PHARMACOGNOSY AND PHYTOCHEMISTRY

31

Unit III

Plant tissue culture:

- **Definition:** Plant tissue culture is an invitro technique to produce clones of a plant by using plant cells, tissues or organs under controlled environment on a nutrient culture medium.

- **Advantages of plant tissue culture:**

- Production of genetically modified plant.
- To quickly produce mature plants.
- Production of disease free plant due to controlled environment.
- Production of exact copies of plant.
- Suitable technique for production of useful primary & secondary metabolites.
- Modification in chemical structure.
- Crop improvement.

Historical development of plant tissue culture :-

Year	Scientists	Historical development
1902	Haberlandt	1st - Proposed the concept of plant tissue culture.
1904	Hanning	Establishment of embryo culture for 1st time.
1909	Kuster	1st observation of fusion cells.
1922	Kotte, Robins	In-vitro cultivation of root tips.
1934	White	1st permanent root cultures.
1934	Gautheret	1st permanent callus culture using vit B & auxins.
1942	Gautheret	Observation of secondary metabolites in plant callus culture.
1955	Mothes & Kala	1st report of secondary metabolite production in liquid media.
1960	Bergmann	Cell clones obtained from single cultured cells on agar medium.

1965	Vasil & Hildebrandt	Regeneration of a plant from one single cell.
1966	Kohlenbach	1st cell division & culture of differentiated mesophyll cells.
1971	Nagata & Takebe	Regeneration of plant from cultured protoplasts.
1977	Noguchi	cultivation of tobacco cells
1983	Barton Brill	Insertion of foreign genes attached to a plasmid. (small, double stranded DNA molecule, exist in bacterial cells)
1988	Simon	obtained success in regeneration of bulky callus, buds & roots.

Nutritional requirements of PTC :-

→ To maintain the vital functions of a culture, the culture media consists of various elements. They are as follows :-

(I) Inorganic nutrients
- (a) → Macronutrients
- (b) → Micronutrients

(II) Organic nutrients
- (a) → Vitamins
- (b) → Carbon / Energy sources.
- (c) → Growth regulators / Phytohormones.

(III) Solidifying agents.

(IV) Antibiotics.

(V) pH

(I) Inorganic nutrients :

(a) Macronutrients : The macronutrients include six major elements :-

- Nitrogen (N)
- Phosphorus (P)
- Potassium (K)
- Calcium (Ca)
- Magnesium (Mg)
- Sulphur (S).

→ These elements are used in large amount as stock solution for plant growth & development.

(b) Microelements: These elements are required in trace or in very small amounts for plant growth & development.

→ Examples: – Manganese – Molybdenum
– Iodine – Iron
– Copper – Zinc.
– Cobalt
– Boron

(II) Organic nutrients :-

(a) Vitamins : Thiamine (Vit B_1)
Pyridoxine (Vit B_6)
Nicotinic acid (Vit B_3)
Calcium pentothenate (Vit B_5).

(b) Carbon/energy source :

– Sucrose (2-5%) – Galactose
– Glucose – Mannose
– fructose – lactose
– Maltose – Sorbitol.

(c) Growth regulators / Phyto hormones :-

→ The growth regulators or plant hormones are required to promote the growth of cells.

→ Examples –
- Auxins
- Cytokinin
- Gibberelline
- Kinetin.

(III) Solidifying agents :-

→ To improve the oxygen supply & support the culture growth, solid media are generally preferred to liquid cultures.

→ for these purpose, substance with strong gelling capacity added into the liquid media.

→ Examples –
- Agar
- Alginate
- Gelatin
- Hydroxyethylcellulose
- Starch
- Silica gel.

(IV) Antibiotics –
e.g. – Streptomycin
– Kanamycin.

(V) pH : The pH of nutritional medium is generally adjusted between 5.0 to 6.0.

General procedure involved in PTC :- (Growth & their maintanance)

(1) Sterilization of glasswares.
(2) Preparation & sterilization of explant.
(3) Production of callus from explant.
(4) Proliferation of cultured callus.
(5) Subculturing of callus.
(6) Suspension culture.

