



# AN INTRODUCTION TO PLANT TISSUE CULTURE

KALYAN KUMAR DE

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## Chapter One

# Laboratory Organization and Techniques in Plant Tissue Culture

### INTRODUCTION

Plant tissue culture is not a separate branch of plant science like taxonomy, cytology, plant physiology etc. Rather it is a collection of experimental methods of growing large number of isolated cells or tissues under sterile and controlled conditions. The cells or tissues are obtained from any part of the plant like stem, root, leaf etc. which are encouraged to produce more cells in culture and to express their totipotency (i.e. their genetic ability to produce more plants). Cells or tissues are grown in different types of glass vials containing a medium with mineral nutrients, vitamins and phytohormones. Therefore, to carry out the experiments using tissue culture techniques, a well-equipped laboratory is first required.

In recent years there has been a large increase in the number of research laboratories using tissue culture techniques to investigate many fundamental and applied aspects of higher plants. However, the use of these techniques is not confined to research alone. Tissue culture techniques are being exploited by many commercial laboratories. Even many horticultural

companies are setting up small units to multiply plants which are difficult to propagate by conventional means.

In this chapter, the general organization of a tissue culture laboratory and the basic techniques will be discussed under different subheadings.

### TISSUE CULTURE LABORATORY

An ideal tissue culture laboratory should have at least two big rooms and a small room. One big room is for general laboratory work such as preparation of media, autoclaving, distillation of water etc. The other big room is for keeping cultures under controlled light, temperature and humidity. The small room is for aseptic work and for keeping autoclaved articles.

### GENERAL LABORATORY

The general laboratory for tissue culture should be provided with the following articles and arrangements—

## A Washing Area

This is very important for a tissue culture laboratory. It should be provided with a large sink, running hot and cold tap water, brushes of various sizes, detergent and a bucket of single distilled water for a final rinse of the washed glass goods. A number of plastic buckets are required for soaking the glass goods to be washed. Another separate bucket with lid is also required for disposing off the used or infected media before cleaning. Only this bucket should be kept outside the room or cleaning area and should be cleaned twice in a week.

## Hot Air Oven

It is necessary for drying the washed glass goods. For this purpose, a number of enamelled trays of different sizes are required for keeping wet glass goods inside the oven.

## Refrigerator

It is essential for storing various thermolabile chemicals like vitamins, hormones, amino

acids, casein-hydrolysate, yeast extract, coconut milk, etc. Stock solutions of salts are also kept to prevent contamination.

## Distillation Plant

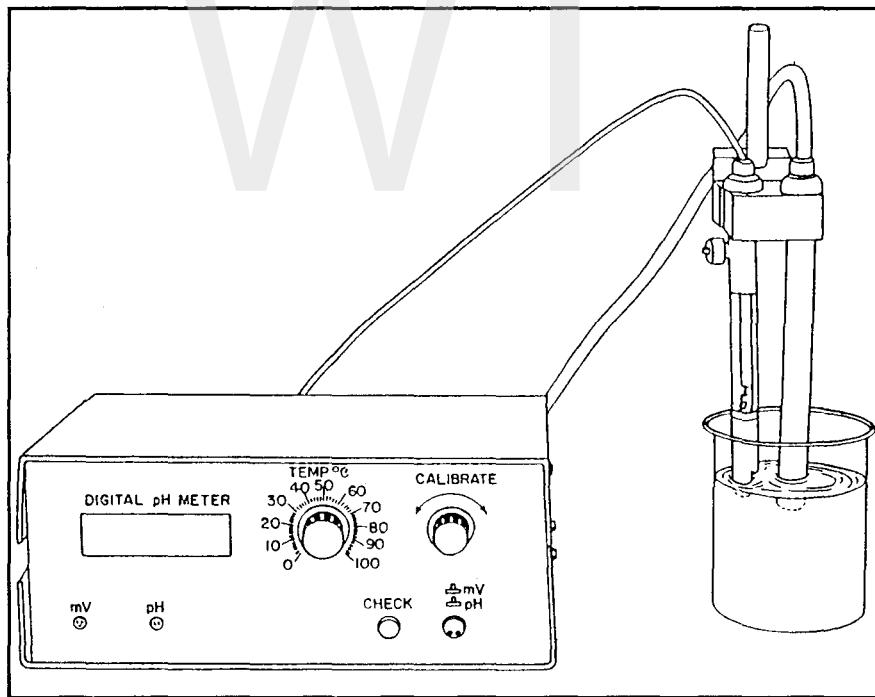
A single distillation and a double distillation water plant are indispensable. Two big plastic containers are required for storing the distilled water.

## Weighing Balance

Three types of weighing balances viz. pan balance, chemical balance and electric balance are required for weighing chemicals, sugars, agar-agar and others.

## pH Meter

It is necessary for the measurement and adjustment of pH of the nutrient medium (Fig 1.1).



□ Fig 1.1

A digital pH meter

## Vacuum Pump

It is required for filtering liquid media, sugar solution etc. through filter apparatus using air suction.

## Autoclave

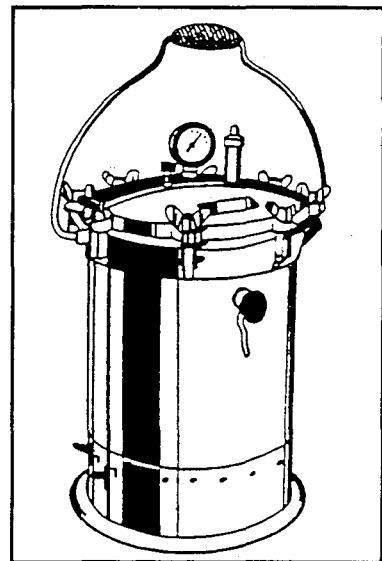
It is very important for sterilization of nutrient media, glass goods, instruments, etc. (Fig 1.2).

## Working Tables

These are necessary for preparation of medium.

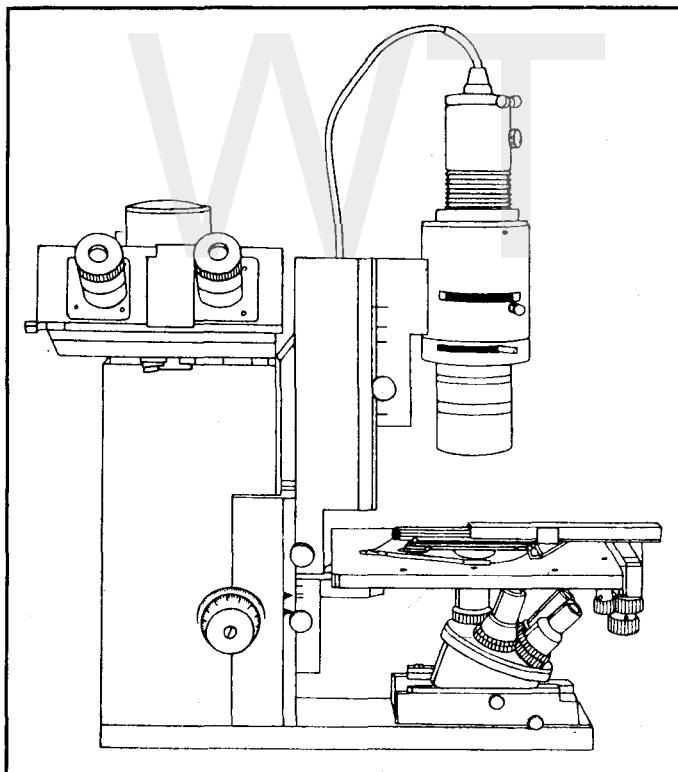
## Heater

It is needed for heating or warming the medium to dissolve agar or to melt the agarified medium.



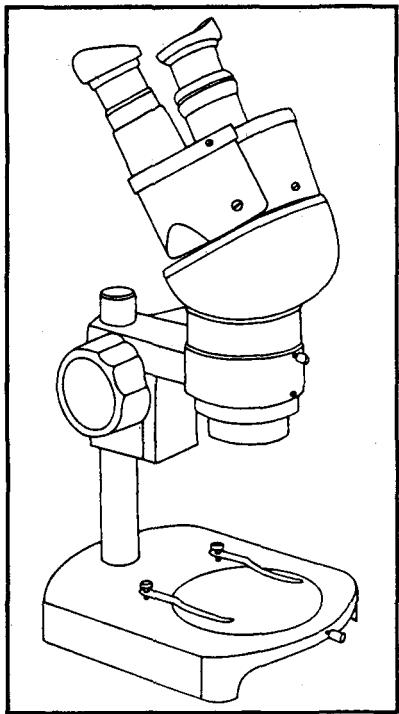
□ Fig 1.2

A simple portable autoclave



□ Fig 1.3

An inverted microscope for the observation of living cell and tissue cultures during experiments



□ Fig 1.4

#### A stereoscopic dissecting microscope

#### Microscope

Simple, compound, inverted binocular dissection microscopes are essential for various purposes. Some of the microscopes (Figs 1.3 and 1.4) should be fitted with a camera for taking photomicrograph.

#### Microtome

It is needed for sectioning the cultured tissue.

#### Wooden Racks

These are required for keeping the various chemicals.

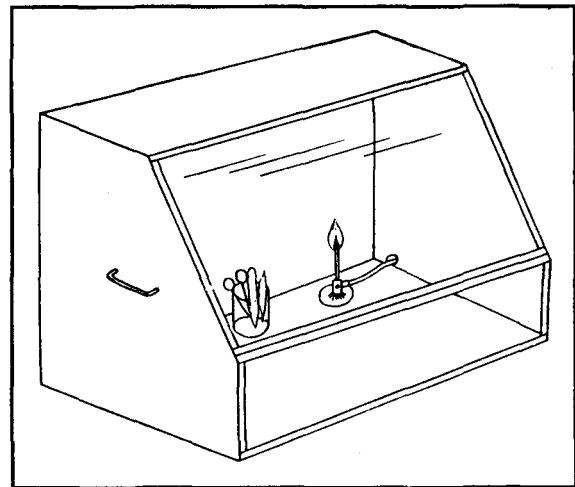
### LABORATORY FOR ASEPTIC INOCULATION

This room should be without any window or ventilator in order to make it dust-free. The room should be provided with double doors. The

doors should have a automatic door closer. Inside floor should be fitted with a rubber mat to facilitate cleaning. For entering into the room, shoes should be left outside. For aseptic work, a large wooden chamber ( $Ca, 4' \times 4' \times 7'$ ) is made for short term work. Upper half of the side walls of the chamber is made of large glass sheets. The chamber should also have double doors provided with a door closer. The chamber is provided with two UV (one small, one big) sterilizing lamps and a fluorescent lamp. The switches to operate them are present outside the chamber so that the lamps can be safely switched on and off. Inside the chamber the working table and shelves are made of thick glass sheets.

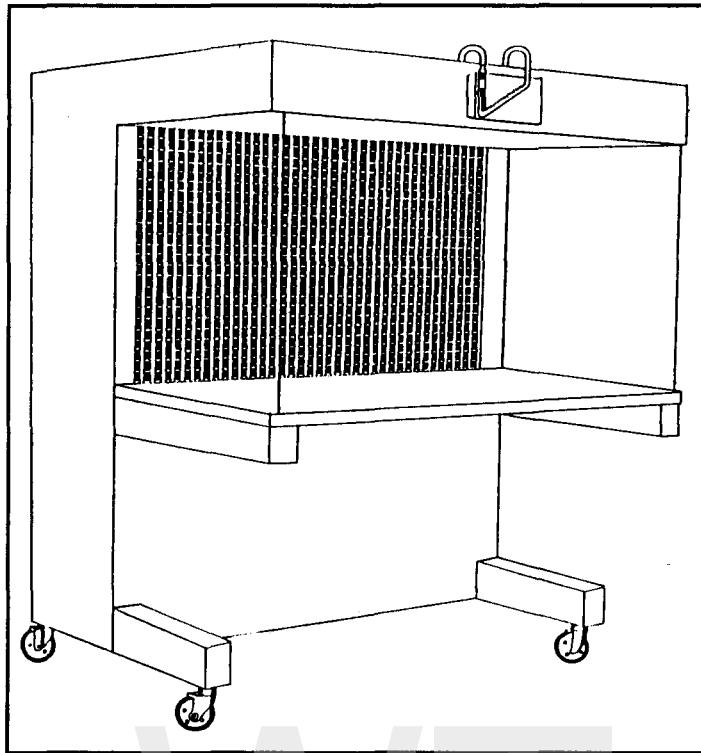
For simple routine work such as aseptic seed germination, harvesting of cultured tissue from the aseptic stock for cytological work or for microtome preparations, a small inoculating hood may be used. This can be placed on a small table at the convenient corner of the room. The figure of an ideal chamber is given here (Fig 1.5).

Laminar air flow cabinet (Fig 1.6) is the most suitable, convenient and reliable instrument for aseptic work. It allows one to work for a longer period which is not possible inside the inoculation chamber. Long hours of work inside the inoculation chamber may also cause suffocation and needs the interruption of work.



□ Fig 1.5

#### Design for a simple inoculating hood



□ Fig 1.6

#### A Laminar air flow

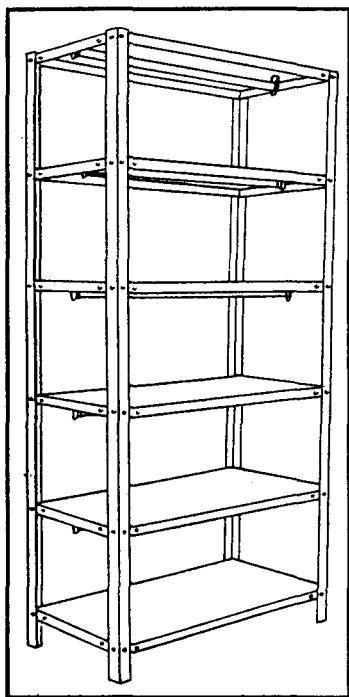
One can work openly and easily for a longer period on the table of laminar air flow.

Laminar air flow has a number of small blower motors to blow air which passes through a number of HEPA (high efficiency particulate air) filters. Such filters remove particles larger than  $0.3 \mu\text{m}$ . The ultraclean air which is free from fungal and bacterial contaminants, flows at the velocity of about  $27 \pm 3\text{m}/\text{minute}$  through the working area. All contaminants are blown away by the ultraclean air and thereby an aseptic environment is maintained over the working area. Before starting work, laminar air flow is put on for 10-15 minutes. The flow of air does not put out the flame of a spirit lamp. Therefore, a spirit lamp can be used conveniently during the work.

#### CULTURE ROOM

The culture room means the room for keeping or incubating the culture under controlled

temperature, light and humidity. The culture room is also fitted with double doors in order to make it dust free and to maintain a constant room temperature. One should enter the culture room keeping the shoes outside the door. To maintain the temperature around  $25 \pm 2^\circ\text{C}$  inside the culture room, air coolers are used. This room is also provided with specially designed shelves (Fig. 1.7) to keep culture vessels. The shelves are made of glass or plywood. Flask, bottles, jars, petriplates can be placed directly on the shelves. Culture tubes can be kept on a support such as empty paper cover of fluorescent lamps. Cultures can be grown in light or in dark. For light arrangement, each culture rack is provided with fluorescent lamps which are photoperiodically controlled by an automatic timer. Racks covered with black curtains are suitable for dark incubation of culture. A thermometer and a hygrometer are fixed on the wall at the safety corner of the room to check temperature and relative humidity respectively. The



□ Fig 1.7

#### Design for a skeleton rack for keeping culture vessels and incubation of culture

relative humidity of the culture room is maintained above 50%. Some small shelves are placed in the culture room for temporarily keeping the autoclaved articles and the culture vials containing the medium.

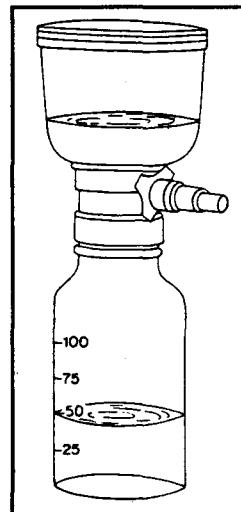
The culture room should also have a shaker for suspension culture or single cell culture in moving liquid medium. The speed of revolution of the shaker can be controlled. The shaker is also provided with light. The platform of the shaker is fitted with clips for holding conical flasks (150 ml to 200 ml).

### GLASS GOODS AND INSTRUMENTS

#### GLASS GOODS

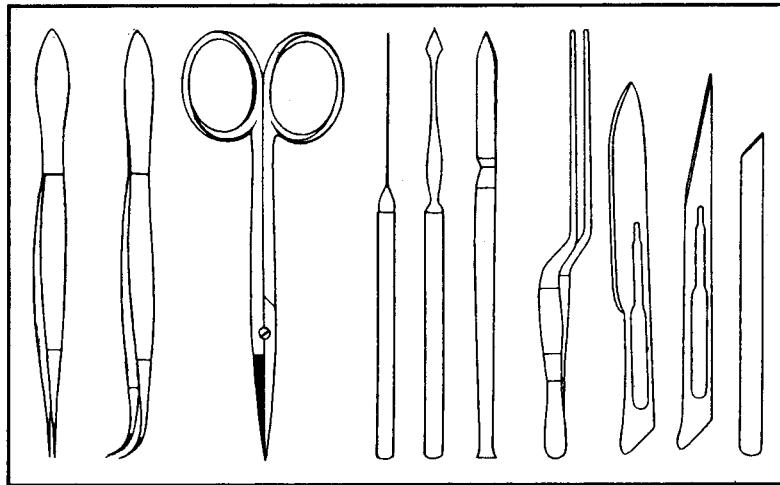
Different types of glass goods are used to culture plant tissues. The conventional and some specific glass goods are required for culture work. Glass goods should be of Corning or Pyrex or similar boro-silicate glass. Measuring cylinder,

conical flask, pipettes, beakers are required for preparation of media. Plant tissues are grown in wide-necked Erlenmeyer conical flask (100 ml, 150 ml, 250 ml etc.), culture tubes (25 mm in diameter and 150 mm in length), pretriplates (50, 90, 140 mm in diameter), screw-capped universal bottles (20 cm<sup>3</sup> capacity). Sometimes Jam bottle, milk bottle may also be used. Particular care must be taken to ensure that glass goods are properly cleaned before use. The traditional method of cleaning new or dirty glass goods is to soak these in soap water followed by brushing and washing well with tap water and finally rinsing with single distilled water. These are dried in the hot air oven and then the clean glass goods are stored in a dust-proof cupboard or drawer. In order to autoclave the culture medium and to culture the plant material, culture vessels particularly culture flasks and culture tubes must be fitted with cotton plugs which exclude microbial contaminants, yet allow free gas exchange. For this, tightly rolled plugs of non-absorbent cotton wrapped in gauge cloth may be used. When in position the exposed part of each plug and the rim of the culture vessel should be covered by brown paper or a cap of aluminium foil. This will keep the plug and vessel rim free from dust and will protect the plug from wetting during autoclaving.



□ Fig 1.8

#### A specially designed glass-made bacterial filtration system



□ Fig 1.9

#### A set of instruments used for tissue culture work

In some laboratories, pre-sterilized, disposable plastic wares are used in order to culture plant tissues. Some of these plastic wares are autoclavable.

For the sterilization of medium containing thermolabile compounds or enzymes for protoplast isolation a specially designed glass made bacterial filter (Fig 1.8) or an autoclavable plastic made bacterial filter is used.

A small spirit lamp made of glass will be required for the flame sterilization of instruments using methylated spirits.

#### INSTRUMENTS

Instruments routinely used for culture work include various sizes of scalpel and forceps, spatula, scissors, etc. (Fig 1.9). All instruments

Instruments	Measurement in length (cms)	Size No.	Use
1. Scalpel	20.32, 25.4 etc.	—	For cutting the plant material or explants
2. Surgical scalpel— (a) Holder	—	3, 4	—do—
(b) Disposable blade (arrow headed)	—	11	
3. Forceps (without corrugated marking at the pointed end)	20.32, 25.5	—	For holding the material
4. Jewellery fine forceps	—	5	For peeling leaf epidermis
5. Arrow headed sharp needle	25.4	—	For dissecting out anthers
6. Spatula (one end spoon headed and opposite end flat)	25.4	—	For transferring or subculturing the material
7. Metal cork borers (1 cm diameter)	—	—	For making tissue cylinder e.g., potato, carrot, etc.
8. Scissors	25.4	—	For cutting roots or shoots from aseptic culture

should be of stainless steel. A list of commonly used instruments, their measurement and uses are given in the previous page.

## CULTURE MEDIUM AND THE PREPARATION OF STOCK SOLUTION

### CULTURE MEDIUM

Excised plant tissues and organs will only grow *in vitro* on a suitable artificially prepared nutrient medium which is known as culture medium. From time to time, many workers (Murashige and Skoog, White, Gamborg, Nitsch and Nitsch, Schenk and Hildebrandt etc.) have proposed the composition of a nutrient medium for the growth of plant tissue (Table 1.1). But no single medium is capable of maintaining optimum growth of all plant tissues. Consequently the most suitable medium for a particular tissue must be determined by trial and error. Proposed composition of a culture medium has often been modified to stimulate the growth of a particular plant material.

A culture medium is composed of inorganic salts, an iron source, vitamins, amino acids, growth substance (hormones) and a carbohydrate supply. Inorganic salts are supplied in two groups—as macro-salts or nutrient and as micro-salts or nutrients. The salts needed in higher amounts are called macro-salts. These include nitrogen, phosphorus, sulphur, magnesium, calcium, potassium. The other essential inorganic salts needed in little amounts are called micronutrients (see Table 1.1). Nitrogen is mostly provided in two forms—as nitrates and as ammonium compounds. When nitrate is used alone, the pH of the medium drifts towards alkalinity, but adding ammonium compounds together with nitrate, checks the drift of pH. In most media, iron may be chelated as ferric-sodium ethylene-amine tetra-acetate (Fe-EDTA). In this state, iron is gradually released into the culture medium as it is utilized by the living cells. Vitamins used in culture media are mesoinositol, nicotinic acid, pyridoxine, thiamine etc. Carbohydrate is supplied usually as sucrose. The most commonly used amino acid is glycine. In addition, phytohormones (auxins and cytokinins) or

their synthetic counterparts are required either singly or in combination to initiate and maintain cell division. The concentration and ratio of hormones may vary from plant to plant and should be standardized for a particular plant tissue. Gibberellic acid ( $GA_3$ ) is rarely used in medium. Reports of the effect of  $GA_3$  in promoting cell growth in general are conflicting, although in some cases stimulation of cambial activity in shoot segments and cell division in cell suspension culture has been noted. The auxins that are commonly used in culture medium are IAA (indole-3-acetic acid), 2,4-D(2,4-dichlorophenoxyacetic acid), BTOA (2-benz-thizolyacetic acid), NAA ( $\alpha$ -naphthaleneacetic acid), IBA (3-indole butyric acid). The cytokinins are kinetin (6-furfurylaminopurine), 6-BAP (6, Benzylaminopurine), Zeatin, 2iPA (2, isopentenyladenine). The hormones are physiologically active in very small quantities.

The inorganic and organic chemicals used in the preparation of media should be analytical grades i.e. 'AR' (Analar or Analytical reagent) or GR (Guaranteed reagent).

Some plant tissues grow in the presence of complex additives such as coconut milk, casein hydrolysate, yeast extract, water melon juice, malt extract, potato extract, ripe tomato extract, orange juice etc. Yeast extract is a good source of organic nitrogen and vitamins. Casein hydrolysate is obtained from milk by removing the cream and acidifying the skimmed milk which causes casein to precipitate without any decomposition. It contains all the common amino acids. Potato extract, ripe tomato extract, orange juice and water melon juice contribute a number of essential nutrients and vitamins. Diphenylurea, a growth factor found in coconut milk, exhibits cytokinin-like responses. So, as a source of cytokinin, 10-15% v/v coconut milk is added to the medium.

On the basis of constituents, culture media are of two types—(i) chemically defined and (ii) chemically undefined. Chemically defined medium is a medium in which the composition and the concentration of all constituents are known. It is mainly prepared from inorganic

**Table 1.1 Chemical Composition of various nutrient media (all values expressed as mg/L)**

Constituents	Gautheret (1942)	Hildebrandt et al (1946)	Burkholder and Nickell (1949)	Nitsch (1951)	Heller (1953)	Reinert and White (1956)	Murashige and Skoog (1962)	White (1963)	Gamborg et al (1968)	Schenk and Hildebrandt (1972)
<b>Macro-nutrients</b>										
$(\text{NH}_4)_2\text{SO}_4$	—	—	—	—	—	—	—	—	134	—
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	125	180	246	250	250	360	370	720	500	400
$\text{Na}_2\text{SO}_4$	—	800	—	—	—	200	—	200	—	—
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	—	—	203	—	—	—	—	—	—	—
KCl	—	65	149	1500	750	65	—	65	—	—
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	—	—	441	25	75	—	440	—	150	200
$\text{NaNO}_3$	—	—	—	—	600	—	—	—	—	—
$\text{KNO}_3$	125	80	202	2000	—	80	1900	80	3000	2500
$\text{Ca}(\text{N}_3)_2 \cdot 4\text{H}_2\text{O}$	500	400	708	—	—	200	—	300	—	—
$\text{NH}_4\text{NO}_3$	—	—	—	—	—	—	1650	—	—	—
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	—	33	—	250	125	16.5	—	16.5	150	—
$\text{NH}_4\text{H}_2\text{PO}_4$	—	—	—	—	—	—	—	—	—	300
$\text{KH}_2\text{PO}_4$	125	—	1088	—	—	—	170	—	—	—
<b>Micro-nutrients</b>										
$\text{NiSO}_4$	0.05	—	—	—	—	—	—	—	—	—
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	3	4.5	—	3	0.1	4.5	22.3	7	10	10
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.18	6	—	0.5	1	1.5	8.6	3	2	1.0
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.05	—	—	0.025	0.03	—	0.025	—	0.025	0.2
$\text{BeSO}_4$	0.1	—	—	—	—	—	—	—	—	—
$\text{H}_2\text{SO}_4$	1	—	—	0.5	—	—	—	—	—	—
$\text{Fe}_2(\text{SO}_4)_3$	50	—	—	—	—	2.5	—	2.5	—	—
$\text{Ti}(\text{SO}_4)_3$	0.2	—	—	—	—	—	—	—	—	—
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	—	—	—	—	0.03	—	—	—	—	—
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.05	—	—	—	—	—	—	—	0.025	0.1
$\text{AlCl}_3$	—	—	—	—	0.03	—	0.025	—	—	—
KI	0.5	3	—	0.5	0.01	0.75	0.83	0.75	—	10
$\text{H}_3\text{BO}_3$	0.05	0.38	—	0.5	1	1.5	6.2	1.5	3	5
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	—	—	—	0.025	—	—	0.25	—	0.25	0.1
$\text{NH}_4\text{H}_2\text{PO}_4$	—	—	—	—	—	—	—	—	—	300
<b>Iron source</b>										
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	—	—	—	—	—	—	27.85	—	—	15
$\text{Na}_2\text{EDTA}$	—	—	—	—	—	—	37.25	—	—	20
<b>(Chelating agent)</b>										
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	—	—	—	—	1	—	—	—	—	—
Ferric tartarate	—	40	—	—	—	—	—	—	—	—
$\text{FeC}_6\text{O}_5\text{H}_7 \cdot 5\text{H}_2\text{O}$	—	—	—	10	—	—	—	—	—	—
Fe (as sequence)	—	—	—	—	—	—	—	—	28	—
<b>Carbohydrate source</b>										
Sucrose	$30 \text{ g}^{1-1}$	$20 \text{ g}^{1-1}$	—	$50 \text{ g}^{1-1}$	$20 \text{ g}^{1-1}$	—	$30 - 50 \text{ g}^{1-1}$	$20 \text{ g}^{1-1}$	$20 \text{ g}^{1-1}$	$30 \text{ g}^{1-1}$
Glocuse	—	—	—	$36 \text{ g}^{1-1}$	—	—	—	—	—	—

Contd.

Constituents	Gautheret (1942)	Hildebrandt et al (1946)	Burkholder and Nickel (1949)	Nitsch (1951)	Heller, (1953)	Reinert and White (1956)	Murashige and Skoog (1962)	White (1963)	Gamborg et al (1968)	Schenk and Hildebrandt (1972)
Contd.										
<b>Vitamins</b>										
Myo-inositol	—	—	—	—	—	100	100	—	100	1000
Nicotinic acid	0.5	—	—	—	—	0.5	0.5	0.5	1	0.5
Pyridoxine HCl	0.1	—	—	—	—	0.1	0.5	0.1	—	0.5
Thiamine HCl	0.1 – 1	0.1	—	1	1	0.1	0.1	0.1	10	5
Calcium pantothenate	—	—	—	—	—	0.1	—	1	—	—
Biotin	—	—	—	—	—	0.01	—	—	—	—
<b>Amino acid source</b>										
Glycine	3	3	—	—	—	—	2	3	—	—
Cysteine HCl	10	—	—	10	—	3	—	1	—	—

and organic chemicals. Chemically undefined medium is a medium in which the exact composition and the concentration of all constituents are not known due to addition of natural products, e.g. coconut milk. The concentration of the constituents of the natural products may vary and depends upon the physiological condition of and the environmental conditions to which a plant is growing.

A medium may be solid or semisolid or liquid. When 6–8% agar agar is dissolved in the liquid nutrient medium, it makes a solid medium. A partially solidified medium is known as semisolid medium where the amount of agar added is reduced. A medium without agar remains in liquid form and is known as liquid medium. Broadly speaking, solid and semisolid media are prepared for callus culture and liquid medium is used for cell suspension cultures. Actually, both types of media are used in different types of culture techniques.

## PREPARATION OF STOCK SOLUTION

It is not possible to weigh and mix all the constituents just before the preparation of medium. It is time-consuming and a tedious job. Again if 100 ml or 200 ml medium is to be prepared, then it is very difficult to weigh some constituents that are used in very small quantity for one litre medium. So it is convenient to prepare the concentrated stock solutions of macro-salts, micro-salts, vitamins, amino acids, hormones etc. All stock solutions should be stored in a refrigerator and should be checked visually for contamination with microorganisms or precipitation of ingredients. Stock solution of vitamins, amino acids and hormones should not be stored for indefinite period and should be kept in a deep freezer chamber. The widely used culture medium was formulated by Murashige and Skoog (commonly called MS medium), so the procedure for the preparation of stock solution of MS medium (1962) is given below—

Constituents	Amount (mg/L) present in original medium	Amount (gm) to be taken for stock solution (X20)	Final volume of stock (ml)
NH <sub>4</sub> NO <sub>3</sub>	1650	33.0	
KNO <sub>3</sub>	1900	38.0	1000
CaCl <sub>2</sub> , 2H <sub>2</sub> O	440	8.8	
KH <sub>2</sub> PO <sub>4</sub>	170	3.4	
MgSO <sub>4</sub> , 7H <sub>2</sub> O	370	7.4	

Particularly macro-salts should be made at ten/twenty times of (X10 or X20) their final concentration in the medium while micro-nutrients can be prepared at thousand time (X1,000) of their final strength. While making the stock solutions, it is advisable to dissolve each constituent completely before adding another otherwise precipitation of salts may occur.

### **Stock Solution of Macro-Salts (X20)**

To make 1,000 ml of this stock solution, dissolve the salts one after another in 800 ml of double distilled water ( $\text{DDH}_2\text{O}$ ) and then make up the volume. The solution is filtered and can be stored in refrigerator ( $10-16^\circ\text{C}$ ) for a long period until the solution is totally used.

### **Stock Solution of KI (X1,000)**

Dissolve 83 mg of KI (0.83 mg/L present in original medium) in 100 ml of  $\text{DDH}_2\text{O}$ . Store in refrigerator ( $10-16^\circ$ ).

### **Stock Solution of Micro-Salts (X1,000)**

Constituents	Amount (mg/L) present in original medium	Amount (X100) to be taken for stock solution (Value expressed in mg)	Final volume of stock (ml)
$\text{H}_3\text{BO}_3$	6.2	620	
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	25	
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	2.5	100
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	2.5	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	860	
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	2230	

### **Stock Solution of MS three Vitamins (X1,000)**

Constituent	Amount (mg/L) present in original medium	Amount (X50) to be taken for stock solution (Value in mg)	Final volume (ml)	Storage temperature ( $^\circ\text{C}$ )	Duration of storage (in days)
Thiamine HCL	0.1	5			
Nicotinic acid	0.5	25	50	0	15
Pyridoxine HCL	0.5	25			

Hormone	Required amount for stock solution (mg)	Amount of solvent required to dissolve	Amount of water to be added	Final concentration	Storage temperature (°C)	Duration (days)
<b>Auxins</b>						
2, 4-dichlorophenoxy acetic acid (2, 4-D)	10	1 ml abs. ethyl alcohol	9 ml	0.5 mg/ml	0	7
Indole acetic acid (IAA)	10	-do-	-do-	-do-	-do-	-do-
$\alpha$ -aphthalene acetic acid (NAA)	10	-do-	-do-	-do-	-do-	-do-
3-indole butyric acid (IBA)	10	-do-	-do-	-do-	-do-	-do-
<b>Cytokinins</b>						
Kinetin (6-furfuryl amino purine)	10	1 ml 1(N) HCl	-do-	-do-	-do-	-do-
BAP (6-Benzyl-amino-purine)	10	-do-	-do-	-do-	-do-	-do-
Zeatin	10	-do-	-do-	-do-	-do-	-do-
2iPA (2, iso-pentenyl-adenine)	10	-do-	-do-	-do-	-do-	-do-

### Stock Solution of Glycine (X1,000)

Dissolve 40 mg glycine in 20 ml of DDH<sub>2</sub>O. Store at 0°C for 15 days.

### Stock Solution of Hormones

These stock solutions are not specific for MS medium. So stock solutions of hormones are general and can be used in any medium at any combinations and concentrations.

Auxins and cytokinins are not directly dissolved in water. So they are at first made soluble in water miscible solvents and then water is added to get the final volume. Auxins and cytokinins are available either in powder or in crystal forms. Preparation of stock solutions of commonly used auxins and cytokinins are given above

## GENERAL TECHNIQUES

Preparation of nutrient medium, sterilization, aseptic manipulation, maintenance of culture are the general techniques.

### Preparation of Culture Medium

#### Principle

*In vivo* plant cells, tissues and organs get their appropriate nutrient and growth requirements from the intact plant body for their organised growth and development. Isolated cell, tissues and organs also need nutrients for their *in vitro* growth and development. So, nutrients are supplied artificially according to the medium formulated by several workers. The main objective of medium preparation is to culture the cell, tissue and organ *in vitro*.

#### Procedure

Media should be prepared with care and the following procedure is recommended.

To make 1 litre of MS medium—

- (i) Dissolve 30 gms cane sugar in 200 ml DDH<sub>2</sub>O. Mix 1–2 gms activated charcoal and filter through filter paper, set inside the Buchner funnel fitted on a special conical flask with small side arm attachment. Filtering is done by using a suction pump.

## TECHNIQUES IN PLANT TISSUE CULTURE

Several techniques have been adopted for *in vitro* plant cell, tissue and organ culture. Among them some are general techniques that are essentially followed in all experiments. There are also some specific techniques that are adopted and designed according to the objectives of experiment by the experimenters.

- (ii) Take DDH<sub>2</sub>O in another flask and add in sequence the appropriate amount of stock solution as follows—

Stock solution of macrosalts	50 ml
Stock solution of microsalts	1 ml
Stock solution of KI	1 ml
Stock solution of Fe-EDTA	5 ml
Stock solution of MS 3 vits	1 ml
Stock solution of Glycine	1 ml
Stock solution of meso-inositol	2 ml

Desired concentration of auxin and/or cytokinin are added from stock solution according to the formula—

Desired concentration

Stock concentration

= amount (ml) of stock solution to be taken for one litre medium.

If the quantity of the medium is less than one litre, then hormones are added using another formula—

Required concentration X Volume of medium

Stock concentration X 1,000

= amount (ml) of stock solution to be added.

- (iii) Pour filtered sucrose solution and salt, vitamins, amino acid, hormone solution mixture into a one litre measuring cylinder. Make the final volume to one litre with DDH<sub>2</sub>O. Shake well to mix up uniformly.
- (iv) Adjust the pH of the liquid medium 5.6–5.8 with the aid of 0.1(N) HCl or 0.1(N) NaOH. This operation is done using the pH metre.
- (v) Add 5% to 8% agar to the liquid medium to make solid medium. Heat to 60°C to dissolve the agar completely. Otherwise, without adding agar, liquid medium can be used for culture.
- (vi) Dispense the culture medium into culture tube (20 ml/tube) or wide mouth conical flask (25–40 ml/flask). Insert non-absorbent cotton plug wrapped with gauge cloth. Cover the plug with the help of brown paper and rubber band.
- (vii) Medium is finally sterilized by autoclaving.

## Sterilization Procedure

### Principle

The culture medium, especially when it contains sugar, will also support the growth of micro-organisms like bacteria, fungi etc. So if they come in contact with medium either in cellular form or in spore form, the micro-organisms grow faster than the higher plant tissue due to their brief life cycle and will kill the tissue. The micro-organisms may come from glass vials, instruments, nutrient medium used for culture and even from plant material itself. Therefore, the surface of plant tissue and all non-living articles including nutrient medium should be sterilized.

### Procedure

- (i) *Sterilization of non-living Articles*—The routine sterilization procedure of non-living articles such as nutrient medium, glass goods, distilled water, instruments (wrapped with brown paper) is by autoclaving under steam at a pressure of 15 lb/in<sup>2</sup> and a temperature of 120°C for 15 minutes.

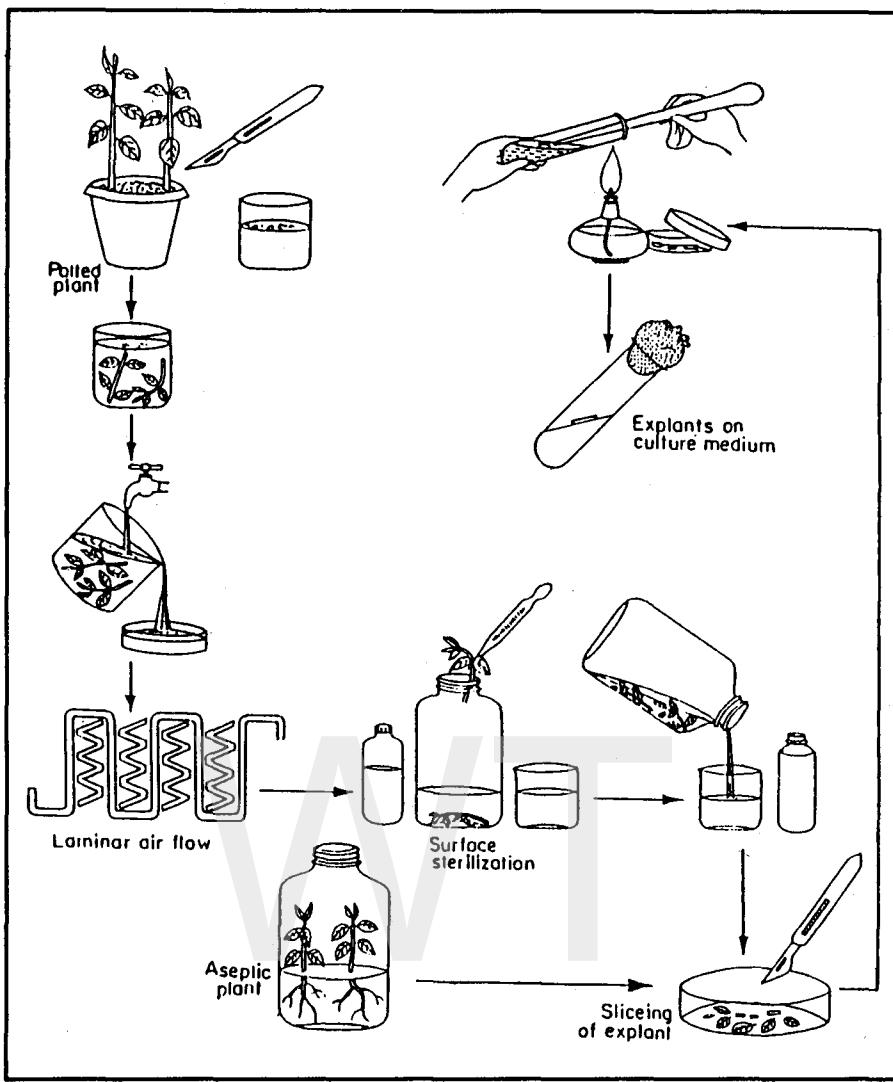
Thermolabile compounds are often added in the medium and such medium is sterilized either at room temperature or in cold by passing through bacterial filter.

An alternative method of sterilizing glass goods and instruments is by heating in an oven at 150°C for 3–4 hrs.

It should be noted that when autoclaving screw capped glass vials, care should be taken to ensure that the caps are not closed too tightly so that gases can expand without the risk of explosion.

- (ii) *Sterilization of Plant Material*—Plant material which is to be cultured, should be surface sterilized to remove the surface borne microorganisms. This procedure is done in front of a laminar air flow or inside the inoculation chamber before the plant material is inoculated onto the culture medium (Fig 1.10).

A typical sterilization procedure is given in the next page.



□ Fig 1.10

**Flow diagram illustrating the procedure for surface sterilization of plant material and inoculation of explant for culture**

- (1) Thoroughly washed plant material or explant in tap water is immersed in 5% v/v solution of liquid detergent such as 'Teepol' for 10–15 minutes. Then wash the material thoroughly in tap water and finally in distilled water. This step can be done in the general laboratory. Subsequent steps are done in front of a laminar air flow or the pre-sterilized inoculation chamber.
- (2) Dip the explants in 70% ethyl alcohol for 60 seconds.
- (3) Immediately transfer the material into an autoclaved jaw bottle and pour 0.1% mercuric chloride ( $HgCl_2$ ) 5–10% Sodium hypochlorite (v/v) solution. Keep them for 10–15 minutes. During that period, the bottle is frequently swirled for shaking so that all surfaces of plant material come equally in contact with sterilant.
- (4) After 10–15 minutes, decant the sterilant and wash the explants thoroughly with sev-

eral changes of autoclaved distilled water to remove all traces of sterilant.

(5) Then the explants are ready for culture.

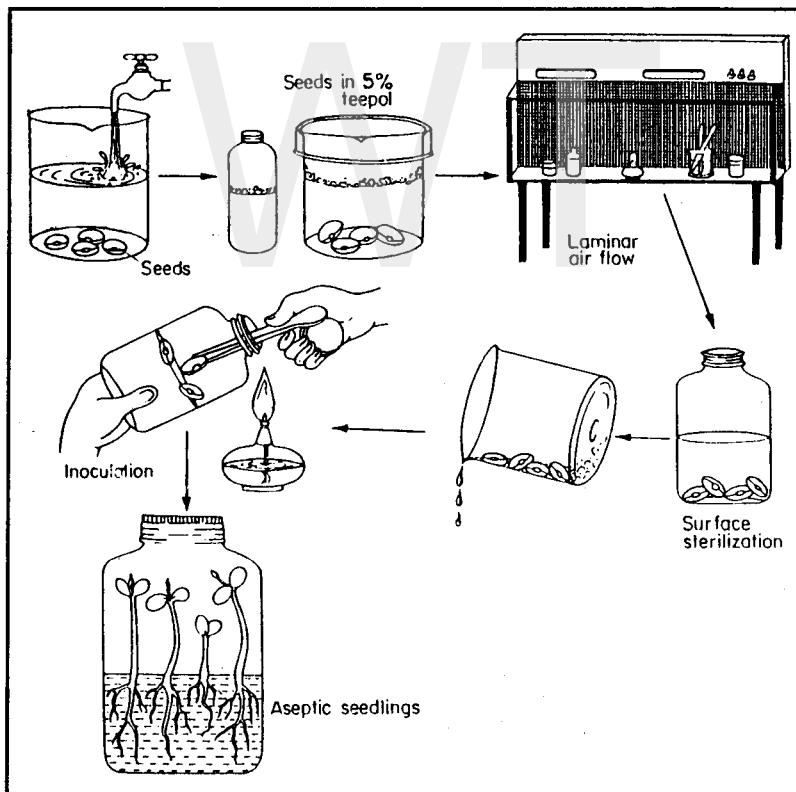
### Preparation of Aseptic Plants

#### Principle

Surface sterilization of plant tissue may cause some deleterious effect because most of the sterilants are toxic chemicals. Seeds can more or less resist such deleterious effect due to the presence of its seed coat. So to avoid the surface sterilization of plant tissue, seeds are surface sterilized and are cultured on simple basal nutrient medium. Seeds in culture germinate and give rise to an aseptic seedling. Explant from such seedlings grown under aseptic and controlled conditions are the most suitable material for culture and need no further surface sterilization.

#### Procedure

- (1) Wash the dry seeds thoroughly with tap water (Fig 1.11).
- (2) Dip the seeds in 5% Teepol solution (v/v) for 10–15 minutes. Decant the Teepol solution and wash the seeds again with tap water and finally with distilled water.
- (3) Rinse the seeds with 70% ethyl alcohol for 1 minute.
- (4) In front of laminar air flow, transfer the seeds into an autoclaved bottle and pour 0.1%  $HgCl_2$  solution (w/v) so that seeds are immersed. Leave for 10–15 minutes. Stir the bottle frequently.
- (5) Decant the sterilant and wash 3–4 times with autoclaved distilled water.
- (6) Transfer the seeds from bottle to autoclaved petridish with the aid of sterile forceps.



□ Fig 1.11

Flow diagram illustrating the preparation of aseptic plants from seeds

- (7) Open the closure of the culture vial containing the basal nutrient medium. Flame the neck of the culture vial and in quick succession transfer a few seeds on to the medium. Replace the closure.
- (8) Incubate the seeds in continuous dark either at room temperature or at 25-28°C.

## Aseptic Techniques

### Principle

Precautions must be taken to prevent the entry of any microorganism at the time of transferring the surface sterilized explants on the nutrient medium (inoculation) using the sterilized instruments. For this reason, manipulation and transfer should ideally be carried out under aseptic condition. Starting from surface sterilization to inoculation, all operations should be done aseptically.

### Procedure

A typical procedure of aseptic technique is given below—

- (1) Put all the sterilized articles (media, instruments, glass goods etc.) for inoculation on the glass racks of the inoculation chamber. Alternatively, if laminar air flow is available, keep all articles on the table of air flow cabinet. Laminar air flow blows bacteria-free air over the working surface.
- (2) Put on the switch of UV lamps of inoculation chamber for one hour before work. In case of laminar air flow, the power switch is put on and allow the air flow to blow air for at least 15 minutes before work.
- (3) Put off the UV lamp before entering inside the inoculation chamber. Do not put off laminar air flow. The working glass table top of the inoculation chamber or the table of laminar air flow is swabbed with alcohol before starting work.
- (4) Wear a clean apron and use a mask. Clean the hands with alcohol and dry it.
- (5) Pour alcohol in a clean coupling jar and dip all instruments into it. Light the spirit lamp. Take the surface sterilized or aseptic plant material in a sterile petri dish.

- (6) Flame the neck of culture tube or flask and in quick succession remove the plug of glass vials. Transfer the tissue onto the medium and replace the closure. Each time, the instruments are passed through the flame of the spirit lamp.

### Precautions

- (1) Always keep away the hands moistened with alcohol from the spirit lamp. So dry the alcohol first.
- (2) Exposure to UV light builds up a high concentration of Ozone gas (toxic) inside the closed chamber. It is, therefore, healthy to enter the chamber only 15–30 minutes after switching off the UV lamp.
- (3) Do not dip hot instruments in alcohol and don't use hot instrument for cutting or holding the plant material.
- (4) Work carefully and try to ensure that media and plant tissues are exposed for the plant material.
- (5) Don't heat the neck of the glass vials excessively.

### Incubation of Culture

### Principle

High temperatures are likely to lead to dissociation of the culture medium and tissue damage while at very low temperatures tissue growth is slow. Again some tissues grow well in dark while others need both light and dark conditions. Low humidity causes the quick desiccation of culture medium and high humidity is favourable for the contamination of culture medium. Therefore, cultures are incubated in a culture room where light, temperature and humidity are controlled.

### Procedure

- (1) After inoculating the tissue onto the culture medium, cultures are incubated on culture rack at 25-28°C constant temperature.
- (2) Culture tubes are placed at 30-45° inclined position. For this purpose a long wooden

stick or an empty paper cover of fluorescence lamp is placed on the middle of culture rack and lay the plugged end of the culture tube on the support.

- (3) Illumination is provided by cool-white fluorescent light placed about 18 inches above the culture to give a light intensity of  $4 - 10 \times 10^3$  lux for 16 hours.
- (4) If light is not necessary, then put off the light and cover the whole rack with a black cloth.

## SPECIFIC TECHNIQUES

A number of specific techniques are as follows—

1. Organ culture	... See ch. 2
2. Callus culture	... See ch. 3
3. Single cell culture	... See ch. 9
4. Cell suspension culture	... See ch. 4
5. Embryo culture	... See ch. 10
6. Anther and Pollen culture	... See ch. 11
7. Plant protoplast culture	... See ch. 12
8. Protoplast fusion and somatic hybridization	... See ch. 13

## Summary

Plant tissue culture is a collection of experimental methods of growing large number of isolated cells or tissues under sterile and controlled condition. To carry out the experiments using tissue culture techniques, a well-equipped laboratory is first required. An ideal tissue culture laboratory should have a big room for general laboratory and a small room for aseptic work and for keeping autoclaved articles. The general laboratory should be provided with a washing area, hot air oven, refrigerator, distillation plant, weighing balance, pH meter, vacuum pump, autoclave, working table, heater, microscope, microtome and wooden racks.

Laboratory for aseptic inoculation should be without any window or ventilator in order to make dust free. The room should be provided with double door having automatic door

closets. For aseptic work, a large wooden chamber is made for short term work. For simple aseptic routine work a small inoculating hood may be used. Laminar air flow is the most suitable, convenient and reliable instrument for aseptic work. It has a number of small blower motor to blow air which passes through a number of HEPA (high efficiency particulate air) filters. Such filters remove particles larger than  $0.3 \mu\text{m}$ . The ultraclean air is free of fungal and bacterial contaminants.

The culture room means the room for keeping the culture under controlled temperature, light and humidity. To maintain the temperature around  $25 \pm 2^\circ\text{C}$  inside the culture room, air coolers are used in tropical countries. This room is also provided with specially designed racks to keep culture vessels. The relative humidity of the culture room is maintained above 50%. The culture room should also have a shaker for suspension culture in moving liquid medium.

Different types of Corning or Pyrex or similar borosilicate glass goods are used to culture plant tissues. Plant tissues are grown in wide necked Erlenmeyer conical flask, culture tube (25 mm in diameter and 150 mm in length), screw-capped universal bottle. In order to autoclave the culture medium and to culture the plant tissue, culture vessels particularly culture tubes and flasks must be fitted with cotton plugs which exclude microbial contaminants. Yet allow free gas exchange. For the sterilization of medium containing thermolabile compounds and enzymes, a specially designed bacterial filter is used.

Instruments routinely used for culture work include various sizes of stainless steel made scalpel, forceps, spatula, scissors etc.

Excised plant tissues and organs will only grow *in vitro* on a suitable artificially prepared nutrient agarified or liquid medium which is known as culture medium. The Murashige and Skoog (MS) based culture media are commonly used for plant tissue culture and have proven effective for growth promotion of both monocotyledons and dicotyledons. A culture medium is composed of inorganic salts, an iron source, vitamins, amino acids, plant hormones and a

carbohydrate supply. Inorganic salts are supplied in two groups—as macrosalts and microsalts. The most commonly used phytohormones are synthetic auxins and cytokinins. The auxins are 2,4D, IAA, BOTA, NAA, IBA etc and the cytokinins are Kinetin, 6-BAP, Zeatin, 2iPA etc. The concentration and ratio of hormones may vary from plant to plant and should be standardized for a particular plant tissue. Some plant tissues grow in the presence of complex natural additives such as coconut milk, casein hydrolysate, yeast extract, water melon extract, malt-extract, potato-extract, ripe tomato extract, orange juice extract etc. Diphenylurea, a growth factor found in coconut milk, exhibits cytokinin-like responses.

On the basis of constituents, culture media are of two types—(i) Chemically defined in which the composition and concentration of all constituents are known. (ii) Chemically undefined in which the exact composition and the concentration of all constituents are not known due to addition of natural additives.

It is not possible to weigh and mix all the constituents just before the preparation of medium. So it is convenient to prepare the concentrated stock solutions of macrosalts, microsalts, vitamins, amino-acids, hormones etc. All stock solutions should be stored in a refrigerator for a limited period.

Several techniques have been adopted for *in vitro* plant tissue culture. Among them some are general techniques such as preparation of nutrient medium, sterilization, aseptic manipulation, maintenance of culture and some are specific techniques such as organ culture, callus culture, organogenesis, embryogenesis, suspension culture, anther and pollen culture, plant protoplast culture, embryo culture etc.

## Questions for Discussion

1. Give an outline of a plant tissue culture laboratory.
2. What types of glass goods and instruments are used for plant tissue culture? Mention

their specific uses and how the glass goods and instruments are sterilized for culture.

3. What is culture medium? State the basic composition of a general plant tissue culture medium. How the culture medium is prepared and sterilized? If the concentration of stock solution of auxins is 0.5 mg/ml then how much amount of stock solution is to be added for the preparation of 700 ml medium with 2.5 mg/L auxin concentration?
4. Give an outline of the principle of tissue culture techniques, methods and its application in biological studies.
5. Define the types of aseptic culture of plants. Discuss the importance of tissue and cell culture.
6. Evaluate the tissue and cell culture techniques in terms of progress that have been made in the field of plant science.
7. Write the brief answers of the following questions—
  - (a) What is surface sterilization?
  - (b) What are complex additives used for the preparation of culture medium?
  - (c) What is the principle of the preparation of aseptic plants?
  - (d) What are the differences between a chemically defined and a chemically undefined medium?
8. Write short notes—
  - (a) Laminar air flow
  - (b) Glass goods and instruments
  - (c) Culture medium
  - (d) General techniques of plant tissue culture
  - (e) Specific techniques of plant tissue culture
  - (f) Culture room
  - (g) Aseptic inoculation
  - (h) Inoculation of culture.

## Chapter Two

### Organ Culture

#### INTRODUCTION

In higher plant body, complex interrelationships are established between different organs like roots, shoot apical meristems, leaf primordia, floral buds, ovules etc. A particular organ can be isolated and cultured aseptically in a chemically defined medium where they retain their characteristic structures and continue to grow in a manner comparable to that of their intact counterparts. In organ culture, organs are not induced to form callus tissue. In this respect, organ culture differs from the callus culture where the organization of the intact tissues is lost.

#### DEFINITION

The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordia or whole or part of an organ in a way that may allow differentiation and preservation of the architecture and/or function.

#### BRIEF PAST HISTORY

**W. Kotte and W. J. Robbins (1922)**—Reported first the culture of excised root tips from the aseptically germinated wheat seedlings.

**P. R. White (1930)**—Reported the successful culture of root segments of aseptically germinated tomato seedlings.

**C. D. LaRue (1942)**—Reported first *in vitro* culture of excised flower buds of *Kalanchoe globulifera* and *Nemesia strumosa*.

**S. W. Loo (1945)**—Reported the culture of 5 mm shoot tips of Asparagus seedlings on a medium.

**L. L. Jansen and J. Bonper (1949)**—Grew the ovaries of *Lycopersicon pimpinellifolium* on a medium. Although ovaries enlarged, viable seeds were not produced.

**J. P. Nitsch (1949–1951)**—Successfully cultured the ovaries of *Lycopersicon esculentum*,

*Cucumis anguria*, *Phaseolus vulgaris*, *Fragaria* sp. and *Nicotiana tabacum*.

**G. Morel** (1952–1955)—Showed that certain virus infection could be eliminated from potato and dahlia by aseptic culture of stem tip. This method allowed the recovery of healthy plants. He also discovered the rapid multiplication of tropical orchid *Cymbidium* using the apical meristem culture.

**N. Maheshwari** (1958)—Isolated ripe pollen and ovules of *Papaver somniferum* and cultured them together. He was able to observe all the stages from pollen germination, through fertilization to the development of mature seeds.

**E. Galun, Y. Yung and A. Lang** (1962–1963)—Tested the effect of IAA and GA<sub>3</sub> upon sex expression of the culture of isolated floral bud of *Cucumis sativus*.

**T. A. Steeves and I. M. Sussex** (1966)—First realised that culture of excised leaf primordia would provide an experimental system to study the complete development of leaves under controlled condition. They successfully cultured the leaf primordia of ferns, particularly *Osmunda cinnamomea*.

## IMPORTANCE OF ORGAN CULTURE

- (i) Organ culture provides an excellent experimental system to define the nutrients and growth factors normally received by the organ from other parts of the plant body and from its external environment.
- (ii) Organ culture is particularly valuable in studies of the interdependence of organs for growth hormones and other growth factors.

- (iii) Cultured organs may be ideally suited for studying specific problems in morphogenesis and for investigating the sites of biosynthesis of specific metabolites and growth compounds.
- (iv) Organ culture also opens up a new avenue for the developments in agriculture and horticulture.

## DIFFERENT TYPES OF ORGAN CULTURE

On the basis of explant, organ culture can be categorised into different types, which are given below.

Each type of organ culture has its own particular advantages and importance for specific research project.

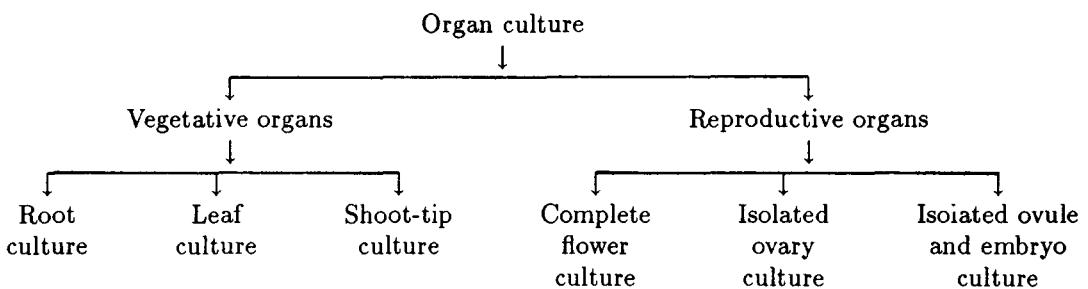
## ROOT CULTURE

### WHAT IS ROOT CULTURE ?

Root culture can be defined as the culture of excised radicle tips of aseptically germinated seeds in a liquid medium where they are induced to grow independently under controlled conditions.

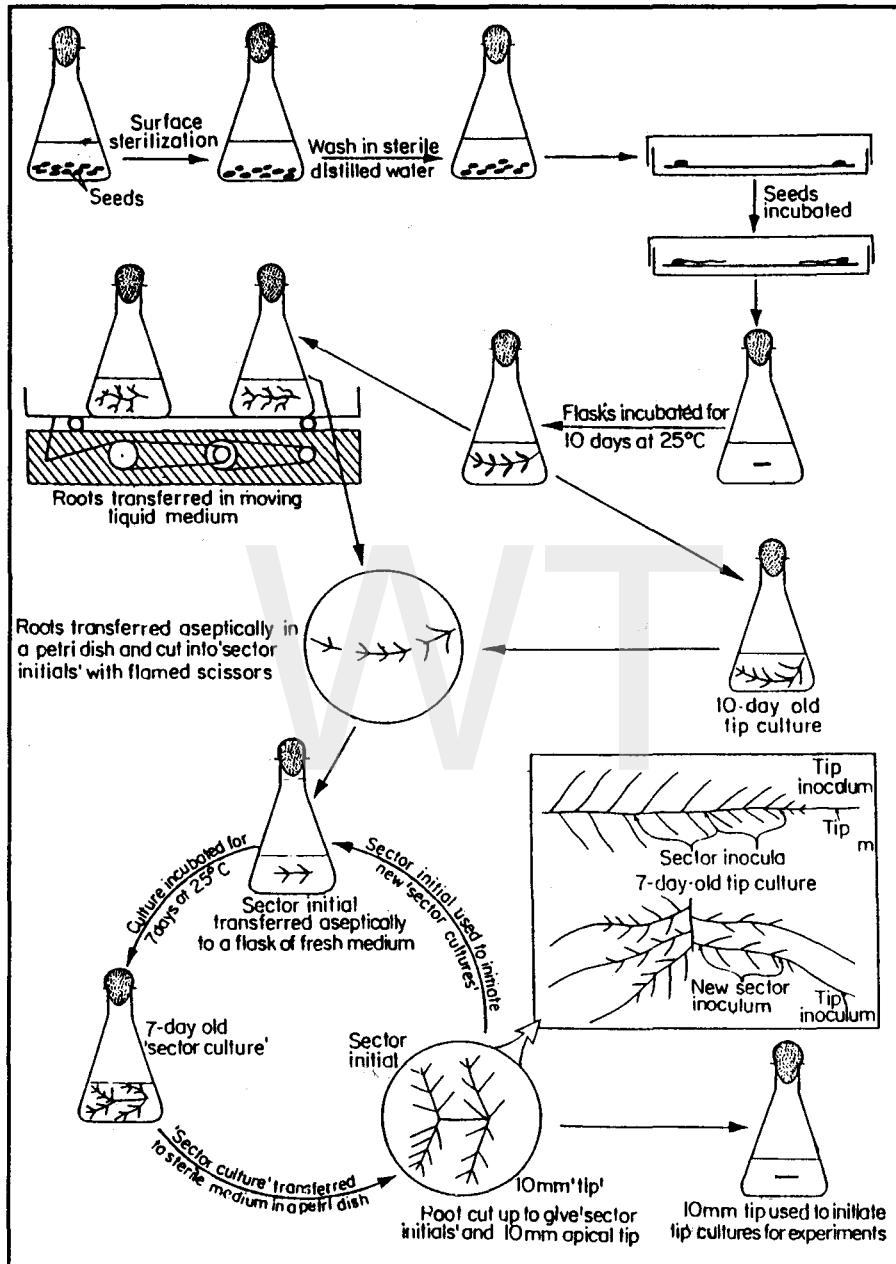
## PRINCIPLE

Intact *in vivo* plants are not suitable for the isolation of intact root tips because the roots of the plant are buried deeply in the soil. Again, root tips from young seedlings are very sensitive to toxic sterilants. So it is better to avoid the surface sterilization of young root tips for the establishment of root cultures. Root cultures can be successfully initiated from the excised radicle tips of aseptically germinated seeds. Root tip



cultures are generally maintained in moving liquid medium. In culture, root tips are induced to grow like that of root system of an intact plant. A clone of excised roots can also be established from a single root culture by repeatedly cutting

and transferring of the main root tips or of lateral tips into fresh medium in every subculture at the interval of definite period. Growth of excised roots can be expressed in terms of fresh and dry weight, increase in length of the main axis,



□ Fig 2.1

#### Procedure for the maintenance of isolated roots in continuous culture

number of emergent laterals and total length of laterals per culture.

## PROTOCOL

### Initiation of Isolated Root Culture

- (1) Seeds are surface sterilized by the conventional methods and germinated on moist filter paper or White's basal medium at 25°C in the dark (Fig 2.1).
- (2) When the seedling roots are 20 to 40 mm in length, 10 mm apical tips (tip inoculum) are excised with a scalpel and each transferred to 40 ml of liquid medium contained in 100 ml wide-necked Erlenmeyer flasks.
- (3) Flasks are incubated at 25°C in the dark.

### Initiation of Clones

The root material derived from a single radicle tip could be multiplied and maintained in continuous culture. Such genetically uniform root cultures are referred to as a clone of isolated roots. Initiation of root clones is a very simple technique. The protocol is given below—

- (1) Establish a root culture from a radical tip of a seed as described above.
- (2) Transfer a 10-day-old established root culture to a sterile petri dish containing sterile medium. Next, using flamed scissors, cut the main axis of root into a number of pieces (each piece is called sector inoculum or initial), each bearing four or five young laterals.
- (3) Transfer the individual sector inoculum aseptically to a flask liquid medium and incubate in dark at 25°C.
- (4) Such sector culture can be used to initiate further tip culture using 10 mm apical tips of laterals of a growing sector inoculum or the growing sector is again cut into 4–5 sectors to initiate the sector culture.

## IMPORTANCE OF ROOT CULTURE

The root of many species cannot be cultured. Studies with successful species (Tomato, Pea, Clover, Carrot, etc.) have contributed a lot of significant informations. The importance of root culture is given below—

### IMPORTANCE OF ROOT CULTURE IN RELATION TO BASIC INFORMATION

- (1) Root cultures have increased our knowledge of carbohydrate metabolism, role of mineral ions, vitamins etc. in root growth.
- (2) Root cultures have provided basic information regarding the dependence of roots on shoots for growth hormones.
- (3) Root clones are ideally suited for the study of the effect of various compounds on root growth.

### SPECIFIC APPLICATIONS OF ROOT CULTURE

#### Study of Nodulation of Leguminous Root in Culture

The process of nodule formation on the roots of leguminous plant by the nitrogen-fixing (NIF) bacteria (*Rhizobium sp.*) is a complex physiological system which is poorly understood. Root cultures of leguminous plant provide an ideal system to study it. When bacteria are inoculated directly in root culture, they quickly grow and spoil the whole culture. Again, nitrate in the medium is required for the root growth but is inhibitory to nodulation. To overcome such difficulties M Raggio, N Raggio and J G Torrey modified the root culture technique for nodulation study. In their study, the base of an excised root of *Phaseolus vulgaris* was supplied with sucrose and vitamins via agar medium in a glass vial. The remainder of the root was in contact with an inorganic nitrate-free medium containing *Rhizobium*. By this process, isolated roots develop nodules in culture. Therefore, *in vitro* nodulation helps to understand the relationship between symbiotic NIF bacteria and higher plants.

## **Regeneration of Shoots on Roots**

Culture of isolated roots can be maintained continuously for many years. However, in some species e.g. *Atropa*, *Convolvulus arvensis*, shoots can be induced to regenerate from cultured roots. The shoot primordia can be derived from callus at the cut ends of the roots, as in case of *Atropa* or endogenously from the internal tissues of the root as in *Convolvulus*. This phenomenon is of practical value as well as theoretical interest.

## **Study of Synthesis of Secondary Metabolites from Root Culture**

The roots of many medicinally important plant species synthesize pharmaceutically important alkaloids as by-products of normal metabolism (secondary metabolites). Root culture have been used to locate the site of biosynthesis of such compounds. Root culture techniques are also used to increase the synthesis of such compounds in cultured root by some nutritional manipulations.

## **Initiation and Development of Secondary Vascular Tissues**

Normally excised cultured roots show only the primary structure of young seedling radicle and, therefore, do not form secondary vascular tissue. But it has been found that excised root tips from pea seedling develop a vascular cambium when cultured in medium containing indoleacetic acid. Attempts have also been made to define the factors that determine the site, time of origin and functioning of such vascular cambium. Torrey studied this phenomenon using a modification of the technique developed by Raggio and Raggio. The basal 5 mm portions of excised 15 mm long pea root were inserted into an agar solidified basal medium supplemented with various test substances e.g., auxins, cytokinins, meso-inositol and extra sucrose and the exposed 10 mm portion of the root was placed in a petri dish of basal agar medium containing inorganic salts, vitamins and sucrose. When the bases of pea roots were fed with extra sucrose (8%)

and IAA ( $10^{-6}$ M) a vasular cambium was initiated. But when the whole portion of roots was allowed to grow in a basal medium, they did not form secondary vascular tissues. These experiments have suggested that auxins, cytokinins, meso-inositol extra sucrose may have an important role in cambial development.

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## **LEAF CULTURE**

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### **WHAT IS LEAF CULTURE ?**

Leaf culture is the culture of excised young leaf primordia or immature young leaf of the shoot apex in a chemically defined medium where they grow and follow the developmental sequences under controlled conditions.

### **PRINCIPLE**

Leaf primordia or very young leaves are excised, surface sterilized and inoculated on an agar solidified medium. In culture leaf remains in healthy condition for a long period. Leaves can be taken from aseptically grown plants for culture. Since leaves have a limited growth potential, so in culture the amount of leaf growth depends upon the stage of maturity at the time of excision. Leaf primordia or very young leaf have more growth potential than nearly mature leaves.

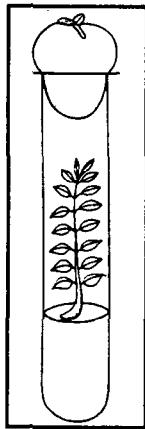
Most of the work on leaf culture has been done with lower plants, particularly fern (*Osmunda*), although higher plant species, such as tobacco and sunflower, have been used. In culture, the fern leaf primordia (1.2 mm), excised from underground buds, develop into leaves having a normal morphology except that they are much reduced in size than *in vivo* leaves due to a reduced number of cells rather than a decrease in cell size. The growth of cultured leaf primordia is also completed earlier than intact leaf.

It has also been found that there is a correlation between leaf primordia size and its mode of development in culture. In *Osmunda cinnamomea*, smallest leaf primordia (300  $\mu\text{m}$  in length) give rise to shoots instead of leaf in culture. However, with increasing size of primordia,

there is an increased tendency to form leaves. These results indicate that some unidentified leaf forming substances gradually accumulate as the primordia develop.

