Plant Tissue culture

Plant tissue culture, also referred to as cell, in vitro, axenic, or sterile culture, is an important tool in both basic and applied studies, as well as in commercial application. Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions in vitro. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in 1902

Principle:

Totipotency ,- The ability to regenerate the entire organism from a single somatic cell, i.e., trigger the use of the genetic information present to direct the entire regenerative and developmental programs needed to create the whole organism from a single cell, Cyto-differentiation: dedifferentiation and re-differentiation are the principles. Dedifferentiation is the capacity of mature cells to return to meristematic condition and development of a new growing point

Competency describes the endogenous potential of a given cell or tissue to develop in a particular way. For example, as embryogenically competent cells are capable of developing into fully functional embryos. The opposite is non-competent or morphogenetically incapable.

Brief history:

1838 - Schwann and Schleiden put forward the theory which states that cells are totipotent, and in principle, are capable of regenerating into a complete plant. Their theory was the foundation of plant cell and tissue culture
1902 - Haberlandt proposed concept of in vitro cell culture
1904 - Hannig cultured embryos from several cruciferous species
1922 - Kolte and Robbins successfully cultured root and stem tips respectively
1926 - Went discovered first plant growth hormone –Indole acetic acid
1934 - White introduced vitamin B as growth supplement in tissue culture media for tomato root tip
1939 - Gautheret, White and Nobecourt established endless proliferation of callus cultures
1941 - Overbeek was first to add coconut milk for cell division in Datura
1946 - Ball raised whole plants of *Lupinus* by shoot tip culture
1954 - Muir was first to break callus tissues into single cells
1955 - Skoog and Miller discovered kinetin as cell division hormone
1957 - Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation
1959 - Reinert and Steward regenerated embryos from callus clumps and cell suspension of carrot (*Daucus carota*)
1960 - Cocking was first to isolate protoplast by enzymatic degradation of cell wall
1960 - Bergmann filtered cell suspension and isolated single cells by plating
1960 - Kanta and Maheshwari developed test tube fertilization technique
1962 - Murashige and Skoog developed MS medium with higher salt concentration

1964 - Guha and Maheshwari produced first haploid plants from pollen grains of Datura (Anther culture)
1966 - Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato
1970 - Power et al.successfully achieved protoplast fusion
1971 - Takebe et al.regenerated first plants from protoplasts
1972 - Carlson produced first inter specific hybrid of *Nicotiana tabacum* by protoplast fusion
1974 – Reinhard introduced biotransformation in plant tissue cultures- starting of genetic engineering
1977 - Chilton et al. successfully integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants
1978- Melchers et al. carried out somatic hybridization of tomato and potato resulting in pomato
1981- Larkin and Scowcroft introduced the term somaclonal variation
1983 - Pelletier et al. conducted intergeneric cytoplasmic hybridization in Radish and Grape
1984 - Horsh et al. developed transgenic tobaccoby transformation with *Agrobacterium*
1987 - Klien et al. developed biolistic gene transfer method for plant transformation
2005 - Rice genome sequenced under International Rice Genome Sequencing Project

Organization of Laboratory

Any laboratory designed for plant tissue culture or biotechnology must focus on cleanliness & maintaining of aseptic condition. The essential 7 fundamental matter is the contamination free condition in all steps of the procedure. Any laboratory, in which tissue culture techniques are performed, regardless of the specific purpose, must contain a number of basic requirements. These are:

a. A general washing area
b. A media preparation, sterilization & storage area
c. Environmentally controlled incubators or culture rooms
d. An observation/data collection area
e. Acclimatization area

a. Washing area:

The washing area should contain good quality basin, large sink & well drainage facilities. It should have access to dematerialized water & double distilled water. Space for drying ovens or racks, automated dishwashers, acid baths, pipette washers & driers & storage cabinets should also be available in the washing area.

