Plant Tissue Culture
Lab Practices Made Easy
(For Beginners)

-Dr. Monica Jain

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PLANT TISSUE CULTURE-
LAB PRACTICES MADE EASY

(FOR BEGINNERS)

By

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Dr. Monica Jain

FOREWORD

Plant tissue culture technique is being utilized as an imperative tool to meet the green demands of the day by day increasing population. The constraints being faced today is the lack of awareness and limited resources in the rural areas of the developing countries which pulls back the swiftness and efficacy of this technique.

For achieving this it is necessary to professionalize and popularize the technology. The manual is an attempt to provide low cost alternatives for expensive physical and chemical methods and to make plant tissue culture practicals economical so that it can be performed at every common lab with minimum basic requirements and help in disseminating the knowledge and practical learning to the young generation. We look forward to disseminate this knowledge to students and develop entrepreneurs in this field contributing to green economy of the country.

The manual provides basic protocols involved for beginners in plant tissue culture. The low cost alternatives recommended are standardized on *Brahmi (Bacopa monnieri)* in our laboratory and can be standardized on different plant systems in practice. Let us all team up and proceed towards a greener lab to land.

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Abbreviations used in the text

- %- Percentage
- 2,4-D- 2,4-dichlorophenoxyacetic acid
- B5- Gamborg's B5 media
- BAP- 6-benylaminopurine
- DW- Distilled water
- EtOH- Ethanol
- h - hour
- l- Litre
- LAF- Laminar air flow
- min- Minutes
- ml- Milli litre
- MS- Murashige and Skoog's media
- NAA- α-Naphthalene acetic acid
- ppm - Parts per million.
- SDW- Sterile distilled water
- sec- Seconds

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DR. SHARAD TIWARI

Plant tissue culture: Prospects

Tissue culture technology is used for the production of doubled haploids, cryopreservation, propagating new plant varieties, conserving rare and endangered plants, difficult-to-propagate plants, and to produce secondary metabolites and transgenic plants. The production of high quality planting material of crop plants and fruit trees, propagated from vegetative parts, has created new opportunities in global trading, benefited growers, farmers, and nursery owners, and improved rural employment. However, there are still major opportunities to produce and distribute high quality planting material, e.g. crops like banana, date palm, cassava, pineapple, plantain, potato, sugarcane, sweet potato, yams, ornamentals, fruit and forest trees.

The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather. However, the technology is capital, labor and energy intensive. Although, labor is cheap in many developing countries, the resources of trained personnel, equipment and infrastructure are often not affordable. In addition, energy, particularly electricity, and clean water are costly. The energy requirements for tissue culture technology depend on day temperature, day-length and relative humidity, and they have to be controlled during the process of propagation. Individual plant species also differ in their growth requirements.

In view of retrospect of various in vitro technologies (as compared to conventional methods) such as higher production cost, infrastructure requirements, technology driven, vulnerability of cultures to contaminations and difficulties during hardening in vitro technique should be adapted only when it is truly essential. Plant tissue culture cannot be solution for all the problems of crop improvement. Unfortunately, lots of efforts in this field are being wasted on indiscriminate superfluous experimentation with usually unrepeatable results. Tissue culture may prove to be indispensable tool in the hands of plant breeders and seed/planting material producers if applied suitably, as described in this article.

A. Seed Culture

It is performed by surface sterilization and *in vitro* culture for increasing efficiency of germination of seeds which that are difficult to germinate *in vivo*. Seed culture can be used for:

1. Precocious germination by application of plant growth regulators.
2. Production of clean seedlings for explants or meristem culture.

B. Embryo culture

Embryo culture is the sterile isolation and growth of an immature or mature embryo in vitro, with the goal of obtaining a viable plant. In plant breeding embryo culture have been valuable tools, especially for the transfer of biotic and abiotic resistance genes from wild relatives into crop plants.

Embryo culture has been used to rescue hybrid plants from wide crosses, which often fail to produce mature viable seeds. In these cases the immature embryo tissue can be removed from the developing seeds and cultured in the laboratory to produce the hybrid plants. Embryo culture enables the breeder to successfully make wide crosses with a greater number of related species of wild plants and have access to a much wider range of genes that can be used for genetic improvement of crop plants.

1. Testing seed viability as functional embryo represent viable seed
2. Overcoming seed dormancy and self-sterility of seeds.
3. Overcoming embryo abortion due to incompatibility barriers
4. Production of haploid plants by embryo rescue after abortion of one set of genome in *bulbosum* technique.
5. Embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor
6. Shortening of breeding cycle culturing immature embryos especially in marker assisted selection (MAS).

C. *In vitro* pollination

When pollen is applied to stigma of ovaries cultured in vitro or directly onto ovules cultured with or without placental tissue, it is called in vitro pollination. Ovaries are collected from emasculated flowers usually 1-2 days after anthesis and cultured intact or with the ovarian, wall removed to expose the placenta. Alternatively, the entire placenta or pieces of placenta bearing ovules may be cultured. This technique is usually carried out when hybrids are difficult to produce by embryo rescue.

D. Haploid Technology

Plant tissue culture have extended the range of crop species from which haploid plants have been produced as well as the efficiency resulting in large-scale haploid plant production by anther and microspore culture techniques Specialized plant tissue culture methods have enabled the production of completely homozygous breeding lines from gametic cells in a shortened time frame compared to conventional plant breeding.

1. Production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce inbred lines
2. Uncovering mutations or recessive phenotypes for selection of desirable traits.
3. Releasing new varieties through F_1 double-haploid system.
4. Developing asexual lines of trees/perennial species
5. Transfer of desired alien genes
6. Developing “Double Haploid (DH)” mapping populations for QTL analysis.

E. Ovary or ovule culture

In flowering plants, the ovule is typically located inside the ovary of the gynoecium which produces the ovules. Ovule consists of three parts: the integument(s) forming its outer layer(s), the nucellus (or megasporangium), and the megaspore-derived female gametophyte (or megagametophyte) in its center.

1. A common explant for the initiation of somatic embryogenic cultures
2. Unfertilized ovary and ovule culture may lead to production of haploid plants
3. Overcoming abortion of embryos of wide hybrids at very early stages of development due to incompatibility barriers
4. In vitro fertilization for the production of distant hybrids avoiding style and stigmatic incompatibility that inhibits pollen germination and pollen tube growth

F. Shoot apical meristems culture

This is a method of asexual propagation used to produce clones of a particular plant in large quantities. The shoot apex explant measures between 100 to 500µm and includes the apical meristem with 1 to 3 leaf primordia. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium. In practice, shoot-tip explants between 100 to 1000µm are cultured to obtain viruses free plants. Meristem cells are grown in nutrient solutions in laboratory flasks until they have recognizable roots and leaves. They are then transplanted into suitable potting medium for hardening. These techniques allow the shortening of the period required to produce large numbers of cloned plants in very less space round the year.

1. Mass *in vitro* propagation for plantation
2. Production of virus free plant
3. Mass production of desirable genotypes
4. Facilitation of germplasm exchange between locations (production of clean material)
5. Cryopreservation (cold storage) or *in vitro* conservation of germplasm

G. Nodal segment or axillary bud culture:

This consists of a piece of stem with axillary bud culture with or without a portion of shoot. When only the axillary bud is taken, it is designated as “axillary bud” culture. These techniques are mostly applied for mass propagation. However, axillary bud culture with some part of shoot does not provide virus free plants.

H. Micro grafting

Tissue culture offers numerous significant benefits over traditional propagation methods by traditional means. It may be possible in vitro to multiply plant that are very difficult to propagate by cuttings or other traditional methods. The key role of micro grafting are:

1. Alternate mass *in vitro* propagation technique especially for orchards
2. Overcoming graft incompatibility
3. Rapid mass propagation of elite scions by grafting onto rootstocks that have desirable traits
4. To allow survival of difficult to root shoots
5. Development of virus free plants

I. Somatic embryogenesis

Somatic embryogenesis has been described to occur in two ways: directly or indirectly. Direct embryogenesis occurs when embryos are started directly from explant tissue creating an identical clone while indirectly occurs from unorganized tissue (callus). Plant regeneration via somatic embryogenesis occurs in five steps: initiation of embryogenic cultures, proliferation of embryogenic cultures, prematuration of somatic embryos, maturation of somatic embryos and plant development on nonspecific media. Some highlights of somatic embryogenesis are:

1. One of the major pathway of regeneration
2. Production of artificial seeds
3. As source material for embryogenic protoplasts
4. Amenable to mechanization and for bioreactors
5. Preferred morphogenesis pathway of regeneration for gene transfer methods

J. Synthetic seeds

Synthetic seeds (somatic embryo as substitutes for true seeds) can be produced either as coated or non-coated, desiccated somatic embryos or as embryos encapsulated in hydrated gel (usually calcium alginate). Successful utilization of synthetic seeds as propagules of choice requires an efficient and reproducible production system and a high percentage of post-planting conversion into vigorous plants. Artificial coats and gel capsules containing nutrients, pesticides and beneficial organisms have long been thought as substitutes for seed coat and endosperm.

