Year	Sem.	Subject Code	Title of the paper	Hours/ Week
2018 -2019 onwards	VI	18BBO66S	SKILL BASED SUBJECT – IV PLANT TISSUE CULTURE	3

OBJECTIVES:

- To create awareness among students about *in vitro* propogation of plants
- To enable the students to enrich their skills in plant tissue cultural techniques.

Unit – I

Introductory history, Laboratory organization, Culture Media (MS medium), Aseptic Techniques.

Unit –II

Micropropagation – Direct and Indirect (Callus culture, Nodal culture, Meristem culture, Shoot tip culture), Somaclonal variation, Suspension culture.

Unit –III

Haploid culture – Anther culture, pollen culture and ovary culture - Triploid production.

Unit –IV

Isolation and culture of protoplast, Somatic Hybridization, Somatic Embryogenesis, Artificial seed production.

Unit –V

Production of secondary metabolites – alkaloids, steroids, and terpenoids (Brief account only). Cryopreservation and germplasm preservation.

PRACTICALS:

1. Sterilization techniques (Fumigation, Flame sterilization, Dry heat, Wet heat and Filter sterilization)
2. MS Medium preparation
3. Callus culture
4. Nodal culture
5. Artificial Seed production

REFERENCES:

1. Plant Cell and Tissue Culture – Narayanasamy, S. Tata Mc- Graw- Hill Publishing & Co Ltd
2. Plant Cell, Tissue and Organ Culture – Edited by J. Renert and Y.P.S.Bajaj, Narosa Publishing House New Delhi First Reprint 1989.
3. An Introduction to Plant Tissue Culture – Razdan, M.K.
4. Biotechnology- U Sathyanarayana Books and Allied (P) Ltd, (2005).

18BBO66S-U1

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PLANT TISSUE CULTURE

Plant tissue culture has a great significance in plant biotechnology especially in the crop improvement programs. The term tissue culture may be defined as the process of in-vitro culture of explants (pieces of living differentiated tissues) in nutrient medium under aseptic conditions. However, in general, the tissue culture includes the term tissue culture as well as cell culture, organ culture and suspension culture also.

Plant tissue culture is fundamental to most aspects of biotechnology of plants. It is evident now that plant biotechnology is one of the most beneficial of all the sciences. The products of plant biotechnology are being transferred rapidly from laboratories to the fields.

Also, the plant tissue culture has become of great interest to the molecular biologists, plant breeders and even to the industrialists, as it helps in improving the plants of economic importance. In addition to all this, the tissue culture contributes immensely for understanding the patterns and responsible factors of growth, metabolism, morphogenesis and differentiation of plants.

Tissue Culture:

The in-vitro culture of the tissue e.g. Callus culture

Cell Culture:

Denotes the in-vitro culture of single or a few cells.

Organ Culture:

This term is used for in-vitro culturing of organs like embryo, root or shoot apices.

Suspension Culture:

Defined as the culture of cell and cell aggregates suspended in a liquid medium.

Ex plant:

The excised piece of differentiated tissue or the organ which is used for culture is called as explant. e.g., embryos, young leaf, bud, etc.

Callus:

The undifferentiated mass of cells is referred to as callus. The cells of callus are meristematic in nature.

INTRODUCTORY HISTORY

G. Haberlandt, a German botanist, in 1902 cultured fully differentiated plant cells isolated from different plants. This was the very first step for the beginning of plant cell and tissue culture. Further contributions were made by the Cell Doctrine which admitted that a cell is capable of showing totipotency.

With the identification of a variety of chemicals like cytokinin, auxin, other hormones, vitamins, etc. and their role in affecting cell division and differentiation, the methods of plant tissue culture developed in a proper manner. Three other scientists Gautheret, White and Nobecourt also made valuable contributions to the development of plant tissue culture techniques.

Later on, a number of suitable culture media were developed, for culturing plant cells, tissues, protoplasts, embryos, anthers, root tips, etc. The discovery and understanding of role of plant growth hormones in the multiplication of cell also provided an extra aid for the development of in-vitro culture methods of plants.

The first plant from a mature plant cell was regenerated by Braun in 1959. Foundation of commercial plant tissue culture was laid in 1960 with the discovery for a million fold increase in the multiplication of Cymbidium (an orchid) which was accomplished by G.M.Morel.

In India, the work on tissue culture was initiated during 1950s at University of Delhi. This initiation is credited to Shri Panchanan Maheshwari who was working there in the Department of Botany. Discovery of haploid production was a land-mark in the development of in-vitro culturing of plants.

Shri S.C. Maheshwari and Sipra Guha made a remarkable contribution in the development of plant tissue culture in India. Later on the development in the composition of nutrient media and genetic engineering served as a basis for further success in the plant tissue culture techniques.

Gottleib Haberlandt was the first person to make attempts for plant tissue culture, i.e., he developed the concept of in-vitro culture of plant cells and is aptly regarded as the father of tissue culture. Thereafter, there happened some dramatic advances in tissue culture techniques.

