

PLANT TISSUE CULTURE TECHNOLOGY

Introduction

What is plant tissue culture?

Plant tissue culture is the method of 'in vitro' culture of plants on a nutrient medium under aseptic conditions usually in a glass container. Tissue culture is sometimes referred to as 'sterile culture' or 'in vitro' culture. The totipotency of plant cells was predicted in 1902 by Haberlandt. The technique has developed around the concept that a cell has the capacity and ability to develop into a whole organism irrespective of their nature of differentiation and ploidy level. By this technique living cells can be maintained outside the body of the plant for a considerable period. By plant tissue culture new plants may be raised in an artificial medium from very small parts of plants, such as, shoot tip, root tip, callus, seed, embryo, pollen grain, ovule or even a single cell, whether the cultured tissue develops into a plant or grows unorganized depends on the genetic potential of the tissue and the chemical and physical environment.

Plant tissue culture techniques can be largely divided into two categories based on to establish a particular objective in the plant species:

I. Quantitative Improvement

- Micropropagation of shoots, Meristem etc
- Somatic embryogenesis & synthetic seeds
- Callus culture

II. Qualitative Improvement

- Anther/ Microspore culture & Ovary/ Ovule culture & Endosperm culture
- Cell culture & Protoplast culture

Plant tissue culture technique has become one of the corner stones of present day agriculture, horticulture , plant breeding forestry etc. It explores conditions that promote cell division and genetic reprogramming in vitro conditions. It forms the backbone of the modern approach to crop improvement by genetic engineering.

Applications of plant tissue culture :

1. Mass propagation of plants
2. Development of virus free plants
3. Preservation of endangered and genetically modified plant species – Germplasm conservation
4. Production of medicinal components –Secondary metabolites
5. Chemical transformation of components – Bio transformation
6. Using plant as bio reactors-Molecular farming
7. Production of resistant plant varieties
8. Production of better yielding somatic hybrids GM crops
9. Development of new varieties to be used as Nutraceuticals
10. Make plants more appealing and a source of bio-degradable plastics

1. MASS PROPAGATION OF PLANTS

A] Micropropagation

The most important advantage of *in vitro* grown plants is that it is independent of geographical variations, seasonal variations and also environmental factors. Growing any part of the plant (explants) like apical shoots, axillary buds & meristems in an artificial medium under controlled conditions (aseptic conditions) for obtaining large scale plant propagation is called micropropagation. It can be done using cutting, budding, grafting, tubers and other vegetative propagules. The main problem with this method is that it is labour intensive, has low productivity and seasonal. By micropropagation millions of clones can be produced within a short span of time. Multiple shoot formation is initiated from the cultured explant. The shoots are separated and rooting is carried out on rooting medium. The tissue culture raised plants are then hardened as they are tender and subsequently transported to the green house for acclimatization and finally to the field.

B] Artificial seeds

Artificial or somatic seeds are generally produced by encapsulating somatic embryos. The somatic embryos germinate and develop into complete plantlets like zygotic embryos. The coating should be mild enough to protect the embryos and allow germination but it must be sufficiently durable for rough handling. Two types

of synthetic seeds are produced. Desiccated synthetic seeds involves encapsulation of somatic embryos followed by their desiccation. Hydrated synthetic seed are generally produced by Ca-alginate encapsulation.

2. Establishment of pathogen free plants

The plants infected with bacteria and fungi can be treated by bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants. A large numbers of viruses are not transmitted through seeds. Therefore, it would be possible to obtain virus free plants from infected individuals by using seeds as propagules. However, genetic variation often occurs from the sexually reproduced plants when propagated by seeds. Generally, clonal multiplication of cultivars can be achieved by vegetative propagation. However, where the entire population of the clone is infected the only way to obtain pathogen-free stock is to eradicate the pathogen from vegetative parts of the plants and regenerate full plants from such tissues. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium, it measures up to about 100µm in diameter and 250µm in length. The apical meristem together with one to three young leaf primordia, measuring 100-500µm, constitutes the shoot-apex. The chances of eradicating viruses is higher through 'meristem' culture. In infected plants the apical meristems are generally either free or carry a very low concentration of the viruses. Viruses readily move in a plant body through the vascular system which is absent in the meristem. The alternative method of cell-to-cell movement of the virus through plasmodesmata is rather too slow to keep pace with the actively growing tip. Also high metabolic activity in the actively dividing meristem cells does not allow virus replication. Thermo-therapy or chemotherapy and physiological stage of the explants also affect virus elimination by shoot-tip culture.

