I Internal Examination (2019-20)

M.Sc. Biotechnology Final Year

Plant Biotechnology

SET-A

Time: 1:30 Hours **Max. Marks-30**

Q1. Answer the following question in very short:

1×7=7

a). Who is the father of Plant Tissue Culture?

Ans: Haberlandt

b). Write the role of auxin in plant Growth?

Ans: Auxins promote stem elongation, inhibit growth of lateral buds (maintains apical dominance).

c). Cite one example of well known cytokinin?

Ans: Kinetin

d). What is the full form of HEPA filter and its standard size?

Ans: High Efficiency Particulate Air Filter and standard 0.3micrometer

e). Which plant hormone control the fruit ripening?

Ans: Ethylene

f). Write the enzyme required to obtain the wall free/naked protoplast?

Ans: Cellulase and Pectinase

g) Define Gametogeneis?

Ans: The process in which cells undergo meiosis to form gametes.

Q2. Write short notes on:

2×4=8

a). Composition of PTC

Ans; Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media.

Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that plants in nature can synthesize their own food material. However, plants growing in vitro are mainly heterotrophic i.e. they cannot synthesize their own food.

Composition of Media:

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

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

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

Major Types of Media:

The composition of the most commonly used tissue culture media is given in Table :

Table 43.1 Composition of commonly used plant tissue culture media

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

b). Enzymatic method of Protoplast Isolation

Ans: Young fully expanded soft leaves, or in vitro grown callus tissue or cell suspension culture grown cells can be used as the source material. The tissues or cells are incubated in plasmolyticum for 1 hr before enzymatic treatment. The intact tissue materials cut into smaller pieces to increase the surface area of enzymatic activity. The enzymes can be used either sequentially in two step method or in a single step by mixed enzymatic method.

The enzymes used are of three main categories:

(i) Cellulases, Cellulysin and Driselase

(ii) Hemicellulase, Helicaseand Rhozyme

(iii) Pectinase, Macerozyme-R-10 and Pectinol

The concentration of enzymes used and the time period of incubation varies greatly depending on the tissue type. In two step method, the pectinase and hemicellulases are applied first and then cellulase is applied for complete removal of cell wall.

After release of protoplast into the suspension, for removal of enzymes the protoplasts are collected in centrifuge tube as pellet and washed several times with the osmoticum.

Q3. Explain the following in short:

2×4=8

a). Bergmann's Cell Plating

Ans: The single cells can be isolated from a variety of tissue and organ of intact plant or from callus tissue or from cell suspension. Single cells can be isolated from intact plant tissue or callus tissue by using either mechanical method or chemical method.

Mechanical isolation means chopping of the tissue material by fine scalpel which releases few intact single cells or by glass homogenizer the tissue may be crushed where the homogenate containing only few intact cells can be cultured. In chemical method the macerozyme or pectinase can be used to dissolve the middle lamella and releasing single cells from intact tissue

The basic technique of plating is to first count the cell number without maceration stage, this will enable a known number of cell units to be established per unit volume of plating media. Both the cell suspension and nutrient medium containing agar are prepared in double concentration separately.

The equal volumes of suspension and the agar medium cooled at 35°C are mixed and then dispersed rapidly into petriplate in such a manner that cells are evenly distributed in a thin layer (~1 mm thick). The dishes are then sealed and incubated for cell division, which will give rise to callus (Fig. 17.4C).

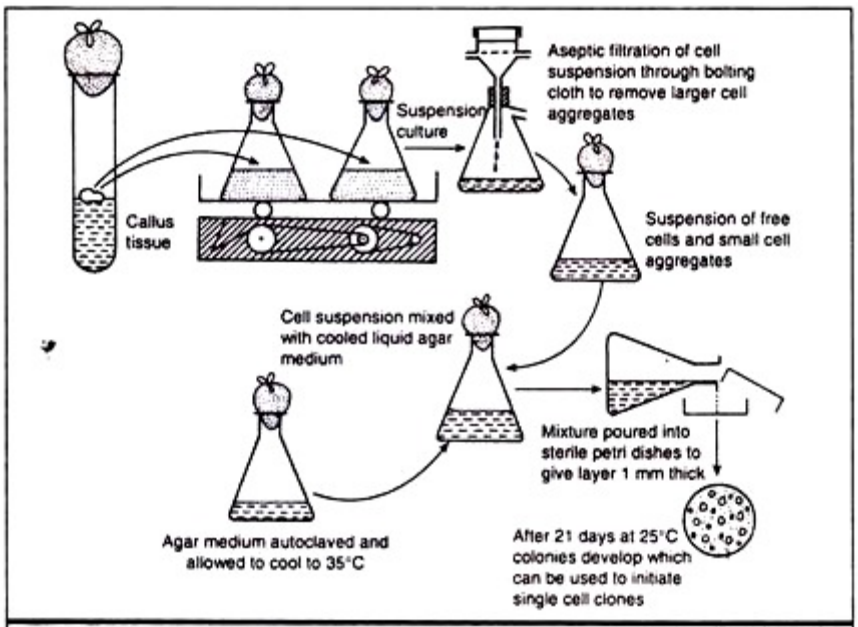

Fig. 17.4C: Procedure for obtaining single cell clones using a petri dish plating technique

Bergmann (1960) first introduced this most popular technique of plating of cell suspension and this technique is very much useful to calculate the plating efficiency.

$$\text{Plating Efficiency (PE)} = \frac{\text{No. of colonies/Plate at the ends of experiment}}{\text{No. of cellular units initially plated/plate}} \times 100$$

The plates may be observed under an inverted microscope and single cells develop into callus, this method ensures the isolation of pure single-cell clones. Usually, plating at cell densities of 103-105 cells/ml or more yields a high plating efficiency.

Efforts have been made to develop a synthetic medium for cells plated at low density. Cells plated in a culture medium synthesize necessary metabolites and threshold conc. of those helpful for cell division to start. At initial high cell density the equilibrium is reached much earlier than at a low cell density.

It has been observed that below a critical cell density the cells fail to divide. This problem can be overcome by supplementing the minimal medium with some undefined factors like coconut milk, casein hydrolysate or yeast extract and following some different culture techniques.

b). Identification of Fused protoplast

Ans: Following fusion treatment the protoplast population consists of parental type proto-plasts, homokaryotic fused products and also heterokaryotic fusion products. The proportion

of viable heterokaryotic fusion generally is lower, identification and recovery of protoplast fusion products have been based on observation of visual characters or hybrid cells may show genetic complementation for some growth requirements, etc.

(a) Visual Selection:

In most of the fusion programme generally the selection procedure involves the fusion between a non-green protoplasts of one parent and the green protoplast of another parent, as this facilitate the visual identification of heterokaryons under light microscope. The non-green protoplasts may be available from callus tissue and the green protoplasts from leaf tissue (Fig. 20.7A-B).