Year	Worker	Advancement
1902	**Haberlandt**	First attempt of *in-vitro* culture of plant cell
1904	**Hannig**	Culture of embryogenic tissue of crucifers
1922	**Robbins**	*In-vitro* culturing of roots
1925	**Laibach**	Zygotic embryo culture in *Linum*
1934	**White**	Culture of roots of tomato plant
1939	**Gautheret, White and Nobecourt**	Successful establishment of indefinite callus culture
1941	**Braun**	Culture of Crown Gall Tissues
1945	**Loo**	Cultures from stem tip
1955	**Miller**	Hormone Kinetin discovered
1957	**Skoog, Miller**	Discovered that Auxin : Cytokinin ratio regulates the organ formation
1960	**Bergmann**	Development of Plating technique for isolation of single cell
1970	**Power**	Successful Protoplast fusion
1970	**Maheshwari and Guha**	Successful Anther Culture
1971	**Takabe**	Plants regenerated from protoplasts
1974	**Reinhard**	Biotransformation in plant tissue culture
1978	**Melchers**	Production of somatic hybrid *Pomato*

The main requirements of plant tissue culture are:

(1) Laboratory Organisation

(2) Culture Media

(3) Aseptic Conditions

LABORATORY ORGANISATION

In a standard tissue culture lab, there must be a few basic facilities like:

i. A Media Room for preparation, sterilization and storage of culture media.

ii. Facilities for washing of lab-wares, explants, etc.

iii. Space for storage of lab-wares.

iv. Culture rooms or incubators where conditions of temperature, humidity and light etc. can be maintained.

v. Observation and Data Collection area.

Any laboratory designed for plant tissue culture or biotechnology must focus on cleanliness and maintaining aseptic conditions. The specific design can vary, but an effective laboratory organization for plant tissue culture must include certain elements irrespective of usage in the hands of either a scientist or group of workers.

A general guideline to set up a facility is to focus on a design wherein different units and activities need to be arranged to make operational steps possible with the least amount of cross-traffic. Thus, a tissue culture facility should have the following features.

WASHING FACILITY

An area with large sinks (some of them lead lined in order to resist acids and alkalies) and a draining area is necessary since the conventional method for cleaning laboratory glassware involves acid soak followed by thorough washing with tap water and subsequent rinsing with distilled water. However, due to the corrosive nature of acids, it is recommended that for routine procedure, glassware should be soaked in a 2% detergent cleaner for 16 h followed by washing with 60- 70°C hot tap water and distilled water. The available wide range of reusable plasticware should be washed with mild detergents followed by rinsing them first with tap water and then with distilled water. The glassware is first air dried and then kept in an oven for 2 hrs at 160°C

PRECAUTIONS

The contents of any containers should be discarded immediately after completion of an experiment.

Contaminated containers should be autoclaved, contents discarded in a waste bottle or container and then washed. The waste bottle containing autoclaved contaminated media should not be emptied in the laboratory sink.

Flasks or beakers used for agar-based media should be rinsed immediately after dispensing the media into culture vessels so as to prevent drying of the residual agar in the beaker prior to washing.

GENERAL PLANT TISSUE CULTURE LABORATORY AND MEDIA PREPARATION AREA

This area comprises a central section of the laboratory, home to most of the activities. The general laboratory section includes the area for media preparation, for autoclaving the media and

also for many of the activities that relate to the handling of tissue-cultured materials. The area to be set aside for media preparation should have ample storage and bench space for chemicals, glassware, culture vessels, closures and other items required to prepare media. The lab tables must be equipped with several taps and wash-basins. It is desirable to install the maximum number of shelves, cupboards, and sideboards for the standard glassware, small material bottles of chemicals and solutions, etc. A separate room for these essentials is also desirable

The following equipment items are essential in a plant tissue culture laboratory:

- Atomizer/ small sprayer
- Autoclave (available as horizontal or vertical) and/or pressure cookers
- Balances, preferably electronic
- Centrifuge tabletop
- Dissecting microscope
- Freezer (-20°C)
- Hot plate/gas plate Lab carts
- Laminar airflow hood
- Magnetic stirrer (with hot plate)
- Microscopes
- Ovens
- Parafilm strips
- pH meter
- Refrigerator
- Shaker, gyratory with platform and clips for different size flasks
- Water distillation unit

The following equipment items are essential in a plant tissue culture-cum-bio technology laboratory at an elementary level:

- Agarose gel electrophoresis apparatus
- Atomizer/small sprayer Autoclave (available as horizontal or vertical) or pressure cookers
- Balances, preferably electronic
- Centrifuge-refrigerated, tabletop.
- Eppendorf microfuge

- Dissecting microscope
- Freezer (-20°. -70°C)
- Gel documentation system Hot plate/ gas plate
- Lab carts
- Laminar airflow hoods
- Liquid nitrogen cans
- Magnetic stirrer (with hot plate)
- Micropipettes
- Microscopes
- Microwave oven for heating agar media
- Milli-Q water purification unit
- Ovens with temperature control (20°-90°C)
- Parafilm strips
- pH meter
- Refrigerators
- Shaker, gyratory with platform and clips for different size flasks
- Vortex
- Water bath with temperature control
- Water distillation unit
- Water purification unit

Of all necessary basic equipments listed above, some may require a wider definition.

pH meter: It is necessary to choose a good laboratory model and not a portable one with an accuracy up to one-tenth of a pH. This apparatus is absolutely essential in any laboratory

Balance: The most modern balances are single pan balances. It is preferable to install an electronic digital balance with sensitivity of 0.001 g.