## PROTOCOL

- (1) Detach vegetative bud or very young leaf from shoot apex at the vegetative phase of the plant. Wash the explants thoroughly with running tap water.
- (2) Immerse the leaf buds or young leaves in 5% Teepol for 10 minutes. Wash the explants to remove Teepol.
- (3) Leaf buds or young leaves are surface sterilized by immersion in 70% v/v Ethanol for 30 seconds. This treatment is followed by 10–15 minutes incubation in sodium hypochlorite solution with 0.8% available chlorine. Rinse the explants 3–4 times in sterile distilled water.
- (4) Excise the leaf primordia from the leaf bud with the help of surgical scalpel.
- (5) Inoculate the leaf primordia or young leaf onto 20 ml of solidified medium in a culture tube (Fig 2.2).



□ Fig 2.2

### Culture of leaf primordia of *Osmunda cinnamomea* producing leaf

- (6) Incubate the culture at 25°C under 16 hrs. light.

## IMPORTANCE OF LEAF CULTURE

- (1) Culture of excised leaf primordia is valuable to study the effects of various nutrients, growth factors and changing environmental conditions on leaf development under conditions divorced from the complexities of the intact plant.
- (2) In case of fern, leaf primordia cultures are used to study the formation of sporangia and the size at which a primordium is destined to become a leaf.
- (3) Young leaves of most of the solanaceous species form numerous shoot buds instead of callus formation when they are cultured in solidified MS medium supplemented with 1–5 µm kinetin or BAP or 2iPA. When shoot have grown to a height 3 cm, they may be removed and subcultured onto MS medium devoid of growth hormones. Root formation is stimulated by this treatment. Therefore leaf culture of solanaceous species can be used as clonal micropropagation.

## SHOOT TIP/MERISTEM CULTURE

### WHAT IS SHOOT TIP CULTURE ?

Shoot tip culture may be described as the culture of terminal (0.1 – 1.0 mm) portion of a shoot comprising the meristem (0.05 – 0.1 mm) together with primordial and developing leaves and adjacent stem tissue.

### WHAT IS MERISTEM CULTURE ?

Meristem culture is the *in vitro* culture of a generally shiny special dome-like structure measuring less than 0.1 mm in length and only one or two pairs of the youngest leaf primordia, most often excised from the shoot apex.

### WHAT IS MERICLONING ?

Mericloning is a popular term. It is not applied in scientific usage. It refers to the *in vitro*

vegetative propagation of orchids from excised shoot tips, axillary buds or floral organs.

## WHAT IS MERISTEMMING ?

Meristemming is also a popular term. It is used to describe the *in vitro* clonal propagation of plants from various explant sources including shoot tips, leaf sections and calli.

## PRINCIPLE

The excised shoot tips and meristem can be cultured aseptically on agar solidified simple nutrient medium or on paper bridges dipping into liquid medium and under the appropriate condition will grow out directly into a small leafy shoot or multiple shoots. Alternatively the meristem may form a small callus at its cut case on which a large number of shoot primordia will develop. These shoot primordia grow out into multiple shoots. Once the shoots have been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves separating the shoot into small segments each containing one node. The axillary bud on each segment will grow out in culture to form yet another shoot. The excised stem tips of orchids in culture proliferate to form callus from which some organised juvenile structures known as protocorm develop. When the protocorms are separated and cultured to fresh medium, they develop into normal plants. The stem tips of *Cascuta reflexa* in culture can be induced to flower when they are maintained in the dark.

Exogenously supplied cytokinins in the nutrient medium plays a major role for the development of a leafy shoot or multiple shoots from meristem or shoot tip. Generally, high cytokinin and low auxin are used in combination for the culture of shoot tip or meristem. Addition of adenine sulfate in the nutrient medium also induces the shoot tip multiplication in some cases. BAP is the most effective cytokinin commonly used in shoot tip or meristem culture. Similarly, NAA is the most effective auxin used in shoot tip culture. Coconut milk and gibberellic acid are also equally effective for the growth of shoot apices in some cases.

## PROTOCOL

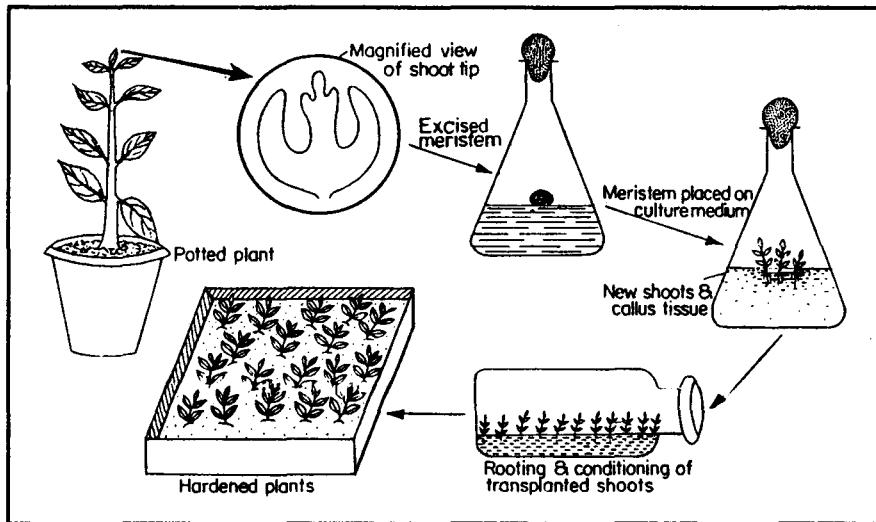
- (1) Remove the young twigs from a healthy plant. Cut the tip (1 cm) portion of the twig.
- (2) Surface sterilize the shoot apices by incubation in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The explants are thoroughly rinsed 4 times in sterile distilled water.
- (3) Transfer each explant to a sterilized petri dish.
- (4) Remove the outer leaves from each shoot apices with a pair of jeweller's forceps. This lessens the possibility of cutting into the softer underlying tissues.
- (5) After the removal of all outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of filterpaper bridge. Flame the neck of the culture tube before and after the transfer of the excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.
- (6) Incubate the culture under 16 hrs light at 25°C.
- (7) As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, develop root, transfer them to hormone free medium.
- (8) The plantlets formed by this way are later transferred to pots containing compost and kept under green house conditions (Fig 2.3).

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## IMPORTANCE OF SHOOT TIP/MERISTEM CULTURE

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The uses of shoot tips and meristem in tissue culture are very varied and include mainly (1) virus eradication, (ii) micro-propagation and (iii) storage of genetic resources.



□ Fig 2.3

**Flow diagram illustrating the technique of shoot tip or meristem culture**

## VIRUS ERADICATION

Many important plants contain systemic viruses which substantially reduce their potential yield and quality. It is, therefore, important to produce virus free stocks which can be multiplied. Generally, highly meristematic tissue of a virus infected plant remain free from virus due to their fast mitotic activity. Therefore, shoot tips and meristems of a virus infected plant are the ideal explants to produce a virus free stock. This technique is also valuable for the maintenance of carefully defined stocks of specific varieties and cultivars in disease free state. The size of the meristem explant is critical for virus eradication. Often so called meristem tip cultures have failed to eliminate virus infection because the explant contains shoot apices with vascular tissue instead of true meristem.

This technique, combined with heat treatment (thermotherapy) or chemical treatment (chemotherapy) has proved to be very effective in virus eradication. Heat treatment is done by placing an actively growing plant in a thermotherapy chamber. Over a period of two weeks the temperature is increased to 38°C inside the chamber and the plants are maintained at this

temperature for two months. After that period, the apical meristem is excised, surface sterilized and transferred aseptically to agar medium. Using this technique 85% to 90% virus free plants have been obtained. Without heat treatment, shoot tips or meristem can be grown on chemotherapeuticants added medium for virus eradication. Commonly used chemotherapeuticants are 2, 4-D, mela-chife green, thiouracil etc.

Shoot tip or meristem culture has enormous horticultural value e.g. in the production of plants for the cut flower industry when stock plants of registered lines must be maintained in as near-perfect condition as possible. Any infection by virus that affect the growth or physical characteristics of size and shape of flowers, is obviously very serious problem from commercial point of view. Meristem culture technique to clean up the stocks could, therefore, avert a commercial disaster. Similarly, in the agricultural world, the production or yield of a crop can fall dramatically as a result of a viral infection and render that particular variety no longer salable or commercial value. Meristem culture could be of value in restoring the original properties of the variety by removing the infection.

## MICROPROPAGATION

A sexual or vegetative propagation of whole plants using tissue culture techniques is referred to as micropropagation. Shoot tip or meristem culture of many plant species can successfully be used for micropropagation.

## STORAGE OF GENETIC RESOURCES

Many plants produce seeds that are highly heterozygous in nature or that is recalcitrant. Such seeds are not accepted for storing genetic resources. So, the meristem from such plants can be stored *in vitro*.

Besides the above-mentioned uses of shoot tip or meristem culture, it can also be utilized in various important fields of plant science such as—

## SHOOT TIP OR MERISTEM CULTURE AND PLANT BREEDING

In many plant breeding experiments, the hybrid plants produce abortive seeds or non-viable seeds. As a result, it makes a barrier to crossability in plants where non-viable seeds are unable to develop into mature hybrid plants. Shoot tip or meristem from such hybrid plant can be cultured to speed up breeding programme.

## PROPOGATION OF HAPLOID PLANTS

Haploid plants derived from anther or pollen culture always remain sterile unless and until they are made homozygous diploid. Meristem or shoot tip culture of haploid plants can be used for their propagation from which detailed genetic analysis can be done on the basis of morphological characters and biochemical assay.

## MERISTEM OR SHOOT TIP CULTURE AND QUARANTINE

There are some strict regulations concerning the international movement of vegetative plant material. After thoroughly checking, the plant materials may be rejected by quarantine

authority if the plant material carries some diseases. Plantlets derived from shoot tip or meristem culture are easily accepted by the quarantine authority for international exchange without any checking. Therefore, using this technique, crop plants can be easily exchanged in crop improvement programmes that are based on materials from different parts of the world.

## FLOWER BUD/COMPLETE FLOWER CULTURE

### INTRODUCTION

The onset of the reproductive phase in the life cycle of an angiosperm is heralded by flower initiation. It is a very important morphogenetic event. Intense morphogenetic activities occur in the short life span of an angiosperm flower. The technique of aseptic culture of flower primordia or flower bud is a potentially useful tool for any study concerned with the control of flower morphogenesis.

### WHAT IS FLOWER CULTURE ?

Flower culture can be defined as the aseptic culture of excised floral bud on a chemically defined nutrient medium where they continue their development to produce a full bloom in a culture vessel.

Young and complete flower culture can also be described as flower culture. In culture medium, the flowers remain healthy and they develop normally to mature seeds.

### PRINCIPLE

Flowers can be cultured at the different stages of development, such as primordial stage, bud stage, prepollination stage and post-pollination stage. Flower primordia and the young flower bud require a complex medium containing inorganic salts, B-vitamins, aminoacids, coconut milk, auxins and cytokinins. The mature flowers at pre or post-pollination stage need comparatively simple media containing inorganic salts, sucrose and a small quantity of hormones.

## PROTOCOL

- (1) Flower buds or mature flowers are collected from the healthy plants.
- (2) Wash them thoroughly and dip them in 5% Teepol solution for 10 minutes and wash.
- (3) Transfer them to laminar air-flow cabinet. Surface sterilize them by immersing in 5% Sodium hypochlorite, wash with autoclaved distilled water.
- (4) Using flamed forceps, transfer the flower bud or mature flower to culture tubes containing 20 ml solid medium.
- (5) Incubate the culture in 16 hrs. light at 25°C.

## IMPORTANCE

- (1) The main application of floral primordia or flower bud culture is in fundamental studies of flower development.
- (2) Flowers put into the culture before pollination do not usually produce fruits. In some cases, parthenocarpic fruit development has been observed, particularly in presence of auxins.
- (3) The culture of pollinated flowers is very important to study the fruit development. Often the *in vitro* fruits are smaller than their natural counterparts, but the size can be increased by supplementing the medium with an appropriate combination of growth hormones such as auxins, gibberellins and cytokinins.
- (4) Flower culture has been used to study the sex expression in flower. In the cucumber (*Cucumis sativus*), there exist different genetic lines that are monoecious (with unisexual male or female flowers on the same plant), gynoecious (purely female) or hermaphrodite.

Under suitable natural conditions, the monoecious types will produce only staminated male flower and the gynoecious types only pistillated female flower.

It has been observed that in culture the potentially male buds tend to form ovaries and this tendency is promoted when IAA is added to the culture medium. In contrast, addition of gibberellic acid counteracts the effect of auxin. Isolated potentially female or bisexual flower buds in culture remain unchanged even in presence of IAA or gibberellic acid or cytokinins. Such culture techniques are also important for experimental studies on floral morphogenesis.

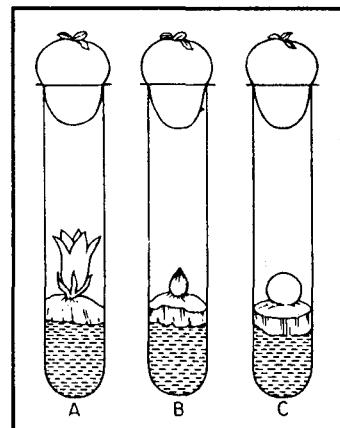
## CULTURE OF ISOLATED OVARY

### WHAT IS OVARY CULTURE ?

Ovary culture is a technique of culture of ovaries isolated either from pollinated or unpolinated flowers.

### PRINCIPLE

Ovary is a ovule bearing region of a pistil. Excised ovaries can be cultured *in vitro*. For many species e.g. tomato, gherkin (*Cucumis anguria*) excised ovaries grow in culture and form the fruits that ripen and produce viable seeds. This development takes place on a simple nutrient medium containing only mineral salts and sucrose, provided the flowers have been fertilized two or more days before excision (Fig 2.4).



□ Fig 2.4

**Ovary culture of *Lycopersicon esculentum*.**  
**A. Just excised flower.** **B. Culture of excised ovary.** **C. Small fruit product on culture**

But the ovaries of unpollinated flowers do not grow on simple nutrient medium. However, use of some synthetic auxins such as 2, 4-D, 2, 4, 5-T (2, 4-5-trichlorophenoxyacetic acid), NOA (2, Naphtoxyacetic acid) in the nutrient medium induces the development of ovaries of unpollinated flowers.

Often, in culture, the ovaries fail to grow into full size fruits in the restricted space of culture vial. To overcome this problem, a partial sterile culture technique is devised in which only the long flower stalk is inserted into the aseptic nutrient medium through an opening in the stopper, thus leaving the ovary free to grow outside the culture vial.

## PROTOCOL

- (1) Collect the pollinated or unpollinated flowers from a healthy plant.
- (2) Wash them thoroughly with tap water, dip into 5% Teepol solution for 10 minutes and again wash to remove the trace of Teepol.
- (3) Transfer the flowers to laminar air flow cabinet. Surface sterilize the flowers by immersing in 5% sodium hypochlorite solution for 5-7 minutes. Wash them with sterile distilled water.
- (4) Transfer the flowers to a sterile petri dish. Using a flamed forcep and a surgical scalpel, dissect out the calyx, petals, anther filaments etc. of the flower to isolate the pistil. During isolation of pistils care should be taken to ensure that the ovaries are not injured in any way. Damaged pistil, should be discarded as they often form callus tissue from the damaged parts.
- (5) Place the ovaries on agar solidified nutrient medium.
- (6) Incubate the cultures at 25°C in a 16 hrs. daylight regime at about 2000 lux. The light is provided by fluorescent lamp.

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## IMPORTANCE

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Ovary culture is an useful technique to in-

vestigate many fundamental and applied aspects. Importances of ovary culture are—

- (1) It is useful to study the early development of embryo development, fruit development, different aspects of fruit physiology including respiration, maturation and disease.
- (2) The effect of phytohormones on parthenocarpic fruit development can be studied from the culture of unpollinated pistil.
- (3) Floral organs play a significant role in fruit development. Role of floral organs can be studied from the *in vitro* culture of ovary. In some cases it has been found that pollinated ovary produce the normal fruits *in vitro* if the sepals are not removed before culture. However, if the sepals are removed before culture, addition of sucrose (5%) is necessary to obtain satisfactory growth of ovary in culture. In barley, lemma and palea are very important. In onion, the growth of ovaries without perianth is markedly inhibitory.
- (4) In hybridization, the plant breeders face many problems such as the failure of pollen germination on the stigma or the slow and insufficient growth of pollen tube as well as precocious abscission of flowers. Ovary or pistil culture, *in vitro* fertilization (test tube pollination) has been used to circumvent these obstacles. In many cases, successful results of *in vitro* fertilization and seed formation has been obtained. *Petunia violacea* is a self-incompatible species. But *in vitro* pollination and seed formation overcome the self-incompatibility. Actually, *in vitro* fertilization has got the immense value in plant breeding where problems of incompatibility or sterility exist as a barrier to normal sexual reproduction. It might be possible, therefore, to produce a hybrid between two species or varieties *in vitro* that could not be produced under normal *in vivo* conditions. Ovary culture has been successfully employed to obtain hybrids of diploid *Brassica chinensis* and autotetraploid *B. pekinensis* which are normally cross incompatible.

- (5) Ovary culture has also been successful in inducing polyembryony. Polyembryo may develop in culture from the various parts of the ovary. These polyembryos give rise to many shoots instead of a single plantlet.
- (6) The process of double fertilization not only brings about the formation of embryo and endosperm, but also stimulates the development of ovary into fruit. In most apomictic plants, although there is no fertilization, pollination alone stimulates the growth of the ovary and seed. The culture of ovaries of apomicts may, therefore, help in understanding the nature of stimulus provided by pollination.

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## CULTURE OF OVULE

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### WHAT IS OVULE CULTURE ?

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions.

### PRINCIPLE

An ovule is a megasporangium covered by integument. Ovules are attached with placenta inside the ovary by means of its funiculus. An ovule contains a megasporangium or an egg cell. After fertilization, a single cell zygote is formed which ultimately leads to form a mature embryo possessing shoot and root primordia.

Ovules can be isolated and cultured in nutrient medium. *In vitro* ovule culture helps to understand the factors that regulate the development of a zygote through organised stages to a mature embryo.

Alternatively, it may be possible to germinate pollen in the same culture as the excised ovule and to induce *in vitro* fertilisation and subsequently embryo production.

### PROTOCOL

- (1) Collect the open flower (unfertilized ovules). If fertilized ovules are desired, collect the open flower where the anthers are dehisced and pollination has taken place. To ensure the fertilization, collect the flower after 48 hrs. of anther dehiscence.
- (2) Remove sepals, petals, androecium etc. from the ovaries containing either fertilized or unfertilized ovules.
- (3) Soak the ovaries in 6% NaOCl solution.
- (4) Rinse the ovaries 3–4 times with sterile distilled water.
- (5) Using sterile techniques, ovules are gently prodded with the help of spoon shaped spatula by breaking the funicles at its junction with placental tissue.
- (6) The spatula with ovules is gently lowered into the sterile solid or liquid medium as the culture vial is slanted about 45°.
- (7) Damaged or undersized ovules are rejected when possible, during transfer.
- (8) Incubate the ovule culture in either dark or light (16 hrs. 3,000 lux) at 25°C

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### IMPORTANCE OF OVULE CULTURE

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Isolated ovule culture as early as the zygote or two to four celled proembryo stage is of considerable importance. Ovule culture is a boon for the plant breeders in obtaining seedlings from crosses which are normally unsuccessful because of abortive embryos. Importance and application of *in vitro* ovule culture are discussed below on different specific aspects—

### TEST TUBE POLLINATION AND FERTILIZATION

An important achievement of research on ovule culture has been the development of the technique of test tube pollination and fertilization. By this technique, it may be possible to germinate pollen in the same culture as the excised ovule and to induce *in vitro* fertilization.

Excised unfertilized ovules of *Argemone mexicana*, *Eschscholtzia californica*, *Papaver somniferum*, *Nicotiana tabacum*, *N. rustica* have been cultured along with their respective pollen grains. All the stages of development starting from the germination of pollen to double fertilization have been observed and the mature seeds containing viable embryos have been obtained by the above experiments. Using the same method, it has been possible to fertilize the ovules of *Melandrium album* with pollen grains from other species of caryophyllaceae and subsequently even with pollen of *Datura stramonium*. Employing ovule culture technique, the incompatibility barrier in *Petunia axillaris* has been overcome.

### APPLICATION OF OVULE CULTURE IN HYBRIDIZATION

In many interspecific and intergeneric crosses, the  $F_1$  hybrid embryos frequently become abortive in the developing seeds or the  $F_1$  seeds are not capable to support the development of embryos. Ovule culture has been successfully employed to obtain hybrid seedlings. It has been observed that in several inter specific crosses, the hybrid embryo of *Abelmoschus* fails to develop beyond the heart or torpedo-shaped embryo. By ovule culture, viable hybrids have been obtained in three out of five interspecific crosses attempted, namely, *A esculentus*  $\times$  *A ficunus*, *A esculentus*  $\times$  *A moschatus* and *A tuberculatus*  $\times$  *A moschatus*. Similarly, a true hybrid between *Brassica chinensis* and *B pekinensis* has been obtained by culturing the fertilized ovule *in vitro*. A hybrid between *Lolium perenne* and *Festuca rubra* has also been obtained successfully by means of ovule culture.

Several attempts have been made to hybridize between different species of the New World and Old World cotton.

Although successful crosses between different species of cotton have been achieved, hybrid plants have not been obtained through fertilized ovule culture. But the seed development and the production of fibre from the cultured ovule have been demonstrated. The *in vitro* growth ovule and the development of fibre from the develop-

ing seed can also be regulated by exogenous hormones and in this respect ovule culture of cotton offers an unique method for the studies on the effect of phytohormones on fibre and seed development.

### PRODUCTION OF HAPLOID CALLUS THROUGH OVULE CULTURE

Uchimiya *et al.* (1971) attempted culturing unfertilized ovules of *Solanum melongena* and obtained vigorous callus formation on a medium supplemented with IAA and kinetin. Although the origin of the callus tissue was not known, a cytological assay revealed it to be haploid in nature. So it is an important attempt to obtain a haploid cell line or plant from an alternative source rather than anther or pollen culture.

### OVULE CULTURE AND ANGIOSPERMIC PARASITES

It is generally believed that in obligate root parasites such as *Striga* or *Orobanche* the formation of seedlings is dependent on some stimulus from the host root. Studies on ovule culture of *Orobanche aegyptica* and *Cistanche tubulosa* have demonstrated that the formation of shoots *in vitro* can be induced in any absence of any stimulus from the host.

### OVULE CULTURE OF ORCHID PLANTS

In nature, the seeds of orchid germinate only in association with a proper fungus. As a result numerous seeds are lost due to unavailability of proper fungus. Beside this, the seed capsule of many orchid takes a long time to mature. To overcome such problems, several attempts have been made to culture the fertilized ovule of orchid *in vitro*. Poddubnaya-Arnoldi (1959, '60) successfully grew the fertilized ovule of *Calanthe veitchii*, *Cypripedium insigne*, *Dendrobium nobile* and *Phalaenopsis schilleriana*.

### INDUCTION OF POLYEMBRYO BY OVULE CULTURE

In horticultural practices, the artificial induction of polyembryo holds a great potential. It

has been observed that the nucellus of monoembryonic ovule of *Citrus* can be induced to form adventive embryos in culture. Therefore, such achievement is very significant.

## VIRUS IRRADICATION THROUGH OVULE CULTURE

In the varieties of *Citrus* which are impossible to free of virus by other means, the ovule culture has proved decisively advantageous to make them virus free.

## EMBRYO CULTURE

See Chapter 10.

## ANTHER AND POLLEN CULTURE

By careful selection of developing anthers at a precise and critical stage, it is possible to establish the anther culture that will give rise to haploid plantlets. Alternatively, the developing pollen grains can be diverted from their normal pathway and are induced to form somatic embryos which subsequently give rise to the haploid plantlets. Haploid plants contain a single set of chromosomes. The phenotype is the expression of single copy of genetic information as there is no masking of characters because of gene dominance.

The use of anther and pollen culture offers an important tool to the conventional plant breeder as a way of obtaining haploid plants for selection of characters and inclusion in the breeding programme.

(Anther and pollen cultures have been discussed in detail in Chapter 11)

## Summary

In higher plant body, complex interrelationships are established among different organs like root, shoot apical meristems, leaf primordia, floral buds, ovules etc. The organ culture refers to

the *in vitro* culture and maintenance of an excised organ primordia or whole or part of an organ in a way that may allow differentiation and preservation of the architecture and/or function. Organ culture provides an excellent experimental system to define the nutrients and growth factors normally received by the organ from other parts of the plant body. On the basis of explant, organ culture is divided into several categories such as root culture, leaf culture, shoot tip culture, flower culture, ovary culture and ovule or embryo culture.

Root culture means the culture of excised radical tip of aseptically germinated seeds in liquid medium where they are induced to grow independently under controlled conditions. Root culture has provided basic information regarding the dependence of roots on shoots for growth hormones. It is also important for the study of nodulation of leguminous root in culture, regeneration of shoots on roots, synthesis of secondary metabolites from root and initiation and development of secondary vascular tissues.

Leaf culture is the culture of excised young leaf primordia or immature young leaf of the shoot apex in a chemically defined medium where they grow and follow the developmental sequences under controlled conditions. Most of the work on leaf culture has been done with lower plants, particularly fern (*Osmunda*). Leaf culture is valuable to study the effect of various nutrients, growth factors, changing environmental conditions on leaf development, the formation of sporangia in fern. Young leaves of most of the solanaceous species form numerous shoot buds. Therefore, leaf culture of solanaceous species can be used as clonal micropropagation.

Shoot tip culture may be described as the culture of terminal (0.1–1.0 mm) portion of a shoot comprising the meristem (0.05–0.1 mm) together with primordial and developing leaves and adjacent stem tissue. Meristem culture is the *in vitro* culture of a generally shiny dome-like structure measuring less than 0.1 mm in length and only one or two pairs of the youngest leaf primordia, most often excised from the shoot apex. Mericloning and meristemming are the popular

terms to describe the *in vitro* clonal propagation of plants from excised shoot tips.

The shoot primordia in culture media grow out into multiple shoots or may form a small callus tissue at the cut end on which a large number of shoot primordia will develop. Once the shoots have been grown from the excised shoot tip or meristem, they can be propagated further by nodal culture. The stem tip of parasitic angiosperm i.e. *cascuta reflexa* in culture can be induced to flower when they are maintained in the dark. Exogenously supplied cytokinin in the nutrient medium plays a major role for the development of a leafy shoot or multiple shoots from meristem or shoot tip. BAP is the most effective cytokinin. Coconut milk and gibberellic acid are also equally effective for the growth of shoot apices in some cases. The uses of shoot tips and meristem in tissue culture are varied and included mainly (a) virus eradication (b) micro-propagation and (c) storage of genetic resources.

Many important crop plants contain systemic viruses which substantially reduce their potential yield and quality. We now know that highly meristematic tissue of both roots and shoots of a virus infected plant remain free from virus due to their fast mitotic activity. Shoot tip or meristem culture has been developed to produce virus free stock. This technique, combined with heat treatment (Thermotherapy) or chemical treatment (Chemotherapy), has provided effective measure for virus eradication. Shoot tip culture is very useful for rapid propagation of hybrid plants which produce abortive or non viable seeds. So this technique is very important for breeding experiments. It is also important for the propagation of haploid plants. Plantlets derived from shoot tip or meristem are easily accepted by the quarantine authority for international exchange without any checking.

Flower culture can be defined as the aseptic culture of excised floral bud on a chemically defined nutrient medium where they continue their development to produce a full bloom in a culture vessel. The culture of flower primordia is very important in fundamental studies of flower development. The culture of pollinated flower is important to study the fruit development and to

study the sex expression in flower.

Ovary culture is a technique of culture of ovaries isolated either from pollinated or unpollinated flowers. Ovary culture is very useful to investigate many fundamental and applied aspects. It is useful to early development of embryo development, fruit development, different aspects of fruit physiology and diseases. Ovary culture and *in vitro* pollination has been used to circumvent many obstacles and problems faced by the plant breeders. Ovary culture has also been successful in inducing polyembryony. The culture of ovaries of apomictic species may help in understanding the nature of stimulus provided by pollination.

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions. *In vitro* ovule culture is very important to study test tube pollination and fertilization and to obtain seedlings from crosses which are normally unsuccessful because of abortive embryos. Several attempts have been made to culture the fertilised ovule of orchid *in vitro* and in many cases successful results have been obtained. The ovule culture has proved decisively advantageous to make a virus free plant in *Citrus*.

## Questions for Discussion

1. Describe the principles and application of root culture techniques mentioning the protocol for root culture.
2. What is leaf culture? Give a general account of the importance and principles of leaf culture.
3. What is shoot tip culture? What are the differences between shoot tip and meristem culture? Describe the method of culturing shoot tip of plants and its uses in different fields of plant science.

4. What is flower culture? Describe the method and discuss the importance and implication of flower culture.
  5. What is ovary culture? Give an outline of the principle of ovary culture technique and its application.
  6. What is ovule culture? Describe the method of culturing ovule of plant and its uses in different fields of plant science.
  7. Evaluate the different types of organ culture techniques in terms of the progress that have been made in the field of plant science.
  8. Write notes on—
    - (a) Study of nodulation of leguminous root in culture.
    - (b) Disease free plants from cultured apical meristems.
  - (c) Ovary culture in relation to plant breeding.
  - (d) Ovule culture of orchid plants.
  - (e) Test tube pollination and fertilization.
  - (f) Flower culture in relation to sex expression.
9. Write brief answers of the following questions—
- (a) What are the importance of organ culture?
  - (b) What are the different categories of organ culture?
  - (c) How are the secondary vascular tissues developed in root culture?
  - (d) How are the viruses eradicated by shoot tip culture?
  - (e) What are the applications of ovule culture in hybridization?



## Chapter Three

### Callus Culture

#### INTRODUCTION

Higher plant body is multicellular and is made of highly organised and differentiated structures-like stem, leaf, root etc. Different tissue systems present in different organs function in a highly co-ordinated manner. Now, if the organised tissue are diverted into an unorganised proliferative mass of cells by any means (like mechanical, chemical or infective agents), they will form the callus tissue.

In nature, sometimes callus or callus-like tissue is found to form in various part of intact plant either due to deep wound or due to some diseases. Deep large wound in branches and trunks of the intact plants results in the formation of soft mass of unorganised parenchymatous tissues which are rapidly formed on or below the injured surface of the organ concerned. Such callus tissue is known as wound callus. Wound callus is formed by the division of cambium tissue. They may also be formed by the same process from the parenchymatous cells of cortex, phloem and xylem rays. The growth of such wound callus is limited and the main function of such callus is to heal up the injured part of the plant.

Again, in some higher plants, sometimes unorganised compact wort-like outgrowths or callus-like tissue masses on stem, leaf, root are formed by the stimulation of cell division in fully differentiated cells due to some diseases. Such diseases are mainly caused by fungus (*Synchytrium endobioticum*), bacterium (*Agrobacterium tumefaciens*), virus (*Aureogenus magnivena*), insect or genetic factors. Such callus-like outgrowth is known as gall or tumour. But the callus tissue which is important to plant tissue culture, is produced experimentally from a small excised portion called the explant of any living healthy plant. The explants are cultured aseptically *in vitro* under controlled condition on a nutrient medium containing specific phytohormones. In culture, the excised plant tissues loses its structural integrity and changes completely to a rapidly proliferative unorganised mass of cells which is called the callus tissue.

#### BRIEF PAST HISTORY

R. J. Gautheret (France) (1934–1937)—He first succeeded in promoting the development of callus tissue from excised cambial tissue of *Salix capraea* and other woody species. He was

able to promote the growth of the callus tissue using simple nutrient medium supplemented with three vitamins (thiamine, pyridoxine and nicotinic acid) and indole-3-acetic acid (IAA) newly discovered by F W Went and K V Thimann (1937).

**P. Nobecourt** (France) (1939)—He first established the callus culture capable of potentially unlimited growth on semisolid agar medium. He started his work using the tap root explant of *Daucus carota*. He was also to maintain the culture by simply transferring portions of the callus to fresh medium at regular interval of four to six weeks.

**J. Van Overbeck, M. E. Conklin and A. F. Blakeslee** (1941)—They first reported the importance of coconut milk in callus culture.

**S. M. Caplin and F. C. Steward** (1948)—They first succeeded in obtaining the growth of differentiated non-cambial cells isolated from *Daucus carota* using coconut milk in medium. Later, they used coconut milk in combination with synthetic auxin such as 2, 4-dichlorophenoxy acetic acid in medium and were able to induce the division of cells in species which had previously been difficult to grow.

**F. Skoog** (1954–1955)—He was able to produce the callus culture from the cut piece of stem of *Nicotiana tabacum* in nutrient medium containing auxins. The callus remained active for some time but failed to grow. Later, he discovered kinetin from old sample of herring sperm DNA. After the addition of kinetin in culture medium, it was possible to renew the growth of tobacco stem callus tissue.

**F. Skoog and C. O. Miller** (1957)—They first put forward the concept of hormonal control of organ formation from callus tissue. They also suggested that equal concentration of auxin and kinetin induced the continuous growth of callus tissue. The inclusion of kinetin in culture media has made it possible to produce callus culture from a large number of plant species.

## WHAT IS CALLUS TISSUE ?

Callus tissue means an unorganised proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions.

## PRINCIPLES OF CALLUS CULTURE

For successful initiation of callus culture, three important criteria should be accomplished—

- (i) Aseptic preparation of plant material,
- (ii) Selection of suitable nutrient medium supplemented with appropriate ratio of auxins and cytokinins or only appropriate auxin, and
- (iii) Incubation of culture under controlled physical condition.

Different plant parts carry a number of surface borne micro-organisms-like bacteria, fungus etc. So, before attempting to initiate a callus culture, it is necessary to surface sterilize the plant parts which is to be cultured. Typical plant parts may be segments of root or stem, pieces of leaf lamina, flower petals etc. The excised plant parts called explants are at first washed with liquid detergent (generally 5% v/v 'Teepol'). Then the explants are surface sterilized by the most commonly used chemicals such as 0.1% w/v mercuric chloride ( $HgCl_2$ ) or Sodium hypochlorite (0.8% to 1.6% available chlorine) for a limited time (generally 10–15 minutes). After surface sterilization, the explants are repeatedly rinsed with autoclaved distilled water.

The surface sterilized plant material is cut aseptically into small segments (a few millimeter in size). Size of explant is a critical factor for the induction of callus tissue. The explants are finally transferred aseptically on a suitable nutrient medium solidified with agar.

Agar solidified or semi-solid nutrient medium after its preparation and sterilization by autoclave at 15 lbs pressure for 15 minutes (see

details in Chapter 1) is used for the induction of callus tissue. In most cases successful callus culture depends upon the inclusion of plant growth hormones in the nutrient medium and for healthy callus growth usually both an auxin and a cytokinin are required.

Incubation of culture under controlled physical conditions such as temperature, light, humidity is indispensable for the proper initiation of callus tissue. The suitable temperature for *in vitro* callus initiation and growth is usually  $25 \pm 2^\circ\text{C}$ . In some plant materials initiation and growth of the callus tissue take place in totally dark condition. However, in case of other plant materials, a particular photoperiod (16 hrs. light and 8 hrs. dark) is necessary for the initiation and growth of callus tissue. Approximately 2,000 to 3,000 lux artificial light intensity is needed. Cool, white fluorescent lamps (4 ft. 2 No.) are generally used for providing light. Generally 55% to 60% relative humidity is maintained in the culture room..

Once the growth of the callus tissue is well established, portions of the callus tissue can be removed and transferred directly onto fresh

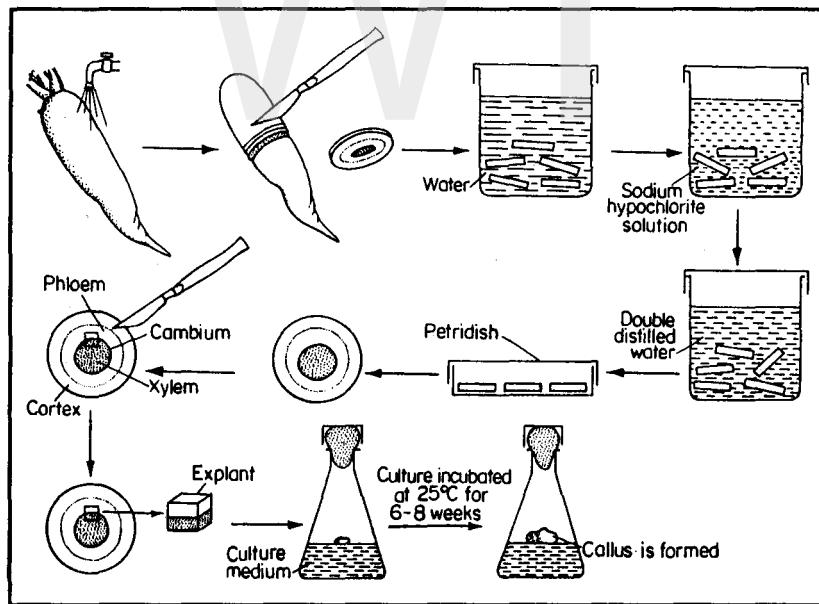
nutrient medium to continue growth. In this manner, callus cultures can be continuously maintained by serial subcultures.

## PROTOCOL OF CALLUS CULTURE

Callus tissue can be induced from different plant tissues of many plant species. Carrot is a highly standardized material. So the callus culture from excised tap root of carrot is described here by the following procedure—

- (1) A fresh tap root of carrot is taken and washed thoroughly under running tap water to remove all surface detritus (Fig 3.1).
- (2) The tap root is then dipped into 5% 'Tee-pol' for 10 minutes and then the root is washed.

The carrot root, sterilized forceps, scalpels, other instruments, autoclaved nutrient medium petridishes are then transferred to laminar air flow or inoculation chamber. Throughout the manipulation sequence forceps, scalpels must be kept in 95% ethanol and flamed thoroughly before use.



□ Fig 3.1

**Procedure for the callus culture from carrot root**

- (3) The tap root is surface sterilized by immersing in 70% v/v ethanol for 60 seconds, followed by 20–25 minutes in sodium hypochlorite (0.8% available chlorine).
- (4) The root is washed three times with sterile distilled water to remove completely the hypochlorite.
- (5) The carrot is then transferred to a sterilized petridish containing a filter paper. A series of transverse slice 1 mm in thickness is cut from the tap root using a sharp scalpel.
- (6) Each piece is transferred to another sterile petridish. Each piece contains a whitish circular ring of cambium around the pith. An area of  $4\text{mm}^2$  across the cambium is cut from each piece so that each small piece contains part of the phloem, cambium and xylem. Size and thickness of the explants should be uniform.
- (7) Always the lid of petridish is replaced after each manipulation.
- (8) The closure (cotton plug) from a culture tube is removed and flamed the uppermost 20 mm of the open end. While holding the tube at an angle of  $45^\circ$ , an explant is transferred using forceps onto the surface of the agarified nutrient medium. Nutrient medium is Gamborg's B<sub>5</sub> or MS medium supplemented with 0.5 mg/L 2, 4-D.
- (9) The closure is immediately placed on the open mouth of each tube. The forceps are always flamed before and after use. Date, medium and name of the plant are written on the culture tube by a glass marking pen or pencil.
- (10) Culture tubes after inoculation are taken to the culture room where they are placed in the racks. Cultures are incubated in dark at  $25^\circ\text{C}$ .
- (11) Usually, after 4 weeks in culture the explants incubated on medium with 2,4-D will form a substantial callus. The whole callus mass is taken out aseptically on a sterile petridish and should be divided into two or three pieces.
- (12) Each piece of callus tissue is transferred to a tube containing fresh same medium.
- (13) Prolonged culture of carrot tissue produces large calluses.

## HOW THE CALLUS TISSUE IS FORMED ?

Formation of the callus tissue is the outcome of cell expansion and cell division of the cells of the explant. During the formation of callus tissue, the explant loses its original characteristic. So, under the influence of exogenously supplied hormone, the explant is triggered off a growth sequence in which cell enlargement and cell division predominate to form an unorganised mass of cells. As a result, the explant undergoes an irreversible change of its shape, size, symmetry, structural organisation and cellular integrity.

For the initiation of callus culture, tissues from young seedling or from juvenile part of the mature plant are generally taken. In such tissue, a growth momentum is already present among the cells due to presence of maximum number of physiologically active meristematic cells. As the explant absorbs the exogenously supplied hormones along with other nutrients, it makes a continuous nutrient gradient among the different cells of the explant on the basis of their location. As a result, the cells divide asynchronously depending upon the availability of the nutrient and hormones and ultimately the callus tissue is formed.

Depending upon the types of explant viz. leaf, stem segment, root segment etc. either enlargement in size or the swelling followed by rupture of the tissue within few days of inoculation takes place. This change indicates the response of explant for callus formation and is followed by the appearance of little irregular cellular masses around the cut edges or from the ruptured surface. It is now explained that initial formation of cellular mass particularly at the cut end may be due to injury during excision. Some endogenous growth substance oozes out through the injured tissue at cut end and stimulates the cell division which is simultaneously induced by the exogenously supplied hormones. There is another

explanation that both endogenous product and exogenous hormones make a threshold level and their interaction results the formation of unorganised cellular growth at the cut end. Whatever may be the fact, it is generally accepted that exogenous hormones play the major role for the formation of callus tissue in culture. Both auxin and cytokinin are required for indefinite growth and cell division in callus culture. Sometimes, only 2, 4-D (an auxin) alone is sufficient for callus formation. Regarding the action of auxin and cytokinin, there is a general concept that auxin is required for growth and cytokinin is required for cell division. This concept is unanimously accepted and is equally applicable for callus formation.

After the formation of visible unorganised mass of cells at the cut end, gradually the whole tissue is involved to form callus tissue as mentioned previously. The callus tissue gradually increases in mass as the new cells are added by mitosis. Estimation of callus growth can be made on the basis of changes in fresh or dry weight.

The type of tissue or cell present in the explant is an important factor for the rapid formation of callus tissue. If any meristematic tissue is present in the explant such as vascular cambium, the existing dividing cell of the vascular cambium under *in vitro* condition continue the rapid growth and proliferate into callus tissue. The quiescent, vacuolated and highly differentiated cells such as pith cortex, vacuolated and highly differentiated cells are also stimulated to divide and proliferate into irregular mass of cells which spread over most of the surface and inner part of the explant.

#### MORPHOLOGY, INTERNAL STRUCTURE AND OTHER CHARACTERISTICS OF CALLUS TISSUE

#### MORPHOLOGY

Callus tissue proliferate as amorphous mass of cells having no regular shape. So, it is very difficult to describe its external morphology. Apparently, all callus tissue derived from different plants looks alike i.e. hazardous mass of cells,

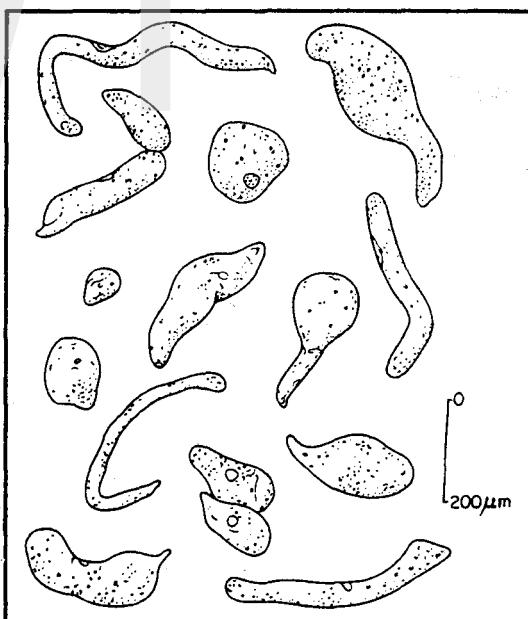
but they can be distinguished on the basis of other characteristics such as texture, colouration, hormone requirements etc. On that basis, even callus tissue initiated from explants (such as stem, leaf, root, petiole, flower etc.) of the same plant species may show considerable variations.

#### INTERNAL STRUCTURE

Internal structure of the callus tissue is revealed by light microscopy and electron microscopy.

#### Light Microscopic Study

Microtome section or squash preparation of the callus tissue shows that the cellular composition of the callus tissue is extremely heterogeneous ranging from small cells with dense cytoplasm to large cells with vacuolated cytoplasm. The shape of the cells within the callus tissue varies from spherical to markedly elongate (Fig 3.2). Large elongated cells are generally nondividing cells having a large central vacuole whereas the small actively dividing cells are with



□ Fig 3.2

**Drawing of cells from a friable callus of carrot showing the diversity in shape and size**

reduced vacuole size and dense cytoplasm. Elongated cells within the callus tissue may differentiate into lignified xylem tracheids or phloem-like cells. Formation of xylem and phloem within the callus tissue is known as cytodifferentiation. It has also been observed that some groups of meristematic cells constituting the active loci develop some small nests, scattered throughout the callus tissue. These are called meristemoids. The meristemoid may differentiate into either shoot or root primordia or embryoids.

### **Electron Microscopic Study**

Electron microscope also reveals that the cells at their non-dividing state have a large central vacuole and thin peripheral cytoplasm. The number of organelles is minimal in the cytoplasm. The synthesis of new cytoplasm and reduction in size of the central vacuole takes place when the cells enter into the dividing state. There is an increase of the endoplasmic reticulum, mitochondria, golgi bodies and ribosome. The endoplasmic reticulum occurs as sheets running parallel with the cell wall. The ribosome occur as polyribosome groups. The cell wall of the actively growing cells is thin. Sometimes cell walls develops irregular cellulosic thickenings which projects inside the cytoplasm. Microtubules are associated with the walls of actively growing cells. Cells from the green coloured zone contains chloroplasts, but generally the internal

system of the grana is poorly developed. More frequently the plastid acts as amyloplasts. Cultured cells contain membranous myelin-like bodies and membrane enclosed groups of vesicles which are known as multivesicular bodies.

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### **OTHER CHARACTERISTICS**

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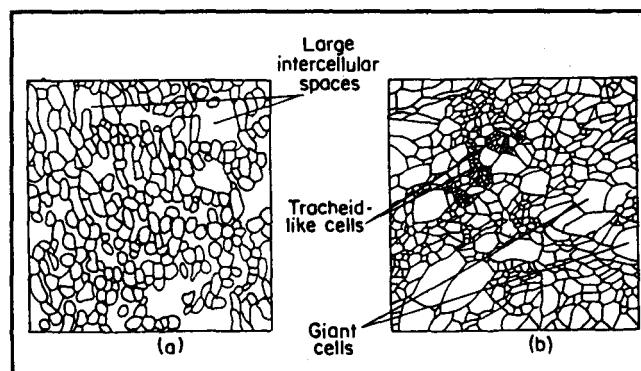
#### **Texture**

Callus tissue can vary considerably in appearance and texture. On the basis of texture, callus tissue can be divided into two categories such as soft callus and hard callus.

Soft callus is friable in nature and is made of heterogenous mass of cells having minimal contact (Fig 3.3). On the other hand, hard callus consists of giant cells, tracheid-like cells and closely packed cells i.e. compact in nature (Fig 3.3b). Hard callus may be nodular in form.

#### **Colouration**

Generally callus tissue is creamish yellow or white in colour. Sometimes callus tissue may be pigmented. Pigmentation may be uniform or patchy. Callus tissue may be green in colour. Sometimes, white callus tissue grown under dark condition turns green after transferring in light condition. Green colour develops due to development of chloroplastid in the cells of callus tissue



□ Fig 3.3

**Drawing of sections of soft, friable callus tissue (a) and compact callus tissue (b)**

e.g. callus tissue from the cotyledons of the soybean.

Callus may be yellow possibly due to synthesis of carotenoid pigments, e.g. callus tissue of *Nigella sativa* grown under dark condition. In some cauliflower cultures, callus tissue is purple in colour due to accumulation of anthocyanin in vacuoles or due to production of oxidized form of DoPA (3, 4 dihydroxy phenylalanine).

Brown colour frequently develops in the explant and subsequently in the callus tissue. This is mainly due to excretion of phenolic substances. Plant tissues contain large number of phenolic compounds and also contains polyphenoloxidase remaining spatially separated from the phenols. When the plant tissue is cut during explant preparation, the enzyme comes in contact with phenols which are then oxidized to quinones. These quinones then subsequently polymerize to form brown products. The formation of such quinones is responsible for the browning of the callus tissue. Excretion of phenols generally inhibit the growth of the callus tissue.

### Habituation of Callus Tissue

Generally, callus tissue needs growth hormones in the nutrient medium in order to grow as long as they are maintained through serial subcultures. But it has been observed that the callus tissue in some plant species may become habituated after prolonged culture. This means that the callus tissue is able to grow on a standard maintenance medium or basal medium which is devoid of growth hormones. This property of the callus tissue is known as habituation and the callus tissue is known as habituated callus tissue. The actual cause of habituation is not fully known. But it has been suggested that the cells in habituated callus tissue appear to have developed the capacity to synthesize adequate amount of auxins and/or cytokinins which probably accounts for their independence of exogenously supplied hormones. Habituated callus tissue cannot be distinguished from the normal callus tissue except in their hormone requirement.

The plant tumour tissue can be isolated from the plant and cultured aseptically. In culture, the tumour tissue is capable of growing on

simple basal medium (i.e. hormone-free)-like the habituated callus. These tissues differ from the habituated callus in their mode of origin. In case of wound tumour, the virus remains and multiplies within the cells and may disappear after prolonged periods in culture.

Crown gall tumour tissue are made free from bacteria artificially for culture. Otherwise the microorganisms soon overgrow the cells in culture. Alternatively, secondary tumours can be cultured directly. Presence of bacterial DNA in the genome of the crown gall tumour cells possibly makes them hormone-independent.

### Chromosomal Variation in Callus Tissue

Chromosomal variation may occur genetically or epigenetically in the cells of the callus tissue.

#### (a) *Genetical basis of Chromosomal Variation*

—Callus tissue is obtained from root, shoot, leaves and other organs. These organs are made of numerous cells which remain in different states of differentiation. Normally, *in vivo* meristematic diploid cells undergo selective division for the growth of an organ. On the other hand, endoreduplication is of frequent occurrence in the differentiated tissues of higher plants and the endoreduplicated cells remain in mitotically blocked condition. The degree of endoreduplication depends upon the degree of cellular differentiation. Therefore, the genomic constituent is heterogeneous in original explant. Callus tissue may get such genomic heterogeneity possibly due to non-selective induction of asynchronous division of both diploid and endoreduplicated cells. So, the pre-existing genomic heterogeneity of explant may be a source of chromosomal variation in the callus tissue. Variation of chromosome number ranges from aneuploidy to different level of polyploidy, such as tetraploid, hexaploid and so on. Occurrence of both diploid and different level of polyploid cells in the same callus tissue is known as mixaploid cell population.

(b) *Epigenetic basis of Chromosomal Variation*

—It has also been observed that at the early stage of callus growth, the percentage of diploid cell is generally higher than the percentage of polyploid cells. The number of polyploid cells may increase or decrease through serial subcultures. Again, highly meristematic cells are expected to be all diploid. But the callus tissue derived from meristem also shows the variation in chromosome number. *Crepis* is a plant in which cellular differentiation occurs without endoreduplication. Callus tissue obtained from the explant of such plant shows that all cells are not diploid. Thus, it appears that variation in chromosome number is not always derived from original explant. But it may come from the interaction of both genome and cytoplasm. Their interaction may bring about mitotic disturbance. In culture of pea, J. G. Torrey showed that kinetin encouraged the development of polyploid cells in culture. There is another common observation that strong auxin-like 2,4-D induces the polyploidy in callus culture.

It is also found that prolonged subculture may lead to the establishment of the one karyotype and others are gradually eliminated. In most of the cases polyploid cells are found. Therefore, whatever may be the cause of chromosomal instability, in fact, it is more or less a common cytological feature in most of the culture.

Sometimes, structural changes of chromosome-like deletion, translocation etc. may occur in culture. Gould (1982) used C-banding technique to show that three years old culture of *Brachycoma dischromosomatica* ( $2n = 4$ ) was pseudodiploid. There are also many other examples where structural change of chromosome occurs.

An ideal callus culture is characterised by the possession of numerical or structural stability in long term culture. But it is very rare. Cells of the callus tissue may be haploid if it is derived from microspore culture.

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## SIGNIFICANCE OF CALLUS CULTURE

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Callus culture as such has no major importance unless and until it is used for other experimental objectives. Still, callus culture has got some importance—

- (i) The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium. This phenomenon is known as plant regeneration or organogenesis or morphogenesis. Similarly, by manipulation of nutrient and hormonal constituents, cluster of embryos can be achieved directly from the somatic cells of callus tissue. These embryos are called somatic embryos. This phenomenon is known as somatic embryogenesis. Somatic embryo directly gives rise the whole plant.
- (ii) Callus tissue is good source of genetic or karyotypic variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue.
- (iii) Cell suspension culture in moving liquid medium can be initiated from callus culture.
- (iv) Callus culture is very useful to obtain commercially important secondary metabolites. If a bit of tissue from a medicinally important plant is grown *in vitro* and produced callus culture, then secondary metabolites or drug can be directly extracted from the callus tissue without sacrificing the whole plant. So, this alternative technique helps the conservation of medicinal plants in nature.
- (v) Several biochemical assays can be performed from callus culture.

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## Summary

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Callus tissue means an unorganised proliferative mass of cells without any differentiation. In nature, sometimes callus or callus-like tissue

is found to form in various parts of intact plant either due to deep wound or due to some diseases. Callus tissue developed from the injured part of the plant is known as wound callus tissue which is formed by the division of either cortical cells or cambium cells. Callus-like outgrowth caused by some diseases is commonly known as gall or tumour. But the callus tissue, which is important to plant tissue culture, is produced experimentally from a small excised portion called explant of any living healthy plant. The explant is cultured aseptically on solid or liquid nutrient medium under controlled conditions.

For successful initiation of callus culture from the explant, three important criteria should be accomplished—(i) aseptic preparation of plant material, (ii) selection of suitable nutrient medium supplemented with appropriate ratio of auxin and cytokinin or only appropriate auxin, and (iii) incubation of culture under controlled physical condition. In most cases, successful callus culture depends upon the inclusion of plant growth hormones in the nutrient medium and for healthy callus growth usually both auxin and cytokinin are required. Incubation of culture under controlled physical conditions such as temperature, light and humidity is indispensable for the proper initiation of callus tissue. The growth of the callus tissue generally takes place in presence of artificial cool light (approximately 2,000 to 3,000 lux for 16 hrs.) or in totally dark condition. The suitable temperature for the growth of callus tissue is usually  $25 \pm 2^\circ\text{C}$ . Generally 55% to 60% relative humidity is maintained in the culture room. Callus culture can be maintained continuously by serial subcultures.

Formation of the callus tissue is the outcome of cell expansion and cell division of the cell of the explant. By the influence of exogenously supplied hormone, the explant is triggered off a growth sequence in which cell enlargement and cell division predominate to form an unorganised mass of cells. Both endogenous product and exogenous hormones make a threshold level and their interaction results in the formation of unorganised cellular growth at the cut end of the explant. There is a general concept that auxin is required for growth and cytokinin is required

for cell division. This concept is applicable for *in vitro* callus formation. The callus tissue gradually increases in mass as the new cells are added by mitosis.

Calluses can vary considerably in appearance and texture. The shape of individual cells within the callus mass ranges from near-spherical to markedly elongated. In some instances, there may be some differentiation within the cell mass with phloem cells and lignified xylem cells becoming apparent. Generally callus tissue is creamish yellow or white in colour. Sometimes callus tissue may be pigmented. Brown colour frequently develops in the explant and subsequently in the callus tissue due to excretion of phenolic substances.

In some plant species the callus tissue may become habituated after prolonged culture. This means that the callus tissue is able to grow on culture medium which is devoid of growth hormones. Callus tissue derived from the plant tumour tissue or crown gall tissue is also capable of growing on simple basal medium-like the habituated callus. Chromosomal variation may occur genetically or epigenetically in the cells of the callus tissue. Sometimes, numerical or structural changes of chromosome may occur in culture.

The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium. This phenomenon is known as plant regeneration or organogenesis or morphogenesis. Similarly, by adjustment of hormonal condition in culture, embryoids can be achieved by the process of somatic embryogenesis. The embryo ultimately gives rise to the whole plant. Callus tissue is a good source of genetic variability, so it may be possible to regenerate a plant from genetically variable cells. Callus culture is very useful to obtain commercially important secondary metabolites.

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## Questions for Discussion

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1. What is callus tissue? Describe the protocol and discuss the significance of callus culture.

2. How the callus tissue is formed *in vitro*? Discuss the morphology, internal structure and other characteristics of the callus tissue.
3. Write brief answers to the following questions—
- Describe the ultrastructure of callus tissue.
  - Why do the explant and the callus tissue of some plants develop brown colour in culture?
  - What is habituated callus tissue? How is it formed?
  - What are the main reasons of chromosomal variation in callus tissue? State the significance of chromosomal variation in callus culture.
- (4) Write short notes on—
- Wound callus tissue
  - Internal structure of callus tissue
  - Difference between soft and compact callus tissue
  - Pigmented callus tissue
  - Habituation of callus tissue
  - Genetic basis of chromosomal variation of callus tissue
  - Significance of callus culture.

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## Chapter Four

### Cell Suspension Culture

#### DEFINITION

Suspension culture is a type of culture in which single cells or small aggregates of cells multiply while suspended in agitated liquid medium. It is also referred to as cell culture or cell suspension culture.

#### BRIEF PAST HISTORY

**W. H. Muir (1953)**—First reported that the fragments of callus of *Tagetes erecta* and *Nicotiana tabacum* could be cultured in the form of cell suspension.

**L. Nickel (1956)**—Described the continuous growth of a variety of *Phaseolus vulgaris*.

**F. C. Steward and E. M. Shantz (1956)**—Reported the suspension cultures from carrot root explant and obtained very large number of plantlets from the culture.

#### PRINCIPLE

Callus proliferates as an unorganised mass of cells. So it is very difficult to follow many

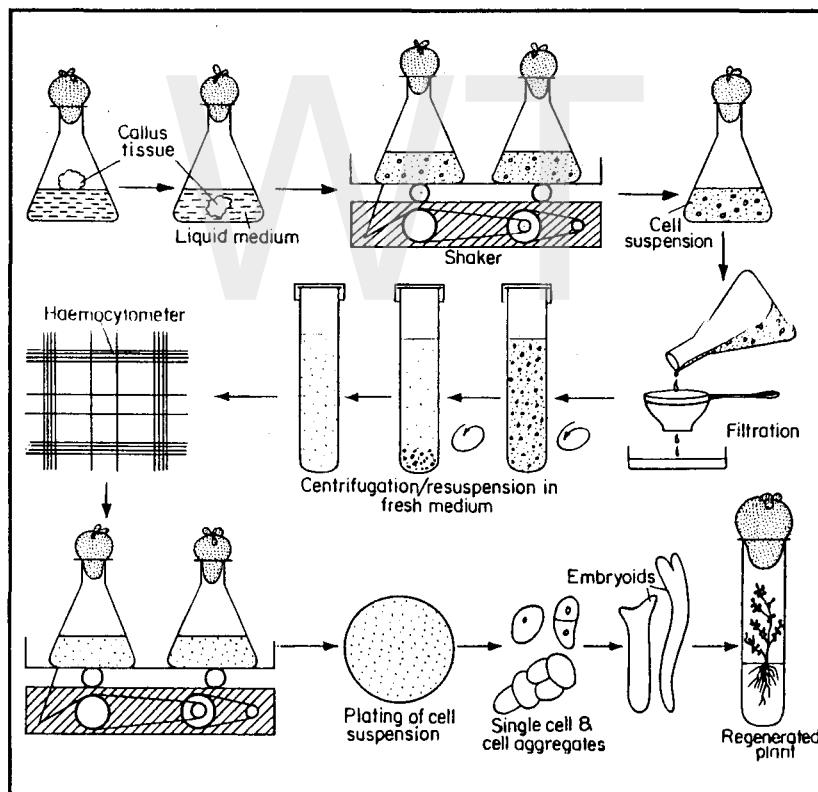
cellular events during its growth and developmental phases. To overcome such limitations of callus culture, the cultivation of free cells as well as small cell aggregates in a chemically defined liquid medium as a suspension was initiated to study the morphological and biochemical changes during their growth and developmental phases. To achieve an ideal cell suspension, most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses. After eliminating the large callus pieces, only single cells and small cell aggregates are again transferred to fresh medium and after two or three weeks a suspension of actively growing cells is produced. This suspension can then be propagated by regular sub-culture of an aliquot to fresh medium. Ideally suspension culture should consist of only single cells which are physiologically and biochemically uniform. Although this ideal culture has yet to be achieved, but it can be achieved if it is possible to synchronize the process of cell division, enlargement and differentiation within the cell population. The culture of single cells and cell aggregates in moving liquid medium can be handled as the culture of microbes. The suspension culture eliminates many of the disadvantages ascribed to the callus culture on agar medium. Movement of the

cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of the cells due to gravity and eliminates the nutrient gradients within the medium and at the surface of the cells.

## PROTOCOL

1. Take 150/250 ml conical flask containing autoclaved 40/60 ml liquid medium (Fig 4.1).
2. Transfer 3–4 pieces of pre-established callus tissue (approx. wt. 1 gm each) from the culture tube using the spoon headed spatula to conical flasks.
3. Flame the neck of conical flask, close the mouth of the flask with a piece of aluminum foil or a cotton plug. Cover the closure with a piece of brown paper.

4. Place the flasks within the clamps of a rotary shaker moving at the 80–120 rpm (revolution per minute)
5. After 7 days, pour the contents of each flask through the sterilized sieve (pore diameter –60 $\mu$ –100 $\mu$  and collect the filtrate in a big sterilized container. The filtrate contains only free cells and cell aggregates.
6. Allow the filtrate to settle for 10–15 min. or centrifuge the filtrate at 500 to 1,000 rpm and finally pour off the supernatant.
7. Resuspend the residue cells in a requisite volume of fresh liquid medium and dispense the cell suspension equally in several sterilized flasks (150/250 ml). Place the flasks on shaker and allow the free cells and cell aggregates to grow.



□ Fig 4.1

**Flow diagram illustrating the method of cell suspension culture and regeneration of plant through embryogenesis**

8. At the next subculture, repeat the previous steps but take only one-fifth of the residual cells as the inoculum and dispense equally in flasks and again place them on shaker.
9. After 3-4 subcultures, transfer 10 ml of cell suspension from each flask into new flask containing 30 ml fresh liquid medium.
10. To prepare a growth curve of cells in suspension, transfer a definite number of cells measured accurately by a haemocytometer to a definite volume of liquid medium and incubate on shaker. Pipette out very little aliquot of cell suspension at short intervals of time (1 or 2 days interval) and count the cell number. Plot the cell count data of a passage on a graph paper and the curve will indicate the growth pattern of suspension culture.

#### **GENERAL ACCOUNT OF SUSPENSION CULTURE**

Suspension culture can be initiated either from pre-established callus culture or from the explant directly into liquid medium which is continuously agitated on a moving elliptical or rotary shaker. The movement of the nutrient medium provides vital aeration of the medium to

sustain cell respiration in the liquid medium and also encourages the callus tissue to break up. As cell division starts in the callus tissue or on the explant surface, they shed and disperse directly into the medium. More friable callus tissue is an ideal material for the dispersion of cells. The dispersion of less friable tissue may be improved by modifying the nutrient medium. Increasing the concentration of auxins or adding very low concentration of cellulase and pectinase enzymes in the liquid medium are also effective for the dispersion of cells. The most dispersed culture so far established e.g. suspension culture of sycamore, consist of cell aggregates as well as single cells (Fig 4.2).

The period of incubation during which the suspension culture is developed from callus tissue is usually called as the initiation passage. During this passage not only do the callus tissues break up but the cells grow and divide until the depletion of some nutrient in the medium. Suspension culture is established in conical flasks (150 ml, 250 ml) and is incubated on a shaker at the speed of 80-120 rpm. The cell suspension developed at the initial passage is passed through a nylon mesh to remove the larger pieces of callus tissue. The filtrate containing small cell aggregates and single cells is transferred into fresh liquid medium to initiate passage 1 suspension.

In the subsequent passages cell suspension is subcultured by pipetting off aliquots of the suspension into new flasks of culture medium. In general, the media suitable for growing callus cultures for a particular species are also suitable for growing suspension cultures providing that agar is omitted. In some cases, the concentration of auxins and cytokinins is often critical for the growth of cell suspension and the concentration of auxins and cytokinins used for callus culture is generally reduced for suspension culture.

The cells within the aggregates are in a different microenvironment from the free floating cells. The cells in suspension may vary in shapes and sizes. They may be oval, round, elongated, coiled etc. Although suspension cultures consist entirely of thin-walled cells, others possess a proportion of lignified, tracheid like elements. These usually arise in the cell aggregates.

Fig 4.2

**Cells from a suspension culture of sycamore**

The cell aggregates within a suspension may give rise to whole plants by formation of root and shoot primordia e.g. suspension culture of *Atropa belladonna*. An alternative pathway of development of cell aggregates can lead to the formation of embryo-like structures or embryoids. e.g. suspension culture of carrot. During embryo formation, a single cell divides and follows two-celled stage, five-celled stage multicellular globular stage, heart-shaped stage etc. Each embryoid is a bipolar structure and results from divisions of a single cell. Embryoids, when mature, can be grown into a plantlet.

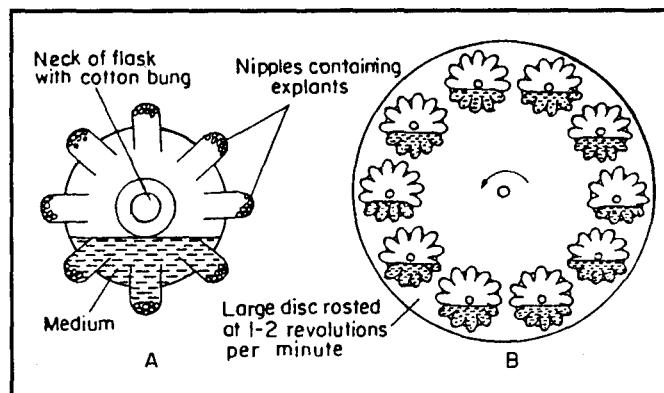
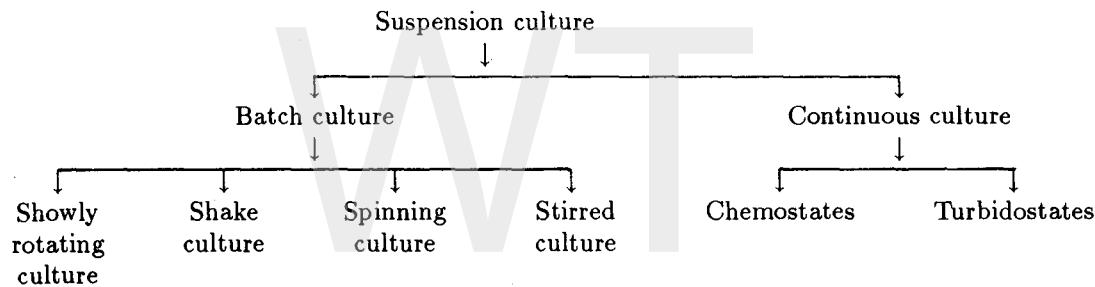
It may be more convenient to transfer cell aggregates from liquid medium and plate them on to solidified medium to grow as a callus. It is possible to plate out single cells from a suspension culture and raise a callus from them. By this way, a whole plant could be regenerated from a single cell.

## DIFFERENT CATEGORIES OF SUSPENSION CULTURE

Broadly speaking, there are two types of suspension cultures (i) Batch cultures and (ii) Continuous culture. Both types are again subdivided into different categories. Each type of culture has its own advantages and all types are being used in practice.

### BATCH CULTURE

Batch culture is a type of suspension culture where the cell material grows in a finite volume of agitated liquid medium. For instance, cell material in 20 ml or 40 ml or 60 ml liquid medium in each passage constitute a batch culture. Batch suspension cultures are most commonly maintained in conical flasks incubated on orbital platform shakers at the speed of 80-120 rpm.



□ Fig 4.3

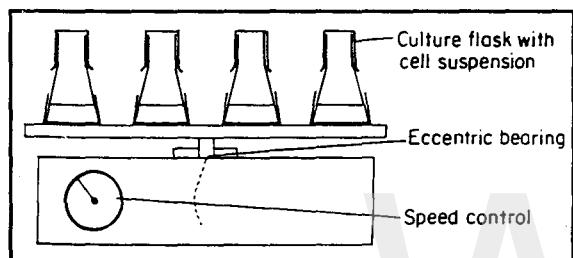
**A. Detail of a nipple flask. B. Large disc loaded with 10 nipple flasks used for growing cell suspension cultures**

## **Slowly Rotating Cultures**

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple-like projections. The capacity of each flask is 250 ml. Ten flasks are loaded in a circular manner on the large flat disc of a vertical shaker (Fig 4.3). When the flat disc rotates at the speed of 1-2 rpm, the cell within each nipple of the flask are alternately bathed in culture medium and exposed to air.