3. Conservation of Germplasm:

By tissue culture plant germplasm can be stored. Many seeds, retain their viability for a short period. These can be conserved by tissue culture. Vegetatively propagated plants (such as, banana, potato) which do not produce seeds, are stored as cuttings or tubers. This requires much labour charge and are expensive to propagate. This problem can be solved by tissue culture. Many fruit trees are propagated by budding, grafting and layering. By tissue culture rapid propagation

of such plants are possible. In many economic plants, such as, coconut, date palm etc. vegetative propagation normally does not occur. The germplasm of such plants can be conserved by tissue culture. Many trees reproduce very slowly. By tissue culture such plants can be multiplied rapidly. For conservation of germplasm the cells should be stored in such a condition which allows minimum cell division. One of the methods attempted is storing of cells in liquid nitrogen having a temperature of -196°C . For germplasm conservation shoot tips or plantlets can be stored. Such stored materials can be used as and when required. Cell division can be suppressed by adding growth retardant like abscisic acid, mannitol, sorbitol, malic hydrazide, succinic acid etc.

4. Plant secondary metabolites

Plant products can be classified into primary plant metabolites and secondary metabolites. Primary plant metabolites are essential for the survival of the plant. It consists of sugars, amino acids and nucleotides synthesized by plants and are used to produce essential polymers. Typically primary metabolites are found in all species within broad phylogenetic groupings, and are produced using the same metabolic pathway.

Secondary metabolites are the chemicals, which are not directly involved in the normal growth and development, or reproduction of an organism. Secondary metabolites are not indispensable for the plants but play a significant role in plant defense mechanisms. Primary metabolites essentially provide the basis for normal growth and reproduction, while secondary metabolites for adaptation and interaction with the environment. The economic importance of secondary metabolites lies in the fact that they can be used as sources of industrially important natural products like colours, insecticides, antimicrobials, fragrances and therapeutics. Therefore, plant tissue culture is being potentially used as an alternative for plant secondary metabolite production. Secondary metabolism in plants is activated only in particular stages of growth and development or during periods of stress, limitation of nutrients or attack by micro-organisms. Plants produce several bioactive compounds that are of importance in the healthcare, food, flavor and cosmetics industries. They are terpenes, phenolics and nitrogen and/or sulphur containing compounds. They are specific to an individual species, genus and are produced during specific environmental conditions which makes

their extraction and purification difficult. Hence Plant cell suspension culture is used to produce them in large quantities.

5. Biotransformation

New chemical compounds can be produced by using plant enzymes for modifications, like methylation, hydroxylation and glycosylation. Stereo- specific biotransformation of the plant cells can be performed for the production of bioactive compounds from economical precursors.

6. Molecular farming

Novel compounds which are not generally found in the parent plants can be produced in the in vitro grown plants through plant tissue culture. It offers a defined production system, continuous supply of products with uniform quality. Efficient downstream recovery of products and rapidity of production are its added advantages.

A] Production of industrial enzymes-The first commercialized 'industrial proteins' produced from transgenic plants were avidin and β - glucuronidase, both of which were produced in maize.

B] Antibodies or immunoglobulins- are the defense proteins produced in mammals. The use of plants for commercial production of antibodies, referred to as plantibodies, is a new approach in biotechnology. The first successful functional antibody produced in plant was mouse immunoglobulin IgG1. For this two transgenic tobacco plants, one synthesizing heavy γ chain and the other light κ chain, are crossed to generate progeny that can produce an assembled functional antibody.

C] Production of edible vaccines- Transgenic plants provide an alternative system for the production of recombinant vaccines. The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration. By the use of edible vaccines the problems associated with the purification of vaccines can be avoided. Edible vaccine provides mucosal immunity against infectious agents. Vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used. Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw. Vaccines, being protein are likely to be

degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response. There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff.