Autoclave/pressure cookers: For a large laboratory, a horizontal model is preferred. In small laboratories, vertical models or even big C domestic pressure cookers serve the purpose. It is

preferable to use two different pressure cookers, one for media autoclaving and other for decontamination purposes.

Laminar airflow hood: Installing a good quality horizontal laminar airflow is a must in the laboratory. The laminar airflow cabinet is the most common accessory used for aseptic manipulations. This cabinet should be designed with horizontal airflow from the back to the front and equipped with gas cocks in the presence of gas burners. In the airflow cabinet air is forced as into the cabinet, through a bacterial HEPA (high an efficiency particulate air) filter. It then flows outwards (forward) over the working bench at a uniform rate. The constant airflow prevents any particles from settling on the bench. One or more laminar airflow cabinets can be housed in such a transfer area. The roof of some of the cabinets house an ultraviolet (UV) other germicidal light which is often used to sterilize the interior of the chamber. These lights are turned on about half an hour prior to using the chamber. Before commencing on any project, it is this desirable to swab down the inside of cabinet, especially the tabletop with 70% alcohol. The air filter should be changed and cleaned front periodically.

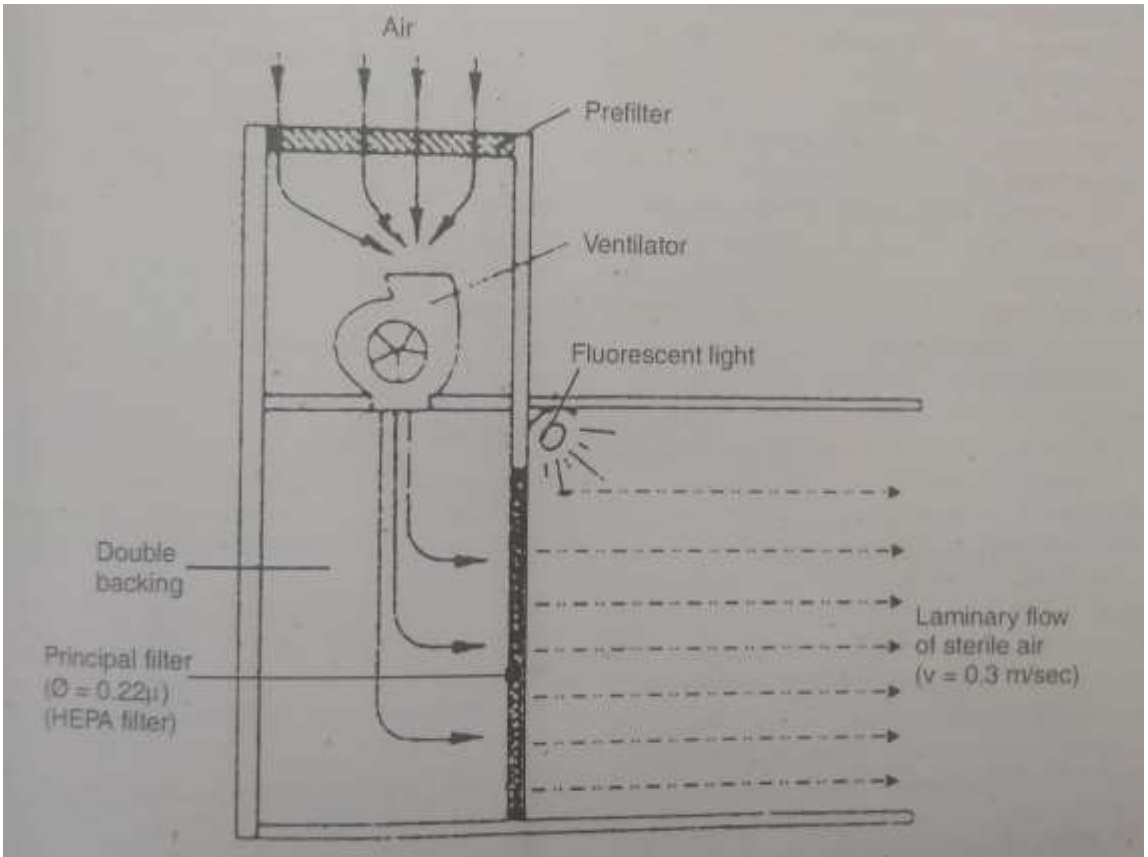


Fig. 2.1 A laminar airflow cabinet

Water distillation assembly: A water distillation force assembly should be installed in the washing area.

