PLANT TISSUE CULTURE

Introduction- Tissue culture is in vitro cultivation of plant cell or tissue under aseptic and controlled environment conditions, in liquid or on semisolid well- defined nutrient medium for the production of primary and secondary metabolite or to regenerate plant.

The whole process requires a well equipped culture laboratory and nutrient medium. This process involves various steps, viz preparation of nutrient medium containing inorganic and organic salts, supplemented with vitamins, plant growth hormone and amino acids as well as sterilization of explants, glassware and other accessories inoculation and incubation.

Advantages – 1) Availability of raw material
2) Fluctuation in supplies and quality
3) Patent right
4) Political reasons
5) Easy purification of the compound
6) Modification in chemical structure
7) Disease-free and desired propagule
8) Crop improvement
9) Biosynthetic pathway
10) Immobilization of cells

Availability of raw material- some plants are difficult to cultivate and are also not available in abundance. In such case, the biochemical/bioproducts from these plants cannot be obtained economically in sufficient quantity. So tissue culture is considered a better source for regular and uniform supply of raw material, manageable under regulated and reproducible conditions in the medicinal plants industry for the production of phytopharmaceuticals.

Fluctuation in supplies and quality- The production of crude drugs is subject to variation in quality due to changes in climate, crop diseases and seasons. The method of collection, drying and storing also influence the quality of crude drug. All these problems can be overcome by tissue culture techniques.

Patent rights- Naturally occurring plants or their metabolites cannot be patented as such. Only a novel method of isolation can be patented. For Rand D purpose, the industry has to spend a lot of money and time to launch a new natural product but can't have patent right. Hence, industries prefer tissue culture for production of biochemical compounds. By this method ,it is possible to obtain a constant supply and new methods can be developed for isolation and improvement of yield , which can be patented.

Political reasons-if a natural drug is successfully marketed in a particular country of its origin, the government may prohibit its export to up-value its own exports by supplying its phytochemical product, e.g. *Rauwolfia serpentina* and *dioscorea spp*.from India. Similarly the production of opium in the world is governed by as such by political

consideration, in such case, if work is going on the same drug; it will be either hindered or stopped. Here also, plant tissue culture is the solution.

Easy purification of the compound- The natural products from plant tissue culture may be easily purified because of the absence of significant amounts of pigments and other unwanted impurities. With the advancement of modern technology in plant tissue culture, it is also possible to biosynthesize those chemical compounds which are difficult or impossible to synthesize.

Modification in chemical structure- some specific compound can be achieved more easily in cultured plant cells rather than by chemical synthesis or by microorganism.

Disease-free and desired propagule- plant tissue culture is advantageous over conventional method of propagation in large scale production of disease free and desired propagules in limited space and also the germplasm could be stored and maintained without any damage during transportation for subsequent plantation.

Crop improvement-Plant tissue culture is advantageous over the conventional cultivation techniques in crop improvement by somatic hybridization or by production of hybrids.

Biosynthetic pathway- Tissue culture can be used for tracing the biosynthetic-pathways of secondary metabolites using labeled precursor in the culture medium.

Immobilization of cells- Tissue culture can also be used for plants preservation by immobilization of cell further facilitating transportation and biotransformation.

History of Plant Tissue Culture

The German Botanist Guttlieb Haberlandt first proposed the importance of plant tissue and cell culture in isolation, in 1902. He is regarded as the father of plant tissue culture. He used tissue of Lamium Puroureum and Eichhornia crassipes, the epidemis of Ornithooalium and epidermal hairs of Pulmonaria Mollissima. He grew them on a particular salt solution with sucrose and observed obvious growth in the cells. The cells remained alive for up to 1 month. They grew in size, changed shape; thickening of cell walls occurred and starch appeared in the chloroplasts, which initially lacked it. However, none of the cells divided. The failure was that he was handling highly differentiated cells and the present day growth hormones, necessary for inducing division in mature cells, were not available to him. Hanning (1940) had initiated a new line of investigation, which later developed into an important applied area of in-vitro techniques. Hanning excised nearly mature embryos of some plants like Raphanus Sativus and successfully grew them to maturity on mineral salts and sugar solution. Van Overbeck (1941) and co-workers demonstrated for the first time the stimulatory effect of coconut milk, which was similar to embryo sac fluid, on embryo development and callus formation in Datura. This proved a turning point in the field of embryo culture, for it enabled the culture of young embryos which failed to grow on a mixture of mineral salts, vitamins, amino acids and sugar. Subsequent detailed work by Raghavan and Torrey