7. Production of resistant plant varieties

Insect resistance

Insects cause serious losses in agricultural products in the field at the time of cultivation and during storage. Insects belonging to the orders, Coleoptera, Lepidoptera and Diptera, are the most serious plant pests which cause agricultural damages. Use of insecticides, bio-pesticides has several harmful side effects. *Bacillus thuringiensis* (Bt), a free-living, Gram-positive soil bacterium, has been employed as insecticide specificity towards lepidopteran pests. It is environmentally safe and thus, is high in demand. On the other hand, the major problems in using Bt sprays for controlling the insect attack on plants are high cost of production of Bt insecticide and instability of the protoxin under field conditions. To avoid these problems transgenic plants expressing Bt toxin genes have been engineered. In the 1980's a lot of work was undertaken by Monsanto to identify and extract the Bt genes and during this decade the gene encoding for the Bt protein Cry1Ac was successfully inserted into a cotton plant.

Three hypotheses have been investigated to engineer development of virus resistance plants

- Expression of the virus coat protein (CP) gene
- Expression of satellite RNAs and
- Use of antisense viral RNA

Long-lasting tomatoes

Long-lasting, genetically modified tomatoes now came in to the market. This is the first genetically modified food available to consumers. The genetically modified tomato produces less of the substance that causes tomatoes to rot, so remains firm and fresh for a long time.

8. Somatic hybrids & GM crops for better yield

Plant regeneration from cultured cells is proving to be a rich source of genetic variability, called “somaclonal variation”. Regeneration of plants from microspore/pollen provides the most reliable and rapid method to produce haploids, which are extremely valuable in plant breeding and genetics. With haploids, homozygosity can be achieved in a single step, cutting down the breeding period to almost half. Pollen raised plants also provide a unique opportunity to screen gametic variation at sporophytic level. This approach has enabled selection of several gametoclones, which could be developed into new cultivars.

9. Nutraceuticals

Golden rice

Golden rice is genetically modified rice which contains a large amount of A-vitamins. Or more correctly, the rice contains the constituent beta-carotene which is converted in the body into Vitamin-A. So when you eat golden rice, so can get more amount of vitamin of A. Beta-carotene is orange colour so genetically modified rice is golden color. For the making of golden for synthesis of beta-carotene three new genes are implanted: two from daffodils and the third from a bacterium.

Advantages:

- The rice can be considered for poor people in underdeveloped countries. They eat only an extremely limited diet lacking in the essential bodily vitamins.

Disadvantage

- Critics terror that poor people in underdeveloped countries are becoming too dependent on the rich western world. Generally, genetically modified plants are developed by the large private companies in the West.
- The customers who buy patented transgenic seeds from the company may need to sign a contract not to save or sell the seeds from their harvest, which raises concerns that this technology might lead to dependence for small farmers.

10. Production of biodegradable plastics

Biodegradable plastics (Bioplastics) are chemically polyhydroxyalkanoates (PHAs). They are intracellular carbon and energy storage compounds, produced by many microorganisms. They are biodegradable polymers, and are elastic in nature. These compounds are currently produced by microbial fermentation. Several experimental studies are in progress to produce bulk quantities of bioplastics in plants. Among the PHAs polyhydroxy butyrate (PHB) is the most important one.

PHA serves as lipid reserve material in bacteria. The granules of PHA, stored within the cells are clearly visible under electron microscope. PHAs are linear polyester polymers composed of hydroxyacid monomers. Structures of PHAs are given in Figure 40.2. The most commonly found monomers are 3 hydroxy acids with a carbon length ranging from C₃ to C₁₄

Laboratory Design and Development

The size of tissue culture lab and the amount and type of equipment used depend upon the nature of the work to be undertaken and the funds available. A standard tissue culture laboratory should provide facilities for:

- washing and storage of glassware, plasticware
- preparation, sterilization and storage of nutrient media
- aseptic manipulation of plant material
- maintenance of cultures under controlled temperature, light and humidity
- observation of cultures, data collection and photographic facility
- acclimatization of in vitro developed plants. The overall design must focus on maintaining aseptic conditions.