Small apparatus: Small apparatus such as forceps of different sizes, scalpels with sterile blades dissection needles, scissors etc. are indispensable. Parafilm strips, glass markers should always be present while working.

General rules

1. A laboratory should have an inventory and a complete up-to-date record of all the equipment along with their operating manuals.
2. A laboratory should have an inventory and a detailed up-to-date record of all the chemicals, including the name of manufacturer and grade.
3. All chemicals should be assigned to specific areas-preferably by their alphabetical order.
4. Special handling or storage procedures 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, etc.)

5. Strong acids and bases should be stored separately.

6. Chloroform, alcohol, phenol, which are 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 refrigerators or freezers should be arranged either alphabetically or in small baskets.

TRANSFER AREA

Tissue culture techniques can be successfully carried out in a very clean laboratory, dry atmosphere with some protection against airborne microorganisms. But it is advisable that a sterile dust-free room should be available for routine transfer and manipulation work.

Precautions

- UV light must be switched off prior to opening the hood of laminar airflow.
- During working in the laminar airflow, the UV light must be switched off.

CULTURING FACILITIES

Plant tissue cultures should be incubated under conditions of well-controlled temperature, illumination, photoperiod, humidity and air circulation. Incubation culture rooms and commercially available incubator cabinets, large plant growth chambers, and walk-in environmental rooms satisfy this requirement. These facilities can also be constructed by developing a room with proper air-conditioning, perforated shelves to support culture vessels, fluorescent tubes and a timing device so as to set light-dark regimes (photopericds), which are the standard accessories. In addition, a dark area simply closed off from the rest of the room with thick black curtains is necessary, provide some cultures require continuous darkness, The type of culture room can also be used to house a variety of liquid culture apparatus, including gyratory and reciprocating shakers or a varie of batch and continuous bioreactors, etc. F most culture conditions, the temperature control should be adequate in order to stay within + 1" in a range from 10°C to 32°C. The photoperiod may be set according to the specific types of culture,

Physical factors

The principal factors in the culture environment are light and temperature. Relative humidity is generally close to 100% in the culture containers

Lighting requirements: Lighting requirements for the cultures grown under in vitro conditions can be divided into different parameters of light intensity, duration of light and quality of light. For cultured tissues, photosynthesis is not a necessary activity as energy is furnished in the form of carbohydrates. However, photosynthesis cannot be eliminated but only considerably reduced because of the presence of sugars in the medium

Light intensity: Lux or foot candles terms have been used but more acceptable terms are: Joule per square metre (j/m^2) is an expression of radiant energy per unit area. Watts per square metre (W/m) is used to express irradiance.

The strength of light per unit of surface (or intensity) is expressed in W/cm (lux unit is also used for measuring the light intensity, but should be discarded as it depends on the physiology of human eye and is thus totally unadapted to the needs of the plant). In tissue culture rooms, light intensities usually vary from 5 to 25 W/cm^2 (1000 to 5000 lux), with the very common use of 10 to 15 W/cm in the initial stages, the cultures should not be kept under excessive light intensity as used in phytotrons (50 W/cm^2 or 10,000 lux)

The preferred term for photosynthetically active radiation units is to use photon flux density which is µmol photons/m^2/s.

A common term for photon flux is based on 1 mol of photos being equal to 1 Einstein (E). Thus, a unit of photon flux density may then be expressed as µE/m^2/s.

Duration of light: It seems that in most cases, it is the quantity of light energy received (intensity x light duration). In practice, most culture rooms have a light duration of 16 to 18 h/day.

Quality of light: Spectral quality of light received by in vitro cultures is very important. In tobacco calluses, blue light (around 467 nm) or violet light (around 419 nm) induces bud formation and that red light (around 660 nm) induces rhizogenesis. Results from various studies indicate that morphogenetic processes seem to be regulated by photoreceptor pigments, phytochrome and others. Normally, light referred to as 'daylight' is richer in blue radiations, and light referred to as "brilliant white light' is richer in red radiations. Generally, commercial white fluorescent tubes are adequate but mixing two types of fluorescent tubes can be tried for optimization.

