stevia rebaudiana/Digitalis purpurea*	Steviocide, steviobiocide

The use of plant cell cultures for biotransformation requires the selection of cell types that express the enzymatic capabilities to catalyze the specific reaction of interest. Another factor taking into consideration in cell selection process is the specificity of enzyme reaction. Plant cells have shown to perform more than one biotransformation with a given substrate. So there is a need to focus on the detection of cell lines that specifically catalyze the desired reaction with little or no contamination from other reaction products.

2.12 MUTANT SELECTION

Cell cultures, especially haploid cell (microspores) cultures have been exploited for mutant selection. Because in haploid cells, the problem of dominance not permitting the expression of mutation in F_1 generation is automatically overcome.

In mutant selection, the cells are often selected directly by adding the toxic substance against which resistance is sought in the mutant cells. Using this strategy, cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins etc., have been isolated.

Auxotrophs: Auxotrophs are mutant plants that will not grow on a mineral medium but they require the addition of some growth regulators.

2.13 SUMMARY

Isolation of single cells can be done mechanically or enzymatically. In enzymatic method, macerozyme has been employed. Tissue is incubated in enzyme mixture which dissolves the middle lamella, so that cells are freed. Cultured tissues have been widely used to isolate single cells. Repeated subculturing of callus on an agar medium improves the fragility of callus, a prerequisite for establishing a cell suspension cultures.

There are two types of cell suspension cultures. These are: (1) **Batch cultures** – the biomass growth in batch cultures follows a fixed pattern, the growth curve shows lag phase, followed by a brief exponential phase and finally stationary phase; these cultures are characterized by a constant change in the pattern of cell growth and metabolism. (2) **Continuous cultures –** the large-scale cultures grown under steady state by adding fresh medium and draining out the used medium in number of specially designed vessels are known as continuous cultures (mass cultures); these may be **open continuous cultures** in which the influx of fresh medium is equal to the efflux of old medium together with cells or **closed continuous cultures** where cells are retained from old medium and reintroduced into the fresh medium.

Cell cultures are mostly asynchronous because of presence of varied shape, size and cell cycle time among individual cells. This situation is not suitable to study the various aspects of cell. So it is necessary to achieve a high degree of synchronization of cell suspension cultures by adopting physical and chemical methods.

Culture of isolated single cells: Isolated single cells can be cloned by Bergamann plating, filter paper-raft nurse and microchamber techniques.

Plating efficiency – If known volume of suspension is transferred to each petridish, it is possible to assess the plating efficiency (PE) quantitatively by using the following formula:

$$PE = \frac{\text{Final number of colonies}}{\text{Initial number of cell units}} \times 100$$

Applications of Cell Cultures: Cell suspension cultures and immobilized cells are being used for the production of secondary metabolites on commercial scale, e.g. Shikonin from *Lithospermum erythrorhizon*, Cephalotaxine from *Cephalotaxus harringtonia* etc. Plant cell cultures are also being used for biotransformation. In this technique, low-cost precursors are used as a substrate and transformed into value-added high-cost product, e.g. digitoxin transforms into digoxin by the immobilized cells of *Digitalis*; hydroquinone into arbutin by *Datura* cell cultures.

2.14 MODEL QUESTIONS

(1) Essay Questions:

(i) Discuss the procedures adopted to establish cell suspension cultures and their role in production of secondary metabolites.

(ii) Discuss techniques adopted to synchronize the cell suspension cultures and cloning of isolated single cells, and add a note on their applications in industry.

(2) Short Answer Questions:

(i) Isolation of single cells
(ii) Batch cultures
(iii) Continuous cultures
(iv) Synchronisation of cell suspension cultures
(v) Plating efficiency
(vi) Viability of cultured cells
(vii) Bergamann's plating technique
(viii) Microchamber technique
(ix) Biotransformations

2.15 REFERENCE BOOKS

1. Bhojwani, S.S.and Razdan, M.K. 1996. **Plant Tissue Culture : Theory and Practice**. Elsevier Science Publishers, New York.
2. Razdan, M.K. 1994. **An Introduction to Plant Tissue Culture**. Oxford & IBH Publishing Co. Pvt.Ltd., New Delhi.
3. Chawla, H.S. 2003. **Plant Biotechnology – Laboratory Manual for Plant Biotechnology.** Oxrod & IBH Publishing Co. Pvt. Ltd., New Delhi.

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Dr. S.M. KHASIM

M.Sc. BOTANY (Final)

Unit-II

Paper-VIII : PLANT BIOTECHNOLOGY

Lesson 3

ISOLATION AND CULTURE OF PROTOPLAST

3.0 OBJECTIVE:

In this chapter, isolation and culture of protoplasts are given.

3.1 INTRODUCTION

3.2 ISOLATION OF PROTOPLASTS

3.2.1 Sources of protoplasts

3.2.2 Methods for isolation of protoplasts

3.2.3 Purification of Protoplasts

3.2.4 Viability of Protoplasts

3.3 CULTURE OF PROTOPLASTS

3.4 SUMMARY

3.5 MODEL QUESTIONS

3.6 REFERENCE BOOKS

3.1 INTRODUCTION

In plant breeding programmes many desirable combinations of characters may not be transmitted through conventional methods of genetic manipulations. Alternate to this, Cocking (1979) has developed the technique, other than the sexual cycle, to get genetic recombinations. In this technique, fusion of two isolated somatic protoplasts is involved and this fusion product known as heterokaryon, is subsequently developed into somatic hybrid. This phenomenon is known as somatic hybridization.

Protoplast x Protoplast
(Wall-less naked cells)
↓ Fusion
Heterokaryon
↓
Somatic hybrid

Through the fusion process, the nucleus and cytoplasm of both parents are mixed together and forms the heterokaryon. This results into formation of various nucleo-cytoplasmic combinations.

Sometimes interaction between plastome (cytoplast carrying cytoplasmic characters) and nuclear genome contributes to the formation of cybrids (cytoplasmic hybrids). Cybrids, in contrast to conventional hybrids, posses a nuclear genome from only one parent but cytoplasmic genes from both parents. The process of protoplast fusion resulting in the development of 'cybrids' is known as 'Cybridization'. So protoplast fusion is an important tool for the induction of genetic variability and combination of traits.

Cytoplast x Protoplast
(enucleated naked cell) (nucleated nacked cell)
↓
Fusion product
↓
Cybrid

The important steps involved in protoplast fusion are: (1) Isolation of protoplasts, (2) Culture of protoplasts, (3) Protoplast fusion, (4) Regeneration of somatic hybrids and cybrids.

3.2 ISOLATION OF PROTOPLASTS

3.2.1 Sources of protoplasts

(A) Leaves - The leaf is the best source of plant protoplasts because it allows isolation of a large number of relatively uniform cells. The following steps involve in the isolation of leaf protoplasts: (a) sterilization of leaves, (b) peel-off the epidermis, (c) incubation in the enzyme mixture, (d) isolation by filtration and centrifugation (Fig. 3.1).

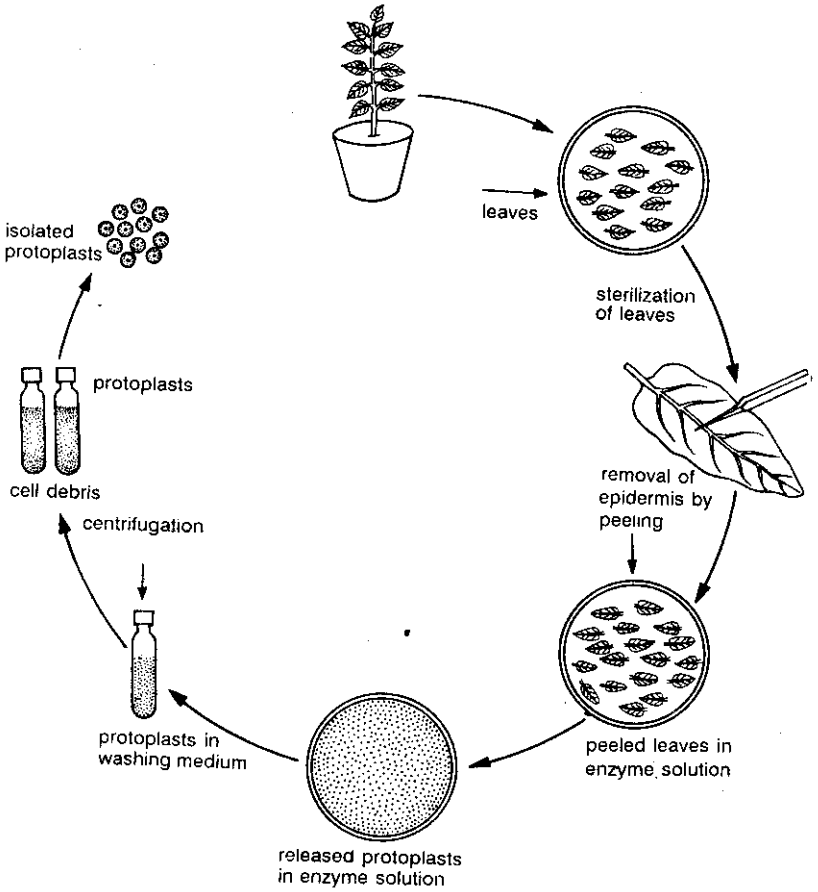

Fig.3.1 Isolation of mesophyll protoplasts (adopted from Gupta, 2000)

(B) Callus cultures – Young callus cultures or actively growing callus cultures are also ideal materials for obtaining large quantities of protoplasts. Young callus is subcultured and it can be used after two weeks for protoplast isolation.

(C) Cell suspension cultures – The cell suspension cultures are also excellent source materials for isolation of protoplasts. A high-density cell suspension is centrifuged. After removing the supernatant, cells are incubated in a enzyme mixture (cellulose + pectinase) in a culture flask placed on the platform shaker for about 6 hr. After incubation protoplast are liberated. Liberated protoplasts are purified subsequently.

3.2.2 Methods of isolation of protoplasts

Mainly there are three methods of isolation of protoplasts. These are: (A) mechanical (non-enzymatic, (B) sequential enzymatic (two-step), and (C) mixed enzymatic (simultaneous) methods.

(A) Mechanical (non-enzymatic) method – Through this method cell walls are removed mechanically with sharp-edged knife. First, cells are kept in hypotonic solution for a few min. This brings about the exosmosis in plant cells, resulting the separation of protoplasts from cell walls. Next the cell walls are broken with the help of knife to release the protoplast from the cell. The isolated protoplasts are transferred to isotonic solution to prevent further damage. This technique has been employed for the isolation of protoplasts from highly vacuolated cells of storage tissues like onion bulbs, raddish root, beet root etc.

(B) Sequential enzymatic (two-step) method – Takebe et al. (1968) had developed the technique of isolation of protoplasts by using commercially available enzymes in two steps. In the first step, leaf segments are treated with pectinase (enzyme mixture **A**) and pectin is digested. In the second step, this tissue is treated with cellulase (enzyme mixture **B**) and cellulose is digested. In this way, whole cell wall is digested and protoplasts are liberated.

I step – **Enzyme mixture** 'A' – 0.5% pectinase (macerozyme) + 0.3% potassium dextran sulphate in 13% mannitol at pH 5.8.

II step – **Enzyme mixture** 'B' – 2% cellulase in 13% mannitol at pH 5.4.

(C) Mixed enzymatic (simultaneous/single step) method – In this method, leaf segments are treated with a mixture consisting of both macerozyme and cellulase enzymes. This can be completed in a single step.

Enzyme mixture consists of both 0.5% macerozyme and 2% cellulase taken in 13% mannitol at pH 5.4. Leaf material is incubated in this mixture for 15-18 hr at 25^{o}C. Protoplasts are isolated in a single step through this method.

3.2.3 Purification of Protoplasts

The protoplasts isolated as above may contain together with broken organelles and cell debris in the medium. So purification of protoplasts is essential before proceeding for the protoplast fusion (Fig. 3.2).

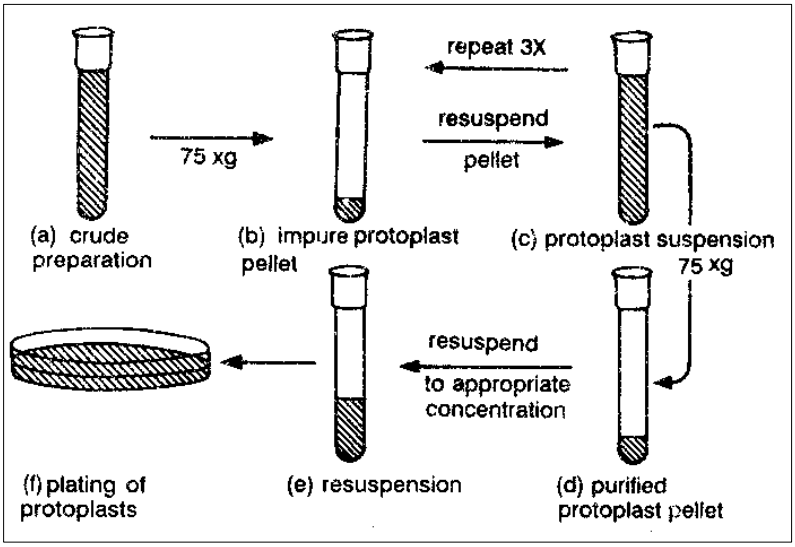

Fig. 3.2 Protoplast purification by repeated gentle pelleting and resuspension

Sedimentation and Washing – In this method, crude protoplast suspension is centrifuged for 5 min. at low speed (50-100 g). The intact protoplasts form a pellet and supernatant containing cell debris can be pipetted off. The pellet is gently resuspended in fresh culture medium and rewashed (Fig. 3.2). This process is repeated two or three times to get relatively clean protoplast preparation.

Floatation – Since protoplasts are lighter (low density) than other cell debris, gradients may be used, which allow the protoplasts to float and the ell debris to sediment. A concentrated solution of mannitol, sorbitol and sucrose (0.3 – 0.6 m) can be used as gradient at appropriate speed. Protoplasts can be pipetted from the top of the solution after centrifugation. This is the most suitable method with less damage. Bobcock bottle is used for floatation (Fig. 3.2), since it facilitatates removal of protoplasts.

3.2.4 Viability of Protoplasts

Isolated protoplasts should be healthy and viable in order to undergo sustained divisions and regeneration. Protoplast viability could be tested by variety of methods including: (A) Presence of cytoplasmic streaming or cyclosis, (B) exclusion of Evans Blue dye by intact membranes, and (C) Presence of photosynthetic and respiratory activity. These tests indicate active metabolism in protoplasts.

Besides these, protoplast viability is tested by using several dyes: (A) staining with fluorescein diaceate (FDA), protoplasts fluoresce green/white; it should be examined within 5-15 min. after the FDA treatment, later FDA dissociates; (B) phenosafranin (0.1%) detects dead protoplasts, turn red whereas viable protoplasts remain unstained even after 2 hr in the stain solution. (C) Calcofluor white (CFW 0.1%) detects onset of cell wall regeneration around plasma membrane of viable protoplasts appears in the form of a ring of flourescence.

3.3 CULTURE OF PROTOPLASTS

Since the isolated protoplasts are devoid of walls, these are very easy to undergo fusion *in vitro*. Protoplast fusion is employed to overcome the incompatibility barriers at interspecific and intergeneric levels. It is an important tool in somatic cell genetics and crop improvement programme.

The first step in the protoplast culture is the development of cell wall around the plasma membrane of isolated protoplast. This is followed by induction of divisions in the protoplast-derived new cell giving rise to a small colony. By providing proper nutritional requirement, cell colonies may be induced to grow callus continuously or to regenerate whole plant.

3.3.1 Culture Media

Generally, protoplast culture media contain 3-5% surcrose but in some systems (tobacco protoplast cultures) 1.5% sugar content is required. Organic nitrogen in the form of CH (caseine

hydrolysate) and inorganic nitrogen NH_4NO_3 are supplementing the medium. Higher concentrations of iron, zinc and ammonia are used in these media. Various combinations of auxins (2,4-D, NAA, IAA) and cytokinins (kinetin, zeatin, BAP) are used to induce cell wall formation and divisions in isolated protoplasts. It has been observed that high auxin/kinetin ratio is suitable to induce divisions in the protoplasts from actively growing cell cultures whereas high kinetin/auxin is for protoplasts derived from differentiated cells (leaf tissue, stem tissue etc.).

3.3.2 Protoplast Culture Techniques

Various techniques have been employed for culturing of protoplasts. These are as follows:

A. Bergman's Cell Plating Technique

For culturing of protoplasts in the solid agar medium, this technique may be followed. About 2 ml aliquots of isolated protoplasts of suitable density (10^3-10^5 cell ml^{-1}) are mixed with equal volume of agar nutrient medium (temperature not to exceed 45^oC). On solidification of agar, the culture plates are sealed and maintained in an inverted position and incubated in diffused light or in dark at 25^oC. Here cell colonies are formed and these can be conveniently observed under microscope.

Plating efficiency is readily determined by the following formula:

$$\text{Plating efficiency} = \frac{\text{Final no. of Cell colonies}}{\text{Initial number of Cell units}} \times 100$$

B. Culturing the Protoplasts at Low Densities

Just like in cell cultures, the initial plating density of protoplasts has a profound effect on the plating efficiency. At high densities, such as 1×10^4 to 1×10^5 protoplasts per 1 ml of medium, the cell colonies arising from individual protoplasts tend to grow into each other at a fairly early stage in culture. This may lead to the formation of chimeral tissue if the protoplast population is genetically heterogeneous. In somatic hybridization and mutagenic studies, cloning of individual cell/protoplast is essential. This can be achieved by plating the protoplasts at very low densities (100-500 protoplasts ml^{-1}). At low density, the development process of individual protoplast or heterokaryon (hybrid protoplast), can be easily monitored, thereby enabling the isolation and identification of hybrid colonies in the absence of a stringent selection system (Bhojwani and Razdan, 1985).

The nutritional components of the most commonly used culture media are not suitable to induce cell division in protoplasts plated at low densities. A complex medium developed by Kao and Michayluk (1975), known as KM 8P (Table 3.1), induces faster divisions in mesophyll protoplasts of alfalfa, pea, potato and potato x tomato fusion products plated at low densities. The

cultures in this medium are kept in darkness because KM 8P medium turns phytotoxic under strong light.

Table 3.1. KM 8P medium for plating the protoplast at low density[a]

Constituents	Amount (mg 1^{-1})
Mineral Salts	
Na_4NO_3	600
KNO_3	1900
$CaCl_2$ $2H_2O$	600
$MgSO_4$. $7H_2O$	300
KH_2PO_4	170
KCl	300
Sequestrene 300 Fe[b]	28
Kl	0.75
H_3BO_3	3.00
$MnSO_4.H_2O$	10.00
$ZnSO_4.7H_2O$	2.00
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Sugars	
Glucose	68400
Sucrose	125
Fructose	125
Ribose	125
Xylose	125
Mannose	125
Rhamnose	125
Cellobiose	125
Sorbitol	125
Mannitol	125
Hormones	
For soyabean x barley	For soyabean x pea or *N. glauca*
2,4-D 1	0.2
Zeatin 0.1	0.5
NAA --	1
Vitamin-free casamino acid[c]	125
Coconut water (from mature fruits, heated to 60^oC for 30 min. and filtered)	10 $ml/1^{-1}$

Organic acids	
(adjusted to pH 5.5 with NH_4O_4)	
Sodium pyruvate	5
Citric acid	10
Malic acid	10
Fumaric acid	10
Vitamins	
Inositol	100
Nicotinamide	1
Pyridoxine-HCl	1
Thiamine-HCl	10
D-Calcium Pantothenate	0.5
Folic acid	0.2
P-Aminobenzoic acid	0.01
Biotin	0.005
Choline chloride	0.5
Riboflavin	0.1
Ascorbic acid	1
Vitamin A	0.005
Vitamin D_3	0.005
Vitamin B_{12}	0.01

[a]Sterilized by filtration
[b]Geigy Chemical Corp., Ardsley, N.Y.
[c]Difco Laboratories, Detroit.