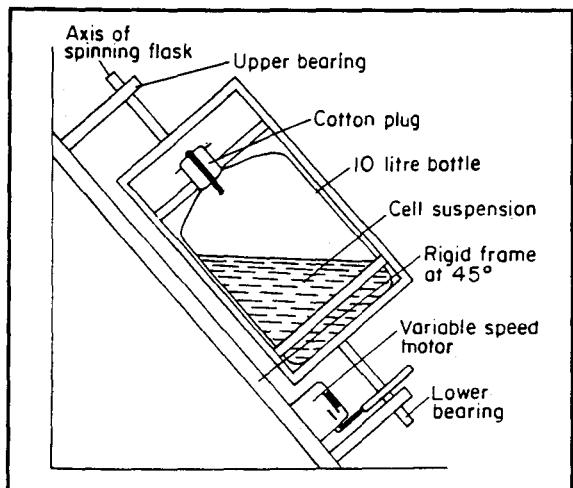
## **Shake Cultures**

It is a very simple and effective system of suspension culture. In this method, single cells



□ Fig 4.4

**Side view of a platform shaker loaded with suspension cultures contained in conical flasks**



□ Fig 4.5

**Diagram of a 10 litre spinning culture apparatus**

and cell aggregates in fixed volume of liquid medium are placed in conical flasks. Conical flasks are mounted with the help of clips on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at the speed of 60-180 rpm (Fig 4.4).

## **Spinning Cultures**

Large volumes of cell suspension may be cultured in 10L bottles which are rotated in a culture spinner at 120 rpm at an angle of 45° (Fig 4.5).

## **Stirred Culture**

This system is also used for large-scale batch culture (1.5 to 10 litres). In this method, the large culture vessel is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through culture medium. The use of an internal magnetic stirrer is the most convenient way to agitate the culture medium safely. The magnetic stirrer revolves at 200-600 rpm. The culture vessel is a 5 to 10 litres round-bottom flask (Fig 4.6).

## **CONTINUOUS CULTURE SYSTEM**

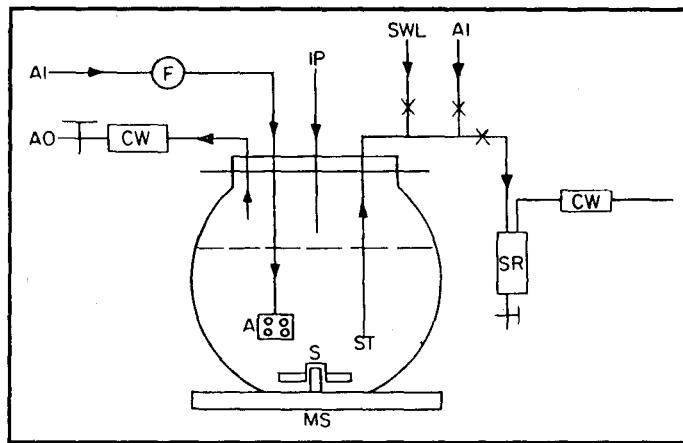
This system is very much similar to stirred culture. But in this system, the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. Normally, the liquid medium is not changed until the depletion of some nutrients in the medium and the cells are kept in the same medium for a certain period. As a result, active growth phase of the cell declines the depletion of nutrient. In continuous culture system, nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase.

## **Chemostats**

In this system, culture vessels are generally cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction of and removal of cells and medium. The

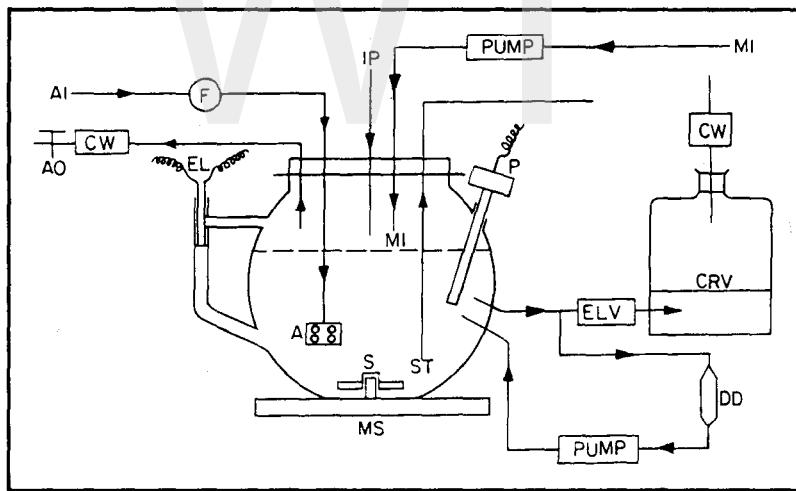
liquid medium containing the cells is stirred by a magnetic stirrer. The introduction of fresh sterile medium, which is pumped in at a constant rate into the vessel is balanced by the displacement of an equal volume of spent or old medium and cells. Such a system can be maintained in a

steady state so that new cells are produced by division at a rate which compensates the number lost in the outflow of spent medium. Thus in a steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant. Such continuous



□ Fig 4.6

**Stirred batch culture unit.** Arrow indicate direction of flow of air; AI = air input; F = sterilizing glass-fibre air filter; AO = air outlet; CW = cotton wool; IP = Inoculation port; A = aerator; S = stirrer magnet; ST = sample tube; MS = magnetic stirrer; SWL = sterile water line; SR = sample receiver; (Diagram after Dr. P. King)



□ Fig 4.7

**Chemostat culture.** Arrows indicate direction of flow of liquid; AI = air input; F = sterilizing glass-fibre; AO = air output; CW = cotton wool; EL = volume-sensing electrodes; ELV = volume controlling outlet valve; MI = medium input; S = stirrer magnet; ST = sample tube; P = probe for oxygen tension; DD = density detector, CRV = culture receiving vessel; MS = magnetic stirrer; IP = Inoculation port (Diagram after Dr. P. King)

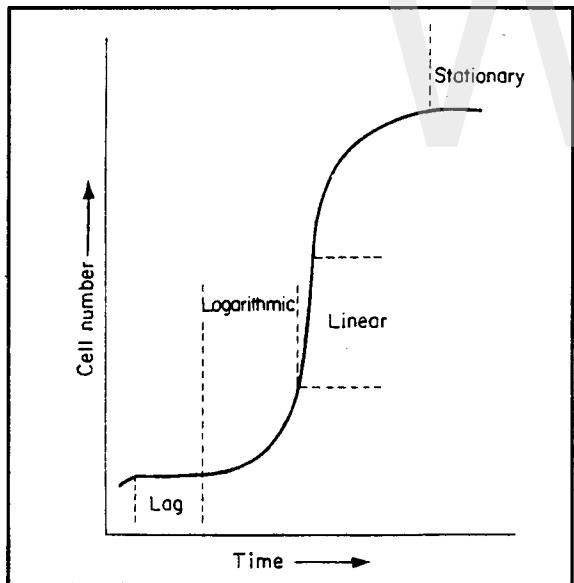
cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants (Fig 4.7).

### Turbidostats

The turbidity of a suspension culture medium changes rapidly when the cells increase in number due to their steady state growth. The changes of turbidity of the culture medium can be measured by the changes of optical density of the medium. Again, the pH of the medium changes due to increase of cell density. In turbidostates, an automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way as to maintain the optical density or pH at a chosen, preset level.

### GROWTH PATTERNS IN SUSPENSION CULTURE

Under appropriate light, temperature, aeration and nutrient medium the growth of suspension culture follows a predictable pattern or growth curve. The growth of suspension culture



□ Fig 4.8

**The growth curve for a typical higher plant suspension culture showing the major phases of growth**

can be monitored very easily by simply counting the cell number per unit volume of culture in relation to days of culture. From such data a typical growth curve can be prepared on a graph paper. The growth curve for a typical higher plant suspension culture consists of lag phase, logarithmic phase or exponential phase, linear phase and stationary phase (Fig 4.8). The lag phase is the period where the cells adjust themselves to the nutrient medium and undertake all the necessary synthesis prior to cell division. This is followed by very rapid cell division causing a logarithmic increase in cell number. This phase is called as logarithmic phase. A further period of rapid cell division results in a linear increase in number and the phase is called linear phase. As nutrients are depleted and some of the cells of the culture begin to show senescent characteristics, the rate of cell division within the culture declines and it passes through the stationary phase. At this stage the growth curve forms a plateau. If the cells are removed just before or just after the entry into stationary phase in each growth cycle and are subcultured to fresh medium, then identical patterns of growth of the cell line can be maintained in each culture passage. Dry weight, total protein, DNA synthesis etc. can also be considered as other parameters for the preparation of identical growth curves. It also indicates that the chemical composition of the cell changes throughout the growth cycle and such changes are closely coupled to the cell division in most of the plant material. However, in some material there is no corresponding increase in dry weight accumulation and consequently the divergence between the rate of cell division and rate of dry weight accumulation increases. From these studies, it has been concluded that there are independent mechanisms for controlling cell division and many biosynthetic pathways. A synchronized cell populations and the continuous changes in physiological property may also cause the divergence between the rate of cell division and the biochemical changes of the cell. It is also important to note that the degree of cellular aggregation is not constant but changes significantly during the growth cycle of the suspension culture. As the culture enters the period of most active growth the cell aggregation is maximum

and during the stationary phase cell aggregation is minimal.

For experimental studies on growth of cell suspension, the inoculum or cell density is an important factor. Very low density or high density of cells in liquid medium are unable to grow. So, to induce the growth, an initial density of  $2 \times 10^6$  cells/ml to  $2 \times 10^8$  cells/ml are inoculated in liquid medium. This initial density increases during growth and attains a higher density at the stationary phase. Most commonly, such high cellular density are diluted on subculture by a factor of *ca.*  $\times 10$ . The particular initial cell density that is able to grow in liquid medium is called critical initial density (CID). The CID may vary from plant to plant.

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### MEASUREMENT OF CELL GROWTH IN SUSPENSION CULTURE

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The cells in suspension culture grow by cell division and the number of cells increases. Growth studies of this kind are very valuable for the characterisation of cell lines, effect of nutrient medium and hormones etc. Growth in such cultures can be monitored by determination of cell number, cell dry weight, packed cell volume, etc.

The rate of increase of cell number can be calculated simply by counting the cell number in haemocytometer under a microscope. Cell count data obtained from haemocytometer is multiplied by a factor  $\times 10^3$  and the result can be expressed in terms of cell number per unit volume of culture. Therefore, by comparing the cell number at the beginning of culture and after certain days of incubation, the growth can be measured.

Again, as the cells increase in number during growth, the liquid medium will be more turbid and as a result the optical density (OD) of the suspension culture will also be altered. The changes of OD value can be detected by a calorimeter. Therefore, from OD value growth can be measured.

Definite volumes of cell suspension can be harvested from multiple replicated sets of culture. Such amount of cell suspension is transferred in a graduated conical centrifuge tube and

is centrifuged at 2,000X g for 5 minutes. The cells will form a pellet after centrifugation. The volume of cell pellet then represents the packed cell volume (PCV). It is also called biomass volume. Therefore, harvesting the cell suspension at definite periods of interval and measuring the PCV, the growth can be monitored and expressed as millilitre cell pellet per millilitre culture. From the same experiments dry weight of cell mass, can also be estimated by drying the pellet in a hot air oven (12 hours at 60°C) after replacing the supernatant and weighing the dried cell mass in a chemical or electrical balance. In this method, growth can be expressed in terms of dry weight in gram or milligram per unit volume of culture.

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### TEST FOR VIABILITY OF CELL

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Cell death may occur in suspension cultures due to several factors. So, for the studies on growth the test for viability of cells is very important. Otherwise, cell count data will be erroneous without testing the viability. The most frequently used staining method for assessing cell viability is fluorescein diacetate (FDA). FDA dissolved in 5 mg/ml of acetone is added to cell population at 0.01% final concentration. Dead cells fluoresces red.

Evans blue also used at a final concentration of 0.01% is specific for dead cells. As soon as the stain is mixed with cell suspension, the inviable cells stain blue and the viable cells remain unstained.

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### IMPORTANCE OF CELL SUSPENSION CULTURE

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The culture of single cells and small aggregates in moving liquid medium is an important experimental technique for a lot of studies that are not correctly possible to do from the callus culture. Such a system is capable of contributing many significant informations about cell physiology, biochemistry, metabolic events at the level of individual cells and small cell aggregates. It is also important to build up an understanding of an organ formation or embryoid formation starting from single cell or small cell aggregates. The

technique of plating out cell suspension on agar plates is of particular value where attempts are being made to obtain single cell clones. Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids and a considerable amount of industrial effort is being placed on the exploitation and expansion of this area.

Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised. Cell population in a suspension can be subjected to a range of mutagenic chemicals e.g. ethyl methane-sulphonate (EMS), N-nitroso-N-methyl urea etc. The mutagens can be added directly in the liquid medium. After the mutagen treatment, cells are plated on agar medium for the selection of mutant cell clones. The hope is that permanent changes in the DNA patterns of some of the cells would be achieved by such treatments. Plants could be raised from the mutant cell clones and the mutant plants are selected from the population either by morphological differences or by metabolic/biochemical differences. The selected plants can then be grown on and propagated further to produce a mutant population for evaluation studies.

## Summary

Cell suspension culture is a type of culture in which cells or small aggregates of cells multiply while suspended in agitated liquid medium. It is also referred to as cell culture or cell suspension culture. To achieve an ideal cell suspension, most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses. Ideally, suspension culture should consist of only single cells which are physiologically and biochemically uniform. Although this ideal culture has yet to be achieved, but it can be obtained if it is possible to synchronize the process of cell division, cell enlargement within the cell population. Therefore, culture of single cells and small cell aggregates in moving

liquid medium can be referred as cell suspension culture. Movement of the cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of the cells due to gravity and eliminates the nutrient gradients within the medium and at the surface of the cells.

Suspension culture can be initiated either from pre-established friable callus culture or from the explant directly into liquid medium which is continuously agitated on a moving elliptical or rotary shaker at the speed of 80-120 rpm. The cell suspension developed at the initial passage is passed through a nylon mesh and the filtrate containing small cell aggregates and single cells are transferred into fresh liquid medium. The concentration of auxins and cytokinins used for callus culture is generally reduced for suspension culture. The cells in suspension may vary in shape and size. Non-friable callus tissue is sometimes used for suspension culture. As the cells do not separate easily, so auxin concentration of the liquid medium is increased or enzymes such as cellulase or pectinase are added to help dissociation. Critical initial density or minimum effective density is the smallest inoculum from which a new suspension culture can be successfully grown.

Broadly speaking, there are two types of suspension cultures—(i) Batch cultures and (ii) continuous culture. Batch culture is a type of suspension culture where the cell material or inoculum grows in a finite volume of agitated liquid medium. It is again subdivided into (i) Slowly rotating culture (ii) Shake culture (iii) Spinning culture and (iv) Stirred culture.

Slowly rotating culture means the culture of single cells as well as cell aggregates in the nipple flasks which are loaded in a circular manner on the large flat disc of a vertical shaker moving at the speed of 1-2 rpm. The cells within each nipple are alternately bathed in culture medium and exposed to air. In shake culture, cell suspension is taken in a conical flask which is mounted on a horizontal large square plate of an orbital platform shaker moving at the speed of 60-180 rpm. In spinning culture, large volumes of culture medium may be cultured in 10L bottles which are rotated in a culture spinner at 120 rpm at an

angle of 45°. Large culture vessel in stirred culture is not rotated but cell suspension inside the vessel is kept dispersed continuously by either bubbling sterile air or using an internal magnetic stirrer moving at the speed of 200-600 rpm.

Continuous culture system is very much similar to stirred culture. But, in this system, the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. In this method nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase. Continuous culture system can be divided into two categories such as—(i) Chemostats and (ii) Turbidostats. Chemostat is an open, continuous culture in which growth rate and cell density are held constant by a fixed rate of input of growth-limiting nutrient. Turbidostat is also an open continuous culture into which fresh medium flows in response to an increase in the turbidity of the culture.

Under suitable conditions, the growth of suspension culture follows a predictable pattern or growth curve. The growth curve for a typical suspension culture consists of lag phase, logarithmic phase or exponential phase, linear phase and stationary phase. The cells in suspension culture grow by cell division and the number of cells increases. The rate of increase of cell number can be calculated by various ways—(i) by counting the cell number in haemocytometer under a microscope, (ii) detecting the changes of optical density of the cell suspension by a colorimeter, (iii) by measuring the packed cell volume (PCV) (iv) by measuring the dry weight of cell mass per unit volume of culture at regular interval. For the studies on growth, the test for viability of cells is very important. It can be determined by the use of some stains like fluoresin diacetate, Evans blue etc.

Suspension culture is a very important experimental technique for a lot of studies. It can be used for obtaining single cell clones by plating cell suspension on agar plates. Plants can be regenerated from such clones by the process of embryogenesis. Cell culture may be used for the whole or partial synthesis of secondary plant

products, such as alkaloids, glycosides etc. Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised.

## Questions for Discussion

1. What do you mean by cell suspension culture? Give a general account of cell suspension culture.
2. Give an outline of the method of cell suspension culture and describe the different categories of suspension culture.
3. Discuss the growth pattern of cells in suspension culture. Describe the methods for the measurement of cell growth in suspension culture. How do you test the viability of cells from culture?
4. Give an outline of the principle of cell suspension culture and its application in biological studies.
5. Write short answers to the following questions—
  - (a) What are the advantages of suspension culture over callus culture?
  - (b) How do you determine the stationary phase from the growth pattern in suspension culture?
  - (c) Suppose, 2ml of  $2 \times 10^5$  cells/ml were inoculated in 18 ml of liquid medium. What will be the density of cells per unit volume of medium after inoculation? If the cells grow just by doubling its initial number at 5 days interval, what will be the final density of cells per unit volume of medium after 20 days?
6. Write short notes on—(a) CID, (b) Turbidostat, (c) Chemostat, (d) Slowly rotating culture, (e) Batch culture, (f) PCV, (g) Mutagenesis studies by the use of cell suspension culture, (h) Cell suspension culture.

## Chapter Five

### Totipotency

#### INTRODUCTION

The higher plant body is a complex, multicellular organisation which is made of a variety of tissue and cell types that are essentially co-ordinated and integrated structurally and functionally. The cells may vary in size, shape, function and degree of differentiation. But, in fact, all these cells have a common origin. This is the single cell of the newly formed zygote. The zygote subsequently divides repeatedly and produces the embryo within the seed. The embryonic shoot and embryonic root meristem develop at the opposite poles of the embryo. Ultimately, the embryo produces the entire plant during seed germination. Thus it is clear that the original single cell (zygote) contains all information required for the production of a functional whole plant. These informations are stored in the chromosomal DNA of the nucleus. Exact copies of such information are duplicated during mitosis and then distributed in the daughter cells in each cell cycle. As each vegetative cell of the adult plant is effectively derived by mitosis from the early embryo, it is obvious that each of these cells must contain all genetic information needed for the growth and development of an entire plant. This inherent potential of a cell is known as totipotency.

#### BRIEF HISTORICAL BACKGROUND

The idea of culture of plant tissues was conceived in 1902 by Gottlieb Haberlandt of Berlin.

He prophesied that plant cells are "tituoitebt" i.e. isolated cells; if given the proper environment and nutrition they have the capacity to regenerate into entire plants. Actually, the concept of totipotency is implicit in the statement of cell theory as Scheiden and Schwann (1838) expressed the view that each living cell of a multicellular organism would be capable of developing independently if provided with the proper external conditions.

During last two decades the varied and continued experimentation by means of tissue culture technique has provided a vast fund of information and such information repeatedly proved the concept of totipotency *in vitro*.

#### WHAT IS TOTIPOTENCY ?

Totipotency is the genetic potential of a plant cell to produce the entire plant. In other words, totipotency is the cell characteristic in which the potential for forming all the cell types in the adult organism is retained.

#### EXPRESSION OF TOTIPOTENCY IN CULTURE

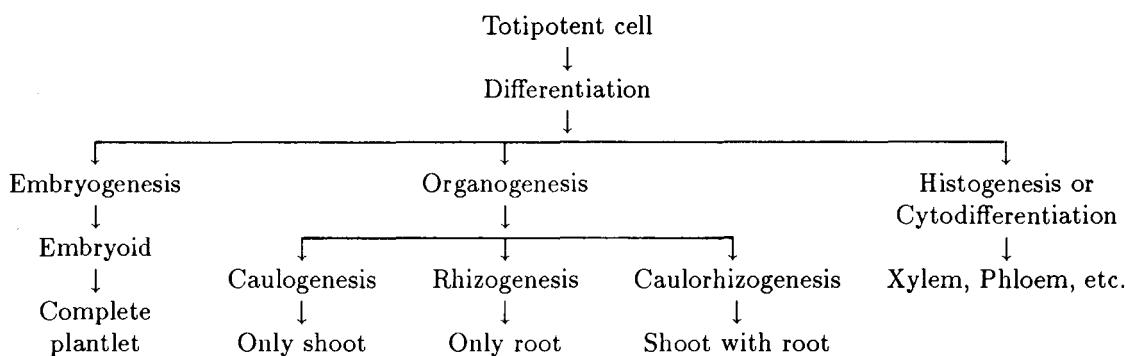
The basis of tissue culture is to grow large number of cells in a sterile controlled environment. The cells are obtained from stem, root or other plant parts and are allowed to grow in culture medium containing mineral nutrients, vitamins and hormones to encourage cell division and growth. As a result, the cells in culture will produce an unorganised proliferative mass of cells which is known as callus tissue. The

cells that comprise the callus mass are totipotent. Thus a callus tissue may be in a broader sense totipotent, i.e., it may be able to regenerated back to normal plant given certain manipulations of the medium and the cultural environment. Truly speaking, totipotency of the cell is manifested through the process of differentiation and the hormones in this process play the major role than any other manipulations. In the fifties, F. Skoog and C.O. Miller of U.S.A. discovered a new plant growth hormone kinetin from herring sperm DNA. With a correct concentration ratio of auxin and cytokinin in tobacco cultures, Skoog was able to demonstrate the role of kinetin in organogenesis. When the ratio of kinetin to auxin was higher, only shoot developed. This is known as caulogenesis. But when the ratio was lower, only roots were formed. This is known as rhizogenesis. Around the same period, F. C. Steward and his colleagues at Cornell University of USA, devised a method for growing carrot tissue by excising small disc, from the secondary phloem region of carrot root and placing them in a moving liquid medium under aseptic conditions. In presence of coconut milk in the medium, the phloem tissue began to grow actively. In moving liquid medium some single cells and small groups of cells were loosened from the surface of growing tissue. When these isolated cells were grown separately it was found that some single cells developed somatic embryos or embryoids by a process that occurs in normal zygotic embryo. It is also observed in some experiment that cells of some callus mass frequently differentiate into vascular elements such as xylem and phloem without forming any plant organs or embryoids. This process is known as

histogenesis or cytodifferentiation. Thus the totipotent cells may express themselves in different way on the basis of differentiation process and manipulation.

Where the totipotent cells are partially expressed or not expressed, it is obvious that the limitation on its capacity for development must be imposed by the microenvironments. The totipotency of cells in the callus tissue may be retained for a longer period through several subcultures. Practically, it is observed that the explant first forms the callus tissue in the callus inducing medium and such callus tissue is maintained through some subcultures. After then it is generally transferred to another medium which is expected to be favourable for the expression of totipotent cells. Actually, the regeneration medium is standarized by trial and error method. In more or less suitable medium, the totipotent cells of the callus tissue give rise to meristematic nodules or meristemoids by repeated cell division. This may subsequently give rise to vascular differentiation or it may form a primordium capable of giving rise to a shoot or root. Sometimes the totipotent cell may produce embryoids through sequential stages of development such as globular stage, heart shaped stage and torpedo stage etc.

After prolonged culture, it has been observed that calluses in some species (e.g. *Nicotiana tabacum*, *Citrus aurantifolia* etc.) may become habituated. This means that they are now able to grow on a standard maintenance medium which is devoid of growth hormones. The cells of habituated callus also remain totipotent and are capable to regenerate a plant without any major manipulation.



A typical crown gall tumour cell has the capacity for unlimited growth independent of exogenous hormones. It shows totally lack of organogenic differentiation. So such tissue is considered to have permanently lost the totipotentiality of the parent cells. In some plant species, the crown gall bacterium (*Agrobacterium tumefaciens*) induces a special type of tumour, called teratomas, the cells of which possess the capacity to differentiate shoot buds and leaves when they are grown in culture for unlimited periods.

Thus it is clear that the mode of expression of totipotency of plant cell in culture varies from plant to plant and also helps us to understand the process of differentiation *in vitro*.

## ARE ALL CELLS IN CULTURE TOTIPOTENT ?

A callus mass contains thousand of cells. Theoretically, if the totipotency of all cells are expressed at a time, then it is expected that equal number of shoots or roots or embryoids will be regenerated from such totipotent cells. But in experiment, such results are not obtained. The reason behind such limited expression of totipotency are many and vary from plant to plant. It has been observed that variation of chromosome number in the cells of callus tissue is one of the main factors that causes the limited expression of totipotency. This variability may be attributed to either pre-existing variation in the somatic cells of the explant (genetic) or variation generated during tissue culture (epigenetic). Changes in chromosome number are aneuploid, polyploid etc. As a result, a mixoploid callus tissue is formed in the subsequent growth. But, very often, from these mixoploid callus cultures, organogenesis and/or embryogenesis occur mostly from diploid cells. Therefore, all cells of the callus tissue are not able to express their totipotency.

Observation on organogenesis or embryogenesis in callus culture have led to propose that an association of cells may be sometimes necessary to provide the appropriate environment for certain individuals to express their totipotency.

It is also proposed that endogenous hormone level of a cell and exogenously supplied hormone makes a threshold level which actually induces the totipotent cell to express in culture.

But the cells that comprise the callus tissue absorb the hormones and the nutrients forming a gradient. Therefore, the availability of hormones is not equal to all cells. As a result, this fact imposes a barrier to reach a threshold level of hormone equally in all cells for their expression of totipotency.

In culture, some cells are highly recalcitrant and in such cases totipotent cells do not respond to any morphogenetic stimuli. These cells can not be easily differentiated.

Besides these factors, heterogeneity in physical structure of the cells make a great difference in the degree of chemodifferentiation of the cells that make an asynchronous situation for which all cells are not able to express their totipotency at a time. Therefore, it seems apparently that all cells are not totipotent. More or less homogeneous thin tissue layer such as epidermal tissue and free unicellular pollen grain, free cells from suspension culture, protoplasts are more efficient system for the synchronous expression of totipotency.

## IMPORTANCE OF TOTIPOTENCY IN PLANT SCIENCE

The ultimate objective in plant protoplast, cell and tissue culture is the reconstruction of plants from the totipotent cell. Although the process of differentiation is still mysterious in general, the expression of totipotent cell in culture has provided a lot of informations. On the other hand, the totipotentiality of somatic cells has been exploited in vegetative propagation of many economical, medicinal as well as agriculturally important plant species. Therefore, from fundamental to applied aspect of plant biology, cellular totipotency is highly important. Recent trends of plant tissue culture includes genetic modification of plants, production of homozygous diploid plants through haploid cell culture, somatic hybridization, mutation etc. The success of all these studies depends upon the expression of totipotency. In many cases, successful and exciting results have been obtained. Plant breeders, horticulturists and commercial plant growers are now more interested in plant tissue culture only for the exploitation of totipotent cells in culture according to their desirable

requirement. Totipotent cells within a bit of callus tissue can be stored in liquid nitrogen for a long period. Therefore, for germplasm preservation of endangered plant species, totipotency can be utilized successfully.

## Summary

As each somatic cell of the mature plant is effectively derived by mitosis from the zygotic cell, so it is obvious that each of these cells must also contain all the genetic information needed for growth and development of a whole organism. This impressive potential of the cell is known as totipotency. Therefore, totipotency is the genetic potential of a plant cell to produce the entire plant. The expression of totipotency of different types of cells such as parenchymatous cells, meristematic cells, leaf mesophyll cells, phloem cells and cambium cells pollen grains can be demonstrated by means of plant tissue culture technique. Truly speaking, totipotency of the cell in culture is manifested through the process of differentiation and the hormones in this process play a major role than any other manipulations. In the fifties, F. Skoog and C. O. Miller of USA advanced an hypothesis that the regeneration of an organ from the cultured cell or tissue is controlled by a balanced ratio between cytokinin and auxin. A relatively high auxin—cytokinin ratio induced root formation in tobacco callus tissue, whereas a low ratio of the same hormones favoured shoot production. The production of root is known as rhizogenesis and the production of shoot is known as caulogenesis. Somatic tissue of carrot has shown evidence of embryo initiation in single cell that are progenitors of bipolar embryos. In this instance, the balance of hormone levels led to the differentiation of embryo or embryoids by a process that occurs in normal zygotic embryos. It is also observed that in some experiment, the cells of some callus mass frequently differentiate into vascular elements without forming any plant organs or embryoids. This process is known as histogenesis or cytodifferentiation. Therefore, the totipotent cells may express themself in different way on the basis of differentiation process and manipulation.

When the totipotent cells are partially expressed or not expressed, it is obvious that the limitation on its capacity for development must be imposed by the microenvironments. The composition of the nutrient medium, which is expected to be favourable for the expression of totipotent cells, is actually standarized by trial and error method. The cells of habituated callus also remain totipotent and are capable of regenerating a plant without any major manipulation. The cells of crown gall tumour show the limited expression of totipotency. The mode of expression of totipotency of plant cell in culture varies from plant to plant.

In callus culture, all cells are not totipotent. Variation of chromosome number in the cells of callus tissue is one of the main factors that causes the limited expression of totipotency. Observation on organogenesis or embryogenesis in callus culture have led to propose that an association of cells may be sometimes required to provide the appropriate environment for certain individuals to express their totipotency. The unavailability of a threshold level of endogenous and exogenous hormone in all cells of callus tissue may impose a barrier for their expression of totipotency. Besides these factors, heterogeneity in physical structure of the cells make a grage difference in the degree of chemodifferentiation of the cells that make an asynchronous situation for which all cells are not able to express their totipotency at a time.

From fundamental to applied aspects of plant biology, cellular totipotency is highly important. The success of different aims and objectives of plant tissue culture depends upon the expression of totipotency.

## Questions for Discussion

1. What is meant by totipotency of plant cells? Give a general account of the expression of totipotency of cells in culture.
2. Why are all cells not totipotent in culture? Mention the importance of totipotency in plant science.
3. Write short notes on — (a) Totipotency; (b) Caulogenesis; (c) Rhizogenesis; (d) Histogenesis; (e) Importance of totipotency.

## Chapter Six

### Cytodifferentiation

#### INTRODUCTION

A multicellular explant is made of different types of cells. Some are dividing cells and some are non-dividing. But all the cells are derived from a single-celled zygote through the process of cell division and cellular differentiation. So the cells of the explant are present in differentiated state. When such an explant is brought into callus culture, most of the cells including the non-dividing mature cells within the explant start to divide and form a mass of undifferentiated callus tissue. This phenomenon is termed as "dedifferentiation" and the cells of the callus tissue are termed as dedifferentiated cells. When such dedifferentiated cell mass is placed in a medium which is suitable for plant regeneration, the cells again form the whole plant or plant organ. This phenomenon is known as "redifferentiation" and the inherent capacity of the plant cell to regenerate the whole plant is known as cellular totipotency. So for a differentiated cell of an explant to express its totipotency in tissue culture, it first undergoes the process of dedifferentiation and finally redifferentiation. During redifferentiation, few dedifferentiated cells of a particular culture system undergo cytoquiescence and cytosenescence. These two changes

are mainly associated with the process of cytodifferentiation which ultimately form the vascular tissue, particularly the xylem elements. Cytodifferentiation may or may not be related with the process of organogenesis and somatic embryogenesis.

#### WHAT IS CYTODIFFERENTIATION ?

In plant tissue culture, during growth and maturation of the callus tissue or free cells in suspension culture, few dedifferentiated cells undergo cytoquiescence and cytosenescence and these twin phenomena are mainly associated with redifferentiation of vascular tissue, particularly tracheary elements. The whole developmental process is termed as cytodifferentiation.

#### BRIEF HISTORICAL BACKGROUND

**C. Camus (1949)**—Grafted small vegetative buds on the upper surface of cultured root tissue of *Cichorium*, and after few days observed the differentiation of vascular strands in parenchymatous tissue below the bud.

**B. E. Struckmeyer (1949)**—Observed the histological effect of growth regulating substances on sunflower tissue of crown gall origin grown *in vitro*.

**R. H. Wetmore and S. Sorokin (1955)**

—Confirmed the work of Camus, using undifferentiated callus tissue (lacking vascular elements) of *Syringa vulgaris*.

**R. H. Wetmore and J. P. Rier (1963)**—

Experimentally induced vascular tissue in callus pieces of *Syringa vulgaris* by varying the sucrose concentration in presence of a low concentration of auxin.

**D. E. Fosket and L. W. Roberts (1964)**

—Observed the induction of wound vessel differentiation in isolated *Coleus* sp. stem segments *in vitro*.

**T. G. Torrey (1975)**—Observed that some

of the single parenchymatous cells differentiated directly into tracheary elements without preceding cell division.

**C. H. Bornman (1976)**—Reported the

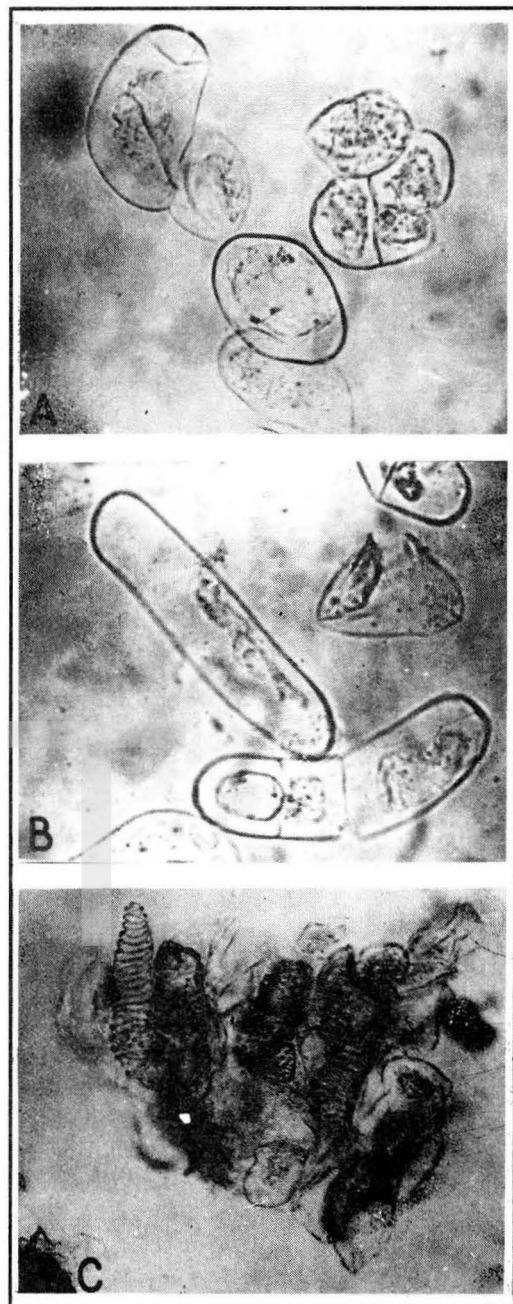
stimulatory interaction between auxin and gibberellin for xylem differentiation.

**K. K. De and S. C. Roy (1984)**—Studied

the role of an acid phosphatase isoenzyme in callus tissue during cytodifferentiation.

#### PRIMARY STEPS IN CYTODIFFERENTIATION

The fate of an individual cell in culture is variable and hence unpredictable. Amongst a group of cells within the callus tissue or free cells in cell suspension culture, a few cells become morphogenetically competent for cytodifferentiation which can not be identified at the early stage in advance. Cytodifferentiation occurs either spontaneously or under the stimulus of specific nutritional or hormonal factors. So, it is not conditioned by a single regular event. Preliminary steps in cytodifferentiation are reflected by a series of histological and biochemical changes in the cell. On the basis of numerous observations and publications, the proposed fate of the cultured cells during cytodifferentiation can be divided into three steps. The callus tissue at the time of its initiation and further growth shows a mixed population of small, more rounded oval and few elongated cells with dense cytoplasm. With an increase in subculture number, few



□ Fig 6.1

Showing morphological and histological changes in the cells of callus tissue during cytodifferentiation. Rounded and oval cells of the callus tissue up to 3 passages (A), elongated cells with thick wall (B) after 3 passages, and xylogenesis after 8 passages of culture (C)

cells become more elongated with a thick wall and the calli are friable. By further increasing the subculture number, callus tissue shows maximum xylogenesis with tracheary elements having a continuous spiral deposition of secondary wall materials (Fig 6.1 A-C). Actually, the initiation of xylogenesis takes place from mitotically blocked and elongated cells.

In ultrastructural studies of cytodifferentiation, it is evident that a chain of intracellular degradative changes are associated with cytodifferentiation. Autodestruction of cellular organelles such as chloroplast, endoplasmic reticulum, dictyosomes, ribosomes and mitochondria leading to loss of entire protoplasmic mass are the main of the degradative changes. The separation of bounding membrane of organelles are the first step in cytoquiescence leading to cytogenesis. These autophagic activity within the cell in turn is closely linked with certain hydrolytic enzymes. Acid phosphatase, a hydrolytic enzyme, is commonly present in the cell and has been detected in association with the cell wall, distycome, plastids and lysosomal systems. It is evident that the synthesis of acid phosphatase is indicative of autolysis of the protoplast during cytoquiescence and cytogenesis of the cell. Therefore, the transformation of living cells into the dead, empty tracheid during cellular differentiation and the biosynthesis of the acid phosphatase enzyme are functionally related to the autolysis of the cell contents and lignin biosynthesis for spiral deposition of secondary wall materials of the developing tracheary elements.

#### IS CELL DIVISION REQUIRED FOR XYLEM DIFFERENTIATION ?

Whether cell division is a prerequisite for xylem differentiation or not is a subject of debate. Studies have shown that tracheary element differentiation may be related to cell cycling activity, although some cells differentiate directly without any expression of mitotic division.

#### Evidences in support of cell cycle activity required for xylem differentiation

1. BUdR is an inhibitor of DNA synthesis. It has been observed that xylem differentiation is completely suppressed in  $10^{-5}$  M BUdR treated *Coleus* stem explants, pea root explant, Jerusalem artichoke tuber explant and lettuce pith tissue. Therefore, DNA synthesis is a critical factor in differentiation phenomenon.
2. High doses of gamma-irradiation also prevents both DNA synthesis and mitosis. So, gamma irradiated cells do not take part in xylem differentiation.
3. Malawer and Phillips (1979) also added some evidences to support the concept that xylem differentiation is preceded by mitotic division. They used  $^3\text{H}$  thymidine in the culture medium and observed the uptake of  $^3\text{H}$  thymidine by the cell up to xylem differentiation. From their experiment, they noted that xylem cell in culture had undergone three rounds of DNA synthesis.

#### Evidences in support of cell division not required for xylem differentiation

In some cases, cell division is not a prerequisite for xylem differentiation. This concept is supported by the following observations—

1. Inhibitor studies with caffeine and colchicine have provided evidence that wound xylem formation in *Pisum* roots is a direct differentiation process.
2. In cell culture of *Centanurea cyanus* some of the single parenchymatous cell differentiates directly into tracheary elements without cell division. But the opponents argued that the cells directly forming xylem elements could have been derived from a recent cell division because the cells were taken from fast-growing suspension culture.

## Evidences in support of both concept

1. In case of *Zinnia elegans*, mesophyll system consists of two different cell populations; some of mechanically isolated cells after a period of extension growth directly differentiate into xylem elements without a cell division, whereas others require cell cycle activity for differentiation.
2. In *Helianthus tuberosus*, direct differentiation of xylem without DNA replication and mitotic division occurs only when the explants are taken from immature tubers. But in mature tuber, xylem differentiation takes place only after cell division.

Therefore, the question of cell division prior to xylem differentiation remain unresolved. To answer the question, the more critical studies on diverse plant systems are required.

## PROTOCOL FOR THE STUDY OF CYTODIFFERENTIATION

Actually there is no specific methodology for the study of cytodifferentiation. During the maintenance of callus culture, or when the callus tissue is transferred in another medium for plant regeneration, differentiation of xylem elements may be frequently observed in the squashed preparation of the callus tissue grown in particular medium. Such medium with some modification can be used as medium for cytodifferentiation of a particular plant material. Differentiation of xylem element in the callus tissue of Cowpea (*Vigna unguiculata*) is described below—

1. The hypocotyl portion of aseptically grown seedling of *Vigna unguiculata* can be used as initial explant.
2. The explant is cultured in Murashige and Skoog's medium supplemented with 2, 4-D (2–4 mg/L) and kinetin (0.5 mg/L) at 25°C with 16 hrs. light.
3. Cultures are maintained in a serial subcultures with 28 days passage duration.
4. Time to time, callus tissue are harvested and macerated in 4% aqueous solution of

NaOH at 50°C. This treatment clears and softens the tissue.

5. NaOH solution is carefully replaced by 0.04% aqueous solution of safranin.
6. After 30 minutes, the dye solution is replaced by 1N HCl at 50°C.
7. After one hour, HCl is removed and glycerol is added. The acid destains the parenchyma cells but the lignified xylem retains the red dye.
8. Finally, a slide is made and observed under microscope.

## FACTORS AFFECTING CYTODIFFERENTIATION

A number of chemical and physical factors have been shown to have a profound effect on cytodifferentiation. All these factors affect the cytodifferentiation qualitatively and quantitatively.

### CHEMICAL FACTORS

Generally phytohormones and sugars influence greatly on cytodifferentiation. It is evident that auxin, cytokinin and gibberellin are involved in the process of cytodifferentiation.

#### Auxin

Auxin at low concentration stimulates xylelogenesis. There is an inverse relationship between the degree of xylem differentiation and auxin concentration. The influence of auxin in xylem differentiation has been demonstrated under *in vitro* by many workers.

Grafting a small vegetative bud on the upper surface of the callus tissue, it has been observed that, after few days, the differentiation of vascular tissue in callus tissue takes place below the bud (Fig 6.2(a)). This observation suggests that the stimulus of vascular tissue differentiation in callus tissue is provided by the vegetative bud. Now, if the direct contact between the bud and the callus tissue is broken by placing a semipermeable membrane at the

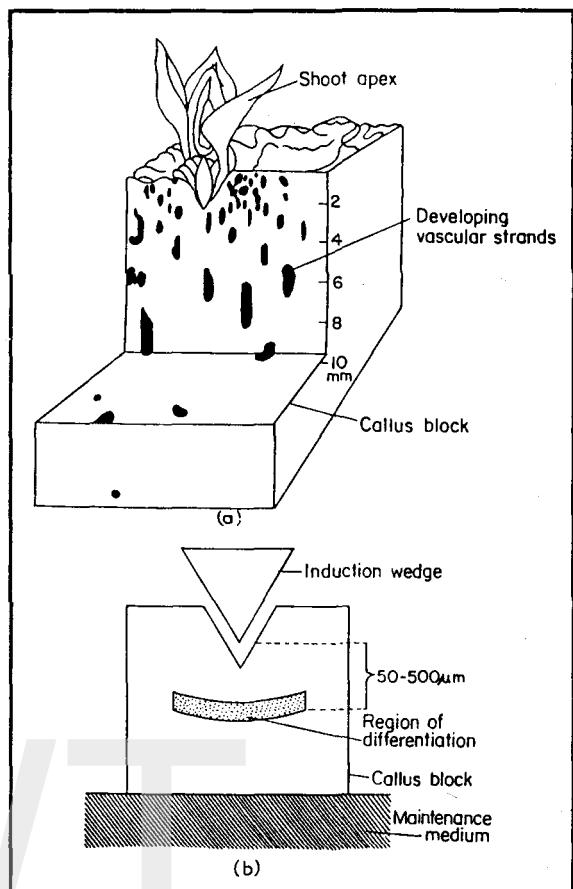
site of the graft, still the differentiation of vascular tissue occurs. This experiment indicates that the nature of stimulus is of diffusible chemicals. Further, a piece of callus tissue is placed on a medium which do not favour the differentiation of vascular tissue. The callus tissue is totally undifferentiated and is free from vascular elements. Now a V-shaped incision is made on the upper surface of the callus tissue and the V-shaped cavity is filled up with 1% agar block containing auxin and sucrose. The appearance of vascular tissue is again noticed in the callus tissue (Fig 6.2(b)). But in the control set the callus tissue is without any vascular tissue. Therefore, the above experimental evidence clearly indicates that auxin plays an important role in cytodifferentiation.

### Cytokinin

There are some evidences to put forward that cytokinin may also be involved in cytodifferentiation. It has been demonstrated that kinetin added medium containing coconut water enhances tracheidal differentiation in the callus tissue of *Nicotiana tabacum*. The stimulatory effect of cytokinin in xylogenesis has also been observed in the cytoledonary callus tissue of soybean. Nitsch and Nitsch (1960) on the other hand, have shown that storage parenchyma of Jerusalem artichoke tuber produced a natural cytokinin and cell division and cytodifferentiation could be the outcome of a complex interaction between exogenous auxin and endogenous level of cytokinins. Similarly, the exogenous cytokinins in combination with an auxin do markedly increase the quantity of tracheary elements has been shown by Bergmann. But in some cases, kinetin shows some inhibitory effect on xylogenesis e.g. *Coleus* stem callus, callus tissue of *Helianthus* and *Linum*. Therefore, the exact role of cytokinin is not clear and remains to be determined in several forms of tissue system.

### Gibberellin

The influence of gibberellin in cell division and xylem differentiation has been investigated under *in vitro* conditions in order to establish



□ Fig 6.2

**Induction of vascularization in callus tissue.**  
**(A)** Callus of *Syringa* into which has been grafted a stem apex bearing 2 or 3 leaf primordia. Drawing made 54 days after grafting. (After Wetmore, R. H. and Sorokin, S. (1955), Journal of the Arnold Arboretum, 36, 305). **(B)** Diagram to show the induction of vascularization in a block of *Phaseolus* callus into which has been inserted an agar wedge containing auxin and sucrose. (After Jeffs, R. A. and Northcote, D. H. (1967), Journal of Cell Science, 2, 77)

some quantitative relationship. Gibberellin interacting with auxin is effective in cell enlargement and tracheidal differentiation but not when used alone. For cultured pea root segments in auxin-cytokinin media, the percentage of xylem differentiation is rarely up to 20 in respect to total cell population, whereas auxin-cytokinin-

gibberellin treatments increase the response giving a higher percentage of differentiated cell compared to other combinations. The hormonal interactions among the three and, in particular, of the role of gibberellin in presence of auxin or auxin-cytokinin in xylogenesis, needs further exploration.

### Effect of Sugars

The sugar, particularly sucrose, in the medium is very essential for cytodifferentiation. Even the effect of auxin on vascular differentiation

seems to be closely dependent on the presence of sugar in the medium. Sucrose as an energy source is very important in cytodifferentiation. At lower levels of sucrose (1.5 to 2.5%) only xylem is formed and concentration above 4% favours a balance of xylem and phloem elements. Only disaccharides which contain an  $\alpha$ -glucosyl radical at the non-reducing end induce vascularised nodules in callus as in *Phaseolus*. As a carbon source, sugar serves a dual purpose—production and deposition of cellulose and concurrently of lignification of cell i.e. deposition of lignin in the lattices of the cellulosic microfibrils in secondary walls during cytodifferentiation of vessels and tracheids. Fructose, mannose, xylose, rhamnose, arabinose, galactose and mannitol (2%) have not shown any positive effect in vascular differentiation as compared to sucrose. Xylem differentiation to some extent occurs with the incorporation of cellobiose, lactose-raffinose and glucose (2%). Maltose (2%) could partially replace sucrose in xylem and phloem nodule differentiation. Glucose used alone causes the development of scattered xylem elements. Soluble starch (4%) stimulates xylem formation. It is interesting to note that only those carbohydrates which support significant cell division also support tracheary element formation. With stem callus of *Parthenocissus tricuspidata* the tissue remains parenchymatous at lower concentration of sucrose (1%) in the medium, but with increase in its concentration (2.5%), the number of xylem arcs and the number of tracheary member in each are proportionally high and each arc is flanked by an internal cambium.

## PHYSICAL FACTORS

There are very few reports of the effect of physical factors i.e. light, temperature etc. on vascular differentiation.

### Light

In general light has proved to be inhibitory in xylogenesis although in exceptional cases as in carrot, it can be a requirement but replaceable by cytokinin. The response to light varies depending on the nature and source of the tissue, being inhibitory in some and promotive in others.

### Temperature

The nature of vascular differentiation is influenced by temperature conditions—whereas high temperature (35°C) proves stimulatory to xylogenesis and formation of compact wood, as in Jerusalem artichoke (*Helianthus tuberosus*), low temperature causes the development of undifferentiated new tissue.

### Pressure

Factors such as increased pressure have been shown to be stimulatory to xylem differentiation. Probably through induced ethylene production.

### Water Stress

Water stress is also a controlling factor in the initiation of experimentally induced xylogenesis in cultured explants of *lactuca*.

## OTHER FACTORS

There are some other factors which play either positive or negative role for cytodifferentiation.

### Morphactins

Chlorflurenol inhibits xylogenesis in pith explants of lettuce possibly through inhibition of auxin transport. However, addition of cysteine

to chlorflurenol medium serves to nullify the inhibitory effect.

### Methionine

The culture medium supplemented with methionine (0.025 to 0.5  $\mu\text{M}$ ) enhances the induction of tracheid differentiation in majority of cases. It has been suggested that enhanced production of ethylene due to the use of methionine as a substrate, plays a role in xylem differentiation.

### Irradiation

Tissues subjected to ionising radiations have shown enhanced xylem differentiation. However, studies on the effect of X-irradiation (4000r) on protein synthesis suggest irradiation might inhibit xylogenesis by disrupting the normal cellular biochemical pathway for protein synthesis as xylem differentiation is thought to be dependent on protein synthesis.

### CONCLUSION

There are still some lacuna in our understanding of the cellular and biochemical aspects of cytodifferentiation. Tissue culture techniques offer not only an excellent opportunity to study the factors that elicit the cytodifferentiation but also allow investigation of factors controlling the differentiation of tracheary elements.

### Summary

In plant tissue culture, during growth and maturation of the callus tissue or free cells in suspension culture, few dedifferentiated cells undergo cytoquiescence and cytogenesis and these twin phenomena are mainly associated with redifferentiation of vascular tissue, particularly tracheary elements. The whole developmental process is termed as cytodifferentiation. Preliminary steps in cytodifferentiation are reflected by a series of histological and biochemical changes in the cell. On the basis of numerous

observations and publications, the proposed fate of the cultured cells during cytodifferentiation can be divided into three steps. The callus tissue at the time of its initiation shows a mixed population of small round oval cells with dense cytoplasm. With an increase in number of subculture few cells become elongated and further increasing the subculture member, callus tissue shows maximum, xylogenesis with tracheary elements. Actually, initiation of xylogenesis takes place from mitotically blocked and elongated cells.

Ultrastructural studies have thrown some revealing light on the plausible claim of intracellular events leading to loss of potential for dedifferentiation. Degradative changes associated with chlorophyll, endoplasmic reticulum, dictyosomes, ribosomes and mitochondria and the separation of bounding membranes are the first steps in cytoquiescence leading to cytogenesis. The transformation of living cells into a dead, empty tracheid during cellular differentiation and the biosynthesis of the acid phosphatase enzyme are functionally related to the autolysis of the cell contents and lignin synthesis.

Whether cell division is a prerequisite for xylem differentiation or not is a matter of debate. It has been observed that xylem differentiation is completely suppressed by the incorporation of BUdR, an inhibitor of DNA synthesis. It indicates that DNA synthesis is a critical factor in differentiation phenomenon. Similarly, gamma irradiated cells do not take part in xylem differentiation. Malawer and Phillips (1979) also added some evidences to support the concept that xylem differentiation was preceded by mitotic cell division. In some cases, cell division is not prerequisite for xylem differentiation. In cell culture of *Centanurea cyanus* some of the single parenchymatous cell differentiates directly into tracheary elements without cell division. In case of *Zinnia elegans*, mesophyll system consists of two different cell populations; some of mechanically isolated cells, after a period of extension growth directly differentiate into xylem elements without cell division, whereas others require cell cycle activity for differentiation. In *Helianthus tuberosus* direct differentiation of xylem

without DNA replication and mitotic division occurs only when explants are taken from immature tubers. Therefore, the question of cell division prior to xylem differentiation remain unresolved.

A number of chemical and physical factors have been shown to have a profound effect on cytodifferentiation. Auxin at low concentration stimulates xylogenesis. The influence of auxin in xylem differentiation has been demonstrated under *in vitro* by many workers. There are some evidences to put forward that cytokinin may also be involved in cytodifferentiation. The influence of gibberellin in cell division and xylem differentiation has been investigated under *in vitro* condition in order to establish some quantitative relationship. The sugar, particularly sucrose in the medium is very essential for cytodifferentiation. There are very few reports of the effect of physical factors i.e. light, temperature, pressure and water stress on vascular differentiation. There are some other factors such as morphactins, methionine, irradiation etc. which play either positive or negative role for cytodifferentiation.

There are still some lacuna in our understanding of the cellular and biochemical aspects

of cytodifferentiation. Quite a number of parameters need consideration before cytodifferentiation in tissue culture can be viewed in its proper perspective.

## Questions for Discussion

1. What is cytodifferentiation? Discuss the primary steps in cytodifferentiation.
2. Is cell division required for xylem differentiation? Comment on the statement with some evidences.
3. Discuss critically the factors affecting cytodifferentiation.
4. Write short notes on—
  - (a) Xylogenesis
  - (b) Role of hormones on cytodifferentiation
  - (c) Role of physical factors on vascular differentiation
  - (d) Explain the possible mechanism of cytodifferentiation

## Chapter Seven

### Organogenesis

#### INTRODUCTION

In culture, the explant develops into callus tissue in a medium containing either a particular concentration of auxin or a definite auxin-cytokinin ratio. Such medium is known as callus inducing or initiation medium. Proliferation of callus mass in a relatively unorganised way will continue for a prolonged period, if the callus tissue is maintained in the same medium through a number of subcultures. But the main objective in plant tissue culture is to regenerate a plant or plant organ from the callus culture. The regeneration of plant or plant organ only takes place by the expression of cellular totipotency of the callus tissue. The callus tissue during its growth in callus inducing medium shows an extremely limited expression of totipotency, but in a certain number of plant species, this potentiality can be enhanced and extended by the adjustment of nutritional and hormonal conditions in culture. Scattered areas of actively dividing cells, known as meristematic centres, develop as a result of differentiation and their further activity results in the production of root and shoot primordia. Skoog and his co-workers at Wisconsin, in their studies with tobacco stem pith culture, demonstrated that the initiation and the type of organ

primordia formed from the resulting callus culture could be controlled by appropriate adjustment of the relative levels of the auxins and cytokinins. With high auxin—low cytokinin roots develop, with low auxin—high cytokinin shoot buds develop; at intermediate levels undifferentiated callus tissue develops (Skoog and Miller, 1957). The expanded expression of totipotency of the callus tissue offers considerable potential for tissue culture technique as it is possible to grow the root or shoot or both. The production of adventitious roots and shoots from cells of tissue culture is called organogenesis.

#### BRIEF PAST HISTORY

**F. Skoog (1944)**—The first indication that *in vitro* organogenesis could be chemically regulated to some extent was given by F. Skoog. He found that the addition of auxin to the culture medium served to stimulate root formation, whereas shoot initiation was inhibited. The latter effect on shoot production could be partially reversed by increasing the concentration of both sucrose and inorganic phosphate.

**F. Skoog and C. Tsui (1948)**—They found that adenine sulphate was active in promoting shoot initiation and this chemical reversed inhibitory effect of auxin.

**F. Skoog and C. O. Miller (1957)**—The studies of Skoog and his colleague led to the hypothesis that organogenesis is controlled by a balance between Cytokinin and auxin. A relatively high auxin—Cytokinin ratio induced root formation in tobacco callus whereas a low ratio of the same hormones favoured shoot production.

**J. G. Torrey (1966)**—He advanced the hypothesis that organogenesis in the callus tissue starts with the formation of clusters of meristematic cells (meristemoids).

**K. Tran Thanh Van, H. Chlyah and H. Trinh (1978)**—The precise regulation of organ formation such as floral buds, vegetative buds and roots has been demonstrated in thin cell layer explants (epidermal and sub epidermal explants) of several species by regulating auxin—cytokinin ratio, carbohydrate supply and environmental conditions.

**T. A. Thorpe (1980)**—He advanced the hypothesis that the endogenous auxin—cytokinin balance is the key factor in the initiation of organogenesis.

**N. Everett (1982)**—Endogenous ethylene was identified as a factor in the induction of shoot buds from cultured tobacco cotyledons.

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### WHAT IS RHIZOGENESIS ?

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Rhizogenesis is a type of organogenesis by which only adventitious root formation takes place in the callus tissue.

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### WHAT IS ORGANOIDS ?

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In some cultured tissues, an error occurs in the development programming for organogenesis and an anomalous structure is formed. Such anomalous organ like structures are known as organoids. Although organoids contain the dermal, vascular and ground tissues present in plant organs, they differ from true organ in that the organoids are formed directly from the periphery of the callus tissue and not from organized meristemoids.

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### WHAT IS MERISTEMOIDS ?

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Meristemoid is a localised group of meristematic cells that arise in the callus tissue and may give rise to shoots and/or roots.

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### GENERAL ACCOUNT OF ORGANOGENESIS

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*In vitro* organogenesis in the callus tissue derived from a small piece of plant tissue, isolated cells, isolated protoplasts, microspores etc. can be induced by transferring them to a suitable medium or a sequence of media that promote proliferation of shoot or root or both. The suitable medium is standarized by trial and error method. The callus may remain in undifferentiated condition regardless of the hormones and nutrients to which they are exposed. Organ neoformation generally follows cessation of unlimited proliferation. Individual cells or groups of cells of smaller dimensions may form small nests of tissue scattered throughout the callus tissue, the so-called meristemoids which become transformed into cyclic nodules from which shoot bud or root primordia may differentiate. In most calli, initiation of shoot buds may precede rhizogenesis or vice-versa or the induced shoot bud

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### WHAT IS ORGANOGENESIS ?

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Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation.

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### WHAT IS CAULOGENESIS ?

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Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue.

may grow as rootless shoots. Shoot bud formation may decrease with age and subculture of the callus tissue, but the capacity of rooting may persist for longer period. In some calli, rooting occurs more often than other form of organogenesis. During organogenesis, if the roots are first formed, then it is very difficult to induce shoot bud formation from the same callus tissue. But if the shoots are first formed, it may form roots later on or may remain as rootless condition unless and until the shoots are transferred to another media or hormoneless medium or conditions that induce root formation. In certain cases, shoot and root formation may occur simultaneously. But the organic connection between two different organ primordia may or may not be established. Therefore, organic connection between shoot and root primordia is essential for the regeneration of complete plantlet from the same culture. Shoot formation followed by rooting is the general characteristic of organogenesis.

The callus tissue may remain unchanged in colour during rhizogenesis or may develop yellow pigmentation. During shoot bud formation, the callus tissue generally develops green or pale green pigmentation.

The callus tissue in many cases shows a high potential for organogenesis when first initiated but gradually a decline sets in as subculture proceeds with eventual loss of organogenic response. The loss of potential for organogenesis may be due to either a genetic or a physiological change induced by either prolonged cultural conditions or the composition of culture media. The genetic effects in a callus tissue are reflected in changes of chromosome structure or number such as aneuploidy, polyploidy, cryptic chromosomal rearrangements etc. Such chromosomal changes may lead to loss of totipotency of the cells. During prolonged culture, totipotent cells of the callus tissue are gradually replaced by non-totipotent cells. It is generally observed that shoot bud formation takes place from the diploid cells of the callus tissue. At the early stage of culture, the callus tissue exhibits maximum number of diploid cells. Eventually at the later stage of culture, the cells of callus tissue become mixaploid due to alteration of chromosome number and

organogenesis could not be induced in such callus tissue. Occasionally, rooting occurs but shoot bud does not develop. But in some cases plantlets could also be regenerated from old subcultured callus tissue and the potential for organogenesis or embryogenesis could be enhanced in the later part of culture. Again, an alteration in karyotype need not always result in organogenetic incapability as, for example, regeneration of extreme aneuploid plants from 20 years old tobacco tumour tissues has been observed. Therefore, it cannot be a generalised the concept that chromosomal changes are the main cause of organogenetic incapability of the callus tissue during prolonged culture. So an alternative physiological hypothesis has been put forward to explain the loss of organogenetic potential of the callus tissue during prolonged culture. According to this hypothesis, subculture often leads to a loss of many endogenous factors or morphogens present at the critical stages of growth. Such factors present in the callus tissue at the initial stage may not be synthesized at all or synthesized only in insufficient quantity at later stages. As a result, callus tissue fails to exhibit the potential for organogenesis or embryogenesis. However, if these factors are supplemented to the medium during subculture, then restoration of organogenetic potential should be regained. It has been reported that addition of kinetin could restore decline in regenerative response in long termed carrot culture, whereas, at the initial stages, no promotive effect of kinetin was observed. But the addition of kinetin or any other additives are not always conducive for the regeneration of plant in other plant species. Therefore, it is plausible that both genetic as well as physiological process are involved in the decline and loss of organogenetic response during prolonged subculture.

The effect of chemical factors or organogenesis, especially those of phytohormones, have been studied in explant from a large number of species. The concept, as propounded by Skoog and Miller (1957), that induction of organogenesis would require, above all, the addition to culture medium of an appropriate balance of known phytohormones such as auxin and cytokinin has

not proved to be so in many experimental materials. In a few cultured tissue, the endogenous regulator complex can be adjusted to the required balance of phytohormones by an exogenous supply of auxin, cytokinin or gibberellin either separately or in combination. Generally high concentration of cytokinin brings about shoot bud initiation, whereas high levels of auxin favours rooting. Therefore, to obtain organogenesis, different permutation and combination of hormones as well as various concentrations of hormones are supplemented in the culture medium.

Certain phenolic compounds also induce shoot initiation in tobacco callus. Phenolic compounds actually inactivate the auxins and consequently rise in the physiologically effective level of cytokinins which ultimately bring about the initiation of shoot buds. The use of auxin inhibitor or auxin antagonist via culture medium also cause the induction of shoot bud. Addition of adenine in the culture medium also induce shoot bud in the callus tissue. Shoot bud initiation takes place in haploid tobacco cultures in presence of chelating agent like 1, 3 diamino-2-hydroxypropane-N.N.N'.N' tetraacetic acid. Addition of abscisic acid in place of cytokinin also induce shoot bud formation in root tuber tissue of sweet potato and stem tuber tissue of potato.

Though the role of hormones and their quantitative interactions has been recognised, it is only recently that some efforts are initiated to gain some insight into the biochemistry of organ differentiation by hormonal interaction. It has tended to rather empirical. During last few years, some indirect studies have been made on organ forming tissues by estimating the level of structural and enzymatic proteins and the changes of isoenzyme pattern through gel electrophoresis during organogenesis.

Of different enzyme systems studied in plants, peroxidase is widely distributed among higher plants and has been investigated in relation to many different activities. One of the most important functions of peroxidase is involvement

in the metabolism of auxin. Plant tissue cultures also require hormones like auxin and cytokinin for growth and differentiation *in vitro*. Hence the study of peroxidase level by estimating the activity and the changes of isoperoxidase patterns during organogenesis is very important. Increases in peroxidase activity in callus tissue have been demonstrated before the differentiation of both shoot as well as root. Distinctive changes in the isoperoxidase patterns have also been demonstrated during organ differentiation in cultured tissue. Differences in isoperoxidase patterns associated with shoot and root differentiation have been elegantly demonstrated. Since cathodic isoperoxidases are considered to be involved in auxin catabolism and the last moving anodic bands have been associated with lignification, the changes in band patterns have been interpreted as creating situations conducive to shoot or root formation. It is also evident that certain isoperoxidase appeared several days prior to the actual emergence of root and shoot primordia from the tobacco callus. Later, these specific peroxidases were detected in the regenerated root and shoot respectively. Such isoperoxidases provide useful biochemical signals for morphogenetic events that follow.

The activities of some enzymes of the carbohydrate metabolism during organogenesis have been looked into. Starch accumulation, which has been known to be conspicuous feature in diverse morphogenetic processes *in vitro*, is also shown to occur prior to shoot differentiation from tobacco callus grown *in vitro*. Starch accumulation reflects high energy requirement for the organogenetic processes as strong correlation has been found between the starch content of the callus, its rate of respiration and shoot formation. Gibberellic acid, which represses starch accumulation by mobilising high amylase synthesis/activity, also inhibits shoot formation.

Comparison of malic dehydrogenase activity under root and shoot forming conditions revealed that this was more pronounced activity prior to shoot and root differentiation.

Developmental patterns of the key Embden-Meyerhof-Parnas (EMP) and Pentose Phosphate

(PP) Pathway enzymes namely phosphoglucose isomerase, aldolase, pyruvate kinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase etc. were investigated in shoot forming and non shoot forming sugarcane callus. As compared with non-shoot forming callus, the shoot forming callus was characterised by higher activity level of these enzymes. Higher activity levels of the EMP and the PP pathway enzymes in the shoot forming sugarcane tissue are indicative of generation of energy molecules, reducing power and pentose sugars vital for energy-dependent reaction and the synthesis of nucleic acids during shoot differentiation.

Since differentiation took place by the synthesis of nucleic acids and proteins, many attempts have been made to correlate the two phenomena. It has been observed that shoot initiation in *Cichorium intybus* was associated with alterations in the pattern of RNA synthesis and nucleotides (Vasseur 1972). An increase in the ratio of RNA/DNA and histone/DNA was related to organogenesis in tobacco and to embryogenesis in carrot with DNA synthesis.

#### PROTOCOL FOR ORGANOGENESIS IN TOBACCO CALLUS

This is an experiment in which mature tobacco stem is initiated to give rise to callus tissue. Under the appropriate hormonal conditions callus is induced to form either root or shoot primordia. The protocol is given below—

1. The upper part of the stem of 3-4 ft tall tobacco plants are harvested and cut into 2 cm long internode segments.
2. Surface sterilization of the tissue is done by immersing the stem pieces in 70% v/v ethanol for 30 seconds, followed by a 15 minutes incubation in sodium hypochlorite (1.0% available chlorine). Then the tissue is washed in several changes of sterile distilled water.
3. The stem explants are taken in a sterilized petri dish and cut longitudinally into two equal pieces and inoculated onto Murashige

and Skoog's (1962) solid medium (MS) supplemented with 2mg/L indole acetic acid (IAA) and 0.2 mg/L kinetin. The cultures are then incubated at 25°C with an illumination of about 2,000 lux (16 hrs. photo period)

4. Callus tissue which is white/yellow in colour, begins to form in two weeks and after six weeks it should be subcultured to fresh medium.
5. Organogenesis in callus culture can be stimulated by transferring tobacco callus onto MS medium with different auxin/cytokinin ratios. Shoot primordia develop within 3 weeks of transfer of callus to MS medium with IAA at 0.02 mg/L and kinetin at 1 mg/L (a high cytokinin/low auxin ratio). Root formation occurs within 2-3 weeks of transfer of callus to MS medium supplemented with 0.2 mg/L IAA and 0.02 mg/L kinetin (a high auxin/low cytokinin).
6. After 6 weeks, rootless shoots can be excised and placed onto the root inducing medium i.e. MS medium with 0.2 mg/L IAA and 0.02 mg/L.
7. It is possible to transplant the tobacco plantlets to soil. It should be noted that aseptic procedures are not required for the transplantation of plantlets. The plantlets are removed from the culture vessels and care should be taken not to damage root or shoot system. The plantlets are carefully washed with tap water to remove the residual agar medium. Individual plantlets are separated out and transplanted into pot (75 mm) containing seedling compost. The soil is watered. The pot is covered with a small inverted polythene bag. This will reduce the amount of water lost by the plantlets due to transpiration. After 7 days, several small holes are made in the polythene bag and gradually enlarged during next 2-3 weeks. At this stage, the tobacco plantlets should be sufficiently "hardened off" to allow the complete removal of plastic bag.

They can be grown to maturity in a green house.

## **FACTORS INFLUENCING ORGANOGENESIS**

*In vitro* Organogenesis is controlled by a number of factors. Such factors are discussed below—

### **Size of Explant**

Organogenesis is generally dependent upon the size of the explant. The large explant consisting of parenchyma, vascular tissue and cambium have greater regenerative ability than the smaller explant. Small groups of homogeneous tissue taken from the epidermal and subepidermal layer could directly give rise to complex organs such as flower or buds or roots. A remarkable capacity to regenerate shoot buds *in vitro* is displayed by certain ferns such as *Davallia*, *Platycerium*. The tissue pieces obtained by aseptically homogenizing the plants in a blender produce numerous new plants.

### **Source of Explant**

The source of explant cultured is important in determining the potential of organogenesis. The most suitable part of the plant for starting culture will depend on the species. Leaves and leaf fragments of many plant species like *Begonia*, *Solanum*, *Nicotiana*, *Crepis* etc. have shown the capacity to regenerate shoot buds. Many of the monocotyledonous species with specialized storage organs possess a profound capacity to produce buds. The bulb scale of *Hilium* sp. regenerates adventitious bulbils in culture. Flower stem explant of *Tulipa* sp. regenerates shoots *in vitro*. Inflorescence axis of *Haworthia* sp.; also forms shoots in culture. Root sections of *Convolvulus* sp., *Ipomea* sp. etc. produce shoot buds in culture. In conifers, the rate of adventitious shoot formation on cotyledons is correlated with the growth rate of the parent tree and with the size of the seed.

### **Age of the Explant**

The physiological age of the explant is another factor which often plays an important role in organogenetic phenomenon. Regeneration of adventitious shoot bud is only noted in case of *Nicotiana* sp. if the leaf explants are collected from the vegetative phase i.e. prior to flowering. Leaf explants of *Echeveria* sp. that are collected from young leaves produce only root, whereas older leaf initiates only shoot buds and leaves of medium age produce both shoots and roots.