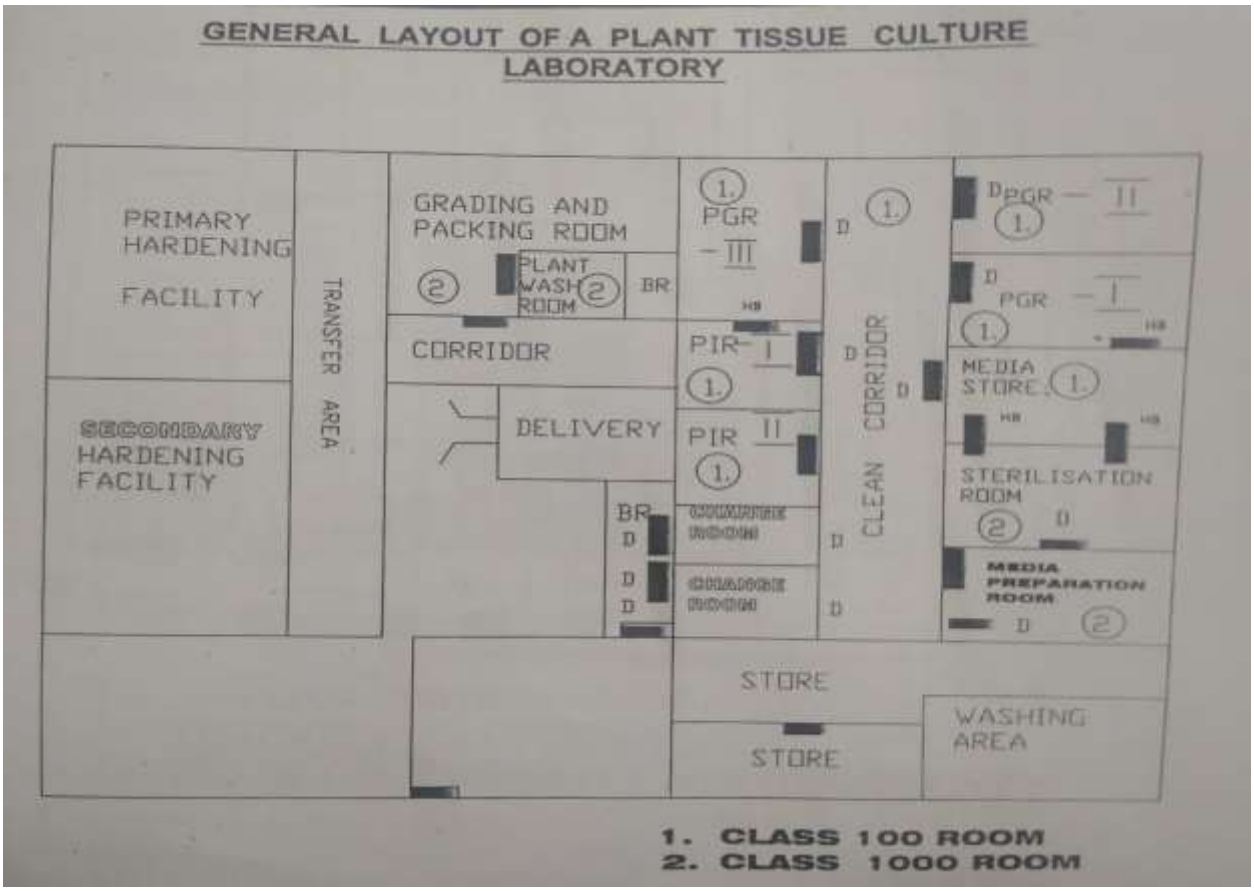
Influence of temperature

The temperature is usually regulated at a constant 22-25°C in tissue culture rooms. This practice is open to criticism as the real temperature of tissues inside culture vessels can be 2 to 4°C greater than that of the room. Practically, the room temperature should be regulated at 2°C lower than that desired for the cultured tissues. Species of temperate climate are habituated to lower temperature as compared to tropical species. Thus, it is advantageous to maintain a temperature of 20 +/- 1°C for temperate and 25 +/- 1°C for tropical species.

GREENHOUSES

Greenhouses have to grow regenerated plants and see them mature for further propagation. This facility is necessary as a transitional step of taking plant materials from culture containers present in the controlled room to the field. Thus, in the greenhouse, plants are acclimatized and hardened before being transferred to the field conditions. In the greenhouse plants develop adequate root systems and leaf structures so as to withstand the field environment. The greenhouse should be equipped with cooling and heating systems to control the temperature, For maximum use, the houses should have an artificial lighting system including a mixture of fluorescent and incandescent lights or contain lamps designed to provide balanced wavelengths of light for plant

growth and photosynthesis. The installation of a misting system is recommended on benches in the greenhouse that will be used to acclimatize the plants.



LABORATORY AND PERSONAL SAFETY

Before starting work, every new worker should be given some orientation along with instructions on how to operate the various items of equipment such as pH meter, balance, laminar airflow, microscope, centrifuges, PCR thermocyclers, gas burners, etc. Special instructions should be given on potential for fire, broken glass, chemical spills or accidents with sharp-edged instruments. The following regulations/instructions should be followed:

1. Do not eat, drink, or smoke in the laboratory

2. Toxic chemicals must be handled with appropriate precautions and should be discarded into separate labelled containers: Organic solvents (e.g. alcohol, chloroform) Mercury compounds (e.g. mercuric chloride) Halogens and mutagenic chemicals.

3. Broken glass and scalpel blades must be disposed into individual marked containers

4. Pipettes, tips, Pasteur pipettes and other things used for genetically-modified organisms and various materials used in the pathogen work should be first collected in autoclavable bags, autoclaved and then disposed of.
5. Always use a pipettor for pipetting any solution
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. Some portable extinguishers should also be placed at convenient sites for easy handling by amateurs.