K. Callus cultures

Any explant *i.e.* any plant parts can be cultured to initiate callus. A callus is a mass of unorganized cells, which in many cases, upon transfer to suitable medium, is capable of giving rise to shoot-buds and somatic embryos, which then form complete plants. In some instances it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis. Some important aspects of callus cultures are:

1. For generation of useful somaclonal variants (genetic or epigenetic)
2. As a source of protoplasts and suspension cultures
3. For production of metabolites
4. Used in *in vitro* selection

L. Suspension Culture

Tissues and cells cultured in a liquid medium produce a suspension of single cells and cells clumps of few to many cells: these are called suspension cultures. Liquid cultures must be constantly agitated, generally by a gyratory shaker, to facilitate aeration and dissociation of cell clumps into smaller pieces. Suspension cultures grow much faster than callus cultures need to be subcultured about every week, allow a more accurate determination of the nutritional requirements of cells and are amenable to scaling up for a large scale production of cells and even somatic embryos (SEs). The suspension cultures are broadly grouped as batch cultures, continuous cultures and immobilized cell cultures. To a limited extent, bioreactors have become popular for somatic embryogenic culture micropropagation and production of secondary metabolites. It is considered that some day robotics could be adapted to bioreactor based technology. Applications of suspension culture are similar to callus culture leave culturing in liquid medium.

M. Protoplast isolation, culture and fusion

A protoplast is a cell that had its cell wall completely or partially removed using either mechanical or enzymatic means. Cell walls are made of a variety of polysaccharides. Protoplasts can be made by degrading cell walls with a mixture of the appropriate polysaccharide-degrading enzymes. Protoplasts of any origin can be fused with each other since they do not have membrane barrier. This unique feature makes it an object of choice for:

1. Studying membrane characteristics including the uptake of macromolecules and viruses.
2. Combining genomes to produce somatic hybrids, asymmetric hybrids or cybrids
3. Production of organelle recombinants
4. Some plants that show physical or chemical incompatibility in normal sexual crosses, may be produced by the fusion of protoplasts obtained from two cultures of different species
5. Cybridization especially for transfer of cytoplasmic male sterility.
6. Widely used for DNA transformation (for making genetically modified organisms), since the cell wall would otherwise block the passage of DNA into the cell.

N. *In vitro* mutagenesis

One of the applications of tissue culture systems is their exploitation for the induction and isolation of mutant cells, which can then be regenerated as mutant plants. While a number of mutations have been recognized in plant cells *in vitro*, few have significance for plant breeding as:

1. Induction of polyploidy
2. Introduction of genetic variability

O. Somaclonal Variations

In plant breeding tissue culture in conventional micro propagation has resulted to a large extent in clonal fidelity, it has become increasingly clear that under the appropriate culture conditions, a great deal of genetic variability can be recovered in regenerated plants. If cultures are established from explants that did not contain a pre-organized meristem, or if cultures are maintained as callus prior to plant regeneration, the regenerated plants are quite variable. In early report, most of the variations were attributed to the readily detected chromosome instability of cultured plant cells. In

many cases, the degree of instability was reported to be proportional to the length of time the cells remained in culture. Reorganization of this spontaneous variation inherent in long- term culture led to the use of cell culture for mutagenesis and selection of genetic variants and for direct recovery of novel genotypes from cell cultures via somaclonal variation. Indications of somaclonal variation in several crop plants have stimulated interest in application of this method for crop improvement.

P. Genetic transformation

Tissue culture is an essential part of many genetic transformation protocols. The ability to move DNA into an organism and thereby alter its genotype or genetic makeup is central to both basic and applied molecular biology. Many different explants can be used, depending on the plant species and its favored method of regeneration as well as the method of transformation. Genes derived from unrelated species and even other kingdoms, such as bacteria, fungi, plants, animals, that would otherwise be inaccessible to an organism, can be combined in the lab using genetic transformation techniques. Many different explants can be used, depending on the plant species and its favored method of regeneration as well as the method of transformation.

1. Introduction of foreign DNA to generate novel (and typically desirable) genetic combinations
2. Used to study the function of genes.

Conclusions

Plant tissue culture is now a well established technology which has made significant contributions to the propagation and improvement of agricultural crops in general. Greater contribution is envisaged from this technology in years to come, both in its own right and as an adjunct to the application of molecular biology. However, most of the experiments conducted are empirical and difficult to repeat. Understanding of the biological processes that permit the manipulation of *in vitro* morphogenesis and investigations on various physiological, biochemical and molecular aspects of plant hormones will greatly advance our knowledge and provide information that will help address the issues of *in vitro* recalcitrance and control of *in vitro* plant growth and development.

Chapter 1

Lab Set up, Equipments and low cost alternatives

A standard tissue-culture laboratory should provide facilities for:

(a) **Wash Area**: For washing and storage of glassware, plastic ware and other lab wares.

(b) **Media Preparation Room**: For preparation, sterilization and storage of nutrient media.

(c) **Culture Transfer Room**: For aseptic manipulation of plant material in Laminar Air Flow.

(d) **Culture Room**: For maintenance of cultures under controlled conditions of temperature, light and, if possible, humidity.

(e) **Green House/Net house**: For hardening and acclimatization of *in vitro* developed plants (Optional).

(f) **Consumables:**

a) <u>Wash Area and its activities</u>

In all instances, glassware must be physically clean; it must be chemically clean; and in many cases, it must be bacteriologically clean or sterile.

- The Wash area should have water facility and minimum basic amenities for cleaning, washing and rinsing of the glass wares and plastic wares used for media preparation and media storage.
- All glassware must be absolutely grease-free.
- For used culture tubes and bottles, acid wash can also be done.
- Do not allow acid to come into contact with a piece of glassware before the detergent (or soap) is thoroughly removed. If this happens, a film of grease may be formed.
- Chromic acid cleaning solution - Use powdered sodium dichromate or potassium dichromate. If the compound is in the form of crystals, grind to a fine powder in a mortar. To 20 grams of the powder in a beaker, add a little water, sufficient to make a thin paste. Slowly add approximately 300mL of commercial concentrated sulphuric acid, stirring well. Transfer to a glass-stoppered bottle. Use the clear supernatant solution. Chromic acid solution can be used repeatedly until it begins to turn a greenish color. Chromic acid solution is strongly acidic and will burn the skin severely. Use care in handling it.

Hot Air Oven:

- Once the glass wares get air dry they are placed in a hot air oven for dry heat sterilization. Proper time and temperature for Dry-Heat sterilization is **160 °C** (**320 °F**) for 2 hours or 170 °C (340 °F) for 1 hour. Instruments should be dry before sterilization since water will interfere with the process
- The d**ry-heat destroys microorganisms by causing coagulation of proteins.**
- The temperature range id 50-**300**°C.
- The thermostat controls the temperature.
- The double walled insulation keeps the heat in and conserves energy.

Fig. 1: A hot air oven

b) <u>Media Preparation Room</u>

A media preparation room is equipped with necessary equipments and facilities to enable the sterile and accurate production and storage of media and reagents for the growth of cells *in vitro*.

It should contain:

1) Autoclave:

- An autoclave is basically a large-sized but sophisticated pressure cooker, and is used for the sterilisation of the medium, glassware and instruments. High-pressure wet heat is needed to sterilise media, water and glassware. Certain spores from fungi and bacteria are killed only at 121°C and 1.05kg/sq.cm (15 pounds per sq. inch) pressure. The time of sterilization depends on the volume to be sterilized.

Volume of Contents	**Time of Sterilization**
500 ml.	**20 min.**
1000 ml.	**25 min.**
2000 ml.	**30 min.**

- **Low cost alternative**: A simple cooker can also solve the purpose if the load is less.

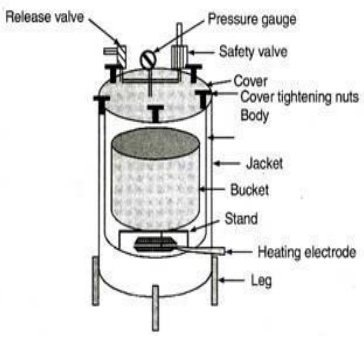


Fig.2: A schematic diagram of laboratory autoclave

2) Water distillation Unit

Distilled water is water from which impurities have been removed through distillation. Distillation involves boiling the water and then condensing the steam into a clean container. Various glass and steel water distillation units are available in market.