### **Seasonal Variation**

The effect of seasonal variation on plant is another factor which exercises an influence on organ formation. Bulb scales of *Lilium speciosum* regenerates bulbils freely *in vitro* when the explant is taken during spring and autumn period of growth. But the same explant collected from summer or winter season does not produce any bulblet.

### **Oxygen Gradient**

Oxygen gradient in a tissue culture often exercises an influence on organ formation. In some cultures, shoot bud formation takes place when the gradient of available oxygen inside the culture vessel is reduced. But rooting requires a high oxygen gradient.

### **Quality and Intensity of Light**

The quality and intensity of incident light on culture may play an effective role in the promotion of organogenesis. Studies on spectral light on organogenesis reveals that the blue region of the spectrum promotes shoot formation and red light induces rooting. The treatment of blue light followed the treatment of red light also stimulates the organogenetic phenomenon. Hence the nature of organogenesis can be regulated by exposure to light of different wavelength. This sort of action of light on organogenesis will help us in understanding the action of auxin and cytokinin on organogenesis. In some

culture, artificial fluorescent light favours rooting and inhibits in others. In case of *Pisum sativum*, shoot bud initiation takes place in dark followed by the sudden treatment of light.

Normally, organogenesis in culture takes place with an illumination of about 2,000–3,000 lux (16 hrs. photoperiod). The callus tissue of *Nicotiana tabacum* also produces shoot bud or embryo when the tissue is exposed to high intensity of light—10,000–15,000 lux.

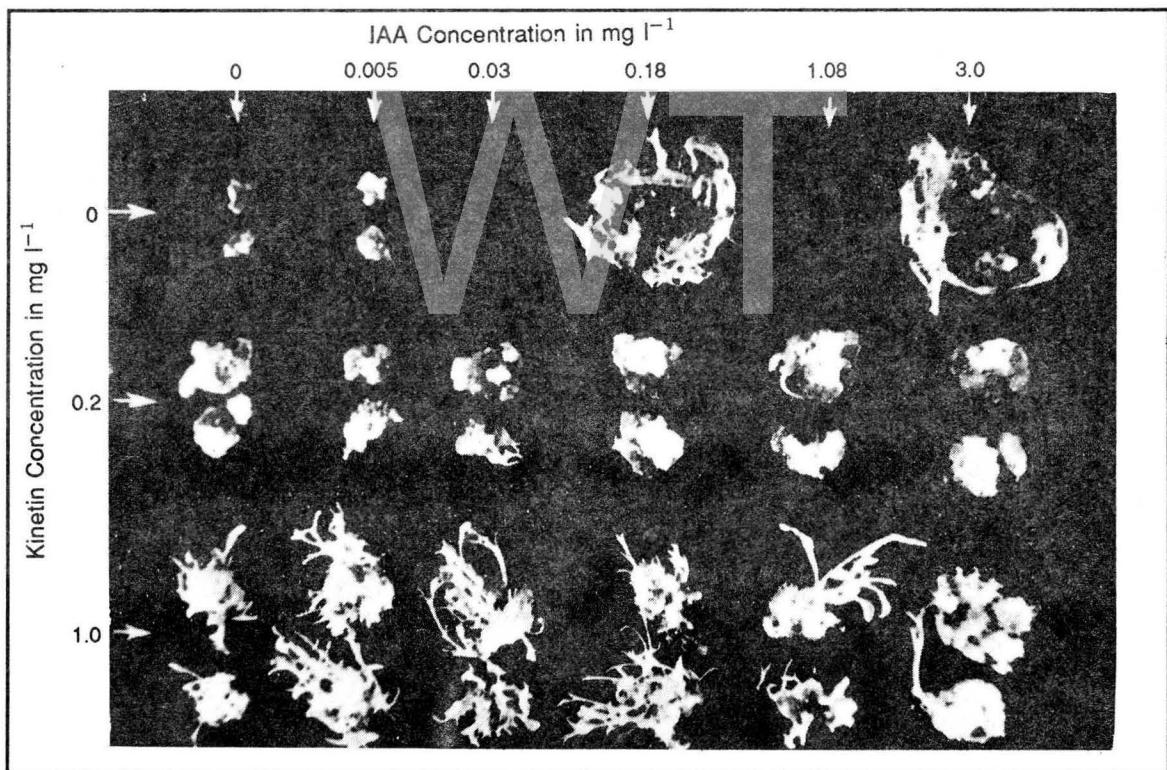
### Temperature

Most tissue cultures are grown successfully at temperatures around 25°C, but the usual environmental temperatures of the species concerned should be taken into account. In a number of bulbous species, the optimum temperature may

be much lower—15°C in case of *Galanthus* and 18°C for some cultivars of *Narcissus* and *Allium*. Tropical species require higher temperatures, the optimum for date palm being 27°C and for *Monsasteria deliciosa* 30°C.

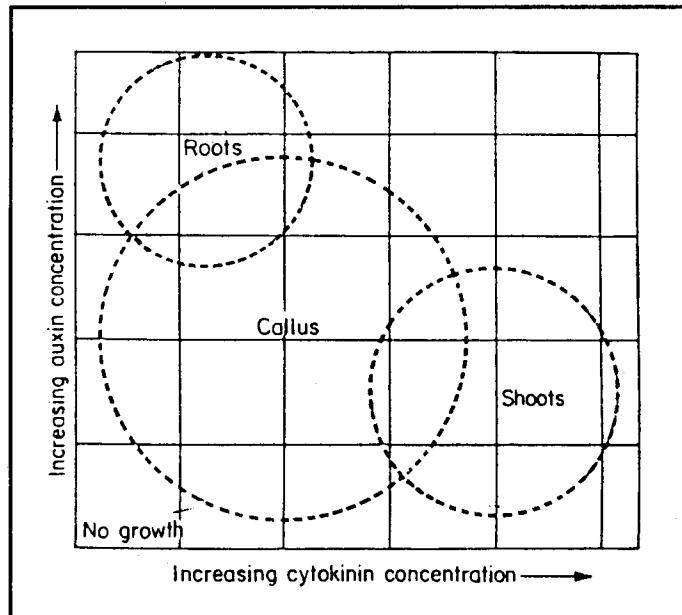
### Plant Hormones

Of the many factors that influence Organogenesis *in vitro*, the most important single factor seems to be the phytohormones. In their classical experiments with cultured stem pith tissue of tobacco, Skoog and Miller (1957) demonstrated that different types of organogenesis (Fig 7.1) can be achieved by varying the concentrations of auxins and cytokinin in the culture medium—when the concentrations of cytokinin are high relative to auxin, shoots are induced; when the concentrations of cytokinin are low relative to



□ Fig 7.1

**Organogenesis in tobacco callus showing the effect of auxin and kinetin at different concentrations. Note root formation in the absence of kinetin and shoot formation in presence of higher concentrations of kinetin (from Skoog and Miller, 1957)**



□ Fig 7.2

### **Relationship of the auxin and cytokinin concentrations of a culture medium to their effects on the growth and development of plant cells**

auxins, roots are reduced; and at intermediate concentrations the tissues grow as unorganized callus (Fig 7.2). This basic concept has been used to regenerate a wide variety of dicotyledonous plants. In general monocotyledonous plants do not show a pronounced response to cytokinins and need high concentration of auxins such as 2, 4-D to obtain changes in the development of cultured tissue. Other plant hormones, particularly abscisic acid and gibberellins have some dramatic action on *in vitro* organogenesis. Endogenous ethylene retards organ initiation during early stages of culture but in the later stages it helps shoot initiation. Very little is known about how phytohormones awake a particular pattern of organogenesis. Various hypotheses have been put forward to explain the mode of action of phytohormones on organogenesis. Such hypotheses have been placed into two broad categories, depending upon how much emphasis is given on the developmental state of the responding cell. In the first category of hypotheses, phytohormones are regarded as primary morphogens. According to these hypotheses, the

responding cell or groups of cells are competent to react to the hormones but are not committed to a particular development fate. When the cells are treated with hormones, the cells start to move a specific developmental pathway. The alternative view is that hormone responsive cells are already determined and that the hormones stimulate the expression of the committed state.

On the basis of large number of available evidences it can be suggested that the hormones act in both ways depending upon the particular experimental system. Histological studies of culture of cereals reveal that organ primordia are arrested in their development by auxin in the culture medium. Again, in dicotyledonous plants, different types of development can be obtained by treating the same cloned line of cells with different combinations of hormones. In some cases, the hormones show different actions at different stages in the same developmental pathway.

## Culture Medium

The essential components of plant cell culture medium are the macro or major salts and micro or minor salts. Besides these, vitamins, amino-acids, carbohydrates etc. are also required for *in vitro* growth and development of plant cells. Inorganic nitrogen's most important role in the plant cells is its presence in the structure of the protein molecule. In addition, nitrogen is found in such important molecules as purines, pyrimidines and coenzymes. Phosphate is found in plants as a constituent of nucleic acids, the co-enzymes NAD and NADP and most important as a constituent of ATP. Increase in phosphate concentration induces shoot formation and suppresses root initiation. One well known role played by calcium in the plant cell is its function as a constituent of cell walls in the form of calcium pectate. Similarly, iron, zinc and molybdenum are parts of certain enzymes. The organic supplements required in plant culture medium include a carbon source and vitamins, sucrose is used as a carbon source but may be substituted with glucose. Other sugars are used less often. Vitamins most often added to culture medium include inositol, nicotinic acid, pyrodoxine, thiamine, calcium pantothenate and biotin. Thiamine is required for plant growth, while others enhance growth in some system. Vitamin E has been shown to regulate cell aggregation and Vitamin D induce root formation. Various complex nutritive mixtures of undefined composition like casein hydrolysate, coconut milk, malt-extract etc. are sometimes added to the culture medium to increase cell growth.

## Agar-Agar

Agar-agar is not an essential component of the culture medium. In plant tissue culture, the culture medium is gelled with agar. The quantity of agar is a factor that may have a determining role in organogenesis. Commercially available agar contains impurities. So "Difco" or Bacto-agar containing less impurities are generally used in plant tissue culture medium. With high concentration of agar, the nutrient medium become hard and does not allow the diffusion of

nutrient to the growing tissue. So the concentration of agar also plays a role in organogenesis.

## pH of the Medium

The pH of the culture medium is generally adjusted between 5.6 to 5.8 before sterilization. The pH is another factor that may have a determining role in organogenesis.

## Ploidy Level

Variation in chromosome number i.e. aneuploidy, polyploidy etc. of plant cell in culture has been well-documented. It is generally observed that with the increase of chromosome instability there is a gradual decline in morphogenetic potentiality of the callus tissue. So the most important factor in maintaining organogenic potential of the callus tissue is the maintenance of chromosome stability. It has been suggested that the frequency of subculture can effect the chromosome stability of cell cultures. So, in order to maintain chromosome stability, cultures are subcultured frequently and regularly.

## Age of Culture

Age of culture often exercises an influence on organ formation. A young culture frequently produces organs. But the organogenic potential may decrease and ultimately disappear in old culture. In certain cultures of some plants, the plant regeneration capacity may retain indefinitely for many years.

## IMPORTANCE OF ORGANOGENESIS

The regeneration of plant from cell and callus culture via Organogenesis is a wide field of plant science. So, the importance and applications of organogenesis are vast and varied. With the discovery of cellular totipotency and the development of methods for regeneration of plant from *in vitro* cell culture, a large number of plant species including economically important crop plants, medicinal plants, horticulturally important plants, timber yielding plants etc. have been successfully regenerated from callus culture

via organogenesis and the reports of regeneration of other plants are still increasing day by day. The production of large numbers of haploids from microspores and the possibility of raising triploids from endosperm cell culture are the dramatic instances of the potential role of organogenesis in genetics and plant breeding. Improvement of crop plants through manipulation at the cellular level such as the vegetative hybridization by the fusion of isolated protoplasts of distantly related plants, the transfer of foreign genetic material in protoplasts etc. is possible only if somatic cells are able to give rise to whole plant; a plant breeder is interested in obtaining modified plants than modified cells. Regeneration of whole plants through organogenesis is of special interest in mutagenic studies. Chemical mutagens and ionizing radiation can be used in bringing about genetic diversity in plants and by means of cell culture and the regeneration of whole plants from somatically mutant cell types, the new strains of mutant plants are obtained through organogenesis. This method is very useful in mutation breeding of both sexually and vegetatively reproducing plants. A new source of genetic variability is also available in plants regenerated from cell culture. This somaclonal variation is a useful source of variability only if plants can be efficiently regenerated from the cell culture via organogenesis.

Variation in chromosome number of cells i.e. aneuploid, polyploid has also been observed in callus culture. By means of organogenesis a wide range of aneuploid plants have been recovered from tissue culture of numerous plants. Reduction in chromosome number has been noted in plants regenerated from callus culture of triploid rye-grass hybrids. A wide range of aneuploid having addition and reduction in chromosome number are also achieved in sugarcane. Each of these chromosomal variations is associated with phenotypic variation, including agriculturally useful characters such as disease resistance. Therefore, variability in chromosome number, if not associated with depression of yield, is particularly valuable in vegetatively propagated medicinal plants and agricultural crops. Freeze preservation of cell culture of many plant

species and the regeneration of plant from them after a desirable time period is another importance of organogenesis for the conservation of endangered plant species. In the application of *in vitro* methods for the improvement of the genetically potential plants for the production of primary and secondary compounds, there generation of plants from cell and tissue cultures attains special significance. Therefore, the success of all available *in vitro* cellular and molecular techniques for the improvement of plants depends upon the ability to regenerate plants from single cells and callus culture via organogenesis.

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## Summary

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*In Vitro* callus tissue may be obtained from many different parts of plants, by plating single cells and cell aggregates of cell suspension culture and from isolated protoplasts. But the main objective in plant tissue culture is to regenerate a complete plant or plant organs from callus culture. The regeneration of plant or plant organ takes place by means of organogenesis which means the development of adventitious organ or primordia from undifferentiated cell mass in tissue culture by the process of differentiation. Individual meristematic cells or group of cells within the callus tissue may form small nests of tissue scattered throughout the callus tissue, the so-called meristemoids which become transformed into cyclic nodules from which shoot or root primordia may differentiate. In most calli, initiation of shoot buds may precede rhizogenesis or vice versa or the induced shoot bud may grow as rootless shoots. Skoog and Miller (1957) demonstrated that the initiation and the type of organ primordia formed from the resulting callus culture could be controlled by appropriate adjustment of the relative levels of auxin and cytokinins. With high auxin-low cytokinin, roots develop; with low auxin-high cytokinin, shoot buds develops. The callus tissue in many cases show a high potential for organogenesis when first initiated but gradually a decline sets in as

subculture proceeds with eventual loss of organogenic response. The loss of potential for organogenesis may be due to either genetic or a physiological change induced by either prolonged cultural conditions or the composition of culture media. The genetic effects in a callus tissue are reflected in changes of chromosome structure or number. It is generally observed that shoot bud formation takes place from diploid cells although the callus tissue becomes mixaploid. Besides genetic changes, long-term culture often leads to loss of many endogenous factors present at the critical stages of growth. Such factors present at the initial stage may not be synthesized all or synthesized only in insufficient quantity at later stage. Phenolic compounds also induce shoot initiation in tobacco callus.

Though the role of hormones and their quantitative interactions has been recognised, it is only recently that some efforts have been made to gain some insight into the biochemistry of organogenesis. Increase in peroxidase activity in callus tissue have been demonstrated before the differentiation of both shoot as well as root. The activities of some enzymes of the carbohydrate metabolism during organogenesis have been looked into. Comparison of malic dehydrogenase activity under shoot and root forming conditions revealed that this was more pronounced activity prior to shoot and root differentiation. Higher activity levels of the EMP and the PP pathway enzymes in shoot forming sugarcane are indicative of generation of energy molecules, reducing power and pentose sugars vital for energy dependent reaction and synthesis of nucleic acid during shoot differentiation. Organogenesis is controlled by a number of factors such as size of explant, source of explant, age of the explant, seasonal variation, oxygen gradient, quality and intensity of light, photoperiod, temperature, phytohormones, components of culture medium, agar-agar, pH of the medium, ploidy level of cells and age of the culture.

The potential importance and applications of organogenesis are vast and varied in the field of plant science. Regeneration of haploid plants from microspore and triploids from endosperm cell culture have the potential use in genetics and

plant breeding. The success of genetic engineering of higher plant, clonal multiplication of new genotype, somatic hybridization via protoplast fusion and isolation of mutants depend upon the ability to regenerate plants from single cell and callus culture via organogenesis. By means of organogenesis a wide range of aneuploid plants with phenotypic variation including agriculturally useful characters such as disease resistances have been realised from tissue culture of numerous plants. Somaclonal variants have also been regenerated from callus culture. Therefore, the uniqueness of cellular and molecular genetics in higher plants is dependent upon the potential for regeneration of the plants from cell cultures via organogenesis.

## Questions for Discussion

1. What is organogenesis? Give a general account of organogenesis.
2. Describe the protocol for organogenesis in tobacco callus and discuss the factors influencing organogenesis.
3. Discusses the importance of organogenesis in different fields of plant science.
4. Write brief answers to the following questions—
  - (a) Why the callus tissue shows a gradual decline in organogenic potentiality after prolonged subculture?
  - (b) What are the biochemical aspects of organogenesis?
  - (c) What is the important role of auxin and cytokinin in relation to organogenesis? Discuss briefly the possible mechanism of action of such hormones during organogenesis.

(d) What is the importance of organogenesis in genetics and plant breeding?

5. Write short notes—

(a) Organogenesis

(b) Caulogenesis

(c) Rhizogenesis

(d) Meristems

(e) Importance of organogenesis in biotechnology

(f) Relationship between organogenesis and variation of chromosome number in callus tissue.

## Chapter Eight

### Somatic Embryogenesis and Artificial Seeds

#### INTRODUCTION

In angiosperms, ovules are developed within the ovary. Within the ovule, a sac-like structure known as embryo sac lies embedded into the nucellus. The embryo sac represents the female gametophyte of angiosperms. The ovule contains a haploid egg cell or ovum which is the female reproductive cell or female gamete. During fertilization, the male gamete fuses with the egg cell or female gamete resulting in the formation of an unicellular zygote or oospore. The zygote gives rise to a multicellular embryo, cells of which are diploid. Embryos derived in this sexual process are known as zygotic embryos and the process of embryo development is called embryogenesis. Sometimes, embryos are formed by the unfertilized egg and such embryos are called parthenogenetic embryos. Again, sometimes, any cell of the female gametophyte (embryo sac) or the sporophytic tissue around the embryo sac may give rise to an embryo and such embryos are called non-zygotic embryos. According to S. S. Bhojwani and S. P. Bhatnagar

in nature there is no instance of ex-ovule embryo development. Therefore, there is no evidence of embryo development *in vivo* from any somatic cells of the plant. That means, *in vivo* somatic plant cells do not express any embryogenic potential to form embryo. In plant tissue culture, totipotency of the somatic cell has been well-established. In modern genetic terms this means that the nucleus of every living somatic cell contains all the genetic information necessary to direct the development of the entire plant. Haberlandt believed that single cultured somatic cells would develop in the same way as the fertilized egg, namely the developmental pathway of embryogenesis. This idea did not find many supporters in Haberlandt's time and it was achieved much later by J. Reinert, a German botanist, in 1959. He found organized small bipolar structures bearing cotyledons and otherwise, resembling zygotic embryos developing from callus cultures derived from carrot storage root tissues. This phenomenon is now called *in vitro* somatic embryogenesis. Embryos derived in this way are termed 'embryoids' to distinguish them from embryos derived from

sexual reproduction. This technique is now well-established and this phenomenon is known to occur in large number of plant species representing most of the major plant families.

## WHAT IS SOMATIC EMBRYOGENESIS ?

In plant tissue culture, the developmental pathway of numerous well-organised, small embryoids resembling the zygotic embryos from the embryogenic potential somatic plant cell of the callus tissue or cells of suspension culture is known as somatic embryogenesis.

## WHAT IS EMBRYOGENIC POTENTIAL ?

The capability of the somatic plant cell of a culture to produce embryoids is known as embryogenic potential.

## WHAT IS EMBRYOID ?

Embryoid is a small, well-organised structure comparable to the sexual embryo, which is produced in tissue culture of dividing embryogenic potential somatic cells.

## BRIEF HISTORICAL BACKGROUND

**J. Reinert** (1958-59)—Reported his first observations of *in vitro* somatic embryogenesis in *Daucus carota*.

**F. C. Steward, M. O. Mapes and K. Mears** (1958)—Also reported the somatic embryogenesis in carrot from freely suspended cells and emphasized the importance of coconut milk for *in vitro* somatic embryogenesis.

**N. S. Rangaswamy** (1961)—Studied in detail the somatic embryogenesis in *Citrus* sp.

**R. N. Konar and K. Nataraja** (1969)—Studied the somatic embryogenesis of *Ranunculus sceleratus* using various floral parts (including anthers) as well as somatic tissues in culture.

**P. V. Ammirato** (1974)—Reported the effect of abscisic acid on the development of somatic embryos from cells of *Carum carvi*.

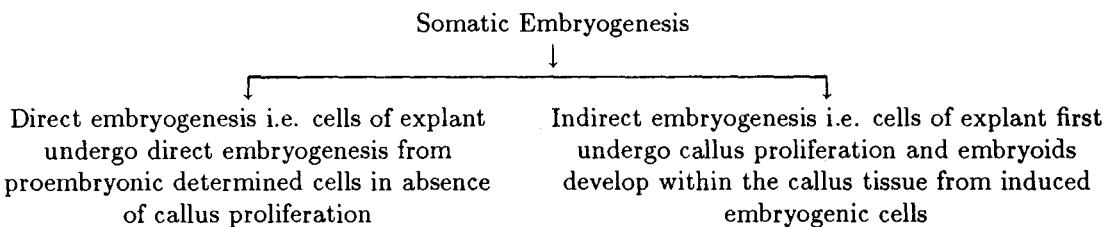
**H. Lang and H. W. Kohlenbach** (1978)—Demonstrated the ability of mechanically isolated, fully differentiated mesophyll cells of *Macleaya cordata* to yield an embryogenic callus.

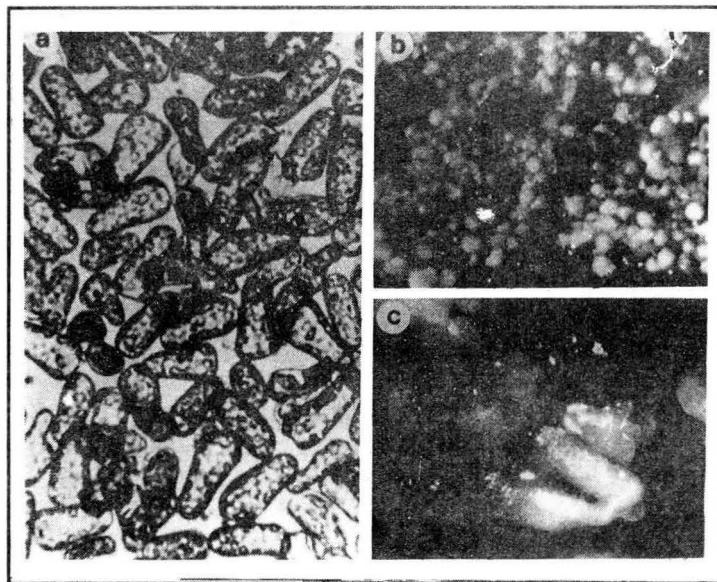
**B. V. Conger, G. E. Hanning, D. J. Gray and J. K. McDaniel** (1983)—Obtained direct embryogenesis from leaf mesophyll cells of orchard grass (*Dactylis glomerata L.*) without an intervening callus tissue.

## PRINCIPLES OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis may be initiated in two different ways—

1. In some cultures somatic embryogenesis occurs directly in absence of any callus production from "proembryogenic determined cells" that are already programmed for embryo differentiation (Fig 8.1). For instance, somatic embryos has been developed directly from leaf mesophyll cells of orchard grass (*Dactylis glomerata L.*) without an intervening callus tissue. Explants, made from the basal portions of two innermost leaves of orchard grass were cultured on a Schenk and Hildebrandt medium supplemented with 30  $\mu$ M 3, 6-dichloro-O-anisic acid (dicamba). Plant formation occurred after subculturing the embryos on the same medium without dicamba (Conger *et al.*, 1983).





□ Fig 8.1

**Photograph showing direct embryogenesis.** A. A suspension of mechanically isolated mesophyll cells. B. Embryogenesis. C. A portion enlarged from B.

2. The second type of somatic embryo development needs some prior callus formation and embryoids originate from "induced embryogenic cells" within the callus tissue.

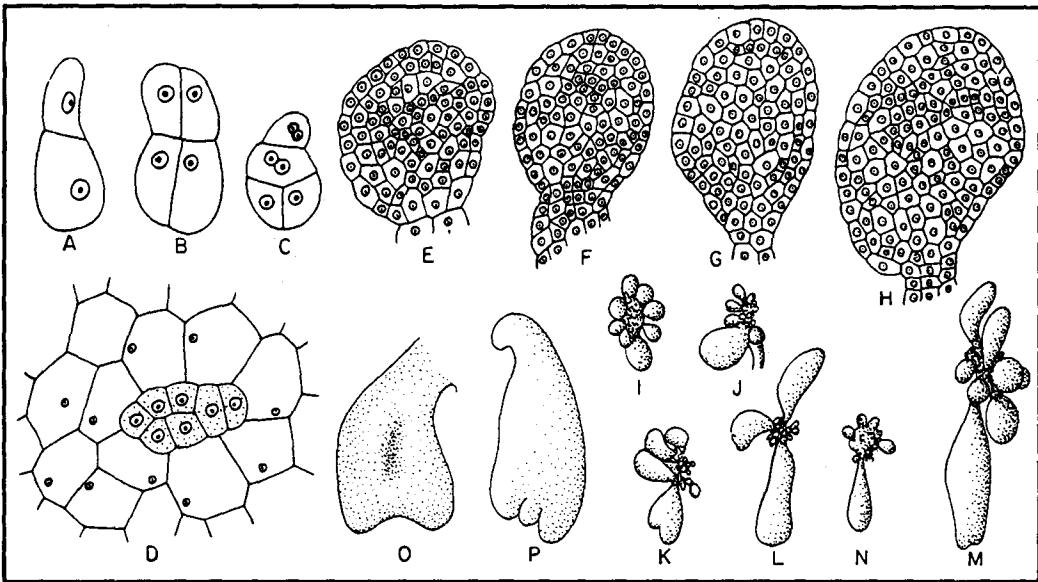
In most of the cases, indirect embryogenesis occurs. For indirect somatic embryogenesis where it has been induced under *in vitro* condition, two distinctly different types of media may be required—One medium for the initiation of the embryonic cells and another for the subsequent development of these cells into embryoids. The first or induction medium must contain auxin in case of carrot tissue and somatic embryogenesis can be initiated in the second medium by removing the hormone or lowering its concentration. With some plants, however, both embryo initiation and subsequent maturation and subsequent maturation occur on the first medium and a second medium is required for plantlet development. In some cases, a given culture may differentiate the embryogenic cells, but their further growth may be blocked by an imbalance of nutrition in the culture medium. According to Kohlenback, (1978), abnormalities known as embryonal budding and embryogenic clump formation may occur, if relatively high

level of auxin is present after the embryogenic cells have been differentiated.

Embryoids are generally initiated in callus tissue from the superficial clumps of cells (primordia) associated with enlarged vacuolated cells that do not take part in embryogenesis. The embryogenic cells are generally characterised by dense cytoplasmic contents, large starch grains, a relatively large nucleus with a darkly stained nucleolus. In suspension culture, embryoids do not form suspended single cell, but form cells lying at or near the surface of the small cell aggregates (Fig 8.2).

Each developing embryoid of carrot passes through three sequential stages of embryo formation such as globular stage, heart-shape stage and torpedo stage (Fig 8.3). The torpedo stage is a bipolar structure which ultimately gives rise to complete plantlet. The culture of other plants may not follow such sequential stages of embryo development.

In general, somatic embryogenesis occurs in short-term culture and this ability decreases with increasing duration of culture. But there



□ Fig 8.2

**Stages in development of embryoids within *Atropa* suspension aggregates.** A–C. Early stages in the development of a proembryo. D. Part of a cell aggregate showing an embedded proembryo. E–H. Progressive stages in development of a globular embryo. I–N. Cell aggregates from suspension culture showing various stages of embryogeny (diagrammatic). O and P. Later stages in embryogeny (diagrammatic).

are some exceptional cultures where embryogenesis has been reported from the callus tissue maintained over a period of year. According to Smith and Street, (1974), changes in ploidy of the cultured cell may lead to loss of embryogenic potential in long term culture. The loss of embryogenic potential in long term culture may also result from loss of certain biochemical properties of the cell.

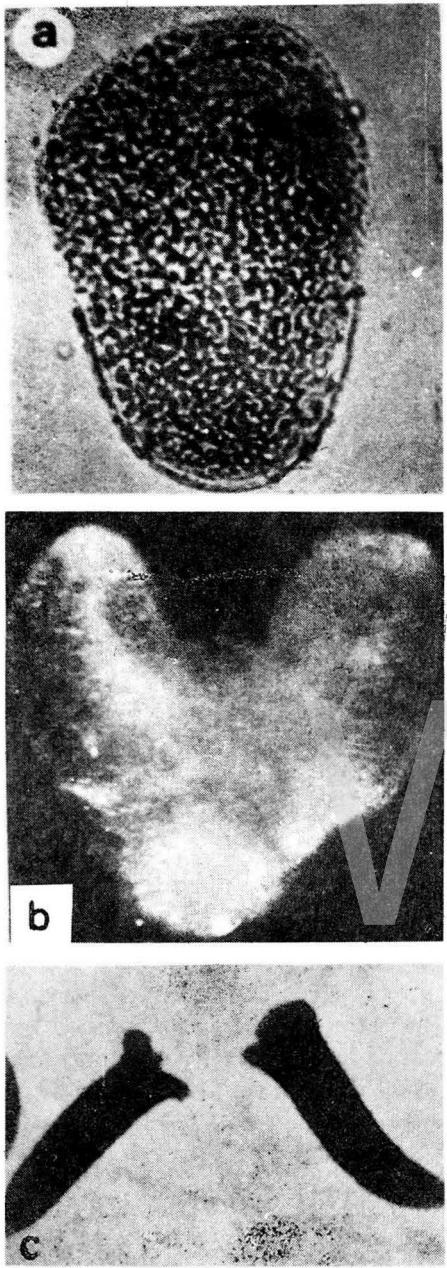
In callus culture or in suspension culture, embryoid formation occurs asynchronously. Some progress has been made in inducing synchronization of somatic embryogenesis in cell suspension culture. A high degree of synchronization has been achieved in a carrot suspension culture by sieving the initial cell population.

#### PROTOCOLS FOR INDUCING SOMATIC EMBRYOGENESIS IN CULTURE

The plant material *Daucus carota* represents the classical example of somatic embryo-

genesis in culture. The protocol is described below—

1. Leaf petiole (0.5–1 cm) or root segments from seven-day old seedlings (1 cm) or cambium tissue ( $0.5 \text{ cm}^3$ ) from storage root can be used as explant. Leaf petiole and root segment can be obtained from aseptically grown seedlings (see details in ‘Specific technique’, Chapter-1). Cambium tissue can be obtained from surface sterilized storage tap root (see details in callus culture, Chapter-3).
2. Following aseptic technique, explants are placed individually on a semi-solid Murashige and Skoog’s medium containing 0.1 mg/L 2, 4-D and 2% sucrose. Cultures are incubated in the dark. In this medium the explant will produce sufficient callus tissue.
3. After 4 weeks of callus growth, cell suspension culture is to be initiated by transferring



□ Fig 8.3

**Embryoid development in tissue culture passes through various stages, namely (a) globular stage, (b) heart shaped stage and (c) torpedo stage before plantlet formation**

0.2 gm of callus tissue to a 250 ml of Erlenmeyer flask containing 20–25 ml of liquid medium of the same composition as used for callus growth (without agar). Flasks are placed on a horizontal gyratory shaker with 125–160 rpm at 25°C. The presence or absence of light is not critical at this stage.

4. Cell suspensions are subcultured every 4 weeks by transferring 5 ml to 65 ml of fresh liquid medium.
5. To induce a more uniform embryo population, cell suspension is passed through a series of stainless steel mesh sieves. For carrot, the 74  $\mu$  sieve produces a fairly dense suspension of single cell and small multiple clumps. To induce somatic embryogenesis, portions of sieved cell suspension are transferred to 2, 4-D free liquid medium or cell suspension can be plated in semi-solid MS medium devoid of 2, 4-D. For normal embryo development and to inhibit precocious germination especially root elongation, 0.1–1  $\mu$ M ABA can be added to the culture medium. Cultures are incubated in dark.
6. After 3–4 weeks, the culture would contain numerous embryos in different stages of development.
7. Somatic embryos can be placed on agar medium devoid of 2, 4-D for plantlet development (Figs 4.1 and 8.4).
8. Plantlets are finally transferred to Jiffy pots or vermiculite for subsequent development.

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#### FACTORS AFFECTING SOMATIC EMBRYOGENESIS

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A number of factors have been shown to have a profound effect on somatic embryogenesis. The most important chemical factors involved in the medium are auxin and reduced nitrogen. Besides this, some other factors are also involved in the process of somatic embryogenesis. The effects of all such factors are described below—

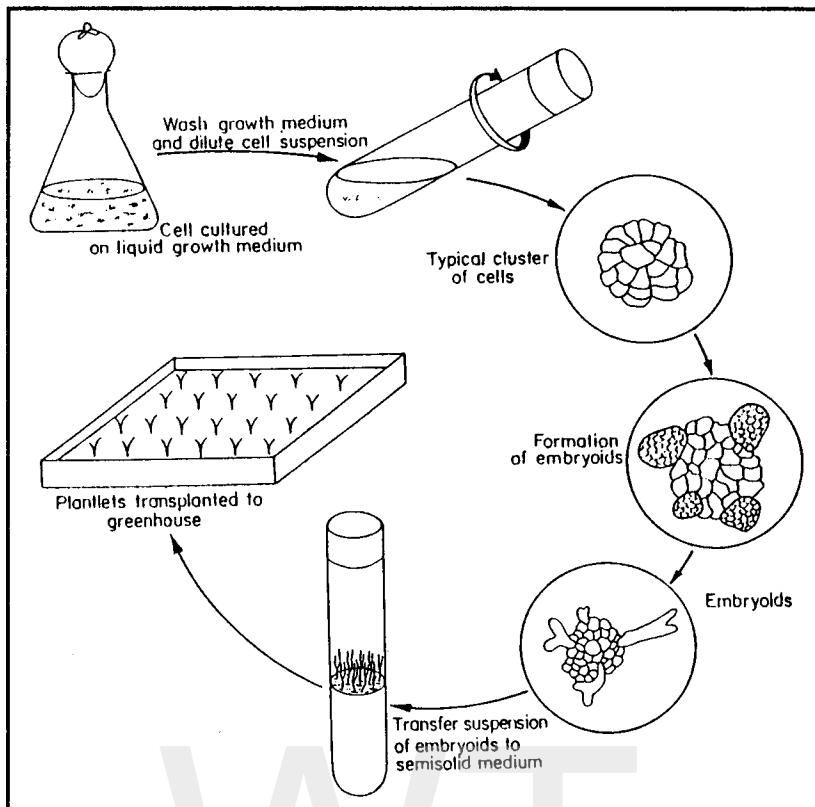


Fig 8.4

Flow diagram illustrating the protocol for inducing somatic embryogenesis in culture

## CHEMICAL FACTORS

### Auxin

Somatic embryogenesis in carrot is a classical example. It is a two-step process. The carrot cells first develop into a callus tissue in the medium containing the auxin, namely 2, 4-D (0.5–1 mg/L). When such callus tissue is transferred to the same medium with a very low level of auxin or no auxin at all, embryos are formed. If the callus tissue is maintained continuously in the medium containing 2, 4-D, embryos would not form. Similarly, if the carrot cells are maintained continuously from the initial step in auxin-free medium, embryos do not develop. Therefore, the presence of auxin in the first step is possibly essential for the proliferation of callus tissue and for the induction of embryogenic

potential cells. In the second step, auxin is no longer required for the embryogenic potential cells to form embryos. Like carrot, two-step process of *in vitro* development of somatic embryo is also found in *Coffea arabica*. Other than 2, 4-D, naphthalene acetic acid (NAA), indole butyric acid (IBA) have also been used in other culture system for the induction of embryogenic potential cells.

In *Citrus sinensis*, the callus tissue is initiated from the nucellar tissue in the medium containing IAA and Kinetin. Such medium is required for the callus growth and embryo differentiation. After repeated subculture in the same medium, the callus tissue shows a gradual decline in somatic embryogenesis. On the other hand, when such callus tissue is transferred to auxin-free medium, it again improves the

callus growth and embryogenesis. At that time even the addition of very low concentration of IAA inhibits the process of somatic embryogenesis. Therefore, it appears that after a prolonged period of culture, the callus tissue may become habituated or phytohormone autonomous. This means that they are now able to grow on a standard medium which is devoid of growth hormones. The cells appear to have developed the capacity to synthesise adequate amount of both auxin and cytokinin which they required for the growth and somatic embryogenesis. But after few subcultures, the habituated culture of *C. sinensis* again shows a decline in the embryogenic potential. When the habituated callus tissue is exposed to irradiation, the process of somatic embryogenesis is again improved. Irradiation is known to breakdown auxin. So this observation reveals that high level of endogenous auxin produced by habituated callus tissue inhibits the process of somatic embryogenesis. But when the tissue is irradiated, the high level of endogenous auxin is lowered and a minimal level of auxin is responsible for the process of somatic embryogenesis.

Thus, from the above experimental evidences, it appears that a minimal level of auxin is essential for the induction of embryogenic potential cells within the cultured tissue but for the organization and maturation of the embryos from the embryogenic potential cell, auxin does not play any positive role.

### Cytokinin

The effect of cytokinins in embryogenesis is somewhat obscure because of conflicting results. In carrot suspension culture, zeatin ( $0.1 \mu\text{M}$ ) a type of cytokinin, stimulates embryogenesis when the cells are subcultured in auxin-free medium. But the process is inhibited by the addition of either kinetin or benzylaminopurine (BAP) to the medium. The inhibitory effect of cytokinins may be due to selective stimulation of cell division of non-embryogenic cells of the culture. Stimulatory effect of cytokinin has also been reported in some specific culture system. Stewart *et al.* (1964) also reported the importance of coconut milk (containing a source of cytokinin) for somatic embryogenesis.

### Gibberellin

Gibberellin has no positive effect. In carrot and *Citrus*, gibberellin inhibits somatic embryogenesis.

### Reduced nitrogen

Substantial amount reduced nitrogen ( $\text{NH}_4^+$ ) are required for embryogenesis. In carrot culture, the addition of  $\text{NH}_4\text{Cl}$  to the embryogenic medium already containing  $\text{KNO}_3$  produces near-optimal numbers of embryos. It is, therefore, convenient to use  $\text{NH}_4^+$  in combination with  $\text{NO}_3^-$ . But no other form of inorganic reduced nitrogen has been as effective as  $\text{NH}_4^+$  for somatic embryogenesis.

Glutamine, glutamic acid, urea and alanine are found to partially replace  $\text{NH}_4\text{Cl}$  as a supplement to  $\text{KNO}_3$ . These various nitrogen sources are not specific for the induction of embryogenesis, although, at low concentration organic forms are much more effective than inorganic nitrogen compounds.

### OTHER FACTORS

The medium supplemented with activated charcoal has facilitated embryogenesis in several culture. The induction of embryogenesis is achieved successfully by the addition of charcoal when auxin depletion in the medium fails to produce the desired results. It has been suggested that charcoal may absorb a wide variety of inhibitory substances as well as hormone.

Optimal level of dissolved oxygen and high potassium in the medium are necessary for embryogenesis. But in *Citrus*, certain volatile and non-volatile substances inhibit embryogenesis.

### IMPORTANCE OF SOMATIC EMBRYOGENESIS

The potential applications and importance of *in vitro* somatic embryogenesis and organogenesis are more or less similar. The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system. The adventitious embryo is a bipolar

structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture. Somatic embryo has no food reserves, but suitable nutrients could be packaged by coating or encapsulation to form some kind of artificial seeds. Such artificial seeds produce the plantlets directly into the field. Unlike organogenesis, somatic embryos may arise from single cells and so it is of special significance in mutagenic studies.

Plants derived from asexual embryos may in some cases be free of viral and other pathogens. For an example, *Citrus* plant propagation from embryogenic callus of nucellar origin are free of virus. So it is an alternative approach for the production of disease-free plants.

## ARTIFICIAL SEEDS

In nature, true seeds are the product of fertilised ovules, consisting of a zygotic embryo (cells of which are diploid) enclosed by protective coat or coats developed from integument or integuments. In a seed, the zygotic embryo is a very important structure and it grows into seedling by seed germination. Now the concept of artificial seed has been developed from somatic embryos which are formed adventitiously from *in vitro* cultured somatic tissue (diploid cells). Somatic embryos are identical with zygotic embryos give rise to plants only under aseptic condition. Somatic embryos are not enclosed by seed coats and due to microbial contamination and desiccation they are not able to survive if these are sown directly into field soil. So, tissue culturists tried to develop a technique by which isolated somatic embryos could be encapsulated by a protective gel-like substance so that embryos were able to survive and would not desiccate even after planting into soil. Such encapsulated embryos could be used as artificial seeds.

Kitto and Janick (1982) first coated clumps of carrot embryos with polyoxethylene to develop artificial seeds. By this technique, some embryos survived the coating process as well as the desiccation step. Redenbangh *et al* (1984 and 1986) discovered that hydrogels such as sodium

alginate could be used to produce single-embryoid artificial seeds. But, in a few experiment, the artificial seeds were planted in the greenhouse with plant production. Later Lawrence *et al* subsequently began to use sodium alginate for encapsulating corrot somatic embryoids. In this, they were successful in obtaining germination of encapsulated carrot somatic embryoids in vermiculite in a growth chamber. Since then, on the basis of such report, research work begins to exploit this new technique for other plant species and to improve the encapsulation process.

## WHAT ARE ARTIFICIAL SEEDS ?

Artificial seeds are the living seed-like structure which are made experimentally by a technique where somatic embryoids derived from plant tissue culture are encapsulated by a hydrogel and such encapsulated embryoids behave like true seeds if grown in soil and can be used as a substitute of natural seeds.

## METHOD FOR MAKING ARTIFICIAL SEEDS

Several steps are followed for makeing artificial seeds—

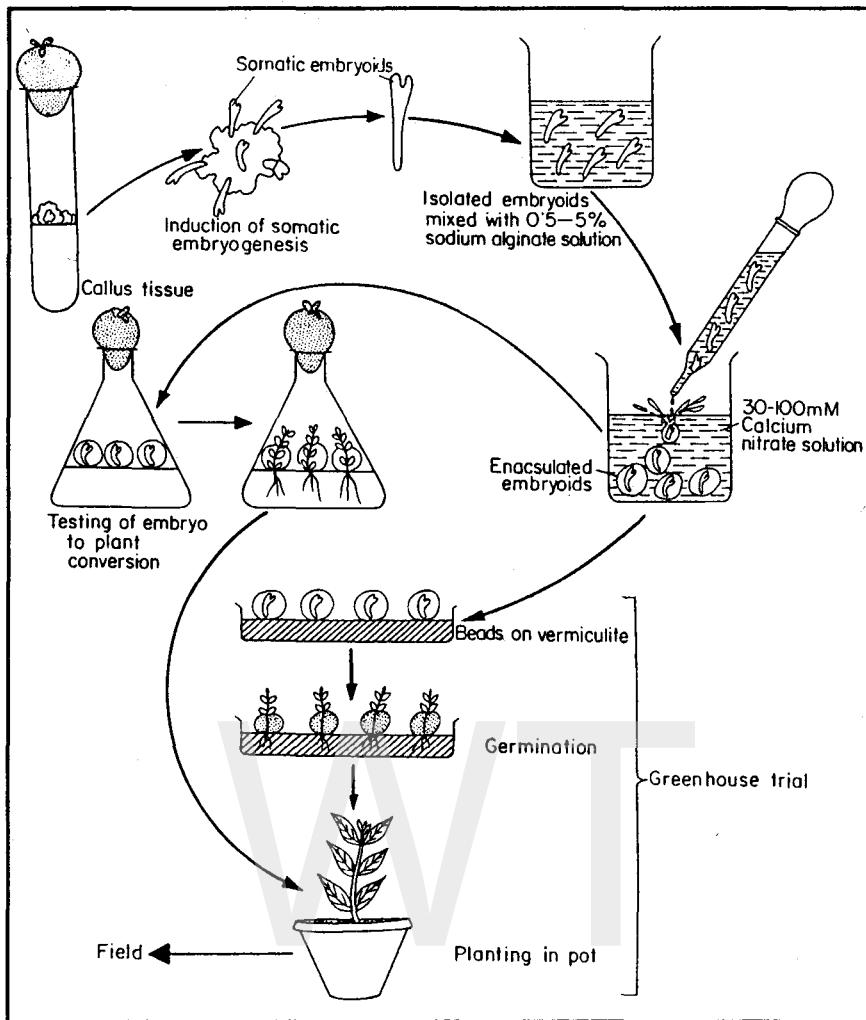
- (1) establishment of callus culture
- (2) induction of somatic embryogenesis in callus culture
- (3) maturation of somatic embryos
- (4) encapsulation of somatic embryos

After encapsulation, the artificial seeds are tested by two steps—

- (1) test for embryoid to plant conversion
- (2) green-house and field planting.

Establishment of callus culture and the induction of somatic embryogenesis in callus culture has already been discussed in details previously.

Maturation of somatic embryos means the completion of embryo development through



□ Fig 8.5

#### Flow diagram showing the method for making artificial seeds

some stages. Initially, embryo develops as globular-shaped stage, then heart-shaped stage and finally torpedo-shaped stage. In the final stage, embryo attains maturity and develops the opposite poles for shoot and root development at the two extremities. This embryo then starts to germinate and produces plantlet. However, in some plant species, such sequential development may not be followed. Again, in some species requiring cold treatment for embryo germination, it may be necessary to chill young or mature embryos for their normal maturation and development into plantlets. Application of GA<sub>3</sub> is also

required for root and shoot development during embryo germination in *citrus*.

Water soluble hydrogels have been found suitable for making artificial seeds. A list of some useful hydrogels for encapsulation of somatic embryos are given in Table 8.1.

Two standardised methods have been used to coat somatic embryos—

- (i) gel complexation via a dropping procedure;
- (ii) Molding.

**Table 8.1 Useful Hydrogel for Encapsulation**

Gel	Conc. % W/V	Complexing agents	Conc. mM
1. Sodium Alginate	0.5–5.0	Calcium salts	30–100
2. Sodium Alginate with Gelatin	2.0 5.0	Calcium Chloride	30–100
3. Carragenan with Locust Beam Gum	0.2–0.8 0.4–1.0	Potassium or Ammonium chloride	500
4. Gel-rite <sup>TM</sup>	0.25	Temperature lowered	

In the first method, isolated somatic embryos are mixed with 0.5 to 5% (W/V) Sodium alginate and dropped into 30–100  $\mu\text{M}$  Calcium nitrate solution. Surface complexation begins immediately and the drops are gelled completely within 30 minutes (Fig 8.5).

In the second method, isolated somatic embryos are mixed in a temperature-dependent gel such as Gel-rite<sup>TM</sup> and placed in the well of a microtiter plate and it forms gel when the temperature is cooled down.

To achieve the satisfactory results, research is required in several areas for making artificial seeds. Somatic embryos need to be produced on a large scale, matured to a stage where germination will be at a high rate and frequency and encapsulated embryos will probably need to be coated to prevent capsule desiccation and allow for singulation during planting.

After encapsulation, initially, the effect of coating on somatic embryos is very difficult to assess because the germination and continued development of the encapsulated embryos are sometimes very inconsistent after planting into soil. So, to overcome this problem, embryo response in terms of embryo to plant development or conversion is tested under aseptic conditions. Embryo conversion frequency is the percent of the somatic embryos that produce green-plants having a normal phenotype. Embryo to plant conversion includes the following steps—

- Encapsulated embryos are placed aseptically on simply agar medium with minimal nutrients.
- Uniform germination of somatic embryos and growth and development of root and shoot systems.
- Production of true leaves.
- Absence of hypostrophy of the hypocotyl.
- A green-plant with a normal phenotype.

This assay should be very critical before showing the artificial seed in green-house or in the field. Otherwise, some modifications are to be required. The final assessment will be the

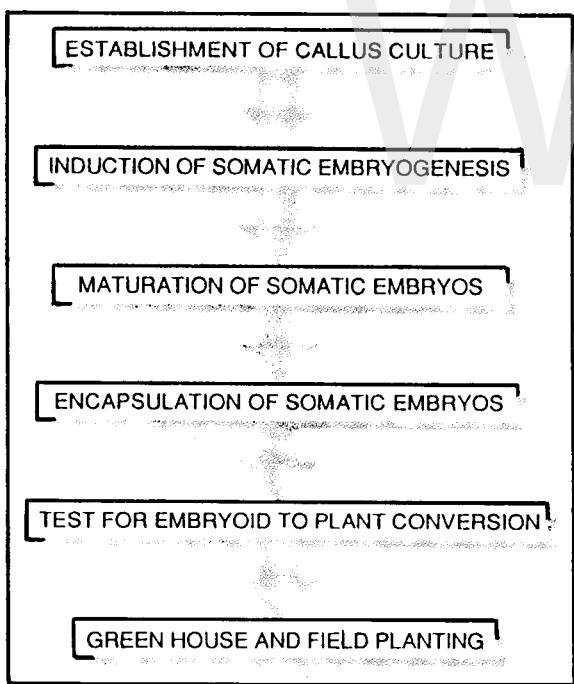


Fig 8.6

**Flow diagram showing the steps of production of artificial seeds**

green-house or field performance of artificial seed and their yield in comparision to plants derived from true seeds.

Storage of artificial seeds is a great limitation. When the artificial seeds are stored at low temperature, the embryos show a characteristic drop in conversion. The limited storage time of artificial seeds is probably due to an anaerobic environment in the capsule. This is a problem for somatic embryos because they are not developmentally arrested and continue very active respiration. To overcome this limitation, two possible solutions are, to have a smaller ratio of capsule volume to embryo volume so that gas diffusion can readily occur or to induce an arrested state in the embryo using growth control agent in the encapsulation medium.

Although the initial cost for artificial seeds i.e. cost of labour and material for the tissue culture processes and encapsulation, is considerably higher than that for true seeds, still there may be some advantages for the use of artificial seeds.

## IMPORTANCE OF ARTIFICIAL SEEDS

The potential importance of artificial seeds are more or less similar to that of somatic embryogenesis, still it has some practical applications—

- (1) True seeds are produced in plant at the end of reproductive phase by the process of complex sexual reproduction. A plant may take a long or short time to attain the reproductive phase. So we have to wait upto the end of reproductive phase of a plant for getting seeds. But artificial seeds are available within at least one month. Nobody has to wait for a long time.
- (2) Plants bear the flower and produce the seeds at particular season of a year. But the production of artificial seed is not time or season dependent. At any time or season, one may get the artificial seeds of a plant.

- (3) Occasionally, the work on some plants is delayed due to presence of long dormancy periods of their seeds. By growing artificial seeds, this period may be reduced. Using artificial seeds, the life cycle of a plant could be shortened.
- (4) Somatic embryogenesis has been observed in a great many species to date, which indicates that it may be possible to produce artificial seeds in almost any desired crops. Successful results have already been obtained in some crops such as *Apium graveolens*, *Daucus carota*, *Zea mays*, *Lactuca sativa*, *Medicago sativa*, *Brassica* sp. *Gossypium hirsutum*.
- (5) Artificial seeds will be applicable for large-scale monocultures as well as mixed-genotype plantations.
- (6) It gives the protection of meiotically unstable, elite genotypes.
- (7) Artificial seed coating also has the potential to hold and deliver beneficial adjuvants such as growth promoting rhizobacteria, plant nutrients and growth control agents and pesticides for precise placement.
- (8) Artificial seeds help to study the role of endosperm and seed coat formation.

## Summary

An alternative pathway of whole plant regeneration is observed in cell cultures of some plant species when they undergo somatic embryogenesis. This means that small, bipolar embryo-like structures develop at the callus surface. These are commonly called somatic or non-zygotic embryoids and have a number of structural similarities to the sexual or zygotic embryo found in the developing seed. They can, under appropriate conditions, be grown on a standard maintenance medium into whole plants. The first non-zygotic embryos in tissue culture were

discovered by Reinert in *Daucus carota*. Somatic embryogenesis may be initiated in two different ways such as direct embryogenesis and indirect embryogenesis. In some cultures, somatic embryogenesis occurs directly from the somatic cells of explant without an intervening callus tissue. The cells of explant undergo direct embryogenesis from proembryonic determined cells in absence of callus proliferation. In the second type of somatic embryogenesis, cells of explant first undergo callus proliferation and embryos develop within the callus tissue from induced embryogenic cells. Embryo formation is more common in liquid-grown cultures. For indirect embryogenesis, two distinctly different types of media may be required. One medium for the initiation of the embryonic cells and another for the subsequent development of these cells into embryos. The first medium must contain auxin in case of carrot tissue and somatic embryo genesis can be initiated in the second medium by removing the hormone or lowering its concentration.

Each developing embryo of carrot tissue passes through three sequential stages of embryo formation such as globular stage, heart-shaped stage and torpedo stage. The torpedo stage gives rise to complete plantlet. Somatic embryogenesis occurs in short-term culture and their ability decreases with increasing duration. Changes in ploidy of the cultured cells may lead to loss of embryogenic potential in long-term culture. Attempts have been made to synchronize the development of somatic embryogenesis in culture.

A number of factors have been shown to have a profound effect on somatic embryogenesis such as auxin, cytokinin, gibberellin, reduced nitrogen, supplementation of charcoal in the medium, optimum level of dissolved oxygen, potassium concentration etc.

The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system. Somatic embryos have no food reserve but suitable nutrients could be packaged by coating or encapsulation to form some kind of artificial seeds. Plants derived from asexual embryos may in some cases be free of viral and other pathogens.

Artificial seeds are the living seed-like structures which are made experimentally by a technique where somatic embryos derived from plant tissue culture are encapsulated by a hydrogel and such encapsulated embryos behave like true seeds if grown in soil and can be used as a substitute of natural seeds. Several steps are followed for making artificial seeds such as (1) establishment of callus culture, (2) induction of somatic embryogenesis in callus culture, (3) maturation of somatic embryos, (4) encapsulation of somatic embryos, (5) testing of encapsulated embryos to plant conversion.

Water soluble hydrogels have been found suitable for making artificial seeds. Two methods have been used to coat somatic embryos. In the first method, isolated somatic embryos are mixed with 0.5–5% w/v. Sodium alginate and dropped them into 30–100  $\mu\text{M}$  calcium nitrate solution. Surface complexion begins immediately and drops are gelled completely within 30 minutes. In the second method, isolated somatic embryos are mixed in a temperature-dependent gel such as Gel-rite<sup>TM</sup> and placed in the well of a microtiter plate and it forms gel when the temperature is cooled down.

Before sowing the artificial seed in greenhouse or in the field, the embryo response in terms of embryo to plant conversion is tested under aseptic conditions.

An artificial seeds has some practical application such as—

- (1) Production of artificial seed is not dependent on reproductive phase of plant.
- (2) It can be produced at any season of a year.
- (3) By growing artificial seed, the life cycle of a plant could be shortened. An artificial seed has no dormancy phase.
- (4) Artificial seed coating has the capacity to hold growth-promoting rhizobacteria growth control agents and pesticides.
- (5) It gives the protection of meiotically unstable, elite genotype.

## **Questions for Discussion**

1. What is somatic embryogenesis? Discuss the principle of somatic embryogenesis.
  2. Describe the protocol for inducing somatic embryogenesis in culture of carrot and discuss the factors affecting somatic embryogenesis.
  3. What is artificial seed? How it is prepared in the laboratory? Discuss the importance of somatic embryogenesis and artificial seeds.
  4. Write the brief answer to following questions—
    - (a) What are the suitable conditions for somatic embryogenesis?
  - (b) Does auxin play any positive role during embryogenesis?
  - (c) Why activated charcoal has facilitated somatic embryogenesis?
  - (d) What is the difference between true seed and artificial seed?
  - (e) What is the advantage of artificial seeds than true seeds?
5. Write short notes on—
- (a) Indirect somatic embryogenesis
  - (b) Role of reduced nitrogen on embryogenesis
  - (c) Direct embryogenesis
  - (d) Artificial seeds.

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## Chapter Nine

### Single Cell Culture

#### INTRODUCTION

A flowering plant body is made of a wide range of innumerable cell types which are successfully integrated both in terms of structure and function. The plant body acquires this cellular diversity either due to permanent alteration in the genetic composition of the cells or due to mere adaptation of the cells to perform a particular function in the plant body without affecting their genetic make-up. It is amazing that all such cell types have been derived from a single-celled zygote by equational division. To understand the complex interrelationships and complementary influences of different cells in multicellular organism, many workers tried to culture the isolated cells to see if each one of them retained the ability to behave like a zygote and could give rise to a complete plant. Faced with the problem of poor techniques available at that time, they could not induce division in the isolated cells in culture.

But with the refinement of methodology, today it is possible not only to culture single cells but also to induce the cell division and to raise a whole plant from it.

The advantage of a single cell culture over callus culture or cell suspension culture or intact organ culture is that single cell culture system is an ideal system for studying cell metabolism, the effects of various substances on cellular responses and to obtain single cell clones.

#### WHAT IS SINGLE CELL CULTURE ?

Single cell culture is a method of growing isolated single cell aseptically on a nutrient medium under controlled condition.

#### BRIEF HISTORICAL BACKGROUND

**G. Haberlandt** (1902)—Made pioneer attempt to isolate and culture single cell from the leaves of flowering plants.

**W. H. Muir** (1954)—Described the paper raft nurse technique for the culture of single cells.

**A. C. Hildebrandt** (1960)—Attempted to culture single cells of a tumerous hybrids tobacco plant (*Nicotiana tabacum* × *N. glutinosa*) using the microchamber technique.

**H. W. Kohlenback** (1966)—Isolated the single cells from the leaf mesophyll of *Mecleaya cordata* and grew them in culture. Later, it was shown that the callus tissue obtained from such highly differentiated cells could be induced to produce embryoids.

**E. Ball and P C. Joshi** (1968)—Isolated cells from the mature leaves of groundnut by first tearing across the leaves to expose the mesophyll cells followed by scraping of the cells with a fine scalpel.

**L. Bergmann** (1968)—Grew the single cells first using petri dish plating technique.

**I. Takebe** (1968)—First reported the large-scale isolation of metabolically active mesophyll cells of tobacco by enzymatic method.

**L. Rossini** (1969)—Describe a method for the large scale mechanical isolation of single cells from the leaves of *Calystegia sepium*.

## PRINCIPLE OF SINGLE CELL CULTURE

The basic principle of single cell culture is the isolation of large number of intact living cells and culture them on a suitable nutrient medium for their requisite growth and development.

Single cells can be isolated from a variety of tissue and organ of green plant as well as from callus tissue and cell suspension. Single cells from the intact plant tissue (leaf, stem, root, cladode etc.) are isolated either mechanically or enzymatically.

Mechanical isolation involves tearing or chopping the surface sterilized explant to expose the cells followed by scraping of the cells with a fine scalpel to liberate the single cells hoping that it remains undamaged. But very few living cells are obtained for a lot of time and effort. Gentle grinding of surface sterilized explant in a sterilized mortar-pestle followed by cleaning the cells by filtration and centrifugation is now widely used for the large-scale mechanical isolation of viable cells.

A considerably more efficient way of large-scale isolation of free cells from the surface steril-

ized is to dissolve the intercellular cementing material, i.e. pectin, by pectinase or macerozyme treatment. The enzyme macerates the tissue from which large-number of variable cells can be obtained. The special feature of enzymatic isolation of cell is that it has been possible to obtain pure preparation of viable cells with less effort and time.

The single cells are traditionally isolated from the established friable callus tissue and cell suspension culture. Mechanically, single cells are carefully isolated from cell suspension or friable callus with a needle or fine glass capillary. Alternatively, the friable tissue is transferred to liquid medium and the medium is continuously agitated by a shaker. Agitation of liquid medium breaks and dispenses the single cells and cell clumps in the medium. As a result, it makes a cell suspension. The cell suspension is first filtered to remove cell clumps and the filtrate is then centrifuged to collect the single cells from the pellet.

The isolated single cell can be cultured either in liquid medium or on solid medium. There are five basic methods that are used for culturing single cells such as paper raft nurse technique, the petri dish plating technique, the microchamber technique, the microdroplet technique, the plating with nurse tissue technique.

In culture, the single cells divide redivide to form a callus tissue. Such callus tissue also retains the capacity to regenerate the plantlets through organogenesis and embryogenesis.

## METHODS OF SINGLE CELL CULTURE

There are five important methods which are widely employed for culturing single cells. The methods are—

- (i) the paper raft nurse technique
- (ii) the petri dish plating technique
- (iii) the microchamber technique
- (iv) the nurse callus technique
- (v) the microdroplet technique.

Before going to follow these techniques, it is very important to isolate the single cells properly as described earlier (see the principle of single cell culture). So isolated single cells are the essential prerequisite item for all such methods.

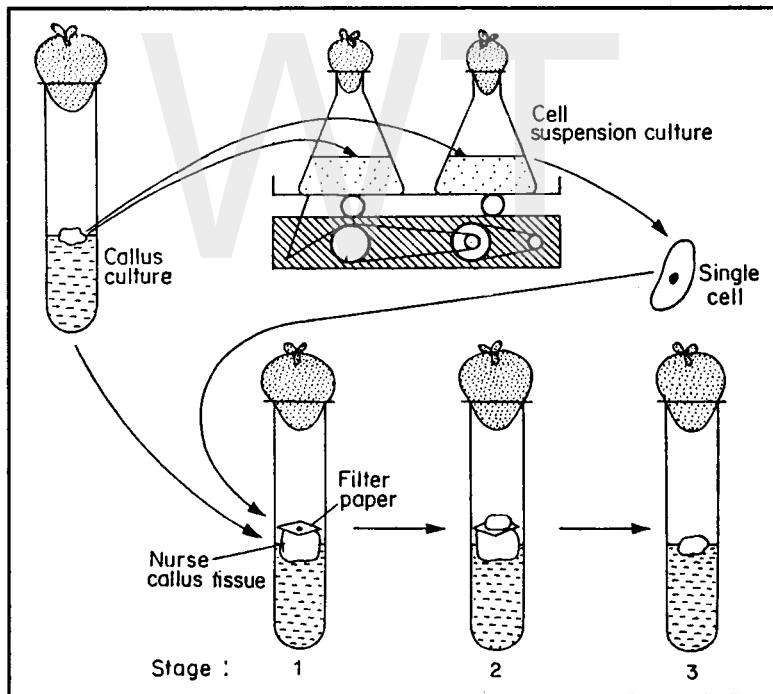
### THE PAPER RAFT NURSE TECHNIQUE

1. Single cells are isolated from suspension cultures or a friable callus with the help of a micropipette or microspatula.
2. Few days before cell isolation, sterile 8 mm x 8 mm squares of filter paper are placed aseptically on the upper surface of the actively growing callus tissue of the same or different species.
3. The filter paper will be wetted by soaking the water and nutrient from the callus tissue.
4. The isolated single cell is placed aseptically on the wet filter paper raft (Fig 9.1).

5. The whole culture system is incubated under 16 hrs. cool white light (3,000 lux) or under continuous darkness at 25°C.

6. The single cell divides and redivides and ultimately forms a small cell colony. When the cell colony reaches a suitable size, it is transferred to fresh medium where it gives rise to the callus tissue.

The callus tissue, on which the single cell is growing, is called the nurse tissue. Actually the callus tissue supplies the cell with not only the nutrients from the culture medium but something more that is critical for cell division. The single cell absorbs nutrients through filter paper. The nutrients actually diffuse upward from culture medium through callus tissue and filter paper to the single cell. A callus tissue originating from a single cell is known as a single cell clone.



□ Fig 9.1

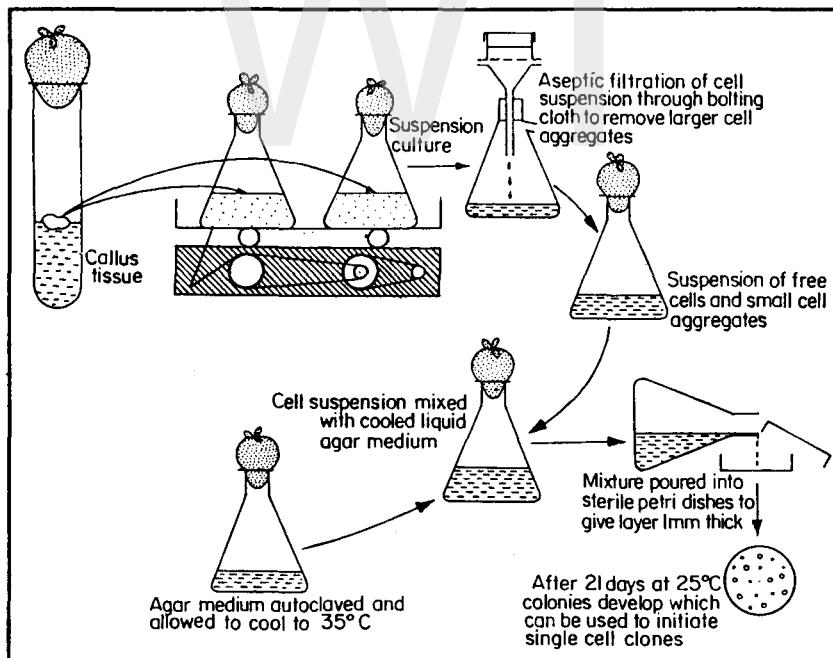
**Growth of single cells using a 'nurse' technique.** Stage 1 : a single cell taken from a friable callus is placed on upper surface of filter paper which is in contact with nurse callus. Stage 2 : the single cell divides and daughter cells proliferate to form colony. Stage 3 : when colony reaches a suitable size it is transferred to fresh medium where it gives rise to a single cell clone.

## THE PETRI DISH PLATING TECHNIQUE

1. A suspension of purely single cells is prepared aseptically from the stock cell suspension culture by filtering and centrifugation. The requisite cell density in the single cell suspension is adjusted by adding or reducing the liquid medium.
2. The solid medium (1.6% 'Difco' agar added) is melted in water bath.
3. In front of laminar air flow, the tight lid of Falcon plastic petri dish is opened. With the help of sterilized Pasteur pipette, 1.5 ml of single cell suspension is put and equal amount of melted agar medium when it cools down at 35°C, is added in the single cell suspension (Fig 9.2).
4. The lid is quickly replaced and the whole dish is swirled gently to disperse the cell and medium mixture uniformly throughout the lower half of the petri dish.
5. The medium is allowed to solidify and the petri dish is kept at the inverted position.
6. The cultures are incubated under 16 hrs. light (3,000 lux, cool white) or under continuous dark at 25°C.
7. The petri dishes are observed at regular intervals under inverted microscope to see whether the cells have divided or not.
8. After certain days of incubation, when the cells start to divide, a grid is drawn on the undersurface of the petri dish to facilitate counting the number of dividing cells.
9. The dividing cells ultimately form pin-head shaped cell colonies within 21 days of incubation.
10. The plating efficiency (PE) can be calculated from the counting of cell colonies by the following formula—

$$PE = \frac{\text{Number of colonies per plate}}{\text{Number of total cell per plate}} \times 100$$

11. Pin-head shaped colonies, when they reach a suitable size, are transferred to fresh medium for further growth.



□ Fig 9.2

Procedure for obtaining single cell clones using a petri dish plating technique

## THE MICROCHAMBER TECHNIQUE

1. A drop of liquid nutrient medium containing single cell is first isolated aseptically from stock suspension culture with the help of long fine Pasteur pipette.
2. The culture drop is placed on the centre of a sterile microscopic slide ( $25 \times 75$  mm) and ringed with sterile paraffin oil.
3. A drop of paraffin oil is placed on either side of the culture drop and a coverglass (called raiser) is placed on each oil drop (Fig 9.3).
4. A third coverglass is then placed on the culture drop bridging the two raiser coverglasses and forming a microchamber to enclose the single cell aseptically within the paraffin oil. The oil prevents the water loss from the culture drop but permits gaseous exchange.
5. The whole microchamber slide is placed in a petri-dish and is incubated under 16 hrs. white cool illumination (3,000 lux) at  $25^{\circ}\text{C}$ .