CULTURE MEDIA

The formulation or the medium on which the explant is cultured is called culture medium. It is composed of various nutrients required for proper culturing. Different types of plants and organs need different compositions of culture media. A number of media have been devised for specific tissues and organs.

The salt composition of Murashige and Skoog 1962) nutrient medium, referred to as MS medium, is very widely used in different culture systems as it gives satisfactory results. But it must be remembered that it is not always the best medium.

Some important media are:

- MS (Murashige and Skoog) Medium
- LS (Linsmaier and Skoog) Medium
- B5 (Gamborg's) Medium
- White's Medium, etc.

Generally, in all the media, the nutritional milieu consists of inorganic nutrients, carbon and energy sources, vitamins, growth regulators, and complex organic supplemena It is desirable to choose a composition according to the knowledge of the physiology of species vis-à-vis mineral nutrition.

MEDIA COMPOSITION

Important constituents of a culture medium are

Inorganic Nutrients:

Mineral elements are very important in the life of a plant. Besides, C, H, N, and O, 12 other elements are known to be essential for plant growth. According to the recommendations of the International Association for Plan Physiology, the elements required by plants in concentration greater than 0.5 mmol/l are referred to as macroelements or major elements (NH), SC and those required in concentration less than Feso, the prescribed amount are microelements or MnSo, minor elements. A variety of salts supply the needed macro and micronutrients that are the same as those required by the normal plant.

Macronutrients include six major elements as Nitrogen (N), Sulphur (S), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg). The salts of potassium (K), nitrogen (N), calcium (Ca), magnesium (Mg). phosphorus (P) and sulphur (S) are required in macro or millimole quantities. Nitrogen is generally used Na, MoO, as nitrate or ammonium salts, sulphur as sulphates and phosphorus as phosphates.

Minor salts: The salts of iron (Fe), manganese (Mn), boron (B), copper (Cu), zinc (Zn), iodine (1), malybdenum (Mo) and cobalt (Co) are required in micromolar concentrations and are considered to be minor salts. These salts are essential for the growth of tissues and are required in trace quantities.

To achieve the maximum growth rate, the optimum concentration of each nutrient can vary considerably. The mineral composition of culture medium is defined precisely by the equilibrium of the concentrations of different ions in a solution. When mineral salts are dissolved in water, they undergo dissociation an ionization. The active factor in the medium is ions of different types rather than the compounds. Therefore, a useful comparison between the two media can be made by looking into the total concentrations of different types of ions in them. To choose a mineral composition and then compare their different ionic balances, one uses ionic concentrations expressed in milliequivalents per litre. Any success with a medium is in all probability due to the fact that the ratios as well as concentrations most nearly match the optimum requirements for the cells or tissues for growth and/or differentiation.

Carbon and energy source

The standard carbon source without exception is sucrose but plant tissues can utilize a variety of carbohydrates such as glucose, fructose, lactose, maltose, galactose and starch. In the

cultured tissues or cells, photosynthesis is inhibited and thus carbohydrates are needed for tissue growth in the medium. Sucrose, at a concentration of 2-5% in the medium, is widely used. The autoclaving process does cause an alteration in the sugars by hydrolysis but presents no drawbacks to the growth plan. Most media contain myo-inositol at a concentration of 100-mg per litre, which improves cell growth.

Vitamins

Normal plants synthesize the vitamins required for growth and development, but plant cells in culture have an absolute requirement for vitamin B, (thiamine), vitamin B (nicotinic acid) and vitamin B, (pyridoxine), Some media contain pantothenic acid, biotin, folic acid, p-amino benzoic acid, choline chloride, riboflavine and ascorbic acid. The concentrations are in the order of one mg/l. Myo-inositol is another vitamin used in the nutrient medium with a concentration of the order of 10-100 mg.

Growth regulators

Hormones now referred to as growth regulators are organic compounds that have been naturally synthesized in higher plants which influence growth and development. These are usually active at different sites from where they are produced and are only present and active in very small quantities. Two main classes of growth regulators of special importance in plant tissue culture are the auxins and cytokinins, while others are of minor importance, viz., gibberellins, abscisic acid, ethylene, etc. Some of the naturally-occurring growth regulators are indole acetic acid (IAA), an auxin and zeatin and sopentenyl adenine (2 iP) as cytokinins, while others are synthetic growth regulators.

Organic supplements

Certain complex substances are also added in the media which supply organic nitrogen, carbon or vitamins. Organic nitrogen in the form of casein hydrolysate (0.2-1 g/l) or certain amino acids such as glutamine and aspargine, nucleotide as adenine are included in the medium. L-glutamine (up to 8 mM, i.e. 150 mg/l) may replace the casein hydrolysate. The amino acids, when added, should be used with caution, since they can be inhibitory. The other amino acids included in the media in mg/l include: glycine (2), aspargine (100), tyrosine (100), arginine (10), cysteine

(10), and aspartic acid, glutamic acid and proline, etc. Only L-isomers are used, while D-isomers have proved to be ineffective. Adenine or adenine sulphate (2-120 mg/l) is added to agar media for morphogenesis. Addition of TCA cycle acids such as citrate, malate, succinate or fumarate permits the growth of plant cells on ammonium as the sole nitrogen source. A variety of extracts, viz., protein hydrolysate, yeast extract, malt extract, coconut milk, orange and tomato juices have also been tested. With the exception of protein hydrolysate and coconut milk, most of the others are used as a last resort. Coconut milk is commonly used at 2-15% (V/V). The present trend is, however, towards fully defined media and the use of complex mixtures is losing favour.