The following techniques are being employed to culture the protoplasts at low densities:

(a) Feeder layer technique: Feeder layer technique is one of the approaches to culture the protoplast at low density. Raveh *et al*. (1973) prepared a feeder cell layer by exposing tobacco cell suspension protoplasts (10^6 cells ml^{-1}) to an x-ray dose of 2 x 10^3 R. This x-ray treatment has inhibited the division of cells but allowed them to remain metabolically active. Irradiated protoplasts were washed thrice to remove toxic substance due to irradiation and then plated on soft agar medium at a low density of about 2.4 x 10^4 ml^{-1}. Non-irradiated protoplasts of low density (10-100 protoplasts ml^{-1}) were plated over this feeder layer (Fig. 3.3). The feeder layer provides the nourishment and initiates divisions in the protoplast-derived new cells.

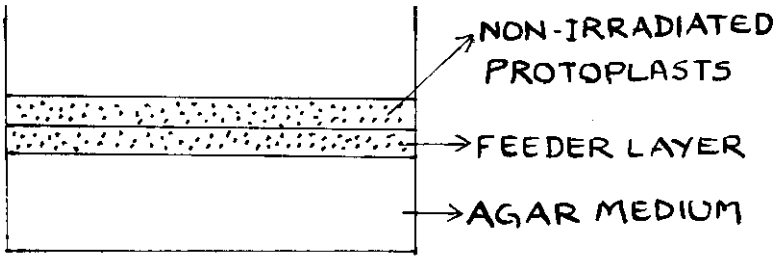

Fig. 3.3 Feeder Layer Technique

(b) Co-culture of protoplasts: Protoplasts of two different species are co-cultured to promote their growth. Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together, so that there is a cross-feeding between these two types of protoplasts. This enables the protoplasts to undergo divisions. This method is generally employed where calli arising from two types of protoplasts can be morphologically distinguishable. For example, mechanically isolated hybrid cells co-cultured with protoplasts isolated from an albino strain will develop green colonies that can be readily distinguishable from non-green colonies of albino types (Menczel *et al*., 1978).

(c) Microdrop culture technique: This technique was employed successfully to culture hybrid cells of *Nicotiana glauca* x *Glycine max* (Kao, 1977) and *Arabidopsis thaliana* x *Brassica campestris* (Gieba and Haffmann, 1979). The technique requires specially designed Cuprak dishes which have a smaller outer chamber and a larger inner chamber. The inner chamber consists of numerous wells, each with a capacity for 0.25 – 25 µl droplet of medium. Individual protoplasts or heterokaryons per 0.25 – 25 µl droplet of nutrient medium are transferred by Drummond Pipette to each wall of the inner chamber. The outer chamber is filled with sterile water to maintain humidity inside the dish. Then cover it with a lid and seal with paraffin wax and provide optimal light and temperature conditions. The size of the droplet is critical for the division of single protoplasts. One protoplast per 0.25 – 05 µl droplet gives a ratio of cell/volume of culture medium equal to a cell density of 2-4 x 10^3 ml^{-1}. An increase in the size of the droplet would decrease the effective plating density (Bhojwani and Razdan, 1984).

(d) The microchamber technique: The microchamber technique was initially designed for culturing the isolated, single cells (De Ropp, 1955; Jones *et al*., 1960). The same technique is also employed for culturing the isolated protoplasts. In this method (Fig. 3.4), a drop of the medium carrying a single isolated protoplast is placed on a sterile microslide and ringed with sterile mineral oil. Again one drop of mineral oil is placed on either side of the ringed culture drop and a cover glass is placed on each oil drop. The third cover glass is placed on the culture drop bridging the two cover glasses. As a result, a microchamber is created enclosing the single cell aseptically within the mineral oil. The microchamber slide is now incubated by placing it in a petridish. The cover glass is removed as soon as the cell colony becomes visible. The cell colony is transferred to media which promotes further proliferation and organogenesis (Fig. 3.4).

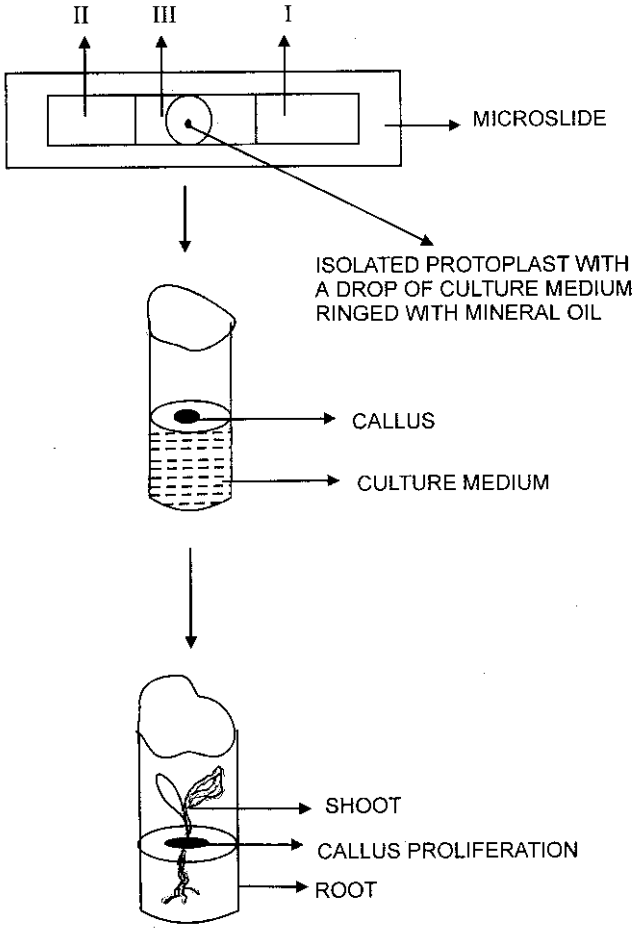

Fig. 3.4. Microchamber Technique

3.3.2 Regeneration of Protoplasts and Plantlet Formation

(i) Formation of cell wall: In the culture media, protoplasts start regenerating cell wall within a few hours after isolation. It takes two to several days to complete the cell wall formation. Protoplasts lose their characteristic spherical shape once the wall formation is completed. There is a direct relationship between wall formation and cell divisions. Protoplasts with poorly developed wall often show budding and those which are not able to regenerate a proper wall fail to undergo normal mitosis.

(ii) Development of callus/whole plant: Soon after the regeneration of wall around the protoplasts, this protoplast-derived new cell show considerable increase in size and first division generally occur within a week. Subsequent divisions give rise to small cell colonies. These small colonies become macroscopic colonies after 2-3 weeks, which can be transferred to osmotic-free medium to develop a callus. The callus may be transferred to medium which promotes

organogenesis, resulted into plantlet formation (Fig. 3.5). Initially protoplast-derived plants (protoclones) were restricted to family Solanaceae but subsequent advancement in protoplast technology enable us to regenerate plantlets from protoplasts in several other families including forage legumes, various tree species, fibre or pulp crops and even cereals such as rice, wheat, pennisetum etc.

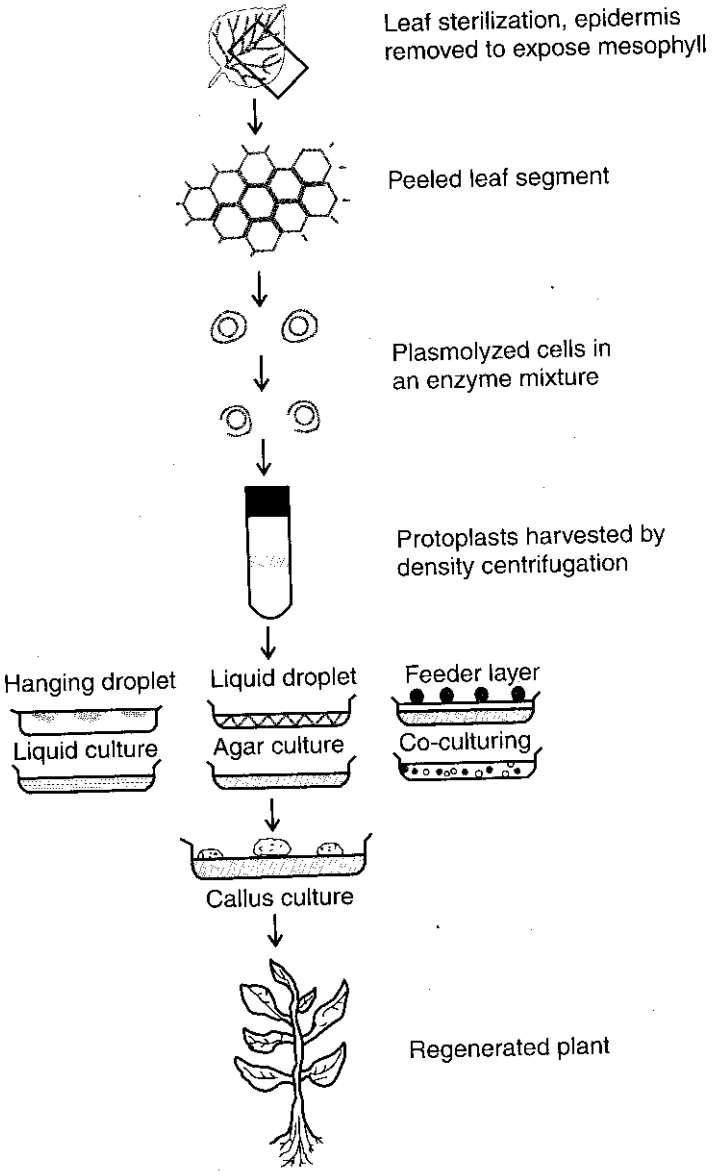

Fig. 3.5 Schematic representation of protoplast isolation and culture procedure

3.4 SUMMARY

Isolation of Protoplasts: Leaves, callus cultures and cell suspension cultures are best sources for protoplast isolation. Various methods have been adopted to isolate protoplast, these are (i) mechanical (non-enzymatic), (ii) sequential enzymatic (two-step), and (iii) mixed enzymatic methods.

Viability of protoplasts is tested by using several dyes, e.g. fluorescein diacetate (FDA), phenosafranin, calcofluor white etc.

Culture of Protoplasts: For culturing of protoplasts several techniques have been adopted, such as Bergmann's plating, feeder layer, microdrop culture and microchamber techniques.

Regeneration of Protoplasts and Plantlet formation: Soon after the regeneration of wall around the protoplasts, this protoplast-derived new cell undergoes divisions and gives rise callus. The callus is transferred to medium which promotes organogenesis, regulating into plantlet formation.

3.5 MODEL QUESTIONS

(1) Essay Questions:

(i) Discuss various methods adopted for culture of protoplasts.
(ii) Explain the isolation and purification of protoplasts

(2) Short answer questions:

(i) Isolation of Protoplasts
(ii) Purification of Protoplasts
(iii) Culturing of Protoplasts at low densities
(iv) Microchamber technique.
(v) Plating efficiency

3.6 REFERENCE BOOKS

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Dr. S.M. KHASIM

M.Sc. BOTANY (Final)

Paper-VIII : PLANT BIOTECHNOLOGY

Unit-I

Lesson 2

Micro Propagation and Anther Culture

2.0 OBJECTIVES
2.1 INTRODUCTION
2.2 METHODS OF MICROPROPAGATION
2.2.1 Auxillary bud proliferation method
2.2.2 Single node culture
2.2.3 Shoot tip culture
2.2.4 Organogenesis
2.2.5 Indirect organogenesis
2.2.6 Direct organogenesis
2.2.7 Applications
2.2.8 Limitations
2.3 ANTHER CULTURE
2.3.1 *In vitro* production of Haploids
2.3.2 Nutrient medium
2.3.3 Culture methods
2.3.4 Hanging drop
2.3.5 Nurse culture
2.4 ANDROGENESIS
2.4.1 Direct Androgenesis
2.4.2 Indirect Androgenesis
2.5 MICROSPORE CULTURE
2.5.1 Factors effecting anther culture
2.5.2 Anther culture for production of Haploids
2.6 SUMMARY
2.7 MODEL QUESTIONS
2.8 REFERENCE BOOKS

2.0 OBJECTIVES

2.1 INTRODUCTION

Since man started cultivation of crops, he had introduced a number of changes as a result of conscious or unconscious selection. Many of these changes in traits of plants have become advantageous to the crop growers. The discoveries of laws of heredity by Mendel, Father of Genetics, the origin of species by Darwin paved the way for refinements in plant breeding techniques. As a result of advancements in plant sciences like toti potency concept, culturing of cells and tissues has become an alternative for the conventional vegetative method of plant propagation.

Micro propagation is one of the types of clonal propagation under *in vitro* conditions. When a population is produced from a single individual through asexual means, this population is called a clone. Compared to the conventional propagation methods, clonal or micro propagation results in the production of large number of plants in a limited space and time. The various explant materials used for micro propagation include auxillary buds, meristems or shoot tips, single nodes etc.

2.2 METHODS OF MICROPROPAGATION

2.2.1 Axillary bud proliferation method

In plants, the axillary buds present in the leaf axils, which are in the dormant state and form a shoot through stimulation. In some cases, the terminal bud results in the breakage of dormancy of the axillary bud and enables it to produce a shoot. Shoots still attached to the explant are separated and rooted and these form the propagules for production of clones. (Fig. 2.1).

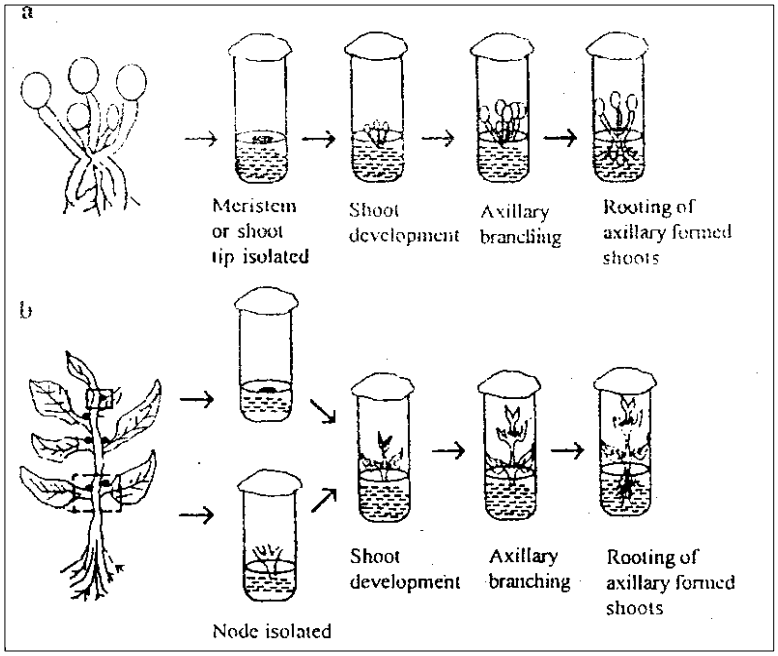

Fig. 2.1 Techniques in major stages *in vitro* clonal progeny

The production of rooted shoots from axillary buds can be achieved through meristem and shoot tip cultures. These explants can be cultured on nutrient medium in the lab for producing adventitious buds, shoots, embryoids or whole plants. This technique involves different steps like explant selection, its sterilization and production of a sterile culture, and multiple shoots from this culture and their transfer to the rooting medium and from there on to the soil.

In plants exhibiting indefinite growth patterns, the leaf axils contain subsidiary meristems which can grow into the shoot resembling the main shoot. Among the buds produced on the stem only a few develop into shoots while other buds do not produce shoots due to apical dominance.

2.2.2 Single node culture

A bud present in the leaf axil when isolated along with a piece of stem, constitutes the node. Such single node explants when cultured on medium produce either single or multiple shoots which develop roots. When the buds produce elongated stem, single nodes with dormant buds are sub cultured and the shoots formed from them can be rooted immediately. This method depends on the number of leaves formed on the stems which are produced in the culture. This method is more suitable for the herbaceous plants and it has been used extensively in plants like Rose, Pea, Vitis, Tomato, *Eucalyptus*, *Asparagus*, *Araucaria* etc. (Fig. 2.2).

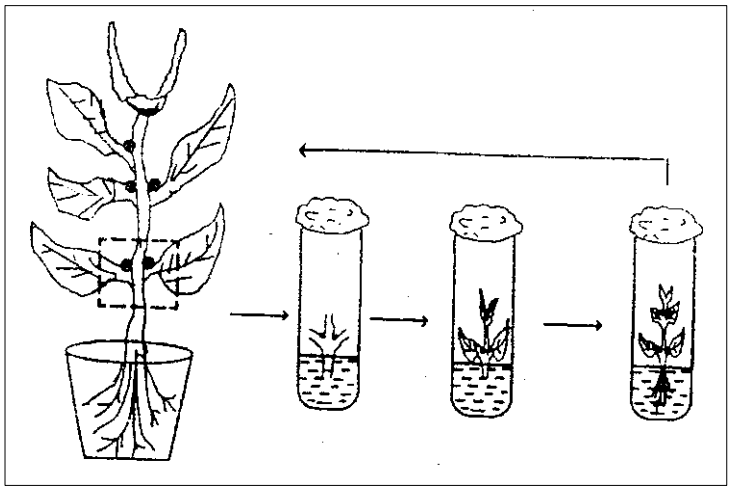

Fig. 2.2 Schematic representation of vegetative propagation by single node technique

2.2.3 Shoot tip culture

This involves the isolation of shoot tips and their culture on medium containing high concentration of cytokinin. The axillary shoots thus formed are allowed to root (Fig. 2.3).

2.2.4 Organogenesis

When plant organs like buds, roots, flowers, embryos are formed from an organized explant, these organs are referred to be of adventitious origin or in other words, adventitious organs are formed from tissues or explants that naturally do not produce these structures (Fig. 2.4).