6. Cell colony derived from the single cell gives rise to single cell clone.

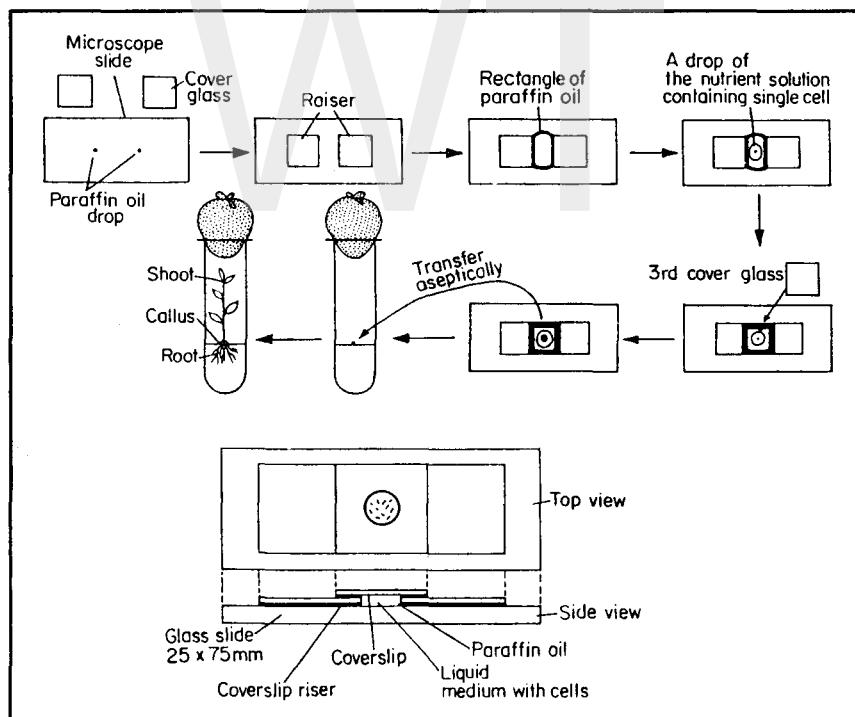
7. When the cell colony becomes sufficiently large, the coverglass is removed and the tissue is transferred to fresh solid or semisolid medium.

The microchamber technique permits regular observation of the growing and dividing cell.

## GROWTH OF SINGLE CELL INDUCED BY NURSE CALLUS

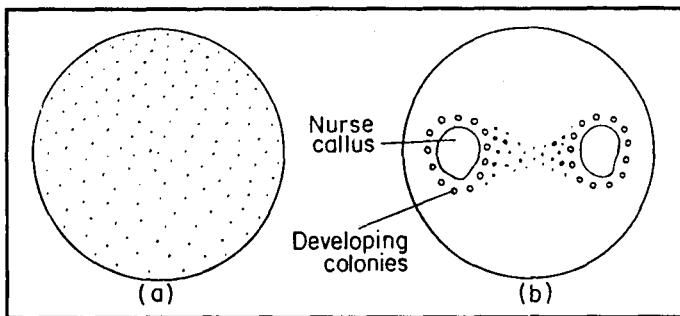
This method is actually a modification of petridish plating method and the paper raft nurse culture method.

In this method, single cells are plated on to agar medium in a petridish as described earlier. Two or three callus masses (Nurse tissue) derived from the same plant tissue are also embedded directly along with the single cells in the same medium (Fig 9.4). Here the paper barrier



□ Fig 9.3

Microchamber used to observe the growth of single cells



□ Fig 9.4

**Growth of colonies from a low density cell suspension in the presence of callus tissue.** A. Petri dish inoculated with low density suspension of cells—no colonies develop. B. Petri dish inoculated with low density suspension plus nurse callus—colonies grow near to nurse calluses only

between single cells and the nurse tissue is removed. Cells first begin to divide in the regions near the nurse callus indicating that the single cells closer to nurse callus in the solid medium gets the essential growth factors that are liberated from the callus mass. The developing colonies growing near to nurse callus also stimulate the division and colony formation of other cells.

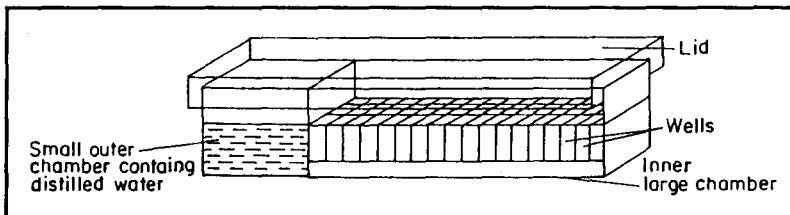
### THE MICRODROPLET TECHNIQUE

1. In this method, single cells are cultured in special Cuprak dishes which have two chambers—a small outer chamber and a large inner chamber. The large chamber carries numerous numbered wells each with a capacity of  $0.25\text{--}25\mu\text{l}$  of nutrient medium.
2. Each well of the inner chamber is filled with a microdrop of liquid medium containing isolated single cell. The outer chamber is filled with sterile distilled water to maintain the humidity inside the dish (Fig 9.5).

3. After covering the dish with lid, the dish is sealed with paraffin.
4. The dish is incubated under 16 hrs. white cool light (3,000 lux) at  $25^\circ\text{C}$ .
5. The cell colony derived from the single cell is transferred on to fresh solid or semisolid medium in a culture tube for further growth.

### FACTORS AFFECTING SINGLE CELL CULTURE

1. The composition of the medium for the growth of single cell culture is generally more complex than callus and cell suspension culture. For example, *Convolvulus* cells require a cytokinin and amino acids that are not necessary for the callus culture of that species.
2. Induction of division of single cells using paper raft technique, indicates that isolated cells get the exact essential nutrient from



□ Fig 9.5

**Diagrammatic view of Cuprak dish used for the microdroplet technique of single cell culture**

the callus mass. It has been suggested that the callus mass leaches out the essential nutrient through plasma membrane of the cells.

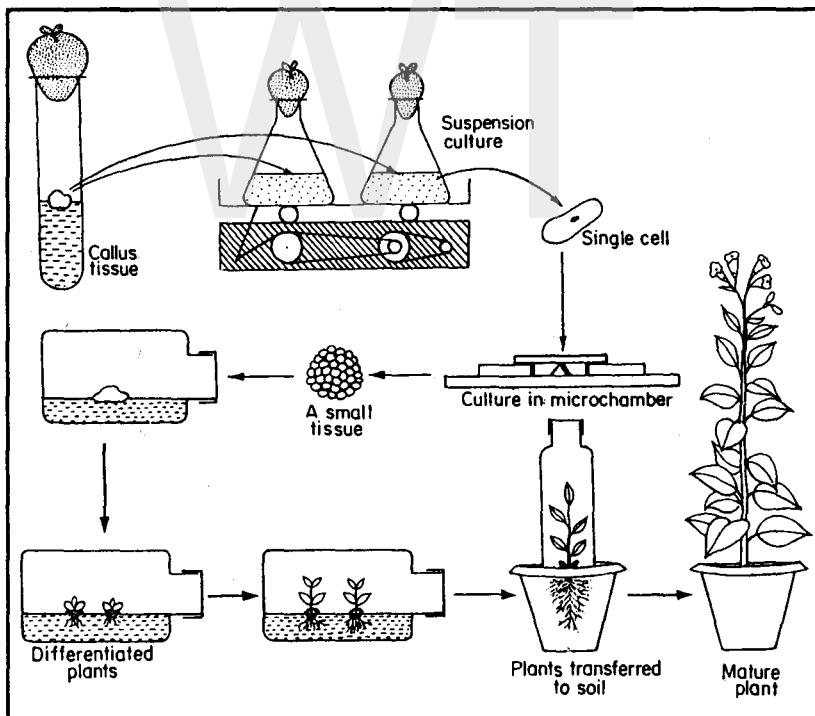
3. In case of petri dish plating technique the initial plating cell density is very critical.

### IMPORTANCE OF SINGLE CELL CULTURE

Single cell culture technique is very important for the fundamental and mutation studies and it has a wide industrial application.

1. Single cell culture could be used successfully to obtain single cell clones.
2. Plants could be regenerated from the callus tissue derived from the single cell clones (Fig 9.6).
3. The occurrence of high degree of spontaneous variability in the cultured tissue and their exploitation through single cell culture are very important in relation to crop improvement programmes.

4. One of the major problem of mutation breeding in higher plants is the formation of chimeras following the mutagenic treatment of multicellular organism. In this respect single cell culture method are more efficient. Isolated single cells can be handled as a microbial system for the treatment of mutagens and for mutant selection. In practice, single cells are grown on a medium containing the mutagenic compounds and the proliferating cell lines are isolated. The mutant nature of the selected cell lines can be confirmed by regenerating the plants and comparing their phenotypes with a normal plant. Many cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins etc. have been selected by this simplest method.
5. Many plants synthesize various important natural compounds in the form of alkaloids, steroids etc. Some of these natural compounds are highly medicinally important. Several workers have reported the synthesis



□ Fig 9.6

Development of a tobacco plant from a single cell

sis of several times higher amounts of alkaloid by cell culture than the alkaloid content in the intact plant. So, from the commercial point of view, single cell culture in large-scale could become a valuable technique for industrial production of such important natural compound.

6. **Biotransformation** means the cellular conversion of an exogenously supplied substrate compound not available in the cell or the precursor of a particular cellular compound to a new compound or the known compounds in higher amounts.

A cell can be described as a metabolic factory where a large-number of enzyme systems are working. When the cells are fed with analogues or intermediate or precursor compounds of a metabolic pathway, the cells place them immediately to the particular metabolic pathway and switch on its machinery for the production of the particular compound. Single cell culture is an ideal system for the study of biotransformation. Generally, two approaches are now being followed for biotransformation studies using single cell culture such as—

- (i) cells are fed with substrate compounds normally not available to the plant. The main objective of such feeding is to obtain a new compound through the biotransformation process.
  - (ii) cells are fed with precursor of a compound available in the plant. The main objective of such feeding is to enhance the production of a compound.
7. **Induction of polyploidy** has been found to be very useful for plant breeding to overcome the problem of sterility associated with hybrids of unrelated plants. Polyploidy can easily be achieved by single cell culture.

A large-number of genetically sterile hybrids exist in the genus *Saccharum*. When cell culture of such sterile hybrid is treated with 50 mg/L colchicine for 4 days, it has been found that about 48% of such treated cells become uniformly polyploid. These polyploid cells are then

induced to regenerate a large-number of fertile plants. In this regard, cell culture is very useful with other crops also.

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## Summary

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With the refinement of methodology, today it is possible not only to culture single cells but also to induce the cell division and to raise whole plants from it. The advantage of a single cell culture over callus culture or cell suspension culture is that a single cell can be handled as a microbial system and an ideal system for studying cell metabolism, the effect of various substances on cellular responses and to obtain single cell clones.

Single cell culture is a method of growing isolated single cell aseptically on a nutrient medium under controlled condition. In 1902, G. Haberlandt made pioneer attempt to isolate and culture single cells from the leaves of flowering plants.

Single cells can be isolated from a variety of tissues of intact plants as well as from cultured tissues. Single cells from the intact plant tissue are isolated either mechanically or enzymatically. Mechanical isolation involves tearing or chopping the surface sterilized explant to expose the cells followed by scraping of cells with a fine scalpel. A considerably more efficient way of large-scale isolation of single cells is the enzymatic method which consists of treating the tissue with pectinase or macerozyme to dissolve the intercellular cementing material, i.e. pectin. The single cells are isolated from the established friable callus tissue by transferring them into agitated liquid medium followed by filtering through nylon mesh and centrifugation.

The isolated single cell can be cultured either in liquid medium or on solid medium. There are five important methods which are widely employed for culturing single cells. The methods are—(i) the paper raft nurse technique, (ii) the petridish plating technique, (iii) the microchamber technique, (iv) the nurse callus technique, (v) the microdroplet technique.

The composition of the culture medium for the growth of single cell is generally more complex than callus and cell suspension culture. In case of petridish plating technique the initial plating cell density is very critical.

Single cell culture technique is very important for the fundamental and mutation studies and it has wide industrial applications.

## Questions for Discussion

1. What do you mean by the term tissue culture and single cell culture? Give an outline of the method of single cell culture.
2. Give a general account of the principle and importance of single cell culture.
3. Write brief answers to the following questions—

- (a) What is the advantage of a single cell culture over callus culture or cell suspension culture?
  - (b) How are the single cells isolated from the intact plant tissue and cultured plant tissue?
  - (c) Suppose, the number of total cell per plate is  $5 \times 10^4$  and the number of colonies formed per plate is  $10^3$ . Calculate the plating efficiency of the culture.
4. Write short notes on—
    - (a) Single cell culture
    - (b) Paper raft nurse technique
    - (c) Nurse callus technique
    - (d) Microdroplet technique
    - (e) Microchamber technique
    - (f) Factors affecting cell culture
    - (g) Importance of single cell culture in mutation breeding.

## Chapter Ten

### Embryo Culture

#### INTRODUCTION

In angiosperms, embryo represents the beginning of sporophyte. Normally, the fertilized egg or zygote undergoes embryogenesis in the post-fertilization stage within the ovule and thus an embryo is formed inside the seed. The typical seed embryo is a bipolar structure consisting of a contrasting meristem at each pole—the primordial shoot or the plumule and the primordial root or the radicle and one or two lateral appendages, the cotyledons. The mature embryo, therefore, possesses the basic organisation of the adult plant. During seed germination a plant is produced through progressive and orderly changes of the embryo. Like any other plant organ, embryo can be used as explant and cultured aseptically in test tube containing medium. For this purpose the embryo is excised without any damage from ovule, seed or capsule. Embryo culture allows one to investigate the factors that influence the embryonic growth under controlled conditions. *In vitro* culture of embryo also facilitates determination of the factors that regulate the growth of the primordial organs of the seedling plant and study of the successive biochemical and metabolic changes of germination which is difficult to undertake in embryos

enclosed within seed without the interference from accessory tissues. From the applied point of view, *in vitro* culture of embryo is very important to obtain viable hybrid plants from non-viable embryos resulted from unsuccessful breeding experiments. Embryo culture is also practically applicable to by-pass the traditional treatments to overcome seed dormancy and accelerate germination in certain types of seeds.

#### BRIEF PAST HISTORY

**E. Hanning** (1904)—first removed mature embryos from seeds of *Raphanus sativus*, *R. landra*, *R. candatus*, *Cochlearia danica* and nurtured them into plantlets in a medium containing mineral salts and sucrose. His work is generally accepted as marking the birth of plant embryo culture.

**M. Dubard, J. A. Urbain, G. D. Buckner and J. H. Kastle** (1913–1917)—studied the role of endosperm in the growth of embryos by separating the latter from the endosperm or by decotylating embryos and planting them in nutrient solutions.

**D. I. Andronescu** (1919)—showed that the graminean embryo devoid of scutellum grew feebly in the nutrient medium.

**L. Knudson** (1922)—first succeeded in germinating orchid embryos into plantlets in the absence of symbiotic fungus by growing them on nutrient agar medium containing sugar.

**K. Dieterich** (1924)—on the basis of the physiological behaviour of cultured embryos of plants belonging to several families, he pointed out that the embryo grown *in vitro* usually skipped a rest period that was observed when it was a part of the intact seed. Dieterich first introduced the term 'precocious germination' in order to explain the malformation of seedling raised from the embryo of immature seed *in vitro*.

**F. Laibach** (1925–1929)—reported the immense application of embryo culture technique in rearing viable seedling from unsuccessful crosses.

**C. D. LaRue** (1936)—successfully grew embryos of a number of dicotyledons, monocotyledons and gymnosperms.

**J. van Overbeek** (1941–1942)—first demonstrated that mature embryos were self-nourishing and grew into seedlings in simple medium, but proembryos failed to grow or grew only feebly. Van Overbeek also showed that the culture of proembryos was possible in highly enriched medium containing coconut milk.

After the successful achievement of embryo culture *in vitro* mentioned above, embryos excised from ovule and seeds of the most varied plant species have been grown under controlled conditions by several workers time to time and much useful information on their nutritional requirements, growth, differentiation, application has accumulated.

### WHAT IS EMBRYO CULTURE?

The embryo of different developmental stages, formed within the female gametophyte through sexual process, can be isolated aseptically from the bulk of maternal tissues of ovule, seed or capsule and cultured *in vitro* under aseptic and controlled physical conditions in glass

vials containing nutrient solid or liquid medium to grow directly into plantlet.

## DIFFERENT CATEGORIES OF EMBRYOCULTURE AND THEIR OBJECTIVES

Culture of embryo (Fig 10.1) may be divided into the following categories—

### CULTURE OF MATURE AND INTACT SEED EMBRYO

The aim of this study is to analyse the various parameters of embryonic growth and the metabolic and biochemical aspects of dormancy and germination.

### CULTURE OF SURGICALLY DISSECTED EMBRYO

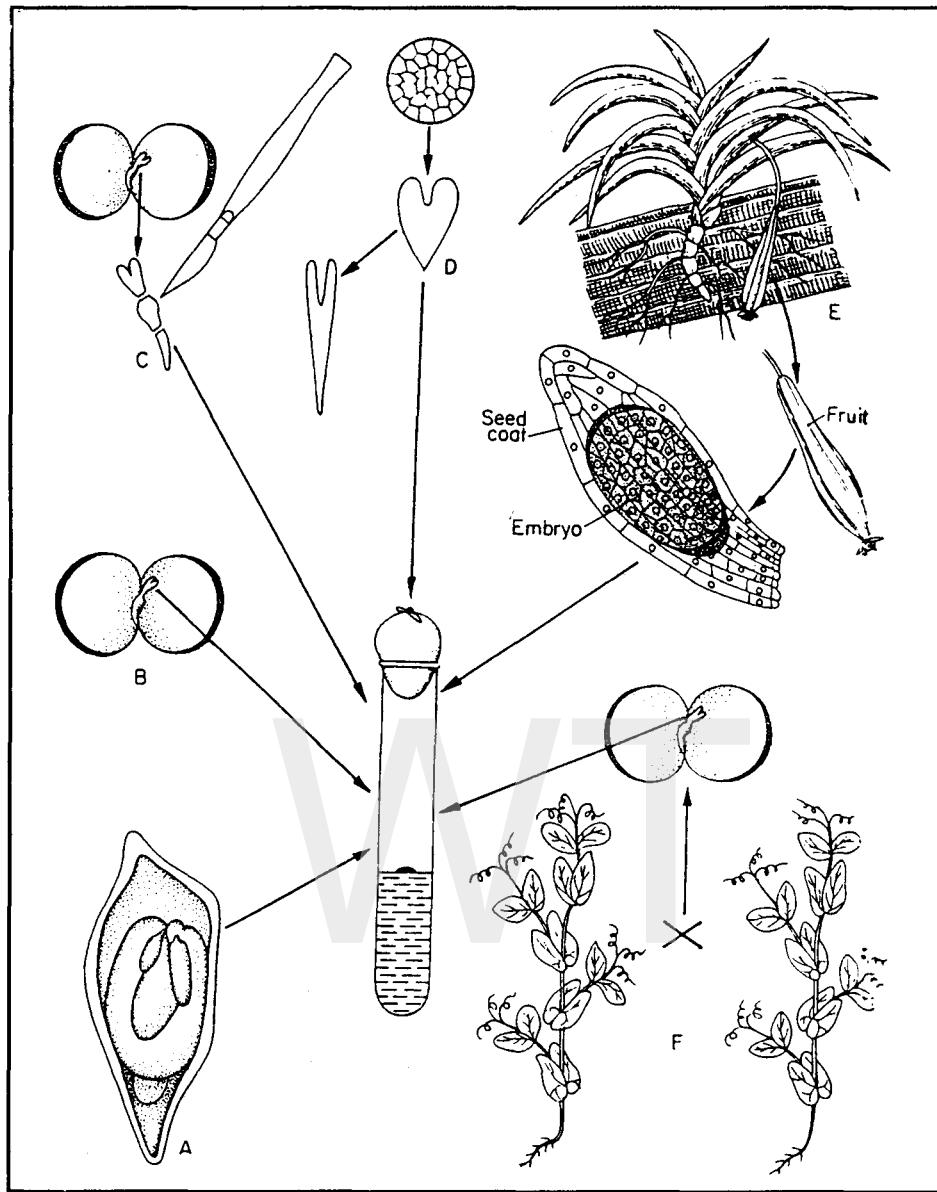
The mature seed embryo can be dissected surgically into a number of segments. Such embryo segments are cultured to analyse the relationship of different parts of the embryo to its final form in culture.

### CULTURE OF IMMATURE EMBRYOS OR PROEMBRYOS

The term proembryo means the early developmental stages of the embryo that precede cotyledon initiation. Globular and heart-shaped stages of embryo are appropriately called as proembryos. The objective of such culture is to understand the control of differentiation and the nutritional requirements of such progressively developing embryos.

### CULTURE OF INTACT SEED CONTAINING UNDIFFERENTIATED EMBRYO

Each fruit of an orchid plant develops several thousand tiny seeds which contain morphologically undifferentiated embryos. These embryos are the spherical mass of tissue lacking both radicle and plumule. There is even no storage tissue in the seeds and the seed coat is reduced to a membranous structure. For this reason, the entire seed of orchid containing undifferentiated embryo is cultured and treated as



□ Fig 10.1

**Different categories of embryo culture.** A. Culture of adventive embryos from polyembryonic seeds. B. Culture of mature and intact seed embryo. C. Culture of dissected embryo. D. Culture of immature embryo. E. Culture of undifferentiated embryo of orchid. F. Culture of abortive or inviable embryos.

embryo culture. In nature, these seeds germinate only in association with a proper fungus or else they perish. As a result, numerous seeds are lost. *In vitro* culture of orchid seeds is routinely employed for orchid propagation.

#### CULTURE OF ADVENTIVE EMBRYOS FROM POLYEMBRYONIC SEEDS

Besides the zygotic embryo produced from egg cell, some additional embryos are produced

from nucellar tissue in polyembryonic seed like lemons and oranges. Such additional abortive embryos can be exploited in culture for clonal propagation.

## CULTURE OF INVIABLE OR ABORTIVE EMBRYOS

In many inter-specific or intergeneric breeding experiments, sometimes inviable or abortive embryos may develop due to unsuccessful crosses. As a result, the non-viable seeds do not germinate normally. But it is now possible to raise a hybrid plant by culturing the inviable embryos *in vitro*.

## PRINCIPLES OF EMBRYO CULTURE

The underlying principle of the method is the aseptic excision of the embryo and its transfer to a suitable nutrient medium for development under optimum culture conditions.

In general, it is relatively easy to obtain pathogen-free embryos, since the embryo is lodged in the sterile environment of the ovule or seed or capsule or fruit. So, surface sterilization of the embryos as such is not necessary. Thus the entire seeds or fruits containing the ovule are surface sterilized and the embryos are then aseptically separated from the surrounding tissues.

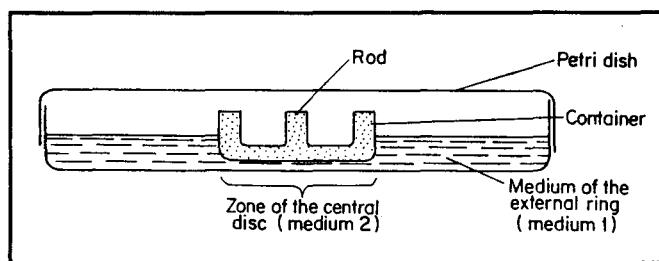
Seeds with hard seed coats are generally surface sterilized and then soaked in sterile water for a few days aseptically so that seed can be cut easily to free the embryo. Although seeds are surface sterilized before soaking they may need to go through a second sterilization before

embryo excision. Splitting open the seeds and transferring embryos to the nutrient medium directly is the simplest technique that can be done with seeds.

In the isolation of comparatively smaller embryos, it is important that they are removed intact carefully from the ovule without any injury. This can be best achieved by carrying out the operation under a specially designed dissecting microscope.

In case of orchid seeds, the entire seed or ovule is cultured because the seed contain morphologically undifferentiated spherical embryo having no functional storage tissues like endosperm and the seed coat is reduced to a membranous structure. Although the entire ovule containing undifferentiated embryos is cultured, but it is referred to as embryo culture. Each fruit of an orchid contains thousands and thousand of tiny seeds, so a large quantity of sample can be cultivated easily just after excision of surface sterilized fruits. It is also important that the freed seeds containing the embryos not become desiccated during above operation.

The most important aspect of embryo culture work is the crucial selection of the medium necessary to sustain continued growth of the embryos. The formulation of nutrient media may vary depending upon the species used for study and many of them have not been rigorously determined. Nevertheless it is possible to make certain generalization i.e. the younger the embryo the more complex is its nutrient requirements. Thus while the mature embryo can be grown in an inorganic salt medium supplemented with



□ Fig 10.2

Device of Monnier for embryo culture

a carbon energy source such as sucrose, relatively young embryos require in addition, different combinations of vitamins, amino acids, growth hormones and in some cases natural endosperm extracts such as coconut milk. Since proembryos are often submerged in the ovular sap under considerable osmotic pressure, culture of such embryo in presence of an osmoticum such as mannitol is often suggested.

The changing nutritional requirement for successful embryo culture has often meant transferring the embryo from one medium to another for optimum growth. Monnier devised a culture method (Fig 10.2) which allowed for the uninterrupted growth of globular embryo to maturity. By this method, embryo can be grown in both solid and liquid medium at the same time. The composition of both media is different. Monnier also stressed the importance of obtaining uninjured embryos with their suspensor for successful culture. Suspensor is important for the growth of immature embryos. But embryo selected at later stages do not require the attached suspensor.

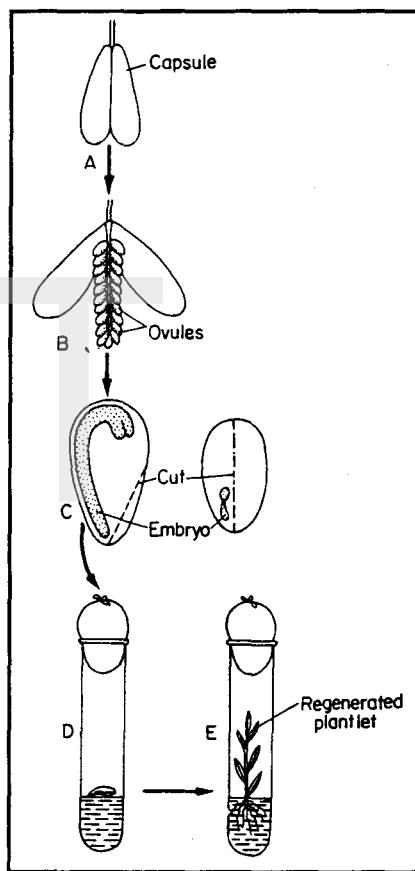
In culture, the embryos are not induced to form callus tissue but they are allowed to form a plantlet. After the embryos have grown into plantlets *in vitro*, they are generally transferred to sterile soil or vermiculite and grown to maturity in the green house.

#### **PROTOCOL FOR EMBRYO CULTURE**

The following protocol for embryo culture (Fig 10.3) is based on the method used for *Capsella bursa-pastoris*. With modification, this basic protocol should be applicable to embryo culture in general. The steps are given below—

1. Capsules in the desired stages of development are surface sterilized for 5–10 minutes in 0.1%  $HgCl_2$ , either in a closed small room previously illuminated by UV lamps or in a Laminar air flow.
2. Wash repeatedly in sterile water.

3. Further operations are carried out under a specially designed dissecting microscope at a magnification of about 90X. The capsules are kept in a depression slide containing few drops of liquid medium.
4. The outer wall of capsule is removed by a cut in the region of the placenta; the halves are pushed apart with forceps to expose the ovules.
5. A small incision in the ovule followed by slight pressure with a blunt needle is enough to free the embryos.



□ Fig 10.3

**Procedure of Isolation of embryo of *Capsella bursa-pastoris* and its culture.** A. a capsule. B. the capsule has been opened. C. Incision of ovule to isolated embryo. D. Culture of isolated embryo. E. Development of plantlet from the cultured embryo

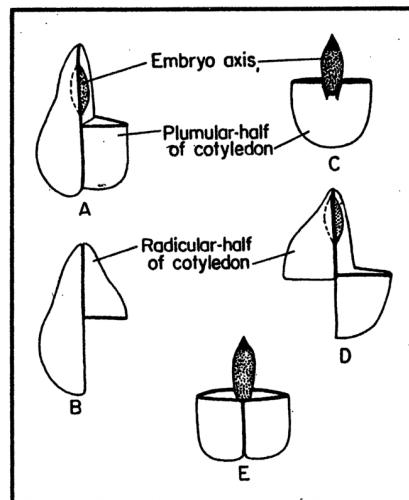
6. The excised embryos are transferred by micropipettes or small spoon headed spatula to standard 10 cm petridishes containing 25 ml of solidified standard medium. Usually 6–8 embryos are cultured in a petridish.
7. The petridishes are sealed with cello tape to prevent desiccation of the culture.
8. The cultures are kept in a culture room at  $25 \pm 1^\circ\text{C}$  and given 16 hrs. illumination by cool white fluorescent tube.
9. Subcultures into fresh medium are made at approximately four weeks intervals.

In case of fresh seeds or dry and imbibed seeds, the schedule is slightly changed. Seeds are cleaned by 5% Teepol (a liquid detergent) for 10 minutes and dipped in 70% ethyl alcohol for 60 seconds. Surface sterilization in 0.1%  $\text{HgCl}_2$  is followed by washing in sterile water, then the seeds are decotylated using a sharp scalpel and embryos are transferred to solid nutrient medium.

In case of orchid seeds, after following the scheduled surface sterilization procedure, the fruits are excised and dusty seeds are gently spread over the medium.

#### **PRECOCIOUS GERMINATION EMBRYO IN CULTURE**

A lot of cellular, physiological and biochemical changes take place during embryo development from the zygote to the fully formed embryo stage. After its full-term development, the embryo becomes dehydrated and enters a phase of metabolic quiescence and developmental arrest (dormancy) which may last from a few days to several months or even years. During this stage, embryo is normally incapable of germination. Embryos of mangroves and some viviparous varieties of cultivated plants (e.g. *Sechium edule*) germinate while still attached to the parent plant. They are able to bypass the stage of dormancy. Similar phenomenon has been observed when excised immature plant embryos are



□ Fig 10.4

#### **Growth of embryos from ovule at different stages of development (after LaRue and Avery 1938)**

grown *in vitro*. In culture, excised immature embryos do not proceed further than the embryogenic development for its maturation and start to germinate. The immature embryos develop into weak seedling showing only those structures which are already present at the time of embryo excision. This phenomenon of seedling formation without completing normal embryogenic development is known as precocious germination. LaRue and Avery (1938) have demonstrated *in vitro* growth of *Zizania aquatica* embryos excised from ovules at different stages of development (Fig 10.4). Embryos 0.05 mm long show very little *in vitro* growth. Older, immature embryos (3.5 mm long) germinate precociously and form seedlings which are not as well developed as those formed by mature embryos in the same culture period. The main objective of culturing immature embryos is to stimulate normal embryological development in order to understand the factor(s) that regulate the orderly development of embryos in nature.

#### **APPLICATION OF EMBRYO CULTURE IN PLANT SCIENCE**

Embryo culture has been presented not only to emphasize its significance to plant science but

also to extend its application to plant breeding and to horticultural problems for plant improvements. For systematic discussion, the importance and application of *in vitro* embryo culture on the basis of available information can be broadly grouped into three categories—

- Importance of embryo culture in relation to biological knowledge.
- Applied aspects of embryo culture.
- Other applications.

### IMPORTANCE OF EMBRYO CULTURE IN RELATION TO BIOLOGICAL KNOWLEDGE

*In vitro* embryo culture has been usefully employed to study some very fundamental problems which are difficult to undertake in embryos enclosed within seeds. Experiments with embryos separated from the seeds without interference from accessory tissues have contributed a lot of informations to our biological knowledge of plant development in the following aspects.

- It helps determining the factors that regulate the growth of the primodial organs of the seedling plant.
- It helps to study the metabolic and biochemical aspects of dormancy and germination.
- It helps in analysis of the various parameters of embryonic growth.
- The culture of proembryo helps to understand the control of differentiation and the nutritional requirements of progressively smaller embryos.
- The culture of surgically dissected embryo segments has facilitated understanding the relationship of the different parts of the embryo to its final form in culture.
- An embryo undergoes a gradual transition from the dependence of the zygote to the relatively autonomy of the mature embryo. Changes in nutritional requirements of embryos at different stages can be demonstrated by embryo culture.

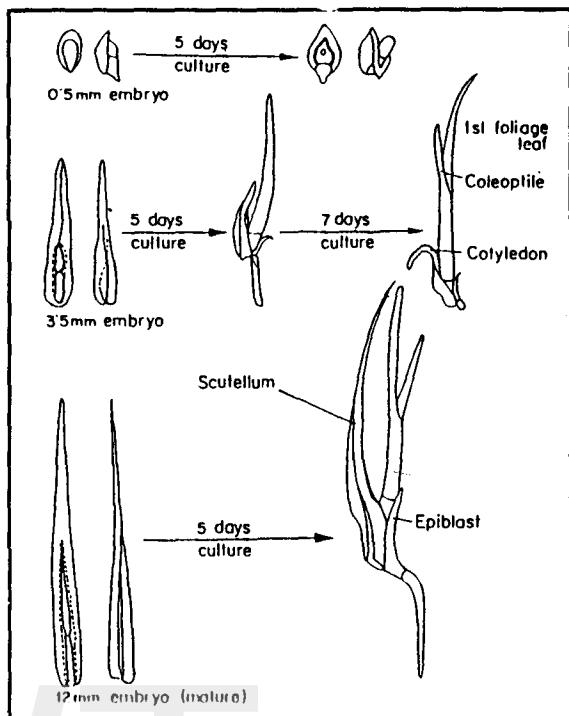


Fig 10.5

**Effect of cotyledon on seedling formation of *Cassytha filiformis* (after Rangaswamy and Rangan, 1971)**

- The role of accessory tissues can be studied by culturing isolated embryos.

*In vitro* microsurgical experiments with embryos of *cassytha filiformis* have shown that cotyledon play an important role in the development of seedling (Fig 10.5). It is evident from the data of Rangaswamy and Rangan (1971) that in *C. filiformis* the growth factor(s) for shoot development resides in the "redicular-halves" of the cotyledon. If the "plumular halves" of both the cotyledons are removed the seedling is well developed but if redicular halves are removed the plumule does not grow.

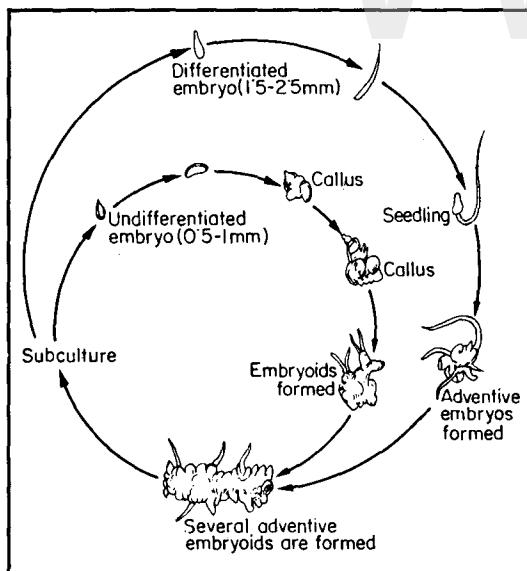
- It has proved difficult to germinate by conventional methods the seeds of obligate phanerogamic root and stem parasites in the absence of contact with the

host plant. Seeds and embryos of several obligate parasites have been grown under aseptic conditions to study their dependence on the host.

In 1962 Johri and Bajaj successfully cultured the young embryo of *Cuscuta reflexa*, a stem parasite in absence of host tissue (Fig 10.6). The mature embryo of *Cuscuta* has a definite plumule but it lacks a radicle. Truscott (1966) cultured mature and immature embryos of *Cuscuta* in order to induce root system by applying adenine, kinetin, GA<sub>3</sub>, coconut milk and casein hydrolysate. But none of the treatments induced rooting. The experiment indicates that *Cuscuta* lacks a root growth potential.

Undifferentiated embryo (0.5–1 mm) of *Cuscuta reflexa* on culture produce callus tissue which later form many embryoids. But differentiated embryos (1.5–2.5 mm) produce seedling and later several adventive embryoids are formed.

In 1963, Rangaswamy and Rangan cultured the embryo of *Cassytha filiformis*, a stem parasite in absence of host.



□ Fig 10.6

#### Culture of embryos of *Cuscuta reflexa*

Root parasites are dependent on the host stimulus. Some root parasites e.g. *Cistanche*, *Orobanche*, *Striga* etc. are also dependent on the host plant for seed germination. In cultures the seeds of root parasites do not germinate in a nutrient medium. However, the addition of extract of host roots to the medium induces the seed germination independent of the host.

*In vitro* seed germination and shoot development independent of host stimulus has also been reported in root parasites *Osyris wightiana*, *Exocarpus cupressiformis* and *Santalum album*. Most of the root parasites do not form haustoria in cultures without a host.

- (ix) The effect of nutrient medium, amino acids, vitamins and plant hormones can be observed on embryo in culture.

### APPLIED ASPECT OF EMBRYO CULTURE

From a practical point of view, *in vitro* embryo culture is very useful in many applied aspects—

- (a) to raise a healthy plant from abortive or non-viable embryos;
- (b) to overcome seed dormancy;
- (c) to shortening the breeding cycle;
- (d) to overcome self sterility of seeds;
- (e) seed testing.

#### Culture of Non-Viable or Abortive Embryo

Embryo abortion or non-viable embryos may develop in some natural seeds or may produce in seeds due to unsuccessful interspecific and intergeneric crosses.

*Culture of abortive embryo from normal plant*—In the seeds of some fruit plants like apple, peach, pear etc. are abortive embryos which do not even respond to stratification treatment. In such cases, embryo culture is the most promising method to obtain a plant from such abortive embryos.

In the polyembryonic seeds like lemons, oranges, the additional embryos are developed from nucellar tissue. Such embryos gradually abort during development and do not germinate under natural conditions. These embryos can be grown under artificial *in vitro* conditions to mature plant for clonal propagation.

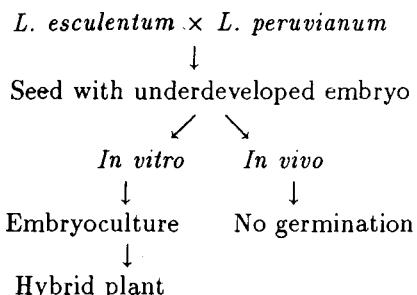
When the goal is to obtain plants from abortive embryos, the embryos should be excised prior to the onset of abortion.

**Culture of abortive hybrid embryo**—In many breeding experiments, embryo abortion is noticed in the seeds of unsuccessful crosses. Although fertilization takes place normally in such crosses and hybrid embryos begin to develop in a relatively normal way, a number of irregularities subsequently set in which results in the formation of non-viable hybrid embryos. As a result, it makes a barrier to crossability in plants where the non-viable hybrid embryos are unable to develop into mature plants. Plant embryo culture has now been used to speed up breeding programme and to overcome the crossability barrier in plants.

In breeding work with crop plant and horticultural plant, embryo culture method is very useful for the production of hybrid plants with desirable characters.

**Crop Plants**—Embryo culture has been successfully employed in many unsuccessful interspecific crosses of crop plants.

**Tomato**—Cultivated tomato (*Lycopersicon esculentum*) is very susceptible to virus, mold and nematodes. But the wild species *L. peruvianum* is relatively resistance. In a cross between the two species, the fruit develops normally but the seeds containing underdeveloped embryos do not germinate. Hybrid plants have been raised from such seeds by embryo culture.



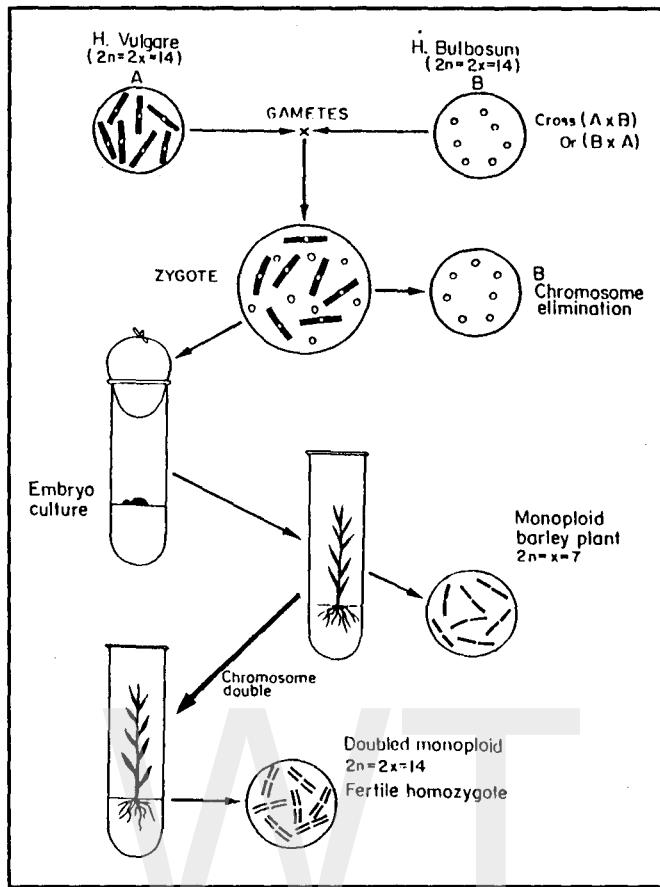
**Barley**—The cultivated winter barley such as *Hordeum vulgare* and *H. sativum* are deficient in winter hardiness and are susceptible to mildew diseases. But wild barley i.e. *H. bulbosum* is winter handy and is resistant to mildew. In a cross between *H. sativum* and *H. bulbosum*, the embryo is formed but fails to grow in the ovule. When the embryo is cultured in nutrient medium, it produces the transplantable hybrid plants. Interspecific hybrid plants have also been obtained in crosses between *H. vulgare* and *H. bulbosum* by *in vitro* embryo culture.

One novel use of embryo culture in barley is the production of monoploid and double monoploid plants. In this process an interspecific cross is made between *H. vulgare* as the female and *H. bulbosum* as a male. Fertilization takes place and zygote is also formed. But the chromosome of *H. bulbosum* are rapidly eliminated from the cells of the developing embryo (Fig 10.7). As a result, a monoploid embryo is formed where the cell division and development are both slow. On the other hand, the developing endosperm disintegrates. Following embryo culture, monoploid plantlets are raised. Double monoploids are also produced by colchicine treatment which actually induces the chromosome doubling. This approach is thus potentially a very useful method in plant improvement.

**Rice**—Embryo culture has been successfully employed to raise hybrid plants. These hybrid plants are capable to withstand adverse environmental conditions and are resistant to several diseases.

**Jute**—Hybridization between *Corchorus capsularis* and *C. olitorius* is a major aim of jute breeders but their attempts have failed due to early abscission of pollinated flowers, resulting a low fruit set. Fruits are associated with premature abortion of embryos. Embryo culture has been successfully applied to raise hybrid plants.

**Cotton**—Hybridization between *Gossypium arboreum* and *G. hirsutum* has almost failed and only marginal success has been achieved in rearing hybrid embryos to maturity in culture.



□ Fig 10.7

### Scheme of monoploid barley production

**Legumes**—*Trifolium* is a forage crop, but hybridization between *Trifolium* species has been impeded by embryo abortion. Embryo culture has been attempted to obtain hybrid combining perennial habit with forage quality.

*Melilotus officinalis* (sweet clover) is another important forage crop. But it has high coumarin content which is harmful to cattle. So attempts have been made to hybridize *M. officinalis* with other low coumarin species like *M. alba*. Some success has only been achieved by culturing hybrid embryos. Similarly, viable hybrid plants have been raised by embryo culture from interspecific crosses in *Lotus* species, *Medicago* species etc.

Employing the similar technique, hybrid plants have been raised from *Phaseolus vulgaris*

× *P. acutifolius* and *Lathyrus clymenum* × *L. articulata*.

Embryo culture is not only useful for unsuccessful interspecific crosses but also equally applicable for intergeneric crosses where embryo abortion, low percentage of seed germination are the chronic problems to plant breeders.

It is, however, now possible to raise the plant by embryo culture from an intergeneric cross between *Hordeum jubatum* and *Secale cereale*. By the same method, hybrids have also been obtained from *H. californicum* × *S. cereale*, *H. vulgare* × *S. cereale*, *H. depressum* × *S. cereale*, *H. jubatum* × *Hordeclymus europaeus*.

*Triticum* and *Elymus* are crossed with a view to achieve a hybrid plant with strong root

system and a higher grain yield. But such cross is unsuccessful due to malformation and low viability of seeds. However, it is now possible to raise a hybrid plant by embryo culture.

In another integeneric cross between *Trip-sacum dactyloides* and *Zea mays*, higher percentage hybrid plants are raised by embryo culture than by normal seed germination.

From the above discussion, it is clear that embryo culture is an effective way of raising hybrid plant where there is a crossability barrier.

**Horticultural Plants**—Embryo culture is an important research tool for the improvement of horticultural plants. Successful results have also been obtained in case of fruit

tree breeding as well as breeding work with flowering garden plant. In some cases hybrid plants raised by embryo culture have proved to be superior with regards to earliness in flowering and the number of flowers per plant. By embryo culture, hybrid plants have been raised from *Lilium henryi* × *L. regale*, *L. speciosum* × *L. auratum*. In *Chrysanthemum*, an interspecific hybrid between *C. boreale* and *C. pacificum* has been raised by embryo culture. Employing the same technique, several *Iris* plants have been reared.

### Seed Dormancy and Embryo Culture

Seed dormancy is a natural phenomenon. Dormant seeds fail to germinate under apparently suitable conditions. In dormant seed, the enclosed embryo is not able to grow. This temporary suspension of growth is definitely due to some internal condition of seeds or due to some environmental causes. Prolonged seed dormancy presents a special problem to plant growers. So several methods have been devised to break the dormancy of seeds. But in some seeds dormancy cannot be broken by any conventional method. Embryo culture has been successfully used to bypass the traditional treatments to overcome seed dormancy and accelerate germination in certain types of seed.

Seeds of tall bearded *Iris* remain dormant varying from a few months to many years after

harvest. But within two to three months, transplantable plants are possible to raise by embryo culture.

Seeds dormancy has been overcome in the seeds of some other plants such as *Lactuca sativa*, *Citrullus colocynthis*, *Phacelia tanacetifolia*, *Nemophila insignis* etc.

Seeds of some of the common cereals become dormant immediately after harvest. *In vitro* culture of excised embryos from such dormant seeds in suitable nutrient medium supplemented with hormone might be advantageously used to raise the seedling immediately after harvest.

In orchids, morphological development of the embryo and their subsequent germination take place in the soil in association with mycorrhizal fungi. Otherwise the seeds remain dormant in absence of symbiotic fungus. The seeds or orchid can be grown asymbiotically by culturing them in nutrient medium. Orchid growers are now commercially utilizing this technique for the production of profuse orchid plantlets.

### Shortening of the Breeding Cycle

In breeding practice, dormant seeds and slow growth of seedling are not suitable material.

Embryo culture is a valuable tool to plant breeders because this method can reduce the breeding cycle.

### Overcoming Self-sterility of Seeds

In some economically important plants like banana, kachoo, seeds are produced, but such seeds are never known to germinate in nature. Such plants propagate very easily by vegetative means. This natural sterility barrier in the seed could be overcome by embryo culture.

### Seed Testing

Embryo culture has provided a rapid means of measuring accurately the viability and germinability of a particular lot of seeds. In seed

testing practices, it is a very useful and reliable method to predict the viability of seeds.

## OTHER APPLICATIONS

### To Study the Evolutionary Relationship

Conventional hybridization technique is an important method for the study of evolutionary relationship, speciation and for determining the taxonomic position of plants. In some cases, hybridization fails at the interspecific level due to formation of abortive embryos. Hybridizing the different species of *Datura* and culturing their abortive embryos in some cases, some interesting results have been obtained about their controversial taxonomic position and evolutionary relationship.

### To Study Host Parasite Interaction

Plant pathogens may infect the flower or flowering spike of host plant e.g. ergot disease of rye plant. This may lead to malformation of seeds or loss of seed. Incorporation of fungal toxin in the culture medium and the culture of embryo facilitate to study the host parasite interaction.

### To Study the Mutagenic Effect

Seeds are often irradiated by X-rays, and gamma-rays to study the mutagenic effect. Embryo culture is a very useful technique to evaluate the mutagenic effect of the irradiated seeds. From the practical point of view, embryo culture is also useful to determine the safety doses of irradiation used for food preservation.

## Summary

In angiosperm, embryo formed inside the seed in the post-fertilization stage represents the beginning of sporophyte. In embryo culture, young embryos are removed aseptically from the developing seeds and are placed on a suitable nutrient medium to obtain seedlings. E. Hanning (1904) first removed mature embryos from

seeds of *Raphanus sativus*, *R. landra*, *R. caudatus* and *Cochleria danica* and nurtured them into plantlets in a medium containing mineral salts and sucrose. His work is generally accepted as marking the birth of plant embryos culture. After the successful achievement of embryo culture *in vitro*, embryo excised from ovule and seeds of the most varied plant species have been grown under controlled condition by several workers time to time and much useful information on their nutritional requirements, growth, differentiation and application has accumulated.

Embryo culture may be divided into different categories such as—(i) culture of mature and intact seed embryo; (ii) culture of surgically dissected embryo; (iii) culture of immature embryos or proembryos; (iv) culture of intact seed containing undifferentiated embryos e.g. embryo of orchid; (v) culture of adventive embryos from polyembryonic seeds and (vi) culture of inviable or abortive embryos.

The underlying principle of the method of embryo culture is the aseptic excision of the embryo and its transfer to a suitable nutrient medium for development under optimum culture conditions. In isolation of comparatively small embryos, it is important that they be removed intact carefully from the ovule without any injury. This can be best achieved by carrying the operation under a specially designed dissecting microscope. In some of orchid seeds, the entire seed or ovule is cultured because the seeds contain morphologically undifferentiated spherical embryo having no functional storage tissue like endosperm and the seed coat is reduced to a membranous structure. The most important aspect of embryo culture work is the crucial selection of the medium necessary to sustain continued growth of the embryos. The formulation of nutrient medium may vary depending upon the species used for the study and many of them have not been rigorously determined. In culture, the embryos are not induced to form callus tissue but they are allowed to form a plantlet. After the embryos have grown into plantlet *in vitro*, they are generally transferred to sterile soil or vermiculite and grown to maturity in the green house.

Embryo culture is now being used in some crop improvement programmes to obtain inter-specific hybrids. The seeds of some fruits like apple, peach, pear etc. contain abortive embryos. In such cases, embryo culture is the most promising method to obtain a plant from such abortive embryos. In many breeding experiments, embryo abortion is noted in the seeds of unsuccessful crosses. As a result, it makes a barrier to crossability in plants where the non-viable hybrid embryos are unable to develop into mature plants. Embryo culture has now been used to speed up breeding programme and to overcome the crossability barrier in plants. Embryo culture has been successful by employed in many inter-specific crosses of crop plants like tomato, barley, rice, jute, cotton, etc. It has some specific uses as well, for example, propagation of orchid, shortening of breeding cycle, overcoming dormancy, seed testing etc. To study the evolutionary relationship of plants, host parasite interaction and mutagenic effect, embryo culture is very important.

## Questions for Discussion

1. What is embryo culture? How could this technique be utilized for the improvement of crops?
2. Describe the principles and application of embryo culture techniques mentioning the protocol for embryo culture.
3. Describe the method of culturing embryos of plants and its uses in different fields of plant science.

4. What are the different categories of embryo culture and mention their objectives. Discuss the importance of embryo culture in agriculture and horticulture.
5. Write the answer to the following short questions—
  - (a) Why the entire seeds of orchid instead of isolated embryo are cultured?
  - (b) How the monoploid and double monoploid plants of barley are produced by embryo culture?
  - (c) What are the importances of embryo culture relation to biological knowledges?
  - (d) Why the embryo in culture are not induced to form callus tissue?
  - (e) How the embryos are dissected out for embryo culture? If any injury occurs during isolation of embryo for culture, they are generally discarded. Why?
  - (f) Discuss briefly the significant achievements to embryo culture.
6. Write short notes on—
  - (a) Embryo culture.
  - (b) Utility of embryo culture.
  - (c) Culture of inviable or abortive embryos.
  - (d) Culture of adventive embryos from polyembryonic seeds.
  - (e) Nutrient medium for embryo culture.
  - (f) Embryo culture in relation of crop genetics.
  - (g) Importance of embryo culture in seed testing.

## Chapter Eleven

### Anther and Pollen Culture

#### INTRODUCTION

In angiosperms, the haploid or gametophytic phase is extremely brief and is represented by pollen grains in anther and one or more cells in embryo sac of an ovule. A typical anther in cross-section shows two anther lobes and each lobe possesses two pollen sacs. During microsporogenesis, pollen mother cells (PMC) inside the pollen sacs form pollen tetrad by meiosis. In each pollen tetrad, four pollens are held together temporarily by their callose wall. Pollens separate as a discrete unit by dissolution of callose wall. Each pollen possesses an unique genome where every gene is present as a single copy.

Exploitation of this unique genetic unit and the totipotency of the plant cell is the basis of anther or pollen culture for the production of haploid plants. On the other hand, egg cell produced within ovule is very difficult to separate from complex tissue integration. In culture, the anther swells and dehisces along its upper margin, lengthwise. This phenomenon helps to expose the pollen grains. Alternatively, huge amount of

pollen grains can be isolated manually and can be cultured aseptically very easily. Therefore, pollen is more suitable material than egg cell for the production of haploid.

The development and production of haploid plant *in vitro* is very important for the study of fundamental and applied aspects of genetics in higher plants. Production of homozygous diploid by doubling the chromosome number of haploids *in vitro* makes a pure line in a single step and such homozygous pure line is of great importance in plant breeding. Thus, from the practical as well as fundamental point of view, anther and pollen culture open up a new avenue in the field of plant science.

#### BRIEF PAST HISTORY

**W. Tulecke (1953)**—First observed that mature pollen grains of *Ginkgo biloba* (a gymnosperm) can be induced to proliferate in culture (pollen culture/microspore culture) to form haploid callus.

**S. Guha and S. C. Maheswari (1964)—**

First reported the direct development of embryos from microspores of *Datura innoxia* by the culture of excised anther (anther culture).

**J. P. Bourgin and J. P. Nitsch (1967)—**

Obtained complete haploid plantlets from anther culture of *Nicotiana tabacum*.

### **WHAT IS ANTER CULTURE ?**

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

### **WHAT IS POLLEN CULTURE ?**

Pollen or microspore culture is an *in vitro* technique by which the pollen grains, preferably at the uninucleated stage, are squeezed out aseptically from the intact anther and then cultured on nutrient medium where the microspores, without producing male gametes, develop into haploid embryoids or callus tissue that give rise to haploid plantlets by embryogenesis or organogenesis.

### **WHAT IS ANDROGENESIS ?**

Androgenesis is the *in vitro* development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation.

There are two modes of androgenesis—(i) direct androgenesis, (ii) indirect androgenesis.

(i) Direct androgenesis—In this type, microspore behaves like a zygote and undergoes change to form embryo which ultimately gives rise to a plantlet.

(ii) Indirect androgenesis—In contrast to the direct androgenesis, the microspore, instead of undergoing embryogenesis, divide, repeatedly to form a callus tissue which differentiates into haploid plantlets.

### **PRINCIPLE OF ANTER AND POLLEN CULTURE**

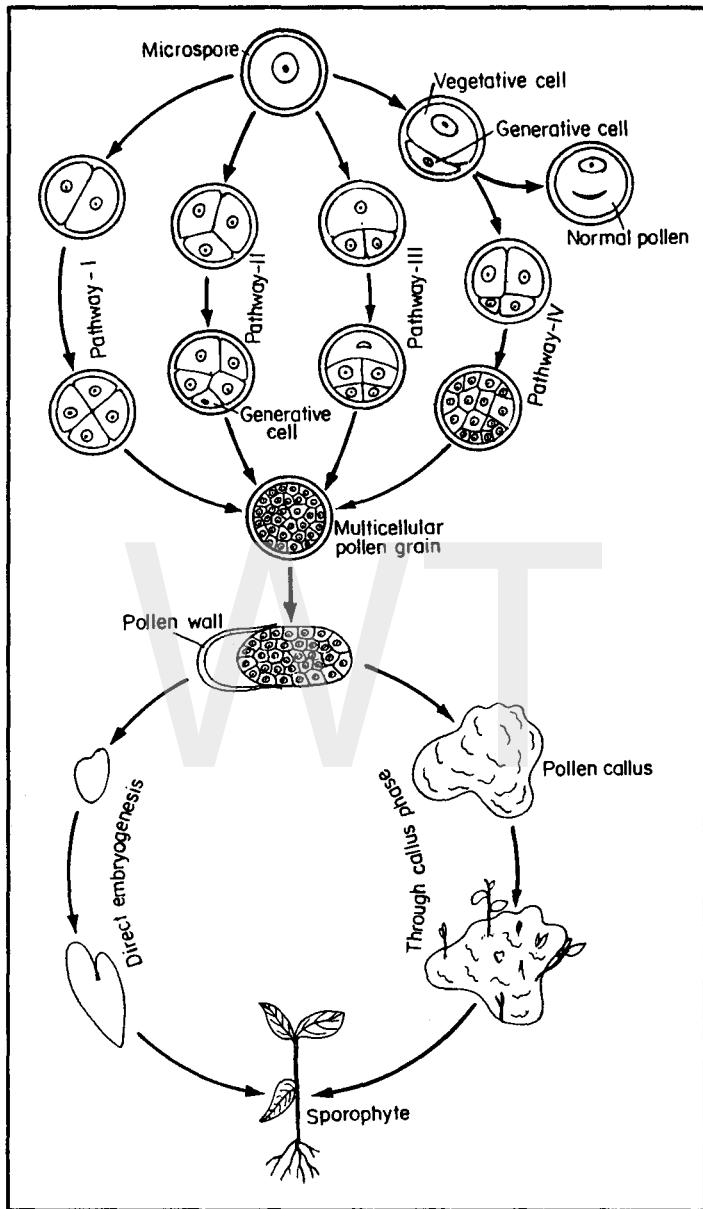
The basic principle of anther and pollen culture is the production of haploid plants exploiting the totipotency of microspore and the occurrence of single set of chromosome ( $n$ ) in microspore. In this process, the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcibly to a new metabolic pathway for vegetative cell division. For this objective, microspores, either within intact anther or in isolated state, are grown aseptically on the nutrient medium where the developing pollen grain will form the callus tissue or embryoids that ultimately give rise to haploid plantlets.

In fact, anther culture is in essence the pollen culture. The principle behind the anther culture is that without disturbing the natural habitat and environment of the enclosed anther, pollen can be grown by culturing the intact anther. In culture condition, the diploid tissue of anther will remain living without proliferation at the selective medium and, at the same time, it will encourage the development of pollen by nursing and providing nutrient. The haploid embryoids or the callus tissue can be seen as the anther dehisces in culture. But there is always the possibility that the diploid somatic cells of the anther will also respond to culture condition and so produce unwanted diploid callus or plantlets. In attempts to avoid this problem, free pollens isolated from the anther are grown in nutrient medium. The knowledge gained so far from anther and pollen culture has established that pollens at the uninucleate stage, just before the first mitosis, or during mitosis are most suitable for the induction of haploids.

Induction of haploids can be enhanced by keeping the anther or flower bud at low temperature. The low temperature has been ascribed to a number of factors such as dissolution of microtubules, alteration in the first mitosis or maintenance of higher ratio of viable pollen capable of embryogenesis. Cold treatment may also act to

help the embryogenesis by repressing the gametophytic differentiation or by lowering the abscisic acid content of the anther which is considered to be inhibitory for the production of haploids.

The important aspect of anther or pollen culture is the nutrient medium. The nutritional requirements of the excised anther are much simpler than those of isolated microspores. In the isolated microspore, it is obvious that certain



□ Fig 11.1

**Diagram showing the origin of sporophytes from pollen grains 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)**

factors responsible for the induction of haploids, which might have been provided by the anther, are missing and these have to be provided through the medium. Rich medium may encourage the proliferation of the diploid tissue of anther wall and should be avoided. Incorporation of activated charcoal into the medium has stimulated the induction of androgenesis. The iron in the medium also plays a very important role for the induction of haploids. Potato extract, coconut milk and growth regulators like auxin and cytokinin are also used for anther and pollen culture due to their stimulatory effect on androgenesis.

In culture, pollen may divide mitotically or can follow the normal pathway of forming vegetative and generative nuclei. The generative nucleus remains quiescent and abort. The vegetative nucleus divides repeatedly, forming a multinucleate pollen. The multinucleate pollen undergoes segmentation which may lead to form either organised embryoid structure or callus tissue (Fig 11.1). Both types of development are utilised to form haploid plantlets.

The haploid plantlets are self-sterile due to presence of single set of chromosome which are not able to participate in meiotic segregation. By colchicine treatment, haploids are made homozygous diploid, or isogenic diploid which are fertile.

Haploids or homozygous diploid grown *in vitro* are transferred to pot and grown to maturity in the glasshouse.

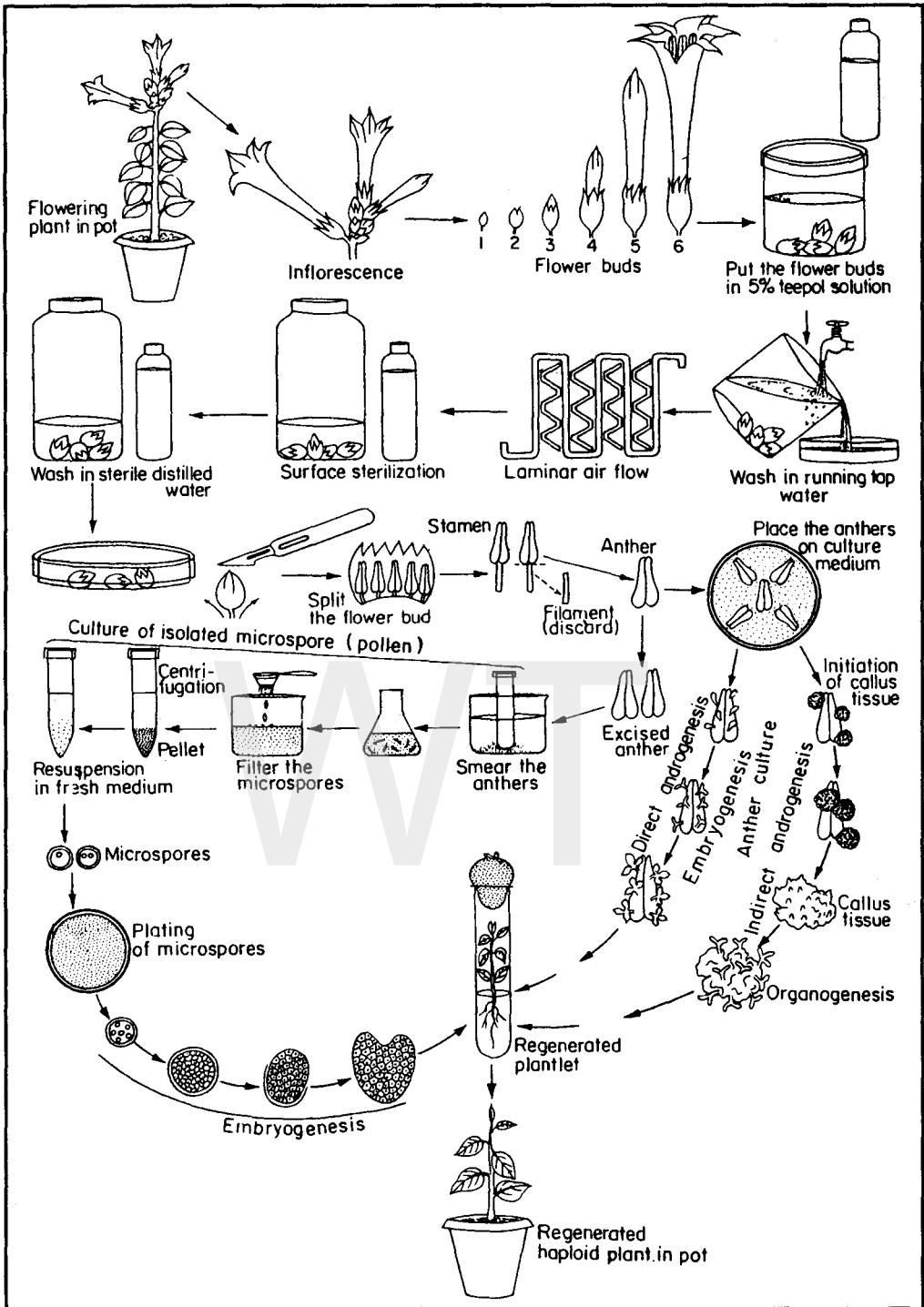
#### PROTOCOL FOR ANTER CULTURE

Tobacco is the ideal material for anther culture. So the basic protocol described below should be applicable to anther culture in general with modification. The immature anthers containing uninucleate pollen at the time of first mitosis are the most suitable material for the induction of haploids. The steps of anther culture are given below—

1. Collect the flower buds of *Nicotiana tabacum* at the onset of flowering (normally 3–4 months old plant). Measure the length

of each individual with a cm ruler. Select the flower bud of 17–22 mm in length when the length of the sepals equals that of the petals. Reject all flower buds which are beginning to open (Fig 11.2).

2. Transfer the selected flower buds to the laminar airflow. Each flower bud contains five anther and these are normally surface sterile in closed buds. The flower buds are surface sterilized by immersion in 70% ethanol for 10 seconds followed immediately by 10 minutes in 2% (v/v) sodium hypochlorite. They are washed three times with sterile distilled water. Finally, transfer the buds to a sterile petridish.
3. To remove the anthers, slit the side of the bud with a sharp scalpel and remove them. With a pair of forceps, place the five anthers with the filaments to another petridish. The filaments are cut gently. During excision of anthers, special care should be taken to ensure that they are not injured in any way. Damaged anthers should be discarded as they often form callus tissue from the damaged parts other than the pollen.
4. Anthers are placed on agar solidified basal MS or White or Nitsch and Nitsch medium.
5. The cultures are kept initially in dark. After 3–4 weeks, the anthers normally undergo pollen embryogenesis and haploid plantlets appear from the cultured anther. In some cases, anther may undergo proliferation to form callus tissue which can be induced to differentiate into haploid plants.
6. At this stage, the cultures are incubated at 24–28°C in a 14 hrs. daylight regime at about 2,000 lux.
7. Approximately 50 mm tall plantlets are freed from agar by gently washing with running tap water and then transferred to small pot containing autoclaved potting compost. Cover each plantlet with a glass beaker to prevent desiccation and maintain in a well-lit humid green-house. After one week, remove the glass beakers and transfer to larger pots when the plants will mature and finally flower.



□ Fig 11.2

Schematic representation of the culture of excised anther and isolated microspores and the development of haploid plant directly by embryo formation or through haploid callus

## **PROTOCOL FOR POLLEN CULTURE**

Isolated pollen can be cultured by two methods—

### **Method 1**

This method is described here for the culture of isolated pollen of tobacco. This technique can be considered as the basic protocol for pollen culture and involves the following steps—

1. Selection of suitable unopened flower bud, sterilization, excision of anther without filament are the same as described previously in anther culture.
2. About 50 anthers are placed in small sterile beaker containing 20 ml of liquid basal medium (MS or White or Nitsch and Nitsch).
3. Anthers are then pressed against the side of the beaker with the sterile glass piston of a syringe to squeeze out the pollens (Fig 11.2).
4. The homogenized anthers are then filtered through a nylon sieve (pore diameter  $40\text{ }\mu$ - $60\text{ }\mu$ ) to remove the anther tissue debris.
5. The filtrate or pollen suspension is then centrifuged at low speed (500–800 revolution per minute) for 5 minutes. The supernatant containing fine debris is discarded and the pellet of pollen is suspended in fresh liquid medium and washed twice by repeated centrifugation and resuspension in fresh liquid medium.
6. Pollens are mixed finally with measured volume of liquid basal medium so that it makes the density of  $10^3$ – $10^4$  pollens/ml.
7. 2.5 ml of pollens suspension is pipetted off and is spread in 5 cm petridish. Pollens are best grown in liquid medium but, if necessary, they can be grown by plating in very soft agar added medium. Each dish is sealed with cello tape to avoid dehydration.
8. Petridishes are incubated at 27–30°C under low intensity of white cool light (500 lux, 16 hrs.).

9. Young embryos can be observed after 30 days. The embryos ultimately give rise to haploid plantlets.
10. Haploid plantlets are then incubated at 27–50°C in a 16 hrs. day light regime at about 2,000 lux. Plantlets at maturity are transferred to soil as described in anther culture.