Antibrowning compounds

Many plants are rich in polyphenolic compounds. After tissue injury during dissection, such compounds will be oxidized by polyphenol oxidases and the tissue will turn brown or black. The oxidation products are known to inhibit enzyme activity, kill the explants, and darken the tissues and culture media, a process which severely affects the establishment of explants. Activated charcoal at concentrations of 0.2 to 3.0% (w/v) is used where phenol-like compounds are a problem for in vitro growth of cultures. It can adsorb toxic brown/ black pigments and also stabilize pH. Besides activated charcoal, polyvinylpyrrolidone (250- 1000 mg/l), citric acid and ascorbic acid (100 mg/l each), thiourea or L-cysteine are also used to prevent oxidation of phenols.

Some of the procedures used by various workers to combat this problem of browning are: (1) Adding antioxidants to culture medium, viz., ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), dithiothreitol, bovine serum albumin, etc.. (ii) Pre-soaking explants in antioxidant before inoculating into the culture medium: (iii) Incubating the initial period of primary cultures in reduced light or darkness because it is known that phenolic oxidation products are formed under illumination; and (iv) Frequently transferring explants into fresh medium whenever browning of the medium is observed.

Gelling agents

Agar, the most popular solidifying agent, is seaweed derivative, Plant tissue culturists ol use Difco Bacto agar at a concentration of to 1.0% (w/v), although other forms of a (agarose, phytagar, flow agar, etc.) are all gaining popularity Solubilized agar forms a go that can bind water and adsorb compounds. The higher the agar concentration, the stronger the water bound. With higher concentrations, the medium becomes hard and does not allow the diffusion of nutrients into

the tissues. Thus in vitro growth may be adversely affected if the agar concentration is too high. Besides agar, the following alternatives can be used.

i. Alginate can be used for plant protoplast culture
ii. Gelrite at 0.2% can be used for solidification of media. Gelrite gels are remarkably clear in comparison to those formed by agar Gelrite requires both a heating cycle and the presence of divalent cations (Mg^{++} or Ca^{++}) for gelation to take place.
iii. Synthetic polymer biogel P200 (polyacrylamide pellets) or a starch polymer can be used.
iv. Agargel: A mixture of agar and synthetic gel has been developed by Sigma Company has the properties of both synthetic gel and agar.

Liquid media with a support can also be used instead of solid media. Such media include:

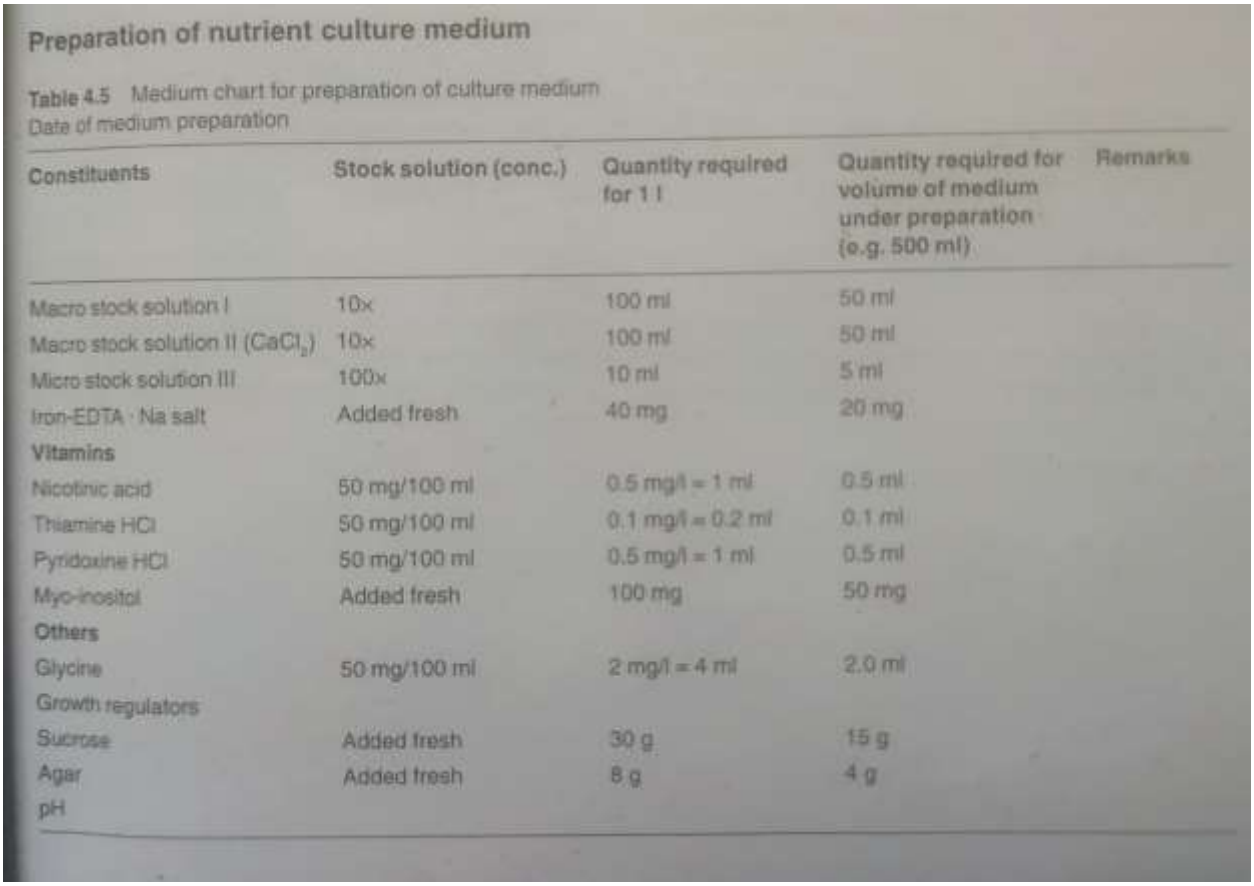
i. Liquid medium without agar using clean foam plastic, glasswool or rockwool as support
ii. Filter paper-bridge, which is hung in a liquid medium.
iii. Growth on a liquid medium containing glass
iv. Viscose sponge underneath the filter paper as a carrier for a liquid medium instead of agar.