(1963), Norstog (1965) and others led to the development of Synthetic media for the culture of younger embryos. Laibach (1925, 1929) demonstrated the practical applicafion of embryo culture in the field of plant breeding. He isolated embryos from nonviable seeds of a particular plant and reared them to maturity on a nutrient medium. In 1922, working independently Robbins (USA) and kotte (Germany) reported some success with growing isolated root tips. White made the first successful report of continuously growing tomato root tips in 1934. During 1939 - 1950 extensive work on root culture was undertaken by Street to understand the role of vitamins implant growth and shoot-root relationship. Gautheret (1934), White (1939) and Nobecourt successfully cultured cells of Salix, Nicotiana-Hybrid and carrot on synthetic media. They, for the first time, demonstrated that growth regulators and vitamins if added to media enhanced the growth forming mess of cells called callus. Skoog (1944), Tsui (1951), and Miller (1955) demonstrated the induction of divisions in isolated, mature and differentiated cells by using synthetic as well as natural compounds. Muir (1953) developed a technique of growing single cells into liquid medium in case of Tenetes Erecta and Nicotiana Tabacum. Vasil and Hildeprandt (1965) raised whole plants starting from single cells of tobacco. Skoog and Miller (1957) showed that changing the relative concentrations of the two substances in the medium could regulate the organ differentiation. The first reports of some embryo formation from Carrot tissue appeared in 1958-59 by Reinert (Germany) and Steward (USA). Ball (1946) successfully raised whole plants of Lupinus and Tropaeolum by culturing shoot tips. Morel and Martin (1952), for the first time, recovered virus-free Dahlia plants from infected individuals by culturing their shoots. Murashige (USA) used this technique to muUiply plants in large number for several species ranging from ferns to foliage, flower and fruit plants. Guha and Maheshwari (1966) demonstrated the possibility of raising large numbers of plantlets from pollen grains of Dhatura. In 1972 Carlson and others produced the first somatic hybrid between two plants by fiising their protoplasts.

Basic Requirement for Plant Tissue Culture

For tissue culture technique a tissue culture laboratory should have the following general basic facilities:

1. Equipment and apparatus
2. Washing and storage facilities
3. Media preparation room
4. Sterilization room
5. Aseptic chamber for culture
6. Culture rooms or incubators fully equipped with temperature, light and humidity control devices
7. Observation or recording area well equipped with computer for data processing

Equipment and apparatus

Culture vessels and glassware- Many different kind of vessels may be used for wing cultures. Callus culture can be grown successfully in large test tubes (25×150mm) or wide mouth conical flasks. In addition to the culture vessels, glassware such as graduated pipettes, measuring cylinders, beaker, filters, funnel, and petri dishes are also required for making preparations. All the glasswares should be of pyrex or corning.

Equipment- scissors, scalpels and forceps for explants preparation from excised plant parts and for their transfer.

- a spirit burner or gas micro burner for flame sterilization of instruments
- an autoclave to sterilize the media
- hot air oven for the sterilization of glassware,
- a p^H meter for adjusting the p^H of the medium
- a shaker to maintain cell suspension culture
- a balance to weigh various nutrients for the preparation of the medium
- incubating chamber or laminar air flow with uv light fitting for aseptic transfer of explants to the medium and for subculturing
- a BOD incubator for maintaining constant temperature to facilitate the culture of callus and its subsequent maintenance

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, p^H meter, water baths, Bunsen burners with gas supply, microwave oven, autoclave or domestic pressure cooker, refrigerator and freezer for storage of preparaed media and stock solutions.

Sterilization room

For the sterilization of culture media, a good quality ISI marks 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 for 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 can be conveniently placed in a quiet corner of the laboratory.
Modern laboratory have laminar air flow cabinet having vertical or horizontal airflow, arrange over the working surface to make it free from dust particle/micro contaminants.

Incubation room or incubator

Environmental factors affect the growth and differentiation of cultured tissues. A typical incubation chamber or area should have both light and temperature controlled devices managed for 24 h period.
AC or room heaters are required to maintain the temperature at 25 ± 2^0c.
Light should be adjusted in the terms of photo period duration.
Humidity should be in the range of 20-90%.
Shelves should be designed in such a way so that the culture vessels can be placed in the shelf or trays in such a ways that there should not be any hindrance in the light, temperature and humidity maintenance. A label should be stick on the each tray and rack to ensure identity and for maintaining the data of experiment. Label should having the full detail about date of inoculation, name of explants, medium and any other special information.
These days BOD incubators with all the requisite environmental condition maintenance are available in the market.
They occupy less space and manageable with small generator or automatic inverter in the case of electricity failure to maintain the necessary light and temperature conditions.
BOD incubators required to maintain the culture conditions should have the following characteristics:

- Temperature range, $2\text{-}40^0$c
- Temperature control$\pm0.5^0$c
- Automatic digital temperature recorder
- 24-h temperature and light programming
- Adjustable fluorescent lighting up to 10,000lux
- Relative humidity range 20-98%
- Relative humidity control±3%
- Uniform forced air circulation
- Shaker
- Capacity up to $0.7m^3$of $0.5m^2$shelf space

  Data collection and recording the observation-

  The growth and maintenance of the tissue culture in the incubator should be observed and recorded at regular intervals. All the observations should be done in aseptic environment, i.e. in the laminar airflow. Separate dust free space should be

marked for microscopic work. All the recorded data should be fed into the computer.