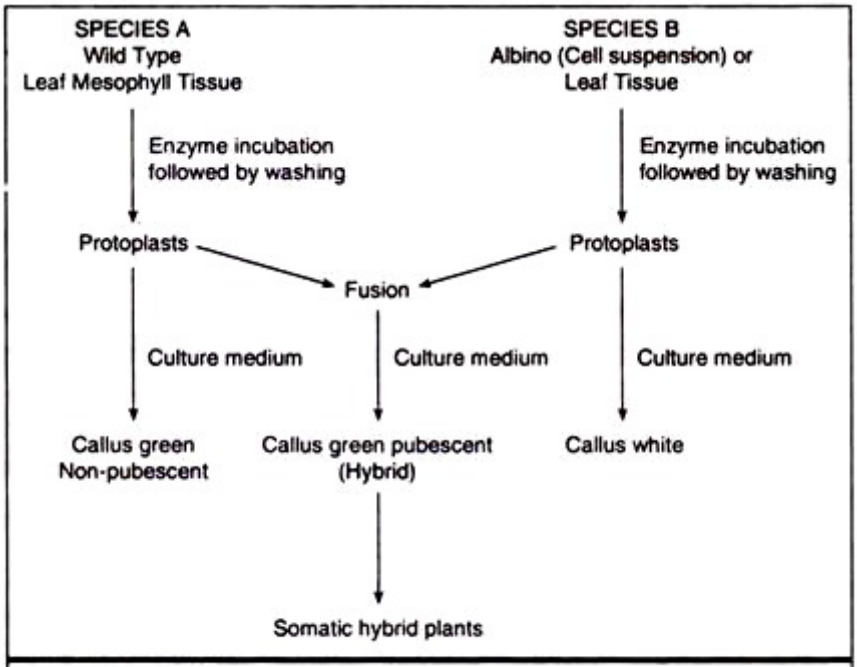

Fig. 20.7A: Selection scheme used in the intergeneric somatic hybridization of *Atropa belladonna* (wild type) with *Datura innoxia* (albino)

(b) Selection by Complementation:

If the parental protoplasts from the two parents can be identified by biochemical marker then the heterokaryons can be selected easily by using the proper growth requirement in media.

(i) Complementation of Resistance Markers:

Dominant characters such as traits conferring resistance to antibiotics, amino acid analogues or toxic com pounds have been selected as potent markers. When the protoplasts from two lines are being fused together then the fused product can be selected in presence of both the metabolites because of double resistance as compared to single parent.

(ii) Use of Metabolic Inhibitors:

The parental protoplasts are treated with irreversible biochemical inhibitor such as iodoacetate or di-ethyl-pyro-carbamate and following treatment only hybrid cells will be capable of division.

(iii) Auxotroph Complementation:

Where two parental protoplasts are both mutated for the same enzyme but both of them are of two different mutational types. So the fused and hybrid protoplasts could be grown easily by complementing each other capable of producing active enzyme.

(iv) Chlorophyll Deficiency Complementation:

This is the frequently used method for selecting the somatic hybrid where the normal plant protoplasts are allowed to fuse with an albino or mutated or chlorophyll deficient type of protoplast. The fused products or somatic hybrids must be able to produce the green colonies by complementation.

Verification and Characterisation of Somatic Hybrid:

Somatic hybrids after regeneration should be verified through clear demonstration of genetic contribution of both the parents.

(i) Morphological Characters:

In most of the cases the somatic hybrids bear the characters from the parents or sometimes they have the somatic or sexual characters intermediate of both the parents. Such traits may be leaf size, leaf surface, pigment, flower shape, pollen character, etc.

(ii) Isoenzyme Analysis:

Electrophoretic banding patterns of isoenzymes have been extensively used to verify hybridity. Somatic hybrids may display isozyme banding of certain enzymes specific to either the parents or sometimes newer type of banding. The enzymes like esterase, isoperoxidase, phosphatase, alcohol dehydrogenase, etc. are studied.

(iii) Chromosomal Constitution:

Counting chromosomes in presumed somatic hybrid is a reliable and easy method of detection. Cytologically the chromosome number should be the sum of chromosome number of two parents. Besides the number, the size and structure of the chromosomes of both the parents are accounted for verification of somatic hybrids.

(iv) Molecular Technique:

With the availability of numerous molecular markers such as RFLP, AFLP, RAPD, microsatellites, etc. the somatic hybrid identification has become more easier. PCR technology are being utilised for hybrid identification. Specific restriction patterns of

chloroplast and mitochondrial DNA have been used with great advantage to characterise the somatic hybrids.

Q.4 What is somatic Hybridization? Explain its method of culturing and its benefits? 7

Ans: Isolated protoplasts are devoid of cell walls which make them easy tools for undergoing fusions in vitro. An important factor is that generally there is incompatibility barrier between two protoplasts of different species or genera. The process of fusion may be spontaneous or it may be induced (Fig. 20.5).

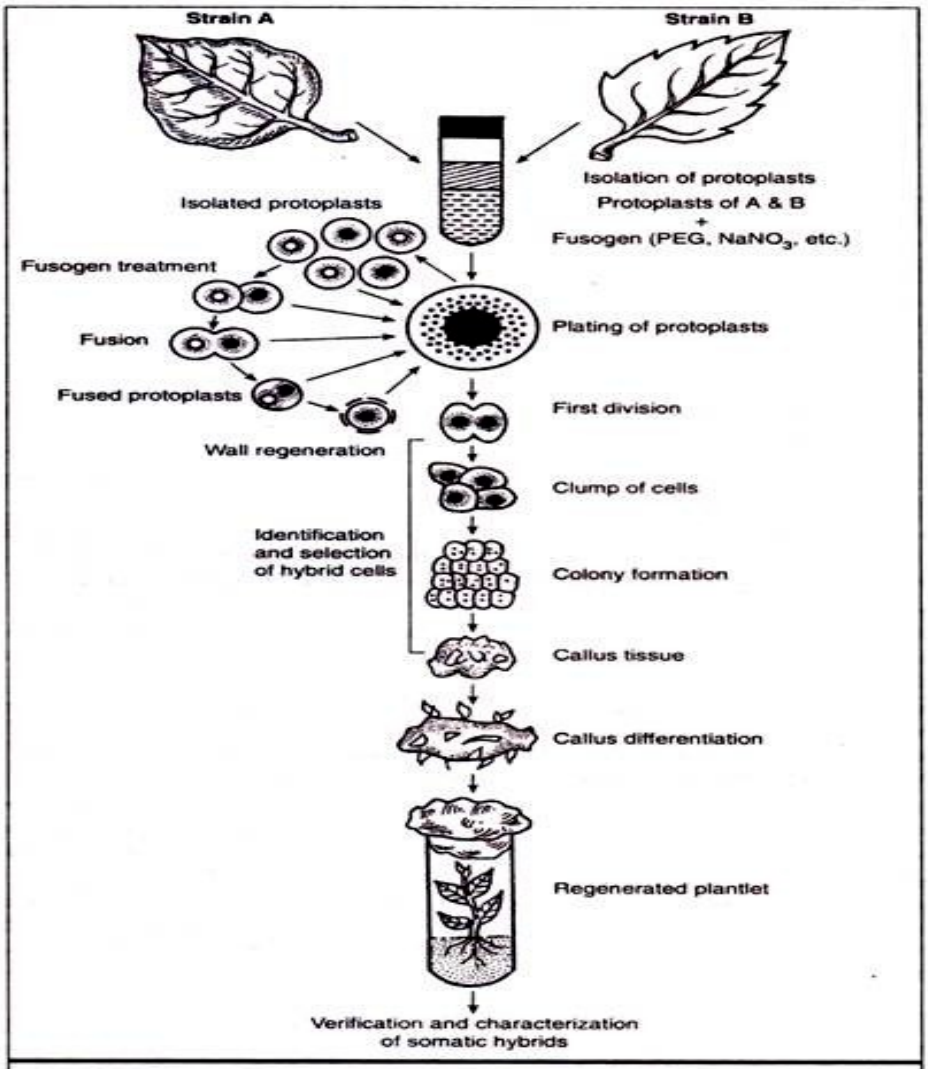

Fig. 20.5: Schematic representation of protoplast fusion and regeneration of plants

Method of Protoplast Fusion:

(a) Spontaneous Fusion Method:

During enzymatic degradation of cell walls some of the adjacent protoplasts may fuse together to form homokaryons, sometimes more than two protoplasts fuse together and form multinucleate cells by a phenomenon where expansion and subsequent coalescence of the

plasmodesmata led to such cases. However the spontaneous fusion products do not regenerate into whole plants except for undergoing a few divisions.