pH

pH determines many important aspects the structure and activity of biologica macromolecules, pH is the negative logarithm of the concentration of hydrogen ions. Nutrient medium pH ranges from 5.0 to 6.0 for suitable in vitro growth of explant. pH higher than 7.0 and lower than 4.5 generally stops the growth and development. The pH before and after are also autoclaving is different. It generally falls by 0.3 to 0.5 units after autoclaving. If the pH falls appreciably during plant tissue culture (the medium becomes liquid), then a fresh medium should be prepared. One should know that a starting pH of 6.0 could often fall to 5.5 or even lower during growth. pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar.

Table 4.4 Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken medium
Macronutrients (10×) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 \cdot 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10×) Stock solution II			
$CaCl_2 \cdot 2H_2O$	440	4400	100 ml
Micronutrients (100×) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 \cdot 4H_2O$	22.3	2230	
$ZnSO_4 \cdot 7H_2O$	8.6	860	
KI	0.83	83	
$Na_2MoO_4 \cdot 2H_2O$	0.25	25	
$CuSO_4 \cdot 5H_2O$	0.025	2.5	
$CoCl_2 \cdot 6H_2O$	0.025	2.5	
Iron source			
Fe · EDTA-Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	
Sucrose	30,000	Added fresh	4 ml
Agar	8000	Added fresh	
pH-5.8			



Preparation of nutrient culture medium

Table 4.5 Medium chart for preparation of culture medium
Date of medium preparation

Constituents	Stock solution (conc.)	Quantity required for 1 l	Quantity required for volume of medium under preparation (e.g. 500 ml)	Remarks
Macro stock solution I	10×	100 ml	50 ml	
Macro stock solution II ($CaCl_2$)	10×	100 ml	50 ml	
Micro stock solution III	100×	10 ml	5 ml	
Iron-EDTA · Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/l = 0.2 ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4 ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

ASEPTIC CONDITIONS

Maintenance of aseptic conditions is the most critical and difficult aspect of in-vitro culturing experiments. Aseptic condition mean the conditions free from any type of microorganisms (so as to prevent the loss of experiment by contamination). For this, sterilization (i.e., complete removal or killing of microbes) is done. The most common contaminants in culture are fungi and bacteria.

Measures to be taken for maintaining asepsis during tissue culture are:

i. Sterilization of the culture vessels using detergents, autoclaves, etc.

ii. Sterilization of instruments like forceps, needles etc. by flame sterilization.

iii. Sterilization of culture medium using filter sterilization or autoclaving methods.

iv. Surface sterilization of explants using surface disinfectants like Silver Nitrate (1%), H_2O_2 (10-12%), Bromine water (1-2%), Sodium Hypochlorite solution (0.3-0.6%), etc.

The whole procedure of plant tissue culture is to be carried out essentially under aseptic conditions. So, the overall design of the laboratory must focus on the maintenance of aseptic conditions. Secondly, the worker is also required to have proper knowledge of operating various equipment's like pH meter, balance, laminar air flow, microscope, etc.

While performing the tissue culture experiments first aid kits and fire extinguishers must be present in the laboratory to avoid any mishap or accident. In addition, proper attention should be given while handling the toxic chemicals and all the chemicals should be kept in correct labeled containers and bottles.