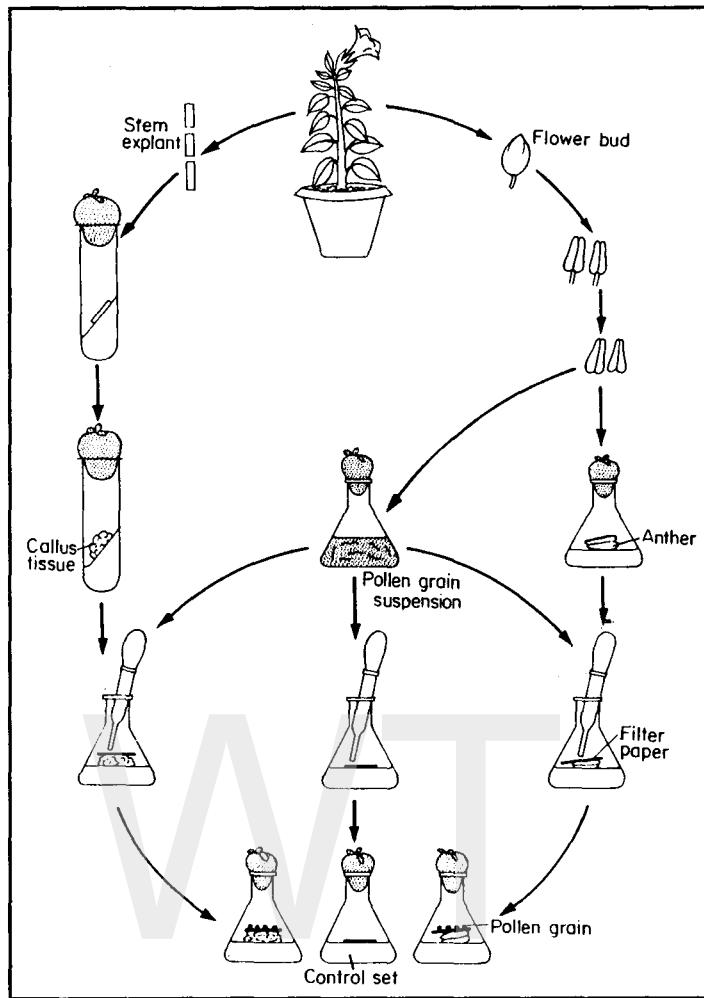
### **Method 2**

This method is known as nurse culture technique. Sharp *et al.* (1972) first introduced this method. The steps are given below—

1. Selection of flower bud, sterilization, excision of anther, isolation of suitable pollen are the same as described previously.
2. In this method, the intact anthers are placed horizontally on the top of solid or semi-solid basal medium within a conical flask.
3. A small filter paper disc is placed over the intact anther and about 10 pollen grains in suspension are then placed on the filter paper disc. Hence the intact anthers are considered as the nurse tissue. A control set is also prepared in exactly the same way except that the pollen grains on filter paper are directly kept on solid medium. Sometimes, callus tissue derived from any part of the plant is used as nurse tissue (Fig 11.3).
4. With this method, pollen grains in the control set did not grow at all. The pollen grains kept on nurse tissue grow and form a culture of green parenchymatous tissue in two weeks. Such tissue ultimately forms the haploid callus tissue.

## **ADVANTAGE OF POLLEN CULTURE OVER ANTER CULTURE**

It has been already proved that anther culture is an efficient way for the production of haploids from the microspores present within the intact anther. In this process, there is always the possibility that somatic cells of the anther that are diploid, will also respond to the culture condition and so produce unwanted diploid calluses or plantlets. Sometimes, the development of microspore inside the anther may be interrupted



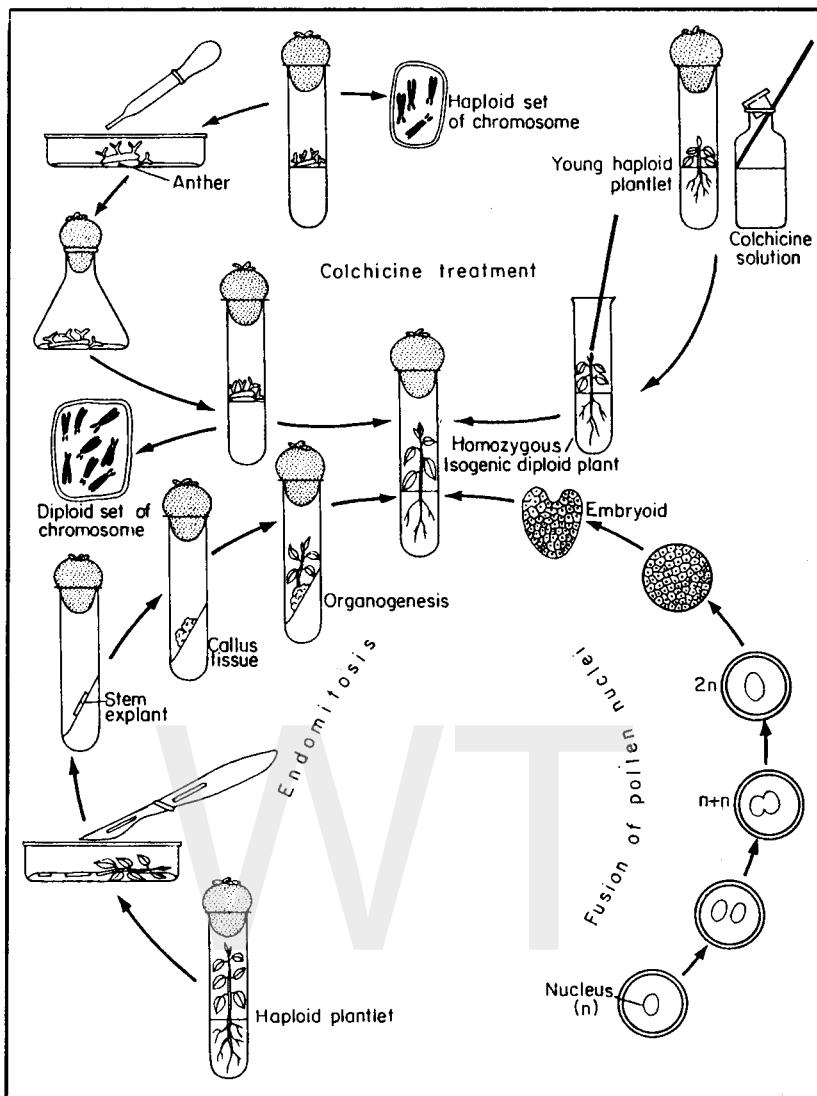
□ Fig 11.3

#### **Diagram showing the technique of nurse culture for raising tissue clones from isolated pollen grains**

due to growth inhibiting substances leaking out of the anther wall in contact with nutrient medium. In attempts to avoid these problem, the culture of free pollen has been investigated. The culture of pollen offers the following additional advantages—

- Overcrowding of pollen grain in anther is eliminated and isolated pollen grains are equally exposed to nutrient medium.
- Unwanted growth of the anther wall and other associated tissue are eliminated.
- The steps of androgenesis can be observed starting from single cell.

- Various factor governing androgenesis can be better regulated.
- Pollen is ideal for uptake, transformation and mutagenic studies as pollens can be uniformly exposed to chemicals and physical mutagens.
- Pollen may be directly transformed into an embryo. So it is very suitable for understanding biochemistry and physiology of androgenesis.
- Higher yields of haploid plants per anther could be expected in pollen culture than the anther culture.



□ Fig 11.4

**Diagram showing the different methods of obtaining homozygous or isogenic diploids**

### HOMOZYGOUS PLANTS

Haploid plants derived from either anther culture or pollen culture are sterile. These plants contain only one set of chromosomes. By doubling their chromosome number, the plants can be made fertile and the resultant plants will be homozygous diploid or isogenic diploid (Fig 11.4). These homozygous diploid plants show the normal meiotic segregation. The fer-

tile homozygous diploid plants are more important than the sterile haploid plants. Homozygous diploid plants can be used as pure lines in breeding programme.

Haploids can be diploidized by a number of methods—

### COLCHICINE TREATMENT

Colchicine has been utilized widely as a spindle inhibitor to induce chromosome dupli-

cation and to produce polyploid plants. This method has been employed for obtaining homozygous diploid plants from haploid culture. The young, plantlets while still enclosed within anther, are treated with 0.5% colchicine solution for 24–48 hrs. Treated plantlets are replanted in the medium after thorough washing. In case of mature haploid plantlets, 4% colchicine-lanoline paste may be applied to the axil of the leaves.

## ENDOMITOSIS

It is known that haploid cells are unstable in culture and have a tendency to undergo endomitosis, i.e., chromosome duplication without nuclear division. This property can be used for obtaining homozygous diploid plants. In this process, a small explant of stem from a haploid plant is cultured on auxin-cytokinin added medium where the segment forms the callus tissue. During callus growth, diploid homozygous cells are produced by endomitosis. Now large number of isogenic diploid plants can be obtained by organogenesis.

## FUSION OF POLLEN NUCLEI

Homozygous diploid callus or embryoids may form by the spontaneous fusion of two similar nuclei of the cultured pollen after first division. In *Brassica*, the frequency of spontaneous nuclear fusion in microspore is high in culture.

## SIGNIFICANCE OF HAPLOID PLANTS

In a diploid cell the chromosomes exist in homologous pairs. The genes for specific characters are also formed in pairs which are known as allelic gene pairs. For an example, T gene (for tallness) is an allele of t gene (for dwarfness) and *vice versa* in heterozygous condition. Each allele is located on one of the pair of homologous chromosome at a particular gene locus. Although each allele controls the same genetic trait (height of the plant), yet, they may control a contrasting phenotypic expression (tall/dwarf) of that trait. In heterozygous condition, the activity of only one of the alleles is expressed phenotypically, the allele is said to be dominant (suppose T gene). On the other hand, the activity of the other al-

lele which is not expressed phenotypically until it is separated from dominant allele, is said to be recessive (suppose t gene).

A chromosome contains a number of dominant and recessive genes whose allelic forms are present on the homologous partner chromosome at the same gene loci. In heterozygous diploid, only dominant alleles are expressed phenotypically. Homologous chromosome separates during meiosis. Pollen grains receive only one set of homologous chromosomes. As a result, in pollen-derived haploid plants, all the recessive genes, along with dominant genes, will be expressed phenotypically as there is no masking of recessive gene by dominant genes. Since the haploid plants are sterile, diploid fertile plant can be made by doubling the same existing chromosome. As a result, the dominant as well as recessive genes will be doubled at their respective loci. So, even in diploid condition, all the recessive genes will be expressed phenotypically. Such diploid plant is also called homozygous plant or isogenic diploid plant. Therefore, comparing the heterozygous diploid plant with homozygous diploid plant, one can easily identify the recessive characters which are not possible to identify in heterozygous condition.

Crossing over is an essential feature in the meiotic cycle by which random exchange of genetic material (genetic recombination) between two homologous chromatids takes place. The exchange is of great importance because it produces a new gene combination. As a result, four haploid nuclei, produced from a single diploid nucleus, differ from one another. Therefore, haploid plants, derived from four haploid pollens of a pollen tetrad, are significant because the plants will differ genetically.

## WHY THE HAPLOID PLANTS ARE STERILE ?

In haploid plants, each chromosome is represented only once and this is the reason there is no zygotene pairing in first meiotic division. Thus, all the chromosomes appear as univalent. During anaphase I, each chromosome moves freely and form generally more than two groups. Gametes with less than the haploid number are gen-

erally not viable, therefore, haploid plants are highly sterile.

## IMPORTANCE AND IMPLICATION OF ANTER AND POLLEN CULTURE

*In vitro* anther and pollen culture for the production of haploid and homozygous diploid have proved an important tool for fundamental and applied plant biology. Haploids are very important for study of fundamental genetics in higher plants because all the recessive genes remain uncovered and the recessive gene controlled traits are expressed phenotypically in regenerated plants. It is also valuable for mutation study. In addition, haploids are very useful for plant breeding programmes.

The importance and implication of anther and pollen culture are given below in details—

## UTILITY OF ANTER AND POLLEN CULTURE FOR BASIC RESEARCH

1. Haploids derived from anther and pollen culture are useful in cytogenetic studies.
2. By comparing the heterozygous diploid with haploid or homozygous diploid population, recessive phenotypic characters can be identified very easily.
3. Critical genetic analysis of haploid population derived from individual microspore of pollen tetrad is very useful for the study of genetic recombination in higher plants.
4. The series of cell division and mode of differentiation (embryogenesis or organogenesis), starting from single cell (microspore) and ending in whole organism, can be studied under microscope.
5. Double haploid, that are homozygous and fertile, are readily obtained, enabling the selection of desirable gene combination.
6. Culture of isolated pollen provides a novel experimental system for the study of factor controlling pollen embryogenesis of higher plants.
7. Study of meiotic behaviour of haploids provides valuable clues to measure chromo-

some duplication within a species and for understanding of phylogenetic relationship between species. It also provides information for the interpretation of chromosome homology.

8. Genetic analysis could be performed on haploid population to establish inheritance patterns.
9. Another application involves the use of haploids in the production of monosomics, nullisomics and other aneuploids. This approach has been used in tobacco for the isolation of nullisomics, trisomics and tetrasomics.

## USE OF ANTER AND POLLEN CULTURE FOR MUTATION STUDY

One unique value of microspore culture lies in the study of somatic cell genetics. In such studies, mutant cell lines are specifically important. Several biochemical mutants have also been reported using haploid cells.

Normally, *in vivo*, the majority of mutation are recessive and, therefore, are not expressed in diploid cells in the presence of immmutated dominant gene. Haploid callus cells have been employed to study the effect of various mutagens, both irradiation as well as chemical. A number of mutant cell lines have now successfully isolated and extensive work is being done to obtain cell lines that are resistant to environmental stresses, herbicides, phytopathotoxin, salts, drought, chilling, various drugs, viruses and nematodes etc. Salt resistant plants of *Datura innoxia* from a cell line have been selected from haploids derived from anther culture.

In *Ginkgo*, arginine-requiring strains from pollen culture have been obtained. With the availability of the technique, a large number of biochemical mutants have been isolated in a number of plant species using haploid cell. Cell line, tissues and complete plants resistant to streptomycin, 5-bromodeoxyuridine, methionine sulfoximine have been obtained. Nitrate reductase mutants have also been reported in *Nicotiana tabacum*. Recovery of auxotrops for pantothenate, adenine and nitrate reductase less

variants have also been reported from *Datura innoxia*.

Subjecting young haploid plantlets while emerging from the anther to 1,500–3,000 Rads of gamma irradiation, a high proportion of mutants have been isolated in *Nicotiana tabacum*. Alternatively, flower buds of *Nicotiana tabacum* are irradiated to X-ray (1,000 R) and the excised anthers are subsequently cultured. By this process, about 50% of haploid plant thus obtained are aberrant phenotypes. Differential radiosensitivity to ultraviolet and gamma-radiation and valine resistant mutants have also been regenerated from haploid cell culture using UV and gamma-irradiation.

#### USE OF HAPLOIDS FOR CRYOGENIC STUDY

1. Cryopreservation of haploid cell, pollen embryos, haploid meristem tips at super-low temperature ( $-196^{\circ}\text{C}$ ) in liquid nitrogen offers a novel approach for the long-term preservation of genetic stability and to establish a haploid germplasm bank.
2. Pollen embryos and haploid meristem are genetically stable and develop into entire plant easily. Therefore, these materials are more suitable for cryogenic studies.

#### USE OF ANTER AND POLLEN CULTURE FOR PLANT BREEDING AND CROP IMPROVEMENT

1. The main and foremost advantage of the *in vitro* production of haploid over the conventional plant breeding method is the saving of time. By anther and pollen culture, homozygous diploid or isogenic diploid plant can be produced within a year as compared to the long inbreeding method which might take four to six years.
2. Isogenic lines are also beneficial where plants are self-incompatible, e.g. rye.
3. In breeding programme, isogenic line facilitates the work in inducing desirable mutation, transformation and biochemical mutation.
4. The success of any crop improvement depends on the extent of genetic variability in

base population. In this regard callus cultures are a rich source of genetic variability. By anther culture, not only haploids but plants of various ploidy level and mutants can be regenerated. The anther culture derived callus of *Arachis hypogea*, *A. villosa*, *Cajanus cajan* and *Cicer arietinum* show a wide range of genetic variability and thus can be incorporated into the breeding programmes.

5. Another advantage to plant breeders which will be within reach, when large number of microspores can be cultured, is the possibility of uncovering new and highly beneficial gene combination. If the  $F_1$  hybrid plant possesses high-yielding qualities than either of parents, the microspore  $F_1$  hybrid can be induced to form plantlets in culture and can be used for breeding with disease resistant variety.
6. In China, by anther culture new varieties of rice Huayu 1, Buayu 2 and Tanfong 1 have been raised. Similarly, new varieties of wheat Lunghua 1 and Huapei 1 have been released. New variety of tobacco, namely Tanyu 1, Tanyu 2 and Tanyu 3 have been obtained by anther culture. A superior variety of tobacco named F 211, which is resistant to bacterial wilt, has been obtained by anther culture in Japan. In China, pollen plants of *Zea mays*, *Populus nigra* (Poplar), *Capsicum annuum* (Pepper), *Beta vulgaris*, *Brassica pekinensis*, *B. chinensis* have been raised for the exploitation in breeding programme.
7. *Hovea brasiliensis* is a rubber-yielding plant. It is perennial cross-pollinating tree and its available varieties are highly heterozygous in nature. Again, 5–7 years are required for sowing to blooming and seed set ratio of inbreeding is usually only a few of ten thousandth. So, it is unreal to obtain pure lines by means of successive inbreeding in many generations. Pollen plants have been produced by anther culture through embryogenesis. Therefore, production of homozygous diploid pollen plants is a new way to obtain pure lines of different geno-

types in a short time and can be utilized in breeding programme.

8. The haploid technique to cereal breeding is a quick route from heterozygotes to homozygotes.
9. Anther derived haploids have been extensively used in protoplast culture and somatic hybridization.

## APPLICATION OF HAPLOID CULTURE FOR HORTICULTURAL PLANTS

In some plants of horticultural importance, haploid culture is highly significant. *Freesia*, a horticulturally important plant, propagates vegetatively by means of underground corms. Normally, it takes 8–10 years to produce a clone which is longer enough for commercial purpose. But in these cases, anther culture can be successfully used to obtain a clone very quickly.

### ANTHER CULTURE AND ALKALOID CONTENT

Amongst other uses, homozygous plants obtained through anther culture have also been employed for the selection of breeding lines of *Nicotinina tabacum* with high alkaloid content. Homozygous recombinants of *Hyoscyamus niger* having higher alkaloid content could be obtained by anther culture.

### HAPLOID CULTURE AND MOLECULAR BIOLOGY

In genetic engineering, haploids can be successfully used to gene transfer. Haploid tissue of *Arabidopsis* and *Lycopersicon* have been used for the transfer and expression of three genes from *Escherichia coli*.

## Summary

In angiosperms, the haploid or gametophytic phase is extremely brief and represented by microspores or pollen grain present inside the anther. By careful selection of developing anthers at a precise and critical stage, it is possible to establish cultures of a number of species that will give rise to haploid cells. Cultured anthers may

give rise to a callus tissue which, after subcultures, can be regenerated into plantlets. Alternatively, in a number of species, the developing pollen grain have been diverted from their normal pathway and have undergone to form haploid embryos which ultimately give rise to haploid plantlets. In 1964, S. Guha and S. C. Masheswari first reported the direct development of embryos from microspores of *Datura innoxia* by the culture of excised anther.

Androgenesis is the *in vitro* development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation. There are two modes of androgenesis —(i) direct androgenesis and (ii) indirect androgenesis.

The basic principle of anther culture is that without disturbing the natural habitat and environment of the enclosed anther, pollen can be grown by culturing the intact anther. But there is always the possibility that diploid somatic cells of the anther will also respond to culture condition and so produce unwanted diploid callus or plantlet.

In attempts to avoid this problem, free pollen isolated from the anther are grown in nutrient medium. The knowledge gained so far from anther and pollen culture has established that pollens at the uninucleate stage just before the first mitosis or during mitosis are most suitable for the induction of haploids. Induction of haploids can be enhanced by keeping the anther or flower bud at low temperature.

In culture, pollen may divide mitotically to form either organised embryo or callus tissue. Both types of development are utilised to form haploid plantlets. The haploid plantlets are self-sterile due to presence of single set of chromosome, which are not able to participate in meiotic segregation. By colchicine treatment, haploids are made homozygous diploid or isogenic diploid which are fertile.

Haploids are very important for the study of fundamental genetics in higher plant, because all the recessive genes remain unmasked and the recessive gene controlled traits are expressed phenotypically in regenerated plants. It is also val-

able for mutation study. In addition, haploids are very useful for plant breeding programmes.

## Questions for Discussion

1. How haploids are produced in tissue culture? Mention the significance of haploids.
2. Describe the method and discuss the importance and implication of pollen culture.
3. Describe the method for the development of androgenic haploids from anther and pollen culture. Discuss the importance of such plants.
4. Write brief answers the following questions—
  - (a) Why the haploid plants are sterile?
- (b) How the haploid plants are made homozygous diploid?
- (c) What are the advantages of pollen culture over anther culture?
- (d) What are the nutritional requirements of anther and pollen culture?
- (e) What is the most important stage of pollen grain or microspore for the induction of haploids?

5. Write short notes on—

- (a) Pollen culture
- (b) Homozygous or isogenic plant
- (c) Endomitosis
- (d) Androgenesis
- (e) Anther culture.



## Chapter Twelve

### Plant Protoplast Culture

#### INTRODUCTION

After the successful establishment of plant tissue culture, it was felt that *in vitro* culture of plant organ or tissue had got some limitations when study of the events occurred at the level of cell in culture. So the culture of single cell separating from the complex tissue integration was developed. But still the presence of inert cell wall as the only barrier between the external environment and the interior of the cell poses some problem for a variety of experimental manipulations that are not possible with walled cells. Such problems can only be overcome if the cell wall of the living cell is removed experimentally for temporary period. Since then, plant scientists made an intelligent attempt to remove the wall of plant cell and culture the naked cell on nutrient medium. Later, with the refinement of methodology plant protoplast culture became successfully established as a novel technique in the field of plant tissue culture. A major reason for rapidly expanding interest in protoplast culture is their potential use in plant cell genetics and specially in cell fusion and transfer of genetic information by DNA uptake and organelle implantation. One basic prerequisite for the potential use of protoplasts in such studies is the

ability to isolate them readily in large number and to culture them *in vitro* to form cell colonies and whole plant.

#### WHAT IS A PROTOPLAST ?

It is known that each and every plant cell possesses a definite cellulosic cell wall and the protoplast lies within the cell wall except some reproductive cells and the free floating cells in some fruit juices like coconut water. Therefore, protoplast of plant cell consists of plasmalemma and everything contained within it. But those of importance to plant protoplast culture are produced experimentally by the removal of cell wall by either enzymatically or mechanical means from the artificially plasmolysed plant cells. Experimentally produced protoplasts are known as isolated protoplasts.

According to Torrey and Landgren (1977) "the isolated protoplasts are the cells with their walls stripped off and removed from the proximity of their neighbouring cells". Vasil (1980) defines that "the protoplast is a part of plant cell which lies within the cell wall and can be plasmolysed and which can be isolated by removing the cell wall by mechanical or enzymatic procedure". Therefore, isolated protoplast is only a

naked plant cell surrounded by plasma membrane—which is potentially capable of cell wall regeneration, cell division, growth and plant regeneration in culture.

## BRIEF PAST HISTORY

**J. Klercker (1892)**—First isolated protoplast mechanically from plasmolyzed cell of water warrior (*Stratiotes aloides*). No attempt was made to culture them.

**E. Küster (1927)**—In the fruits of several plants like *Solanum nigrum*, *Lycopersicon esculentum* etc. the cell wall are hydrolysed during fruits ripening process so that free protoplasts and protoplasmic units are left. Kuster preferred such physiological method for isolating protoplasts. No report of culture was available.

**R. Chambers and K. Hofler (1931)**—Were able to isolate few protoplasts by using thin slices of epidermis of onion bulb immersed in 1M sucrose until the protoplast shrunk away from their enclosing walls and then cutting sheets of epidermis with a sharp knife. Report of culture was not available.

**E. C. Cocking (1960)**—First reported the enzymatic method for isolation of protoplast in a large number from root tip cells of *Lycopersicon esculentum* by using a concentrated solution of cellulase enzyme, prepared from cultures of the fungus *Myrothecium verrucaria* to degrade cell wall.

**I. Takebe, Y. Otsuki and S. Aoki (1968)**—First employed the commercial preparation of cellulase and macerozyme sequentially (in two steps) for the isolation of mesophyll protoplast of tobacco.

**J. B. Power and E. C. Cocking (1968)**—Demonstrated first that the mixture of such two enzymes (cellulase + macerozyme) can be used simultaneously (one step method) for the isolation of protoplasts.

**I. Takebe, G. Labib, G. Melchers (1971)**—First reported the plant regeneration from isolated protoplast in *Nicotiana tabacum*.

**P. S. Carlson, H. H. Smith, R. D. Dearing (1972)**—First reported a somatic hybrid in higher plants involving two different sexually compatible species of mesophyll protoplast (*N. glauca* × *N. langsdorffii*).

## DIFFERENT SOURCES OF PLANT TISSUE AND THEIR CONDITION FOR PROTOPLAST ISOLATION

Protoplast can be isolated either directly from the different parts of whole plant or indirectly from *in vitro* cultured tissue.

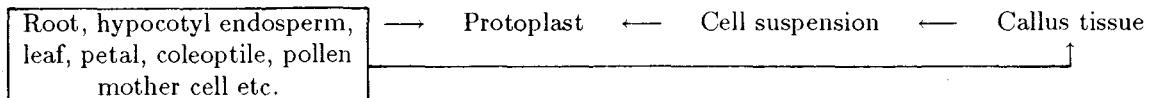
Convenient and suitable materials are leaf mesophyll and cells from liquid suspension cultures. Protoplast yield and viability are profoundly influenced by the growing conditions of plants serving leaf mesophyll sources. The age of the plant and of the leaf and the prevailing conditions of light, photoperiod, humidity, temperature, nutrition and watering are contributing factors.

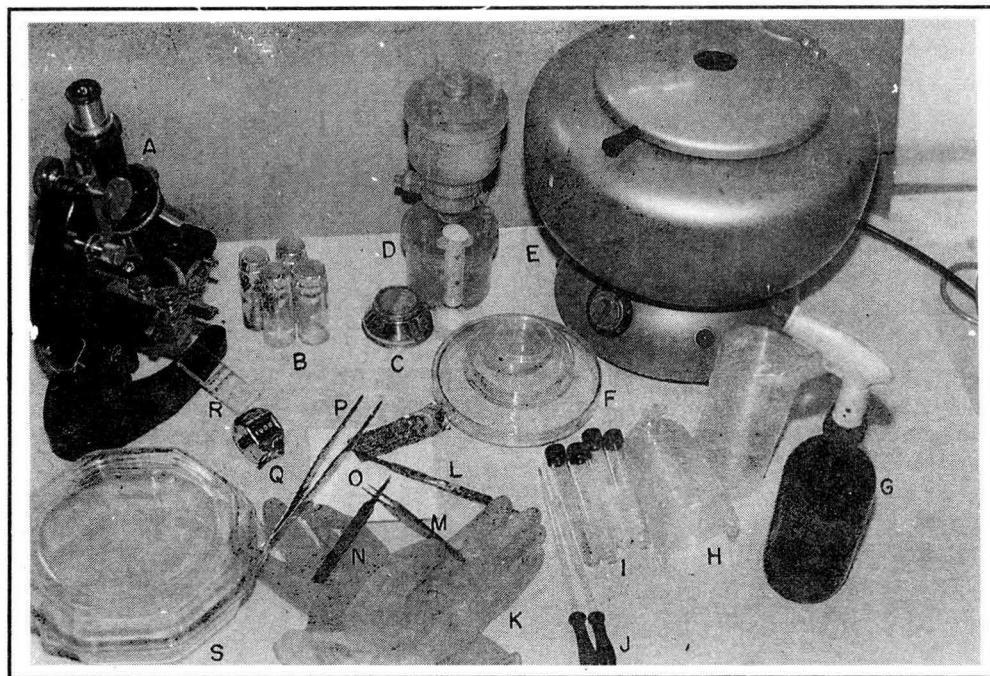
Cell suspension cultures may provide a more reliable source for obtaining consistent quality protoplasts. It is necessary, however, to establish and maintain the cells at maximum growth rates and utilize the cell at the early log phase.

## PRINCIPLES OF PROTOPLAST CULTURE

The basic principle of protoplast culture is the aseptic isolation of large number of intact living protoplasts removing their cell wall and culture them on a suitable nutrient medium for their requisite growth and development.

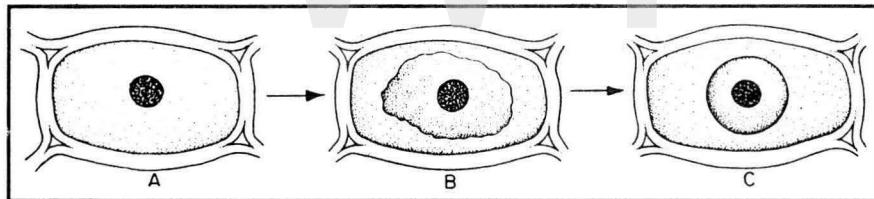
Protoplast can be isolated from varieties of plant tissues. Convenient and suitable materials are leaf mesophyll and cells from liquid





□ Fig 12.1

**Instruments required for a plant protoplast culture.** A. Compound microscope. B. Screw topped bottle. C. Nylon mesh. D. Bacterial filter. E. Centrifuge machine. F. Petridishes. G. Alcohol sprayer. H. Disposable sterile petridishes. I. Screw capped centrifuge tube. J. Pasteur pipette. K. Hand gloves. L. Disposable sterile scalpel. M & N. Jewellery fine forceps. O. Tile. P. Long forceps. Q. Counter. R. Haemocytometer. S. Casserole. (Photograph taken by Mr. T. K. Bera)



□ Fig 12.2

**A – C. Showing the stages of plasmolysis. A. normal cell, B. Shrinking of protoplasm. C. complete plasmolysis**

suspension culture. Protoplast yield and viability are greatly influenced by the growing condition of the plant as well as the cells.

The essential step of the isolation of protoplast is the removal of the cell wall without damaging the cell or protoplasts. The plant cell is an osmotic system. The cell wall exerts the inward pressure upon the enclosed protoplasts. Likewise, the protoplast also puts equal and oppo-

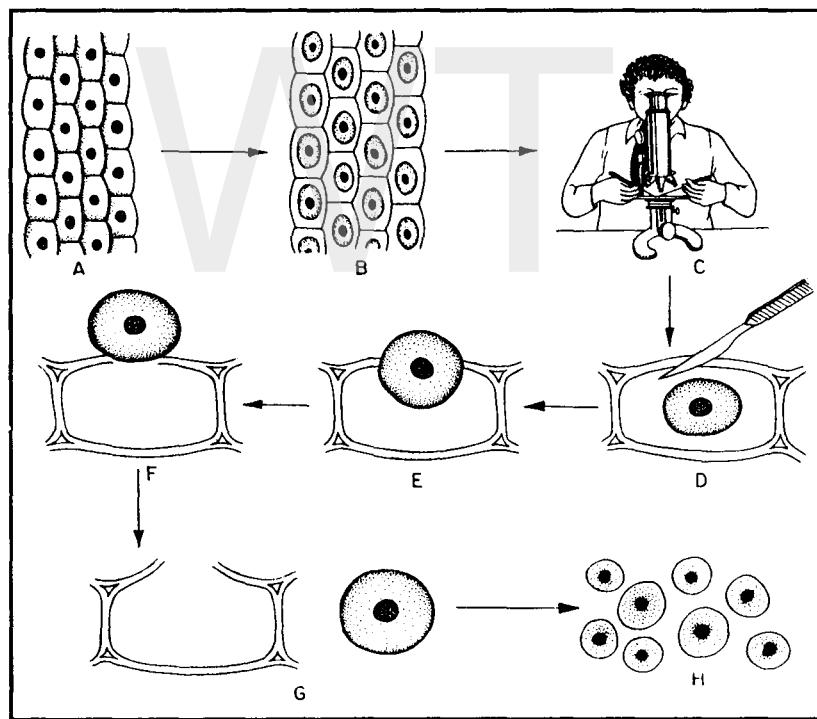
site pressure upon the cell wall. Thus, both the pressures are balanced. Now if the cell wall is removed, the balanced pressures will be disturbed. As a result, the outward pressure of protoplast will be greater and at the same time in absence of cell wall, irresistible expansion of protoplast takes place due to huge inflow of water from the external medium. Greater outward pressure and the expansion of protoplast cause it to burst.

So, the isolated protoplast is an osmotically fragile structure at its nascent stage. Therefore, if the cell wall is to be removed to isolate protoplast, the cell or tissue must be placed in a hypertonic solution of a metabolically inert sugar such as mannitol at higher concentration (13%) to plasmolyse the cell away from the cell wall (Fig 12.2). Mannitol, an alcoholic sugar, is easily transported across the plasmodesmata, provides a stable osmotic environment for the protoplasts and prevents the usual expansion and bursting of protoplast even after loss of cell wall. That is why, this hypertonic solution is known as osmotic stabilizer or plasmolyticum or osmolyticum.

Once the cells are stabilized in such a manner by plasmolysis the protoplasts are released from the containing cell wall either mechanically or enzymatically. Mechanical isolation (Fig 12.3) involves breaking open each cell compartment to liberate the protoplast. This operation can be

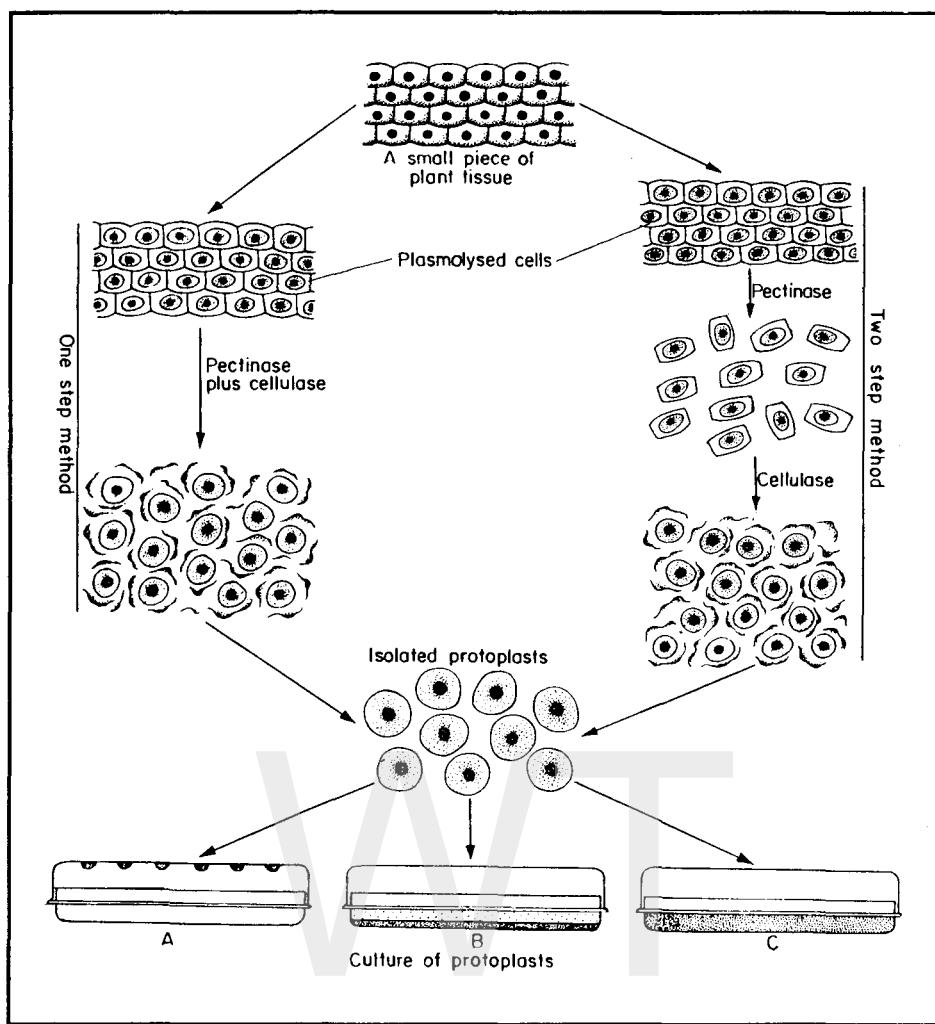
done carefully on small pieces of tissue under a microscope using a microscalpel. But very few protoplasts are obtained for a lot of time and effort. Large-scale attempts at mechanical isolation involves the disrupting tissue with fine stainless steel-bristled brush. This process may liberate more protoplasts with less efforts, but the percentage of yield of intact protoplasts is still very low. A considerably more efficient way of liberating the protoplasts is to digest the cell walls away around them, using cell wall degrading enzymes such as cellulase, hemicellulase, pectinase or macerozyme etc. These enzymes are isolated from fungi and available commercially (Table 12.1).

Period of treatment and concentration of enzymes are the critical factors and both factors should be standardized for particular plant tissue. Intact tissue can be incubated with a pectinase or macerozyme solution which will dissolve



□ Fig 12.3

**Method of mechanical isolation of protoplasts.** A. A small piece of plant tissue. B. Plasmolysis of cells. C – D. Cutting of cell wall by microscalpel under microscope. E – F. Subsequent stages of liberation of protoplasts. G. Isolated protoplast and empty cell. H. Isolated protoplasts



□ Fig 12.4

**Methods of enzymatic isolation of a large number of protoplasts and their culture. A. Hanging-droplet method of culture. B. Co-culture. C. Plating of protoplasts**

the middle lamella between the cells and so separate them. Subsequent treatment with cellulase will digest away the cellulosic layer of the cell wall. This process is known as sequential enzyme treatment or two step method as opposed to a mixed enzyme treatment (one step method) in which both cellulase and pectinase or macerozyme are mixed so that the entire wall is broken down in a single operation (Fig. 12.4).

The isolated protoplasts can be cultured either static liquid or agarified medium. The protoplast media consist of mineral salts, vitamins,

carbon sources and plant growth hormones as well as osmotic stabilizers and possibly organic nitrogen sources, coconut milk and organic acids. In culture protoplast can reform a new cell wall around them. Once the wall is formed, the protoplast becomes a cell. The cells from protoplasts subsequently enter cell division which is followed by the formation of callus and cell cultures. Such callus also retain the capacity for morphogenesis and plant regeneration.

A brief list of plant regeneration from plant protoplast culture is given below (Table 12.2).

## PROTOCOL FOR ISOLATION AND CULTURE OF PROTOPLAST

Protoplasts can be prepared from a variety of tissue but among them leaf mesophyll tissue from a wide range of plants has been proved to be the most ideal source of plant material for protoplast isolation (Fig 12.5). Leaves of *Nicotiana tabacum* is a highly standardized material for easy entry into the art of protoplast isolation and culture. Now-a-days the mixed enzyme method (single step) is very popular and this procedure is followed as routine work in most of the laboratories of the world. The protocol for isolation of protoplast from the mesophyll cells of tobacco using mixed enzyme method and its culture are described below—

## ISOLATION OF PROTOPLAST

1. Young fully expanded leaves from the upper part of 7-8 weeks old plants growing in a greenhouse are detached and leaves are washed thoroughly with tap water.
2. Surface sterilization of leaves is done by first immersing in 70% ethanol for 60 seconds followed by dipping into 0.4-0.5% sodium hypochlorite solution for 30 minutes. For this purpose, a sterile casserole dish is used as a sterilizing container. Sterilization is done in front of laminar air flow.
3. After 30 minutes, the sterilant is poured off and leaves are washed aseptically 3-4 times with autoclaved distilled water to remove every trace of hypochlorite.

Table 12.1 Commercial enzymes, their commercial name and source

Enzyme	Source organism
A. Cellulose degrading enzymes Cellulysin (Onozuka R10) Driselase	<i>Aspergillus niger</i> <i>Trichoderma reessei</i> (formally <i>T. viride</i> ) <i>Irpea lactescens</i>
B. Hemicellulose degrading enzymes Hemicellulase Rhozyme HP150	<i>Aspergillus niger</i> <i>A. niger</i>
C. Pectin degrading enzymes Pectinase Macerase (Macerozyme) Pectinol AC, Pectolyase Y23 Pectic-acid acetyl transferase (PATE)	<i>A. niger</i> <i>Rhizopus spp.</i> <i>A. niger</i> <i>A. japonicus</i>

Table 12.2 Species in which plant regeneration has been achieved from cultured protoplasts

Common name	Species	Family	Cell origin
Tobacco	<i>Nicotiana tabacum</i>	Solanaceae	Leaf, cell culture
Potato	<i>Solanum tuberosum</i>	Solanaceae	Leaf
Datura	<i>Datura innoxia</i>	Solanaceae	Leaf
Petunia	<i>Petunia hybrida</i>	Solanaceae	Leaf
Carrot	<i>Daucus carota</i>	Umbelliferae	Cell culture
Rape seed	<i>Brassica napus</i>	Cruciferae	Leaf
Orange	<i>Citrus sinensis</i>	Rutaceae	Nucellus callus
Asparagus	<i>Asparagus officinalis</i>	Liliaceae	Cladodes
Bromegrass	<i>Bromus inermis</i>	Poaceae	Cell culture

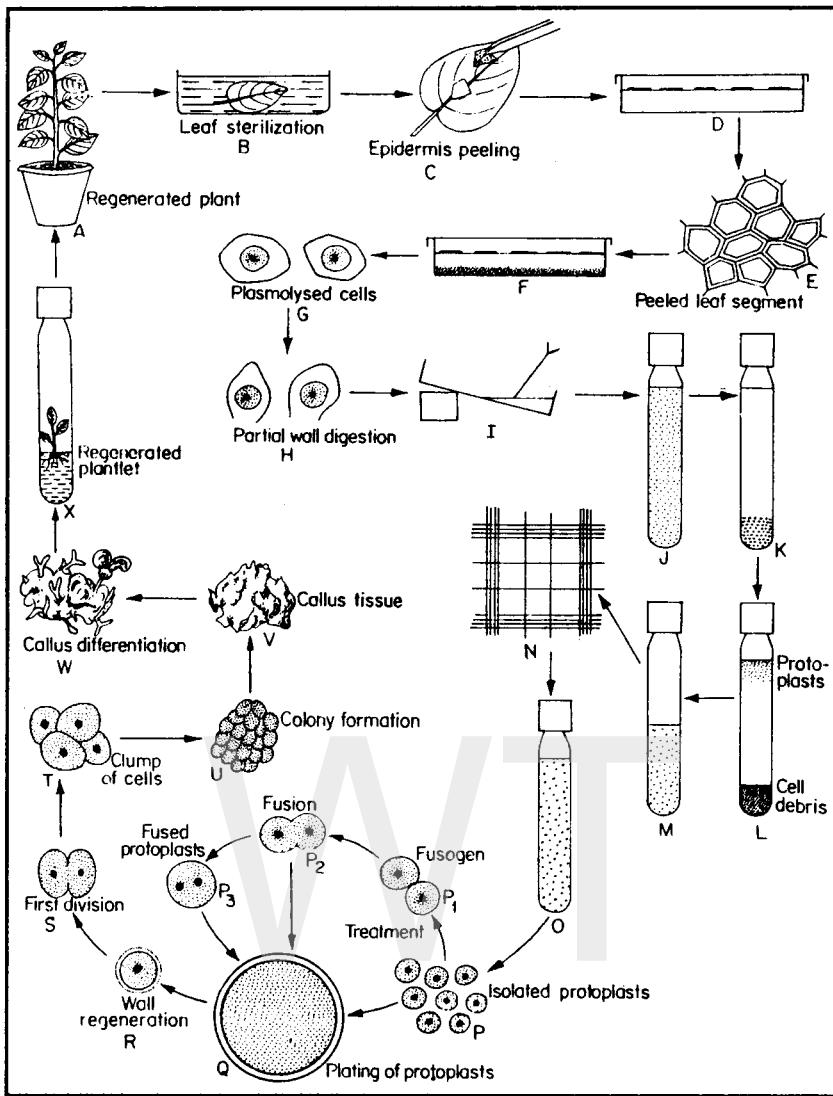


Fig 12.5

**Flow diagram of Isolation, culture and fusion of leaf-cell protoplasts.** A. Potted plant. B. Leaf sterilization. C. Epidermis peeling. D. Leaf piece floated on enzyme solution plus osmotic stabilizer. E. Peeled leaf segment. F. Protoplast sink to bottom of petridish. G. Plasmolysed cells in enzyme mixture. H. Partial wall digestion. I. Removal of enzyme solution. J. Protoplast in CPW 13M. K. Pellet of protoplasts after centrifugation. L. Protoplasts in CPW 21S. M. Resuspended in culture medium. N. Counting of protoplast density by haemocytometer. O. Resuspended in culture medium to give correct pre-plating density

4. With the help of long sterilized forceps (8 inches), one leaf is transferred on a sterilized floor tile. The impermeable lower epidermis of the surface sterilized leaves are peeled off as completely as possible. During

this process, a sterilized fine jeweller's forcep is inserted into a junction of the midrib and a lateral vein and the epidermis is carefully peeled away at an angle to the main axis of the leaf.

**N.B.—**Where peeling of the leaf is not possible, slicing of the leaf into thin strips may be sufficient to allow entry of the enzymes through the cut edges of the strip.

5. Peeled leaf pieces are placed lower surface down onto 30 ml sterilized CPW 13M\* solution in a 14 cm petridish. When the liquid surface is completely covered with peeled leaf pieces, then the CPW 13M solution is pipetted off from the beneath of leaf pieces. The CPW 13M solution is replaced by bacterial filter sterilized solution of enzyme containing 2% cellulase (Onozuka R1O), 0.5% macerozyme in 13% manitol added CPW (pH 5.5).
6. Leaf pieces in enzyme solution are incubated in the dark at 24-26°C for 16-18 hrs.
7. Without disturbing the digested leaf pieces the enzyme solution is gently replaced by CPW 13M. Then digested leaf pieces are gently agitated and squeezed with sterile fine forceps to facilitate the release of the protoplast. The protoplast suspension is then allowed to pass through a 60 μ-80 μ nylon mesh to remove the larger pieces of undigested tissue.
8. The filtrate is transferred to screw-capped centrifuge tube and is spun for 5 minutes at 100 g.
9. The protoplasts form the pellet. The supernatant is pipetted off and the pellet is resuspended in CPW 21S solution. It is again centrifuged for 5-7 minutes at 200 g. The viable protoplasts will float at the top surface of CPW 21S in the form of dark green band while the remaining cells and debris will sink at the bottom of the tube.
10. The viable protoplasts are collected from the surface and are resuspended again in CPW 13M to remove the sucrose. Centrifugation are repeated two-three times for washing.
11. Finally the protoplasts are suspended in measured volume of liquid Nagata and Tabeke medium (1971) supplemented with NAA (3 mg/L), 6-BAP (1 mg/L) and 13% mannitol.

\* CPW means cell and protoplast washing medium. The composition of CPW is given below—

KH <sub>2</sub> PO <sub>4</sub>	...	27.2 mg/L
KNO <sub>3</sub>	...	101 mg/L
CaCl <sub>2</sub> , 2H <sub>2</sub> O	...	1480 mg/L
MgSO <sub>4</sub> , 7H <sub>2</sub> O	...	246 mg/L
KI	...	0.16 mg/L
CuSO <sub>4</sub> , 5H <sub>2</sub> O	...	0.025 mg/L
pH	...	5.8

CPW 13M = CPW + 13% mannitol (13M)

CPW 21S = CPW + 21% sucrose (21S)

## ISOLATION OF PROTOPLAST FROM CELL SUSPENSION CULTURE

Rapidly growing cell suspension culture are the most suitable material for protoplast isolation. A new cell suspension does not yield many protoplast until it has been subcultured at least twice. For protoplast isolation, suspension cultures are generally harvested at its early exponential growth phase or log phase.

Older suspension culture have a tendency to form elongated giant cells with thick wall. So it is very difficult to isolate the protoplast from such culture. Again, the presence of large number of cell aggregates in suspension culture is not desirable for the isolation of protoplast. Addition of colchicine and some chelating chemicals in suspension culture generally prevents the formation of cell aggregates. Sometimes very low concentration of cellulase (0.1%) is added in cell suspension culture two days before use to discourage the formation of thick wall.

### Step 1

*Filtering of cell suspension*—The harvested cell suspension is passed through a coarse nylon sieve so that filtrate contains single cells as well as very small cell clumps.

### Step 2

*Preparation of liquid medium free cells for plasmolysis*—The filtrate is allowed to settle out of the medium. Most of the medium is decanted off and the cells are transferred (by pouring) to a flask. Using the Pasteur pipette, all of the culture medium is removed. This is best achieved

by drawing off medium from the base of the cell layer.

### Step 3

*Preplasmolysis*—The cells are suspended in CPW13M solution for 1 hr. After 1 hr., the plasmolyticum is pipetted off.

### Step 4

*Enzyme incubation*—The enzyme solution is added to the cells. The flask containing cells and enzyme are placed on the platform of a slowly rotating gyratory shaker (ca 30–40 rpm) for standardized period (4–6 hrs.).

### Step 5

*Washing and purification*—Protoplast suspension is filtered through 60  $\mu$ –100  $\mu$  stainless steel sieve to remove the larger debris. The filtrate is transferred into centrifuge tubes and is spun at 80 g for 5–10 minutes so as to sediment the protoplasts and then supernatant is pipetted off. The pellet is resuspended in CPW 21S solution. The protoplast suspension is again spun at 100 g for 5–7 minutes. Viable and debris free protoplasts are collected at the surface. The protoplasts are washed with CPW 13M by repeated centrifugation and finally protoplasts are resuspended in measured volume of liquid culture medium. Finally, the yield of protoplast is measured by counting in haemocytometer.

### Sample Protocol—Isolation of Protoplast from Cell Suspension Culture of *Daucus carota*

1. Allow the cells to settle out of the medium and remove the supernatant with Pasteur pipette.
2. Add 20–30 ml of enzyme solution (Onozuka R10 cellulase – 2% and hemicellulase – 1% in 10% mannitol added CPW solution, pH 5.5) and incubate the cells at room temperature on the platform of slowly rotating gyratory shaker (30 rpm) for 4 hrs.
3. Filter protoplast suspension through 67  $\mu$  stainless steel sieve and transfer the filtrate in centrifuged tube. Spin at 80 g for 5 min.

4. Discard in supernatant and resuspend in CPW 21S. Spin 100 g for 5–7 minutes.

5. Collect the protoplast from the surface of CPW 21S solution.

6. Wash the protoplast with CPW 13M solution by repeated centrifugation (3–4 times).

7. Finally transfer the protoplast to a measured volume of liquid B<sub>5</sub> medium supplemented with 2, 4-D (1 mg/L), kinetin (2 mg/L) and casein hydrolysate (1 gm/L).

## ISOLATION OF HAPLOID PROTOPLAST

Pollen mother cell (PMC), pollen tetrad (PT) and mature pollen are the natural source of haploid cells. Since they are produced in large number in anther and is very easy to squeeze out from anther, they are suitable material for the isolation of protoplast. Egg cell is also haploid cell, but generally single egg cell is produced per ovule and is very difficult to separate them from complex tissue integration.

Isolation and yield of protoplast from haploid male cells depends upon the composition of cell wall. The cell wall of PMC and PT is made up of callose (unbranched 1-3 glucan) whereas the exine of mature pollen is coated with sporopollenins which is very difficult to digest by enzymes. The freshly isolated protoplast from mature pollen tends to fuse spontaneously to form multinucleate giant protoplast. Therefore, isolation and culture of protoplast have yielded only limited success. Better preparation of protoplasts is obtained from pollen mother cell or pollen tetrad than the mature pollen. The isolated protoplast from haploid cell is known haploid protoplast or gametoplast. Isolation and culture of haploid protoplast are useful for the induction mutation and to study the effect of irradiation as well as chemical mutagens.

## ISOLATION OF PROTOPLAST FROM PMC AND PT

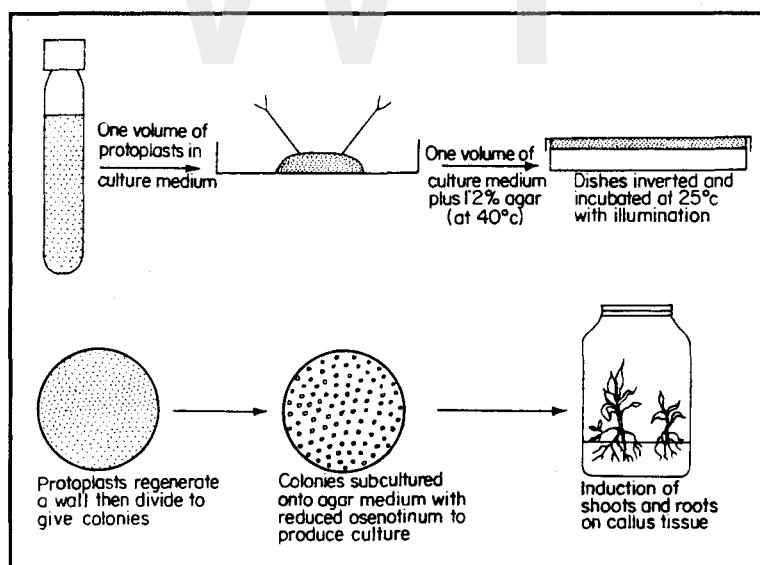
To ascertain the presence of PMC and PT in anther in relation to flower bud size, the anthers are smeared with 2% acetocarmine before using them. Like leaf mesophyll tissue and cell suspension culture, the isolation of haploid protoplast is also multistep process.

Approximately, the anticipated size of anther containing PMC and PT are aseptically removed with fine forceps. The content of this anther are squeezed out with the help of glass spatula. The pollen mother cell and the pollen tetrad comes out as a milky fluid. It is treated with 0.75-1% helicase enzyme in 8-10% sucrose for about 30-45 minutes. Helicase is obtained from snail gut. Sometimes Zymolase, an enzyme isolated from *Arthobacter luteus* is used for the isolation of protoplast from pollen tetrad. After incubation enzyme is carefully replaced by 10% sucrose and the protoplasts are allowed to settle. Protoplasts are rinsed by conventional washing medium. Finally, protoplasts are cultured in either liquid or solid medium.

## CULTURE OF PROTOPLAST

Isolated protoplast can be cultured in several ways of which agar embedding technique in small petridish is commonly followed. In this technique, protoplast suspension is mixed with equal volume of melted 1.6% 'Difco' agarified medium ( $37^{\circ}\text{C}$ ) and the protoplast-agar mixture are poured into small petridish. In petridishes, embedding of protoplasts in solid agar medium is known as plating of protoplasts. The plated protoplast can be handled very easily and the agar medium provides a good support to the protoplast. *In situ* developmental stages of embedded protoplast can be studied under compound microscope. Besides this, separated clones derived from individual protoplast can be monitored. The method is described below—

1. The protoplasts in liquid NT medium\* are counted with the help of haemocytometer. The protoplast density is adjusted to  $1 \times 10^5$  to  $2 \times 10^5$  protoplast/ml.
2. Agar solidified (1.6% 'Difco' agar) NT medium is melted.
3. The tight lid of Falcon plastic petridish (35 mm diameter 5 mm thickness) is opened



□ Fig 12.6

Diagram showing the culture of protoplast by agar embedding technique in small petridishes (after Bhojwani and Razdan 1983)

and 1.5 ml of protoplast suspension is taken. To this equal aliquot of melted agar medium is added when it cools down at 37°C to 40°C (Fig 12.6).

4. The lid is quickly replaced tightly and the whole dish is swirled gently to disperse the protoplast-agar medium mixture uniformly throughout the dish.
5. The medium is allowed to solidify. The petridish is then inverted.
6. The culture is incubated at 25°C with 500 lux illumination (16 hrs. light) initially.
7. The cultures are subcultured periodically in the same solid medium (0.8% agar) with gradually reducing mannitol.

Several other methods have been described for the culture of protoplasts, such as droplet culture, Coculture, feeder layer, hanging droplets and immobilized/bead culture.

### Droplet Culture

Suspending protoplasts in liquid culture media are placed on petri dishes in the form of droplet (Fig 12.7) with the help of micropipette. This method enables the subsequent microscopic examination of protoplast development. In this method, cultured protoplast clump together at the centre of droplets.

### Coculture

Sometimes a reliable fast growing protoplast is mixed in varying ratio with the less fast growing protoplast. The mixed protoplasts are plated in solid medium as described previously. The fast growing protoplast presumably provides some growth factors which induces the growth and development of the desirable protoplasts. This is known as coculture technique.

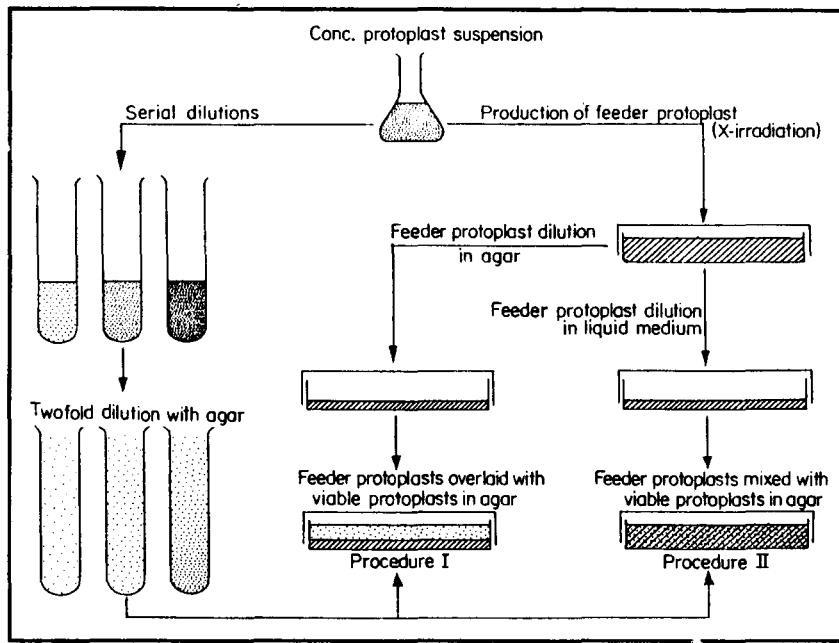
### Feeder Layer Technique

Fast growing protoplasts are sometimes made mitotically blocked protoplast by low doses (1-2 Krad) of X-ray treatment. Such irradiated protoplasts are plated with agar medium. Upon

this thin solidified layer of irradiated protoplast, desirable protoplasts are again plated at a low density with agar medium. As a result, it makes two agar layers containing irradiated protoplast in lower layer and desirable protoplast in upper layer. The lower irradiated protoplast is known as feeder layer which improves the growth and development of normal protoplasts even at lower density (Fig 12.7).

\* Composition of Nagata and Takebe (NT) medium (1971) for protoplast culture.

Constituents	Amounts in mg/L
<b>Macro-nutrients</b>	
NH <sub>4</sub> NO <sub>3</sub>	825
KNO <sub>3</sub>	950
CaCl <sub>2</sub> , 2H <sub>2</sub> O	220
MgSO <sub>4</sub> , 7H <sub>2</sub> O	1233
KH <sub>2</sub> PO <sub>4</sub>	680
<b>Micro-nutrients</b>	
KI	0.83
H <sub>3</sub> BO <sub>3</sub>	6.2
MnSO <sub>4</sub> , 4H <sub>2</sub> O	22.3
ZnSO <sub>4</sub> , 4H <sub>2</sub> O	8.6
Na <sub>2</sub> NoO <sub>4</sub> , 5H <sub>2</sub> O	0.25
CuSO <sub>4</sub> , 5H <sub>2</sub> O	0.025
<b>Iron source</b>	
FeSO <sub>4</sub> , 7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA, 2H <sub>2</sub> O	37.3
<b>Vitamins</b>	
Meso-inositol	100
Thiamine HCl	1
<b>Carbohydrate source</b>	
Sucrose	1%
<b>Growth substances</b>	
α-naphthalene-acid (NAA)	3
6-Benzylaminopurine (6-BAP)	1
<b>Plasmolyticum</b>	
Mannitol	13%-0%
pH	5.8
For solid medium 1.6% or 0.8% agar is added	



□ Fig 12.7

**The technique of using X-irradiated protoplasts as feeder cells to stimulate growth of viable protoplasts at low density**

#### Hanging Droplet Method

Culture of protoplasts in an inverted liquid droplet ( $0.25\text{--}0.50\ \mu\text{l}$ ) is known as hanging droplet method. Each droplet contains very small group of protoplasts. A number of droplets are generally placed on the inner surface of the lid of a petri dish. Very thin layer of water is generally kept on the lower part of the petri dish to make a humid condition inside the petri dish as well as to prevent the dessication of the droplets. This technique facilitates to observe the development of protoplast under microscope. Protoplasts also gets better aeration as they go down to the hanging surface of the droplets.

#### Bead Culture

Sometimes protoplast suspension are mixed with several polymer like alginate, carrageenan etc. as well as melted standard difco agar. Small beads are made by dripping the mixture into liquid medium. After that, beads in liquid medium are put on moving shaker. Entrapped proto-

plasts culture have shown several advantage over static liquid culture or slowly moving liquid culture where the protoplast suffers the mechanical breakage. This technique increases the mechanical stability, aeration and viability with biochemical activity.

#### TESTS FOR VIABILITY OF PROTOPLAST

Viability of protoplasts after isolation and during culture in liquid medium is very important. Cell wall formation, cell division, callus formation etc. depend upon the viability of protoplast.

The most frequently used staining methods for assessing protoplast viability are fluorescein diacetate (FDA), phenosafranine. FDA dissolved in 5.0 mg/ml acetone is added to the protoplast culture at 0.01% final concentration. The chlorophyll from broken protoplasts fluoresces red. Therefore, the percentage of viable protoplasts in a preparation can be easily calculated.

Phenosaftranin, also used at a final concentration of 0.01% is specific for dead protoplast. As soon as the stain is mixed with protoplast preparation, the inviable protoplasts stain red and viable protoplasts remain unstained.

## WALL FORMATION, CELL DIVISION AND CALLUS FORMATION

The viable protoplast in culture regenerates its own wall around them. Once the wall is formed, the protoplast becomes essentially a regenerated cell. Depending upon the species, the protoplast remains in naked condition hardly for 10 minutes or a day. Generally protoplast begins to deposit cellulose microfibril immediately after washing and enzyme removal. Cell membrane of newly isolated protoplast contain protruding microtubules that function in the orientation of newly synthesized cellulose microfibrils. The rate and regularity of cell wall regeneration depend on the plant species and the state of differentiation of the donor cell used for protoplast isolation.

Calcafluor white (CFW) is the most commonly used stain to detect the onset of cell wall regeneration. CFW binds to the-linked glucosides in the newly synthesized cell wall. Optimum staining is achieved when 0.1 ml of protoplasts is mixed with 5.0  $\mu$ l of a 0.1% v/v solution CFW. Cell wall synthesis is observed by a ring of white fluorescence around the plasma membrane.

Cell wall regeneration is prerequisite for nuclear and cell division. After the formation of cell wall, the walled cells expand and divide into two cells. At this stage it looks like '8'. Cell division stages can also be stained using CFW. In most of cases, first cell division usually takes place with 2-7 days of culture. After the first division, each daughter cell divides into two cells. Repeated division results the formation of cell clump or cell aggregates. All the cells derived from the protoplasts do not divide and form the cell colonies. Therefore, the percentage of cells which give rises cell colonies, to known as plating (colony forming) efficiency. Several factors such as genotype of the donor plant, culture medium, hormones as well as physical factors are important for the division of protoplast and callus formation.

The small callus mass can be handled in the conventional manner which means that it can be subcultured at regular interval and can be used for organogenesis. For subculturing, the plate containing dividing protoplasts are sliced into several agar blocks. Each block is transferred to the surface of fresh medium. The plasmolyticum level in the culture medium is progressively reduced to zero by repeated subculturing.

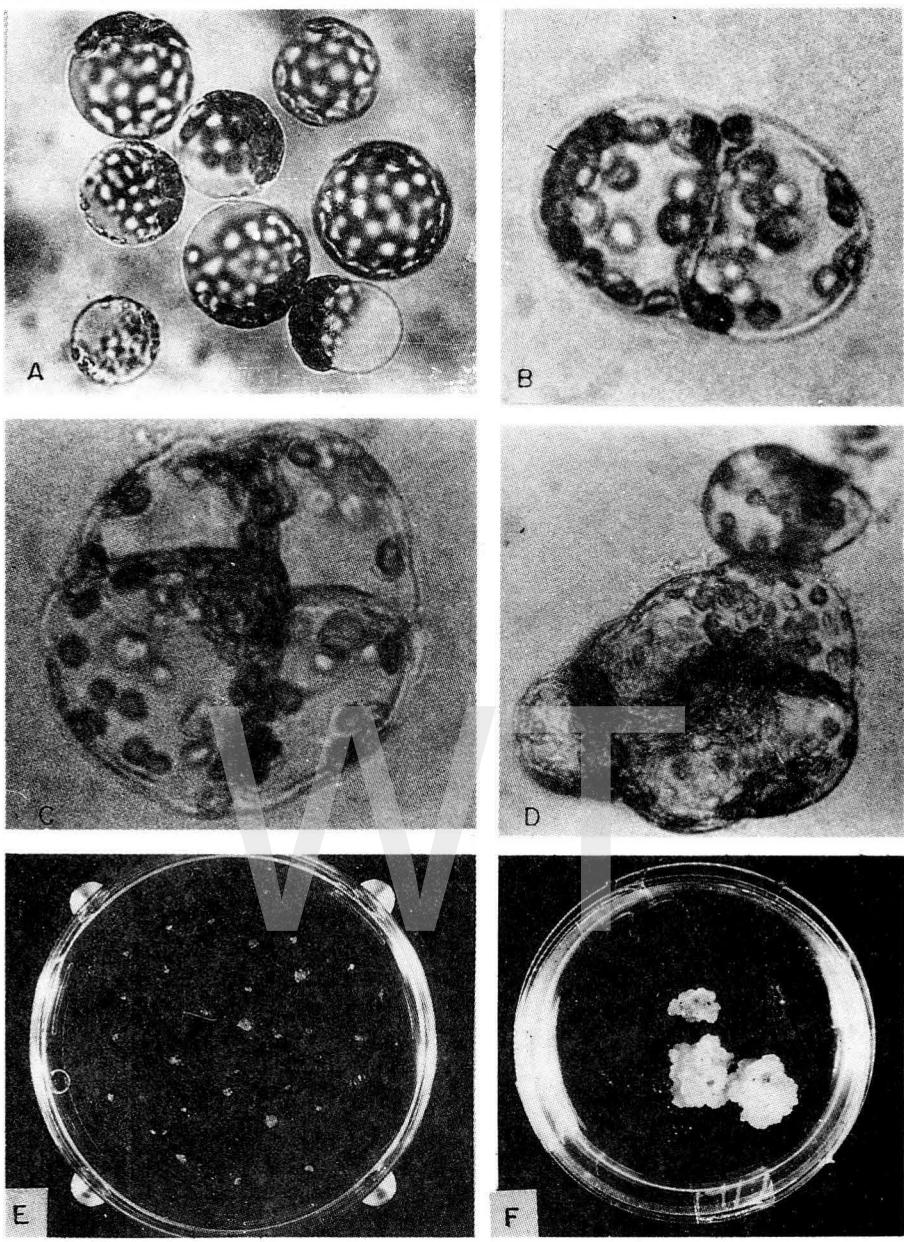
## PLANT REGENERATION

The ultimate objective in protoplast culture is the reconstruction of plant from the single protoplast. The strategy for plant regeneration has been to recover rapidly growing callus from protoplasts and to transfer the callus to a species specific regeneration medium (Fig 12.8). It is generally noted that plant regeneration occurs very easily in some plant species while others are recalcitrant. Plant regeneration from protoplast derived callus tissue have been reported mainly from solanaceous species. It includes 17 *Nicotiana* species, 6 *Petunia* species and 6 *Solanum* species. In recent years the list of non-solanaceous species capable of plant regeneration from protoplasts has been steadily expanding. The list contains several species monocot and dicot including carrot, endive, cassava, alfalfa, millet, clover, rapeseed, asparagus, cabbage, citrus etc.

## ACTION OF CELLULASE AND PECTINASE OR MACEROZYME ON PLANT CELL

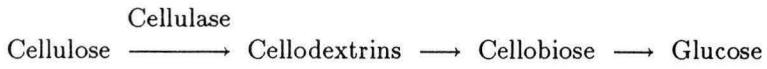
Cellulose is a fundamental component of the plant cell wall. It is straight chain polymeric molecule composed of 10,000 or more D-glucose units bound together with  $\beta(1 \rightarrow 4)$  glycosidic links. The  $\beta(1 \rightarrow 4)$  linkage of cellulose are hydrolyzed by cellulase enzyme complex. This enzyme attacks the polymeric chain of cellulose at random on the  $\beta(1 \rightarrow 4)$  linkage. The cellulose molecule is reduced sequentially to cellobextrins, cellobiose and eventually to glucose molecule.

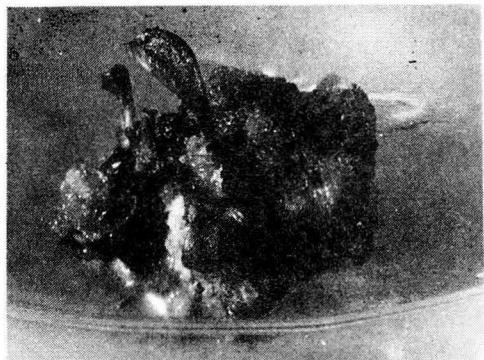
Pectin is a polysaccharide substance present in the middle lamella of the cell walls of all plant tissues which functions as an intercellular cementing material. It is a straight chain molecule



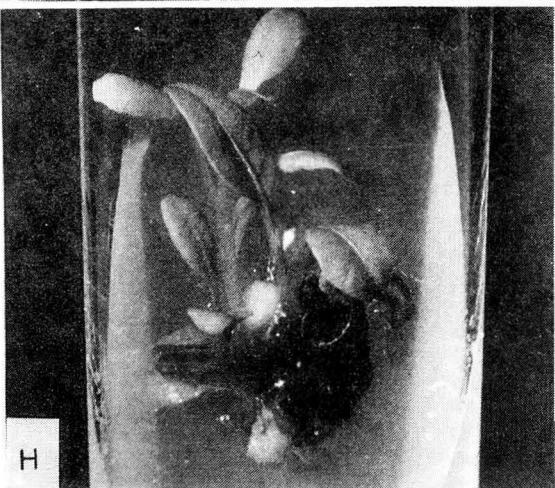
□ Fig 12.8

**A. Protoplasts isolated from mesophyll tissue. B – C. First, second and third divisions of protoplast respectively. D. Small pin head shaped calli from protoplasts. E. Masses of calli after second subculture.**

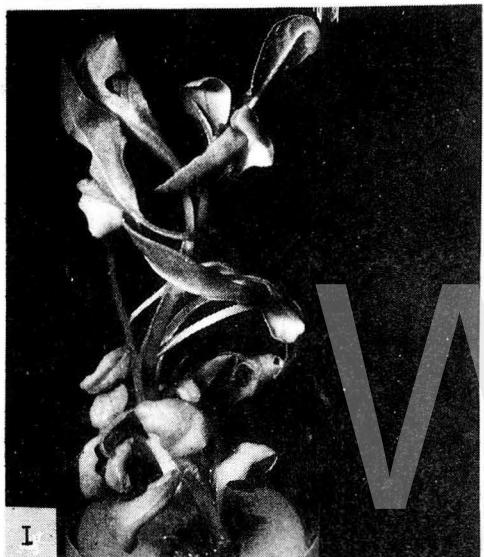




G



H



I



J

□ Fig 12.8

**G. Shoot buds from calli. H. Development of shoots. I – J. Mature plantlets.**

consisting of about 100 D-galacturonic acid residues bound together by  $\alpha(1 \rightarrow 4)$  linkage. This linear sequence of D-galacturonic acid is interrupted with  $(1 \rightarrow 2)$  L-rhamnose residues. Hydrolysis of the  $\alpha(1 \rightarrow 4)$  linkage of pectin is catalyzed by the pectinase or macerozyme enzyme complex.