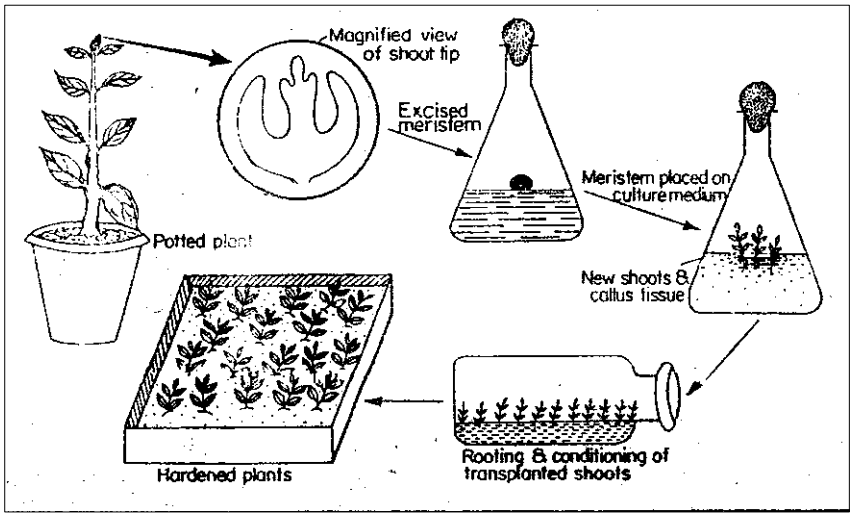

Fig. 2.3 Flow diagram illustrating the technique of shoot tip or meristem culture

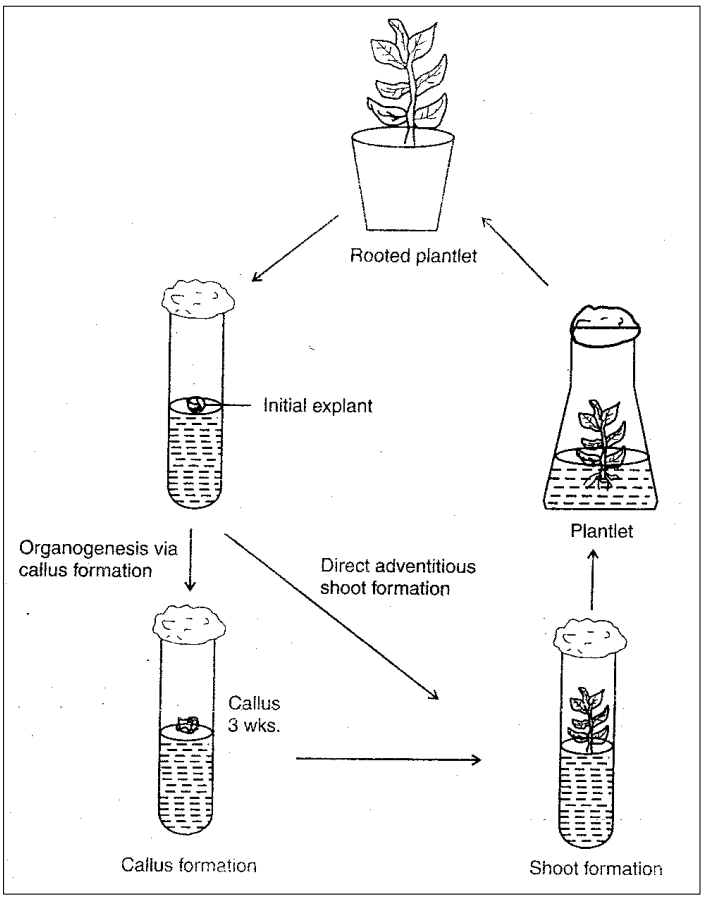

Fig. 2.4 Protocol for plant regeneration via organogenesis

2.2.5 Indirect organogenesis

The explants like cotyledons, hypocotyls, stem, leaf, shoot apex, root, immature inflorescence, floral organs immature or mature embryos of any plant when inoculated on to the nutritive medium produce a homogeneous mass of cells called as callus which produces plantlets via somatic embryo genesis.

Callus production under *in vitro* conditions could be the result of wounding of explant or due to the effects of hormones either present originally in the explant (endogenous) or those added (exogenous) to the medium.

During callus formation, the different tissues present in the explant get disorganized into a mass of cells and this phenomenon is called de differentiation and the development of shoot and root from the somatic embryos is called re differentiation. Addition of hormones in higher concentrations induce callus from the explant while low concentration of auxin and higher concentration of cytokinins induce shoot formation from the callus. These shoots when transferred into a media with higher auxin concentration and low cytokinin concentration induces root formation. Some of the cells of the callus will organize into multicellular structures called the embryoids which pass through different stages of the somatic embryos like the globular, heart and torpedo which are bipolar structures finally produce the shoot and root (Fig. 2.5).

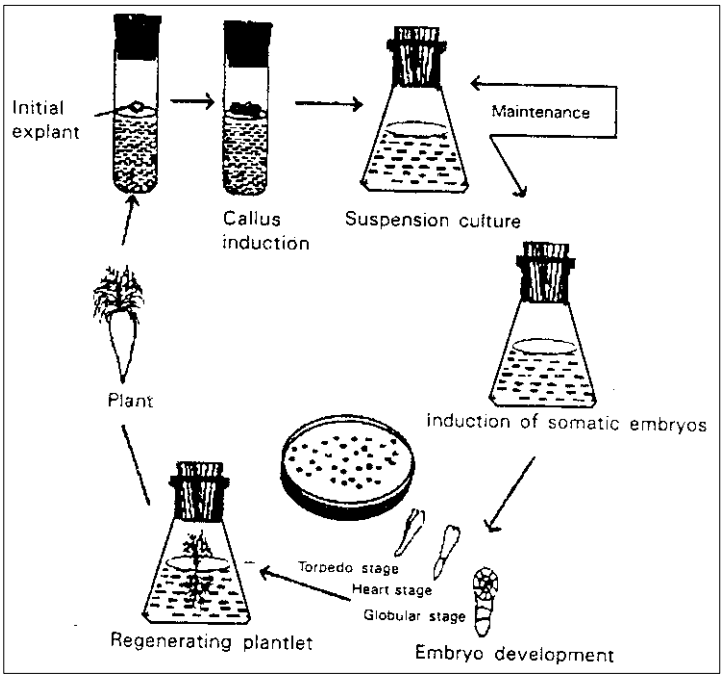

Fig. 2.5 Procedure for the callus culture from carrot root

2.2.6 Direct organogenesis

Adventitious shoot production from roots, leaves, bulb scales, inflorescence axes is commonly seen in nature, while, such adventitious shoots can be produced without a callus phase from an explant in culture. This method is more suitable for herbaceous plants. The adventitious bud formation is promoted by the addition of growth regulators to the medium.

2.2.7 Applications

(i) Production of large number of plants in limited space and time.
(ii) Production of plants throughout the year especially in herbaceous species.
(iii) Production of pathogen free plants.
(iv) Through storage of the explants / somatic embryos at low temperature conditions (cryo preservation), the material for clonal propagation *in vitro* can be made available.
(v) The *in vitro* cloning enables genetic manipulation (genetic engineering) which depends on the regeneration abilities of the callus or shoot buds.

2.2.8 Limitations

(i) *In vitro* production of plants requires sophisticated and expensive facilities.
(ii) Loses caused in plant production due to pathogenic contamination in the cultures.
(iii) Hormonal effects result genetic variations due to which aberrant plants are produced.
(iv) Vitrification effects caused in the cultures result in water soaked translucent leaves on the regenerated plants and these show reduced growth and finally die.

Inspite of the disadvantages associated with micro propagation, this technique implemented carefully at sterilization level can help in the large scale production or multiplication of clones in short time and limited space compared to the conventional vegetative propagation methods.

2.3 ANTHER CULTURE

Anther represents the male reproductive part in the Angiosperms and contains one or two anther lobes which contain the pollen sacs. The microspores produced from the pollen mother cells due to meiosis develop into pollen grains which represent the male gametophytic phase. Culturing of anthers or microspores leads to production of plants having gametic number of chromosomes and these are called Haploids. Haploids originating from diploid parents are called monoploids while those arising from allopolyploids having more than one genome are called poly haploids.

Tulecke (1953) produced haploid callus from *Cinkgo biloba*, a gymnosperm, but this callus did not produce plantlets. Guha and Maheswari (1964), recorded the formation of somatic embryos from the uninucleate pollen grains from *Datura,* while, Bourgin and Nitsch (19) produced haploids from anther cultures of *Nicotiana* (tobacco*).*

2.3.1 Anther culture and *In vitro* production of Haploids

2.3.2 Nutrient medium

The nutrient media used for culturing anthers/microscopes are specific to each of the crop species. For plants belonging to the family Solanaceae, M.S. White (1963), Nitsch and Nitsch (1969) and N_6 media proved to be suitable, B_5, N_6 and Linsmaier and Skoog (1965) and potato medium of Chuang *et al*., (1978) are in use.

2.3.3 Culture Methods

Pre culture treatment

For anther culture the flower buds/inflorescences are kept in dark for 48-72 hours at -5^oC; anthers are dissected out under sterile conditions and inoculated on culture medium solid/liquid. (Nitsch medium for tobacco, N_6 medium for rice etc.) and left for 3 to 4 days at 28^0C and 2000 for 18 hours.

The anthers are then pressed to isolate the microspores into the culture medium; the medium along with microspores is sieved using mesh of 48 m pore size and subjected to centrifuge at 500g. The pellet of microscopes is washed twice and transferred to fresh medium 2.5 ml of this medium is transferred to 5 cm Petriplates which are sealed with para film and left in dark at 28^oC.

2.3.4 Hanging drop

This method involves introducing a drop of the medium with pollen grains on to a cover slip and inserting it into the depression in the cavity slide and sealing the edges of the cover slip (Fig. 2.6).

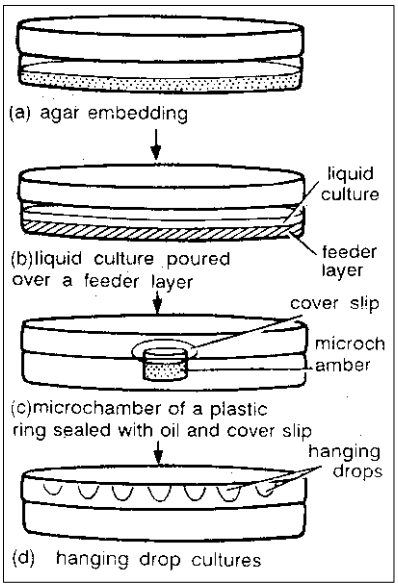

Fig. 2.6 Different culture methods: (a) agar embedding. (b) liquid culture poured over a feeder layer of medium or medium and inactivated protoplasts of another species, (c) micro-chamber constructed of a small plastic ring with oil and a cover slip, (d) hanging drop cultures.

2.3.5 Nurse culture

An anther is placed on the agar medium and a filter paper disc containing pollen suspension is placed on the anther and incubated at 28^0C when cell colonies form after a month (Fig. 2.7).

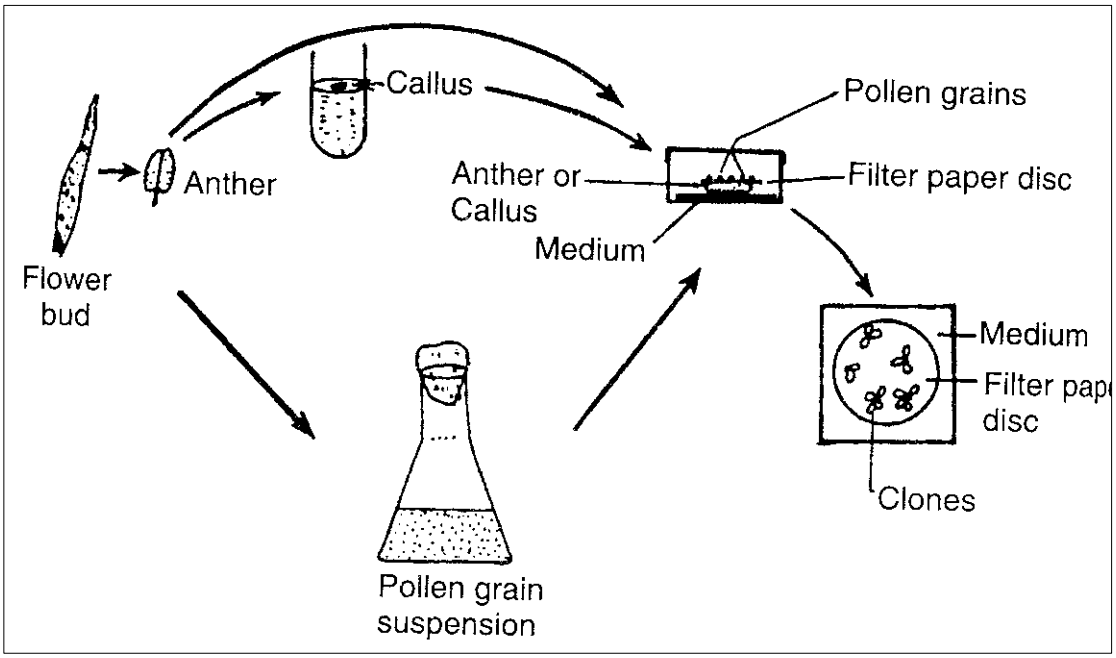

Fig. 2.7 Illustration showing the technique of nurse culture for cloning isolated pollen grains

2.4 ANDROGENESIS - Direct and Indirect

Androgenesis occurs in two ways viz., the direct and indirect types.

2.4.1 Direct androgenesis

The microspore behaves like a zygote and forms an embryoid which produces the plant let.

2.4.2 Indirect androgenesis

The microspore divides and forms callus tissue and somatic embryos formed from the callus cells, give rise to the plant lets.

2.5 MICROSCOPE CULTURE

When plants are produced from the microspores in the anthers, they will be haploids or when cold pretreatment is given they may produce homozygous diploids (Fig. 2.8).

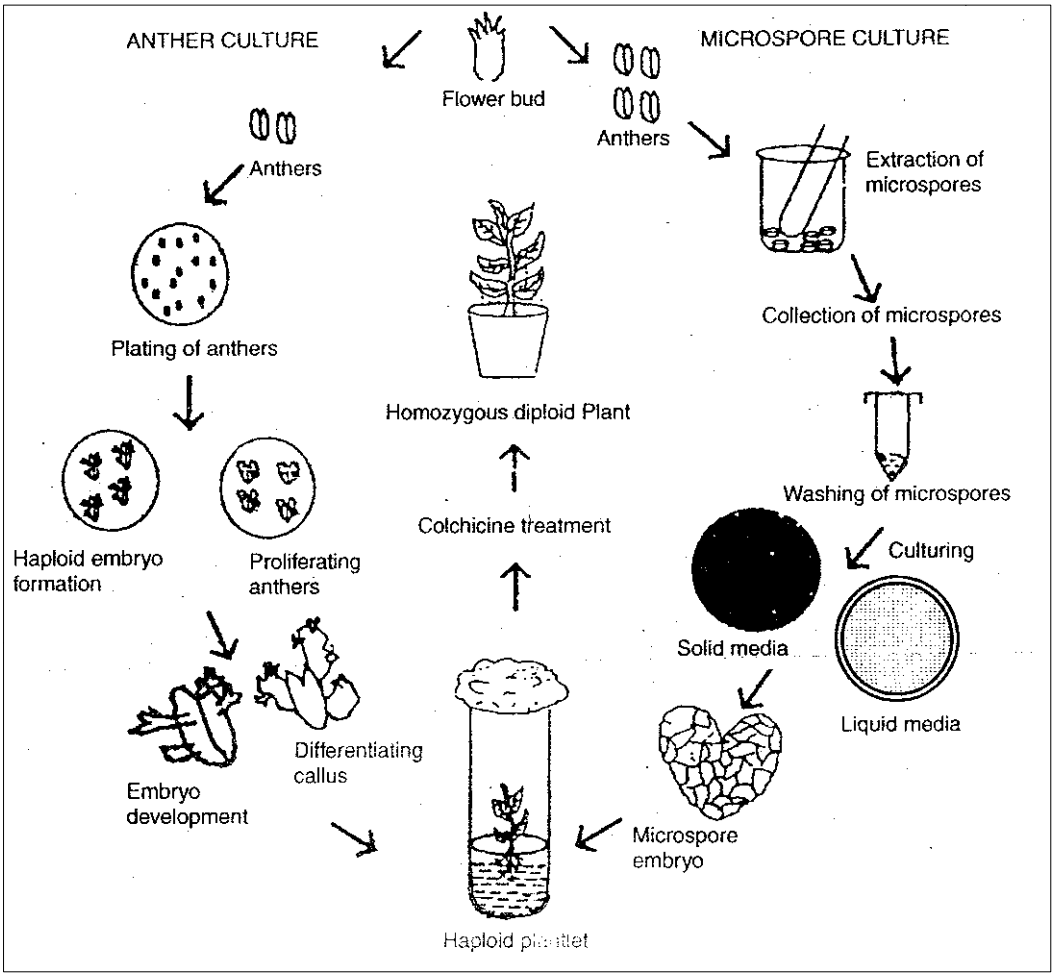

Fig. 2.8 Diagrammatic illustration of anther and microspore culture for production of haploid plants and diploidization

There is every possibility for the production of plants either through organogenesis or via callus phase from the somatic cells of the anther like the wall cells, connective tissue or anther filament and there will be heterozygous diploids. Thus isolated microspore culture is likely to produce haploid plant lets.

When the flower buds or inflorescences (as in rice) are pretreated in dark for 2-3 days before culturing the anthers, it has been found that the pollen grain nucleus produces 2 equal sized nuclei during first mitotic division. These two nuclei may fuse and the embryo produced will be a homozygous diploid or isogenic diploid which is highly useful as pure line in plant breeding programs (Fig. 2.9).

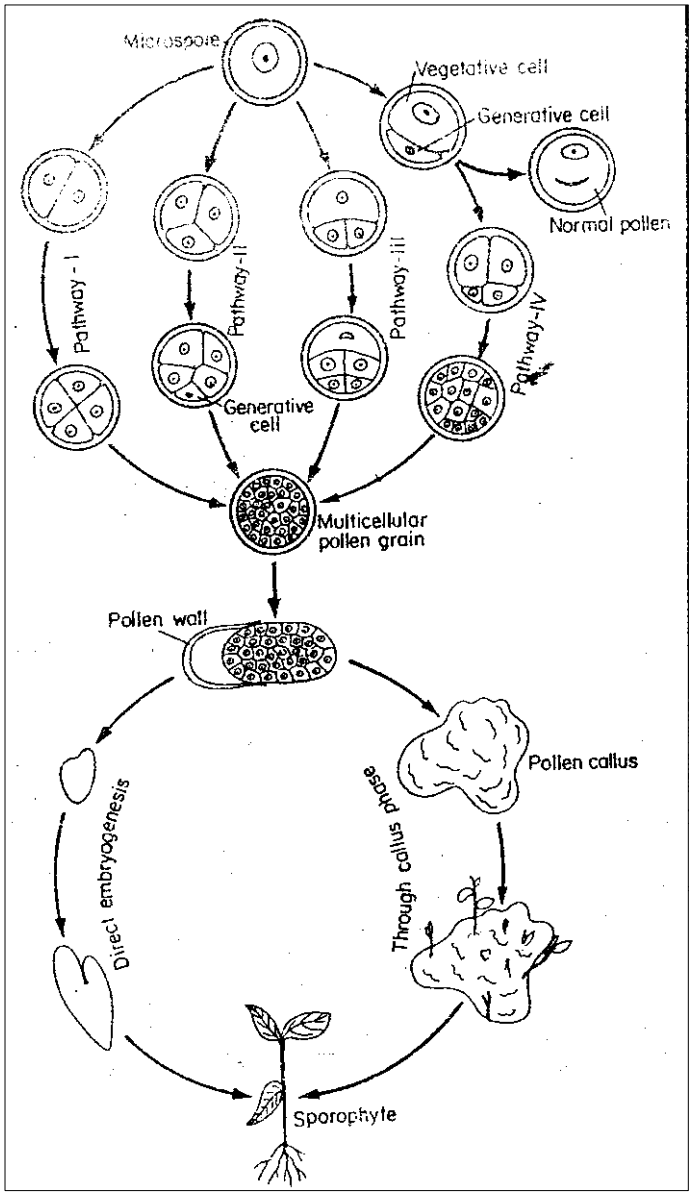

Fig. 2.9 Diagram showing the origin of sporophytes from pollen gains in anther cultures. A microspore may follow any one of the four pathways to form a multicellular pollen grain. The latter may directly form an embryo or produce callus tissue (After Bhojwani and Razdan, 1983).

In culture the microspore/pollen grain nucleus may divide mitotically and follows the normal path giving rise to vegetative or tube and generative nuclei. Due to the degeneration of one of these nuclei, the other divides and forms a multi-nucleate pollen grain. This may form an organized embryoid which produces the plant let or results in callus formation, the cells of which produce haploid plantlets via somatic embryogenesis.