Proliferation
↓
rapid rise in no. of something

(1) Sterilization of glasswares :-

→ All the glasswares to be used in tissue culture are firstly washed & sterilized properly.

→ To make them free from any dirt, waxy material or bacteria all the glasswares should be kept overnight dipped in sodium dichromate soln / Sulphuric acid sol^n & next day glasswares should be washed with fresh tap water & then distilled water.

→ For sterilization, glasswares were placed in hot air oven at 120°C for 1/2 - 1 hour.

(2) Preparation & sterilization of explant :

Explant - Explant is a portion of plant body which has been taken from any part of plant like root, stem, leaf, stamen, anthers, etc to establish a culture.

→ for the surface sterilization of explant, chromic acid (H_2CrO_4), mercuric chloride (0.11%), calcium hypochlorite, sodium hypochlorite (1-2%) & alcohol (70%) are used.

(3) Production of callus from explant :

→ **Callus** - It is an unorganized & undifferentiated cell mass.

→ In this step, the sterilized explant is transferred aseptically on to the nutrient medium contained in flasks.

↓

now, the flask is transferred to BOD incubator for maintenance of culture.

↓

Sufficient amount of callus is produced within 3-8 days of incubation.

→ In this, the temperature is adjusted to 25±2°C.
→ Some amount of light is necessary for callus production.

(4). Proliferation of callus :-

→ If callus is well developed, it should be cut into small pieces & transferred to another fresh medium containing some amount of hormones ~~for~~ which supports growth.

→ The medium used for the production of more amount of callus is called proliferation medium.

(5) Subculturing of callus :-

→ After sufficient growth of callus, it should be periodically transferred to fresh medium to maintain the viability of cells.
→ This subculturing will be done in an interval of 4-6 weeks.