General guidelines for washing area;

1. Reusable glassware for tissue culture should be emptied immediately & need to be soaked in water. Media or agar must never be allowed to dry on the glassware
2. All glassware containing corrosive chemicals or fixatives should be separated from the rest of the tissue culture glassware.
3. All glasswares contaminated or coming into contact with microorganisms should be autoclaved before washing.
4. The contents of any containers should be discarded immediately after completion of an experiment.
5. Flaks or beakers used for agar based media should be rinsed immediately after dispensing the media into culture vassels so as to prevent drying of the residual agar in the beaker

prior to washing.

b. Media Preparation Area:

This area comprises the central section of the laboratory, home to most of the activities. This area should have ample storage space for the chemicals, culture vessels & glassware required for media preparation & dispensing. The general laboratory section includes the area for media preparation for autoclaving the media & also for many of the activities that relate to the handling of tissue culture materials. Laboratory equipments required for media preparation room are as follows

1. Gas, water & electric supplies & compressed air & vacuum line.
2. Water heater
3. Different types of glasswares
4. Hot plate with magnetic stirrer
5. Coarse & sensitive balance
6. Spatula for use during weighing
7. Microwave oven for rapid heating media & agar mixture
8. pH meter
9. Distillation unit
10. De-ionizer
11. Metal racks for holding test tubes in the autoclave
12. Test tubes, flasks, plastic containers
13. Autoclave or cooker
14. Storage tank for distilled and /or de-ionized water.

Chemicals for culture media:

A. Inorganic elements:

a. Macro nutrient:

The need of macro nutrients is higher in tissue culture media. It provides both anion & cation for the plant cell. The name of each element with available form & important functions are given below:

Sl. No.	Name of the macro nutrient	Available form	Function
1.	Nitrogen (N)	KNO_3 ,NH_4NO_3	Both structural & functional role in protein synthesis
2.	Phosphorus (P)	KH_2PO_4	Activation in nucleotide synthesis
3.	Potassium (K)	KNO_3	Essential for activation of many enzymes, maintenance of ionic balance of the cell.
4.	Calcium (Ca)	$CaCl_2.2H_2O$	Acts as a cofactor & largely bound to the cell wall & cell membrane, Essential for cation-anion balance by counteracting organic inorganic anions.
5.	Magnessium (Mg)	$MgSO_4.7H_2O$	Essential for photosynthesis & many other enzymatic reactions.
6.	Sulphur (S)	$MgSO_4.7H_2O$,K_2SO_4	Functional role in protein synthesis.

b. Micro nutrients

Micro nutrient is essential for plant cell tissue growth. The name of elements, available salt

combination & function are given below:

Sl. No.	Name	Available form	Function
1.	Zinc (Zn)	$ZnSO_4.7H_2O$	Act as a component of a number of enzymes, plays active role in protein synthesis, specially in the synthesis of tryptophan
2.	Manganese (Mn)	$MnSO_4.4H_2O$	Help in photosynthesis
3.	Copper (Cu)	$CuSO_4.5H_2O$	Plays an important role in electron transport chain at the time of photosynthesis
4.	Molybdenum (Mo)		It participates in the conversion of nitrate to ammonium
5.	Boron (B)	H_3BO_3	It is required for the synthesis of cell wall & cell membrane
6.	Iron (Fe)	$FeSO_4.5H_2O$	Formation of protein, important for biosynthesis of chlorophyll
7.	Cobalt (Co)	$CoCl_2.6H_2O$	Helpful for nitrogen fixation
8.	Chlorine (Cl)	$CaCl_2.2H_2O$	To control the osmoregulation of cell development.

B. Organic Components

a. Vitamins:

Normally plants synthesis vitamins endogenously. When plant cells & tissues are grown on in vitro condition some essential vitamins are absolutely required.