2.5.1 Factors effecting anther culture

Some of the important factors include:

(i) Physiological status of the donor plant. This requires growing the donor plants under best environmental conditions avoiding the use of any pesticide sprays on the plant.

(ii) The success in anther culture is dependent on the genotype of the donor anther plant since anther culture ability is genetically controlled.

(iii) **Stage of pollen:** Uninucleate stage of the microspore – just before or during first mitosis is considered to the correct one. Pollen at *or after* pollen mitosis in *Datura innoxi* and *Nicotiana tabaccum* and early bicellular stage in *Atropa belladonna and Nicotiana sylvestris* and mid to late uninucleate stage in cereals have been found to be suitable in the production of haploids from anther culture (Fig. 2.8).

(iv) **Pretreatment of anthers:** Pretreating the flower buds containing anthers at temperatures range between 3 to 6 for 3 to 15 days. The extent of cold temperature to be given depends on the plant species.

2.5.2 Anther culture for production of haploids

1. For the production of Haploid plants which find their use in fundamental and applied biological research.

2. In Haploids each chromosome is represented only ones and hence the recessive genes present on these chromosomes find their expression in the phenotype of the regenerated plants and thus find their use in fundamental genetics.

3. Anther culture derived haploids on chromosome doubling through agents like colchicine produce homozygous diploids which are highly used for plant breeding and crop improvement programs.

4. Haploids are useful in mutation studies.

2.6 SUMMARY

Micropropagation is one type of *in vitro* clonal propagation. Explants like nodes, axillary buds, meristems or shoot tips are used for this purpose. Plantlets can be produced either through direct or indirect organogenesis. If it is the later, plantlets are formed through somatic embryogenesis involving callus formation. Micropropagation is an inexpensive technique resulting in production of large number of plants within a short time.

Haploid organisms have gametic chromosome numbers. Haploids produced spontaneously or through mutagenic treatments occur in very low frequencies while they can be produced in large numbers through anther culture and these are called androgenic haploid Homozygous diploids, which are useful for any plant breeding or crop improvement programs can be produced in a single step from haploids through chromosome doubling. Haploids can be produced from the microspore culture while, other parts of anther like the wall cells, connective tissue or the filament give rise to heterozygous diploids. Haploids can be of immense use in the mutant selection experiments.

2.7 MODEL QUESTIONS

1. Give an account of the various methods of micropropagation.
2. What is organogenesis? Describe the method of production of plantlets via indirect organogenesis.
3. Explain the advantages and disadvantages of micropropagation.
4. Give an account of haploid production from anther microspore culture and mention their applications.
5. What is androgenesis? How many types of androgenesis are there / add a note on the advantages of microspore culture over anther culture?
6. Write short notes on:
   (a) *In vitro* production of Haploids.
   (b) Factors affecting anther culture.

2.8 REFERENCE BOOKS

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3. **Plant Biotechnology** by S. Ignacimuthu, S.J., Oxford & IBH Pvt. Co. Ltd., New Delhi.

4. **Introduction to Plant Biotechnology** by H.S.Chawla, Second Edition, Oxford & IBH Pvt. Co. Ltd., New Delhi.

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Prof. V. Manga

M.Sc. BOTANY (Final)

Paper-VIII : PLANT BIOTECHNOLOGY

Unit-III

Lesson 3

Genomic and CDNA libraries

3.0 **OBJECTIVE:**
To provide technical information about the importance and method of preparation of genomic and CDNA libraries..
3.1 INTRODUCTION
3.1.1 Construction of a genomic library
3.1.2 Vectors
3.1.3 Construction of a cDNA library
3.2 SCREENING PROCEDURES
3.2.1 Colony and Plaque Hybridization
3.2.2 Expression Screening by immunodetection
3.2.3 Chromosome walking
3.3 SUMMARY
3.4 TECHNICAL TERMS
3.5 MODEL QUESTIONS
3.6 REFERENCE BOOKS

3.1 INTRODUCTION

A gene library is a collection of different DNA sequences from an organism that have been cloned into vector molecules for ease of storage and analysis. If the library has been prepared from genomic DNA, it is called a genomic library. If the DNA used to prepare the library is a cDNA (copy of an mRNA); then the library is called a cDNA library. A library that contains all the clones is a representative library. Genomic libraries can be representative but cDNA libraries will lack most sequences and is limited to only a few sequences. Hence, they are not representative when dealing with eukaryotic organisms, the first major decision is whether to begin with messenger RNA (mRNA) or genomic DNA. Although, the DNA represents the complete genome of the organism, it may contain non-coding DNA such as introns, control regions and repetitive sequences. This can sometimes present problems, particularly if the genome is large and the aim is to isolate a single-copy gene. If the control regions consisting of regulatory sequences have to be isolated, then genomic DNA is the only alternative.

Messenger RNA represent the genetic information that is being expressed by the particular cell type that it has been isolated from. Messenger RNA and the CDNA prepared from it represent the coding sequence of the gene with the introns having been removed during RNA processing. Thus if production of a recombinant protein is the goal, preparation of CDNA is more beneficial.

3.1.1 Construction of a genomic library

For the construction of a genomic library, the total genomic DNA of an organism is extracted (Fig. 1). The DNA is then cut into fragments by either physical shearing or restriction enzyme digestion. Physical shearing is carried out by pipeting, mixing or by sonication. DNA fragments of 15-25 kb are convenient, to be cloned in λ or cosmid vectors. Complete digestion with restriction enzymes is avoided because it generates fragments of great variation in size. Partial digestion, by using limited amounts of restriction enzyme results in the DNA being cut only at a few restriction sites, thus producing DNA molecules of lengths greater than in a complete digest. The enzymes **Sau3A, Alu I** and **Hae III** with recognition sequences of four nucleotides are

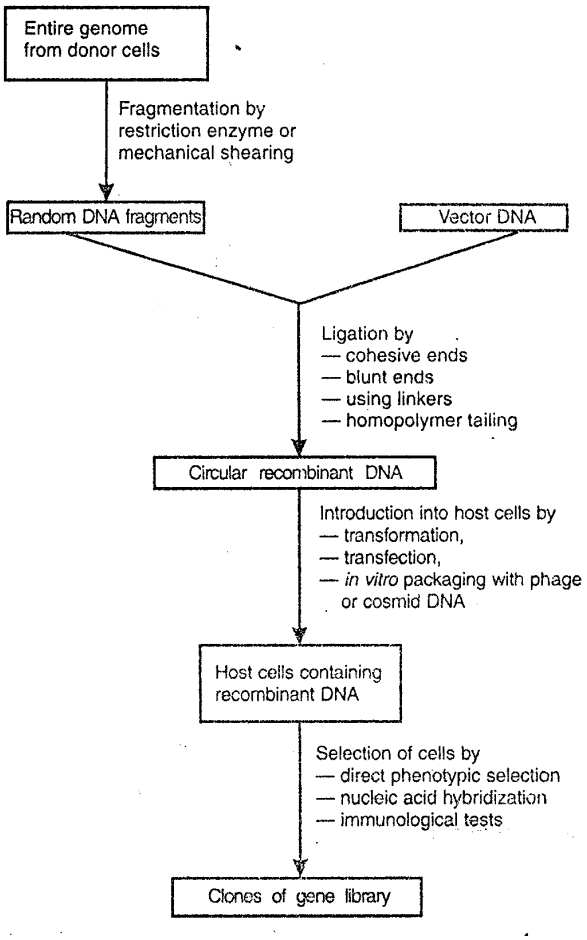

Fig. 1 Methods of construction a gene library / Genomic DNA Library

most commonly used (with/GATC, AG/CT and GG/CC respectively). The partial digests of genomic DNA are subjected to agarose gel electrophoresis. The separated fragments are then

inserted into suitable vectors for cloning. This constitutes the shotgun approach to gene cloning. The λ vectors and cosmids have been mostly used for constructing gene libraries. The vectors containing the inserts are cloned in a suitable bacterial host.

3.1.2 Vectors

For cloning the E. coli genome, a plasmid vector could be enough since its genome size is small. Libraries from organisms with larger genomes are constructed using phage λ, cosmid or yeast artificial chromosome (YAC) vectors.

3.1.3 Construction of a cDNA library

Generally, cDNA libraries are not prepared from prokaryotic mRNA but are the most preferred for eukaryotic mRNA because the cDNAs do not have intron sequences and therefore can be used to express the encoded protein in *E. coli*. The cDNA molecules represent the transcribed parts of the genome (i.e. the genes rather than the non-transcribed DNA). The mRNA can be readily isolated from lysed eukaryotic cells by adding magnetic beads which have oligo (dT) conalently attached. The mRNA binds to the origo (dT) via its poly (A) tail and can thus be isolated from the solution. The integrity of an mRNA can be checked by gel electrophroesis. For the synthesis of cDNA, the enzyme reverse transcriptase is used to make a cDNA copy of the mRNA by extending a primer, usually oligo (dT), by the addition of deoxyribo nucleotides to the 3' end (Fig. 2). The enzyme reverse transcriptase is also called a RNA dependent DNA polymerase was discovered by Temin and Battimore (1970) and uses the mRNA molecule as a template. The RNA-DNA hybrid molecule is then treated with RNase H or subjected to alkaline hydrolysis to free the single stranded cDNA molecule. The 3' prime end of this cDNA acts as a template for a primer. This primer is generated spontaneously by hairpin formation or folding back on itself. There are certain other methods of inducing the synthesis of the second strand. The spontaneously primed strands with the hairpin are treated with a single-strand specific Sinuclease so that the phosphodiester bond within the loop is broken, leaving the double-stranded cDNA molecule amenable to cloning.

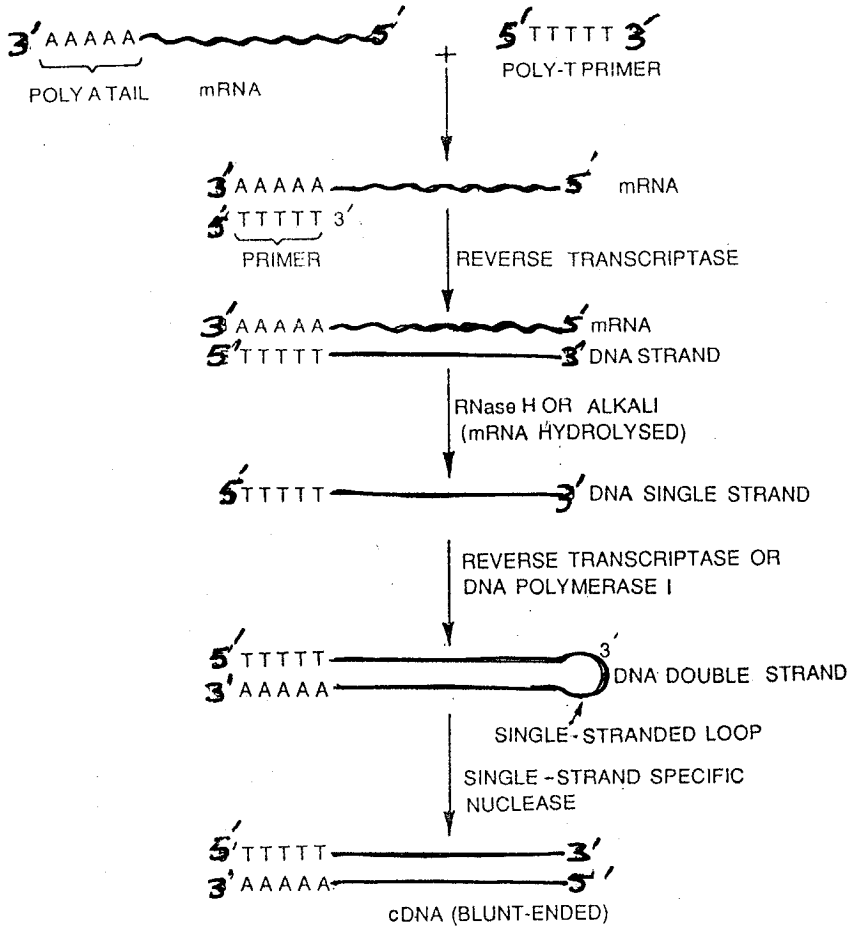

Fig. 2. Production of cDNA (complementary or copy DNA) from an mRNA molecule used as template by the enzyme reverse transcriptase.

Any vector with an **Eco RI** site would be suitablefor cloning the cDNA. Plasmid vectors are used mostly since the cDNAs are relatively short (0.5 to 1.0 kb). λ phase vectors are used to clone expression cDNA. Ligalion of vector to cDNA is carried out using T4 DNA ligase and the recombinant molecules are either packaged or transformed to create the cDNA library.

3.2 SCREENING PROCEDURES

The method of identifying are particular clone containing a specific gene of interest from among the total collection of clones of a gene library is called screening. To carry out this screening, some information regarding the sequence of the gene, or the knowledge of the gene product is essential. This information can be used to prepare a nucleic acid probe (a short single stranded RNA or DNA sequence that is complementary to a part of the target gene present in the clone) or to prepare antibodies to the specific gene product (protein). The nucleic acid probe is

used for location of the clone by hybridization and the antibodies are used for immuno-detection where the clone to which an antibody binds (it binds to the protein expressed by the target gene in the clone) is in turn detected by other antibodies.

3.2.1 Colony and Plaque hybridization

The gene libraries prepared with plasmid vectors and transformed into bacterial cells produce colonies on agar plates (petri dishes containing the culture medium solidified with agar). The λ phage gene libraries however produce plaques instead. The screening methods for both colonies and plaques (Fig. 3) are essentially the same, but the bacterial colonies must be lysed first to release their DNA, whereas, the phage DNA is directly available since plaques are actually lysed bacterial areas on the lawn (bacterial culture). The method of colony/plaque hybridization is given below:

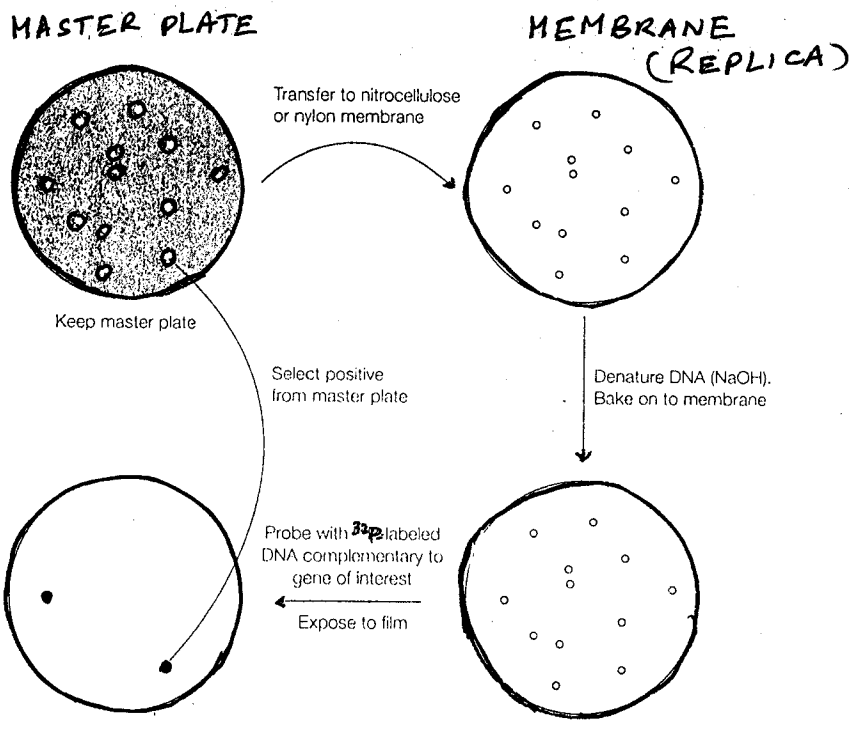

Fig. 3. Screening by plaque hybridization.

1) The bacterial cells subjected to transformation are plated on to a suitable agar plate. This plate is called the '**master plate'**.

2) The bacterial cells containing different genes grow into colonies. The process involves the selection of a specific clone (colony with recombinant plasmid/phage) carrying a particular gene.

3) The colonies/plaques on the '**master plate**' are replica plated onto a nitrocellulose filter membrane placed on the agar plate. The membrane is gently pressed and removed carefully to reveal some cells of the bacterial colonies stuck to it in the same pattern as that on the master plate. The master plate is retained for future use. Proper markings are made on the membrane filter for comparison later.

4) The membrane filter is treated with sodium dodecyl sulphate and a protease to lyse the bacterial cells and release the DNA. This is not required for plaques since the DNA will be already released due to bacterial cell lysis.

5) The DNA on the membrane is then denatured with alkali to produce single strands which are then bound to the membrane by baking at 80oC or by U.V. irradiation.

6) The membrane is now immersed in a solution containing a nucleic acid probe (labelled radioactively or non-radioactively) and incubated to allow the probe to hybridize to its complementary target sequence present in bacterial cells of one or more colonies on the filter.

7) The membrane is then washed with buffer to remove the unhybridized probe.

8) The colonies, whose DNA has hybridized with the probe are detected by autoradiography (by placing a X-ray film over it and developing the same to prepare an autoradiograph). The positions of the positive colonies are compared to the colonies on the master plate. These colonies are then picked up for further studies and multiplied.

3.2.2 Expression screening by immunodetection

This is an alternative screening procedure which relies on gene expression and is generally applicable for identification of the clone synthesizing a particular polypeptide. The most important requirement for this test is the availability of a suitable antibody. Antibodies are used to identify the colonies developed on masterplates that synthesize antigens (proteins) encoded by the foreign DNA present in plasmids of the bacterial clones. The vectors used for this purpose are called as expression vectors. The method of immunodetection (Fig. 4) is given below:

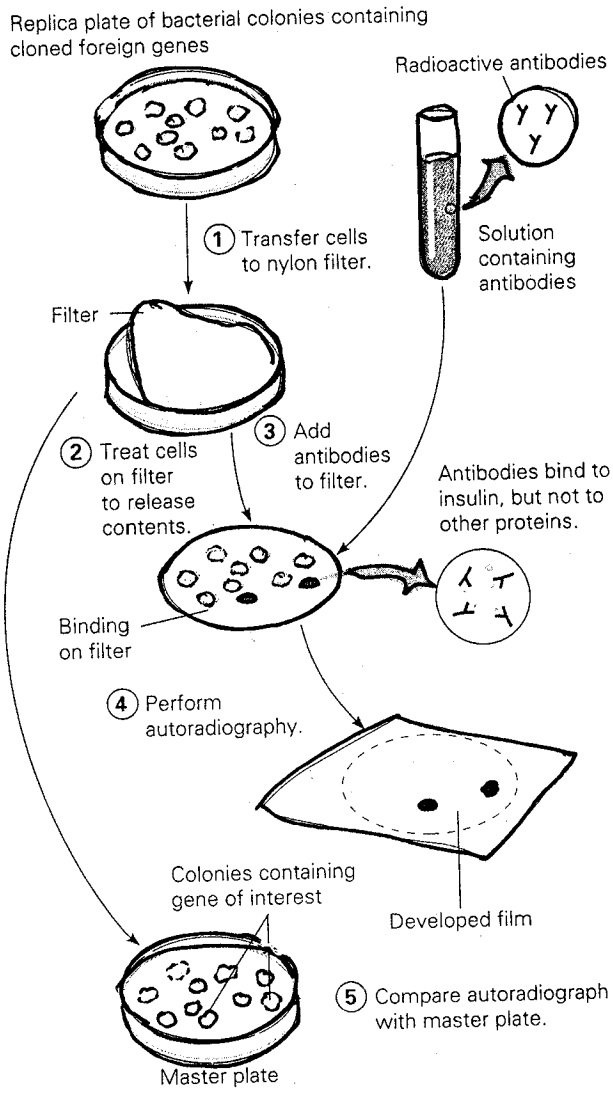

Fig. 4. Immuno detection

1) The bacterial cells subjected to transformation are plated onto a suitable agar plate. the clones (colonies) are allowed to grow on this '**master plate**'.