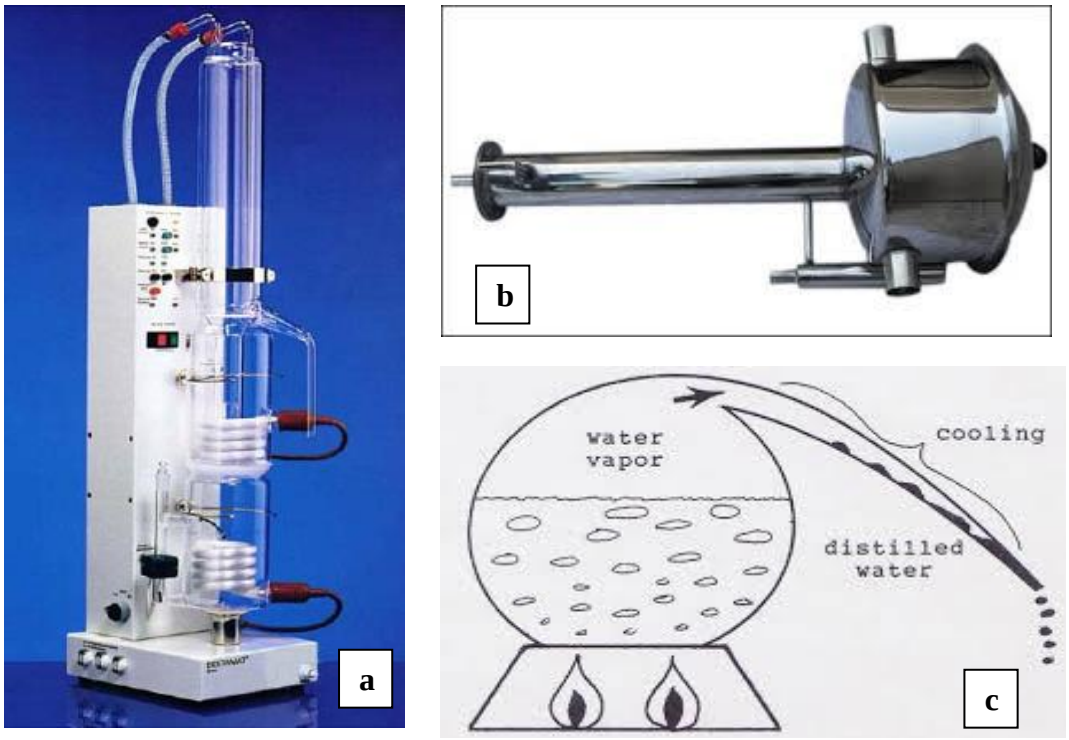


Fig. 3: a) Glass distillation unit b) Steel distillation unit c) low cast alternative

3) Hot plate with magnetic stirrer

Hot plate with magnetic stirrer is used for effective stirring and mixing fluid samples. The mixing is made faster by heating the samples under study during the stirring operation. The hot plate heating surface temperature is adjustable up to 250°C (480°F) and reaches maximum temperature in a few minutes.

Fig.4: Hot plate with magnetic stirrer

- **Low cost alternative**: Mechanical shaking the liquid samples effectively can solve the purpose.

4) pH meter:

A pH Meter is a scientific instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its acidity or alkalinity. It is necessary equipment for plant tissue culture lab as the media required for plant tissues should be slightly acidic (usually 5.8) and it becomes imperative to set the pH of the medium for making the nutrients available for plant tissues. The pH meter displays the initial pH of the solution with the help of its electrode. The electrode is specially designed and manufactured technically containing a silver based electrical wire suspended in a solution of potassium chloride (neautral solution). This thin bulb is made up of a special glass membrane through which an electric potential difference is sensed based on the difference of H^+ ions on both the sides of the glass. This potential difference is displayed as pH.
The pH can be set as follows: For eg., if the initial pH of the medium is basic (that is above 7) then few drops of 0.1N of HCl is added until the pH drops down to 5.8 and if the pH of the medium is acidic that is below 5.8, then 0.1 N of NaOH is added until it reaches to 5.8.

- **Low cost alternative**: If digital pH meter is not available then the pH can be tested by litmus paper strips and then require pH can be set by adding alkali or a base. One precaution should be taken with this method, that the colour change of the litmus paper should be minutely observed and analysed matching it with the colour indicator affixed with the paper strips.

Fig. 5: a) pH meter, b) Low cost alternative (Litmus paper with colour indicator)

5) Weighing balance (1 to 200g): For weighing large amounts of chemicals over 10 g For eg., Macronutrients for plant tissue culture media like, Ammonium nitrate, Calcium Chloride, Magnesium sulphate, Potassium nitrate etc.

6) **Weighing balance (1.0-000.1g)**: Analytical balance for accurate weighing of minute quantities. For eg. Micronutrients for plant tissue culture media like, Boric acid, cobalt chloride, cupric sulphate etc.

7) Refrigerator-freezer to store chemicals and stock solutions.

8) Trolley for carrying hot media flasks and containers (Optional).

9) Cupboards along the walls for storage of chemicals and presses underneath for storage of glassware and containers.

c) Culture Transfer room

A culture transfer room facilitates the aseptic inoculations of the plant tissue cultures. It should contain following equipments:

1) Laminar Flow cabinets (LAF)

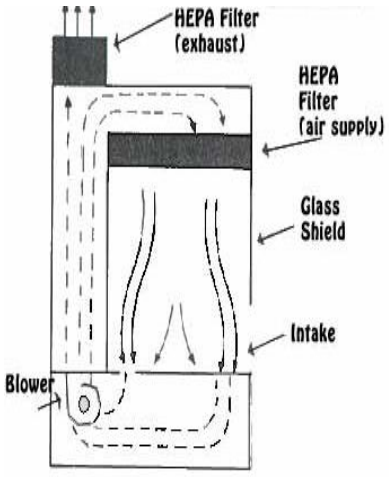
The LAF provides a UV light which is bactericidal and is kept on for 15-20 min prior to inoculation. This creates environment free from bacteria and contamination in the cultures can be avoided. During inoculation, the air blower is kept on in the cabinet. It is a small motor to blow air which first passes through a coarse filter, where it loses large particles, and subsequently through a fine filter. The fine filter, known as the 'high efficiency particulate air' (HEPA) filter, removes particles larger than 0.3/~m, and the ultraclean air (free of fungal and bacterial contaminants) flows through the working area. The velocity of the air coming out of the fine filter is about 27 ± 3 m min^{-1} which is adequate for preventing the contamination of the working area by the worker sitting in front of it. All contaminants are blown away by the ultraclean air flow, and a completely aseptic environment is maintained in the working area as long as the cabinet is kept on. The flow of air does not in any way hamper the use of a spirit lamp or a Bunsen burner. The LAF provide clean filtered air and a fluorescent white tube light allows cultures to be handled under contamination-free environment.

Laminar air flows are of two types:

i) Vertical LAF's

PROS

- Hood not as deep: requires less floor space.
- **Safety**: Air not blowing directly at operator, and sash provides a barrier in front of operator's face.
- Filter on top: easier to access.
- Less turbulent effect from air striking large objects.
- Less cross-contamination of items placed on the work surface.



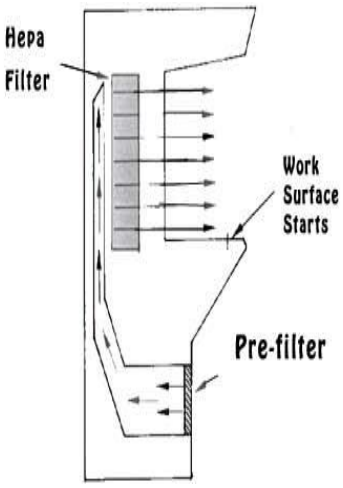
CONS

- Overhead clearance requirement; changing filters or servicing unit may require a step ladder.
- Cannot place items or hands on top of other items: obstructs airflow.
- Increased turbulent effect of air striking work surface.

ii) <u>Horizontal LAF's</u>

PROS

- Reduced turbulent effect of air striking work surface.
- No sash: easier to work and position equipment, **but air blows directly on operator.**
- Hands and gloves are generally less contaminating since they're downstream of the sample.



CONS

- Filter change or service usually requires repositioning hood for rear access.
- Large samples obstruct laminar air flow, may contaminate downstream samples.
- Blows fumes and/or powders in operator's face.

Low Cost Alternative: If Laminar air flow is not available, the same experiment can be performed between two flames produced by two separate burner sets, placed in a vertical line on a platform. The vicinity of the flames produced by burners is considered aseptic, and the experiments can be performed in that limited area between the two flames..

2) Glass bead sterilizers: Glass bead sterilizer is kept in laminar air flow and is used for the sterilisation of instruments, such as the forceps, scalpel holders and blades.The sterilization time should be minimum 60 sec to sterilize the surgicals.

Low cost option: A spirit lamp or Bunsen burner can be used, and flame sterilization should be done for the above said instruments, by dipping the forceps or blades in absolute alcohol and flame it. This is called as **flame sterilization.**

Fig. 7: (a) Glass bead sterilizer (b) Spirit lamp

3) **Binocular microscope** to observe histological variations in the cultures.

Fig. 8: Binocular microscope

4) **Stereoscopic microscope** (optional) to observe morphology of tissues and/ or embryos.

Fig. 9: Stereoscopic microscope

d) <u>Culture room</u>

A culture room is used to keep plant cell cultures under optimum temperature, light and humidity conditions. The room should have:

1) **An Air-conditioner**: Air conditioner is used to provide optimum temperature conditions. Generally for plant cell cultures, 25±2°C is optimum.

Fig. 10: Air Conditioner

2) **Culture Racks and Tube Lights**: The culture racks can be prepared as per requirement. For artificial lighting, use two 40W cool daylight fluorescent tubes and two yellow fluorescent tubes per shelf. The height of the tube lights above the shelf should be set, such that the light intensity measured should be less than 1 kLx on the cultures. The intensity of light can be measured by a lux meter.

Fig. 11: Culture racks with tube lights

3) **Photo timer:** A photo timer clock can be connected with the tube lights to provide photo period of 14 h day and 8 h night by switching the tube lights on and off at the set time.

Low cost alternative: The lights can be switched on and off mechanically.

4) Thermometer with maximum-minimum temperature recording.

5) Green House/Net house

A green house or a net house is required only in such cases where the *in vitro* regenerated plants are transferred from lab to land. In the initial phases of establishment of cultures this facility is not required.