Sl. No.	Name of the vitamin	function
1.	Thiamine (vitamin-B1)	Promotion of cell growth & development.
2.	Nicotinic acid B20	
3.	Pyridoxin-HCl (B6)	
4.	Folic acid	
5.	Biotin	
6.	Riboflavin	
7.	Retinol (vitamin-A)	

b. **Myo-inositol:**

It has several functions like sugar transport, carbohydrate metabolism, membrane structure & cell wall formation.

c. Sugar:

It can be supplied in the form of sucrose, glucose, and fructose. It is a source of carbon.

d. Amino acid:

Cultured tissues are normally capable of synthesis of amino acid. In spite of this, the addition of amino acids to the media is important for stimulating cell growth. Unlike inorganic nitrogen, amino acids are taken up more rapidly by plant cells. Glycine is the most common amino acid used in different tissue culture media. Some of the other amino acids like glutamine, asparagines, cystine etc. are also required for cell culture.

e. Plant growth regulators:

Plant growth regulators are the organic molecules which have different regulatory effects on growth & development in whole plants & plant tissues. It is the most critical component of any culture media accepted that without regulators, in vitro culture is often impossible. Plant growth regulators which are often used in plant tissue culture are the following.

i. Auxin: The major functions of auxin are cell division, cell elongation, organogenesis. It is frequently used as a rooting hormone. The most frequently employed auxins are IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), NAA (Napthalene acetic acid), 2, 4-D (2 ,4-Dichlorophenoxy acetic acid). IAA is a naturally occurring auxin is added in concentration of 0.01-10 mg/l. The most effective auxin of callus proliferation for most cultures is 2, 4-D, but unfortunately it strongly suppresses organogenesis & should not be used in experiments involving root & shoot initiation.

ii. Cytokinin: Cytokinins are derivatives of adenine, which promote cell division, regulate growth and development in plant tissues. It is known as shooting hormone essential for induction of auxillary branching and adventitious shoot formation. The most widely used cytokinins are kinetin, zeatin, BAP (Benzyladenine), 2iP (2- isopentenyladenine).

iii. Other regulators: Other types of hormones which may be used in plant tissue culture include gibberellins (GA3), which promotes shoot elongation, and internodal elongation, ethylene and abscisic acid.

Aseptic Transfer Area/Inoculation room:

All the activities of sterile transfers are performed in this room. There must be a laminar air flow cabinet where all the precautions should be taken to prevent entry of any contaminant into the culture vial during the process of inoculation or subculture. Laminar air flow hoods are usually sterilized by switching on the hood and wipping the working sueface with 70% ethyl alcohol for 15 minute before initiating any operation under the hood. Ultraviolet light (UV) is sometimes installed to disinfect the area; this light should only be used when people and plant materials are not in the room. This room is provided with:

1. Laminar air flow cabinet: Inoculation & subculture by maintaining aseptic condition.
2. Steribed sterilizer, Sprit lamp/Bunsen burner: Sterilization of the knives, scalpels, forceps etc.
3. Stereo-microscope: observe for specific part.
4. Ethyl alcohol: sterilization and flaming of small instruments.
5. Tiles/glass plates use during sterile cutting.
6. Hypochloride solution: sterilization of plant material.

c. Incubation Room/Culture Room: This is the room where light, temperature, humidity are maintained. All of these environmental considerations will vary depending on the size of the growth room.
Temperature: is an important consideration for the tissue culture and other factors like light, relative humidity, and shelving depend on it. Generally temp. of the growth room remain in the range of 25± 2°C. Temp. in the primary growth room can be maintained by air conditioner.

Lighting facility: Intensity of light in the room can easily be maintained by using fluorescent light with timer. However, most culture rooms are lighted at the 1000 lux (for 1000cft) with some going up 5000-10000 lux.

Light duration: 16-18 h/day.

Light quality: Spectral quality of light received by in vitro cultures is very important.

Relative humidity: Relative humidity (RH) is very difficult to control inside the room but humidifier can be used to control humidity. Humidity inside the room should be 70-75%

Shelves: Shelving with primary growth rooms can vary depending upon the situations & explants grown. Wood is recommended for the inexpensive easy to build shelves.

This room is provided with

1.Temperature control (25± 2°C)
2.Electricity supply essential for lighting, cooling and heating
3.Shelves for culture racks
4.Fluorescent tubes for lighting
5.Timer for regulating day length
6.Racks for culture vials
7.Rotary shaker for suspension cultures
8.Observations table.

d. Data collection Area: Culture room is prepared by glsswall. Qualititative data could be collected from out side of the culture room through the glasswall. The quantitative data could be collected from inside the culture room by following aseptic rules and regulation.

e. Acclimatization area:

Plants regenerated from in vitro tissue cultures are transplanted to vermiculite pots. The potted plants are ultimately transferred to greenhouses or growth cabinets and maintained for further observations under controlled conditions of light, temperature and humidity.