Therefore, pectinase or macerozyme, by complete or partial hydrolysis of pectin, loses the cell mass from tissue and exposes the cellulosic layer to cellulase enzyme for further action.

#### IMPORTANT PROPERTIES OF ISOLATED PROTOPLAST

1. Freshly isolated protoplasts in plasmolyticum solution are always spherical in shape.
2. The protoplasts are highly fragile in structure.
3. Newly released protoplasts show high pinocytic activity. This property helps to intro-

duce foreign particle like DNA, cell organelles, virus, bacteria etc. into the protoplasts.

4. Plant protoplasts carry a negative surface charge. Depending on the species this charge may vary from -10 to -30 mV.
5. One of the exciting and potential properties of protoplasts is that each isolated protoplast is entirely separated from another and hence a population of protoplast provides best possible approach to single isolated cell system. This permits the handling of protoplasts in much the same way as with micro-organism.
6. Newly formed protoplast begins to synthesize a new cell wall immediately after washing and enzyme removal. Deposition of cellulose microfibril around the plasma membrane can be detected in some material within 10 minutes of culture after washing.
7. After the formation of cell wall, it enters the cell division and ultimately forms the callus tissue from which complete plant can be regenerated.
8. Freshly isolated protoplasts do not fuse and they repel each other due to carrying similar charges. But in presence of some fusion inducing chemicals e.g. PEG (polyethylene glycol) in the medium, the protoplasts isolated from distantly related plant groups can be induced to fuse. Such fusion may lead to form a somatic hybrid plant.
9. Protoplast fusion takes place when the molecular distance between the protoplasts is 10 Å or less. This indicates that protoplast fusion is highly a traumatic event.

#### **IMPORTANCE OF PROTOPLAST CULTURE**

The technique of cultivation of isolated protoplast in a mass scale has opened a new vista in plant science. Freshly isolated protoplasts with their high pinocytotic activity combined with their totipotent nature are especially important because of the far-reaching application in studies

of fundamental and applied research in experimental biology, somatic hybridization, genetic engineering and somatic cell genetics. The production of new hybrids without recourse to sexual reproduction is one of the greatest use of protoplast for crop improvement.

Major applications of protoplast technology are given below—

#### **STUDY OF OSMOTIC BEHAVIOUR**

Influence of different environmental factors on the osmotic behaviour can be studied using plant protoplasts.

#### **STUDY OF IAA ACTION**

When growth promoters like IAA are applied to plants, they act directly on plasma membrane of the cell and increase the permeability of the membrane to water resulting in cell elongation. This can be established by the use of protoplast *in vitro*. When IAA is applied to the plasmolyticum containing protoplasts they expand rapidly and finally burst due to too much vacuolation (Cocking and Hall, 1974). Further, it can be verified by using anti-auxins that suppress this bursting, indicating that the site of action of IAA is the plasmalemma of the plant cell.

#### **STUDY OF PLASMALEMMA**

When newly released protoplasts are placed in hypotonic solution or plain water, the protoplasts burst within a second or if the protoplast are dropped from a certain height on a glass slide, the same result will happen. So by this process, plasma-membrane can be isolated very easily from protoplast and a number of study on plasma-membrane can be investigated.

#### **STUDY OF CELL WALL FORMATION**

The early deposition of cellulosic microfibril and their orientation at the protoplast surface can be followed using both light and electron microscope and has also provided much basic information concerning cell wall biology.

## ORGANELLE ISOLATION

Protoplasts are very convenient material for the isolation of chloroplasts, mitochondria, nuclei and even chromosomes. It has been demonstrated that chloroplasts particularly isolated from cereal protoplast have higher capacity for CO<sub>2</sub> fixation than those obtained by mechanical grinding.

## STUDY OF MORPHOGENESIS

Isolated protoplast provides an ideal single cell system. Under suitable condition, protoplast regenerates its own wall and become the walled cells. Cell division followed by plant regeneration may occur from such unique single cell system either through organogenesis or embryogenesis.

Protoplasts → Walled cells



## ISOLATION OF BACTERIODS FROM ROOT NODULE PROTOPLAST

Viable bacterioids from root nodules of legumes has been isolated by first preparing nodule protoplast and then rupturing them either mechanically or by lowering suddenly the concentration of the plasmolyticum in the surrounding medium. This method ensures the freedom of the preparation of bacteria from the infection thread.

## GENETIC ENGINEERING OR GENE TRANSFER IN PLANT THROUGH PROTOPLAST

Since the isolated protoplast shows high pinocytic activity, so it can uptake some specific genes or foreign DNA and this property

Callus Tissue → Organogenesis



Embyroids → Plant

brings some genetic modification in plants. Genetic modification by DNA uptake implies that DNA from one source be taken up and incorporated into the recipient genome and that genetic information encoded in the exogenous DNA be expressed as a new and stable characteristic in the recipient protoplast and subsequently in the regenerated plants. This approach provides an alternative way to increase genetic diversity to the existing genetic make-up of the plant.

Several approaches has been employed for transferring exogenous DNA or specific genes into the protoplasts such as—

- (a) Direct gene transfer.
- (b) Indirect or liposome mediated delivery of DNA to plant protoplasts.
- (c) Gene transfer using biological vectors.
- (d) Microinjection of DNA into protoplasts.
- (e) Fusion between the bacterial spheroplasts and plant protoplast.

Direct uptake of foreign DNA has been investigated in protoplast. The anticipated steps

## VIRUS UPTAKE AND REPLICATION

The plant virus interrelationships in the past were not clearly known due to lack of suitable experimental systems that can easily infect the cells. But after the innovation of protoplast isolation and its culture, this problem is almost solved. Protoplast can directly be inoculated with pathogenic virus in the medium. The process of uptake of virus particle, their replication inside the protoplasts and their mode of action at the molecular and cellular level are made possible by the aid of protoplasts.

## STUDY OF PHOTOSYNTHESIS FROM ISOLATED PROTOPLAST

Elegant experiments to investigate various biophysical and biochemical aspects of photosynthesis in C<sub>3</sub> and C<sub>4</sub> plants have been carried out by a number of workers using protoplasts.

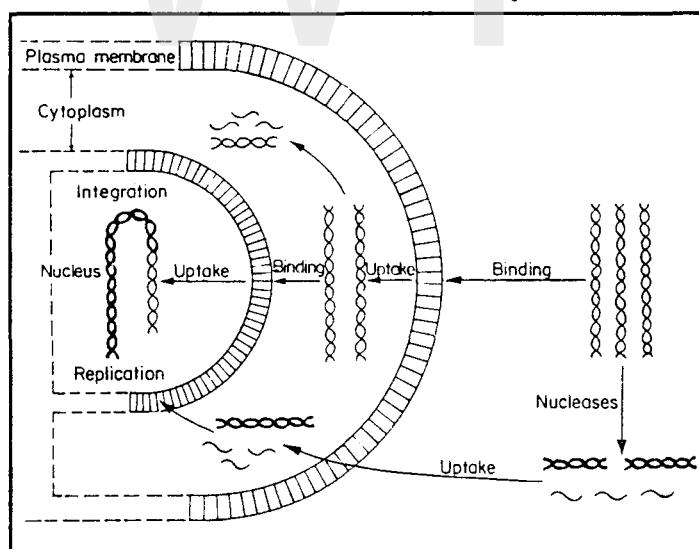
in the uptake process into protoplasts include binding to specific sites on the plasma membrane and passage through the membrane and cytoplasm. This is followed by binding to specific binding sites, passage through the nuclear membrane into the nucleus and ultimately integration into the host genome (Fig 12.9).

A major obstacle in feeding isolated DNA directly into the protoplasts is the presence of enzymes that degrade DNA. So it needs a good protection for exogenous DNA so that DNA makes a safe journey from external medium to the recipient nucleus and such protection will facilitate its stabilization in recipient cytoplasm.

The use of liposome is a new innovation to facilitate the uptake of nucleic acid without any degradation. Liposomes are the liquid crystalline structure obtained when amphipathic lipids such as phospholipids are dispersed in water or aqueous salt solution. Each liposome is a bilayered completely enclosed sac-like vesicle (250 Å to 10 µm diameter). It is possible to enclose the nucleic acid within liposome which can readily transport through biological membrane of the protoplast. The result indicates that liposome can protect the enclosed nucleic acid from the degradation by the enzymes of the recipient protoplast.

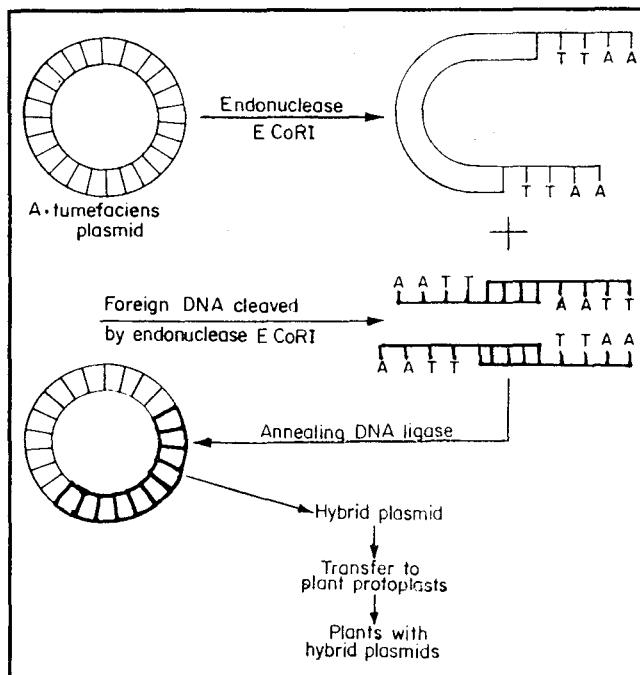
The incorporation of DNA by vectors may also reduce the degradation of DNA and improve the gene transfer process. Two types of vectors are being considered, plant viruses and bacterial plasmids. Using the DNA virus as a vector, it may be possible to insert the foreign DNA into the viral genome. Since the foreign DNA in association with viral DNA can be incorporated safely in the nucleus of the recipient cell. So DNA viruses are more or less suitable vectors for gene transfer.

Plasmids are double stranded, closed circular extra-chromosomal DNA found in bacteria. Plasmids can integrate into the chromosome of the recipient cells. The research with plasmids has developed rapidly in the recent years and has resulted new and revolutionary techniques for gene transfer. On the other hand, a series of novel enzymes called restriction endonucleases have been discovered. Such enzymes could be used as 'molecular scalpel'. The enzymes cut the DNA at points with specific nucleotide sequences. At the same time ligase enzyme used as 'molecular adhesive' can join the cleaved DNA. The restriction enzymes cleave the plasmid DNA to linear, double stranded sections with overlapping and complementary nucleotide sequences. The linear plasmid DNA section can be mixed



□ Fig 12.9

Events in DNA uptake by protoplasts (after Gamborg *et al* 1979)



□ Fig 12.10

#### Plasmids for gene transfer in protoplasts (modified after Gamborg *et al* 1979)

with endonuclease-cleaved section of foreign DNA from other source. With the help of ligase, reconstituting the plasmid DNA carrying the genes or section of foreign DNA can be mixed with the protoplasts. After the hybrid plasmid is taken up by the protoplast, it is replicated and the genetic information that is encoded in the foreign DNA can be transcribed and eventually expressed in the walled cells and subsequently in the plants derived from the protoplasts (Fig 12.10).

It is well known that the pathogenic bacteria *Agrobacterium tumefaciens*, causes the plant tumour crown gall upon wounding and infection. The agents responsible for tumour induction are plasmid called tumour inducing or Ti plasmids. Crown gall cells have their capacity to grow *in vitro* in absence of phytohormones. It has been established that the autonomous growth of the tumour cells is caused by the integration of a piece of DNA (T-DNA) form a Ti plasmid. This naturally transformed system or Ti plasmid DNA can be used in experiments to transform plant protoplasts into hormone independent

opine synthesizing cells. Thus genetic manipulation of plant protoplast by means of *Agrobacterium tumefaciens* can be achieved by three different approaches—(a) fusion of crown gall protoplasts with normal protoplast, (b) infection of cell wall regenerating protoplast with virulent strains of *A. tumefaciens*, (c) transformation of protoplasts with Ti plasmid DNA.

With the help of above genetic engineering or plasmid technology, intensive efforts are being made in various laboratories, all over the world to transfer the 'nif' gene (nitrogen fixing gene) in the protoplast of non-legumes. It may well usher us into an era of "self fertilized farming"

Microinjection of foreign DNA into the nuclei of protoplasts has also been developed into an efficient and reproducible method of gene transfer.

Recently, the fusion of protoplasts with bacterial spheroplasts are also used as a method of introducing DNA into plant cell.

Genetical change that occurs in bacteria due to absorption of foreign DNA or extract of

other bacteria, is known as bacterial transformation. Transgenesis is a new term for the type of transformation in higher plant system.

## INDUCTION OF MUTATION AND GENETIC VARIABILITY

It has been repeatedly observed that plant cell in culture show a wide range of genetic diversity. This phenomena can be exploited by plant breeders and geneticists for inducing variability in protoplast culture. The recessive characters can be detected in the regenerated plants derived from haploid protoplasts. Therefore, haploid protoplast would make an ideal system for studying the effect of irradiation and for the induction of mutation by plating them in media supplemented with various chemical mutagens.

From this method, mutant line can be selected.

## MICROORGANISM TRANSPLANTATION

Incorporation of microorganisms like bacteria, blue-green algae, yeast etc. into protoplasts has been attempted with the immediate objective of establishing endosymbiotic association with higher plant cells which may eventually yield a plant having some beneficial activity. Bacterial cell uptake by plant protoplasts has been investigated with species of *Rhizobium* and *Spirillum*. There are reports based on ultra-structural examinations that bacteria enter the cells by endocytosis and may become embedded in vesicles in the cytoplasm of protoplasts. Similar uptake studies were performed with yeast and blue green algae cells. Introduction of *Anabaena variabilis* and nitrogen fixing blue green algae *Gleocapsa* sp. into protoplasts has also been reported. However, nothing is known about the fate of the introduced microorganism, because there has been no reported evidence of survival or development of any organisms within the protoplasts.

## IMPLANTATION OF CHLOROPLAST

Plant protoplasts have ability to uptake the isolated chloroplasts by the process of endocy-

tosis. Several reports have described uptake of chloroplasts. Chloroplasts isolated from *Vaucheinia dichotoma* were implanted into carrot cell culture protoplasts. The chloroplasts may enter the cytoplasm enclosed in membrane-bound vesicles, although the enclosing membrane in some cases is absent. Biological evidence of chloroplast gene expression was presented but the experiments have not been confirmed. The inability and ability of chloroplasts to survive and multiply in recipient protoplasts have not been unequivocally demonstrated, although limited replication has been reported. Potentially the chloroplast uptake procedure offers an excellent approach to study chloroplast/cytoplasm and nuclear interrelationships, genetics and physiological autonomy and specificity of functions of the organelles.

## TRANSPLANTATION OF NUCLEI

Isolated nuclei can be introduced into the protoplasts. Both intra and inter-specific nuclear transplanatation have been observed in *Petunia hybrida*, *Nicotiana tabacum* and *Zea mays*. Retention, normal function or degradation of the incorporated nuclei is not known. But it is really opening up new avenues for the study of nuclear-cytoplasmic interaction if fertile plants with foreign nuclei could be regenerated from such protoplasts.

## TRANSPLANTATION OF CHROMOSOME

The uptake of isolated metaphase chromosomes has proven successful in plant protoplast. This procedure provide a valuable method for genetic information transfer and gene analysis.

## SOMATIC HYBRIDIZATION

The main objective of protoplast culture lies in the possibilities of fusion of one protoplast with another. Normally isolated protoplasts do not fuse with each other, but in presence of fusion inducing agent like PEG, a protoplast of one species can be fused with one of a different species, thus a hybrid protoplast will be produced. Now this hybrid protoplast regenerates a

cell wall and its two nuclei fused together, then a somatic hybrid cell will be produced. Sometimes nuclei do not fuse and one nucleus of any one parent may be eliminated in the subsequent development stages. Thus hybrid cell is produced with the nuclear genome of any one partner and the cytoplasm of both parent. A somatic hybrid plant can be regenerated from these cells. Thus the production of new hybrid without recourse to sexual reproduction will constitute one of the greatest potentials in plant genetics for crop improvement. A number of crosses including both inter and intraspecific ones are not possible by normal pollination and so the hybrids cannot be produced. This obviously limits the scope of the plant breeder. Somatic hybridization or protoplast fusion will provide an alternative way to overcome the limitations of conventional breeding. It is also possible to manipulate hybrid production such that desirable characters e.g. disease resistance, protein quality, nitrogen fixation and cold tolerance which are genetically controlled, could be transferred from one species to another. On the other hand, hybrid plant is also useful where some desirable characters are controlled by cytoplasmic genome e.g. male sterility. A brief list of somatic hybrid plants raised through protoplast fusion is given below—

### INTERSPECIFIC HYBRIDIZATION

#### Sexually Compatible Combination

*Daucus carota + D. capillifolius, Nicotiana glauca + N. langsdorffii, N. tabacum + N. alata, Petunia parodii + P. hybrida, Solanum tuberosum + S. chacoense.*

#### Sexually Incompatible Combination

*Datura innoxia + D. candida Nicotiana, sylvestris + N. knightiana, N. tabacum + N. neosophila, Petunia parodii + P. parviflora.*

### INTERGENERIC HYBRIDIZATION

*Arabidopsis thaliana + Brassica campestris, Daucus carota + Aegopodium podagraria, Solanum tuberosum + Lycopersicon esculentum.*

Aseptic isolation and *in vitro* culture of protoplast of higher plant is a novel technique in the field of plant tissue culture. The protoplast includes the plasmalemma and everything contained within i.e. the entire cell without its inherent cellulosic cell wall. Plant protoplast can be isolated either mechanically or enzymatically from plasmolysed plant tissue or cells. A number of different sugar such as sucrose, glucose and sugar alcohol such as mannitol, sorbitol are used to plasmolyse the cells. Mannitol at the concentration of 0.45–0.80M is widely used as plasmolyticum. Convenient and suitable materials for protoplast isolation are leaf mesophyll and cells from liquid suspension culture.

Protoplasts were first isolated using mechanical methods. In most cases, very few protoplasts are obtained for a lot of time and effort. Later, Prof. E. C. Cocking in 1960 first introduced enzymatic method for the isolation of large number of protoplast. By this technique, protoplasts are isolated using cell wall degrading enzymes such as cellulase and pectinase. Both the enzymes are of fungal origin. Intact tissues can be incubated with a pectinase in a plasmolysing solution which will dissolve the middle lamella between the cells and separate them. Subsequent treatment with cellulase will digest away the remaining cellulose fibres of cell walls. This is referred to as a sequential enzyme (two-step) treatment as opposed to a mixed enzyme treatment in which both pectinase and cellulase are mixed into the plasmolysing solution so that the entire wall is broken down in a single treatment.

Population of isolated protoplasts at a definite density can be cultured by embedding them in an agar based solid medium or placing them in a static liquid medium. Cultured protoplasts regenerate a new cell wall around them in culture. Once the wall is formed, the ability of protoplast to divide is restored. After repeated division a small callus mass is formed that can be handled

in the conventional manner. Viability of the protoplast can be tested by 0.01% fluorescein diacetate or phenosafranin. The early development of cell wall can be detected by 0.1% calcofluor white. From a single protoplast, a number of plants can be regenerated through callus formation.

A major reason for the protoplasts is their potential use in plant cell genetics and specially in cell fusion and transfer of genetic information by DNA uptake and organelle implantation. The use of protoplast in plant physiology, plant pathology and many other fundamental research is significant.

## Questions for Discussion

1. Give a general account of the principle, method and importance of plant protoplast culture.
2. What is protoplast culture? Describe the protocol for isolation and culture of leaf cell or mesophyll protoplast.
3. Describe the methods of isolation and culture of protoplast from cell suspension culture, Pollen mother cells and pollen tetrad.
4. Discuss the use of protoplast culture for genetic modification in plant.
5. Discuss the factors affecting yield and viability of protoplasts.
6. Write the answers to the short questions—
  - (a) What are the different sources of plant tissue and their conditions for protoplast isolation?

- (b) Why plasmolyticums or osmolyticums are used essentially for protoplast isolation and culture?
  - (c) What are the important tests for viability of protoplasts?
  - (d) What are the important properties of isolated protoplasts?
  - (e) What is the action of cellulase and pectinase or macerozyme on plant cell?
  - (f) How do you demonstrate that the cell wall has been formed around the protoplast during culture?
  - (g) What is the main objective of plant protoplast culture?
  - (h) Is there any possibility of transformation of higher plant cell like bacteria?
7. Write short notes on—
- (a) Plasmolyticum or Osmolyticum
  - (b) Cell wall degrading enzymes
  - (c) Haploid protoplasts
  - (d) CPW 13M and CPW 21S
  - (e) Culture of protoplast
  - (f) Gene transfer in plants through protoplast
  - (g) Induction of mutation and genetic variability in protoplast culture
  - (h) Importance of protoplasts in biotechnology
  - (i) Transgenesis.

## Chapter Thirteen

### Protoplast Fusion and Somatic Hybridization

#### INTRODUCTION

Sexual hybridization in higher plant is a valuable tool for the conventional plant breeding to improve cultivated crops. It involves the artificial cross fertilization between the genetically dissimilar individuals to combine several desirable traits present in different varieties into one single variety. Unfortunately, conventional hybridization is limited to only very closely related species. Until recently, sexual hybridization was a total failure for distantly related plant species as well as sexually incompatible plant species. Therefore, if a system could be developed for transferring genetic information between widely different plant species, it would provide the basis for a technology to overcome the limitation of conventional sexual hybridization. In protoplast technology, from any two genotypically different plants protoplasts are isolated from the somatic cells (diploid) and are experimentally fused to obtain parasexual hybrid protoplasts. The hybrid protoplast contains heteroplasioic cytoplasm and two fused parental nuclei. The fused proto-

plast is grown *in vitro* with an aim to obtain a hybrid plant. So the *in vitro* fusion of plant protoplasts derived either from somatic cell of same plant or from two genetically different plant is called somatic hybridization. Sometime the protoplasts from vegetative cell and gametic cell are fused and such fusion is called somato-gametic hybridization. Protoplast fusion and somatic hybridization in plant are based on the following basic points—

- (i) Protoplast isolation.
- (ii) Fusion of protoplast, obtained from the different species, to produce viable heterokaryons.
- (iii) Cell wall regeneration by the fused product.
- (iv) Fusion of nuclei.
- (v) Division of hybrid cells and their subsequent growth.
- (vi) Identification, selection or isolation of hybrid cell.

- (vii) Induction of organogenesis in the callus tissue derived from hybrid cell.
- (viii) Raising mature plants from regenerated shoots.

Experimental fusion of plant protoplasts was reported by Küster (1909) and Michel (1937). Although fusion products were obtained, they failed to survive because of shortenings in the understanding of environmental, nutritional and hormonal requirements of isolated protoplasts. After protoplasts became available in large quantities with the aid of enzymes, renewed attempts were made at protoplast fusion to obtain somatic hybridization.

### METHODS OF PROTOPLAST FUSION

Protoplast fusion is a physical phenomenon. During fusion, two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing chemicals. After adhesion, membranes of protoplasts fuse in some localized areas and eventually the cytoplasm of the two protoplasts intermingle.

Broadly speaking, protoplast fusion can be classified into two categories, such as—

- A. Spontaneous fusion
- B. Induced fusion.

In somatic hybridization, spontaneous fusion is of little significance. The methods used for induced fusion can again be sub-categorised.

### SPONTANEOUS FUSION

Protoplasts during isolation often fuse spontaneously and this phenomenon is called spontaneous fusion. Simply physical contact is

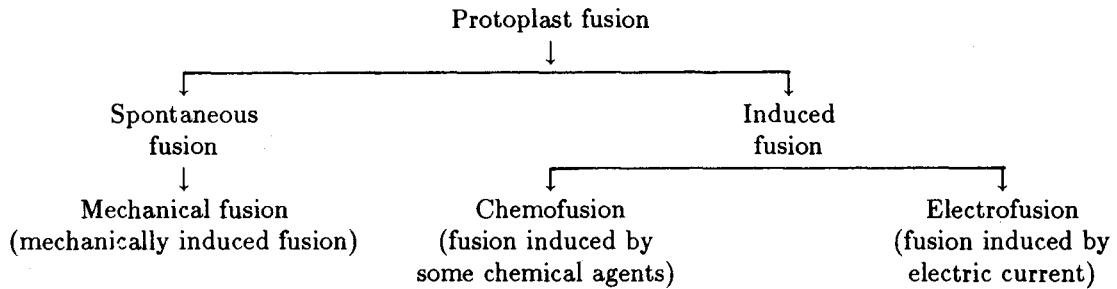
sufficient to bring about the spontaneous fusion among the similar parental protoplasts. During the enzyme treatment for the isolation of protoplasts, it is found that protoplasts from adjoining cells fuse through their plasmodesmata to form a multinucleate protoplast. Electron microscopic studies have shown that as the cell wall are enzymatically degraded, the plasmodesmatal connection between the adjacent cells enlarge due to removal of its constriction and the enlargement of pit fields. Eventually, the greater enlargement of plasmodesmata allow the entry of organelles into neighbouring cells. Finally a complete coalescence of adjacent cell takes place. Spontaneous fusion is strictly intraspecific and give rise to homokaryon.

The protoplasts, once they are freely isolated, do not fuse spontaneously with each other. An exception is the protoplast from microsporocytes of some plants of lily family where the freely isolated protoplast fuse spontaneously. This type of spontaneous fusion has been used to produce intergeneric fusion, e.g. the spontaneous fusion of microsporocyte protoplast of *Lolium longiflorum* and *Trillium kamtschaticum*.

Spontaneous fusion of two or more adjoining somatic protoplasts is of no practical use, but this may be important in studies of the nature and function of plasmodesmata, the physiology and control of mitosis in multinucleated cells and nuclear fusion. Perhaps spontaneous fusion has some practical importance for chromosome doubling.

### INDUCED FUSION

Fusion of freely isolated protoplasts from different sources with the help of fusion induc-



ing chemical agents is known as induced fusion. Normally, isolated protoplasts do not fuse with each other because the surface of the isolated protoplast carries negative charge ( $-10\text{ mV}$ — $-30\text{ mV}$ ) around the outside of plasma membrane and thus there is a strong tendency for protoplasts to repel one another due to their same charges. So this type of fusion needs a fusion inducing chemical agent or system which actually reduces the electronegativity of the isolated protoplasts and allow them to fuse with each other.

Actually, induced fusion is a highly important and a valuable technique because the protoplast from widely different and sexually incompatible plants can be fused by this procedure. This technique has the possibility and ability to combine different genotype beyond the limits imposed by sexual process. The fundamental objectives of somatic hybridization are mainly based on induced protoplast fusion.

The isolated plant protoplasts can be induced to fuse by three ways—

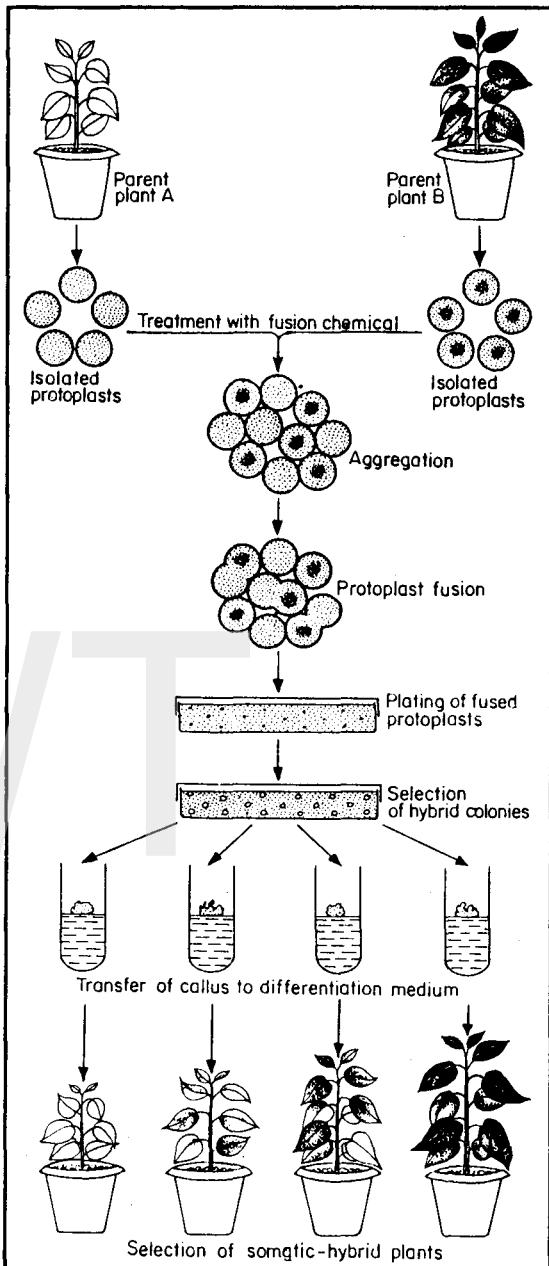
### Mechanical Fusion

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

### Chemofusion

Several chemicals have been used to induce protoplast fusion. Sodium nitrate ( $\text{NaNO}_3$ ), polyethylene glycol (PEG), Calcium ions ( $\text{Ca}^{2+}$ ), Polyvinyl alcohol etc. are the most commonly used protoplast fusion inducing agents which are commonly known as chemical fusogens. Generally, chemofusion techniques are followed in most of induced fusion experiments. Chemical fusogens cause the isolated protoplasts to adhere to one another and leads to tight agglutination followed by fusion of protoplast (Fig 13.1). The adhesion of isolated protoplast takes place either due to reduction of negative charges of protoplast or due to attraction of protoplast

by electrostatic forces caused by chemical fusogens.



□ Fig 13.1

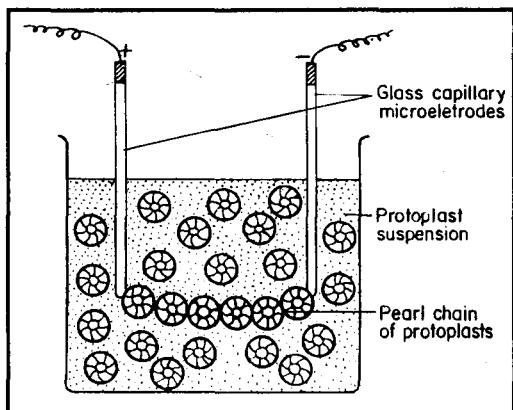
**Diagrammatic illustration for the fusion of protoplasts from two different plant species and later plating and selection of hybrid colonies and the regeneration of "Somatic-Hybrids" (after Rehnert and Bajaj 1977)**

## Electrofusion

Recently, mild electrical stimulation is being used to fuse protoplasts. This technique is known as electrofusion of protoplasts. Two glass capillary microelectrodes are placed in contact with the protoplasts. An electrical field of low strength ( $10 \text{ kV m}^{-1}$ ) gives rise to dielectrophoretic dipole generation within the protoplast suspension. This leads to pearl chain arrangement of protoplasts. The number of protoplasts within the pearl chain depends upon the population density of the protoplast and the distance between the electrodes (Fig 13.2). Subsequent application of high intensity electric impulse ( $100 \text{ kV m}^{-1}$ ) for some microseconds results in the electric breakdown of membrane and subsequent fusion. Zimmermann and Scheurich (1981) improved the method for the large scale fusion of plant protoplast. There are indications that this electrical method may increase fusion frequency and reproducibility.

### Chemofusion Procedures

Several chemofusion procedures have been proposed time to time to improve the fusion frequency and reproducibility of the fused product. Each and every method has its own merits and limitations also. Some chemofusion methods are described below—



□ Fig 13.2

Diagrammatic illustration for the electrofusion of protoplasts

### (i) Fusion induced by Sodium or Potassium Nitrate

Fusion of isolated onion subprotoplasts plasmolysed with Sodium salts was achieved for the first time by Küster (1909). Subsequently, Michel (1937) demonstrated fusion between protoplasts using potassium nitrate as plasmolyticum. Power *et al.* (1970) reported sodium nitrate induced fusion of cereal root protoplasts.

By this method, equal densities of protoplast from two different sources are mixed and then centrifuged at 100g for 5 minutes to get a dense pellet. This is followed by addition of 4 ml of 5.5% sodium nitrate in 10.2% sucrose solution to resuspend the protoplast pellet. The suspended protoplasts are kept in waterbath at 35°C for 5 minutes and again centrifuged at 200g for 5 minutes. The pellet is once again kept in waterbath at 30°C for 30 minutes. Fusion of protoplast takes place at the time of incubation. The pellet is again suspended by 0.1% sodium nitrate for 5–10 minutes. The protoplasts are washed twice with liquid culture medium by repeated centrifugation. Finally, the protoplasts are plated in semisolid culture medium.

Using the above principle, intra and inter-specific fusions have been achieved by several workers. However, sodium nitrate is toxic to cell at fusogenic concentration.

The frequency of fusion is not very high in this method. Yet it is useful only for the protoplasts derived from meristematic cells.

### (ii) Fusion induced by Calcium ions at high pH

In 1973, Keller and Melcher from Germany, developed a method to effectively induce fusion of tobacco protoplast at high temperature (37°C) in media containing high concentration of  $\text{Ca}^{2+}$  ions, (i.e. calcium chloride) at a highly alkaline condition (pH 10.5). Equal densities of protoplasts are taken in a centrifuge tube and the protoplasts are spun at 100g for 5 minutes. The pellet is suspended in 0.5 ml of medium. 4 ml of 0.05M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  in 0.4M mannitol at pH 10.5 is mixed to the protoplast suspension. The centrifuge tube containing protoplasts

at high pH/Ca<sup>2+</sup> is placed in the water bath at 30°C for 10 minutes and is spun at 50g for 3–4 minutes. This is followed by keeping the tubes in water bath (37°C) for 40–50 minutes. About 20–30% protoplasts are involved in this fusion experiment.

### (iii) Fusion induced by PEG

In 1974, Kao and Michayluk from Canada discovered another fusion inducing chemical polyethylene glycol (PEG) which is the most effective agent discovered so far. Many fusion experiments are performed by a polyethylene glycol. PEG induces protoplast aggregation and subsequent fusion. But the concentration and molecular weight of PEG are important with respect to fusion. A solution of 37.5% w/v PEG of molecular weight 1,540 or 6,000 aggregates mesophyll and cultured cell protoplasts during a 45 minutes incubation period at room temperature. Fusion of protoplast takes place during slow elution of PEG with liquid culture medium. Carrot protoplast can be fused by 28% PEG 1540 and the fusion can be promoted by Ca<sup>2+</sup> ion at the concentration of 3.5 mM. But higher concentration of Ca<sup>2+</sup> ion (10 or 50 mM) has been considered beneficial. In some studies, high pH/Ca<sup>2+</sup> and PEG method have been combined. By this method, the agglutination of protoplasts can be brought about using sufficient quantities (0.1–5 ml) of protoplast in centrifuge tube or microden-sities (150  $\mu$  l) of protoplast on a coverslip. The PEG method has been modified slightly to fuse higher plant protoplast.

The modifications are given below—

- (a) PEG is more effective when it is mixed with 10–15% dimethyl sulfoxide (DMSO).
- (b) Addition of concanavalin A (Con A) to PEG increases protoplast fusion frequency.
- (c) Sea water has been used alone or in combination with PEG to fuse protoplasts.

### (iv) Fusion induced by Other Chemicals

Some other chemicals have also been observed to promote protoplast fusion—

- (a) 15% solution of Polyvinyl alcohol (PVP) in combination with 0.05 CaCl<sub>2</sub> and 0.3 M mannitol are used to fuse plant protoplasts.
- (b) Lectins are also known to agglutinate protoplasts.
- (c) Various protein are also used for agglutination of protoplast.

## MECHANISM OF PROTOPLAST FUSION

The mechanism of protoplast fusion is not fully known. Several explanations have been put forward to understand the mechanism of protoplast fusion. Some important explanations are given below—

- (i) When the protoplasts are brought into close proximity, this is followed by an induction phase whereby changes induced in electrostatic potential of the membrane result in fusion. After fusion, the membrane stabilizes and the surface potential returns to their former state.
- (ii) When the protoplasts are closely adhered, the external fusogens cause disturbance in the intramembranous proteins and glycoproteins. This increases membrane fluidity and creates a region where lipid molecule intermix, allowing coalescence of adjacent membranes.
- (iii) The negative charge carried by protoplasts is mainly due to intramembranous phosphate groups. The addition of Ca<sup>2+</sup> ions causes the zeta-potential of plasma membrane to be reduced and under that condition the protoplasts aggregate.
- (iv) The high alkaline solution used in chemofusion induces the intramembranous production of lysophospholipid which may be linked with membranous fusion.
- (v) The high molecular weight (1,000–6,000) polymer of PEG acts as a molecular bridge connecting the protoplasts and Ca<sup>2+</sup> ions link the negatively charged PEG and membrane surface. On elution of the PEG, the surface potential are disturbed, leading to

intermembrane contact and subsequent fusion. Besides this, the strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity, thus inducing fusion.

- (vi) PEG itself induces aggregation, but  $\alpha$ -tocopherol present as an impurity in commercial grade PEG, actually promotes membrane fusion.

## HYBRID IDENTIFICATION

Following fusion of protoplasts, identification of protoplast fusion product is necessary to quantitate fusion frequency and to monitor the fusion products. The fusion frequency may vary due to either protoplast quantity or fusion conditions. The preliminary identification of fusion product is done under microscope. The microscopic identification is based on differences between the parental cells with respect to pigmentation, presence of chloroplast, nuclear staining, cytoplasmic marker etc. A system that has been used successfully consists of fusing protoplasts of leaf mesophyll cell containing chloroplasts with those from cell cultures lacking chloroplasts. At the initial stage, the fusion products at the light microscope level are seen to contain chloroplast in one half and colourless starch granules in other half. As a result, the fused cell can easily be distinguished from unfused parental protoplasts. Similarly, the protoplast of flower petal are usually vacuolated and pigmented. So the protoplast fusion products between petal-mesophyll or petal-cell culture protoplast can readily be identified.

If both types of parental protoplasts look alike, i.e. either colourless or pigmented, then the fusion products can be distinguished using nuclear staining technique. A hybrid cell contains two nuclei of two different parental protoplasts. Such dikaryotic cells can be identified using conventional aceto-orcein or aceto-carmine staining procedure. But the presence of two different parental nuclei in the hybrid cell, i.e. heterodikaryotic condition, can more precisely be distinguished using carbol-fuchsin staining technique because carbol-fuchsin stains differently two parental nuclei.

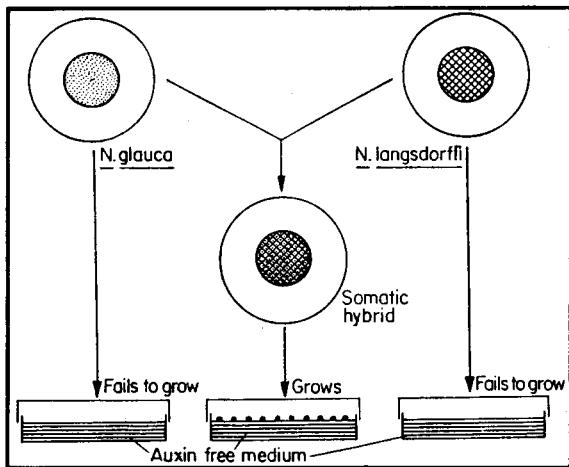
Non-toxic fluorochromes are often used for the identification of heterokaryon or fusion products. For example, fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) or rhodamine B are used as fluorochromes. The advantage of using fluorochromes for the identification of fusion products is that it does not depend upon the types of protoplast being used. For an example, in a fusion experiment, heterokaryons between FITC labelled suspension cell protoplast and unlabelled mesophyll protoplasts exhibit an apple-green fluorescence of FITC and a red fluorescence from chlorophyll of the mesophyll partner.

## HYBRID SELECTION

In the mixture of both fused and unfused protoplasts, the latter usually predominate. So, after plating these mixed protoplasts in the solid medium, it is very difficult to identify the hybrid cells microscopically. On the other hand, in most fusion experiments, the division rate of fused protoplast is relatively low. At the same time one or both unfused parental protoplast may also divide and very shortly the hybrid protoplast can no longer be distinguished from parental cells. So some types of selection technique are required at the level of culture to recover hybrid cells and its callus tissue following fusion. Since the cultural behaviour of protoplasts and their nutrient and hormone requirements may vary from plant to plant, several selection procedures have been developed. Some important selection procedures are discussed below—

## AUXIN AUTOTROPHY

The selection of the hybrids of *Nicotiana glauca* and *N. langsdorffii* is based on auxin autotrophy of the hybrid cells (Fig 13.3). The parental protoplast or cell requires an auxin compound in order to proliferate, whereas hybrid callus tissue needs no such requirement because the cells are auxin autotrophic. Therefore, somatic hybrid cells can be isolated selectively by growth on auxin free culture medium. Auxin autotrophy of the hybrid cell is expressed only as a result of



□ Fig 13.3

#### **Isolation of somatic hybrids formed by fusion of protoplasts of *N. gauca* and *N. langsdorffii* on a auxin free medium**

the genetic combination of the two parental protoplasts.

#### **USE OF GENETIC COMPLEMENTATION**

Melcher and Lalib (1974) first used genetic complementation to isolate green somatic hybrids following fusion of two distinct homozygous haploid recessive albino mutants of *Nicotiana tabacum*. A population of albino protoplasts are fused with either a population of protoplasts isolated from a second non-allelic albino mutant or with a population of normal green mesophyll protoplasts. In this process, the parental protoplasts forms the albino colony whereas the hybrid protoplast will produce either light green or green colony. This can be usually distinguished at the cultural level.

Sometimes a single recessive albino mutation as one parental line is not always sufficient to distinguish hybrid protoplasts. So morphological markers have also been used in combination with genetic complementation. For an example, when albino, *Daucus carota* protoplasts are fused with wild type of *D. capillifolius* protoplasts, then *D. capillifolius* and hybrid protoplasts are both able to regenerate green shoots which are apparently looked alike. However, origin of shoots can be

traced as the morphology of the leaves of the hybrid plant are more closely resembled with *D. carota* leaves.

#### **USE OF UNCOMMON AMINO ACIDS**

Attempts have also been made to utilize uncommon amino acids as selective agents. Conavaline which is present in some legume, inhibits division of soyabean and pea cells but sweet clover and alfalfa are unaffected. Heterokaryon obtained by the fusion of protoplast from soyabean with those from any one of the resistant plant will divide in presence of the conavaline.

#### **USE OF CELLS RESISTANCE TO AMINO ACID ANALOG**

A number of cell line resistant to amino acid analogs have been isolated and are used routinely for the selection of hybrid cells following protoplast fusion.

For an example, using cell lines resistant to 5-methyl-tryptophan (5-MT) and S-2 amino ethyl-cysteine (AEC), the interspecific hybrids of *Nicotiana sylvestris* are selected after protoplast fusion using medium containing both amino acid analogs.

In case of *Daucus*, two different cell lines have been raised for the selection of hybrid cells. A non-regenerating cell line of *D. carota* is resistant to 5-MT and azetidine 3-carboxylate (AZC), whereas a totipotent wild type line of *D. capillifolius* is sensitive to 5-MT. Hybrid colonies are selected by growth on 5-MT added medium and their ability to form plant through embryogenesis.

#### **USE OF PHYTOTOXIN**

Some of the well known fungal toxin may be used in selecting the fusion product. For an example, the protoplast of cultured soyabean cells resistant to HmT toxin produced by *Helminthosporium maydis* race T, whereas the leaf protoplasts of *Zea mays* are sensitive to this toxin. It has been observed that fusion products of soyabean and *Zea mays* survive on toxin containing

medium. On this it is suggested that toxin may be a useful selective agent in fusion experiment.

## USE OF ANTIBIOTICS

Cell lines or strains resistant to antibiotics are easy to obtain and their usefulness is being employed in hybrid selection. For instance, the drug actinomycin D has been used in the selection of somatic hybrids of two *Petunia* species. Cells from fusion products of protoplasts from *P. parodii* and *P. hybrida* can give rise to the complete plant via callus formation. The cells of *P. hybrida* fails to grow in the presence of actinomycin D. Adjustments in the medium results preferential growth of the hybrid cells and subsequent plant regeneration, whereas *P. parodii* fails to regenerate plants.

Similarly, a Kanamycin resistant cell line *Nicotiana sylvestris* has been used as a genetic marker to identify the fusion products between *N. sylvestris* and *N. knightiana*. Streptomycin resistant mutant of *N. tabacum* are also used to recover interspecific hybrids with *N. sylvestris*. Cyclohexamide resistant cell line of *Daucus carota* can be used as marker for the fusion with albino cell line of *D. carota*.

## USE OF AUXOTROPHIC MUTANT

Nutritional or auxotrophic could be the most attractive material because hybrid could be selected at the cellular level and plant regeneration would not be an essential part of this selection procedure. Auxotrophic mutants has been successfully used to isolate hybrid protoplast in *Spherocarpus donnelii*. Hybrids obtained by fusion of protoplasts from nicotinic acid and glucose requiring mutants are selected on minimal media. The regenerated hybrid plants are identified on the basis of morphology and karyotype. Nutritional mutants also have been used in somatic hybridization with *Physcomitrella petens*.

## USE OF METABOLIC MUTANT

A series of nitrate reductase deficient mutants have been obtained from mutagenized haploid cells of *Nicotiana tabacum* cultured on me-

dium containing chlorate and with amino acids as the nitrogen source. Cells with nitrate reductase convert chlorate to chlorite which is cytotoxic. The isolated mutants are unable to grow on nitrate containing medium and lack nitrate reductase and other molybdenum-protein containing enzymes. Such mutants may be suitable for hybrid selection.

Chlorophyll deficient mutants have also been selected from haploid cells of *Datura innoxia* after radiation treatment.

Metabolic mutants of *Arabidopsis* and a proline requiring mutant of corn have been reported. Threonine deaminase and nitrate reductase deficient mutants have been obtained using haploid plants of *Nicotiana plumbaginifolia*.

## USING ISOENZYME ANALYSIS

Isoenzymes are multiple molecular forms of an enzyme with similar or identical substrate specificity occurring within the same organism. Now-a-days, isoenzyme analysis has been extensively used to verify hybridity. Isoenzymes of different constitutive enzymes exhibit the unique banding pattern or zymograms in polyacrylamide gel electrophoresis. The number of band and  $R_f$  value of isoenzyme are constant and specific for each parental plant species. The summation or intermediate banding pattern of isoenzyme may be found in the hybrid callus tissue. This analysis thus help to select the hybrid cells.

## USE OF HERBICIDES

Plants possess differences in their capacity to metabolize herbicides. This property can be utilized effectively for selection. For an example, rice plants are resistant to propanil (3, 4-dichloropropionanilide). This resistance is based on the ability of rice cells to metabolize propanil.

## CHROMOSOME ANALYSIS OF HYBRID CELL

Chromosome preparation from activly growing small cell colonies derived from protoplasts and their karyotype assay clearly indicate the hybridity.

## HYBRID ISOLATION

Several selection methods, as described above, are not applicable for the selection of all types of fusion products at the cultural level. Sometimes selection is so specific for a particular intergeneric somatic hybridization. Various mutant cell lines are often used in some selection methods. But such methods are limited by the fact that mutant cell lines are not easy available in plants. Again, it has been observed that in fusion product, chromosome elimination may occur from the fused products. Therefore, use of mutant or genetic complementation may fail in attempts for the selection of hybrid produced from widely divergent sexually incompatible genera. To overcome the limitation of selection methods, recently specifically fusion products following fusion are isolated physically before culturing them in either solid or liquid medium.

Hybrid isolation methods are given below—

### MICROPIPETTE TECHNIQUE

Kao (1977) first developed this technique. By this technique, heterokaryons are isolated from the fusogen treated protoplast suspension, under a microscope using micropipette. But very few heterokaryons are obtained for a lot of time and efforts.

### DENSITY GRADIENT FRACTIONATION OF PROTOPLAST SUSPENSION AFTER FUSION

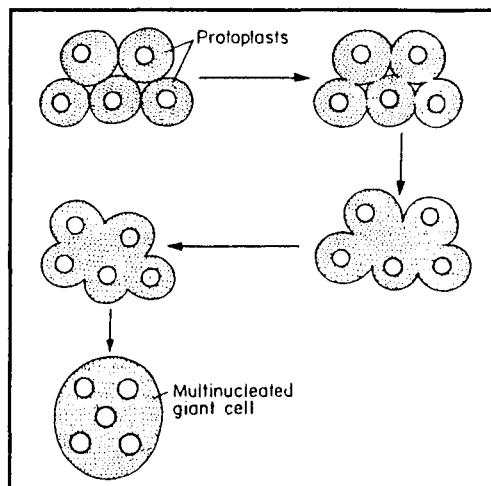
Harms and Potrykus (1978) used this technique to isolate heterokaryons from protoplasts on a large scale. Protoplast suspension after fusion is suspended in KMC solution (equal volume of 0.35M KCl, 0.245 M MgCl<sub>2</sub>, 0.254 M CaCl<sub>2</sub> pH 6.0, 660 ± 20 mOs/kg H<sub>2</sub>O) and is placed on the top of iso-osmotic KMC/sucrose-density gradients. Gradients are centrifuged at 20°C for 5 minutes at 50–100g. The fused protoplasts will form a band in intermediate density position. Heterokaryons are carefully pipetted off using Pasteur pipettes and are examined under the microscope to determine the number.

Finally, the heterokaryons are washed once with liquid culture medium before plating.

## POST FUSION EVENTS

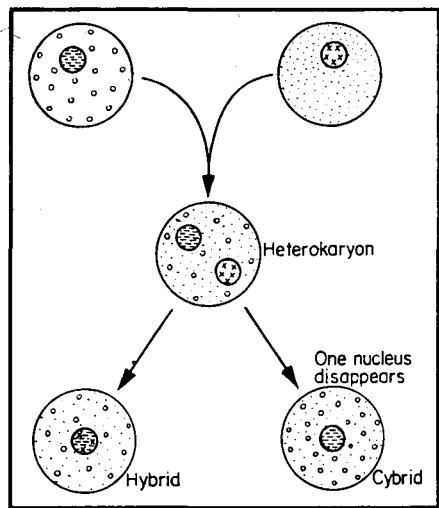
Following membrane fusion, cytoplasm and its organelles of both parental protoplasts are intermixed with each other and such mixing forms a heteroplasmic cytoplasm. It offers an opportunity of obtaining heterozygosity of extrachromosomal material. This fusion differs from a zygote in that there is no strict maternal inheritance of cytoplasmic organelles.

In fused protoplast, normally, a dikaryotic condition is established. It means that the ratio of 1 : 1 nucleus of each species occurs most frequently in heteroplasmic cytoplasm. Two types of dikaryotic condition may be observed. Few fused protoplasts may be homokaryons which result from the fusion of similar parental protoplasts, but are of little significance in somatic hybridization. Others are heterokaryons which are formed by the fusion of dissimilar parental protoplasts. Thus the protoplast population in culture is composed of a mixture of unfused parental protoplasts and fused homokaryotic and heterokaryotic protoplasts. Sometimes more than two protoplasts are involved in fusion and produces multinucleated giant cells incapable of mitosis and subsequent development (Fig 13.4).



□ Fig 13.4

Fusion of isolated protoplasts



□ Fig 13.5

#### Fusion products of two different protoplasts

Heterokaryon can produce either hybrid or cybrid cells. Only nuclear fusion takes place in case of hybrid cells. This event can be detected one day after fusion and requires several hours to complete. Nuclear fusion possibly occurs through the formation of nuclear membrane bridges. Nuclear fusion forms a syncaryon which contains a mixed chromatin. Sometimes, nuclei of the heterodikaryotic condition do not fuse to form a syncaryon and one nuclei of any one parent may be eliminated in the subsequent developmental stages. Thus a cybrid cell is produced with the nuclear genome of any one partner and the cytoplasm of both parent (Fig 13.5).

The hybrid or cybrid protoplasts regenerate a wall around them and enter the mitotic cycle.

Since diploid protoplasts are generally used for somatic hybridization, tetraploid somatic hybrid should be expected. But particularly at the wide cross level (intergeneric, interspecific), such tetraploid cell is considered as amphidiploid.

In wide crosses, a few or several of chromosomes of one parent may be eliminated during segregation. It has been found that in hybrid cell between *Glycine max* and *Nicotiana glauca* where most of the larger chromosomes of *N. glauca* are eliminated. In certain crosses some chromosomes of both parent are eliminated as in

hybrid between *Arabidopsis thaliana* and *Brassica campestris*. Difference in mitotic cycle times in species which are not compatible sexually, may result in such chromosome elimination.

A hybrid or cybrid cell undergoes mitotic divisions and ultimately forms callus tissue. Complete hybrid or cybrid plants can be regenerated from such callus tissue. But plant regeneration has to date been achieved successfully to only a small number of plant species and is mainly confined to some interspecific sexually compatible species. In most of the other cases, particularly sexually, incompatible species, reports of plant regeneration is very limited.

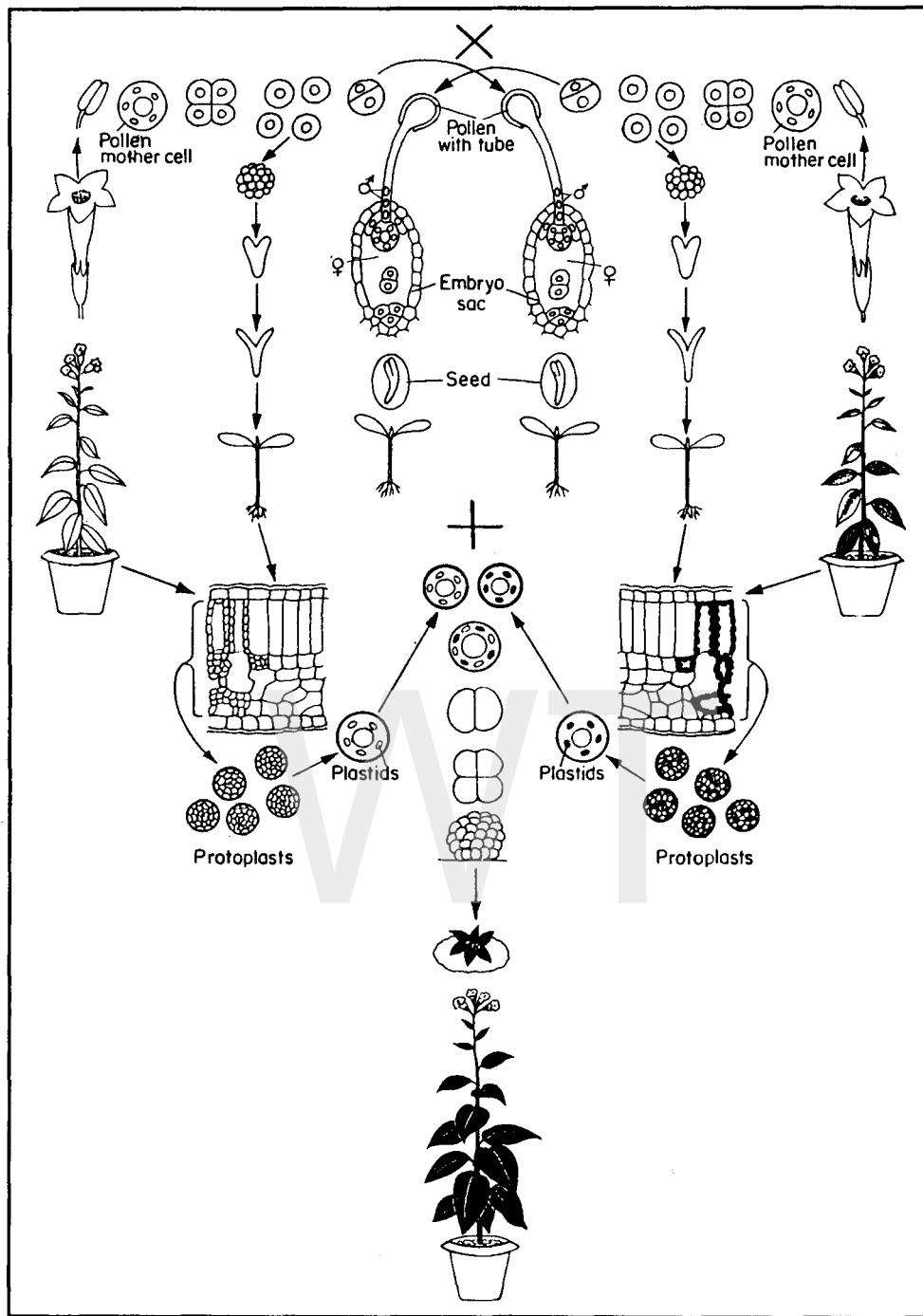
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#### IMPORTANCE OF PROTOPLAST FUSION AND SOMATIC HYBRIDIZATION

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Protoplast fusion and somatic hybridization have opened up a new avenue in plant science. It is now a well known fact that the somatic hybridization in plants can be used in the improvement of plants. One of these is the production of hybrids which is not possible through normal sexual fusion or fertilization process. In other words, it includes the formation of somatic hybrids between two species which are sexually incompatible. Thus protoplast fusion provides a method of combining the different genomes of different genera and species, with the potential of overcoming sexual incompatibility barrier between plants.

The cytoplasmic mix obtained from protoplast fusions is novel with the opportunity for the production of cybrids coupled with the opportunity for the formation of mitochondrial recombinants. Mitochondria can segregate or recombine their DNAs to form a new type of mitochondria. Chloroplasts segregate but do not appear to undergo recombination. Thus, with the production of hybrid or cybrid, the mixing of cytoplasm of both parental protoplast can improve the extranuclear genetic elements. In sexual hybridization, only maternal cytoplasm, i.e. the cytoplasm of egg cell take part in the formation of hybrid (Fig 13.6).



□ Fig 13.6

#### Diagram showing the comparison between normal sexual hybrid production and somatic hybridization

Cybrids are generally produced due to elimination of total genome of one parent after the

fusion of two protoplasts. If one parental nucleus completely disappears, the cytoplasm of the two

two parental protoplasts are still hybridized and the fusion product is known as cybrid or cytoplasmic hybrid or heteroplast. But the use of a certain compound like cytochalasin has been found to completely extrude the nucleus from the protoplast thus producing enucleate protoplasts. The fusion of the enucleate protoplast with nucleate protoplast may lead to the production of male sterile somatic cybrid where male sterility is present in the cytoplasm. In the experiment, cybrid may also arise by the following ways—

- (a) fusion between a normal nucleate protoplast and a protoplast containing a non-viable nucleus.
- (b) elimination of one of the nuclei after heterokaryon formation.
- (c) selective elimination of chromosomes at the later stage.

The formation of cybrid has some application in plant improvement programme. The importance of cybridity has been confirmed by breeding experiments. The transfer of cytoplasmic male sterile cytoplasm by protoplast fusion to somatic hybrid should be of interest to the plant breeders. This may be of critical importance in male sterility based hybrid seed production.

Chromosome elimination in fusion product can be used for gene mapping as in fusion products of animal cells.

Studies of fusion product can give information about compatibility or incompatibility of the nuclei or cytoplasm.

It has in recent years been repeatedly emphasized that plant tissue culture *per se* appears to be an unexpectedly rich source of genetic variation; this has stimulated effort to find out whether such genetic variation can be enhanced by protoplast cloning. This opportunity will undoubtedly lead to the production of new genetic variation.

By protoplast fusion, it is possible to transfer some useful genes such as disease resistance, nitrogen fixation, rapid growth rate, protein quality, frost hardiness, drought resistance etc.

from one species to another and thereby widen the genetic base for plant breeding.

Hybrid vigour is well known in sexual hybridization and it has been suggested that somatic hybridization may produce even greater vigour in hybrids. A critical evaluation of this suggestion is required since it could result in enhanced yields in many crops.

In case of vegetatively reproducing plants, the genetic variation can be induced through protoplast fusion of two species, varieties or two different genera. In case of sugarcane, which is vegetative, the production of somatic hybrids between different varieties followed by regeneration of whole plants can produce improved varieties which may be highly beneficial to the sugarcane industry. Similar advantage may be obtained in potato and other horticultural plants for their improvement.

## LIMITATION OF SOMATIC HYBRIDIZATION

It was once suggested that somatic hybrids would be of great value in crop improvement. But the experimental reports are not very encouraging. At present, techniques for selection and manipulation of somatic hybrid cells and regeneration of hybrid plants from them is limited to a few special cases where they can be manipulated very easily in culture. So far the production of somatic hybrid of agronomically important plants are not possible.

The main objective of protoplast fusion and somatic hybridization was to overcome the pre-fertilization barrier to sexual incompatibility or any genomic incompatibility. Therefore, it would be undoubtedly expected to achieve very wide crosses through protoplast fusion and it will solve many problems relating to crop improvement. But practically intergeneric crosses between widely related plants, which are not compatible sexually, are not possible.

Few interspecific somatic hybridization where plants are sexually compatible or incompatible due to natural reproductive isolation, are only achieved.

In certain wide crosses, elimination of chromosomes from the hybrid cell is another limitation of somatic hybridization. So, desirable hybrids are no longer available.

In protoplast fusion experiment, the percentage of fusion between two different parental protoplast is very low. Although some attempts has been made to increase the percentage of fused cells, still it is also a limitation of somatic hybridization.

Lastly, for hybrid identification, selection and isolation at the culture level, there is no standardized method which is applicable for all material.

## Summary

Protoplast can be isolated from almost every plant species and cultured to produce callus tissue from which a whole plant may be regenerated. If a protoplast of one species could be fused with one of a different species, a hybrid protoplast would have been produced. If this hybrid regenerated a cell wall and its two different nuclei fused together during cell division, then a hybrid cell would have been produced. If, by the techniques of micropropagation, a plant could be regenerated from this cell it would be a hybrid between two species involved, but one that was produced completely by passing sexual reproduction which is the traditional method for hybridization. The plant would be, in fact, a somatic hybrid and the technique of protoplast fusion is often referred to as somatic hybridization. This technique would be able to make successful hybridization of species that are less closely related and sexually incompatible.

Protoplast fusion is a physical phenomenon. During fusion, two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing chemicals. Spontaneous fusion takes place among the similar parental protoplasts. It is strictly intraspecific fusion and gives rise to homokaryon. Spontaneous fusion has no practical use.

Normally, isolated protoplasts from different sources do not fuse with each other because they carry negative charges outside and thus repel one another due to their same charges. So a fusion inducing chemical or agent is needed to reduce the electronegativity and allow them to fuse with each other. Sodium nitrate, polyethylene glycol (PEG), calcium ions, polyvinyl alcohol etc. are the most commonly used protoplast fusion inducing chemicals which are commonly known as chemical fusogens. Recently, mild electrical stimulation is being used to fuse protoplasts. This technique is known as electrofusion.

The exact mechanism of protoplast fusion is not fully known. The possible explanation of PEG induced fusion is that the high molecular weight polymer, PEG, acts as a molecular bridge connecting the protoplasts and  $\text{Ca}^{2+}$  ions link the negatively charged PEG and membrane surface leading to intermembrane contact and subsequent fusion. Besides this, the strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity, thus inducing fusion.

Identification of fusion product is essential to estimate the fusion frequency and to monitor the fusion products. The microscopic identification of fusion product is based on differences between the parental cell with respect to pigmentation, presence of chloroplast, nuclear staining, cytoplasmic marker etc. Non-toxic fluorochromes such as fluorescein isothiocyanate or rhodamine isothiocyanate are often used for the identification of heterokaryon or fusion products.

Some types of selection technique are also required at the level of culture to recover hybrid cells and its callus tissue following fusion. Several selection procedures such as auxin autotrophy, use of genetic complementation, use of uncommon amino acids, use of cell resistance to amino acid analog, use of phytotoxin, use of antibiotics, use of auxotrophic mutants, use of metabolic mutant, isoenzyme analysis, use of herbicides, chromosome analysis have been employed to confirm the hybridity. Micropipette technique and density gradient fractionation have also been employed to isolate only the fu-

sion products from the unfused parental protoplasts population.

Following membrane fusion, cytoplasm and its organelles of both parental protoplasts are intermixed. In this, process of dikaryotic condition is established. The fusion of nuclei in a binucleate heterokaryon results in the formation of a true hybrid protoplast. The fusion of two protoplasts from the same culture results in a homokaryon. Frequently, genetic information is lost from one of the nuclei. If one nucleus completely disappears, the cytoplasms of the two parental protoplasts are still hybridized and the fusion product is known as cybrid. Certain genetic factors are carried in the cytoplasmic inheritance system instead of in the nuclear genes. The formation of cybrids has application in a plant breeding programme.

Protoplast fusion and somatic hybridization have opened up a new avenue in plant science. Protoplast fusion provides a method of combining the different genomes of different genera and species, with the potential of overcoming sexual incompatibility barrier between plants. Besides this, studies of fusion product can give information about compatibility or incompatibility of the nuclei or cytoplasm. By protoplast fusion, it is possible to transfer some useful genes such as disease resistance, nitrogen fixation, rapid growth rate, protein quality, frost hardiness, drought resistance etc. This opportunity will undoubtedly lead to the production of new genetic variation and thereby widen the genetic base for plant breeding. In case of vegetatively reproducing plants, the genetic variation can be induced through protoplast fusion.

There are some limitations of protoplast fusion and somatic hybridization.

At present, practically intergeneric crosses between widely related plants which are not compatible sexually, are not possible. Elimination of chromosomes from the hybrid cell is another limitation of somatic hybridization. In protoplast fusion experiments, the percentage of fusion product is very low. Lastly, for hybrid identification, selection and isolation at the culture

level, there is no standardized method which is applicable for all materials.

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## Questions for Discussion

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1. What is somatic hybridization? What are the advantages of protoplast fusion over traditional methods of sexual hybridization? What are the basic criteria required for protoplast fusion?
2. Discuss the different types of protoplast fusion. What chemical compounds have been used as fusogen agents?
3. Describe the procedures of chemofusion. State the possible mechanisms of protoplast fusion.
4. What types of procedures can be used for the identification, selection and isolation of hybrid cells?
5. What is cybrid? How does this phenomenon occur, and does it have any significance in the breeding of plants?
6. Discuss the importance of protoplast fusion and somatic hybridization.
7. Write brief answers to the following questions—
  - (a) What is somato-gemetic hybridization?
  - (b) In somatic hybridization, what will be the ploidy level of hybrid cell or plant?
  - (c) What are possible explanations of chromosome elimination from the hybrid cell?
  - (d) What is the possible role of PEG as fusogen agent in protoplast fusion.
8. Write short notes—
  - (a) Protoplast fusion/cell fusion
  - (b) Somatic hybridization
  - (c) Cybrid
  - (d) Fusogen agents.

## Chapter Fourteen

### Somaclonal Variation

#### INTRODUCTION

It has been recognised that all plants regenerating from a tissue culture are not exact replicas of a parental form. Phenotypic variations are frequently observed amongst regenerated plants. For a long time, the frequently observed variability in plant populations has been ignored and normal plant regeneration from the tissue culture was the ultimate objective for plant propagation. But we know from our classical genetics that the phenotypic changes are associated with genetic changes of an organism. It is also now well established that frequently genetic changes could result from mutations, epigenetic changes or a combination of both mechanisms. The distinction between the two mechanisms is an important one, since genetic mutations are irreversible and are likely to persist in the progeny of regenerate plants whereas epigenetic changes are not transmitted by sexual reproduction. Therefore, when the cells of callus tissue with some genetic variations take part in the process of *in vitro* organogenesis, the regenerated shoots will be formed of cells containing some genetic variations. Due to presence of such cells, the regenerated plants will express some changed or new quantitative or qualitative phenotypic traits.

Thus, a population of plants which are derived from somatic cells of the explant via callus culture and show some new heritable traits, are known as somaclonal variants, and the variations displayed by such plants are known as somaclonal variations.