At least three separate rooms should be available one for washing up, storage and media preparation (the media preparation room); a second room, containing laminar-air-flow or clean air cabinets for dissection of plant tissues and subculturing (dissection room or sterilization room); and the third room to incubate cultures (culture room). This culture room should contain a culture observation table provided with binoculars or stereozoom microscope and an adequate light source.

Additionally, a green house facility is required for hardening-off in vitro plantlets. For a commercial set-up, a more elaborate set-up is required.

Media preparation room

The washing area in the media room should be provided with brushes of various sizes and shapes, a large sink, preferably lead-lined to resist acids and alkalis, and running hot and cold water. It should also have large plastic buckets to soak the labware to be washed in detergent, hot-air oven to dry washed labware and a dust-proof cupboard to store them. If the preparation of the medium and washing of the labware are done in the same room, a temporary partition can be constructed between the two areas to guard any interference in the two activities. A continuous supply of water is essential for media preparation and washing of labware. A water distillation unit of around 2 litre/h, a Milli-Q water purification systems needs to be installed.

Culture room

The room for maintaining cultures should be maintained at temperature $25 \pm 2^\circ\text{C}$, controlled by air conditioners and heaters attached to a temperature controller are used. For higher or lower temperature treatments, special incubators with built-in fluorescent light can be used outside the culture room. Cultures are generally grown in diffuse light from cool, white, fluorescent tubes. Lights can be controlled with automatic time clocks. Generally, a 16-hour day and 8-hour nights are used. The culture room requires specially designed shelving to store cultures. Some laboratories have shelves along the walls, others have them fitted onto angle-iron frames placed in a convenient position. Shelves can be made of rigid wire mesh, wood or any building material that can be kept clean and dust-free. Insulation between the shelf lights and the shelf above will ensure an even temperature around the cultures. While flasks, jars and petridishes can be placed directly on the shelf or trays of suitable sizes, culture tubes require some sort of support. Metallic wire racks or polypropylene racks, each with a holding capacity of 18-24 tubes, are suitable for the purpose.

Sterilization room

This area should have restricted entry, which is needed to ensure the sterile conditions required for the transfer operations. For sterile transfer operations, the

laminar-air-flow cabinets are used. Temperature control is essential in this room as the heat is produced continuously from the flames of burners in the hoods. The room should be constructed in a way to minimize the dust particles and for easy cleaning. Several precautions can be taken including the removal of shoes before entering the area.

The laminar horizontal flow sterile transfer cabinets are available in various sizes from many commercial sources. They should be designed with horizontal air flow from the back to the front, and equipped with gas cocks if gas burners are to be used. Electrical outlets are needed for use of electric sterilizers and microscopes, and if weighing is to be done in the hoods. A stainless steel working platform is most durable, easy to keep clean and to prevent the unwanted damage due to accidental fire. Sometimes it is fitted with Ultraviolet light to maintain sterility inside the cabinet. UV light is a source of ozone, which can be mutagenic, therefore, utmost care is to be taken while using this. Although UV light is not necessary, a short exposure time of 3-5 min to cabinet is fine sometimes. Work can be started after 10-15 min of switching on the air flow, and one can work uninterrupted for long hours.

A Laminar-air-flow cabinet has small motor to blow air which first passes through a coarse filter, where it loses large particles, and subsequently through a fine filter known as 'high efficiency particulate air (HEPA). The HEPA filters remove particles larger than $0.3 \mu\text{m}$, and the ultraclean air flows through the working area. The velocity of the ultra clean air is about $27 \pm 3 \text{ m min}^{-1}$ which is adequate for preventing the contamination of the working area as long as the flow is on. The flow of the air does not in any way hamper the use of a spirit lamp or a Bunsen burner. A

Greenhouse

The greenhouse facility is required to grow parent plants and to acclimatize in vitro raised plantlets. The size and facility inside the green house vary with the requirement and depends on the funds available with the laboratory. However, minimum facilities for maintaining humidity by fogging, misting or a fan and pad system, reduced light, cooling system for summers and heating system for winters must be provided. It would be desirable to have a potting room adjacent to this facility.