1. *In vitro* derived plants need to be gradually hardened to field conditions. Plant hardening is usually carried out under greenhouse that ensures high survival of the tissue-cultured plants in the field.
2. Greenhouse can be of net, glass or polyethylene films or sheets of polycarbonate or acrylic can also be used. Appropriate light, shading and blackout systems can be achieved with supplementary lighting. Drip irrigation systems, misting and fogging can be installed as needed. Greenhouses erected in warm climates should have fan-assisted drip pad cooling especially during summer.

Fig. 12: A Green House

Consumables

- Glass wares – Duran bottles with rims (100, 250, 500, 1000 ml), Test tubes (25X150") glass cylinders (10, 50, 250, 500 1000 ml), beakers (25, 500, 1000 ml); Erlenmeyer flasks (250, 500, 2000 ml).
- Surgical blades (24 number sterile blades are available) and blade holder (for 24 number blade 4 number blade holder is used), forceps (8 inches in length).
- Pipettes (0.1, 0.5, 1.0, 5 ml).

Chapter 2

Nutrient media

A plant nutrient is a chemical element which can be categorised as an **essential** nutrient if:

- The nutrient has a direct effect on plant growth and reproduction. In the absence of it or with severe deficiency, the plant will die before it completes the cycle from seed to seed.
- The nutrient plays a unique role in plant metabolism or physiology such that no other element substitutes fully for this function. A partial substitution might be possible. For example, a substitution of manganese for magnesium in enzymatic reactions may occur, but no other element will substitute for magnesium in its role as a constituent of chlorophyll.

Macro nutrients: Elements which generally accumulate to 0.1% and upward of the dry mass of plant tissues are categorised as macro nutrients.

Micro nutrients: Elements which generally accumulate to amounts less than 0.01% of the dry mass of plant tissues are categorised as micro nutrients.

Plant cell culture media are composed of many different compounds, including inorganic macronutrients (salts of N, P, S, K, Na, Ca, Mg) and micronutrients (salts of e.g., I, Ni, Fe, Cu, B, Mn, Co), as well as vitamins and a carbohydrate source (usually 2–5% sucrose). The most common media for plant cell culture are those devised by Murashige and Skoog (1962), Gamborg et al. B5 (1968), Schenk and Hildebrandt (1972), and White (1934).

Major Constituents

- Salt mixtures
- Organic substances
- Natural complexes
- Inert supportive materials
- Growth regulators

Salt Mixtures

- M.S. (Murashige and Skoog)
- Gamborg's B5
- Nitsch and Nitsch
- White

Macronutrient Salts

- NH_4NO_3 (Ammonium nitrate)
- KNO_3 (Potassium nitrate)
- $CaCl_2$-$2H_2O$ (Calcium chloride, anhydrous)

- $MgSO_4$-$7H_2O$ (Magnesium sulphide, Epsom salts)
- KH_2PO4 (Potassium di hydrogen phosphate)

Micronutrient Salts

- FeNaEDTA or (Na_2EDTA and FeSO4)iron remains available upto pH7.6-8.0
- H_3BO_3 (Boric acid)
- MnSO4-$4H_2O$ (Manganese sulphate)
- ZnSO4-$7H_2O$ (Zinc sulphate)
- KI (Potassium iodide)
- Na_2MoO_4-$2H_2O$ (Sodium molybdate)
- $CuSO_4$-$5H_2O$ (Cupric sulphate)
- $CoCl_2$-H_2O (Cobalt chloride)

Carbon Sources

- Sucrose (1.5 to 12%)
- Glucose (Sometimes used with monocots)

Role of different elements

Nitrogen:

Nitrogen is the major component supplied in the form of nitrates or ammonium salts. Nitrogen is an important part of amino acids, proteins, nucleic acids. Inorganic nitrogen is utilized in order to synthesize organic molecules. For most purposes, a nutrient medium should contain from 25 to 60 mM inorganic nitrogen. The cells may grow on nitrate alone, but often there is a distinct beneficial effect and requirement for ammonium or another source of reduced nitrogen as nitrate itself drifts the pH towards alkalinity. Nitrate cannot be simply used to synthesize organic molecules but has to be reduced to ammonia first. Most plants prefer nitrate to ammonium, although the opposite is also true in some cases.

Potassium:

Potassium is required at concentrations of 2 to 26 mM. It is a monovalent cation with high mobility in the plant. Potassium salts have an important function in the osmotic regulation of the cell. Potassium ion is essential for the activation of many enzymes. In photosynthesis, K+ regulates the ion balance and pH of chloroplasts.

Calcium:

Calcium is essential for cation-anion balance by counteracting organic and inorganic anions. A concentration of 1-3 mM of calcium is usually adequate. Calcium is also important for cell and root multiplication. Calcium, a component of the cell wall, is largely bound to the cell wall and membrane. This is because of the large number of Ca++ binding places on the cell wall and limited

mobility of calcium through the membrane into the cytoplasm. The stability of cell membrane is highly influenced by Ca++.

Phosphorus:

Phosphorus is present in the plant in the form of inorganic phosphate (iP). A concentration of 1-3 mM phosphate is usual adequate. The high-energy pyrophosphate bond of phosphorus, when bound to another P atom as in ATP, is very important for the energy metabolism in the cell. Phosphorous is an essential element in DNA and RNA nucleic acids. In phospholipids, this element is very important for the energy metabolism of the plant in form energy-rich phosphate esters.

Magnesium:

This element is an essential component for many enzymes reactions and is very important in photosynthesis. Magnesium is indispensable for the energy metabolism of the plant because of its importance in the synthesis of ATP.

Sulphur:

These have to be reduced first for the synthesis of sulphur containing compounds such as amino acids, proteins and enzymes. Sulphur in its non-reduced form is incorporated in sulpholipids and polysaccharides.

Boron: Boron is required for the synthesis of cell wall as well as in the stabilization of the constituents of cell wall and cell membrane.

Chlorine:

Chlorine is taken up as a chloride and is very mobile in the plant. The main functions of the ions are in osmoregulation. Chlorides play a role in photosystem II during the Hill reaction. Chlorine also regulates the opening and closing of stomata and is thus very important in the regulation of the osmotic potential of vacuoles as also to turgor-related processes.

Copper:

Copper is taken up by the plant as Cu++ or as a copper chelate complex. Within the cell, copper is mostly part of the enzyme complexes and important in redox reactions executed by these enzymes. It is useful in photosynthesis.

Cobalt:

Cobalt is assumed to be important in nitrogen fixation. In higher plants the function of this element is not very clear.

Manganese:

Manganese is taken up by the plant as bivalent unbound Mn++ ions. The element is strongly bound to several metalloproteins. The ion is involved in the Hill reaction of photosystem II in which water is split into oxygen and protons.

Molybdenum:

Molybdenum is used as a cofactor in many enzymes, including nitrogenase and nitrate reductase. It is also directly involved in the reduction of N2.

Zinc:

Zinc is taken up by the roots as Zn++. It is neither oxidized nor reduced in the plants. It is an important component of a number of enzymes, e.g. alcohol dehydrogenase in the meristem zone of the plant. Zinc is also very important for protein synthesis.

Iron:

Iron is generally added as a chelate with ethylene diamine tetra acetic acid (EDTA). In this form, iron remains available up to a pH of 8.0. It is mainly bound to chelators and complex compounds in plants. Most plants absorb only ferric ions (Fe3+). The main function of iron is to form iron chelates and two kinds of proteins: haeme proteins and iron sulphur proteins. The most well-known haeme proteins are the cytochromes, functioning as intermediates for electrons required for the reduction of nitrate to nitrite by the enzyme nitrate reductase in nitrogen assimilation. The second group of iron-binding proteins are the iron sulphur proteins. The iron is bound to a thiol group (-SH) of cystine and/or inorganic sulphur. Ferridoxin is the most common iron sulphur protein. It functions as a carrier in the electron transport reaction catalyzed by nitrate reductase, sulphate reductase, the synthesis of NADP+ during photosynthesis and nitrogen reduction by nitrogenase complex. Iron is also important in the biosynthesis of chlorophyll.

Carbon and energy source

The standard carbon source 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..

Organic Compounds

Vitamins

- Thiamine 1.0 mg/L
- Nicotinic acid and pyroxidine 0.5 mg/L
- Glycine 2.0 mg/L
- Vitamin C (antioxidant) 100.0 mg/L
- Amino acids and amides: Amino acids can be used as the sole source of nitrogen, but are normally too expensive.

2 most commonly used amino acids:

- L-tyrosine, enhances adventitious shoot formation.
- L-glutamine, may enhance adventitious embryogenesis.

Other Organic Compounds

Inositol: Stimulates growth, Use at the rate of 100 mg/L

Purine/pyrimidine

• Adenine stimulates shoot formation; Can use adenine sulphate at 100 mg/L

Organic acids

• Citric acid (150 mg/L) typically used with ascorbic acid (100 mg/L) as an antioxidant.

Phenolic compounds

• Phloroglucinol-Stimulates rooting of shoot sections.
• L-tyrosine-stimulates shoot formation.