Major equipments and their function

Sl. No.	Name of the equipments	Function
1.	Autoclave machine, Pressure cooker	Sterilization of media, glassware &small instrument.
2.	Balance	Measurement of chemical from the range of µgm to Kg
3.	Hot plate magnetic stirrer	To mix the chemical & other ingredient of media

4.	pH meter	To determine the pH of various chemicals & media
5.	Refrigerator	To store all sorts of temperature-sensitive chemical & stock solution.
6.	Micro oven	To melt agar, agarose & other gelling agents.
7.	Hot air oven	For dry heat sterilization of cell & suspension culture
8.	Shaker	Use for gentle rotation of cell& suspension culture
9.	Filter sterilization unit with vacuum pump	Filtration of thermoliable compound like growth regulator, vitamin, amino acid etc.
10.	Microscope	To study the cell & tissue culture material at different stages of development
11.	Luxmeter	To measure the light intensity of the culture room
12.	Thermometer	To record the temperature reading of laboratory & culture room
13.	Centrifuge machine	To sediment cell & clean supernatant
14.	Laminar air flow cabinet	To avoid air remaining contaminant

General rules to be followed in a tissue culture laboratory

1. A laboratory should have an inventory & a complete up-to-date record of all the equipments along with their operating manual.
2. A laboratory should have an inventory & a complete up-to-date record of all the chemicals including the name of manufacturer & grade.
3. All chemicals should be assigned to specific areas preferably by their alphabetical order.
4. Strong acid & bases should be stored separately.
5. Special handling or storage procedure should be posted in the records so that retrieving of chemical is easy, because chemicals need storage at different temperatures (for example room temperature 4°, -20° C)
6. Chloroform, alcohol, phenol, which is volatile or toxic in nature, must be stored in a fume hood.
7. Chemicals which are hygroscopic in nature must be stored in desiccators in order to avoid caking.
8. Chemicals kept in refrigerator or freezers should be arranged either alphabetically or in small baskets.

Safety rules:

1. Eating, smoking and drinking is strictly prohibited in the tissue culture laboratory.
2. Toxic chemical must be handled with appropriate precautions and should be discarded into separate labeled containers. e.g. Organic compounds, halogens etc.
3. Broken glass and scalpel blades must be disposed into individual marked containers.
4. Pipettes, tips, Pasteur pipettes and other things used in the lab should be first collected in autoclavable bags and then it should be finally autoclaved and disposed in safe place.
5. Pipetting any solution should not be conducted without using any pipette.
6. First aid kits should be placed in every laboratory and every individual working in the laboratory should know its location and how to use its contents.
7. Fire extinguishers should be provided in each laboratory.

Steps involved in general techniques.

Regeneration of Plantlets:

1. Preparation of Suitable Nutrient Medium:

Suitable nutrient medium as per objective of culture is prepared and transferred into suitable containers.

2. Selection of Explants:

Selection of explants such as shoot tip should be done.

3. Sterilisation/ surface decontamination of Explants:

Surface sterilization of the explants by disinfectants and then washing the explants with sterile distilled water is essential.

4. Inoculation:

Inoculation (transfer) of the explants into the suitable nutrient medium (which is sterilized by filter-sterilized to avoid microbial contamination) in culture vessels under sterile conditions is done.

5. Incubation:

Growing the culture in the growth chamber or plant tissue culture room, having the appropriate physical condition (i.e., artificial light; 16 hours of photoperiod), temperature (-26°C) and relative humidity (50-60%) is required.

6. Regeneration:

Regeneration of plants from cultured plant tissues is carried out.

7. Hardening:

Hardening is gradual exposure of plantlets to an environmental conditions.

8. Plantlet Transfer:

After hardening plantlets transferred to the green house or field conditions following acclimatization (hardening) of regenerated plants.

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