(b) Induced Fusion Method:

Freshly isolated protoplasts can be induced to undergo fusion, irrespective of their origin with the help of different kinds of fusogen (fusion inducing agents) e.g., $NaNO_3$, lysozyme, High pH/Ca^{++}, polyethylene glycol (PEG), concavalin, polyvinyl alcohol, electro-fusion, dextran sulphate, etc.

Some of these treatments are discussed here:

(i) $NaNO_3$ Treatment:

Isolated protoplasts are suspended in mixture of 5.5% $NaNCO_3$ and 10% sucrose soln. and incubated in a water bath at 35°C for 5 min. and centrifuged for 5 min. at 200 x g, following centrifugation the supernatant is decanted and the pellet is incubated at 30°C for 30 min. During this period most of the protoplasts undergo cell fusion. After sometimes these are washed with osmoticum and then plated properly in culture medium.

(ii) Calcium Ions at High pH:

Here the isolated protoplasts are incubated in a solution of osmoticum containing 0.05 M $CaCl_2$ at pH 10.5 (using 0.05 µ glycine— NaOH buffer) and at temperature 37°C. Aggregation of protoplasts generally takes place at once and fusion occurs within 10 mins. After this treatment, 20-50% of the protoplasts have been found to be involved in fusion.

(iii) PEG Treatment:

PEG has been found to be used as a fusogen in most of the successful cases of protoplast fusion. 1 ml of protoplast suspension is equally mixed with 1 ml of 28-56% PEG (1500-6000 MW) solution. The tube is then allowed to settle for 10 min to 40 min at room temperature.

Both the mol. wt. and the conc. of PEG used is critical in inducing successful fusions. After PEG treatment the elution of fusogen is done by using high pH/Ca^{++} containing solution which is most effective in enhancing the fusion frequency and survivability of protoplasts (Fig. 20.6A).

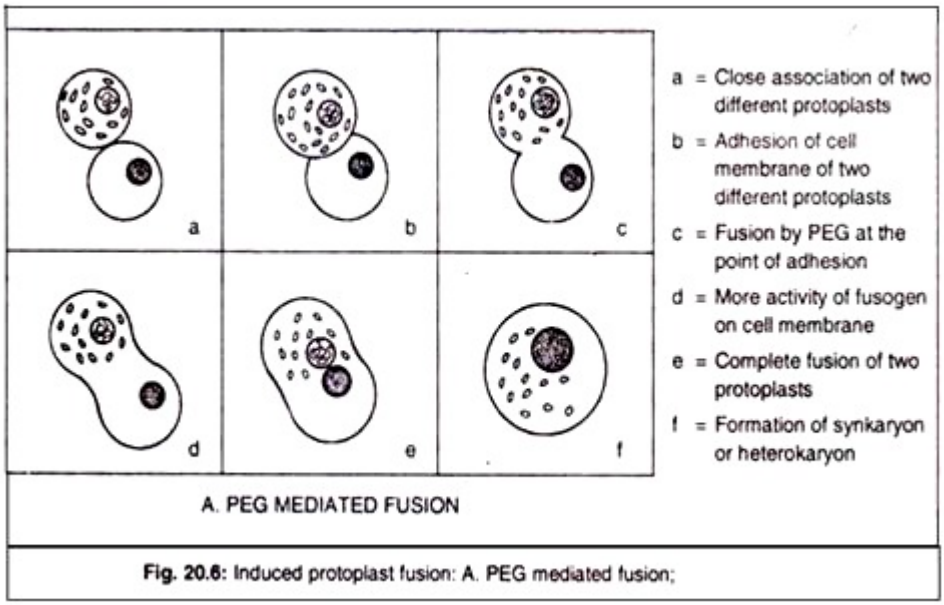

Fig. 20.6: Induced protoplast fusion: A. PEG mediated fusion;

(iv) Electro-fusion:

Protoplasts are placed into a small cell culture containing electrodes and a potential difference is applied due to which protoplasts line up between the electrodes. In this fusion method, two step procedures is followed: a low voltage and rapidly oscillating AC field is applied, which causes the protoplasts to become aligned into chains by cell to cell contact.

Second step is brief application of a high voltage DC pulse which induces reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and consequent membrane reorganisation. Heterokaryons produced by this electro-fusion divide normally in culture medium and have the capability of regenerating the plantlet (Fig. 20.6B).

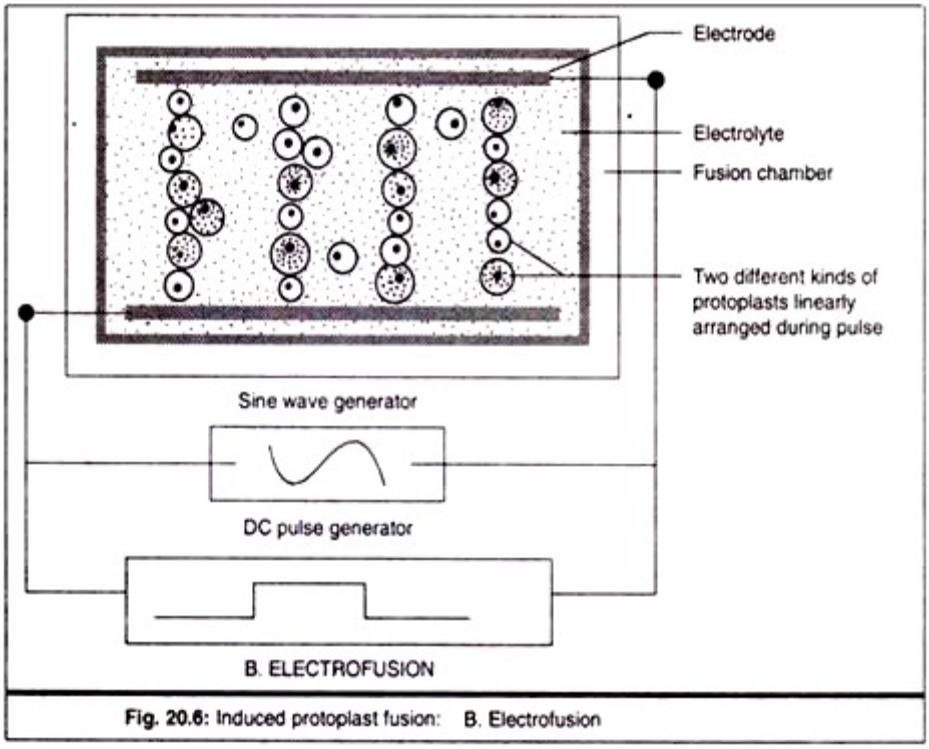

Fig. 20.6: Induced protoplast fusion: B. Electrofusion

Mechanism of Protoplast Fusion:

Protoplast Fusion Consists of Three main Phases:

(i) Agglutination or Adhesion:

Two or more protoplasts are brought into close proximity by using a variety of treatment like PEG, high pH, high conc. of Ca^{++} ions, etc.