Natural Complexes

- Coconut endosperm
- Fish emulsion
- Protein hydrolysates
- Tomato juice
- Yeast extracts
- Potato agar

Nutritionally Inert Complexes

- Gelling agents
- Charcoal
- Filter paper supports
- Other materials

Gelling Agents

- Agar-extract from Marine red agar

Solubilized agar forms a gel that can bind water and adsorb compounds. The higher the agar concentration, the stronger is 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.

- Phytagar

- Agarose
- Hydrogels
- Gelatin
- Gelrite

Charcoal

Activated charcoal is used as a detoxifying agent. Detoxifies wastes from plant tissues, impurities. The concentration normally used is 0.3% or lower. Never reuse.

Growth Regulators

Hormones now referred to as growth regulators 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 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 isopentenyl adenine (2 iP) as cytokinins, while others are synthetic growth regulators.

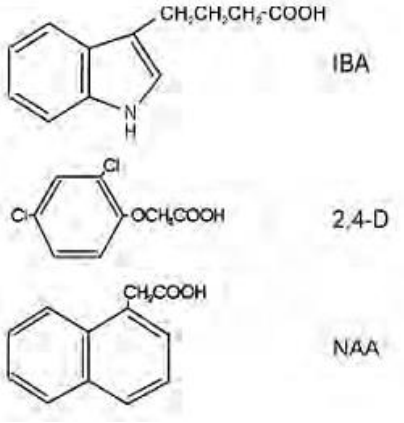
- Auxin
- Cytokinin
- Gibberellin
- Abscisic acid
- Ethylene

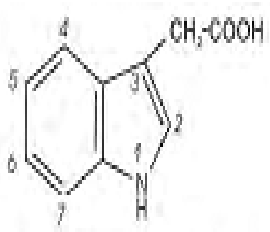
Auxins

The word auxin has a Greek origin; *auxein* means to enlarge or to grow. Auxins are effective in callus formation, rooting of cuttings, and the induction of adventive embryogenesis. At the cellular level, auxins control two main processes in collaboration with cytokinins, i.e., cell cycle and cell division on one hand, and cell elongation on the other. The auxin:cytokinin ratio represents an important signal in the formation of cell phenotype and also in the onset and maintenance of the process of cell division. Activation of cell elongation by auxin is mediated by increased proton efflux. Normal cell divisions require synchrony between the S phase and cell division, suggesting that auxin and cytokinin levels in cultures need to be carefully matched.
Since they are capable of initiating cell division they are involved in the formation of meristems giving rise to either unorganised tissue, or defined organs. Auxins stimulate differentiation of vascular bundles. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide. Auxins cause DNA to become more methylated than usual which might be necessary for the re-programming of differentiated cells. In organised tissues, auxins are involved in the establishment and maintenance of polarity and in whole plants their most marked effect is the maintenance of apical dominance.

- Indole 3-Acetic acid (IAA)
- Indole 3-Butyric acid (IBA)

- α- Naphthalene acetic acid (NAA)
- 2,4-D (2,4-Dichloro phenoxy acetic acid)
- 2,4,5-Trichloro phenoxy acetic acid (2,4,5-T)





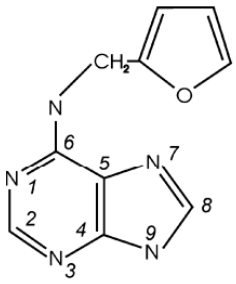
Indole 3- Acetic acid

Fig. 13: Different types of Auxins

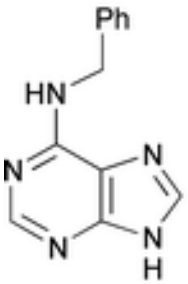
Cytokinins

Cytokinins encourage the growth of axillary buds, and reduce apical dominance in shoot cultures of broad leafed plants. They activate RNA synthesis, stimulate protein synthesis and the activities of some enzymes. Cytokinins, together with auxins, take part in the regulation of the cell cycle and formation of adventitious shoots in plant cells. High levels of cytokinin are deleterious; produce many small shoots, which typically fail to elongate; they may also cause the leaves of some species to have an unusual shape, and/or induce shoots to become hyperhydric. High concentrations also inhibit or delay root formation. This prevents root growth and the promotive effects of auxins on root initiation.

- 6 benzyl amino purine
- Kinetin
- 2iP
- Zeatin
- PBA



Kinetin



BAP

Fig. 14: Different types of Cytokinins

Gibberellin

Not generally used in tissue culture.
Tends to suppress root formation and adventitious embryo formation.

Abscisic Acid

Primarily a growth inhibitor but enables normal development of embryos, both zygotic and adventitious.

Ethylene

Natural substance produced by tissue cultures at fairly high levels, especially when cells are under stress. It enhances senescense and supresses the embryogenesis and development in general.

Preparation of MS medium

Preparation of stock solutions:

To weigh the necessary products each time a medium is prepared, is a time consuming and tedious process, hence concentrated solutions of the desired composition of a medium are used which can be diluted adequately. These concentrated solutions are called stock solutions.

Stock solutions of macro and micronutrients, vitamins and growth regulators are prepared in distilled or high purity demineralized water.

i. **Macronutrient stock solution(s):** Usually, the stock solution of macronutrients is prepared as 10x. Dissolve all the macronutrients one by one except ($CaCl_2$ for macronutrient stock solution. The stock solution of $CaCl_2$ should be prepared separately. Another way is to dissolve the different macronutrients one after the other and $CaCl_2$ is dissolved separately and later added to the rest of the stock solution in order to avoid precipitation.

ii. **Micronutrient stock solution:** A stock solution of all the micronutrients with 100x is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of these two salts (100x) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution.

iii. I**ron-EDTA**: Iron EDTA should be added fresh. If stock solution (100x) is prepared, then $FeSO_4$ and Na_2EDTA should be dissolved separately, can be heated for effective solubility, then mix both of them and set the pH at 5.5. Then it should be stored in an amber bottle or a bottle covered with an aluminium foil.

iv. **Vitamins and growth regulators stock solutions**: These are simple stock solutions. All the growth regulators are not soluble in water. The compound should be dissolved in a few ml of solvent and then water is slowly added to make the requisite volume. Concentrations of compounds can be taken as mg/l or in molarity.

Stock solutions for MS Medium

Constituents	Amount (mg l^{-1})
Stock solution I	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2 \cdot 2H_2O$	8800
$MgSO_4 \cdot 7H_2O$	7400
KH_2PO_4	3400
Stock solution II	
KI	166
H_3BO_3	1240
$MnSO_4 \cdot 4H_2O$	4460
$ZnSO_4 \cdot 7H_2O$	1720
$Na_2MoO_4 \cdot 2H_2O$	50
$CuSO_4 \cdot 5H_2O$	5
$CoCl_2 \cdot 6H_2O$	5
Stock solution III[b]	
$FeSO_4 \cdot 7H_2O$	5560
$Na_2EDTA \cdot 2H_2O$	7460
Stock solution IV	
Inositol	20000
Nicotinic acid	100
Pyridoxine·HCl	100
Thiamine·HCl	20
Glycine	400

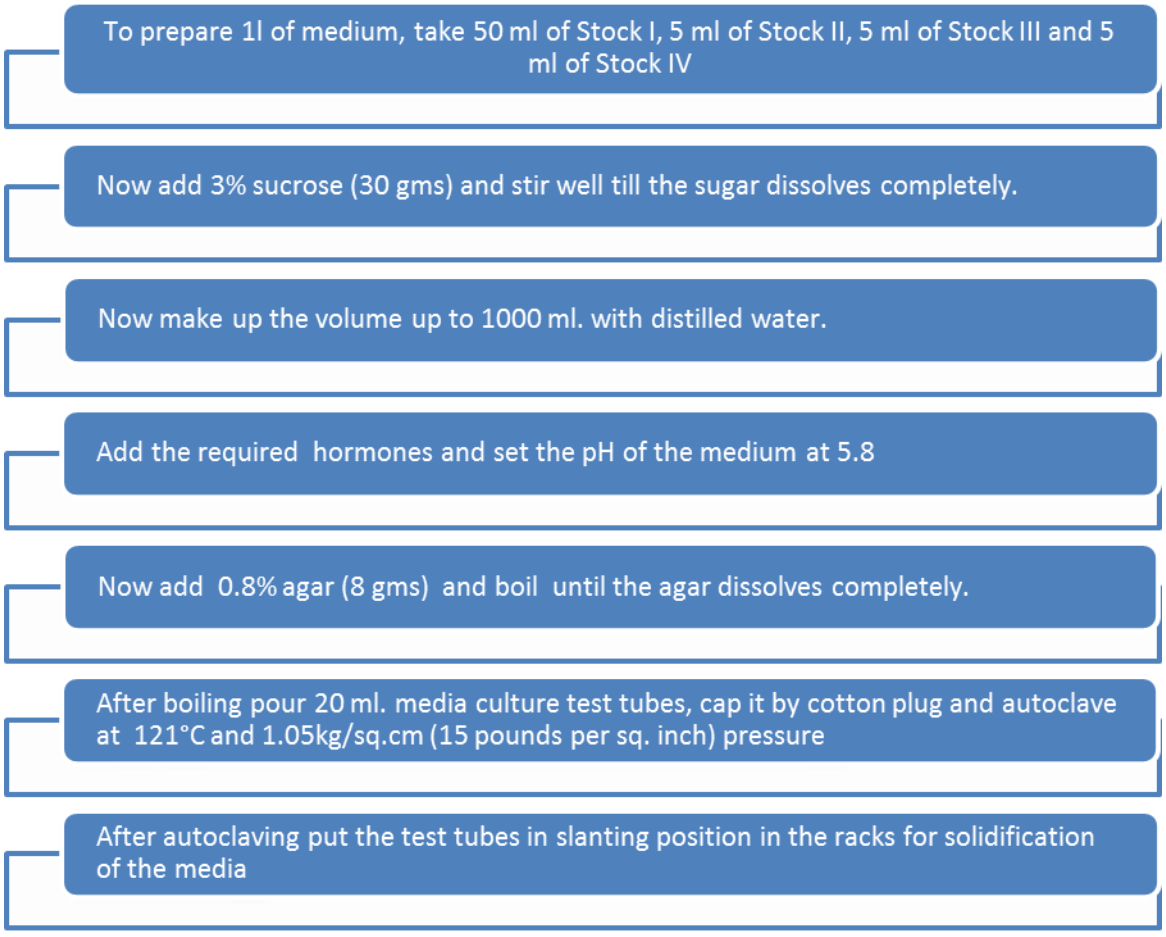


Fig. 15: Stock solutions and preparation of MS medium

Chapter 3

Germination of seeds *in vitro.*

Theory:

The embryos are formed with a placenta-like interface of intervening tissues between parental vascular supply and the embryo proper during the development of seed. This circumstance depresses passive migration of microorganisms into the developing embryo. Hence, the developing embryo tends to exclude pathogens and foreign materials that may be in the parent plant. If the embryo is released from the seed by aseptic germination procedures, then aseptic seedlings are resulted. Any part of such aseptic seedling can be used as "*in vitro*" experimental material required for the establishment of primary culture. Since aggressive chemicals (e.g., hypochlorite or hydrogen peroxide) are generally used for decontamination, it may be of advantage to treat the robust seeds with such chemicals instead of the treating much softer explants, such as leaf or stem pieces.

Materials Required:

Experimental material: Seeds (Soybean, Moong, Cucumber, Radish, Amaranthus)

Apparatus: Flasks and beakers, forceps (8 inches), petri dishes, distilled water; **all should be sterilized.**

Media: Agar gelled water for the above mentioned seeds.

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$

Gelling agent: Agar (0.8%)

Preparation of Agar gelled water

- Weigh agar (8gms).
- Add it to one litre of distilled water.
- Heat until boiling begins.
- Dispense 20 ml. aliquots in 25X150˝ culture tubes or accordingly if bottles/jars are selected.
- Label the media details on to the test tubes with the help of glass marker.
- Cap the test tubes with cotton plugs, wrap them with paper and autoclave at 15 lbs/inch^2 (121°C) for 15 minutes.
- Prepare the slants of media (in order to get more surface area) in the test tubes and allow it to solidify.

Pre-treatment of the seeds

- Discard the damaged seeds and select the healthy ones.
- Seeds with an uneven seed coat or with a cover of hairs may cause problems. It may be of help to wash them with a few drops of a detergent, e.g., Tween 80.

- Wash the seeds with tap water followed by distilled water.

Check list: (Before starting the inoculation)

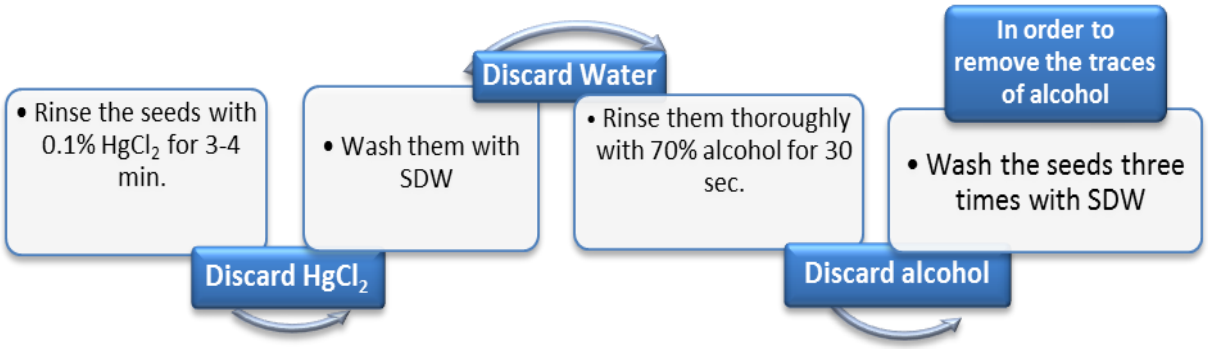
The LAF/transfer area should contain the following before putting on the UV light

- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.
- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps dipped in a beaker of ethanol.
- Sterile petri dishes which can be used for preparation of the explants.
- Media tubes.

Treatment inside the hood

a) Surface sterilization of the explants

1. Swab the chamber and hands with 70% ethanol.
2. Place the pre treated seeds in a sterilized beaker inside the hood.



b) Inoculation

1. Flame sterilize the forceps.
2. Inoculate the seed with the help of the forceps in the media tube.
3. Label the test tubes with name of the seed, date of inoculation and the type of media.

Culture Conditions

The seeds are kept in dark for germination at 25±2°C. Once sprouting is observed, the germinated seeds can be transferred to light for further growth.

Observations

Number of seeds inoculated (SI)	Number of seeds Germinated (SG)	% seed germination $\frac{SI}{SG} X100$

Result:

The seeds show……% germination after……days of culture.

Precautions: Appendix- 1

Chapter 4

Establishment of primary culture from leaves/stem sections of *Bacopa monnieri* (*Brahmi*)

Theory:

The tissues of selected explants are differentiated, mature and non dividing. After the transfer of freshly cut explants into growth-promoting conditions, usually on the cut surface cell division is initiated, and as a form of wound healing, unorganized growth occurs—a callus is initiated. The differentiated tissues also undergo modifications to become meristematic. This phenomenon of mature cells reverting back to meristematic state to form undifferentiated callus tissue is called dedifferentiation. Callus cells continue to proliferate without differentiating leading to the establishment of primary culture. Eventually differentiation occurs within the tissue mass with the help of growth regulators leading to the formation of complete plants. Callus can therefore be used as a starting material for experimenting totipotency of tissues to organ and plant regeneration.

Materials Required:

Experimental material: Brahmi (*Bacopa monnieri*) Leaves or stem sections.

Apparatus: Flasks and beakers, forceps (8 inches), Scalpel or blade holder (no.4) with surgical blade (24 no.), petri dishes, distilled water **all should be sterilized**.

Medium: (The protocol is subjected to change with different species)

- Gamborg's B5 medium (Himedia) without growth regulators (as control for comparing the effects of growth regulators.
- Gamborg's B5 medium with growth regulator: 2,4-D (0.5-1.0 mg.l^{-1}) (For experiments)

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$

Gelling agent: Agar (0.8%)

Composition and Preparation of Gamborg's B5 medium:

Ingredients	milligrams/litre
Potassium nitrate	2500.00
Ammonium sulphate	134.00
Calcium chloride.$2H_2O$	150.00
Magnesium sulphate	122.09
Sodium phosphate monobasic	130.42
Manganese sulphate.H_2O	10.00
Boric acid	3.00
Potassium iodide	0.75
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	2.00
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.80
EDTA disodium salt.$2H_2O$	37.30
myo - Inositol	100.00
Thiamine hydrochloride	10.00
Pyridoxine hydrochloride	1.00
Nicotinic acid (Free acid)	1.00
Sucrose	20000.00