General procedures for plant tissue culture –

- Sterilization of glassware tools/vessels
- Preparation and sterilization of explants
- Production of callus from explants
- Proliferation of cultured callus
- Sub culturing of callus
- Suspension culture

Sterilization of glassware tools/vessels- All the glassware should be of Pyrex or corning. All the glassware should be kept overnight dipped in sodium dichromate-sulphuric acid solution. Next morning, glassware should be washed with fresh running tap water, followed by distilled water and placed in inverted position in plastic bucket or trays to remove the extra water. For drying the glassware, it is placed in hot air oven at high temperature about 120^0c for ½- 1 h.

In the case of plastic lab ware, washing should be carried out with a mild nonabrasive detergent followed by washing under tap water or the plastic ware after general washing with dilute sodium bicarbonate and water followed by drainage of extra water, rinsed with an organic solvent such as alcohol, acetone and chloroform. Washed and dried glassware or plastic ware should be stored in dust proof cupboards.

To prevent reinfection following sterilization, empty containers are wrapped with aluminium foil. Stainless steel, metal tools (knives, scalpels, forceps, etc) are also wrapped with aluminium foil and pads o cotton wool are stuffed into the opening of the pipettes, which are either also wrapped in aluminium or placed in an aluminium or stainless steel box. The period of sterilization usually ranges between 1 and 4 hour.

Preparation of Explant-

Explants can be defined as a portion of plant body, which has been taken from the plant to establish a culture. Explant can be obtained from plants, which are grown in controlled environmental conditions. Such plants will be usually free from pathogens and are homozygous in nature. Explant may be taken from any part of the plant like root, stem, leaf or meristematic tissue like cambium, floral parts like anthers, stamens etc.

Age of the explant is also an important factor in callus production. Young tissues are more suitable than mature tissues. A suitable portion from the plant is removed with the help of sharp knife, and the dried and mature portion are separated from young tissue. When seeds and grains are used for explants preparation, they are directly sterilized and put in nutrient medium. After germination, the obtained seedlings are to be used for explants preparation.

Surface sterilization of explant-

For the surface sterilization of the explant, chromic acid, mercuric chloride(0.11%), calcium hypochlorite(1-2%) and alcohol(70%) are used. Usually the tissue is immersed in the solution of sterilizing agent for 10 s to 15 min, and they are washed with distilled water. Repeat the treatment with sodium hypochlorite for 20 min, and the tissue is finally

washed with sterile water to remove sodium hypochlorite. Such tissue is used for inoculation.

The explants are sterilized by exposing to aqueous sterilized solution of different concentration.

Different types of surface sterilizing agent are-

Name of chemical	**Concentration%**	**Exposure(min)**
Bromine water	1-2	2-10
Benzalkonium chloride	0.01-0.1	5-20
Sodium hypochlorite	0.5-51	5-30
Calcium hypochlorite	9-10	5-30
Mercuric chloride	1-2	2-10
Hydrogen peroxide	3-10	5-15
Silver nitrate	1-2	5-20

In the case of leaf or green fresh stem the explant needs pretreatment with wetting agent (70-90% ethyl alcohol, Tween20), 5-20 drops in 100 ml of purified water or some other mild detergent to be added directly into the sterilization solution to reduce the water repulsion (due to waxy secretion)

Procedure to be followed for respective explants is as follows:

Seeds-

- Dip the seeds into absolute ethyl alcohol for 10s and rinse with purified water.
- Expose seeds for 20-30 min to 10% w/v aqueous calcium hypochlorite or for 5 min in a 1% solution of bromine water.
- Wash the treated seeds with sterile water followed by germination on damp sterile filter paper.

Fruits-

- Rinse the fruit with absolute alcohol.
- Submerge into 2% (w/v) solution sodium hypochlorite for 10 min.
- Washing repeated with sterile water and remove seeds of interior tissue.

Stem-

- Clean the explants with running tap water followed by rinsing with pure alcohol.
- Submerge into 2% (w/v) solution sodium hypochlorite for 15-30 min.
- Wash three times with sterile water.

Leaves-

Clean the leaf explant with purified water to make it free from dirt and rub the surface with absolute ethyl alcohol. Dip the explants in 0.1%(w/v) mercuric chloride solution, wash with sterile water to make it free from chloride and then dry the surface with sterile tissue paper.

Production of callus from explants-

The sterilized explant is transferred aseptically onto defined medium contained in flasks. The flasks are transferred to BOD incubator for maintenance of culture. The temperature

is adjusted to 25 ± 2^0c. Some amount of light is necessary for callus (undifferentiated amorphous cell mass) production. Usually sufficient amount of callus is produced within 3-8 days of incubation.

Proliferation of callus-

If callus is well developed, it should be cut into small pieces and transferred to another fresh medium containing an altered composition of hormone, which supports growth. The medium used for production of more amount of callus is called proliferation medium.

Sub culturing of callus-

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

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 the incubator at a rate of 50-150 rpm. After the production of sufficient number of cells subculturing can be done.