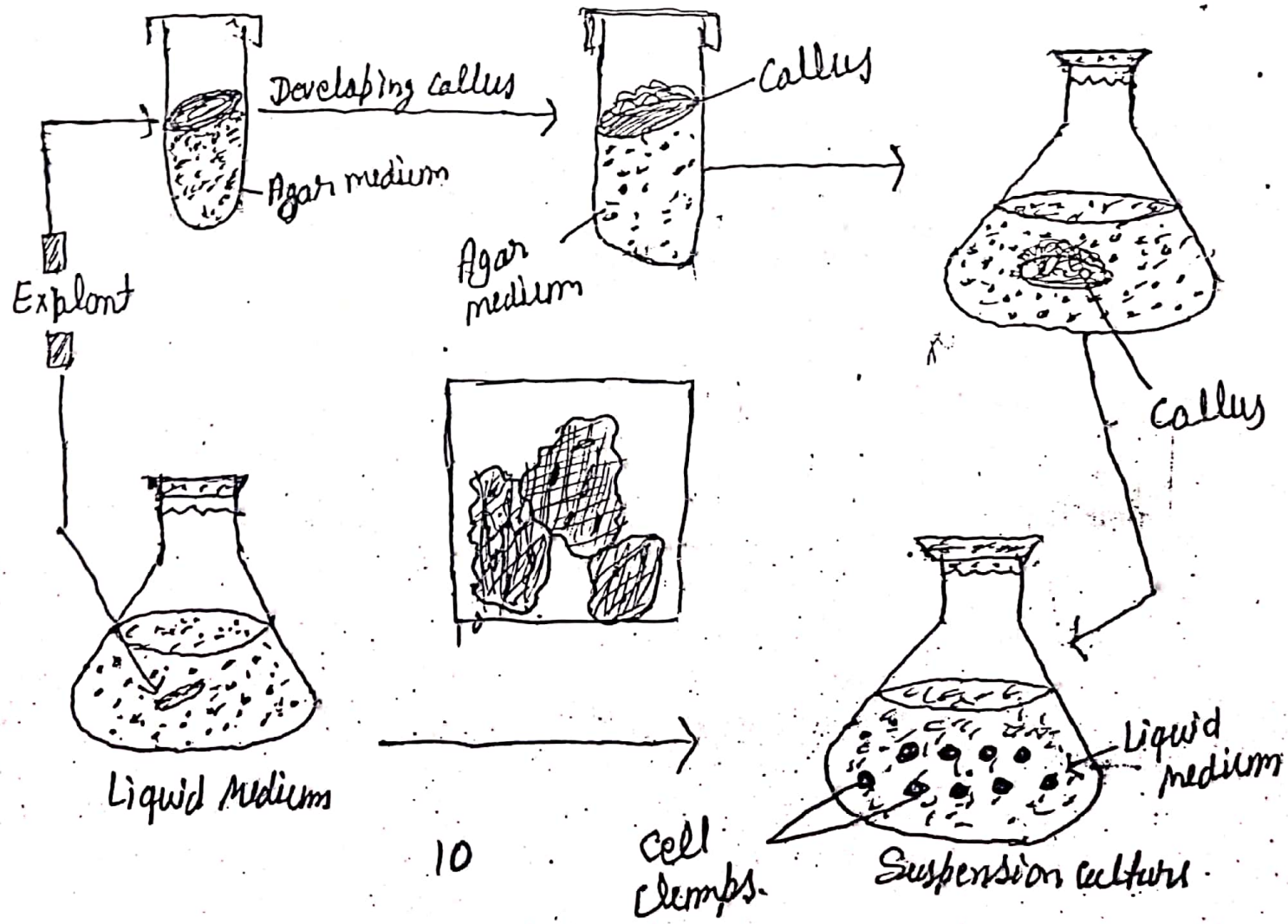
(6) Suspension culture :-

→ Suspension culture contains a uniform suspension of separate cells in liquid medium.

→ For the preparation of suspension culture, callus is transferred to liquid medium, which is agitated continuously to keep the cells separate.

→ Agitation can be achieved by rotary shaker system attached within incubator at 50 – 150 rpm.

fig :→ Growth & their maintenance of PTC



Types of culture

UNIT → 3 Part → 2

(1) Flower organ culture
(2) Anther & pollen culture
(3) Shoot tip culture
(4) Root culture.
(5) Protoplast culture
(6) Embryo culture
(7) Ovule culture.
(8) Leaves culture.

(1) Flower culture:

→ flower buds or mature flowers are collected
↓
washed thoroughly & dipped in 5% Teepol sol? for 10 min.
↓
rewashed
↓
Transfer them to laminar air flow cabinet.
↓
Surface sterilization is done by immersing it in 5% Sodium hypochlorite
↓
washed with distilled water.
↓
using sterilized forceps, transfer the flower bud or mature flower to culture tubes containing media
↓
now incubate the culture for 16 hrs at 25°C under light.

(2) Anther & pollen culture :

2) ~~Pollen grains &~~ anthers are excised from flower.

↓

They are superficially sterilized & washed with double distilled water

↓

now, they are directly transferred on nutrient agar or liquid medium.

↓

Embryogenesis occur.

(b) Pollen grains are aseptically removed from the anthers & cultured on liquid medium. They gives rise to haploid embryos or callus & it is called as pollen culture.

(3) Shoot tip culture :

→ Shoot tips (100–1000 Hm long) excised from plant species.

↓

transferred into nutrient media containing growth hormone

↓

After some days, root forms

↓

develop into whole plants.

(4) Root tip culture :-

Roots are excised from plant
↓
Sterilized & transferred to fresh medium.
↓
The lateral roots continue to grow & provide several roots
↓
maintained in continuous culture
↓
develop into whole plant after some days.

(5) Leaf culture :-

Leaves (800 µm) detached or excised from shoot
↓
Surface sterilization is done
↓
placed on solidified medium.
↓
~~Long~~ Young leaves have more growth potential
↓
maintained a culture & develop into plant.

(6) ~~Ovule &~~ Embryo culture :-

Embryo is obtained or excised or isolated from ovule, seed or fruit
↓
now, they are aseptically transferred to the simple nutrient medium.

the nutrient media containing mineral salts, sugar & agar for growth & development.

↓

cultures are kept at 25 ± 1°C for 16 hours in a culture room

↓

sub-culturing is done in fresh medium in the interval of 4 weeks.