(ii) Fusion of Plasma Membrane at Localised Sites:

Membranes of protoplasts agglutinated by fusogen get fused at the point of adhesion, which results in the formation of cytoplasmic bridges between the protoplasts and fusion requires less than 10A distance between two membranes. The high pH and high Ca^{++} ions have shown to neutralize the normal surface charge so that the agglutinated protoplasts can fuse due to intermingling of lipid molecules in membranes.

(iii) Formation of Heterokaryon:

Rounding off of the fused protoplasts occur due to the expansion of cytoplasmic bridges forming spherical heterokaryon or homokaryon.

Identification and Selection of Hybrid Cells:

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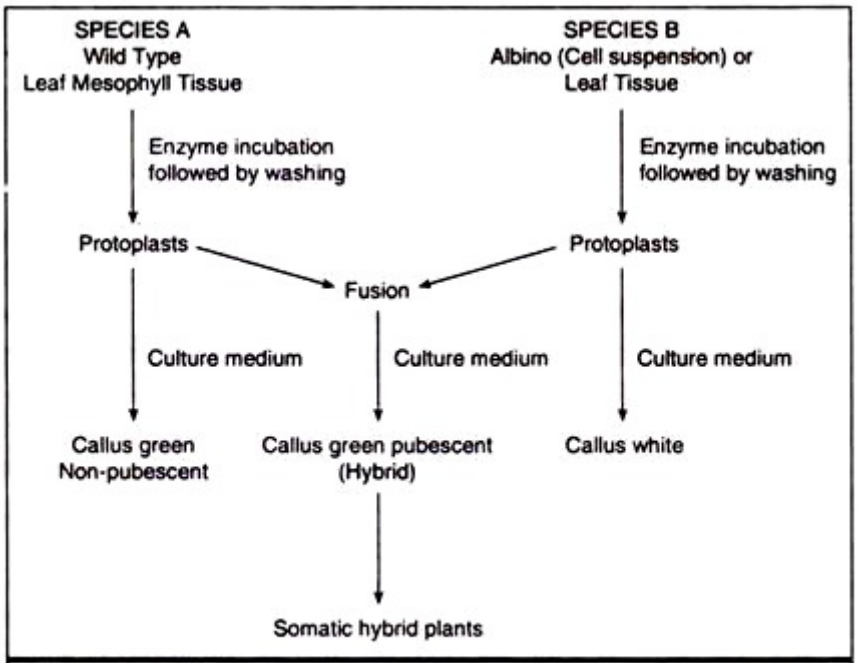

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Plant Biotechnology

SET-B

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Q1. Answer the following question in very short: **1×7=7**

a). Cite one example of well known Auxin?

Ans: 2,4-D

b). Write the name of scientist who first invented the enzymatic method of protoplast isolation?

Ans: Cocking

c). Define Electroporation?

Ans: Electroporation, or electropermeabilization, is a microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (also called electrotransfer).

d). Write the names of environmental factors which interfere with PTC?

Ans: Light source, temperature and osmotic Pressure

e). What do you mean by Totipotency?

Ans: Totipotency is the genetic potential of a plant cell to produce the entire plant.

f). Write the name of two fusogens?

Ans: Sodium nitrate ($NaN0_3$), polyethylene glycol (PEG)

g). Define osmoticum with one example?

Ans: Any substance that acts to supplement osmotic pressure in a plant or a culture of plant cells, Ex: Mannitol

Q2. Write short notes on: **2×4=8**

a). Sterilization Technique

Ans: ffective sterilisation techniques are essential for working with isolated cell lines for obvious reasons you don't want bugs from the environment growing in your nice culture medium, and equally, cultures must be sterilised before disposal.

So what are the most common methods of sterilisation, and how do they work? Unsure? Read on…

WET HEAT (Autoclaving)

The method of choice for sterilisation in most labs is autoclaving; using pressurised steam to heat the material to be sterilised. This is a very effective method that kills all microbes, spores

and viruses, although for some specific bugs, especially high temperatures or incubation times are required.

Autoclaving kills microbes by hydrolysis and coagulation of cellular proteins, which is efficiently achieved by intense heat in the presence of water.

The intense heat comes from the steam. Pressurised steam has a high latent heat; at 100degC it holds 7 times more heat than water at the same temperature. This heat is liberated upon contact with the cooler surface of the material to be sterilised, allowing rapid delivery of heat and good penetration of dense materials.

At these temperatures, water does a great job of hydrolysing proteins… so those bugs don't stand a chance.

DRY HEAT (Flaming, baking)

Dry heating has one crucial difference from autoclaving. You've guessed it – there's no water, so protein hydrolysis can't take place.

Instead, dry heat tends to kill microbes by oxidation of cellular components. This requires more energy than protein hydrolysis so higher temperatures are required for efficient sterilization by dry heat.

For example sterilisation can normally be achieved in 15 minutes by autoclaving at 121degC, whereas dry heating would generally need a temperature of 160degC to sterilize in a similar amount of time.

FILTRATION

Filtration is a great way of quickly sterilizing solutions without heating. Filters, of course, work by passing the solution through a filter with a pore diameter that is too small for microbes to pass through.

Filters can be scintered glass funnels made from heat-fused glass particles or, more commonly these days, membrane filters made from cellulose esters. For removal of bacteria, filters with an average pore diameter of 0.2um is normally used.

But remember, viruses and phage can pass through these filters so filtration is not a good option if these are a concern.

SOLVENTS

Ethanol is commonly used as a disinfectant, although since isopropanol is a better solvent for fat it is probably a better option.

Both work by denaturing proteins through a process that requires water, so they must be diluted to 60-90% in water to be effective.

Again, a it's important to remember that although ethanol and IPA are good at killing microbial cells, they have no effect on spores.

RADIATION

UV, x-rays and gamma rays are all types of electromagnetic radiation that have profoundly damaging effects on DNA, so make excellent tools for sterilization.

The main difference between them, in terms of their effectiveness, is their penetration.

UV has limited penetration in air so sterilisation only occurs in a fairly small area around the lamp. However, it is relatively safe and is quite useful for sterilising small areas, like laminar flow hoods.

X-rays and gamma rays are far more penetrating, which makes them more dangerous but very effective for large scale cold sterilization of plastic items (e.g. syringes) during manufacturing.

b). Inorganic Supplement for PTC Media

Ans: **Inorganic Nutrients:**

The inorganic nutrients consist of macronutrients (concentration >0.5 mmol/l^{-}) and micronutrients (concentration <0.5 mmol/l^{-}). A wide range of mineral salts (elements) supply the macro- and micronutrients. The inorganic salts in water undergo dissociation and ionization. Consequently, one type of ion may be contributed by more than one salt. For instance, in MS medium, K^+ ions are contributed by KNO_3 and KH_2PO_4 while NO_3^- ions come from KNO_3 and NH_4NO_3.