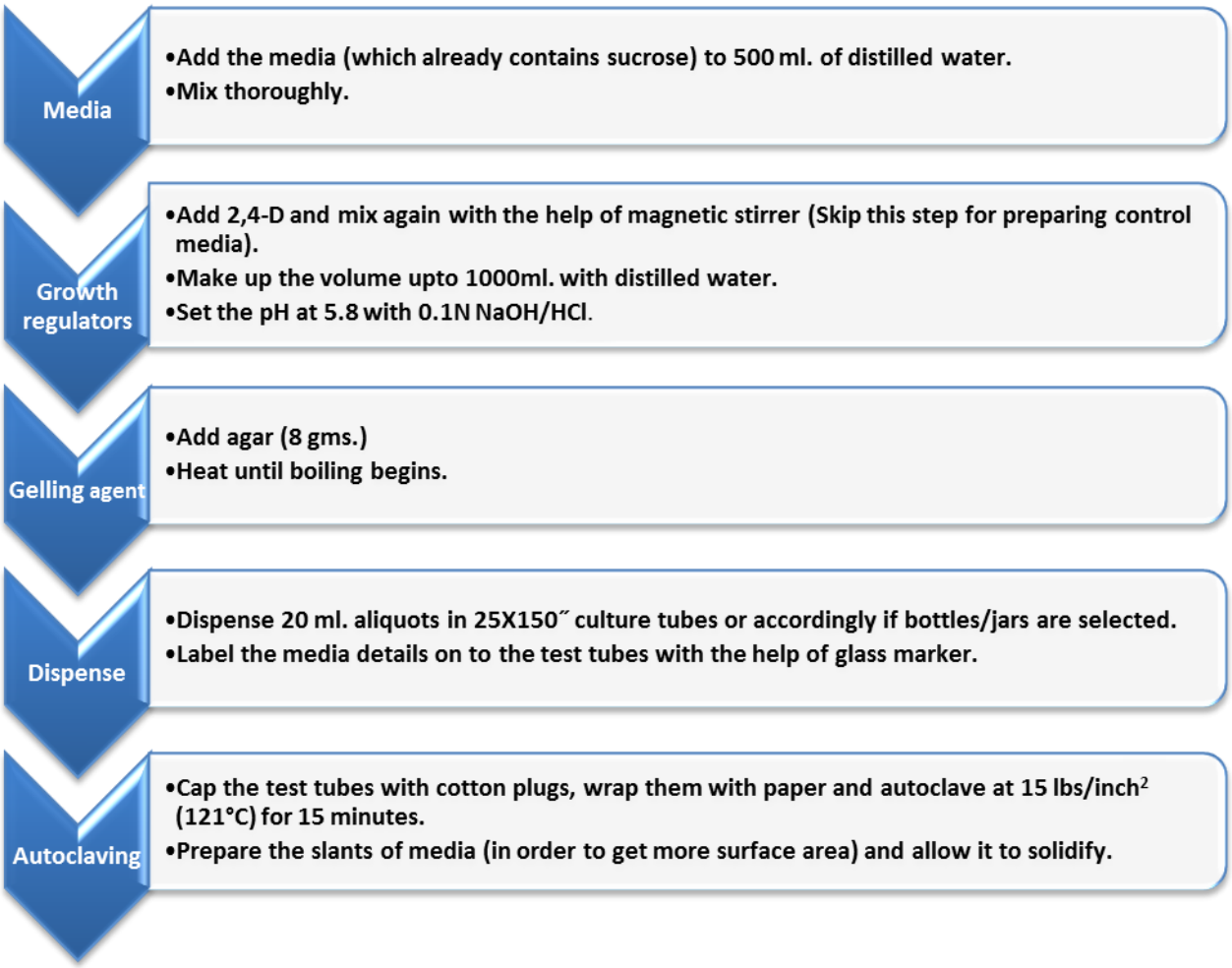


Fig. 16: Composition and preparation of Gamborg's B5 medium

Selection of the explants (*Brahmi* Leaves/stem sections)

- The explants can be selected from the plants growing in natural conditions or from the pre grown *in vitro* shoots.
- Discard the damaged leaves/stem, select the healthy ones.
- The explants should be collected from plants neither too young nor too old.
- Wash the explants with tap water followed by distilled water.
- Cut short the stems if they are too lengthy and collect it in a beaker.
- Detach the leaves from the stem with petiole intact and collect it in a beaker.

Note: If the explants are collected from *in vitro* grown shoots then all the steps of surface sterilization are omitted and inoculation is performed directly inside the hood.

Check list: (Before starting the inoculation)

The hood/transfer area should contain the following before putting on the UV light

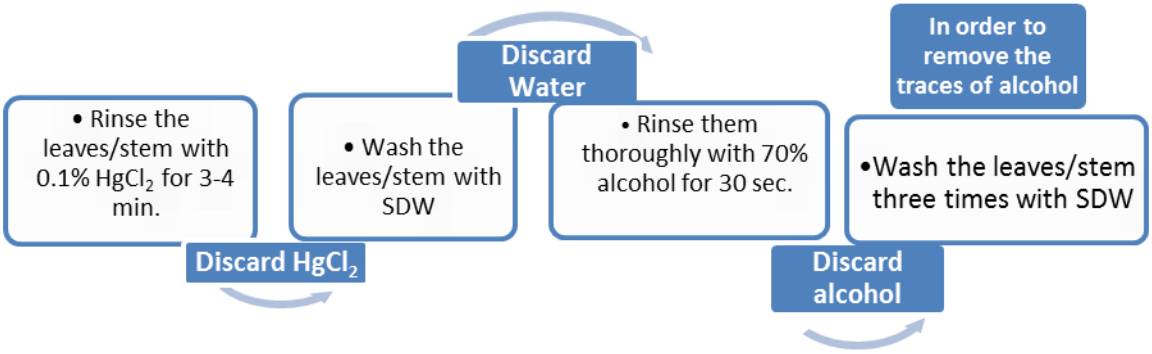
- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.

- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps and blade holder/scalpel dipped in a beaker of ethanol.
- Surgical blades.
- Sterile petri dishes which is used for preparation of the explants.
- Media tubes.

Treatment inside the hood

a) Surface sterilization of the explants

1. Swab the chamber and hands with 70% ethanol.
2. Place the pre treated explants in a sterilized beaker inside the hood.



b) Inoculation

1. Flame sterilize the forceps.
2. Fix the surgical blade tightly on to the blade holder (do not touch it with hands).
3. Cut the petiolar end and apex of the leaf with the help of forceps, resulting into approx. 1cm sized leaf explants.
4. Similarly cut the lower and top portion of the stem with the help of forceps, resulting into stem sections of 1cm size.
5. While cutting the stem sections remove the nodal portion of the stem and select the internode section of the appropriate size.
6. Inoculate the leaf/stem with the help of the forceps placing them horizontally in separate media tubes including the control tubes.
7. Press the explants gently against the surface of the agar to make good contact.
8. Label the test tubes with name and type of the explant, date of inoculation and the type of media.

Culture Conditions

The culture tubes are kept at 25±2°C in light conditions (50µEm^{2}s^{-l}) under 16/8 hrs of photoperiod (16 hrs light and 8 hrs dark).

Observations

Stages	Number of explants	Details (write your observations including the mentioned characteristics comparing with control)
Initiation of Callus	• Inoculated • Got contaminated • Remained • Showing growth symptoms	• Swollen surfaces. • Callus starting from the cut ends/from the areas which are in contact of the media/from whole surface etc. • Colour of cells (Pale green/yellow/ etc.) • Fresh weight (I) of the mass.
Proliferation of Callus		• Callus gradually covering the entire surface of the explants. • Friable/compact/vitrified mass of cells. • Colour of cells (Pale green/yellow/ etc.) • Texture smooth/rough • Increase in fresh weight (F) of the mass (F-I X100).

Results:

The explants exhibit initiation of callus after……. days of culture as compared to control.

Initial weight of callus……in …….days of culture.

Final weight of callus……in ……..days of culture.

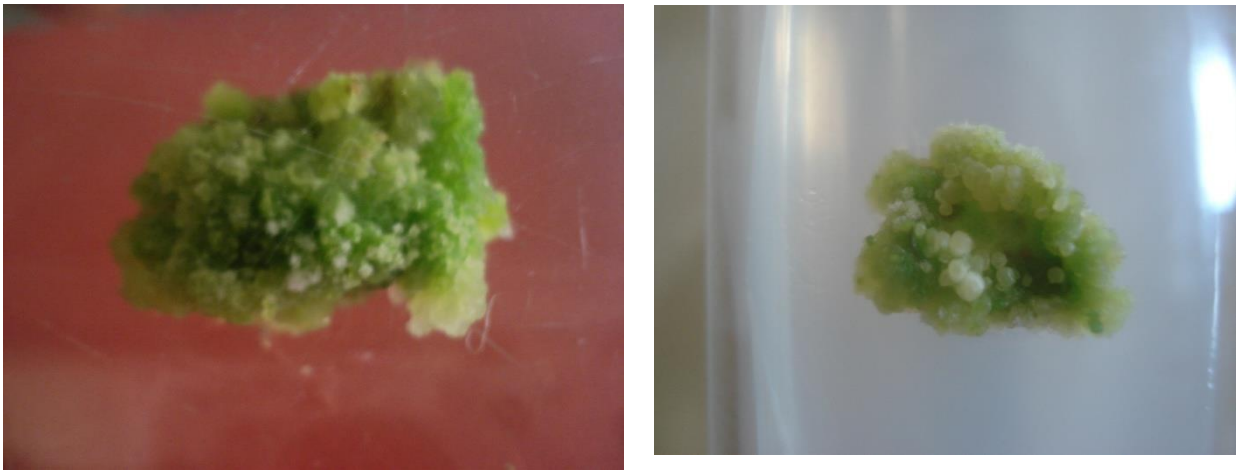


Fig. 17: Different type of callus obtained from leaf sections.