2) The colonies on the master plate are replica plated onto a nitrocellulose filter membrane and relevant markings are made (as explained under colony hybridization).

3) The membrane filter is treated with high temperature or exposed to chloroform vapour to lyse the cells.
4) A suitable antibody is immobilized onto a cellulose filter and this is placed in gentle contact with the membrane filter carrying lysed cells to allow absorption of antigen to antibody.

5) The antigen – antibody complex is detected by incubating the cellulose filter with a radio labelled or non-radio labelled second antibody.

6) The unreacted antibodies are washed off and the position of antigen-antibody complex is determined by autoradiography.

7) The position of the positive colonies are compared to the colonies on the master plate.

8) The positive colonies are then picked up for further studies and multiplied.

3.2.3 Chromosome walking:

This is the repeated screening of a genomic library to obtain overlapping clones and hence build up a collection of clones covering part of a chromosome, for chromosome mapping.

3.3 SUMMARY

A gene library is a collection of different DNA sequences from an organism that have been cloned into vector molecules for storage and analysis. If the library has been prepared from genomic DNA, it is called a genomic library. If the DNA used to prepare the library is a cDNA (copy of an mRNA called as complementary DNA), then the library is called a cDNA library. Genomic DNA or genomic library represents the complete genome of the organism including the non-coding regions such as introns, regulatory regions and repetitive sequences. The cDNA or CDNA library is prepared from mRNA which represents the coding sequence of the gene, excluding the introns etc. The cDNA is preferred in cases where the recombinant protein needs to be expressed and genomic DNA is preferred if there is a need to study the control of gene expression i.e. study of regulatory sequences.

For preparation of genomic library, the genomic DNA is partially digested by using limited amounts of restriction enzymes such as **Sau 3A, Alu I** and **Hae III** which recognize four nucleotide sequences. The fragments thus generated are inserted into suitable vectors for cloning. This method is called the shot gun approach to gene cloning and the fragments are cloned in λ vectors, cosmids or yeast artificial chromosomes.

The cDNA libraries are prepared and preferred for ukaryotic organisms. The mRNA which represents the transcribed parts of the genome is readily isolated and cDNA is synthesized from it by reverse transcription by the enzyme reverse transcriptase also called as RNA dependent DNA polymerase. Suitable primers are used for the cDNA synthesis along with SI nuclease enzyme (to

finally prepare the double stranded DNA). Plasmid vectors are mostly used to clone cDNA. Lambda λ phage vectors are also used especially for cloning expression cDNA.

The method of identifying one particular clone containing a specific gene of interest from among the total collection of clones of a gene library is called screening. There are two most important screening procedures viz. Colony/Plaque hybridization and expression screening by immuno detection, colony/plaque hybridization uses a nucleic acid probe (a short single stranded sequence labelled radio actively or non-radio actively) that is complementary to a part of the gene of interest. The immuno-detection uses an antibody to the specific gene product (protein or antigen) appropriately labelled whereas the nucleic acid probe hybridizes with the gene of interest, the antibody forms a complex with the protein product (antigen). For both the screening procedures, a replica of the colonies on the master plate is taken and the hybridization/complex formation patterns on the replica are compared to the colonies on the master plate and the required colony is selected for further studies and multiplied.

3.4 TECHNICAL TERMS

Genomic library: A collection of clones which together represent the entire genome of an organism.

cDNA: DNA that is made by copying mRNA using the enzyme reverse transcriptase. Its sequence of nucleotides are complementary to those of the mRNA. Hence, it is called complementary DNA or cDNA.

cDNA library: A collection of clones prepared from the cDNA (which is prepared from mRNA).

Nucleic acid probes: A labelled nucleic acid molecule (DNA or RNA) used in DNA hybridization procedures. The labelling is carried out by radioactive or non-radioactive methods.

Antibody: Proteins (immunoglobulins) that specifically recognises and binds to an antigenic determinant on an antigen (protein) that is bound by an antibody.

Nucleic acid hybridization: The joining together of artificially separated nucleic acid molecules **via** hydrogen bonding between complementary bases.

Chromosome Walking: Repeated screening of a genomic library to obtain overlapping clones and hence map the chromosome.

3.5 MODEL QUESTIONS

1. Briefly describe the procedure for constructing a genomic library.
2. Define CDNA. Explain the various steps involved in the synthesis of cDNA.

3. Explain the procedure of preparation of a cDNA library
4. What are the different screening procedures used to identify clones?
5. Explain the procedure of colony/plaque hybridization.
6. How is expression screening carried out by immunodtection?

3.6 REFERENCE BOOKS

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Unit-III

Lesson 4

Paper-VIII : PLANT BIOTECHNOLOGY

Molecular Analysis of DNA by blotting techniques

4.0 **OBJECTIVE:**
To provide technical information of the different blotting techniqu4es used for molecular analysis of DNA.
4.1 INTRODUCTION
4.1.1 Southern blotting – hybridization
4.1.2 Dot blot technique
4.1.3 Northern blot-hybridization
4.1.4 Western blotting
4.2 PROBES AND LABELLING OF PROBES
4.3 SUMMARY
4.4 TECHNICAL TERMS
4.5 MODEL QUESTIONS
4.6 REFERENCE BOOKS

4.1 INTRODUCTION

During the course of several molecular biology experiments (e.g. characterization of transgenic organisms or transformed cells), it becomes necessary to carry out molecular analysis of DNA to confirm the integration, presence and expression of a specific gene. The presence and integration of the specific/foreign gene is confirmed through Southern blotting – Hybridization and dot blots. The expression of the gene is confirmed through northern blotting and western blotting procedures.

4.1.1 Southern blotting – hybridization:

Southern blotting hybridization refers to a technique of blotting electrophoresed DNA fragments from the gel onto a nitrocellulose filter (membrane) and subjecting it to a hybridization between a specific DNA fragment and a DNA probe. The procedure was invented by E.M. Southern, hence the name of the technique. In this procedure, the DNA sample is digested with restriction enzymes and the fragments subjected to agarose or polyacrylamide gel electrophoresis. Agarose gel is used to separate DNA fragments of few hundred to 20 kb in size while polyacrylamide is preferred for smaller fragments. Large DNA fragments of 1000 to 2000 kb are separated in agarose gel with pulsed electrical fields. The gel forms a network (mesh) of polymeric molecules through which DNA fragments migrate according to their size under an electric field

since DNA molecules are negatively charged and migrate towards the positive electrode. Smaller DNA molecules migrate faster and reach the bottom ahead of the larger molecules. Marker DNA fragments are run on the side so that the size of the separated fragments are determined by interpolation. The gels are stained with ethidium bromide which intercalates in between the DNA base pairs and emits fluorescence with UV light. Photographs of the gel are taken with appropriate filter and analysed.

To detect and identify specific DNA fragments or genes that are complementary to a given DNA sequence e.g. presence of a gene in transgenics or to detect Restriction Fragment Length Polymorphism (RFLP) etc. Southern blot hybridization is carried out. This can be explained in the following steps (Fig. 1) given below:

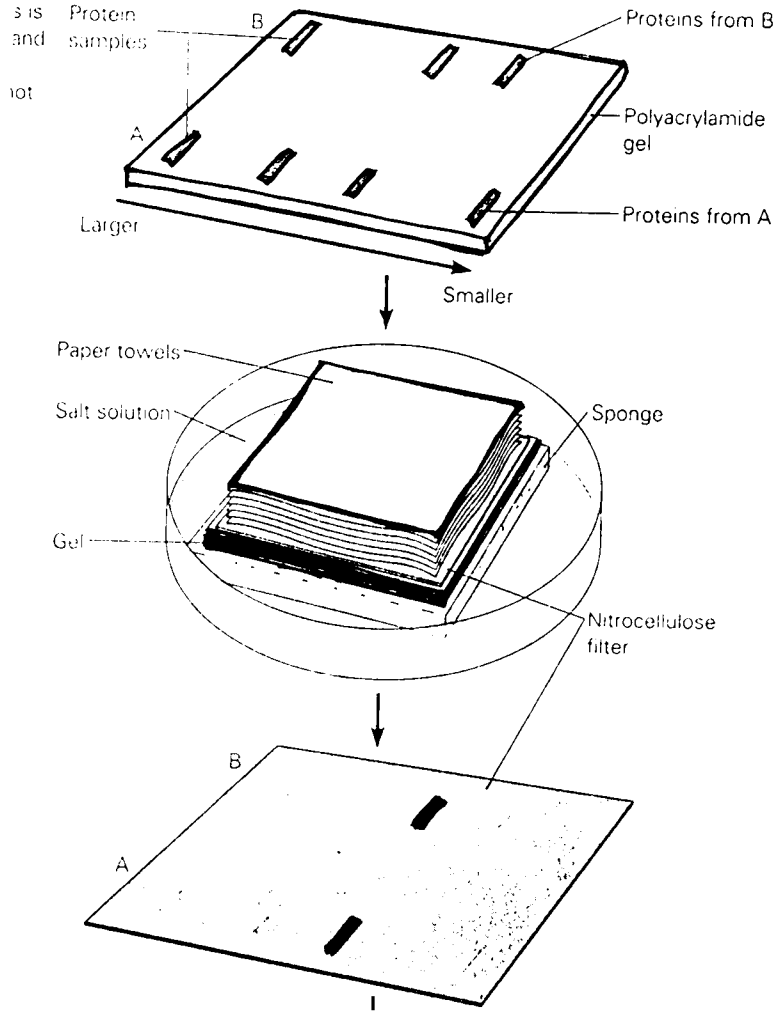

Fig.1.

1) The electrophoresed gel containing the separated DNA fragments is treated with alkali to denature the DNA into single-stranded form.

2) The DNA fragments are transferred to a nitrocellulose filter by placing the gel on top of a buffer saturated filter paper, then laying the nitrocellulose filter membrane on top of the gel. A stack of dry filter papers placed over the filter and a firm weight placed over them ensures the transfer of the DNA fragments from the gel onto the filter through capillary action by the moving buffer. The DNA (which was denatured to single stranded form) becomes trapped in the filter membrane as the buffer passes through it. The relative position of the bands on the membrane remain the same as that on the gel.

3) The nitrocellulose filter is baked in an oven at 80^{o}C in vacuum to permanently fix the DNA onto it.

4) The nitrocellulose filter is treated with special buffer solutions to prevent the non-specific binding of the labelled probe (explained below).

5) The pre-treated membrane filter is placed in a solution of single stranded labelled DNA probe (a short DNA sequence that is complementary to a part of a DNA fragment on the membrane filter). The probe is c labeled by radioactive or non-radio active method to aid the identification of the hybridization signal through autoradiography (on an X-ray film).

6) The hybridization process is carried out by using high stringency to ensure perfect complementary pairing of the probe to the related sequence on the membrane.

7) The membrane is washed to remove unhybridized probe and placed in contact with a X-ray film and incubated for a specific time to allow images (due to the emissions from the probe) to be formed on the film.

8) The X-ray film is then developed to reveal distinct bands indicating the DNA fragments with the complementary (to probe) sequence. The photograph of the autoradiogram is taken, documented and analyzed. Of late, Nylon membranes are being used in place of nitrocellulose membranes. Using nylon membranes saves time since a brief exposure to U.V. is enough to cross-link the DNA to the membrane, instead of the time-consuming baking process.

The genomic DNA fragments look like a smear after electrophoresis since innumerable fragments are formed with a continuous distribution as per their size, whereas the hybridization results in distinct bands in only one or more fragments.

The Southern blotting technique is extremely sensitive and is used for DNA fingerprinting, preparation of RFLP maps, detection of foreign genes in transgenics etc.

4.1.2 Dot blot technique

Dot blot technique is used to test for the presence of a gene in a DNA sample without the need for electrophoresis. It serves as a preliminary test which can be carried out and if it tests positive, will be followed by Southern blot hybridization. It is used to confirm the presence of a gene or specific mRNAs in a number of samples within a short time. The procedure of Dot blot is explained below:

1) The sample DNAs/RNAs of several organisms are applied as dots on the nitrocellulose filter.

2) The DNA is denatured with alkali and baked at 80^{o}C to fix it to the filter membrane.

3) The membrane is pre-treated to prevent non-0specific binding of the probe to the filter.

4) The membrane is incubated in the probe solution (refer Southern blotting) to allow hybridization between the probe and related sequence in the DNA samples (dots).

5) The dots having the specific DNA sequence (or RNA) will produce positive signals on the X-ray film after autoradiography.

6) Southern blot-hybridization is then carried out on the positive samples to find the exact fragment on which the gene is actually located.

4.1.3 Northern blot-hybridization

Northern blot hybridization s simply an extension of the Southern blotting technique. In this technique, RNA fragments are separated by gel electrophoresis, transferred to a nylon membrane, immobilized by U.V. cross linking and hybridized with radioactive or non-radioactive single-stranded DNA probes. The hybridization bands are detected by autoradiography. The Nylon membranes or special Diazo Benzyloxy Methyl (DBM) papers are used. Northern hybridization produces RNA : DNA hybrids. It is useful in the identification of specific sequences on the RNA and is mainly used for detection of transcription of a DNA sequence actually indicating the gene expression.

4.1.4 Western blotting:

Western blotting refers to the electrophoresis of proteins on a polyacrylamide gel followed by their transfer onto a nitrocellulose or nylon membrane and finally the detection of specific

protein bands by their specific interaction with antibodies or other compounds. The procedure of western blotting (Fig. 2) is described below:

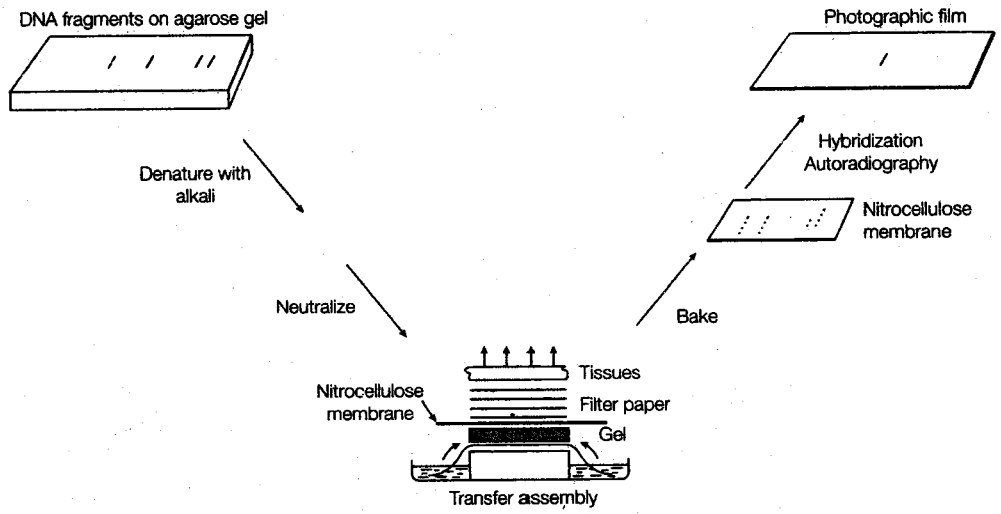

Fig. 3. Southern blotting.

Fig. 2.

a) Protein bands are separated on a polyacrylamide (vertical) gel by electrophoresis.

b) The protein bands on the gel are transferred onto a nitrocellulose or nylon membrane by capillary movement of the buffer similar to Southern botting. Of late, an electro blotting or electrophoretic blotting is carried out in special apparatus, which is much more efficient and faster.

c) Specific protein bands are identified by use of antibodies (used in place of probes) for detecting specific antigens. Lectins are used as probes for identification of glycoproteins. a second antibody (labelled with radioactive method or with biotinylated fluorescent tags) is used to bind with the first antibody that is bound to a protein. This identification involving the binding of the first antibody to the protein band followed by the binding of a second antibody (labelled specially – e.g. Streptovidin) to the first antibody that is bound to the protein band is called as the '**Sandwich'** reaction.

4.2 EXPANSION OF THE STRUCTURE – PROBES AND LABELLING OF PROBES

Probes are short, single-stranded nucleotide sequences (about 15 to 30 bases long) labelled radioactively or non-radio actively to aid detection after their hybridization with complementary sequences in the target gene under investigation. Both DNA and RNA can be used as probes.

Probes are prepared in various ways. Highly purified mRNA (which are naturally single-stranded) molecules can be used as probes or the CDNA molecules prepared from mRNA by reverse transcription by limiting the copying to only one strand can be used. Probes can also be prepared by PCR by the generation of single-stranded copies of the DNA sequence. Synthetic origonucleotides can be prepared for use as probes, if the nucleotide sequence of the target gene is known.

Labelling of the probes is carried out by radioactive means or by non-radioactive means. There are various techniques for labelling of nucleic acids like: (1) nick translation; (2) primer extension, (3) end labelling, and (4) direct labelling. Direct labelling is carried out by including a nucleotide labelled with ^{32}P during the production of the probe, so that it gets incorporated into the sequence of the probe. All these methods belong to the radioactive labelling method.

The non-radioactive labelling involves the labelling with: (1) biotin, (2) Digoxigenin, and (3) fluorescent molecules. Nick translation is commonly used for non-radioactive labelling method. The radioactive isotopes have short half-life periods and therefore need to be used within a short period of time after labelling. In contrast, non-radioactively labelled probes can be stored at $-20^{o}C$ for long periods of time.

The hybridized samples are detected by autoradiography by virtue of the radioactive output in case of the radioactively labelled probes. Further, the unhybridized probe can be easily washed off and other probes can be used for hybridization on the same membrane filter for detection of other genes.

The hybridized samples are detected by colour development after cytochemical reactions in case of the non-radioactively labelled probes. Further, the filter cannot be reused for detection of other genes (with other probes).

Since probes permit a highly reliable and extremely efficient detection of nucleic acid sequences complementary to them, they are used extensively in molecular biology research.

4.3 SUMMARY

During the course of several molecular biology experiments, it becomes necessary to carry out molecular analysis of DNA to confirm the integration, presence and expression of a specific gene. Southern blotting and dot blots are used to confirm the presence of the target gene whereas the northern blotting and western blotting are used to check the expression of the gene. Probes (short complementary single-stranded, labelled nucleic acid molecules) are used to hybridize with the target gene in case of Southern, dot and northern blotting probes can be labelled radioactively or non-radioactively. The western blotting uses antibodies to bind with specific antigens of the protein band on the membrane. Southern and dot blotting deals with the electrophoresed DNA fragments or DNA on the membrane being hybridized with DNA probes. Northern blotting deals with the

hybridization of RNA fragments on the membrane being hybridized with DNA probes. Western blotting uses a primary antibody which hybridizes with the protein band on the membrane. A second antibody is then used to bind to the primary antibody to produce a cytochemical reaction.

All the nucleic acid blotting methods use the DNA/RNA fragments (produced by digestion with restriction enzyme) by electrophoresing on gels and then transferring onto nitro cellulose membrane or nylon membrane before taking up the hybridization with probes. The western blotting however deals with protein electrophoresis and hybridization of the blotted protein bands with antibodies.

4.4 TECHNICAL TERMS

Southern blotting: Method for transferring DNA fragments onto a membrane for detection of specific sequences by hybridization

Northern blotting: Transfer of RNA molecules onto membranes for the detection of specific sequences by hybridization.