Equipments and apparatus

Media preparation area, benches at a height suitable to work while standing, pH meter to determine the pH of various media [standard media pH is maintained at 5.8], hot-plate-cum-magnetic stirrer for dissolving chemicals and during media preparation, an autoclave to sterilize media, water, labware, forceps, needles etc. [Certain spores from fungi and bacteria can only be killed at a temperature of 121°C and 15 pounds per square inch (psi) for 15-20 min], high quality microbalance to weigh smallest of the quantities & top pan balance less sensitive quantities, hot-air oven for the dry heat sterilization of clean glassware like, Petridishes, culture tubes, pipettes etc. [Typical sterilizing conditions are 160-170 °C/1hr], provision for Reverse osmosis water & sink for cleaning glass pipettes in running water. Binocular microscope to observe surface details and morphogenic responses of cultures and their possible contamination & low speed table-top centrifuge to sediment cells or protoplasts. Culture room should have heating / cooling system) to maintain 25±2 °C temperature, racks for holding test-tubes, lights to provide diffuse light and to maintain photoperiod, shakers with various sized clamps for different sized flasks to grow cells in liquid medium, time clock for lights, wall cabinets for dark incubation of cultures. Other apparatus like beakers, measuring cylinders, graduated pipettes, reagent bottles for storing liquid chemicals and stock solutions (glass or plastic), culture tubes and flasks (glass or polypropylene or disposable), plastic baskets, filter membrane of sizes 0.22 µm and 0.45 µm & filter assembly for solutions requiring filter sterilization, large forceps (blunt and fine points) and scalpels for dissecting and subculturing plant material, chemicals and reagents for preparing culture media, disposable gloves and masks & Micropipettes of various volumes.

Preparation and handling of culture media

The simplest method of preparing media is to use commercially available, dry, powdered media containing mineral elements and growth regulators. By following the procedure written on the packets, dissolve the powder in distilled or demineralized water (10% less than the final volume of the medium). After adding sugar and other desired supplements like, plant growth regulators, make up the final volume with distilled water, adjust the pH, add agar and then autoclave the

medium. An alternative method of media preparation is to prepare a series of concentrated stock solutions which can be combined later as required. For preparing stock solutions and media, use glass-distilled or demineralized water and chemicals of high purity, analytical reagent (AR) grade.

Macronutrients are required in large amounts (concentrations greater than 0.5 mmole l⁻¹) by cultured plant cells. Macronutrients are usually considered to be carbon, nitrogen, phosphorous, magnesium, potassium, calcium and sulphur.

Micronutrients are required in trace amounts (concentrations less than 0.5 mmole l⁻¹) for plant growth and development. These include, manganese, copper, cobalt, boron, iron, molybdenum, zinc and iodine.

Organic supplements (vitamins) essential are myo-inositol and thiamine. Myo-inositol is considered to be vitamin B and has many diverse roles in cellular metabolism and physiology. It is also involved in the biosynthesis of vitamin C. Carbon source is supplied in the form of carbohydrate. Plant cells and tissues in the culture medium are heterotrophic and are dependent on external source of carbon. Sucrose is the preferred carbon source.

Dilutions:

$$\frac{\text{required concentration} \times \text{medium volume}}{\text{concentration of stock solution}} = \text{volume of stock required}$$

S.No.	Elements	Functions
1	Oxygen	Common cell component, electron acceptor.
2	Carbon	Common cellular component, forms basic backbone of most biochemicals.
3	Nitrogen	Part of proteins, vitamins, amino acids and coenzymes.
4	Sulphur	Part of some amino acids and some coenzymes.
5	Potassium	Principal inorganic cation
6	Magnesium	Important coenzyme factor and part of chlorophyll molecules
7	Manganese	Important cofactor
8	Calcium	Important constituent of cell wall and enzyme cofactor
9	Iron	Part of cytochromes
10	Cobalt	Part of some vitamins
11	Copper	Enzyme cofactor
12	Zinc	Enzyme cofactor
13	Molybdenum	Enzyme cofactor

The composition of Murashige and Skoog (MS) Medium (1962)