(7) Protoplast culture:

→ A cell without cell wall is called as protoplast.

→ Leaf is excised

↓

Surface sterilization is done

↓

Removal of epidermal tissue

↓

cutting into small fragments.

↓

~~For~~ Cell wall is removed by enzymatic method (treating the cells with enzyme like cellulose & pectinase)

↓

Protoplast

↓

obtained protoplast is cleaned by centrifugation.

↓

plating of protoplast is done by transferring protoplast & in petridish containing ~~neo~~ nutrient medium & incubated in BOD incubator

wall regeneration

↓

Cell division occurs & forms callus within

& 2-3 weeks

↓

The callus is then subcultured on fresh medium containing plant hormones.

↓

Develop into plantlets.

Totipotency:

→ The ability of a plant cell to develop into a whole plant is called as totipotency.

Applications of plant tissue culture :-

1 → To study the respiration & metabolism of plants.

2 → for the evaluation of organ functions in plants.

3 → To study the various plant diseases.

4 → In the production of plant clones ~~cells~~ with new characteristics.

→ Production of haploids for improving crops.

→ Production of genetically modified plants.

7 → To conserve rare or endangered plant species.

8 → To study the molecular basis for physiological, biochemical & reproductive mechanisms in plants.

9 → Virus eradication.

10 → Production of phytoconstituents in increased amount.

11 → Micropropagation (regeneration of whole plant through tissue culture).

12 → for the improvement in crops.

Edible vaccines :-

→ Vaccine - A vaccine is a biological preparation that contains an antigenic material to stimulate an individual's immune system to develop an adaptive immunity to a ~~pathogen~~ particular disease.

→ Edible - "capable of being eaten". -खाने योग्य

1 → Edible vaccine: The vaccines that one can eat are called edible vaccines.

2 → These are the mucosal targeted vaccines, which cause stimulation of both systematic & mucosal immune response.

3 → In the edible vaccine, transgenic plants are used as vaccine production systems.

4 → Initially, it was useful only for preventing infectious disease, But now, it has also found application in prevention of autoimmune diseases, cancer therapy, etc.

5 → The edible vaccine is also known as "plant-based vaccines".

6 → The concept of edible vaccines was developed by Charles Arntzen in the 1990.

- **Advantages of edible vaccines :-**

2 → Ease of administration.
Z → No need of medical personnel.
3 → Avoidance of sterile injections.
4 → Economical in mass production & transportation.
5 → ~~Strong~~ Easy to store.
C → Heat stable, ~~during~~ eliminating the need for refrigeration.
7 → Generation of systemic & mucosal immunity.
8 → They are cheap.

- **Limitations of edible vaccines :**

→ Selection of best plant is difficult.
→ Evaluating dosage requirement.
→ Certain foods like potato are not eaten raw, & cooking the food might weaken the medicine present in it.

- **Working phenomenon of edible vaccines :-**

→ Edible vaccines contain DNA fragments from the original pathogen. These fragments code for a protein & responsible for giving body's immune response.

- **Plants used for edible vaccine :-**

– Tobacco	– Carrot	– Soyabean
– Potato	– Peanut	
– Banana	– Wheat	
– Rice	– Corn	

- **Examples of edible vaccine :**

S.No.	Vaccines	Vector used	Disease.
(1)	Hepatitis B virus	Tobacco Potato	Hepatitis B.
(2)	Norwalk virus	Tobacco potato	Diarrhoea Nausea Stomach cramps
(3)	Rabies virus	Tobacco	Rabies
(4)	HIV virus	Tomato	AIDS
(5)	Vibrio cholerae	Potato	Cholera.

Washing and storage facilities

First and foremost requirement of the tissue culture laboratory is provision for fresh water supply and disposal of the waste water, and space for distillation unit for the supply of distilled and double distilled water and de-ionized water. Acid and alkali resistant sink or wash basin for apparatus/equipment washing and the working table should also be acid- and alkali-resistant.