Dot-blot technique: Small spots or dots of nucleic acid are immobilized on a nitrocellulose or nylon membrane for hybridization.

Probe: A labelled molecule used in hybridization procedures. It is a single-stranded labelled nucleic acid molecule complementary to a part of the target sequence/gene.

Antibody: An immunoglobulin that specifically recognizes and binds to an antigenic determinant on an antigen.

Hybridization: The joining together of artificially separated nucleic acid molecules **via** hydrogen banding between complementary bases.

4.5 MODEL QUESTIONS

1. Explain Southern blot hybridization in detail.
2. Discuss the similarities between Southern and northern blotting.
3. Write short notes on:
   a) Dot-blot technique
   b) Probes
   c) Antibodies in Western blotting

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Unit-III

Lesson 5

Paper-VIII : PLANT BIOTECHNOLOGY

Amplification of DNA by Polymerase Chain Reaction

5.0 **OBJECTIVE:**
To provide an explanation of the technique of amplification of specific DNA sequences by in vitro synthesis by polymerase chain reaction.
5.1 INTRODUCTION
5.1.1 Denaturation
5.1.2 Primer annealing
5.1.3 Polymerization
5.2 EXPANSION OF THE STRUCTURE - VARIATIONS OF P.C.R. AND APPLICATIONS
5.3 SUMMARY
5.4 TECHNICAL TERMS
5.5 MODEL QUESTIONS
5.6 REFERENCE BOOKS

5.1 INTRODUCTION

The Polymerase Chain Reaction (PCR) is a specialized technique used for amplification of selective DNA fragments *in vitro*. This technique developed by Kary Mullis in 1985, makes it possible to synthesize large quantities of a DNA fragment without cloning it in a vector. It is especially suited in situations where very little quantity of biological specimens are available (e.g. a single hair or a tiny drop of blood stain left at the site of the crime) which yield very minute quantity of DNA. The PCR technique uses a specially designed machine called '**Thermocycler'** to amplify the DNA. Suitable primers (one primer for each strand) flank the target sequence on either ends (primers have complementary nucleotides to the ends of the two strands on opposite sides). The primers are synthesized as oligonucleotides and added in excess to be sufficient for each cycle of amplification. A thermostable form of DNA polymerase isolated from *Thermus aquaticus* (a thermophilic bacterium which inhabits hot springs) is added in excess to be sufficient for each cycle involving a high rise of temperature (to 80^{o}C or more) followed by drop in temperature. Sufficient amounts of deoxynucleoside triphosphates (dATP, dGTP, dCTP and dTTP) are also added to the reaction mixture for amplification of the target DNA sequence.

The PCR technique includes three essential steps viz. (i) Denaturation, (ii) Primer annealing, and (iii) Polymerization (Fig. 1).

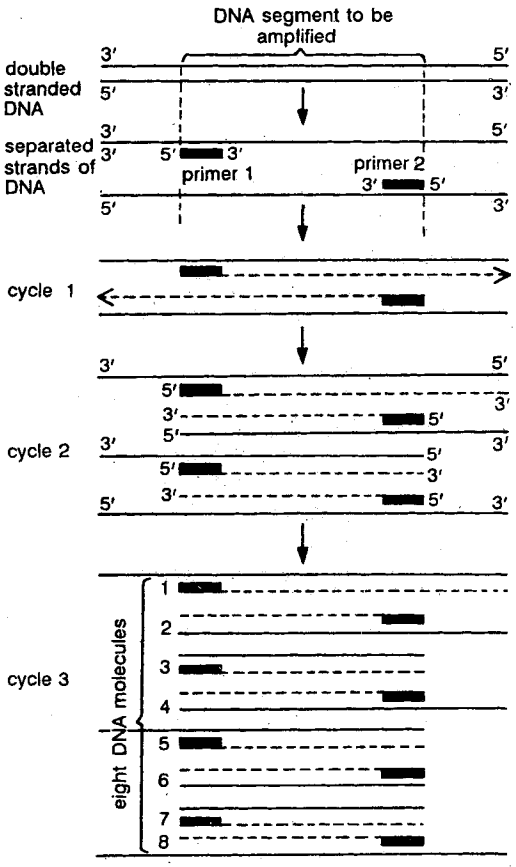

Fig. Basic reaction of the PCR (only three cycles of PCR are shown; in each cycle primers are shown by solid boxes, template strands are shown by continuous lines and newly synthesized strands are shown by broken lines).

5.1.1 Denaturation: This actually means melting of DNA by heating it to high temperatures upto 95°C. Denaturation results in separation of the two DNA strands. The DNA strands would be carrying the target sequence that needs to be amplified.

5.1.2 Primer annealing: The second step is the annealing of two origo nucleotide sequences (primers) on the ends (of opposite strands of DNA) of the target sequence by complementary pairing. This annealing is facilitated by lowering the temperature to around 55°C. Annealing takes place by formation of hydrogen bonds between the complementary bases.

5.1.3 Polymerization: The enzyme, DNA polymerase (Taq DNA polymerase) that is added in the reaction mix accelerates the polymerization process of primers and therefore, extends the primers with the help of deoxynucleotide triphosphates (dNTPs) in the reaction mix and requires Mg^{2+}. The temperature is increased to $72^{o}C$ to facilitate the polymerization, thus completing the synthesis of copies of target DNA sequence by the end of the third step. Thus, after completion of step 3 (of one cycle), the targeted sequences on both strands are copied and four strands are produced.

The three step cycle that produces four copies is then repeated which yields 8 copies from 4 strands. Similarly, the third cycle produces 16 strands. This cycle is then repeated to about 20-40 cycles. If **PCR** is 100% efficient, i.e. when suitable temperatures are used at each step and suitable primers are used, one target molecule would become $\mathbf{2^n}$ after **n** cycles. To achieve a perfect **PCR**, the reaction conditions must be varied accordingly. The target sequence can be a gene from genomic DNA or from a cDNA prepared from mRNA by reverse transcription. The latter process where reverse transcription is followed by PCR is called as RT-PCR. After PCR cycles, the amplified DNA segment is purified by gel electrophoresis and used as desired.

5.2 EXPANSION OF THE STRUCTURE – VARIATIONS OF P.C.R. AND APPLICATIONS

The PCR technique has been modified in a variety of ways to suit specific situations and applications. Some of these variations are **inverse PCR**, anchored PCR, nested PCR etc.

PCR has many exciting applications.

(i) PCR can be used to amplify a specific gene present in different individuals of a species or from a crime spot.

(ii) PCR has been used to study DNA polymorphism in the genomes or related genotypes of a species using known random primers. Such an application of PCR generates random amplified polymorphic DNA (RAPD) which is detected as bands after elecrtrophoresis. The bands of different species or strains can be compared and polymorphism analyzed.

(iii) PCR can be used to detect the presence of a gene transferred into an organism (transgene) for the amplification of DNA from the transgenic organism.

(iv) PCR is used for diagnosis of diseases and causal micro-organisms and also for detection of genetic diseases such as sickle cell anaemia, phenyl ketonuria and muscular dystrophy.

(v) PCR can be used to determine the sex of embryos that have been fertilized *in vitro* (in cattle) using Y-chromosome specific primers before their implantation in the uterus.

5.4 SUMMARY

The Polymerase Chain Reaction (PCR) is a specialized technique used for amplification of selective DNA fragments *in vitro*. This technique makes it possible to synthesize large quantities of a DNA fragment without cloning it in a vector. The PCR technique uses a specially designed machine called '**Thermocycler'** to amplify the DNA. Suitable primers are used to anneal to complementary sequences on either ends of the target DNA sequence. Special thermostable Taq DNA polymerase is used to synthesize copies of the target sequence by polymerization by using a mixture of dNTPs and Mg^{2+}.

The PCR comprises three steps viz. (1) Denaturation, (2) Primer annealing, and (3) Polymerization. The target sequence denatures to separate into two copies which are amplified into four copies at the end of the first cycle. Eight copies are produced by the end of the second cycle and so on. This cycle is then repeated to about 20-40 cycles and one target molecule would become $\mathbf{2^n}$ after **n** cycles. The target sequence can be a gene from genomic DNA or from a cDNA prepared from mRNA by reverse transcription. The latter process where reverse transcription is followed by PCR is called as RT-PCR. After PCR cycles, the amplified DNA segment is purified by gel electrophoresis and used.

PCR has many exciting applications comprising the amplification of specific DNA sequences from the genomic DNA of a species, evaluation of DNA polymorphism in related genotyeps of a species by use of random primers (called RAPD), confirmation of the presence of a transgene in a transgenic organism, diagnosis of crime diseases caused by micro-organisms and genetic diseases and determination of sex of *in vitro* fertilized cattle embryos before implantation in the uterus.

5.4 TECHNICAL TERMS

Polymerase Chain Reaction: A method for the selective amplification of DNA sequences.

DNA polymerase: An enzyme that synthesizes a copy of a DNA template.

Deoxy nucleoside triphosphate: Triphosphorylated precursor required for synthesis of DNA, where N refers to one of the four bases (A, G, T or C).

Random amplified polymorphic DNA: This method called RAPD is a PCR performed with random primers to detect polymorphism of DNA samples/genotypes of a species.

5.5 MODEL QUESTIONS

1. Write in detail, about the PCR technology.
2. Discuss the applications of PCR.
3. What are three steps in a PCR. Explain in detail.
4. Write short notes on:
   a) DNA polymerase
   b) Deoxy ribonucleoside triphosphate.
   c) Primers
   d) Thermus aquaticus
   e) RAPD

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M.Sc. BOTANY (Final)

Paper-VIII : PLANT BIOTECHNOLOGY

Unit-I

Lesson 3

MUTANT SELECTION FOR BIOTIC AND ABIOTIC STRESSES

3.1 INTRODUCTION
3.2 TYPES OF DEFINITION OF STRESSES
3.3 TYPES OF CULTURES AND THEIR SUITABILITY FOR *IN VITRO* MU PRODUCTION
3.3.1 Callus cultures
3.3.2 Protoplast cultures
3.3.3 Limitations of protoplasts for *in vitro* mutagenesis
3.3.4 Cell suspension cultures: Limitations and advantages of cell suspension culture
3.4 GENERAL METHODOLOGY FOR *IN VITRO* INDUCTION AND SELECTION OF MUTANTS USING SUSPENSION CELL CULTURES
3.5 TYPES OF MUTANTS SELECTED USING IN VITRO CULTURES
3.5.1 Herbicide resistance
3.5.2 Salt tolerance
3.5.3 Amino acid and Amino acid analogue resistance
3.6 IMPORTANCE OF IN VITRO MUTANT SELECTION
3.7 SUMMARY
3.8 MODEL QUESTIONS
3.9 REFERENCE BOOKS

3.1 INTRODUCTION

Identification of spontaneous mutants or induction of mutants for desirable characters through conventional means is a time taking and space consuming process. Development of protocols for successful regeneration of plants from tissue, cell and protoplast cultures have led to the induction, screening and isolation of mutants using in vitro techniques.

The establishment and handling of large populations and improving selection under in vitro conditions (which follow the growth of only the mutant cells and their identification) becomes very easy compared to the field evaluation.

Thus tissue culture systems offer efficient means for generating and selecting different types of stresses including the biotic and abiotic types. The chances for isolation of desirable mutants for biotic and abiotic in cell cultures can be further enhanced through mutagenic treatments.

3.2 TYPES OF STRESSES

There are two major categories of stresses in plants viz., the Biotic and Abiotic stresses

Biotic stress: The stress caused by living organisms that harm plants which include the viruses, fungi bacteria, parasitic weeds and insects

Abiotic stress: The effect of non-living factors like drought, low and high temperatures, salt minerals etc. on living organisms.

3.3 TYPES OF CULTURES AND THEIR SUITABILITY FOR *IN VITRO* MUTANT PRODUCTION

3.3.1 Callus cultures

Calli growing on solid medium are not suitable for induction and isolation of mutants due to (i) slow growth of callus cells (ii) the crowded nature of growth of cells which does not allow for their uniform exposure to any mutagen (iii) tested cells located at the base or those that are deep seated are tolerant.

Since cells grow in large masses, those resistant/tolerant for the desirable traits may not be able to regenerate, since they are deep seated and are surrounded by normal cells.

3.3.2 Protoplast cultures

(i) Even though protoplast derived callus cultures as well as regenerates have been reported, they are limited to a very few cases and the protocols are not available for a wide range of plants.

(ii) Protoplast are obtained in low numbers and the isolation process proves to be very costly.

(iii) Handling of protoplasts for mutant selection becomes difficult due to the fragile nature of protoplasts.

3.3.3 Limitations of protoplasts for *in vitro* mutagenesis

(i) Techniques of protoplast isolation and culture are not available in wide number of protoplasts.

(ii) Isolation of large quantities of protoplasts may prove very costly.

(iii) Protoplasts are very fragile due to their extreme sensitivity osmotic imbalances and hence difficult to handle.

However, protoplasts offer an advantage over callus cultures in that they form reliable single cell culture system and hence the possibility of selected calli/regenerates not being chimeric in nature is more. Because of these factors, protoplasts have been used only in plant systems like tobacco and *Petunia* only for in vitro selection of mutants.

3.3.4 Cell suspension cultures: Limitations and advantages of cell suspension cultures

Cell suspension cultures show faster growth rate than callus and can be manipulated similar to microorganisms. The most common limitation is frequent presence of cell aggregates unlike the protoplasts. However the limitation can be overcome by filtering the cultures through a carefully chosen mesh (200 – 400 um) so as to increase the proportion of single cells or a few celled aggregates (Fig. 3.1).

3.4 GENERAL METHODOLOGY FOR IN VITRO INDUCTION AND SELECTION OF MUTANTS USING SUSPENSION CELL CULTURES

(i) Select the cell culture which has high regenerating ability.

(ii) Subculture the cells such that a high density of single cells in exponential growth phase are available. This is important because when large number of cells exist in exponential phase, they are actively dividing and the possibility of mutagenesis during action synthesis perish (S phase of inter phase) is more.

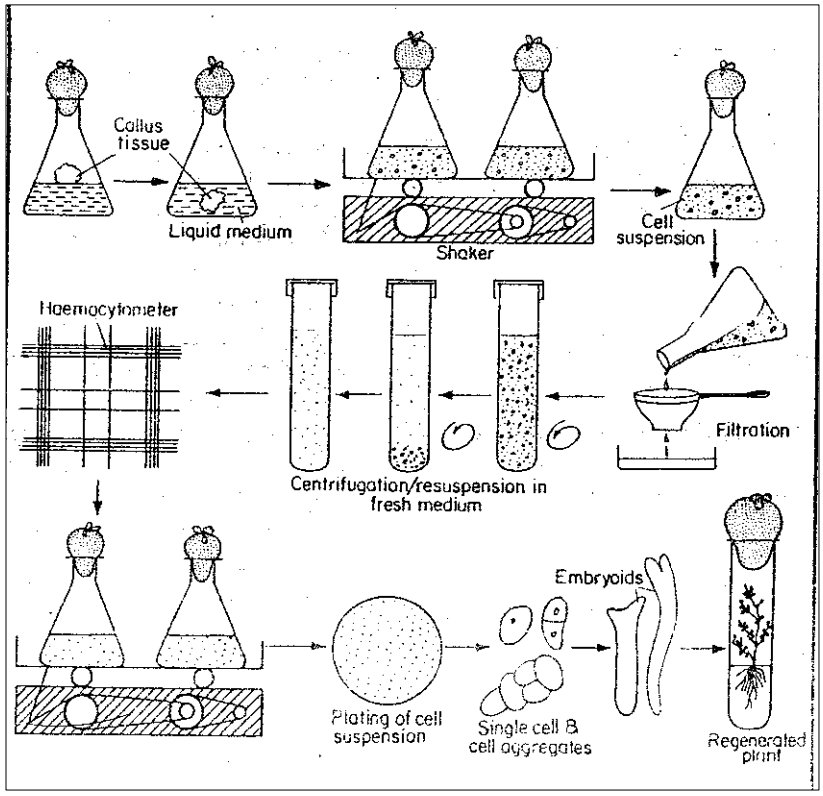

Fig. 3.1 Flow diagram illustrating the method of cell suspension culture and regeneration of plant through embryogenesis

(iii) Concentrate the cells through centrifugation and expose them to physical mutagenes like Ethyl methane sulfonate (EMS) or diethyl sulfate (DES) etc. which are used to make suitable concentrations of the chemical of the culture medium and incubate the cells on shaker for the required period.

(iv) Wash the cells with fresh liquid medium devoid of the chemical mutagenes and finally suspend them in fresh medium. If physical mutagenes are used this step may not be necessary.

(v) Grow the cells in dark for few replication cycles either before or after plating them on a suitable solid medium.

(vi) Cells should be grown for several generations to allow segregation and expression of the recessive mutations before actual selection is undertaken.

(vii) Method of selection – most of the mutants have been selected by plating the suspension of cells following mutagenesis on a selective medium containing (or devoid of) a particular

chemical, the tolerance/resistance of which is under study. Only those cells that can tolerate the presence/absence of that chemical will survive and develop into cells while others will not survive.

3.5 TYPES OF MUTANTS SELECTED USING *IN VITRO* CULTURES

3.5.1 Herbicide resistance

Regenerants showing tolerance for herbicides like glyphosate, sulphonyl urea, Imadazolenoues and for 2,4-D, picloram paraquat and asulum have been produced in soybean, wheat, maize, tobacco, *Lotus corniculatus, Tripolium repens* and *Apium graveolens* etc.

3.5.2 Salt tolerance

Cell lines exhibiting resistance or relative tolerance to salt stress have been produced in a large number of plant species which can be used for plantation in degraded lands having salinity problems. The successful plant systems revealing salt tolerance include species of *Brassica, Capsicum, Avena, Medicago, Cicer, Citrus, Daucus Hordeum, Nicotiana, rice* etc.

3.5.3 Amino acid and Amino acid analogue resistance

The majority of mutants isolated from *in vitro* cultures of higher plants have been selected for resistance to amino acid analogues or growth inhibitory naturally occurring amino acids. Most mutants have been isolated as resistant to either aromatic amino acid analogues (5 methyltrypophan, p-fluorophenyl alanine, 6-fluorotryptophan) or lysine and threonine or their analogues (S-amino ehyl cysteins)

Resistance to antimetabolites can arise through one or more of several mechanisms:

(i) Reduced uptake of analogue – by changing the membrane permeability.

(ii) Decreased sensitivity of an amino acid biosynthetic enzyme to feed back inhibition leading to ex: 5 methyl tryptophan resistance in *N. tabaccum* may arise through resistance of the first enzyme in the tryptophan pathway, Anthranilate synthetase, to feed back inhibition by tryptophan or its analogue. (Wilton, 1972)

(iii) Overproduction of naturally occurring amino acid

(iv) Excess of amino acid diluted effect of analog in protein synthesis.

(v) Detoxification of the compound through degradation. e.g.: paraflurophenyl can be degraded by phenylalanine ammonialyase (Berlin, 1980)

(vi) Conversion of the excess chemical into other forms (secondary metabolites like phenolic compounds) e.g.: S-amino ethyl, L-cysteine resistance in *Arabidopsis thaliana* and *Oryza sativa* induced by EMS, Hydrorpline resistance in *Hordeum vulgare* induced by sodium azide, valine resistance induced in naploid *N. tabacum* by UV.

B) **Purine and Pyrimidine analogues:** Spontaneously arising aminoptesin resistance in haploid *Datura innoxia,* 8-Ayaguanine resistance induced by EMS in *Haplopappus gracilis* 5-BUDR (a thymidine analogue) resistance – spontaneous in toboco EMS induced in Medicagosativa.