Macronutrient elements:

The six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of nitrogen and potassium is around 25 mmol l^{-1} while for calcium, phosphorus, sulfur and magnesium, it is in the range of 1-3 mmol l^{-}. For the supply of nitrogen in the medium, nitrates and ammonium salts are together used.

Micronutrients:

Although their requirement is in minute quantities, micronutrients are essential for plant cells and tissues. These include iron, manganese, zinc, boron, copper and molybdenum. Among the microelements, iron requirement is very critical. Chelated forms of iron and copper are commonly used in culture media.

Q3. Explain the following in short: **2×4=8**

a). Factor affect the haploid production

Ans: A good knowledge of the various factors that influence androgenesis will help to improve the production of androgenic haploids. Some of these factors are briefly described.

Genotype of donar plants:

The success of anther or pollen culture largely depends on the genotype of the donor plant. It is therefore important to select only highly responsive genotypes. Some workers choose a breeding approach for improvement of genotype before they are used in androgenesis.

Stage of microspore or pollen:

The selection of anthers at an ideal stage of microspore development is very critical for haploid production. In general, microspores ranging from tetrad to bi-nucleate stages are more responsive. Anthers at a very young stage (with microspore mother cells or tetrads) and late stage (with bi-nucleate microspores) are usually not suitable for androgenesis. However, for maximum production of androgenic haploids, the suitable stage of microspore development is dependent on the plant species, and has to be carefully selected.

Physiological status of a donar plant:

The plants grown under best natural environmental conditions (light, temperature, nutrition, CO_2 etc.) with good anthers and healthy microspores are most suitable as donor plants. Flowers obtained from young plants, at the beginning of the flowering season are highly responsive. The use of pesticides should be avoided at least 3-4 weeks preceding sampling.

Pretreatment of anthers:

The basic principle of native androgenesis is to stop the conversion of pollen cell into a gamete, and force its development into a plant. This is in fact an abnormal pathway induced to achieve in vitro androgenesis. Appropriate treatment of anthers is required for good success of haploid production.

Treatment methods are variable and largely depend on the donor plant species:

1. Chemical treatment:

Certain chemicals are known to induce parthenogenesis e.g. 2-chloroethylphosphonic acid (ethrel). When plants are treated with ethreal, multinucleated pollens are produced. These pollens when cultured may form embryos.

2. Temperature influence:

In general, when the buds are treated with cold temperatures (3-6°C) for about 3 days, induction occurs to yield pollen embryos in some plants e.g. Datura, Nicotiana. Further, induction of androgenesis is better if anthers are stored at low temperature, prior to culture

e.g. maize, rye. There are also reports that pretreatment of anthers of certain plants at higher temperatures (35°C) stimulates androgenesis e.g. some species of Brassica and Capsicum.

Effect of light:

In general, the production of haploids is better in light. There are however, certain plants which can grow well in both light and dark. Isolated pollen (not the anther) appears to be sensitive to light. Thus, low intensity of light promotes development of embryos in pollen cultures e.g. tobacco.

Effect of culture medium:

The success of another culture and androgenesis is also dependent on the composition of the medium. There is, however, no single medium suitable for anther cultures of all plant species. The commonly used media for anther cultures are MS, White's, Nitsch and Nitsch, N6 and B5. These media in fact are the same as used in plant cell and tissue cultures. In recent years, some workers have developed specially designed media for anther cultures of cereals.

Sucrose, nitrate, ammonium salts, amino acids and minerals are essential for androgenesis. In some species, growth regulators - auxin and/or cytokinin are required for optimal growth. In certain plant species, addition of glutathione and ascorbic acid promotes androgenesis. When the anther culture medium is supplemented with activated charcoal, enhanced androgenesis is observed. It is believed that the activated charcoal removes the inhibitors from the medium and facilitates haploid formation.

b). Culture techniques for protoplast Culturing

Ans: There are different methods of protoplast culture such as liquid culture, agar culture, droplet culture, co-culture, hanging droplet culture, immobilised/bead culture and feeder layer technique (Fig. 20.4A-D).

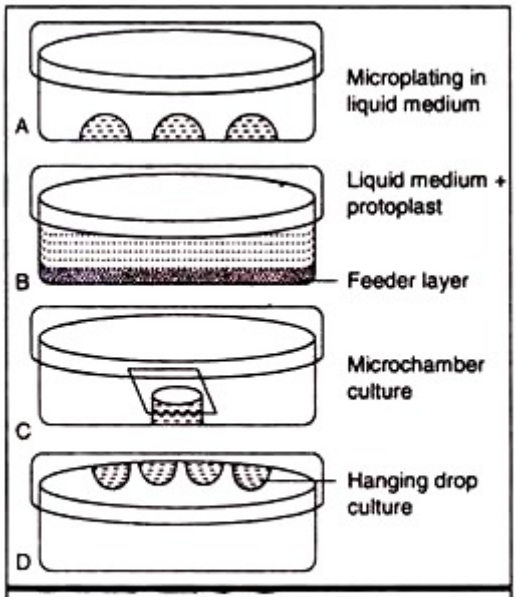

Fig. 20.4A-D: Different cultural techniques for protoplast culture

1. Liquid Culture:

This method is generally preferred in most cases during early developmental stages of protoplasts, because it allows easy dilution and transfer, protoplasts easily get divide in liquid media, osmotic pressure of the medium can be regulated and can be effectively reduced during further growth of protoplasts. The disadvantage of this method is that it does not permit the isolation of single colonies derived from one parent cell.

2. Agar Culture:

Agarose is most frequently used to solidify protoplast culture media. Protoplast suspension is taken at double density and mixed with melted agar medium at 45°C and mixed well and plated in small petridish. Here the protoplasts remain in same position and immobilised, proper plating efficiency can be obtained but the medium change can be done only after visible calli formation.

3. Droplet Culture:

Suspending protoplasts in liquid culture media are placed on petridishes in the form of droplet, the cultured protoplasts clump together at the centre of droplets. The liquid medium can be changed at regular interval.

4. Co-Culture:

Sometimes to induce division the newly isolated protoplast suspension is mixed with a reliable fast growing protoplast suspension and mixed protoplasts are plated. Some growth factors help to induce the proper growth and development of the isolated protoplasts.

5. Hanging Droplet Technique:

Culture of protoplasts can be done in an inverted droplet on the inner surface of the lid of petridish, a very small number of protoplasts can be cultured in this way. A thin layer of liquid medium is kept in the petridish to keep the environment inside the petridish humid.

6. Bead Culture:

The protoplasts suspension can be mixed with any kind of polymer like alginate, carrageenan, etc. and then small beads are made by dripping into the liquid medium and then cultured into liquid medium with slow shaking condition.

7. Feeder Layer:

In many cases it is desirable to reduce the plating density, then a feeder layer consisting of X-irradiated non-dividing but living protoplasts are plated in agar medium and on this layer the isolated protoplasts are plated in a thin layer of liquid medium. Here the living but non-dividing protoplasts provide necessary growth requirement for the isolated less number of protoplasts.