Component	Concentration in stock (mg l ⁻¹)	Concentration in medium (mg l ⁻¹)	Volume of stock per litre of medium (ml)
Macronutrients			
NH ₄ NO ₃	33000	1650	50
KNO ₃	38000	1900	
CaCl ₂ ·2H ₂ O	8800	440	
MgSO ₄ ·7H ₂ O	7400	370	
KH ₂ PO ₄	3400	170	
Micronutrients			
KI	166	0.83	5
H ₃ BO ₃	1240	6.2	
MnSO ₄ ·4H ₂ O	4460	22.3	
ZnSO ₄ ·7H ₂ O	1720	8.6	
Na ₂ MoO ₄ ·2H ₂ O	50	0.25	
CuSO ₄ ·5H ₂ O	5	0.025	
CoCl ₂ ·6H ₂ O	5	0.025	
Iron Source			
FeSO ₄ ·7H ₂ O	5560	27.8	5
Na ₂ EDTA·2H ₂ O	7460	37.3	
Vitamins			
Myo-inositol	Add freshly to the medium	100	5
Nicotinic acid	100	0.5	
Pyridoxine-HCl	100	0.5	
Thiamine-HCl	100	0.5	
Glycine	400	2	
Carbon Source			
Sucrose	Add freshly to the medium	30g l ⁻¹	
Adjust pH to 5.7-5.8 before autoclaving			

- Stock concentration of macronutrients is for 20 litres of medium, while micronutrients, iron and vitamins stock concentrations are prepared for 200 litres of medium.

- Myoinositol and sucrose are added freshly to the medium.
- Dissolve 5.56 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 350 ml of water. Apply heat if needed. Dissolve 7.46 g of Na_2 EDTA in 350 ml of water. Apply heat if needed. When both solutions are dissolved, combine and bring to 1 litre final volume. The chelation reaction is forced to completion by autoclaving. The final stock solution should be deep golden yellow in color.

The steps involved in preparing a medium are summarized below:

- ✓ Add appropriate quantities of various stock solutions, including growth regulators and other special supplements. Make up the final volume of the medium with distilled water.
- ✓ Add and dissolve sucrose.
- ✓ After mixing well, adjust the pH of the medium in the range of 5.5-5.8, using 0.1 N NaOH or 0.1 N HCl (above 6.0 pH gives a fairly hard medium and pH below 5.0 does not allow satisfactory gelling of the agar).
- ✓ Add agar, stir and heat to dissolve. Alternatively, heat in the autoclave at low pressure, or in a microwave oven.
- ✓ Once the agar is dissolved, pour the medium into culture vessels, cap and autoclave at 121°C for 15 to 20 min at 15 pounds per square inch (psi). If using pre-sterilized, non-autoclavable plastic culture vessels, the medium may be autoclaved in flasks or media bottles. After autoclaving, allow the medium to cool to around 60°C before pouring under aseptic conditions.
- ✓ Allow the medium to cool to room temperature. Store in dust-free areas or refrigerate at 7°C (temperature lower than 7°C alter the gel structure of the agar).

Gelling agents

The media listed above are only for liquids, often in plant cell culture a 'semi-solid' medium is used. To make a semi-solid medium, a gelling agent is added to the liquid medium before autoclaving. Gelling agents are usually polymers that set on cooling after autoclaving. Agar, Agarose or Gelrite can be used.

Plant growth regulators

In addition to nutrients, four broad classes of growth regulators, such as, auxins, cytokinins, gibberellins and abscisic acid are important in tissue culture. The growth, differentiation, organogenesis and embryogenesis of tissues become feasible only on the addition of one or more of these classes of growth regulators to a medium. In tissue culture, two classes of plant growth regulators, cytokinins and auxins, are of major importance. Auxins are found to influence cell elongation, cell division, induction of primary vascular tissue, adventitious root formation, callus formation and fruit growth. The cytokinins promote cell division and axillary shoot proliferation while auxins inhibit the outgrowth of axillary buds. Besides, cytokinin in tissue culture media, promote adventitious shoot formation. The ratio of plant growth regulators required for root or shoot induction varies considerably with the tissue and is directly related to the amount of growth regulators present at endogenous levels within the explants. In general, shoots are formed at high cytokinin and low auxin concentrations in the medium, roots at low cytokinin and high auxin concentrations and callus at intermediate concentrations of both plant growth regulators.