C) Antibiotic resistance: It is most extensively used in the analysis of somatic hybrids Antibiotic resistance is expected to be cytoplasmically inherited. However, some cases are known where they are inherited in Mendelian manner.

D) Disease or pathotoxin resistance: This type of resistance has been reported in *Solanum tuberosum* for *Phytopthora infestanse* and in the same species for *Fusarium oxysporum* and *In Zea mays* for *Helminthosporium maydis*

E) Cold Tolerance: The mutants can resist frost and winter hardiness. EMS induced cold tolerance mutants have been reported in *Datura innoxia* and such mutants in *Nicotiana sylvestris* were of spontaneous origin.

F) Miscellaneous mutant types: Mutants for Aluminum tolerance in tomato as well as Zinc and Copper tolerance in *Agrostis stolonifera* were selected using callus cultures

3.6 IMPORTANCE OF IN VITRO MUTANT SELECTION

Cell cultures offer many advantages over mutagenesis of seed / meristems. Screening of large numbers of cells becomes possible in a limited space Reproducible selection programs can be operated as the cells are grown in a uniform culture environment. Uniform exposure of cells to mutagenes becomes possible. Mutants obtained from cell level are highly useful as markers in somatic hybridization experiments for selection of somatic hybrids at an early stage. Disease resistant plants can be produced and screened directly under in vitro condition itself. The *in vitro* mutants are important in agriculture like those involving indirect selection for a particular phenotypic trait like Proline accumulation as indicator of stress/ drought tolerance and Sodium chloride tolerance as indicator of salinity

3.7 SUMMARY

Production selection and identification of mutants for stresses would be much easier through use of tissue culture as compared to their field evaluation. Of the different types of cultures, suspension cell cultures happen to be better for mutant selection experiments. The mutants include those resistant to herbicides, antibiotics, salt, amino acids and their analogues, various diseases, cold drought etc. For mutant selection, cell suspension cultures have many advantages over the mutants produced through seed mutagenesis.

3.8 MODEL QUESTIONS

1. Give an account of the role of cell cultures in isolation and selection of mutants for abiotic stress.
2. What are biotic and abiotic mutants? Explain different types of mutants that come under these two categories.
3. Explain the role of cell cultures in the induction and selection of mutants for biotic stress.
4. Write short notes on:
   a) Amino acid analogue resistance
   b) Selection mutants
   c) Cell suspension culture

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Paper-VIII : PLANT BIOTECHNOLOGY

Unit-IV

Lesson 1

Molecular Markers – FFLP, RAPD and their applications in crop improvement

1.1 OBJECTIVE
1.2 INTRODUCTION
1.3 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)
1.4 OTHER METHODS
1.5 APPLICATIONS OF MOLECULAR MARKERS IN CROP IMPROVEMENT
1.6 SUMMARY
1.7 TECHNICAL TERMS
1.8 MODEL QUESTIONS
1.9 REFERENCE BOOKS

1.1 OBJECTIVE

To provide basic technical description of the subject of Molecular markers viz., RFLP and RAPD and explain their application in crop improvement.

1.2 INTRODUCTION

The production of domestic crops has evolved through the collective efforts of agronomists since the dawn of agriculture. The study of Genetics involving the study of resemblance between relatives has made a major contribution to improved agricultural productivity. In recent years, the field of molecular biology has provided tools suitable for rapid and detailed genetic analysis of higher organisms including agricultural species. The most fundamental of these are the DNA markers. DNA markers referred to as molecular markers are, actually simply detected difference in the genetic information carried by two or more individuals. Information from molecular markers serves many purposes such as forensic science, paternity testing, identifying the genes responsible for genetic diseases and inferring evolutionary relationships (Phylogeny) among organisms or related species. The most widespread application of DNA markers is in the construction of genetic maps which can be used to determine the chromosomal location of genes affecting either simple or complex traits. By knowing the 'map position' of the gene, one can use nearby DNA markers to diagnose the presence of the gene without having to wait for the gene's effects to be seen.

Estimates of genetic relatedness are important in designing crop improvement programmes and molecular markers are the best tools to study genetic diversity. The properties desirable for ideal DNA markers are:

- Highly polymorphic nature.
- Frequent occurrence in genome.
- Easy access (availability).
- Easy and fast assay.

Depending on the type of study to be undertaken, a marker system that fulfills at lest a few of the properties (given above) can be identified.

Although DNA sequencing is a straight-forward approach for identifying variations at a locus, it is expensive and laborious. A wide variety of techniques using molecular markers have been developed in the past few years for visualizing DNA sequence polymorphism. The term DNA-finger printing was introduced for the first time by Alec Jeffrey in 1985 to describe the bar-code-like DNA fragment patterns generated by probes after electrophoretic separation of genomic DNA fragments. DNA finger printing essentially involves the display of a set of DNA fragments from a specific DNA sample and is used in investigating genetic variability, forensics and paternity tests. The molecular markers can be generally classified as hybridization-based markers and polymerase chain reaction-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA to a labelled probe. The PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme, followed by electrophoresis to detect the banding patterns. The restriction fragment length polymorphism (RFLP) technique is based on the hybridization-based markers while the PCR-based marker techniques include the random amplified polymorphic DNA (RAPD) and the amplified fragment length polymorphism (AFLP) methods. These methods are described below.

1.3 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted onto a nitro cellulose membrane via Southern blotting, followed by hybridization with labelled probe and visualized after auto radiography. RFLP markers first used by Botstein et al in 1980 to construct genetic maps have been mainly used to detect genetic polymorphisms. One must understand that mutations in non-coding DNA do not result in varying phenotypes, but can be detected by RFLP analysis. Imagine two individuals X and Y who contain two genes **A** and **B** which are separated by a non-coding region of the chromosome (Fig. 1). Both the genes have a single **Hind III** restriction site. These are **Hind III** restriction sites between genes A and B also: X has two sites and Y has only one site in between the genes. A mutation occurred in Y when a single base changed causing the loss of the **Hind III** restriction site. Can an RFLP analysis detect this mutation in Y?

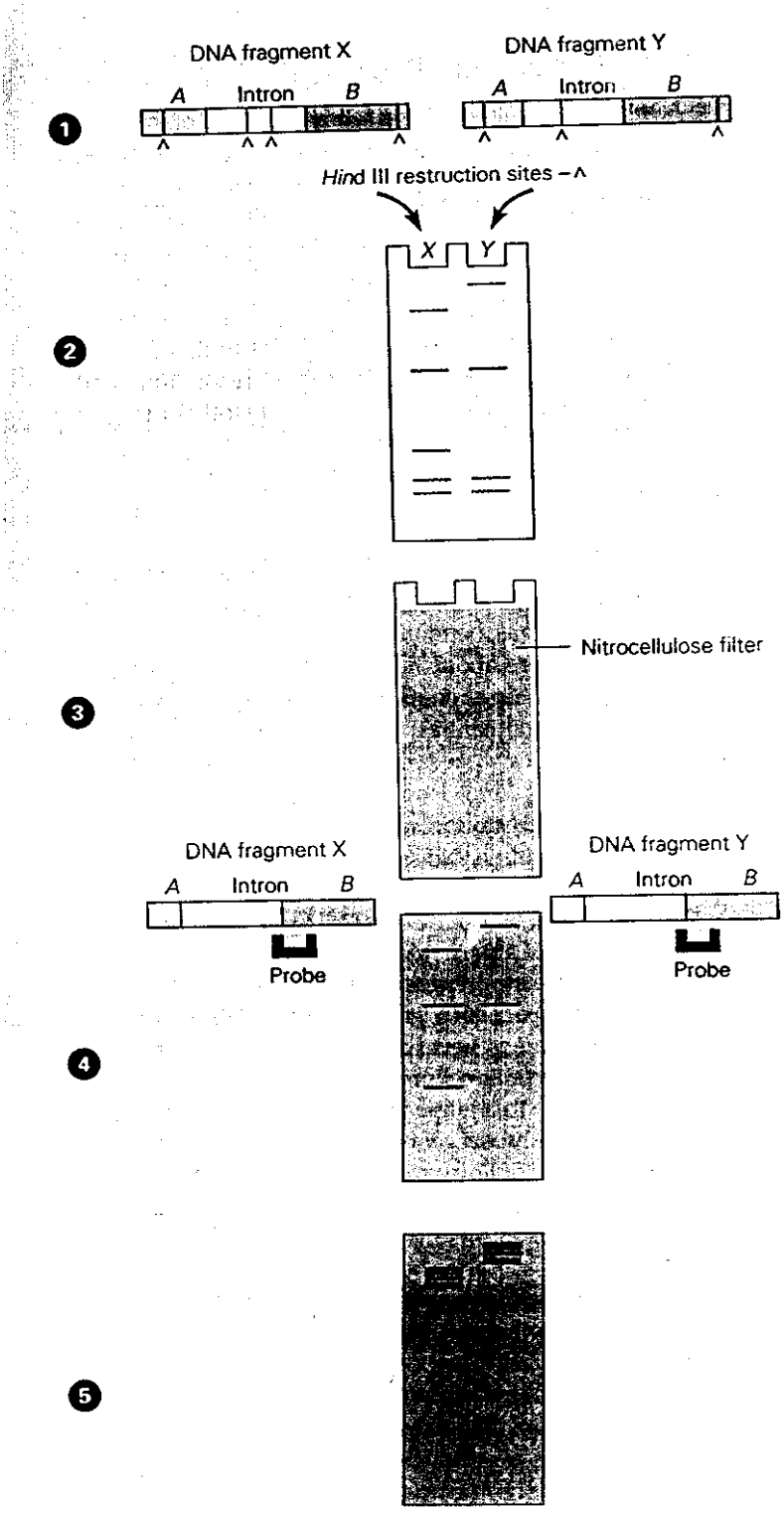
DNA fragment X
A
Intron
B
DNA fragment Y
A
Intron
B
1
Hind III restruction sites
X
Y
2
Nitrocellulose filter
3
DNA fragment X
A
Intron
B
Probe
DNA fragment Y
A
Intron
B
Probe
4
5

Fig. 1.

DNA is isolated from both individuals, and specific fragments are separated and then digested with **Hind III**. The resulting fragments are separated by gel electrophoresis and subsequently transferred onto a nitro-cellulose filter. The DNA bands are not actually visible. After the DNA on the gel is denatured (strands separated) by chemical treatment, the single strands are transferred onto the filter by Southern blotting method (explained in Unit-III). A probe that is complementary to the left side of the gene 'B' is hybridized to the DNA on the filter. After the probe is hybridized, the excess is rinsed away and a photographic film is laid on the filter on which, the radio activity of the hybridized probe forms an image corresponding to the DNA bands containing the left side of gene 'B'. The probe hybridizes on a small fragment containing the left side of gene B plus a small part of the in between region in individual X. The probe hybridizes on a large fragment containing the left side of gene B plus a large part of the in between region in individual Y. Hence there is a difference in the length of the hybridized fragments generated by the restriction enzyme digestion in the two individuals which is referred to as the restriction fragment length polymorphism. These RFLP markers are heritable and can be passed from one generation to the next. The utility of RFLP has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. The requirement of labelled isotopes makes the analysis expensive and the whole assay is time-consuming and labour intensive. The led to the development of PCR-based marker systems.

1.4 RANDOM BY AMPLIFIED POLYMORHPIC DNA (RAPD)

This PCR-based genetic marker assay developed by Welsh and McClelland in 1991 detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these two primary sites are within a short distance of each other, a discrete DNA product is formed by thermocyclic amplification of this region (Fig. 2). Each primer directs amplification of several discrete loci in the genome, thus making it easy for comparison or different genomes. It is however important to optimize reaction conditions for reproducible results. RAPD assay has been used as efficient tools for identification of markers linked to agronomically important tracts which are introgressed during plant breeding crosses. RAPD analysis has been used to screen variability and for individual-specific genotyping but is hampered with problems such as poor reproducibility and difficulty in scoring bands due to the faint or fuzzy products.

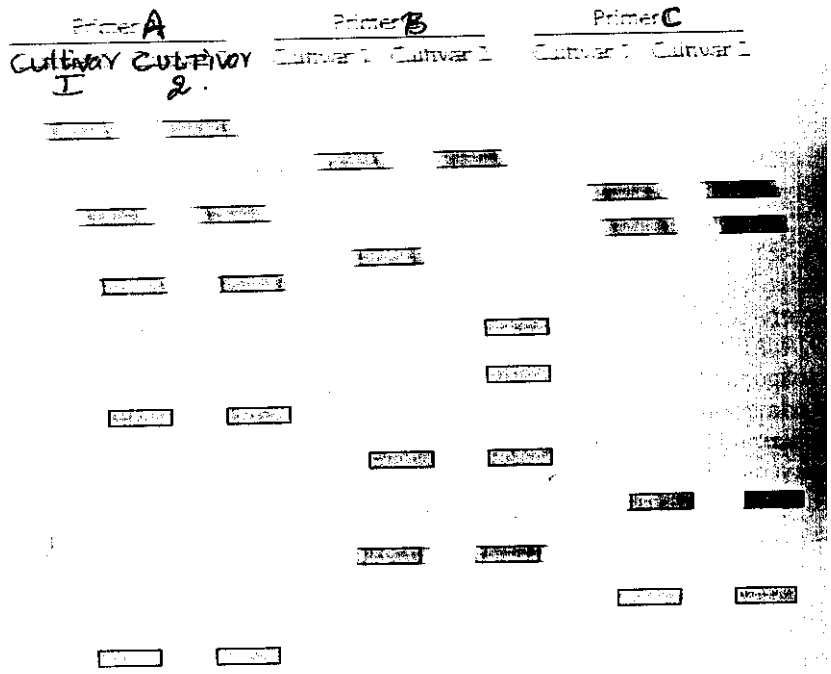

Fig. 2

Some variations in the RAPD technique used of late are the

a) DNA amplification finger printing(DAF).
b) Arbitrary primed polymerase chain reaction (AP-PCR).
c) Sequence characterized amplified regions for amplification of specific band (SCAR).
d) Cleaned amplified polymorphic sequences (CAPS).
e) Randomly amplified micro satellite polymorphisms (RAMPO).
f) Amplified fragment length polymorphism (AFLP).

Of all the recent variations listed above, the most recent and popular approach is the AFLP technique. The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The finger prints are produced without any prior knowledge of sequence, using a limited set of generic primers. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection or polymorphism between closely related genotypes.

1.4 OTHER METHODS

Apart from the AFLP method, other methods have been developed by conversion of RFLP markers to PCR-based markers. They are:

a) Sequence tagged sites (STS).

b) Expressed sequence tag markers (EST).

c) Use of multilocus probes to hybridize with Repetitive DNA sequences. These can exist as Micro Satellites and Mini Satellites. Other types are the simple sequence repeats (SSRs) and Alu-repeats. Transposable elements (repetitive elements) have also been exploited as molecular markers.

1.5 APPLICATIONS OF MOLECULAR MARKERS IN CROP IMPROVEMENT

Molecular markers have been used as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They are also being used for phylogenetic analysis. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to store it.

Genome maps were constructed in maize, rice, *Arabidopsis* etc using RFLP markers. Later, maps of Potato, barley, banana and other Brassicaceae members were constructed. Once the maps were available several molecular markers were used to saturate them. Using this method, several micro satellites have been assigned to five linkage groups in *Arabidopsis*. Once these markers are mapped, they are efficiently employed in tagging several individual traits like yield, disease resistance, stress tolerance, seed quality etc. Several such monogenic and polygenic loci have been identified in a number of plants which are currently being used by breeders and molecular biologists for crop improvement RFLR markers have proved their importance as markers for gene tagging and are very useful in locating and manipulating quantitative trait loci (QTLs) in a number of crops. The RFLP markers have been used to detect gene introgression in a back cross breeding programme and synteny mapping among closely related species.

Molecular markers are extensively being used for reconstructing phylogenies of various species. These studies provide information regarding the time-scale on which closely related species have diverged from a single species. These phylogenetic studies also provide information about the pattern of genetic variation within species. One of the most recent applications has been shown in sex identification in dioecious species of plants, wherein a micro satellite probe (GATA) is found to reveal sex-specific differences in Southern analysis and can be used as a diagnostic marker to screen at young seedling stage itself.

Extensive research using DNA markers is in progress in many institutions all over India. Several genes have been identified so far. A Rockefeller Foundation assisted EST based marker assisted mapping programme is ongoing in rice at University of Hyderabad. Early identification of sex in dioecious *papaya* using molecular marker is an achievement of National Chemical Laboratory in Pune. Efforts are underway in the use of molecular markers in several crop plants and horticultural plants.

1.6 SUMMARY

DNA markers are actually simply detected differences in the genetic information carried by two or more individuals. Information from molecular markers serves many purposes such as forensic investigations, paternity tests, identification of genes responsible for genetic diseases and phylogenetic studies to infer evolutionary relationships among related species.

Molecular markers are used to map the position of a gene on a worked out molecular genetic map. The molecular markers are basically of two types: the hybridization-based markers e.g. the RFLP markers and the PCR-based markers e.g. the RAPD technique especially the latest method of AFLP markers. These are several other types of markers based on the repetitive DNA sequences like the micro satellite markers and SSRs, besides the transposable element repetitive sequences.

Molecular markers have been used as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They are also being used for phylogenetic analysis and to map genomes of plants to generate vast data for construction of databases. RFLP markers have been used to tag quantitative trait loci (QTLs) in several crop plants. Extensive research using DNA markers is in progress in many institution of India.

1.7 TECHNICAL TERMS

RFLP: Restriction Fragment length polymorphism is a difference in restriction fragment length between very similar DNA molecules: RFLP analysis is used to detect relatively small differences between DNA molecules; used in DNA typing and genetic disease detection. The differences in DNA lengths are due to the presence or absence of a specific restriction endonuclease recognition site detected by DNA hybridization with DNA probes (Southern hybridization).

RAPD: Randomly amplified polymorphic DNA. RAPD is a diagnostic procedure in which chromosomal DNA (usually from plants but sometimes from micro organisms or animals) is characterized by the DNA fragments that are synthesized when PCR is initiated after the addition of a single primer to the reaction mixture.

1.8 MODEL QUESTIONS

a) What is a molecular marker? Describe the different molecular markers being used by Scientists.
b) What is a RFLP? Describe the technique of RFLP analysis.
c) What is RAPD procedure? How is it used to characterize plant cultivars?
d) Describe how AFLP is an extension of RAPD.
e) Write an essay on the use of molecular markers for crop improvement.

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M.Sc. BOTANY (Final)

Unit-IV

Lesson 3

Paper-VIII : PLANT BIOTECHNOLOGY

Direct gene transfer methods

3.0 OBJECTIVE
To provide basic technical description of the subject of Direct technical gene transfer.
3.1 INTRODUCTION
3.2 METHODS OF DIRECT GENE TRANSFER
3.2.1 Chemical methods
3.2.2 Electroporation
3.2.3 Microinjection
3.2.4 Fibre-mediated DNA delivery
3.2.5 Laser-induced DNA delivery
3.2.6 Pollen Transformation
3.2.7 Macroinjection
3.2.8 Microprojectile bombardment
3.2.9 Integration of transformed genes
3.3 SUMMARY
3.4 TECHNICAL TERMS
3.5 MODEL QUESTIONS
3.6 REFERENCE BOOKS

3.1 INTRODUCTION

Conventional plant breeding techniques have contributed to crop improvement by producing increased yields, resistance to disease and enhanced nutritional value foods. Recombinant DNA technology is also an important modern tool for the direct genetic manipulation of plants. Several DNA-delivery systems and expression vectors have been used for transfer of desirable genes to crop plants, horticultural plants and other economically important plants. The use of the natural genetic engineer, *Agrobacterium* for gene transfer was explained in Lesson-2. Here, the direct gene transfer methods are explained with special emphasis on the micro projectile bombardment method.