#### WHAT IS SOMACLONAL VARIATION ?

Plants derived from tissue culture has been variously referred to as somaclones or calliclones or protoclones and the variations displayed by such plants are simply called 'somaclonal variation'. According to Larkin and Scowcroft (1986), 'somaclonal variation is the genetic variability which is regenerated during tissue culture.' Variations for karyotype, isoenzyme characteristics and morphological variations in somaclones have been commonly observed. Such variations manifest themselves as heritable mutation and persist in the plant population even after transplantation to the field. Sometimes, phenotypic variations may arise in the progeny of plants regenerated from culture, but after the transplantation to the field, the plants exhibit the parental characteristic during their further growth and development. Such variations are not considered as somaclonal variations.

## BRIEF PAST HISTORY

**S. K. Pillai** (1969)—Observed variability in plant populations raised from tissue cultures of *Geranium*.

**D. J. Heinz and G. W. P. Mee** (1971)—Isolated morphologic, cytogenetic and enzymatic variation in *Saccharum* clone derived from callus tissue without mutagenic treatment.

**M. Krishnamurthi** (1974)—Isolated disease resistant variants of sugarcane from tissue culture.

**K. Oono** (1978)—Isolated 800 somaclones from rice callus.

**J. F. Shepard, D. Bidney and E. Shahin** (1980)—They screened about 10,000 somaclones from leaf protoplasts of potato.

**P. J. Larkin and W. R. Scowcroft** (1980)—Worked on somaclonal variation in sugarcane and first proposed a general term "Somaclonal variation" for plant variants obtained from tissue cultures.

**D. A. Evans and W. R. Sharp** (1983)—Analysed somaclonal variations which occurred in a large number of plants regenerated from leaf explants of a cultivar of a typical diploid seed propagated tomato.

## MECHANISMS CAUSING SOMACLONAL VARIATION

The somaclonal variation may be attributed to either (i) pre-existing variation in the somatic cells of the explant (genetic) or (ii) variation generated during tissue culture (epigenetic). Often both factors may contribute.

The original ploidy level of the plant or plant organ from which the explant is taken, may play an important role in somaclonal variation. Meristematic explants such as apical meristem, derived from either shoot apex or axillary bud, have a lesser degree of genetic variability as compared to plants regenerated from non-meristematic explants which generally produce genetic

variability. Cells of meristematic explant divide by normal mitosis and cells are maintained at a uniformly diploid level. However, the cells in non-meristematic explant are the derivatives of the meristematic part of the plant and during their subsequent differentiation, do not divide by normal mitosis, but undergo DNA duplication and endoreduplication. The varying degrees of endoreduplication results in the cells. Endoreduplication leads to the formation of chromosomes with four chromatids (diplochromosomes), chromosomes with eight chromatids (quadruplochromosomes) and polytene condition (polychromosomes).

When the cells of various genomic constitution of the initial explants are induced to divide in culture, the cells may exhibit changes in chromosome number such as aneuploids and polyploids, but very often from these mixoploid callus cultures. Organogenesis and/or embryogenesis occur mostly from diploid cells. Therefore, pre-existing variations in the explant tissue does not always rule out the somaclonal variation in culture. In sugarcane somaclonal variants, correlation between changes in chromosome number and certain traits such as Fiji disease resistance and morphological modification could not be found. Again normal karyotype was found amongst most of the cauliflower somaclonal variation. The presence of several chromosomal aberrations such as reciprocal translocation, deletions, inversion, chromosome breakage, reunion, multicentric, acentric fragments, heteromorphic pairing etc. were found among the somaclones of barley, ryegrass, garlic and oat. Besides these relatively large change in chromosome constitution, there are examples of phenotypic variation which can be observed in plants regenerated from cultured cells or protoplasts where no apparent chromosomal abnormalities are seen. This variation often occurs at a high frequency. The apparent non-chromosomal variation in regenerated plants has a genetic basis. There is now sufficient direct as well as circumstantial evidence to indicate that most of the variations seen in culture or in regenerants occur during the culture phase. This does not preclude pre-existing variation in the original explant as providing some of the variations, but

this seems to be a small component. Not only the source of explant, explant age are the cause of variation among the regenerants in culture, cultural environment, duration of culture, chemical additives and growth stimulants or regulators are also associated with the genetic variability of regenerated plants from tissue cultures derived from such explants. Even under most favourable conditions, mutations will occur at a low frequency in a growing culture. This variation may not only be attributed to the many varied nutrient media used and different cultural conditions adopted but also to mutagenic effects of metabolic products that accumulate in the medium. We are still largely ignorant of the fundamental cause of chromosomal mutation. Particularly, extended culture periods can result in cell lines and regenerated plants with chromosomal abnormalities. A component of the culture medium that is capable of inducing chromosomal variation could also cause nuclear gene mutation. Apart from karyotypic abnormalities, there is little hard evidence to support or favour any one of several possible mechanisms to account for somaclonal variation. Chromosomal abnormalities are part of the spectrum of somaclonal variation and they, as well as other classes of somaclonal variants, may have a more fundamental biological basis. DNA sequence amplification could be one of the mechanisms responsible for somaclonal variation. Such amplification could lead either to increased synthesis of a specific gene product or to perturbations in developmental timing of gene activity if the repeat sequences function in new chromosomal locations. Barbara McClintock postulated the existence of genetic elements which transpose from one location in the genome to another in eukaryotes. Transposition occurs in both somatic and germ lines cells. By virtue of their movement, transposable elements can inactivate structural genes, other gene regulation, possibly reactivate silenced genes and can generate duplications and deficiencies. Though not understood, genomic and developmental shock can induce the transposition of mobile elements. Concrete evidence has yet to be presented that transposition events are a cause of somaclonal variation in plants. A greater understanding of somaclonal variation

will only come from the molecular analysis of mutants. The cloning and sequencing of variant genes will provide information on changes in copy number, integrity of the structural gene and its regulator sequences.

## **ISOLATION OF SOMACLONAL VARIATION**

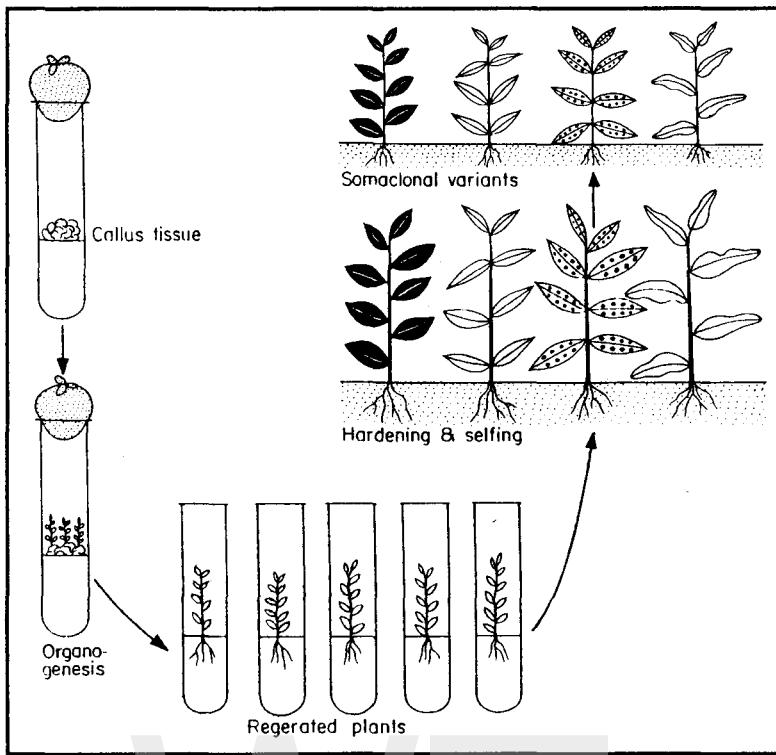
Isolation and selection of somaclonal variation is an important task. Since several changes are involved in producing somaclonal variation in different plant species, it is very difficult to sort out the somaclonal variants using a single selection system. A number of selection systems are now being used to select the variants. A brief outline of commonly used techniques for detecting variations are given—

## **ANALYSIS OF PHENOTYPIC CHARACTERS**

Phenotypic variations may arise among the regenerants during culture. Such variant characters are observed thoroughly. A detailed report on observed characters are to be prepared for each variant plants at the active level. After that, variants are transferred from culture flask to the field. In the field, such variants plants are observed during their successive growth and development. Such qualitative and quantitative phenotypic characters viz., plant height, maturity date, leaf size, flowering date, yield, seed fertility, waxiness in different plant parts, flower morphology etc. are used as a parameter to sort out the variants. Variants are also compared thoroughly with parental plants in all possible qualitative and quantitative phenotypic characters. Several consecutive seed generations of the variants are analysed to pursue whether the variant characters persist or not among the progeny (Fig 14.1).

## **CYTOTOLOGICAL STUDY OF THE VARIANTS**

The traditional methods of acetocarmine and Feulgen-stained squashes of meristematic tissue (root tip, leaf tip cells) of the variants permit the study of the number and gross morphology of chromosomes. So any change in chromosome number or gross structures of chromosomes



□ Fig 14.1

#### Steps involved in the induction and selection of somaclonal variation

can be detected by this method. To have a better assessment of minor structural changes of chromosomes, banding techniques can be used.

#### DNA CONTENT OF THE VARIANTS

DNA content of the Feulgen stained interphase nuclei can be measured by cytophotometer. An uniformly diploid state of cells always maintains its fixed amount of DNA. Any numerical changes of chromosome will show either higher or lower values of DNA content. So the measurement of DNA content can be used as a parameter for rapid screening of variants.

#### GEL ELECTROPHORESIS OF PROTEINS OR ENZYMES

A somaclone could be variant for a number of biochemical characters. Among them gel electrophoresis of the proteins or enzyme extract from the homogenised plant parts is a reliable

parameter for detecting the variants. Any alteration in electrophoretic pattern of proteins or enzymes indicates that the variants have lost or gained some specific proteins or enzyme fractions. The relative concentration of particular proteins or enzymes may alter relative to the parental proteins or enzymes. Assay of other biochemical products like pigments, alkaloids, amino acids etc. using the sophisticated instruments have also revealed the extent of variations among the regenerants.

#### SELECTION FOR DISEASE RESISTANCE

Sometimes, disease resistance character may appear among the somaclonal variants where the parent is highly susceptible to a particular disease. The pathogen or its toxin can be used as a selection agent during culture. If the *in vitro* selection is not feasible on cell, tissue or protoplast culture level, screening at the seedling

level is frequently possible. Behnke (1979) regenerated potato plants from callus selected for resistance to the toxin culture filtrate of *Phytophthora infestans*. Field resistance of some of the sugarcane variants has also been established.

## SELECTION FOR HERBICIDE RESISTANCE

Selection for herbicide resistant phenotypes among the somaclonal variants has also been made. The herbicide is generally added to the cell culture system and the regenerated plantlets showing the tolerance to herbicide are selected. Chaleff (1980) has reported the selection of several *Nicotiana tabacum* mutants with increased tolerance to picloram.

## SELECTION FOR ENVIRONMENTAL STRESS TOLERANCE

Salt, water-logging and drought, low and high temperatures and mineral toxicity and deficiency are frequently cited as environmental stresses. Many attempts have been made to isolate stress-tolerant phenotypes in tissue culture. The persistence of stress-tolerant trait in regenerated plants has also been demonstrated.

Selection of high sodium chloride tolerant cell lines in tobacco and the regeneration of plants have been reported by Nabors *et al.* (1975). Regenerated plants showed salt tolerance through two successive sexual generations. But this may be due to physiological adaptation rather than genetic modification.

Few attempts have been made to select for water-logging and drought resistance in cell culture. Handa *et al.* (1983) has reported somaclonal variation for resistance to polyethylene glycol in tomato cells.

Temperature stress is an unattractive *in vitro* selection agent. Breideubach and Waring (1977) studied the chilling response in tomato suspension culture and cell lines with enhanced chilling tolerance have been demonstrated by Dix and Street (1976) in tobacco. Wu and Wallner (1983) studied the heat response in suspension culture of pear cells in an effort to provide

a biochemical basis of *in vitro* selection for tolerance to high temperature.

Mineral toxicities and deficiencies are frequently problems in strongly acidic and strongly alkaline soils. Aluminium toxicity at low pH is one of the best known examples. Meredith (1978) reported the stable aluminium resistant variant cell lines in tomato tissue culture. Sorghum plants have also been regenerated after selection against aluminium (Smith *et al.*, 1983).

## APPLICATION OF SOMACLONAL VARIATION

In tissue culture, somaclonal variation has been presented a lot of significant contributions to plant science. Somaclonal variation among regenerated plants from callus and protoplast culture has been suggested as a useful source of potentially valuable germplasm for plant breeding and plant improvement. The major benefit of somaclonal variation is to create variation in adapted genotypes. Recognition of new genotypes at the whole plant level and their efficient exploitation would however be very useful in breeding programme. Agronomically desirable many traits in several crop plants have been raised from tissue culture. Many of these traits will find a place in new improved varieties. Some examples of somaclonal variation in crop plants as well as in some horticulturally important plants has been discussed below—

**Rice**—The occurrence of somaclonal variation in rice (*Oryza sativa*) had been independently reported several times. Numerous variations have been observed in somaclones derived from an assumed homozygous parent; a dihaploid derivative (a self-doubled haploid or homozygous diploid) from anther culture. About 1,121 somaclones, selected progeny were examined immediately in three successive selfed generations and these segregated for characters such as plant height, maturity, heading date and grain yield. Within two generations of selfing, many of the variants had become true breeding. Based on progeny analysis of five characters, 72% of the regenerants differed from the parent in at least one character; 28% differed for two or more traits. Schaeffer *et al.* (1984) found significant

variation among anther-derived dihaploid of rice. Significant improvements relative to the parent were observed for seed weight, seed proteins percentage, tiller number, panicle length and time of flowering. At the International Rice Research Institute, recent research has involved analysis of somaclonal variation in several rice cultivars. Mutants were observed for many characters such as panicle, grain and leaf morphology and tiller arrangement. Several kinds of chlorophyll-defective mutants were also observed. The progeny have also been screened for tolerance to salinity and aluminium toxicity.

**Wheat**—It has recently become possible to regenerate variant plants from tissue cultures of wheat. Variations were manifested for both morphological characters and for traits under simple genetic control such as gliadin proteins in seed, grain colour etc. and polygenic control such as plant height, heading date and yield. Both heterozygous and homozygous mutants were screened in the primary regenerants of wheat.

**Maize**—The classical genetic, chromosomal and recently molecular research in maize has enabled a penetrating analysis of somaclonal variation. In one analysis of 77 somaclones regenerated from maize tissue cultures, 17 defective endosperm or seedling mutants were identified. In another analysis of 51 somaclones, eight segregated for recessive kernel mutations and one segregated for a mutation which caused premature wilting. Maize studies have also provided conclusive evidence that the mitochondrial genome can undergo genetic changes during cell culture. Cytoplasmic male sterile line of maize is very sensitive to T-toxin produced by the causal organism of southern corn leaf blight, *Drechslera maydis*. Normal cytoplasm plants are male sterile and resistant to the T-toxin. The toxin has been used as a selective agent in tissue culture of T-cytoplasm maize lines. Plants regenerated from the selected cell lines were resistant both to the toxin and to infection by *D. maydis*. Toxin resistant, male fertile plants were regenerated from Cms - T maize culture grown without exposure to toxin. The conversion during tissue culture to toxin resistance, male fertility was maternally inherited and shown to be associated with the mitochondria.

**Potato**—For the improvement of potato crop Shepard et al. (1980) suggested that it will be more profitable to improve a popular variety selectively rather than to create a new one.

The potato somaclones were also screened for both late and early blight resistance. The parent 'Russet Burbank' is highly susceptible to both these diseases. From among more than 800 plants, a range of variation to late blight (*Phytophthora infestans*) was found. About 2% of the somaclones were also able to transmit the disease resistant character through subsequent tuber generations. In addition, several other disease resistant variants were recovered. Such variants are resistant to early blight (*Alternaria solani*) and to multiple races of *Phytophthora infestans*.

**Tomato**—A large number of somaclonal variations have been raised from leaf derived callus tissue of tomato. Variants were screened for a number of characters such as male sterility, jointless pedicel, fruit colour, indeterminate growth etc.

**Oat**—Many somaclonal variations have been observed among plants regenerated from cultured immature embryo, apical meristem of oat. Variants were selected for plant height, heading date, leaf striping, awns, etc.

**Brassica spp.**—The occurrence of somaclonal variation in *Brassica* spp. has been independently reported several times. Variants were found which affected flowering time, growth habit, waxiness glucosinolates, *Phoma lingam* tolerance.

**Nicotiana sp.**—In *Nicotiana* sp., plant regeneration was possible from anther culture, protoplast culture and leaf callus culture. From regenerated plant population, somaclonal variants were selected for a number of characters such as plant height, leaf size, yield grade index, alkaloids, reducing sugars, specific leaf chlorophyll loci etc.

**Lolium**—A triploid hybrid was obtained by crossing a diploid *Lolium perenne* with a tetraploid *L. multiflorum*. Callus tissue derived from this hybrid produced more than 2,000

plants in five years. These somaclones exhibited a wide variation in leaf shape, size, floral development, growth vigour and longevity. Some variants possessed characteristics of both the parents which were agronomically valuable and their progenies also showed the same variations, whereas these characteristics were not observed in triploid hybrids reared up in a conventional way.

**Pelargonium**—A high degree of variability in tissue culture regenerated plants from 5 cultivars of *Pelargonium zonale* was observed. Changes were found in plant and organ size, leaf and flower morphology, essential oil constituents, fasciation, pubescence and anthocyanin pigmentation. One of the variants had been released, as a new cultivar known as 'Velvet Rose'.

**Geranium**—Tokumasu and Kato (1976) using somatic callus cultures of a dihaploid *Geranium* plant had obtained homogenous plants amongst which two were variants for essential oil constituents.

The potentiality of somaclonal variation has increased dramatically with clear evidence of expression of selected traits in selected plants and seed progeny. Among the economically important traits which have been selected *in vitro* and recognized as having potential impact are now being employed to enhance the efficiency of plant breeding. Somaclonal variations provide also a powerful option for plant improvement and this may be the best approach of plant improvement over conventional methods.

## Summary

It has been recognised that all plants regenerated from a tissue culture are not exact replicas of a parental form and the variations displayed by such plants are simply called somaclonal variation. P. J. Larkin and W. R. Scowcroft (1980) first proposed the term somaclonal variation and according to them, 'Somaclonal variation is the genetic variability which is regenerated during tissue culture'. Sometimes, phenotypic variations may arise in the progeny of plants regenerated from culture, but after the transplantation

to the field, the plants exhibit the parental characteristics during their further growth and development. Such variations are not considered as somaclonal variation.

There are several possible mechanisms causing somaclonal variation. The mechanisms are—(i) Chromosomal aberrations (ii) DNA amplification and (iii) transposable elements.

Chromosomal aberrations may be attributed to either (a) pre-existing variation in the somatic cells of the explant, i.e. genetic cause or (ii) variation generated during tissue culture or epigenetic cause. Often, both factors may contribute. Chromosomal abnormalities are the part of the spectrum of somaclonal variation and they, as well as other classes of somaclonal variants, may have a more fundamental biological basis. DNA sequence amplification could be one of the mechanisms responsible for somaclonal variation.

Such amplification could lead either to increased synthesis of a specific gene product or perturbations in developmental timing of gene activity if the repeat sequences function in new chromosomal location. Transposable elements occur both in somatic and germ lines cells. By virtue of their movement, transposable elements can inactivate structural genes other gene regulation, possibly reactivate silenced genes and can generate duplications and deficiencies. A greater understanding of somaclonal variation will come only from the molecular analysis of mutants. The cloning or sequencing of variant genes will provide information on changes in copy number, integrity of the structural gene and its regulator sequences.

Isolation and selection of somaclonal variation is an important task. A number of selection systems are now being used to select the variants such as—(1) analysis of phenotypic characters, (2) Cytological study of the variants, (3) DNA content of variants, (4) gel electrophoresis of proteins or enzymes, (5) selection for disease resistance, (6) selection for herbicide resistance and (7) selection for environmental stress tolerance etc.

Somaclonal variation among regenerated plants from callus and protoplast culture has been suggested as a useful source of potentially valuable germplasm for plant breeding and plant improvement. The major benefit of somaclonal variation is to create variation in adapted genotype without the treatment of mutagenic substances. Agronomically desirable many traits in several crop plants such as rice, wheat, maize, potato, tomato etc. have been raised from tissue culture. A large number of somaclonal variation of some horticulturally important plants namely *Lolium*, *Pelargonium*, *Geranium* etc. have also been raised from tissue culture.

## Questions for Discussion

1. What is somaclonal variation? Discuss the possible mechanisms causing somaclonal variation.
2. How do you isolate and select the somaclonal variants from regenerated plants?

3. Discuss the application of somaclonal variation.
4. Write brief answer to the following questions—
  - (a) What are the differences between genetic change and epigenetic change?
  - (b) What is the scope of somaclonal variation in plant breeding?
  - (c) What is agronomic usefulness of somaclonal variation?
5. Write notes on—
  - (a) Somaclonal variation.
  - (b) Role of DNA amplification and transposable elements to account for somaclonal variation.
  - (c) Difference between genetic mutation and somaclonal variation.
  - (d) Selection of somaclonal variation.

## Chapter Fifteen

### Plant Tissue Culture in Forestry

#### INTRODUCTION

Forest is an important renewable natural resources for man, because it provides several forest products like fuel, timber, lumber, paper, fodder etc. Forest has also other uses such as recreation, wildlife habitat, air and water sheds. From ecological viewpoint, forest regulates the level of rainfall necessary for the existence of vegetation on earth. It also helps in recycling moisture. Forest checks flood, drought and soil erosion. But the increasing world population requires more food, fuel and space for habitation. Hence indiscriminate deforestation by man for his own interest i.e. for agriculture, construction and habitation purposes ultimately reduces the forest covered area on earth. It is feared that in the coming decades, there is going to be an acute shortage of forest products and the ecological balance will be lost. So deforestation should be stopped. One method of avoiding this situation is aforestation with superior genotype and the conservation of forest by adopting some scientific and technical measures. The most common traditional measure is the rapid propagation and plantation of forest trees. Many forest trees are propagated vegetatively by cuttings, graftings, layerings etc.

These methods produce the plant genetically alike to the parent plants, but there are some difficulties. Only a small number of plants can be produced by this way and it takes years to build up enough stock for planting in fields or forests. Somtimes the method proves to be impossible. The long life cycle of trees also makes development of superior varieties a very lengthy and tedious process. Traditional methods of tree breeding and improvement, particularly in the area of genotype evolution with respect to growth rates, cold hardiness, disease resistance, tolerance to draught or chemicals may take many years to produce a desired hybrid and sometimes it is not easy to raise such improved tree hybrid.

Again there are some problems to propagate forest trees from seeds. Even storage of seeds for longer period is not feasible to preserve the germplasm.

In recent years, another technique called plant tissue culture has been developed which could be an answer to problems faced by the traditional procedures mentioned above. Plant tissue culture has a great potential to improve traditional methods of tree breeding. This technique may dramatically reduce the time to produce new varieties. Plant tissue culture is very

useful for mass clonal propagation of forest trees. *In vitro* the rate of multiplication cannot be expected by any of the *in vivo* methods of clonal propagation. Multiplication cycle is very short. In tissue culture, plant multiplication can continue throughout the year irrespective of the season. It is also feasible to preserve just the germplasm in a deep-freeze as mass of the cells and later grow a complete plant in tissue culture. So million of potential forest trees could be stored in a few test tube.

### **SCOPE OF TISSUE CULTURE IN FORESTRY**

Some aspects of plant tissue culture have already been employed in forestry such as—

1. Micropropagation
2. Apical meristem culture
3. Embryo culture
4. Protoplast and somatic hybridization
5. Isolation of haploidy.

Micropropagation is employed for the production of rapid clonal propagation of selected plant species. Micropropagation generally involves several steps—

- (a) The mother plant is selected for yield and quality
- (b) the growing tissue (e.g. vegetative buds, nodal cuttings, petioles etc.) is dissected
- (c) Surface sterilization of tissue
- (d) Inoculation of tissue on a defined medium
- (e) Resulting shoots are multiplied by subculture in fresh medium the operation being repeated many times
- (f) Shoots are then rooted
- (g) After development of root system plants are transferred to small pot and to the field.

By this technique a millionfold annual increase in the rate of artificial plant propagation over conventional methods of vegetative propagation is possible. This technique has now been exploited in accelerating tree improvement programmes for forest trees.

Apical meristem culture is very useful for the production of virus-free plants. Generally the apical meristem is free from virus infection. Plants obtained from such meristem through tissue culture are usually free of viruses. This technique has now been commercialized for production of virus free forest trees.

One of the procedures commonly employed in plant breeding programmes for improvement of trees is by hybridization which in many cases involves wide crosses between plants belonging to different varieties or species. However, due to several reasons, embryos abort at an early stage. The use of embryo culture can overcome post-pollination incompatibility to enable rescue of interspecific hybrids and allow the genetic base of tree species to be significantly broadened. There are some reports on breeding of forest tree hybrids by embryo culture.

Protoplasts are the naked plant cells (cells without cell wall) and plantlets can be regenerated from a protoplast. Somatic hybridization is a technique by which somatic or gametic cells of different plants are fused and plantlets raised from fused protoplasts. Somatic hybridization also provides a mechanism to broaden the germplasm base. Protoplast fusion enables the reciprocal exchange of cytoplasmic organelles and possible genetic recombination between genetically dissimilar mitochondria or chloroplast genomes. Though extensive studies are in progress few works have been done on isolation and regeneration of protoplasts from forest trees.

Plants can also be raised from isolated microspore or from anthers by tissue culture. This technique has enhanced the capacity to generate large number of haploid plants. Haploids enable the achievement of rapid homozygosity, enhanced selection efficiency for recessive genes and breeding at a diallelic stage for autopolyploid species. This technique appears to have the best prospect for utilization in forest tree improvement plan in the near future.

## BRIEF PAST HISTORY

**R. J. Gautheret** (1934)—First cultured cambium tissue of some tree species (*Salix caprea*, *Populus nigra*) on Knop's solution containing glucose and cysteine hydrochloride and noted that the tissues proliferated for a few months. The addition of B-vitamins and IAA enhanced considerably the growth of *Salix* cambium.

**R. J. Gautheret** (1940)—First reported the organogenesis in cambial tissue of *Ulmus campestris* in culture. He obtained buds *in vitro* from cambial tissue.

**E. A. Ball** (1950)—First obtained buds from callus tissue of *Sequoia sempervirens* (a gymnosperm) having greater life span.

**E. C. Stone and J. W. Duffield** (1950)—Have described a case of breeding hybrid seedling from *Pinus lambertiana* × *P. armandi* and *P. lambertiana* × *P. koraiensis* crosses by planting embryos encased in the gametophytic tissue on agar slant. As both *P. armandi* and *P. koraiensis* are blister rust resistant, these attempts constitute a significant achievement towards breeding a disease-resistant line.

**P. G. Haddock** (1954)—Reported a successful rearing of excised embryos of *Pinus lambertiana* completely free of megagametophyte by tissue culture.

**L. L. Winton** (1968)—First reported the regeneration of true triploid plant from the callus tissue of *Populus tremuloide*.

**H. E. Sommer** (1975)—The first gymnosperm plantlets were obtained by Sommer from cultured excised cotyledons and hypocotyls of *Pinus palustris*.

**W. J. Kaiser and L. R. Teemba** (1979)—Reported the virus free plant from apical meristem culture of Cassava (*Manihot esculenta*).

**E. A. Shahim and J. F. Shepard** (1980)—They reported mesophyll protoplast isolation, proliferation and shoot formation from Cassava (*Manihot esculenta*).

**P. K. Gupta, A. F. Mascarenhas, A. L. Nadgir and V. Jagannathan** (1980, 1981)—Obtained the clonal propagation of *Tectona grandis* and *Eucalyptus citriodora*.

**C. H. Chen** (1982)—Obtained haploid plants from anther culture of rubber tree.

## APPLICATION OF PLANT TISSUE CULTURE IN FORESTRY

Plant tissue culture technique can be used to produce, maintain, multiply and transport pathogen free forest plants safely and economically.

### MICROPROPAGATION

Efficient methods for micropropagation has been used for many trees (Fig 15.1). So far much success has been achieved in micropropagating some tree plants which are difficult to clonally multiply by the conventional methods. Success of micropropagation is judged on the basis of rate of shoot multiplication and the success of transplantation to the field. A list of tree plants which have been micropropagated *in vitro*, is given in table 15.1.

The advantage of micropropagation is that the shoot multiplication cycle is very short i.e. within 2–6 weeks, each cycle resulting in a logarithmic increase in the number of shoots. AL AFOCEL (France) micropropagation of *Sequoia sempervirens* are regularly applied for the production of 30,000 plantlets from approximately 200 selected clones. Similarly, a small production laboratory has been built at the Forest Research Institute at Rotorua (New Zealand) where tissue culture techniques are applied to produce 100,000 radiata pine plantlets annually.

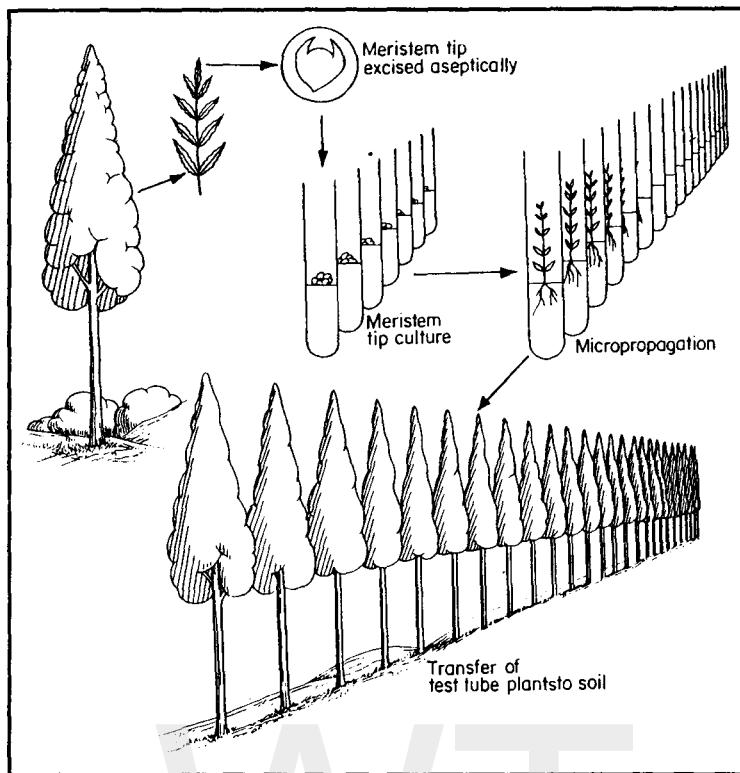
Teak is one of the most important timber trees of India and in neighbouring countries. The wood of teak is highly superior for its strength, durability and insect resistance. Similarly *Eucalyptus citriodora* is commercially valued for its wood and the essential oil of its leaves. The main component of oil is citronella. Suitable methods

Common Name	Scientific Name	Rate of shoot multiplication
Birch	<i>Betula pendula</i>	not known exactly
Birch	<i>B. papyrifera</i>	15-30 fold in 6 weeks
Birch	<i>B. platyphylla</i>	15-30 fold in 4 weeks
Birch	<i>B. schezuanica</i>	15-30 fold in 4 weeks
	<i>Rhododendron sp.</i>	3.2 fold in 5 weeks
	<i>Rhododendron simsii</i>	not known exactly
	<i>Rhododendron sp.</i> (Hybrid Azalea)	8 fold in 100 weeks
Feijoa	<i>Feijoa selloiana</i>	3 fold in 4 weeks
Date palm	<i>Phoenix dactylifera</i>	not known exactly
Cassava	<i>Manihot utilissima</i>	not known exactly
Paper Mulberry	<i>Bronssonetta kazinoki</i>	not known exactly
Chestnut	<i>Castanea sativa</i>	not known exactly
Shisham	<i>Dalbergia sissoo</i>	4 fold in 5 weeks
Citron-scented gum	<i>Eucalyptus citriodora</i>	5-8 fold in 2 weeks
Flooded gum	<i>E. grandis</i>	not exactly known
Mountain Ash	<i>E. reganans</i>	not exactly known
Silver Poplar	<i>Populus alba</i>	10 fold in 4 weeks
Poplar	<i>P. euroamericana</i>	not exactly known
Poplar	<i>P. nigra</i>	$10^6$ fold in 52 weeks
European aspen	<i>P. tremula</i> <i>(P. deltoides and P. nigra)</i>	10 fold in 4 weeks
American aspen	<i>P. tremuloides</i>	$10^6$ fold in weeks
	<i>P. vunnanensis</i>	not exactly known
	<i>Paulownia tomentosa</i>	not exactly known
Sandal wood	<i>Santalum album</i>	not exactly known
Hybrid willow	<i>Salix matsudana</i>	4 fold in 4 weeks
Teak	<i>Tectona grandis</i>	2-3 fold in 2-3 weeks.

are not available for vegetative propagation of such elite trees by cutting. Micropropagation of such plants have been developed by which about 500 plants of teak and 100,000 plants of *Eucalyptus citriodora* can be obtained from a single bud in a year. This preliminary experiment clearly demonstrates vast potentials of micropropagation techniques for clonally multiplying elite trees. The production of vast quantity of plant obtained through micropropagation will fulfil the requirements of reforestation and in future provide the timber, oil, fuelwood and the raw materials for paper industry.

Micropropagation has got some disadvantages such as—

1. In some cases multiple shoot formation takes place as usual. But the rooting of such shoot is very difficult. It depends upon so many factors viz. correct choice of explant, age of the plant, culture medium, hormones etc. As for example, in *Feijoa* terminal and subterminal cuttings taken from 4 year old plant developed multiple shoots *in vitro* but rooting takes place rarely. On the other hand, nodal cutting of the same plant developed multiple shoots *in vitro* and gave 100% rooting under non-sterile conditions.
2. Browning of medium and browning of explant in culture is a serious problem for the micropropagation of tree plant. Browning



□ Fig 15.1

#### Flow diagram showing the technique of meristem tip culture for rapid propagation of forest plant

takes place due to oxidation of phenolic substances that come out from the cut surface of the explant which turns the medium dark brown and is toxic to the explant. It inhibits the growth of explant in culture and sometimes the tissue dies. Several methods have currently been adopted to reduce the browning problems.

#### APICAL MERISTEM CULTURE

*Manihot esculenta*, commonly known as Cassava, is systematically infected with one or two viruses such as African Cassava Mosaic virus, Cassava Brown Streak virus. Virus attack does not always lead to death of the plant but it can reduce the yield and quality of the plant. Eradication of viruses is highly desirable to optimize the yield. Apical meristem culture is widely used to eliminate virus and to raise a number of virus free plants using a single meristem tip. In

Cassava, microscopic size of apical meristem ( $200\text{--}500\ \mu\text{m}$ ) has been cultured in nutrient medium supplemented with  $\text{GA}_3$ , NAA and BAP. By this technique, a number of virus free plants have been raised. This experiment demonstrates the great potentials of apical meristem culture for making disease free tree plants.

#### EMBRYO CULTURE

Embryo culture is generally applied for obtaining rare hybrids. Conventional interspecific or intergeneric breeding of forest plants has been done for improvement of timber quality, oil content etc. and also for making disease resistant line. Sometimes fertilization occurs but germinable seeds are not obtained due to premature death of hybrid embryo. If the hybrid embryos are cultured before the onset of abortion, it is easy to get a hybrid plant. Some *Pinus* sp. e.g. *P. lambertiana* is very susceptible to blis-

rust disease whereas *P. armandi* and *P. koraiensis* are resistant to such disease. An attempt has been made to cross *P. lambertina* × *P. armandi* and *P. lambertiana* × *P. koraiensis* to bring the disease resistant character in hybrid. In both cases, fertilization occurs, but seeds are not germinable. In such cases by successful rearing of excised premature hybrid embryos through tissue culture it is possible to grow a hybrid disease resistant *Pinus* plant.

### ENDOSPERM CULTURE

It is well known that endosperm tissue of the seed of angiosperm is cytologically triploid in nature. Now, if the endosperm tissue is used as explant for tissue culture to raise a plant, the plant will be triploid. Triploid plants are seed sterile and usually undesirable. But sometimes triploids are desirable for plant improvement. Quaking aspen (*Populus tremuloides*) is an important pulpwood yielding plant. It has been seen that triploid plants raised through endosperm culture have better pulpwood qualities as compared to their diploid plants obtained from seeds. Again, seed sterility of the triploid plant is not a serious setback because seedless triploid quaking aspen plant can be propagated vegetatively. So it is commercially very useful.

### HAPLOID PLANTS

Haploid plants are obtained by isolated microspore and anther culture. Haploids are very important for mutation research on forest tree. Mutant varieties can be achieved very easily from homozygous diploid plants by treatment of mutagenic agent. *Hevea brasiliensis* is a rubber yielding plant. In China, haploid plants of *Hevea brasiliensis* have been obtained from microspores in culture.

### Conclusion

It is well known that our underground fossil fuel is limited and unrenewable. So forest products are the only alternative way to meet our fuel requirement. At the same time, deforestation is continuously destructing our ecological balance

and causes natural calamities in every year. So major efforts should be given to improve the field performance of tissue culture derived plantlets for rapid clonal propagation so that the cost and benefit become easily accessible for commercial purposes. On the other hand, the future of forest tree tissue culture does not stop at merely producing clonal plants. Continuing progress in the field of genetic engineering should soon enable scientists to carry out breeding within the test tube. New genetic information could be introduced directly into cells by using protoplasts or cells of different chosen strains.

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### Summary

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Forest is an important renewable natural resource for man because it provides several forest products like fuel, timber, lumber, paper, fodder etc. But with the spread of industrial civilisation and the rapid growth of population, unhappily, forests tend to disappear. This should be immediately stopped on one hand and, on the other hand, aforestation and the conservation of forest should be done by adopting some scientific and technical measures. The most common traditional measure is the rapid propagation and scientific plantation of forest trees. But there are some problems to propagate forest trees by the conventional methods. To overcome such problems, plant tissue culture technique has been employed and exploited for rapid multiplication of forest trees within very short time and to produce new varieties. By this technique, millions of potential forest trees could be stored in a few test tubes.

Some aspects of plant tissue culture have already been employed in forestry such as—(1) micropropagation, (2) apical meristem culture, (3) embryo culture, (4) protoplast and somatic hybridization and (5) isolation of haploidy.

Micropropagation is employed for the production of rapid clonal propagation of selected plant species. Apical meristem culture is very useful for the production of virus-free plants.

The use of embryo culture can overcome post-pollination incompatibility to enable rescue of interspecific hybrids and allow the genetic base of tree species to be significantly broadened. Somatic hybridization by protoplast fusion also provides a mechanism to broaden the germplasm base of tree plants. Plants can also be raised from isolated microspores or from anther culture. This technique has enhanced the capacity to generate large number of haploid plants. Haploids enable the achievement of rapid homozygosity, enhanced selection efficiency for recessive genes and breeding at a diallelic state for autopolyploid species.

Plant tissue culture technique can be used to produce, maintain, multiply and transport of pathogen free forest plants safely and economically.

The advantage of micropropagation of tree plants is that the shoot multiplication cycle is very short, each cycle resulting in a logarithmic increase in the number of shoots. For an example, micropropagation of teak and *Eucalyptus* plant have been developed by which about 500 plants of teak and 100,000 plants of *Eucalyptus citriodora* can be obtained from a single bud in a year. This preliminary experiment clearly demonstrates vast potentials of micropropagation technique for clonally multiplying elite trees. Micropropagation has some disadvantages such as 1) the rooting of multiplied shoots is sometimes very difficult, 2) browning of explant due to secretion of phenolic substances in culture is a serious problem. In Cassava (*Manihot esculenta*) apical meristem has been cultured and a number of virus-free plants have been raised.

Embryo culture is generally applied for obtaining rare hybrids. Some *Pinus* sp. e.g. *P. lambertiana* is very susceptible to blister rust disease whereas *P. armandi* and *P. koraiensis* are resistant to such disease. An attempt has been made to cross *P. lambertiana* × *P. armandi* and *P. lambertiana* × *P. koraiensis* to bring the disease resistant character in hybrid. In both cases, fertilization occurs, but seeds are not germinable. By means of embryo culture, it is possible to raise a hybrid disease resistant *Pinus* plant.

Triploid plants of *Populus tremuloides* has been raised through endosperm culture. The quality of pulpwood of the triploid plants is better than the pulpwood obtained from diploid plant.

*Hevea brasiliensis* is a rubber yielding plant. In China, haploid plants of *Hevea brasiliensis* have been obtained from microspores in culture.

It is well known that our underground fossil fuel is limited and unrenewable. So forest products are the only alternative way to meet our fuel requirement. So major efforts should be given to improve the field performance of tissue culture derived plantlets for rapid clonal propagation.

## Questions for Discussion

1. How far have tissue culture techniques been employed for the rapid propagation and improvement of forest tree?
2. Discuss the application of different techniques of plant tissue culture in forestry.
3. Write brief answer to the following questions—
  - (a) What are the disadvantages of micropropagation of forest plants?
  - (b) How could the technique of embryo culture be utilised for the improvement of tree plants?
  - (c) How far virus-free trees are raised in tissue culture?
4. Write short notes on—
  - (a) Scope of tissue culture in forestry.
  - (b) Advantage of micropropagation of forest plant.
  - (c) Endosperm culture of forest plant.
  - (d) Possible role of protoplast culture in forestry.

## Chapter Sixteen

# Applications and Importance of Plant Cell and Tissue Culture in Plant Science

### INTRODUCTION

The importance and application of plant cell and tissue culture in plant science are vast and varied. The last few years of research into plant cell, tissue and organ culture have seen the emergence of technology from technique. So now plant tissue culture has been included as an important tool under biotechnology. The establishment of micropropagation for rapid propagation, the use of shoot-tip culture to produce nuclear stock free from parasites especially viruses and the application of a variety of procedures including anther and pollen culture to speed up the process of producing better varieties, protoplast culture for hybrid plant production, and genetic manipulation have all contributed to the acceptance of plant tissue culture as valuable tool for plant improvement. Nevertheless, there is now considerable commercial interest in the exploitation of this new technology.

The principal applications of plant tissue culture are now discussed under the following sub-headings—

### MICROPROPAGATION

The regeneration of whole plant through tissue culture is popularly called "micropropagation". This is a technique where a callus mass has been initiated from a single explant taken from any living part of a donor plant and within very short time and space, a large number of plantlets can be produced from such callus tissue. Again, it is possible to make a large number of callus pieces from the original stock culture during subculturing. Then it is possible to produce hundreds of plantlets that develop on each of these callus pieces. Therefore, the most obvious advantage of micropropagation is the numerical one. As discussed previously, suspension cultures can also be used to exploit this numerical advantage as they produce numerous cell aggregates relatively rapidly, generally growing faster than callus tissues. The numbers of plantlet production depends upon the number of shoot primordia that can be induced to form within these cell aggregates. Alternatively, if the cell suspension culture happens to be embryogenic, then

this propagation potential depends upon the rate at which embryoids are formed by the cell aggregates and the rate at which new embryogenic aggregates are formed in culture. Again, the shoot tip and nodes of the regenerated plantlets derived from callus culture and cell suspension culture can be multiplied further following organ culture method. As a result, large numbers of a plant species or variety can be propagated all the year around. The plant breeder or grower is no longer restricted by season in the production of large numbers of plants.

## CLONAL PROPAGATION

*In vitro* clonal propagation is a type of micropropagation. The cultured plants raised from tissue culture are derived asexually and also multiply within culture vessel by asexual means. A sexual reproduction, on the otherhand, gives rise to plants which are genetically identical to the parent plant. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation and a plant population derived from a single donor plant in tissue culture constitutes a clone. So, the variability that can arise from sexual reproduction and seed formation in a crop plant is omitted. More specifically, a single plant with desirable characters can be selected from a breeding programme and propagated so that further trials and selections can be carried out as quickly as possible. The plants with long seed dormancy can be raised faster by *in vitro* clonal propagation than *in vivo* seed propagation. The undesirable juvenile phase associated with seed raised plants in some variety does not appear in the vegetatively propagated plants from adult material. For the orchids, *in vitro* clonal propagation is the only commercially viable method of micropropagation. Clonal multiplication of cultivar is very important in horticulture and silviculture.

## PRODUCTION OF GENETICALLY VARIABLE PLANTS

In some callus culture, there is a major tendency of the callus tissue towards the numerical

variation of chromosomes in the cells that occurs after a number of serial subcultures. Such chromosomal variations in culture may arise because of two factors. First, the cells of various ploidy and genetic constitution of the initial explant may be induced to divide and secondly, culture condition may contribute new irregularities. The chromosomal instability in the cultured cells play an important role in polyploidization of cells and genetically variable plants can be raised from such polyploidized cells by subsequent micropropagation. Thus, tissue culture is proving to be rich and novel sources of variability with a great potential in crop improvement without resorting to mutation or hybridization. Variants selected through tissue culture has been variously termed to as calliclones (from callus culture) or protoclones (from protoplast culture). Larkin and Scowcroft have proposed a general term "somaclones" for plant variants achieved from tissue cultures, irrespective of their origin. Such variant plants may show some useful characters such as resistance to a particular disease, herbicide resistance, stress tolerance etc. Such changes are valuable for crops which are normally propagated by vegetative methods. Moreover, plant breeders can exploit such variants for their breeding programme.

## PLANT PATHOLOGY AND PLANT TISSUE CULTURE

There have been many valuable contributions of plant tissue culture to problems concerning plant pathology. One outstanding success is the virus eradication by apical meristem culture and the second success of tissue culture in plant pathology is the result of its application to the problems of plant tumors, especially crown gall.

## VIRUS ERADICATION

In virus infected plants, the distribution of viruses in plant body is uneven. It is well known that the apical meristems are generally either free or carry a very low concentration of viruses. The apical meristem culture (see details in Chapter 2) is the only way to obtain a clone of virus free plant which can be multiplied vegetatively

under control conditions that would protect them from the chance of reinfection. The elimination procedure of virus can usually be improved by combining it with heat therapy of the host plant or the culture. Virus eradication by apical meristem culture has enormous horticultural and agricultural value e.g. in the production of plants for the cut flower industry when stock plants of registered line must be maintained in as near-perfect condition as possible. Any infection by virus that affects growth rate or physical characteristics of size and shape is obviously very serious if it afflicts these nuclear stock, for they are the basis of all propagation and breeding.

In the agricultural world, the production or yield of a crop can fall dramatically as a result of viral infection and render that particular variety no longer saleable or commercially viable. Tissue culture techniques could be of value in restoring the original properties of the variety, by removing the infection and so bringing it back into the commercial market. These virus tested stocks could provide ideal material for the national and international distribution of plants, either for further propagation or use as breeding material. It is hoped that these selected virus free cultures would be acceptable to quarantine authorities.

### STUDY OF CROWN GALL BY PLANT TISSUE CULTURE

Smith and Townsend (1907) discovered that crown gall or plant tumor formation was induced by a bacterium, *Agrobacterium tumefaciens*. Braun (1941) showed that in sunflower *Agrobacterium* could induce tumors not only at the inoculated point but at a considerable distance, where secondary tumors free of bacteria are formed. Cells of these secondary tumors could be cultivated by tissue culture technique and multiplied on a medium without adding auxin and cytokinin, whereas normal tissue required auxins and, in some cases, cytokinins. Crown gall tissues deprived of bacteria give rise to tumors by grafting. Kulescha (1952) established that

they synthesized more auxin than normal tissues. Braun demonstrated that crown gall tissues free of bacteria contain a tumor-inducing principle (TIP) which may be a macromolecule. The biochemistry of the crown gall problem was studied by Lidret (1957) who discovered an amino acid called lysopin which might be specific for the tumor. Menage and Morel (1965), Goldmann-Menage (1970) and Morel (1971) isolated two substances of the same chemical fairly octopine and nopaline and concluded that these opines were not characteristic of the plant but of the bacterium that had induced the tumor. Crown galls induced by some strains of *A. tumefaciens* synthesize octopine, while tumors produced by other strains elaborate nopaline. Following these observations, Morel (1971) suggested that the synthesis of opines depends on the presence of TIP in the tumor cells of genes coming from the bacterium. In other words, the TIP consists of DNA. Zwnen *et al.* (1974) discovered the segment of bacterial DNA which is responsible for the tumoral transformation and opine synthesis. This segment belongs to a large plasmid. Only a small part (about 8%) of the plasmid is stably incorporated and replicated in plant cells. Therefore, we can say that this transferred DNA (T-DNA) contains the genetic information which promotes the tumoral transformation and gene coding for octopine or nopaline. Finally, geneticists and molecular biologists were able to establish the map of the crown gall plasmid. But the basic mechanism of tumoral transformation has not been clarified. It is hoped that plant tissue culture technique can throw some light on the basic mechanism of tumoral transformation.

### PLANT BREEDING, PLANT IMPROVEMENT AND PLANT TISSUE CULTURE

The conventional breeding methods are the most widely used for crop improvement. But in certain situations, these methods have to be supplemented with plant tissue culture techniques either to increase their efficiency or to be able to achieve the objective which is not possible through the conventional methods.

Embryo culture is now routinely used in recovery of hybrid plants from distant crosses. Some examples are recovery of hybrids from *Hordeum vulgare* × *Secale cereale*, *Triticum aestivum* × *Agropyron repens*, *H. vulgare* × *Triticum* species. In case of *Triticale*, a rare hybrid between *Triticum* and *Secale* develop viable seeds. But most of the tetraploid and hexaploid wheat carry two dominant genes  $K_{r_1}$  and  $K_{r_2}$  which prevent seed development in crosses with *Secale*. The hybrid seeds are minute, poorly developed and show very poor germination. By embryo culture, 50–70% hybrid seedlings has been obtained. Hybrid seedlings from *T. aestivum* × *H. vulgare* are not obtained. But it has been achieved by embryo culture.

When *H. vulgare* or *T. aestivum* (used as male) is crossed with *H. bulbosum* (used as female) the chromosome complement of *H. bulbosum* is eliminated from the developing embryo. Most of the seedling obtained from such crosses are haploid, having only one set of chromosomes either from *H. vulgare* or *T. aestivum* parent.

Embryo culture is also useful for propagation of orchids, shortening the breeding cycle and overcoming seed dormancy (see Chapter 10 'Embryo culture' for additional informations and examples).

In meristem culture, shoot apical meristem along some surrounding tissue is grown *in vitro*. It is used for clonal propagation and recovery of virus free plants and is potentially useful in germplasm exchange and long-term storage of germplasm through freeze preservation. (See Chapter 2 'Shoot tip or meristem culture' for additional information and examples).

Anther and pollen culture has a potential application in plant breeding and plant improvement programme for the production of haploid as well as homozygous diploid plant (see Chapter 11 'Anther and pollen culture' for additional information).

All-year-round rapid clonal propagation using plant tissue culture techniques has highlighted possibilities for new plant improvement techniques.

Protoplast culture and somatic hybridization is a promising line for plant breeding and plant improvement techniques. But, at present, techniques for selection and multiplication of somatic hybrid and regeneration of hybrid plants is very limited to a few classical plant species. So it is expected that in near future it would be possible to use this technique for a wide variety of plant species.

Another most important approach is the mutation of tissue culture cells to produce a mutant line from which plants can be raised. Production of mutant line is highly desirable for plant breeding. Callus cells, produced either from vegetative cell or reproductive tissues, can be subjected to a range of mutagenic chemicals e.g. N-nitroso-N-methyl urea or ionizing radiations e.g. gamma rays. The hope is that permanent changes in the DNA pattern of some of the cells would be achieved by such treatment. Plants could be raised from the treated cultures and any mutant whole plants selected from the population either by physical differences or by metabolic/biochemical differences. Biochemical mutants could be selected for disease resistance, resistance to phytotoxin, improvement of nutritional quality, adaptation of plants to stress conditions e.g. saline soils and to increase the bio-synthesis of plant products used for medicinal or industrial purposes.

## PRODUCTION OF USEFUL BIOCHEMICALS

Man depends on plants for many compounds other than food such as medicines, pigments, vitamins, hormones, flavoring agents, latex and tanins. If most plant somatic cells are totipotent, it should be possible to take a culture of cells from a plant that naturally produces a certain biochemical and cause the culture to produce that chemical under *in vitro* conditions. The main difficulty is that we do not yet understand the regulation mechanisms that control the production of most biochemical substance and so cannot manipulate them.

Even so, a surprising number of cell cultures have been found that do produce specialized biochemicals found in the intact parent, including

alkaloid such as nicotine, atropine, ephedrine, caffeine and codeine and their precursors and derivatives. Production of cardiac glycosides and other steroids, benzoquinones, latex, phenolics, anthocyanins, organic acids, anti-tumor agents, anti-microbials and various flavors and odours have also been reported.

The first patents for producing biochemicals commercially by large-scale plant cell culture was issued in 1956. The general approach since then has been to select high product-yielding cell lines, preferably as suspension cultures and to enhance their efficiency either by feeding them inexpensive product precursors or by manipulating their biosynthetic control mechanisms. Here the expertise of the microbial product industry is valuable. Large-volume automated culture vessels called fermenters have been used successfully to mass produce cultured plant cells. This technology will soon be producing selected pharmaceuticals and other high-cost biochemicals commercially.

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#### PRESERVATION OF PLANT GENETIC RESOURCES OR GENE CONSERVATION BANKS

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The need for a programme for the conservation of plant genetic resources arise from the rapid changes that are occurring in modern agriculture practice. The primitive cultivars and wild relatives of crop plants constitute a pool of genetic diversity which is invaluable for future breeding programmes. But these have already led to the replacement by new cultivars which encompass a much narrow range of genetic diversity. As a result, there is a very real danger of future breeding being impeded by the shrinking genetic bases of some crops. Therefore, storage of this sort of irreplaceable breeding material or germplasm (gene combinations available for breeding) and establishment of a centralised gene bank are the practical ways to solve these problems. Conventionally, germplasms are stored in the form of seeds because they occupy a relatively small space and can be stored for many years. But there are a number of important species, particularly root and tuber crops, which

are normally propagated vegetatively. These include potato, sweet potato, yam and cassava. So conventional preservation methods is not applicable to vegetatively propagated plants. On the other hand, the cost of maintaining a large proportion of the available genotype of these plant in nurseries or field is very high and there is a risk of the plants being lost as a result of disease or environmental hazards.

It is now possible with modern tissue culture techniques to provide a germplasm storage procedure which uniquely combines the possibilities of disease elimination and rapid clonal multiplication.

In addition, the possibility of using liquid nitrogen freeze-storage techniques for the preservation of cell, tissue and apical meristem is being studied. The advantages of this technique are that cell division and normal cellular reactions are totally arrested at the very low temperature of liquid nitrogen (-196°C), which means that there should be a high level of genetic stability and that the chemical reaction responsible for cellular damage will not occur. The plant materials can be stored in liquid nitrogen for desirable period. This technique could be particularly valuable for storage of any germplasm which needs to be maintained in a clonal form. This technique is known as cryopreservation or freeze preservation of tissue or cell. It has already been shown to be successful with a range of cell cultures e.g carrot cell and sycamore suspension cells and meristem tips from a number of crop plants including asparagus, tomato and potato. Plant tissue or meristems of such plants have been successfully recovered from liquid nitrogen and grown into normal, mature fertile plants.

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#### IMPORTANCE OF TISSUE CULTURE IN BIOTECHNOLOGY

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In 1981, the European Federation of Biotechnology defined "biotechnology as the integrated use of biochemistry, microbiology and

chemical engineering in order to achieve the technological application of the capacities of microbes and cultured tissue and cells". Some people equate it with the new field of genetic engineering, while others take a broader viewpoint defining it as the evaluation and use of biological agents and materials in the production of goods and service for industry, trade and commerce. To reduce confusion we will limit our interpretation to the two areas most often equated with biotechnology. One of these, the genetic engineering of organisms, is the endeavor that inspired the coinage of the term "biotechnology" in the 1970's. The other area consists of recent developments in the fields of tissue and cell culture, most notably those that have enabled us to fuse two different eukaryotic cells into a single cell that possesses the combined properties of both.

Cell suspension culture in liquid medium is a relatively young field of biotechnology. This technology involves the large-scale culture of isolated plant cell under condition which induces them to synthesize the natural secondary metabolites characteristic of parent plants from which they were obtained. In recent years, the technique of callus culture and cell suspension culture have also been viewed, particularly from the view-point for the study of biosynthesis and metabolism of steroids and cardiac glycosides. Biotechnologists are also trying to increase the synthesis of natural compounds or new compounds by higher plant cells culture as a result of mixing or feeding transformable precursors in the culture medium. The metabolic process of transformation or conversion of such added precursors into the natural or new compound within the cell is known as biotransformation. Biotechnologists are also trying to augment the synthesis of medicinally important alkaloids in culture by means of fungal elicitor. This means that cells are cultured in a liquid medium by adding required quantity of the bacterial filter sterilized extract of certain fungi. It has been observed that the fungal extract in certain cases helps to increase the synthesis of a desirable compound by the higher plant cell.

Biotechnologists are also trying to modify the genetics of the cultured cells by three ways such as—(i) mutagenesis and selection of cell lines in cell suspension culture, (ii) transplantation of foreign genetic material in protoplasts by means of genetic engineering and (iii) somatic hybridization by the fusion of distantly related plant proplast just to widen the genetic diversity of hybrids.

In the field of mutagenesis and selection of cell lines *in vitro* and the exploitation of totipotency, biotechnologists are trying to improve plants, for example by producing crop species that are more resistant to draught, disease, poor soil condition, chemical pesticides and herbicides.

Transplantation of foreign genes in protoplast has already been discussed in Chapter 12 (see importance of plant protoplast culture under the subheading genetic engineering or gene transfer in plant through protoplast). Somatic hybridization by the fusion of protoplast brings together, in a single plant, genes of different species too unrelated to allow mixing, thereby allowing creation of hybrid or cybrid plants for the improvement of crop species. It has also been discussed in Chapter 13 (See importance of protoplast fusion and somatic hybridization). Another goal of biotechnology using plant tissue culture technique is to produce self-fertilizing plants that would provide their own usable nitrogen. Plants rely on a few types of bacteria and cyanobacteria to fix atmospheric nitrogen into a biologically usable form. Genetically engineered plants that contain the bacterial genes for nitrogen fixation could grow well even in nitrogen-poor soil.

Genetic engineers are also developing plants that produce their own pesticides (one bite and pest dies). In 1986, the first of such genetically engineered plants successfully passed a field test.

The value of plant protein to human diet is being improved by creating corn or beans that manufacture a complete protein, one with all the amino acids essential to the human diet.

In another effort (W. David *et al.*, *Science*, Vol. 234, pp. 856, 1986) the luciferase gene from

the firefly, *Photinus pyralis* was used as a reporter of gene expression by light production in transfected plant cells and transgenic plant. A complementary DNA (cDNA) clone of the firefly luciferase gene under the control of a plant virus promoter (cauliflower mosaic virus 35 S RNA promoter) was introduced into plant protoplast cells (*Daucus carota*) and into plants (*Nicotiana tabacum*) by the use of the *Agrobacterium tumefaciens* tumor-inducing plasmid. In this experiment, stable expression of the firefly luciferase gene in plant cell and transgenic plants has been achieved. The transgenic plant incubated in luciferin also emits light like firefly. The successful introduction of animal DNA into plant genome has opened a new avenue in the field of biotechnology. With the help of this technology production of antibody has also been possible in plant (Nature 342 No. 6245, 1989).

N.B.—Besides the above mentioned importance of plant tissue culture, readers are also advised to consult the importance of individual techniques in different Chapters.

## Summary

The importance and application of plant cell and tissue culture in plant science are vast and varied. There have been many valuable contribution of plant tissue culture in the field of fundamental and applied botany.

The regeneration of whole plants through tissue culture is popularly called "micropropagation". By this method, a large number of plant species can be propagated all the year round. The plant breeder is no longer restricted by season in the production of large numbers of plants.

*In vitro* clonal propagation is a type of micropropagation. By this method the variability that can arise from sexual reproduction and seed formation in a crop plant, can be omitted. The plant with long seed dormancy can be raised faster by *in vitro* clonal propagation. For the orchid, *in vitro* clonal propagation is the

only commercially viable method of micropropagation. Clonal multiplication of a cultivar is very important in horticulture and silviculture.

The plant tissue culture is also proving to be rich and novel sources of variability with a great potential in crop improvement without resorting to mutation or hybridization. Larkin and Scowcroft have proposed a general term "Soma-clones" for plant variants obtained from tissue cultures, irrespective of their origin. Such variant plants may show some useful characters such as resistance to a particular disease, herbicide resistance, stress tolerance etc. Plant breeders can exploit such variants for their breeding programme.

There have been many valuable uses of plant tissue culture to solve the problems concerning plant pathology. One outstanding success is the virus eradication by apical meristem culture and the second success of tissue culture in plant pathology is the result of its application to the problems of plant tumors, especially crown gall.

The conventional breeding methods are most widely used for crop improvement. But in certain situations, these methods have to be supplemented with plant tissue culture technique either to increase their efficiency or to be able to achieve the objective which is not possible through conventional methods. Embryo culture is now routinely used to recovery of hybrid plant from distant crosses. Meristem culture, anther and pollen culture, plant protoplast culture and somatic hybridization contribute a lot of potential applications in plant breeding and plant improvement programmes.

Plant tissue culture, particularly cell suspension culture, has been exploited for the production of useful alkaloids, cardiac glycosides and other steroids.

Preservation of plant genetic resources can be achieved by plant tissue culture. Cryopreservation of plant tissue, cell or meristem in liquid nitrogen for desirable period and the recovery of whole plant via organogenesis is also a valuable tool for the preservation of very rare germplasm and to maintain a clone for crop improvement.

Plant tissue culture is relatively a young field of biotechnology. Biotechnologists are trying to modify the genetics of the cultured cells by three ways—mutagenesis and selection of cell lines; transplantation of foreign genetic material in protoplast by genetic engineering, and somatic hybridization by the fusion of distantly related plant protoplasts just to widen the genetic diversity among the existing plants.

## Questions for Discussion

1. Write an essay on the application of plant tissue culture in plant science.
2. Discuss the importance of tissue and cell culture in biological studies.
3. Discuss the importance of plant tissue culture in plant pathology.
4. Discuss the application of plant tissue culture in plant breeding and plant improvement.
5. Write short notes on—
  - (a) Micropropagation
  - (b) Clonal propagation
  - (c) Importance of tissue culture in biotechnology
  - (d) Production of genetically variable plants.
  - (e) Cryopreservation
  - (f) Gene conservation bank.



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## Glossary of terms used in Plant Tissue Culture

**Adventitious**—Development of shoots, roots or any other organs from unusual point of origin. In plant tissue culture, shoots or roots are developed from callus tissue and embryos from sources other than zygote.

**Adventive embryony**—Embryo formation and development resulting from asexual cells as occurs in certain member of Rutaceae.

**Androgenesis**—Development of plant *in vitro* from male gametophyte.

**Apical meristem**—Small group of meristematic cells that are located at the tips of the shoot and root axis and are progenitors of these structure.

**Aseptic culture**—A culture without undesired or foreign life form. An aseptic culture means the purposeful culture of different types of cells, tissues of higher plant without any contamination of bacteria, fungi etc.

**Aseptic technique**—Techniques used to prevent the entry of fungi, bacteria or other micro-organisms into cell, tissue and organ culture.

**Auxins**—A group of growth substances produced in the zone of elongation and cause cell elongation, apical dominance, root initiation etc. 2, 4-D (2, 4-dichlorophenoxyacetic acid), IAA

(Indole-3-acetic acid),  $\alpha$ -naphthaleneacetic acid (NAA), Indole butyric acid (IBA) are some of the synthetic auxins commonly used in plant tissue culture.

**Batch culture**—A type of cell suspension culture in which cell or small cell aggregates grow in a definite volume of nutrient medium.

**Callus tissue**—An unorganised mass of proliferative cells produced either in culture or in nature.

**Cell culture**—Term used to define the maintenance or culture of cells *in vitro* including the culture of single cells. In cell cultures, the cells are no longer organized into tissues.

**Chemostat**—A type of cell suspension culture in which growth rate and cell density remain constant by a fixed rate of input of growth-limiting nutrient.

**Chimera**—Organism or tissue that is not genetically homogeneous.

**Clonal propagation**—*In vitro* propagation of plants that are considered to be genetically uniform and originated from a single individual or explant.

**Clone**—A group of genetically identical individuals produced from a single explant.

**Cybrid**—When two or more protoplasts fuse then their cytoplasms always fuse, but the nuclei may or may not fuse. If, in a binucleate heterokaryon, one of the nuclei disappears then it is called a cybrid or a cytoplasmic hybrid as the cytoplasms remain in a fused state.

**Cytodifferentiation**—Means the *in vitro* vascular differentiation particularly the xylem elements within the callus tissue.

**Cytokinins**—A group of growth hormones which stimulates cells to divide, although in some cases they affect only cell enlargement. Another more spectacular property of cytokinins is their ability to induce the formation of organs by undifferentiated callus tissue.

**Differentiation**—Process of change in cell, tissue or organ resulting in the variety of structure and function found in the adult or other phases in the life history.

**Disease free**—A plant certified through specific pathological tests as being free of pathogens or infections micro-organisms.

**Embryo culture**—*In vitro* culture of isolated zygotic mature or immature embryos.

**Embryogenesis**—The development of embryoids in plant tissue culture.

**Embryoid**—A structure comparable to the zygotic embryo which is produced in tissue culture by dividing somatic cells.

**Epigenetic variation or change**—Phenotypic variation or change which has a non-genetic basis. Epigenetic changes are not transmitted by sexual reproduction.

**Explant**—The piece of tissue isolated from the intact plant that is used to initiate the culture.

**Friable or Friability**—The tendency of plant cell or cells of callus tissue to separate from one another.

**Gene bank**—A store of germplasm.

**Genetic change**—Phenotypic variation or change which has a genetic basis. Genetic changes are transmitted by sexual reproduction.

**Germplasm**—Gene combinations available for breeding.

**Habituated**—It is the ability of culture tissue or organ to grow on nutrient medium without added plant hormones.

**Haploid**—A cell or nucleus containing a single set of chromosomes.

**Heterokaryon**—A cell in which two or more genetically unlike nuclei are present.

**Heterozygous**—A term applied to organism that possesses two different alleles for a trait.

**Homozygous**—A term applied to an organism that has two identical alleles for a particular trait.

**In vitro**—Cells and tissues removed from the intact organism and placed in an artificial situation for experimentation. Literally it means “in glass”.

**In vitro propagation**—Propagation of plants in artificial nutrient medium under controlled condition.

**In vivo**—Cell and tissue when they remain integrated into the whole plant. Literally it means “in life”.

**Inoculum**—A portion or volume or aliquot of suspension culture used for its subculture.

**Meristemoid**—A localized group of meristematic cells appeared within the callus tissue during culture and such cells may give rise to roots and/or shoots.

**Micropropagation**—The asexual or vegetative propagation of whole plants using tissue culture technique.

**Morphogenesis**—The process of growth and development of a form or structure.

**Nutrient medium**—A nutritive solution for culturing cells and tissue. It is prepared by mixing several inorganic and organic chemicals, a carbohydrate, vitamins, amino acids and phytohormones. Nutrient medium is solidified by adding definite amount of agar-agar.

**Nurse culture**—An isolated cell which generally fails to divide when plated directly on the medium used for callus cultures is able to divide under the nursing effect of the callus.

**Organ culture**—The maintenance or growth of organ primordia *in vitro* without forming callus tissue.

**Organogenesis**—The development of organs or primordia from undifferentiated cell masses in tissue culture.

**Passage**—The transfer of cell or tissue from old culture medium to fresh culture medium within a definite time period. Maintenance of culture in a medium for a definite period before transferring to fresh medium constitute a passage. This term is synonymous with the term “subculture”.

**Plantlet**—The small regenerated plant while it is still within the culture tube or flask.

**Plant tissue culture**—It is an experimental technique for culturing plant protoplast, cell, tissue and organ in artificial nutrient medium under aseptic and controlled conditions.

**Plating efficiency**—It is a quantitative assessment of the percentage of cell colonies per total number of cells or protoplasts plated at a definite density for a specific time period. It can be determined by counting cell colonies under microscope at the end of experiment and calculated by using the formula:

**Plating efficiency**—(PE)=

$$\frac{\text{No. of Colonies/plate}}{\text{No. of cell units}} \times 100$$

**Primary culture**—A culture started from explants taken directly from plant body.

**Primordium**—Earliest stage in the development of a plant organ.

**Protoplast**—The entire plant cell without its cellulosic cell wall.

**Protoplast fusion**—Technique in which two or more protoplasts are fused into a single cell.

**Somaclonal variation**—It is the genetic variability which is generated during tissue culture.

**Somatic hybridization**—The *in vitro* fusion of plant protoplasts derived from somatic cells which differ genetically.

**Sterile** — To make free from microorganisms.

**Sub culture**—See ‘passage’.

**Suspension culture**—Cell or cell aggregates cultured in moving liquid medium.

**Totipotency**—Totipotency is the genetic potential of a plant cell to produce the entire plant.

**Transfection**—In culture, the transfer of naked, foreign DNA into cells for the purpose of genomic integration.

**Transgenesis**—In plant cell culture, the introduction and stable genomic integration of foreign DNA into a plant cell by any means, resulting in a genetic modification.

**Virus-free**—A plant certified through specified tests as being free of specified plant viruses.

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