Establishing aseptic cultures

Plant tissue culture media contain sugar and so support the growth of many microorganisms (bacteria and fungi). When these microorganisms reach a medium, they generally grow much faster than the cultured plant materials. Their growth and toxic metabolites will affect, and may even kill, the tissue cultures. It is, therefore, essential to maintain a completely aseptic environment inside the culture vessels.

There are several possible sources of contamination of the medium- the culture vessel, the medium itself, explant, the environment of the transfer area, the instruments used to handle plant material during establishment and subculture & the environment of the culture room.

Autoclaving media will eliminate contamination from the culture vessel or the medium. In some cases, substances such as gibberellic acid, abscisic acid (ABA), urea and certain vitamins are thermolabile and break down upon autoclaving. These chemicals can be sterilized by membrane filtration using microfilters of pore size 0.22-0.45 μm which is suitable enough to exclude pathogens. Later the filter sterilized compound can be added to autoclaved medium cooled to around 40°C.

To prevent the environment of the culture room from being the source of contamination, keep the culture room as dust-free as possible and remove contaminated cultures from the area as soon as they are detected. Ideally, the culture room should be clean, filtered air which has passed through high efficiency particulate air (HEPA) filters.

The transfer area in most laboratories is within a laminar air-flow cabinet. A laminar air-flow cabinet has a small fan which blows air through a coarse filter to remove large dust particles and then through a fine HEPA filter to remove microbes, their spores and other particles larger than 0.3 μm . The velocity of the air coming out of the fine filter is about 27 ± 3 m/min, which keeps airborne microorganisms out of the working area. The working area is swabbed with 70% alcohol (or equivalent) and instruments dipped in 70% alcohol, flamed and cooled before use.

Take care with ultraviolet light as it can permanently damage eyes and promote skin cancer. Laminar flow cabinets equipped with ultraviolet light for surface sterilization should be fitted with safety doors which can be closed when ultraviolet light is used.

Plant surfaces carry a wide range of microorganisms. The tissue must be thoroughly surface-sterilized before being placed on the nutrient medium. Discard cultures with fungal or bacterial contamination. Solutions of sodium or calcium hypochlorite are usually effective in disinfecting plant tissues. Placing tissues in a 0.5 to 1% solution of sodium hypochlorite for 10 to 15 minutes will disinfect most tissues. Surface sterilants are toxic to plant tissues. Choose the concentration of the sterilizing agent and the length of time to minimize tissue damage, which shows up as white, bleached areas. Other techniques for surface sterilisation include dipping plant material for a few seconds in 90% ethanol or placing in running water for 30 minutes and 2 hours before disinfection.

A summary of the six steps commonly involved in establishing and maintaining aseptic plant tissue culture follows.

- i. Collect pieces of plant material (ex-plants) in a screw-cap bottle. Immerse them in a dilute solution of the disinfectant containing a wetting agent. Replace the lid and store the bottle in the laminar air flow cabinet. Shake the bottle two or three times during the sterilization period.

- ii. Remove the lid and drain carefully. Thoroughly rinse the plant material in sterilized distilled water and replace the lid. After shaking a few minutes, discard the water. Rinse two or three times more.
- iii. Transfer the material to a pre-sterilized Petri-dishes or test-tubes.
- iv. Sterilize the required instruments by dipping them in 70% ethanol and flamed them. Allow to cool. Sterilize the instruments after each time they are used to handle tissue.
- v. Prepare suitable explants from the surface sterilized material using sterilized instruments (scalpels, needles, forceps, etc.).
- vi. Quickly remove the lid of the culture vessel, transfer the explants on to the medium, flame the neck of the vessel (only if glass) and replace the lid.

If handling aseptic plant materials during routine subculture, omit the first two steps.

BIBLIOGRAPHY

1. Biotechnology – changing life through science
2. Invitation to biotechnology
3. HI media plant tissue culture manual
4. Lecture notes on workshop on biotechnology education