Although earlier reports indicated that monocotyledons could not be transformed with the *Agrobacterium* method, there are several recent reports of successful genetic transformation in several cereals including rice, wheat and corn. Due to the initial inefficient transformation of mono cotyledons with *Agrobacterium* infection; several direct methods of gene transfer were developed with high rates of success. The spontaneous uptake of DNA by plant cells is quite low. Therefore, different physical and chemical treatments are employed to facilitate the entry of DNA into plant cells. The gene construct need not be specific as in the *Agrobacterium* method (i.e. Ti-plasmid) and it may range from plasmid or cosmid vectors, bacterio phages or even in yeast artificial chromosomes. The various methods of direct gene transfer are explained below.

3.2 METHODS OF DIRECT GENE TRANSFER

i. Chemical methods,
ii. Electroporation,
iii. Microinjection,
iv. Fibre-mediated DNA delivery,
v. Laser-induced DNA delivery,
vi. Pollen transformation,
vii. Macroinjection,
viii. Micro projectile bombardment,
ix. Integration of transformed genes.

3.2.1 Chemical methods

This method is useful for gene transfer to protoplasts which need to be cultured with care of regeneration of transgenic plants. Certain chemicals e.g. Polyethylene glycol (PEG), Poly vinyl alcohol and calcium phosphate are added to the protoplast suspension to enhance the uptake of linearized plasmid gene construct. A heat-shock of 45^{o}C for 5min is given to the mixture to facilitate the gene transfer (transformation). A selectable marker gene can be included in the gene construct so that the transformed cells can be selected by culture on a culture medium to which the marker is added.

3.2.2 Electroporation

This method also uses the gene transfer technique through protoplasts. Plant protoplasts are suspended in suitable isotonic liquid culture medium to which, the linearized plasmid DNA is added and subjected to electric pulses for the desired duration. Either low-voltage is used for long duration (300 to 400 V cm^{-1} for 10-50 milliseconds) or high-voltage is used for short pulses (1000 to 1500 V cm^{-1} for 10ms (micro seconds) (Fig. 3.1). The protoplasts take up the DNA through the pores in the membrane (created due to electric pulses) after which they are cultured on culture medium containing the selection agent to selectively and thereby regenerate transgenic plants.

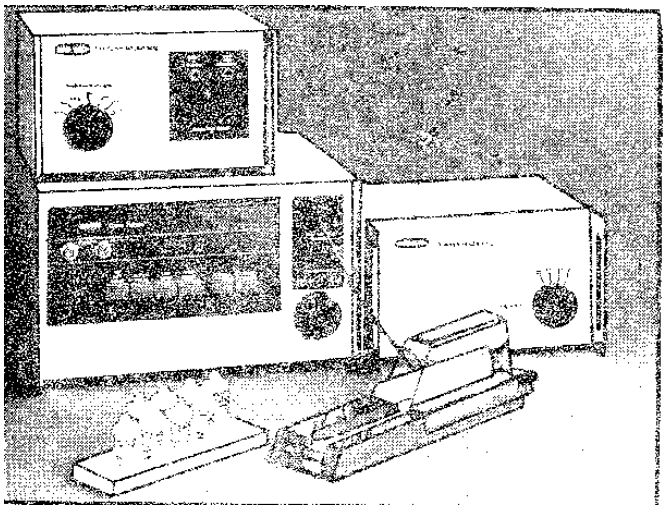

Fig. 3.1 An electroporation system, suitable for transformation of bacteria, mammalian cells and plant cells

3.2.3 Microinjection

This method is also used as the transfer method of DNA to protoplasts. The DNA solution is injected directly inside the protoplast using capillary glass micropipettes with the help of micro manipulators of a micro injection assembly (Fig. 3.2). The protoplasts are immobilized in agarose or are held under section by a micropipette. The procedure is very slow and yields very few transformed plants after successful culture on selection.

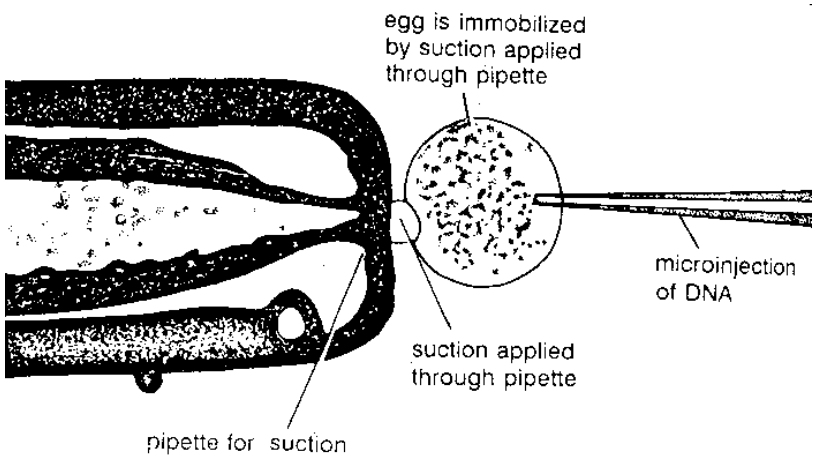

Fig. 3.2 Microinjection of foreign DNA into a fertilized egg for production transgenic animals

3.2.4 Fibre-mediated DNA delivery

This method is used to transfer DNA directly into cells through Silicon carbide fibres of 0.6μm diameter and 10 to 80 μm length. The mixture containing the cell suspension, Silicon carbide fibres and DNA is vortexed to yield transformed cells which could be successfully cultured on selection. However, only transient expression was observed with rare occurrence of stable transformation.

3.2.5 Laser-induced DNA delivery

This method has been used for transformation of animal cells by the Lasers puncturing temporary holes in the cell membranes. Lasers have been in use to transform plant cells only recently and information regarding stable transformation is not yet available.

3.2.6 Pollen transformation

This method uses pollen grains for transformation of foreign DNA. Pollen soaked in DNA solution is then used to pollinate to yield transformed seeds. Reports of stable transformation have not yet been published so far.

3.2.7 Macro injection

This method uses injection of DNA solution (by a hypodermic syringe) into inflorescences. A low frequency of transformation has been reported, but stable transformation has not yet been reported so far.

3.2.8 Micro projectile bombardment

This is the most successful of all the techniques of direct gene transfer and it has become a very popular method for transformation of both dicotyledonous and monocotyledonous plants in view of innumerable reports of stable transformation. This method uses tiny (1-2 μm) tungsten or gold particles coated with the gene construct or DNA, which are shot into plant tissue with suitable velocities to enable their entry into plant cells (into nuclei). The particles are accelerated by the use of helium gas under pressure. The method is also called as the biolistic method of gene transfer. This method is used to transform shoot apical meristems, leaf blades, immature and mature embryos (both somatic and zygotic), mature pollen, root and shoot sections etc. Meristematic cells are generally targeted to achieve a very high transformation frequency. The main components of the micro projectile gun (Fig. 3.3) are the gas acceleration tube, rupture disc, stopping screen, macro carrier (membrane) which carries the micro projectile metal particles that are coated with DNA and the target tissue into which the micro projectiles are shot. The macro projectile is stopped by a stopping screen and the micro projectiles pass through the mesh on the screen to get embedded in the target tissue under suitable helium pressure of 1000 psi approx. The plasmid DNA construct is precipitated on the micro projectiles by use of calcium chloride and spermidine.

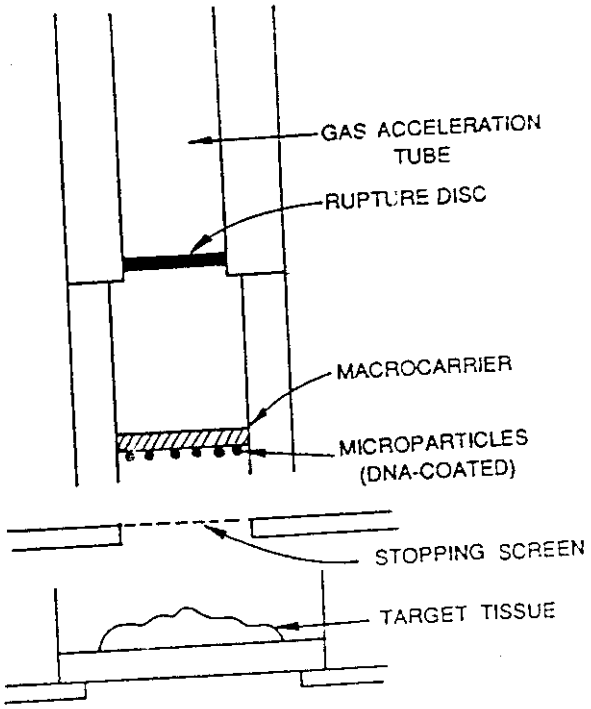

Fig. 3.3 A simplified representation of those components of a helium pressure pesticide gun device which are involved in DNA delivery. The microprojectiles are coated with the DNA to be delivered in the target tissue.

Micro projectile bombardment has been used successfully in several crop plants to recover transgenic plants by the culture of bombarded tissue on selection. Micro projectile method has also been used to transform chloroplast DNA genome by the use of specific markers that can be expressed only in the chloroplast.

3.2.9 Integration of transformed genes

Genetic transformation generally occurs with low frequency since a major part of the introduced (bombarded) DNA gets degraded in the target cells and only a small portion of it gets integrated into the plant genome. The foreign DNA construct may become rearranged, deleted or become joined end to end in a repetitive sequence. Multicopy integrations occur in tandem at one site. The integration frequency can be increased by using higher DNA concentrations, but this can also result in increased number of gene copies. A lower DNA concentration will result in single copy of the gene integration which is therefore preferable.

The stably integrated transgenes are inherited by later generations of transgenic plants in a Mendelian Fashion. However, the inherited transgenes may also become non-functional in subsequent generation due to methylation or rearrangements in the transgene. Molecular confirmation of the integration, presence and functionality of the transgene of every generation is therefore essential (by carrying out PCR, Southern and Western blots etc.), along with the assays for the expression of the gene product and effectiveness of the transgenic plant's inherited trait.

3.3 SUMMARY

Recombinant DNA technology is an important modern tool for the direct genetic manipulation of plants to contribute to crop improvement. Apart from the *Agrobacterium*-base method of gene transfer (explained in Lesson-2), there are several direct gene transfer methods that have been used to produce transgenic plants. The direct gene transfer methods include chemical methods, electroporation, micro injection, fibre-mediated DNA delivery, laser-induced DNA delivery, pollen transformation, macro injection and micro projectile bombardment. Of all these methods, the most successful and popular direct gene transfer method is the micro projectile gun method that bombards the gene construct-coated micro projectiles (tungsten or gold particles of 1-2μm) into target tissue comprising mostly meristematic cells. The transformed tissue is cultured on selection medium containing the selection component in relation to the marker gene that is adjacent to the main gene that is transferred.

The transgene is inherited by later generations of transgenic plants in a Mendelian Fashion. Proper molecular analysis (PCR, Southern and Western blots etc.) is essential for confirmation of transgenics at every level. Proper bioassays are also essential.

3.4 TECHNICAL TERMS

Gene construct: A vector in which the genes to be transferred are integrated for use in genetic transformation.

Direct-gene transfer: Transfer of gene by using methods other than the Agrobacterium based method.

Micro projectile gun: A device driven by helium gas that bombards DNA coated micro-projectiles (tungsten or gold particles of 1-2μm) into target tissue to recover transformed plants.

Molecular analysis: Comprises a series of tests for confirming the presence, integration and functionality of a gene (such as PCR, Southern and Western blots).

3.5 MODEL QUESTIONS

a) What is Genetic transformation?

b) Describe briefly, the different methods of direct-gene transfer.

c) Explain the apparatus and working principle of the micro projectile gun.

d) Why are molecular tests performed on the transformed tissue and plants?

e) How is a transgene integrated? How are transgenes inherited?

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M.Sc. BOTANY (Final)

Paper-VIII : PLANT BIOTECHNOLOGY

Unit-I

Lesson 4

Meristem Culture and Embryo Culture

4.0 OBJECTIVES:
The importance of meristem and embryo culture, culture media and the uses of the above methods are described here.

4.1 INTRODUCTION

4.2 TYPES OF MERITSTEM

4.3 STRUCTURE OF MERISTEM

4.4 VIRUS INDEXING

4.5 CULTURE MEDIUM

4.6 APPLICATIONS

4.7 MATURE EMBRYO CULTURE

4.8 IMMATURE EMBRYO CULTURE or EMBRYO RESCUE

4.9 NUTRIENT MEDIUM

4.10 SUMMARY

4.11 MODEL QUESTIONS

4.12 REFERENCE BOOKS

4.1 INTRODUCTION

In many plant breeding experiments, the hybrid plants produce abortive or non viable seeds and hence the mature hybrid plants can not be produced. The shoot tip or meristem from such hybrids plants can be cultured to speed up the breeding program.

In flowering plants, embryo is a post fertilization product and it gives rise to the sporophyte. It is a bipolar structure having one meristem at each pole, which produce the shoot primordium or plumule (in dicots) at one pole and the root primordium or radicle (in monocots) at the other pole and one or two lateral appendages.

Hannig (1904) obtained viable plants from isolated embryo cultures of *Cochleria* and Raphanus. Dietrich cultured both immature and mature embryos of different plants and observed that mature embryos showed normal growth. The immature embryos directly produced the seedlings bye-passing the normal stages of embryogenesis and dormancy condition of the embryo.

The embryos whether fully formed or premature do not show normal growth and produce the seedling due to various causes and can be isolated aseptically and cultured on nutrient media to produce the seedlings from which plants can be regenerated.

4.2 TYPES OF MERISTEMS

Two types of mertistems occur in plants i.e., the apical meristems which occur at the tips of stems and roots and (ii) the intercalary meristems occurring between the xylem and phloem of vascular bundles, in the cortex and in grasses at the bases of internodes and leaves.

The technique of meristem culture was introduced by Morel and Martin (1952 for *in vitro* virus iradication in Dahlia, while Georges Morle (1965)applied shoot tip culture in an orchid *Cymbidium* for micro propagation *in vitro*.

4.3 STRUCTURE OF MERISTEM

Meristem is an undifferentiated but determined tissue whose cells divide and differentiate into specialized tissues or organs such as shoots and roots. In general the meristems occur at the tips of the stems and roots which are called the apical meristems.

The apical meristem is a dome shaped tissue located at the tips of shoots measuring about 0.25 to 0.3 mm in length. This is surrounded by one or two leaf primordia which constitutes the shoot apex. The meristem is to be cut below the tip of the dome, explant is removed and transferred to the medium. For production of virus free plants, the meristem to be cultured should be 0.2 to 0.5 mm long.

The success of shoot tip culture depends on the season and actively growing shoot tips are that selected as explants. In plants showing dormancy, the explants should be cultured at the end of dormancy period and actively growing shoot tips are selected as explants.

4.4 VIRUS INDEXING

Testing plant for the presence or absence of virus is called virus indexing. Every meristem tip or calls-derived plant must be tested before using it as a mother plant to produce virus-free stock. Following methods have been adopted for virus indexing:

(i) Sap transmission test: Leaves are taken from the test plant and grind in an equal volume of buffer solution (0.1 mol l^{-1} sodium phosphate) using a mortar and pestle. The filter-leaf extract is then surcared onto the leaf of an **indicator** plant predusted with 600-grade carborandum powder. If the indicator plant develops the characteristic symptoms, that shows the presence of virus in the sap. Some of the indicator plants used are *Chenopodium amaranticolor* and *Gomphrene* globosa.

(ii) Serology: The enzyme-linked immunosorbant assay (ELISA) is one of the serological methods used to identify virus based on antibody reaction. This test is simply performed by adding a drop of centrifuged sap from the test plant to a drop of antiserum taken from the blood of a rabbit. If the virus is present, the precipitation will take place due to the presence of specific antibodies in the blood.

(iii) Electron Microscopic studies: EM studies have been conducted for identifying latent viruses (viruses do not exhibit visible symptoms).

4.5 CULTURE MEDIUM

Murashige and Skoog's medium, Whites as well as Gautheret's media are used. The growth regulators added to the medium vary from species to species. For plant species possessing phenolic compounds (i) PVP, Ascorbic acid, or citric acid are to be added to the medium or the explants are to be presoaked in these antioxidant solutions before culturing. (ii) the primary cultures are to be incubated in darkness to avoid the oxidation of phenolic compounds. (iii) The explants are to be frequently transferred into fresh medium when the medium turns brown in color.

4.6 APPLICATIONS

(1) Virus eradication - Meristem culture finds its wider application in the large scale production of horticultural plants especially in the cut flower industry, where the stock plants of commercial value are to be maintained in virus free state.

(2) Shoot tip or meristem culture can be used for micro propagation of many plants.

(3) In some plants, seeds produced are heterozygous or they are recalcitrant and seed material cannot be used as the genetic resource for future use.

Hence, *in vitro* culture of meristems from such plants can serve this purpose. Culturing of isolated embryos help in contributing our knowledge on plant development factors such as (i) factors regulating the growth of primordial organs of the seedlings, (ii) different aspects of dormancy and germination, (iii) nutritional requirements of the embryo, (iv) the role of tissue surrounding the embryo, and (v) the effects of nutrients in the medium on embryo development.

4.7 MATURE EMBRYO CULTURE

In certain cases, mature embryos from ripe seeds under normal conditions do not germinate immediately but become dormant or do not survive. When these embryos are excised from the seed coat and cultured on nutrient medium, they germinate and produce the seedling. Mature embryos grow on simple inorganic medium having energy source and develop into plants.

4.8 IMMATURE EMBRYO CULTURE OR EMBRYO RESCUE

In inter-specific or inter-generic crosses, the embryo produced become abortive mainly due to post fertilization barriers. In some cases pre fertilization barriers like incompatibility between pollen and stigma operate which hinder the pollen germination at the post zygotic level due to incompatibility between embryo and endosperm, the toxins produced by endosperm kill the embryo. In such cases embryos are removed and are grown *in vitro* to raise such hybrid plant.

4.9 NUTRIENT MEDIUM

The medium used is important for embryo culture. It is the standard basal medium supplemented with major salts and trace elements. While mature embryos can be grown on basal medium with carbon source like sucrose, while immature ones need vitamins, amino acids, hormones and high sucrose content. After the embryo ceases to grow, it should be transferred to a medium containing sucrose, low auxin and moderate cytokinin content, whereby the embryo regenerates shoots. In some cases the regeneration is achieved through callus formation and somatic embryogenesis.

4.10 SUMMARY

Meristem is an undifferentiated tissue, localized at the tips of stem and roots of plants and used mainly for colonal propagation under *in vitro* conditions. The technique is highly useful for virus eradication and production of progenies from intergeneric or inter specific hybrids, when they cannot produce viable seeds (micro propagation). Two types of Meristems viz., the apical and intercalary occur in the plants. Meristem or shoot tip culture is practiced in horticultural plants and agricultural crops.

Embryo culture requires the correct selection of the nutrient medium for the continued growth of the embryo and the formulation of the medium may vary depending on the plant species used. In general, the embryos are not induced to form callus in culture but are allowed to form plants.

4.11 MODEL QUESTIONS

1. Give an account of meristem culture
2. Explain the structure of meristem, types of meristem and applications of meristem culture.
3. Mention the different types of embryo cultures used and mention their objectives.
4. Describe the principles and applications of Embryo culture techniques.

4.12 REFERENCE BOOKS

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