Q.4 Define Haploid? Describe the Process of Haploid Production? 7

Ans: There are two approaches for the production of haploid plants. The two approaches are: (1) In Vivo Approach and (2) In Vitro Approach.

Haploid plants are characterized by possessing only a single set of chromosomes (gametophytic number of chromosomes i.e. n) in the sporophyte. This is in contrast to diploids which contain two sets (2n) of chromosomes. Haploid plants are of great significance for the production of homozygous lines (homozygous plants) and for the improvement of plants in plant breeding programmes.

Brief History:

The existence of haploids was discovered (as early as 1921) by Bergner in Datura stramonium. Plant breeders have been conducting extensive research to develop haploids. The Indian scientists Cuha and Maheswari (1964) reported the direct development of haploid embryos and plantlets from microspores of Datura innoxia by the cultures of excised anthers. Subsequently, Bourgin and Hitsch (1967) obtained the first full-pledged haploid plants from Nicotiana tabacum. Thereafter, much progress has been made in the anther cultures of wheat, rice, maize, pepper and a wide range of economically important species.

Grouping of Haploids:

Haploids may be divided into two broad categories:

1. Monoploids (monohapioids):

These are the haploids that possess half the number of chromosomes from a diploid species e.g. maize, barley.

2. Polyhaploids:

The haploids possessing half the number of chromosomes from a polyploid species are regarded as polyhaploids e.g. wheat, potato. It may be noted that when the term haploid is generally used it applies to any plant originating from a sporophyte (2n) and containing half the number (n) of chromosomes.

In Vivo and in Vitro Approaches:

The importance of haploids in the field of plant breeding and genetics was realised long ago. Their practical application, however, has been restricted due to very a low frequency (< 0.001%) of their formation in nature.

The process of apomixis or parthenogenesis (development of embryo from an unfertilized egg) is responsible for the spontaneous natural production of haploids. Many attempts were made, both by in vivo and in vitro methods to develop haploids. The success was much higher by in vitro techniques.

In vivo techniques for haploid production:

There are several methods to induce haploid production in vivo.

Some of them are listed below:

1. Androgenesis:

Development of an egg cell containing male nucleus to a haploid is referred to as androgenesis. For a successful in vivo androgenesis, the egg nucleus has to be inactivated or eliminated before fertilization.

2. Gynogenesis:

An unfertilized egg can be manipulated (by delayed pollination) to develop into a haploid plant.

3. Distant hybridization:

Hybrids can be produced by elimination of one of the parental genomes as a result of distant (interspecific or inter-generic crosses) hybridization.

4. Irradiation effects:

Ultra violet rays or X-rays may be used to induce chromosomal breakage and their subsequent elimination to produce haploids.

5. Chemical treatment:

Certain chemicals (e.g., chloramphenicol, colchicine, nitrous oxide, maleic hydrazide) can induce chromosomal elimination in somatic cells which may result in haploids.

In vitro techniques for haploid production:

In the plant biotechnology programmes, haploid production is achieved by two methods.

1. Androgenesis:

Haploid production occurs through anther or pollen culture, and they are referred to as androgenic haploids.

2. Gynogenesis:

Ovary or ovule culture that results in the production of haploids, known as gynogenic haploids.

Androgenesis:

In androgenesis, the male gametophyte (microspore or immature pollen) produces haploid plant. The basic principle is to stop the development of pollen cell into a gamete (sex cell) and force it to develop into a haploid plant. There are two approaches in androgenesis—anther culture and pollen (microspore) culture. Young plants, grown under optimal conditions of light, temperature and humidity, are suitable for androgenesis.

Anther Culture:

The selected flower buds of young plants are surface-sterilized and anthers removed along with their filaments. The anthers are excised under aseptic conditions, and crushed in 1% acetocarmine to test the stage of pollen development.

If they are at the correct stage, each anther is gently separated (from the filament) and the intact anthers are inoculated on a nutrient medium. Injured anthers should not be used in cultures as they result in callusing of anther wall tissue.

The anther cultures are maintained in alternating periods of light (12-18 hr.) and darkness (6-12 hrs.) at 28°C. As the anthers proliferate, they produce callus which later forms an embryo and then a haploid plant (Fig. 45.1).

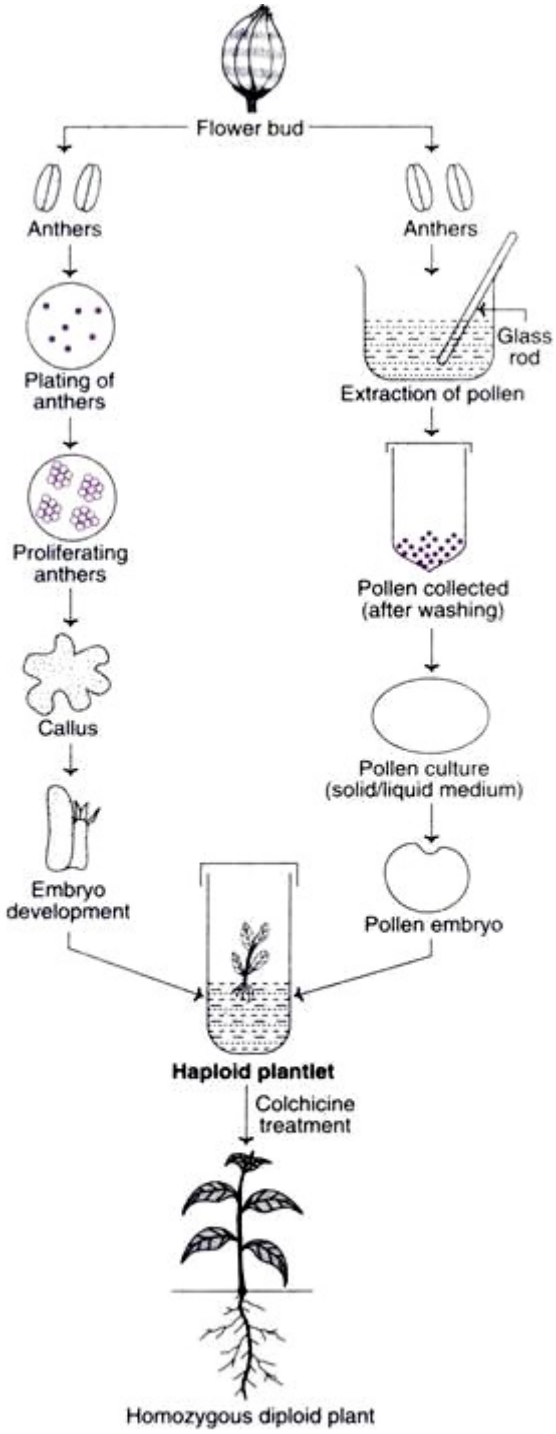

***Fig. 45.1** : Diagrammatic representation of anther and pollen cultures for the production of haploid and diploid plants.*

Pollen (Microspore) Culture:

Haploid plants can be produced from immature pollen or microspores (male gametophytic cells). The pollen can be extracted by pressing and squeezing the anthers with a glass rod against the sides of a beaker. The pollen suspension is filtered to remove anther tissue debris. Viable and large pollen (smaller pollen do not regenerate) are concentrated by filtration, washed and collected. These pollen are cultured on a solid or liquid medium. The callus/embryo formed is transferred to a suitable medium to finally produce a haploid plant (Fig. 45.1), and then a diploid plant (on colchicine treatment).