Sufficient space is required for placing hot air oven, washing machine, pipette washers and the plastic bucket or steel tray for soaking or drainage of the detergent bath or extra water. For the storage of dried glassware separate dust proof cupboards or cabinet should be provided. It is mandatory to maintain cleanliness in the area of washing, drying and storage.

Media preparation room

Media preparation room should have sufficient space to accommodate chemicals, lab ware, culture vessels and equipments required for weighing and mixing, hot plate, pH meter, water baths, Bunsen burners with gas supply, microwave oven, autoclave or domestic pressure cooker, refrigerator and freezer for storage of prepared media and stock solutions.

Sterilization Room

For the sterilization of culture media, a good quality ISI mark autoclave is required and for small amount domestic pressure cookers, can also serve the purpose. For the sterilization of glassware and metallic equipments hot air oven with adjustable tray is required.

Aseptic chamber/area for transfer of culture

For the transfer of culture into sterilized media, contaminant-free environment is mandatory. The simplest type of transfer area requires an ordinary type of small wooden hood, having a glass or plastic door either sliding or hinged fitted with UV tube. This aseptic hood can be conveniently placed in a quiet corner of the laboratory.

These days, modern laboratory have laminar airflow cabinet having vertical or horizontal airflow, arrange over the working surface to make it free from dust particles/micro-contaminants.

The air coming out of the fine filter (a 0.3-μm HEPA filter) is ultra-clean (free from fungal or bacterial contaminant) and having adequate velocity (27±3 m/min) to prevent micro-contamination of the working area by worker sitting in front of the cabinet.

Inside the cabinet, there is arrangement for Bunsen burner and a UV tube fitted on the ceiling of the cabinet (to make area free from any live contamination). The advantage of working in the laminar airflow cabinet is that the flow of air does not hamper the use of Bunsen burner and moreover, the cabinet occupies relatively small space within the laboratory (Fig. 26.1).

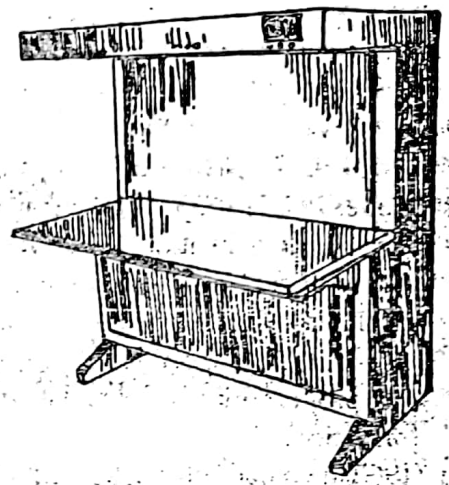


Fig. 26.1 Laminar air flow

Incubation room or incubator

Environmental factors have great effect on the growth and differentiation of cultured tissues. Therefore, it is very much essential to incubate all types of cultures in well-controlled environmental conditions, like temperature, humidity, illumination and air circulation. A typical incubation chamber or area should have both light and temperature controlled devices managed for 24 h period. Air conditioners or room heaters are required to maintain the temperature at 25±2°C. Light is adjusted in the terms of photo period duration (specified period for total darkness as well as for higher intensity light). Further the requirement for humidity range of 20–90% controllable to ±3% and uniform forced air circulation can be achieved.

The incubation chamber or room should have the provision for storing the culture vessels (flask, jars and petri dishes). Shelves should be designed in such a way so that the culture vessels can be placed in the shelf or trays in such a way that there should not be any hindrance in the light, temperature and humidity maintenance. A label having full detail about date of inoculation, name of the explant, medium and any other special information should stuck on each tray and rack to ensure identity and for maintaining the data of experiment. In the case of suspension culture arrangement for shaker should also be made.

These days BOD incubators (Fig. 26.2) with all the requisite environmental condition maintenance are available in the market, they occupy less space and manageable with small generator or automatic invertor in the case of electricity failure to maintain the necessary light and temperature conditions. Failure of electricity may spoil important experiment and in the case of suspension culture the whole culture may get damaged due to stoppage of the shaker.

BOD incubators required to maintain the culture conditions should have the following characteristics:

- Temperature range, 2–40°C
- Temperature control ±0.5°C