Comparison between anther and pollen cultures:

Anther culture is easy, quick and practicable. Anther walls act as conditioning factors and promote culture growth. Thus, anther cultures are reasonably efficient for haploid production. The major limitation is that the plants not only originate from pollen but also from other parts of anther. This results in the population of plants at different ploidy levels (diploids, aneuploids). The disadvantages associated with anther culture can be overcome by pollen culture.

Many workers prefer pollen culture, even though the degree of success is low, as it offers the following advantages:

i. Undesirable effects of anther wall and associated tissues can be avoided.

ii. Androgenesis, starting from a single cell, can be better regulated.

iii. Isolated microspores (pollen) are ideal for various genetic manipulations (transformation, mutagenesis).

iv. The yield of haploid plants is relatively higher.

Development of Androgenic Haploids:

The process of in vitro androgenesis for the ultimate production of haploid plants is depicted in Fig. 45.2.

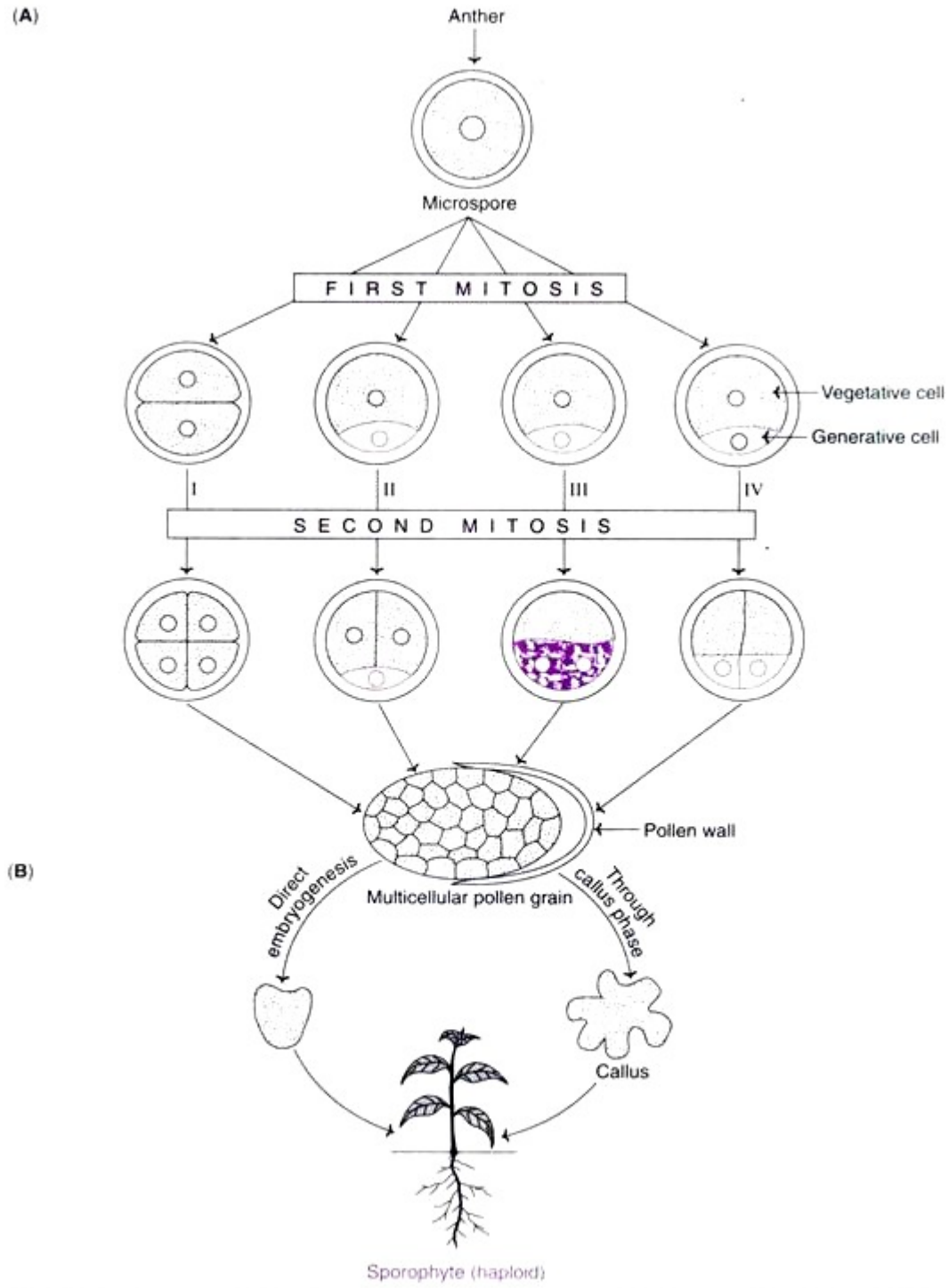

***Fig. 45.2** : Diagrammatic representation of microscope divisions leading to the formation of a multicelluar pollen grain (**A**), followed by the formation of haploid sporophyte (**B**) (**Note** : I, II, III and IV indicate respective pathways).*

The cultured microspores mainly follow four distinct pathways during the initial stages of in vitro androgenesis.

Pathway I:

The uninucleate microspore undergoes equal division to form two daughter cells of equal size e.g. Datura innoxia.

Pathway II:

In certain plants, the microspore divides unequally to give bigger vegetative cell and a smaller generative cell. It is the vegetative cell that undergoes further divisions to form callus or embryo. The generative cell, on the other hand, degenerates after one or two divisions—e.g., Nicotiana tabacum, Capsicum annuum.

Pathway III:

In this case, the microspore undergoes unequal division. The embryos are formed from the generative cell while the vegetative cell does not divide at all or undergoes limited number of divisions e.g. HyoScyamus niger.

Pathway IV:

The microspore divides unequally as in pathways I and II. However, in this case, both vegetative and generative cells can further divide and contribute to the development of haploid plant e.g. Datura metel, Atropa belladonna.

At the initial stages, the microspore may follow any one of the four pathways described above. As the cells divide, the pollen grain becomes multicellular and burst open. This multicellular mass may form a callus which later differentiates into a plant (through callus phase). Alternately, the multicellular mass may produce the plant through direct embryogenesis (Fig. 45.1).

Factors Affecting Androgenesis:

A good knowledge of the various factors that influence androgenesis will help to improve the production of androgenic haploids. Some of these factors are briefly described.

Genotype of donar plants:

The success of anther or pollen culture largely depends on the genotype of the donor plant. It is therefore important to select only highly responsive genotypes. Some workers choose a breeding approach for improvement of genotype before they are used in androgenesis.

Stage of microspore or pollen:

The selection of anthers at an ideal stage of microspore development is very critical for haploid production. In general, microspores ranging from tetrad to bi-nucleate stages are more responsive. Anthers at a very young stage (with microspore mother cells or tetrads) and late stage (with bi-nucleate microspores) are usually not suitable for androgenesis. However, for maximum production of androgenic haploids, the suitable stage of microspore development is dependent on the plant species, and has to be carefully selected.

Physiological status of a donar plant:

The plants grown under best natural environmental conditions (light, temperature, nutrition, CO_2 etc.) with good anthers and healthy microspores are most suitable as donor plants. Flowers obtained from young plants, at the beginning of the flowering season are highly responsive. The use of pesticides should be avoided at least 3-4 weeks preceding sampling.

Pretreatment of anthers:

The basic principle of native androgenesis is to stop the conversion of pollen cell into a gamete, and force its development into a plant. This is in fact an abnormal pathway induced to achieve in vitro androgenesis. Appropriate treatment of anthers is required for good success of haploid production.

Treatment methods are variable and largely depend on the donor plant species:

1. Chemical treatment:

Certain chemicals are known to induce parthenogenesis e.g. 2-chloroethylphosphonic acid (ethrel). When plants are treated with ethreal, multinucleated pollens are produced. These pollens when cultured may form embryos.

2. Temperature influence:

In general, when the buds are treated with cold temperatures (3-6°C) for about 3 days, induction occurs to yield pollen embryos in some plants e.g. Datura, Nicotiana. Further, induction of androgenesis is better if anthers are stored at low temperature, prior to culture e.g. maize, rye. There are also reports that pretreatment of anthers of certain plants at higher temperatures (35°C) stimulates androgenesis e.g. some species of Brassica and Capsicum.

Effect of light:

In general, the production of haploids is better in light. There are however, certain plants which can grow well in both light and dark. Isolated pollen (not the anther) appears to be sensitive to light. Thus, low intensity of light promotes development of embryos in pollen cultures e.g. tobacco.

Effect of culture medium:

The success of another culture and androgenesis is also dependent on the composition of the medium. There is, however, no single medium suitable for anther cultures of all plant species. The commonly used media for anther cultures are MS, White's, Nitsch and Nitsch, N6 and B5. These media in fact are the same as used in plant cell and tissue cultures. In recent years, some workers have developed specially designed media for anther cultures of cereals.

Sucrose, nitrate, ammonium salts, amino acids and minerals are essential for androgenesis. In some species, growth regulators — auxin and/or cytokinin are required for optimal growth. In certain plant species, addition of glutathione and ascorbic acid promotes androgenesis. When

the anther culture medium is supplemented with activated charcoal, enhanced androgenesis is observed. It is believed that the activated charcoal removes the inhibitors from the medium and facilitates haploid formation.

Gynogenesis:

Haploid plants can be developed from ovary or ovule cultures. It is possible to trigger female gametophytes (megaspores) of angiosperms to develop into a sporophyte. The plants so produced are referred to as gynogenic haploids.

Gynogenic haploids were first developed by San Noem (1976) from the ovary cultures of Hordeum vulgare. This technique was later applied for raising haploid plants of rice, wheat, maize, sunflower, sugar beet and tobacco.

In vitro culture of un-pollinated ovaries (or ovules) is usually employed when the anther cultures give .unsatisfactory results for the production of haploid plants. The procedure for gynogenic haploid production is briefly described.

The flower buds are excised 24-48 hr. prior to anthesis from un-pollinated ovaries. After removal of calyx, corolla and stamens, the ovaries (see Fig. 45.3) are subjected to surface sterilization. The ovary, with a cut end at the distal part of pedicel, is inserted in the solid culture medium.

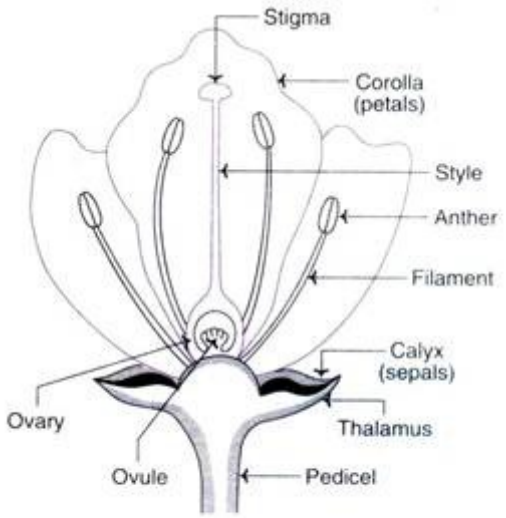

***Fig. 45.3** : A diagrammatic representation of important parts in a flower.*

Whenever a liquid medium is used, the ovaries are placed on a filter paper or allowed to float over the medium with pedicel inserted through filter paper. The commonly used media are MS, White's, N6 and Nitsch, supplemented growth factors. Production of gynogenic haploids is particularly useful in plants with male sterile genotype. For such plant species, this technique is superior to another culture technique.

Limitations of Gynogenesis:

In practice, production of haploid plants by ovary/ ovule cultures is not used as frequently as anther/ pollen cultures in crop improvement programmes.

The major limitations of gynogenesis are listed:

1. The dissection of unfertilized ovaries and ovules is rather difficult.

2. The presence of only one ovary per flower is another disadvantage. In contrast, there are a large number of microspores in one another.

However, the future of gynogenesis may be more promising with improved and refined methods.

Identification of Haploids:

Two approaches based on morphology and genetics are commonly used to detect or identify haploids.

Morphological Approach:

The vegetative and floral parts and the cell sizes of haploid plants are relatively reduced when compared to diploid plants. By this way haploids can be detected in a population of diploids. Morphological approach, however, is not as effective as genetic approach.

Genetic Approach:

Genetic markers are widely used for the specific identification of haploids. Several markers are in use.

i. 'a_1' marker for brown coloured aleurone.

ii. 'A' marker for purple colour.

iii. 'Lg' marker for ligule less character.

The above markers have been used for the development of haploids of maize. It may be noted that for the detection of androgenic haploids, the dominant gene marker should be present in the female plant.

Diploidizatioim of Haploid Plants (Production of Homozygous Plants):

Haploid plants are obtained either by androgenesis or gynogenesis. These plants may grow up to a flowering stage, but viable gametes cannot be formed due to lack of one set of homologous chromosomes. Consequently, there is no seed formation.

Haploids can be diploidized (by duplication of chromosomes) to produce homozygous plants. There are mainly two approaches for diploidization— colchicine treatment and endomitosis.

Colchicine Treatment:

Colchicine is very widely used for diploidization of homologous chromosomes. It acts as an inhibitor of spindle formation during mitosis and induces chromosome duplication. There are

many ways of colchicine treatment to achieve diploidization for production of homozygous plants.

1. When the plants are mature, colchicine in the form of a paste is applied to the axils of leaves. Now, the main axis is decapitated. This stimulates the axillary buds to grow into diploid and fertile branches.
2. The young plantlets are directly treated with colchicine solution, washed thoroughly and replanted. This results in homozygous plants.
3. The axillary buds can be repeatedly treated with colchicine cotton wool for about 2-3 weeks.

Endomitosis:

Endomitosis is the phenomenon of doubling the number of chromosomes without division of the nucleus. The haploid cells, in general, are unstable in culture with a tendency to undergo endomitosis. This property of haploid cells is exploited for diploidization to produce homozygous plants.

The procedure involves growing a small segment of haploid plant stem in a suitable medium supplemented with growth regulators (auxin and cytokinin). This induces callus formation followed by differentiation. During the growth of callus, chromosomal doubling occurs by endomitosis. This results in the production of diploid homozygous cells and ultimately plants.