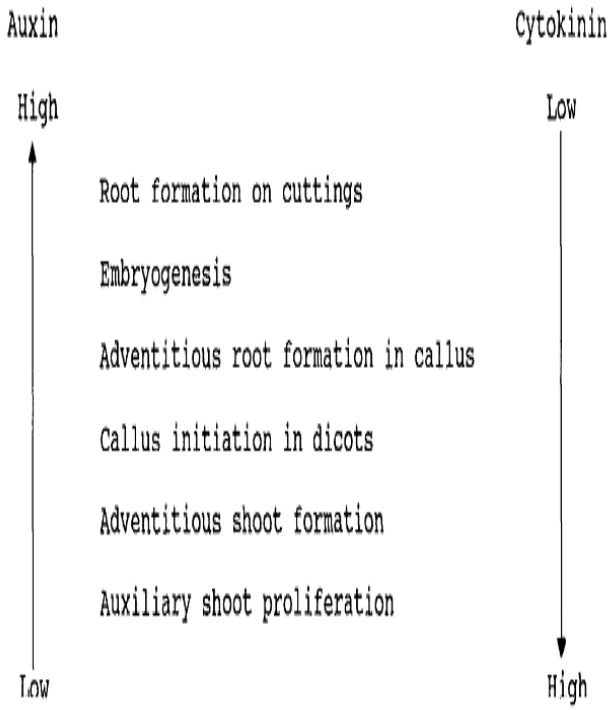
Precautions: Appendix-1

Chapter 5

Establishment of organogenesis from leaves/stem sections of *Bacopa monnieri* (*Brahmi*)

Theory:

Organogenesis is a pathway acquired by group of cells in callus/explants, stimulated by auxin:cytokinin ratio to differentiate, form organs and ultimately complete plants. Growth and organogenesis *in vitro* is highly dependent on the interaction between naturally occurring endogenous growth substances and an analogous growth regulator added to the medium. Organogenesis can be direct (development of shoots/roots directly on the surface of the explants, skipping the callus phase) or indirect (development of shoots/roots from callus; an intervening phase). The shoot bud developed from callus is a monopolar structure which develops procambial strands and establishes a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explants.



(extracted from George and Sherrington, 1984,) (1).

Materials Required:

Experimental material: Brahmi (*Bacopa monnieri*) Leaves or stem sections

Apparatus: Flasks and beakers, forceps (8 inches), Scalpel or blade holder (no.4) with surgical blade (24 no.), petri dishes, distilled water **all should be sterilized**.

Medium:

- Gamborg's B5 medium without growth regulators (as control for comparing the effects of growth regulators.
- Gamborg's B5 medium with growth regulators for experiments
    - For direct organogenesis: BAP (0.5-1.0 $mg.l^{-1}$)
    - For indirect organogenesis: 2,4-D (0.25 $mg.l^{-1}$) and BAP (0.5 $mg.l^{-1}$)

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$
Gelling agent: Agar (0.8%)

Preparation of B5 medium:

Refer Chapter 4 with following modifications: Add the given concentration of BAP along with 2,4-D keeping the rest of the procedure same.

Selection of the explants (Brahmi Leaves/stem sections):

Refer Chapter 4
Check list: (Before starting the inoculation)

The hood/transfer area should contain the following before putting on the UV light

- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.
- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps and blade holder/scalpel dipped in a beaker of ethanol.
- Surgical blades.
- Sterile petri dishes which is used for preparation of the explants.
- Media tubes.

Treatment inside the hood

Surface sterilization of the explants: Refer Chapter 4.

Inoculation: Refer Chapter 4 points 1-8.

Culture Conditions

The culture tubes are kept at 25±2°C in light conditions ($50\mu Em^2s^{-l}$) under 16/8 hrs of photoperiod (16 hrs light and 8 hrs dark).

Observations

Stages	Number of explants	Details (write your observations including the mentioned characteristics comparing with control)
Initiation of Callus/Direct Organogenesis	• Inoculated • Got contaminated • Remained • Showing growth symptoms	• If callus is obtained {Swollen surfaces, callus starting from the cut ends/from the areas which are in contact of the media/from whole surface etc., Colour of cells (Pale green/yellow/ etc.), Fresh weight (I) of the mass } • If direct organogenesis is obtained (Initiation of shoots from cut ends/from the margins/ from all over the surface of the explants, number of shoots emerged/ accompanied with rooting etc.)
Proliferation of Callus and organogenesis		• Callus gradually covering the entire surface of the explants, friable/compact/vitrified mass of cells, colour of cells (Pale green/yellow/ etc. texture smooth/rough, fresh weight (F) of the mass. • Emergence of shoot buds from callus surface, number of shoots eventually formed/ accompanied with rooting, length of the shoots etc.

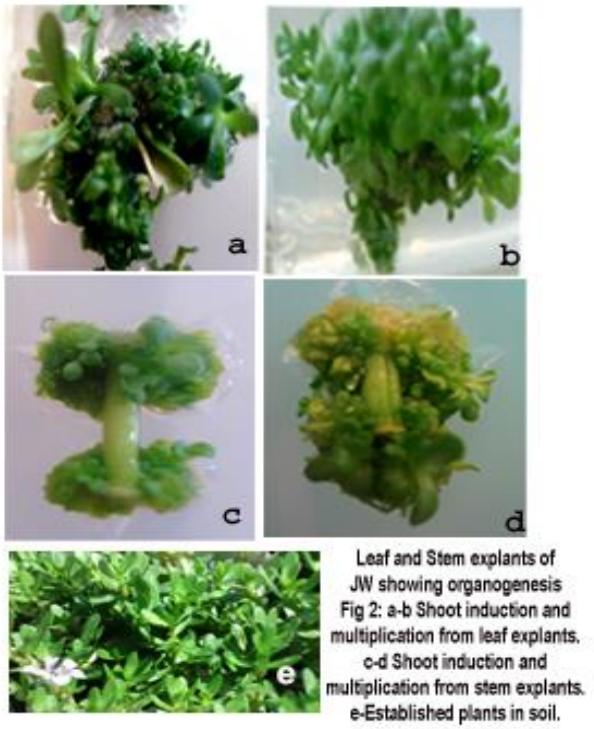
Results:

The explants exhibit direct organogenesis and developed……..number of shoots in……. days of culture as compared to control.

The explants formed callus and developed.……number of shoots in …….days of culture as compared to control.

Total ……. regenerated plants are obtained after……..days of culture.

Fig. 18: Leaf and stem explants showing organogenesis a,b: Shoot induction and multiplication from leaf explants c,d: Shoot induction and multiplication from stem explants e: Established plants in soil



Leaf and Stem explants of JW showing organogenesis
Fig 2: a-b Shoot induction and multiplication from leaf explants. c-d Shoot induction and multiplication from stem explants. e-Established plants in soil.

Precautions: Appendix-1

Chapter 6

Establishment of somatic embryogenesis from leaves/stem sections of *Bacopa monnieri* (*Brahmi*)

Theory:

Embryogenesis is a pathway acquired by cells or callus cultures to form embryo-like structures called somatic embryos, which on germination produce complete plants. Embryo is a bipolar structure with a closed radicular end. During development it breaks any vascular connections with the maternal callus tissue or the cultured explants and become isolated. Therefore somatic embryos are easily separable from the explants.
Somatic embryos are produced as adventitious structures directly on explants (direct somatic embryogenesis) or from callus (indirect somatic embryogenesis). The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos usually do not develop further until the auxin concentration is reduced. During differentiation the somatic embryos in dicotyledonous plants progress through four stages of development: globular stage, heart stage, torpedo stage, and cotyledonary stage. The stages are based on the overall embryo shape. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis.

Materials Required:

Experimental material: Brahmi (*Bacopa monnieri*) Leaves or stem sections
Apparatus: Flasks and beakers, forceps (8 inches), Scalpel or blade holder (no.4) with surgical blade (24 no.), petri dishes, distilled water **all should be sterilized**.
Medium:

- Gamborg's B5 medium without growth regulators (as control for comparing the effects of growth regulators.
- Induction media: Gamborg's B5 medium with growth regulators 2,4-D (0.5 $mg.l^{-1}$) and BAP (0.25 $mg.l^{-1}$).
- Maturation media: Gamborg's B5 medium with growth regulators 2,4-D (0.25 $mg.l^{-1}$).
- Conversion media: Gamborg's B5 medium with growth regulators BAP (0.25 $mg.l^{-1}$).

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$
Gelling agent: Agar (0.8%)

Preparation of B5 medium:

Refer Chapter 4 with following modifications: Add the given concentration of BAP along with 2,4-D keeping the rest of the procedure same.

Selection of the explants (Brahmi Leaves/stem sections): Refer Chapter 4

Check list: (Before starting the inoculation)

The hood/transfer area should contain the following before putting on the UV light

- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.
- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps and blade holder/scalpel dipped in a beaker of ethanol.
- Surgical blades.
- Sterile petri dishes which is used for preparation of the explants.
- Media tubes.

Treatment inside the hood

Surface sterilization of the explants: Refer Chapter 4.

Inoculation: Refer Chapter 4 from points 1-8.

Culture Conditions

The culture tubes are kept at 25±2°C in light conditions (50µEm^2s^{-l}) under 16/8 hrs of photoperiod (16 hrs light and 8 hrs dark).

Observations

Stages observed/days of culture	Medium	Number of explants	Details (write your observations including the mentioned characteristics comparing with control)
Induction	B5+2,4-D+BAP (0.5/0.25 mg.l^{-1})	• Inoculated • Got contaminated • Remained • Showing growth symptoms	• Small globular structures developed from callus in the areas which are in contact of the media/from the outer edges /from whole surface etc. • The structures appear shiny/translucent/watery etc. Possessing hairy outgrowths/which look glossy etc.
Maturation	B5+2,4-D (0.25 mg.l^{-1})		• The embryos change their shape from globular to heart, followed by torpedo to cotyledonary shape.
Conversion	B5+BAP (0.25 mg.l^{-1})		• The embryos show bipolar germination forming complete plantlets.

Results:

The explants exhibit somatic embryogenesis and developed……..number of embryos in……. days of culture as compared to control.
The embryos show..…… changes in their morphological shapes during maturation.
Total ……. regenerated plants are obtained after ……. days of culture.

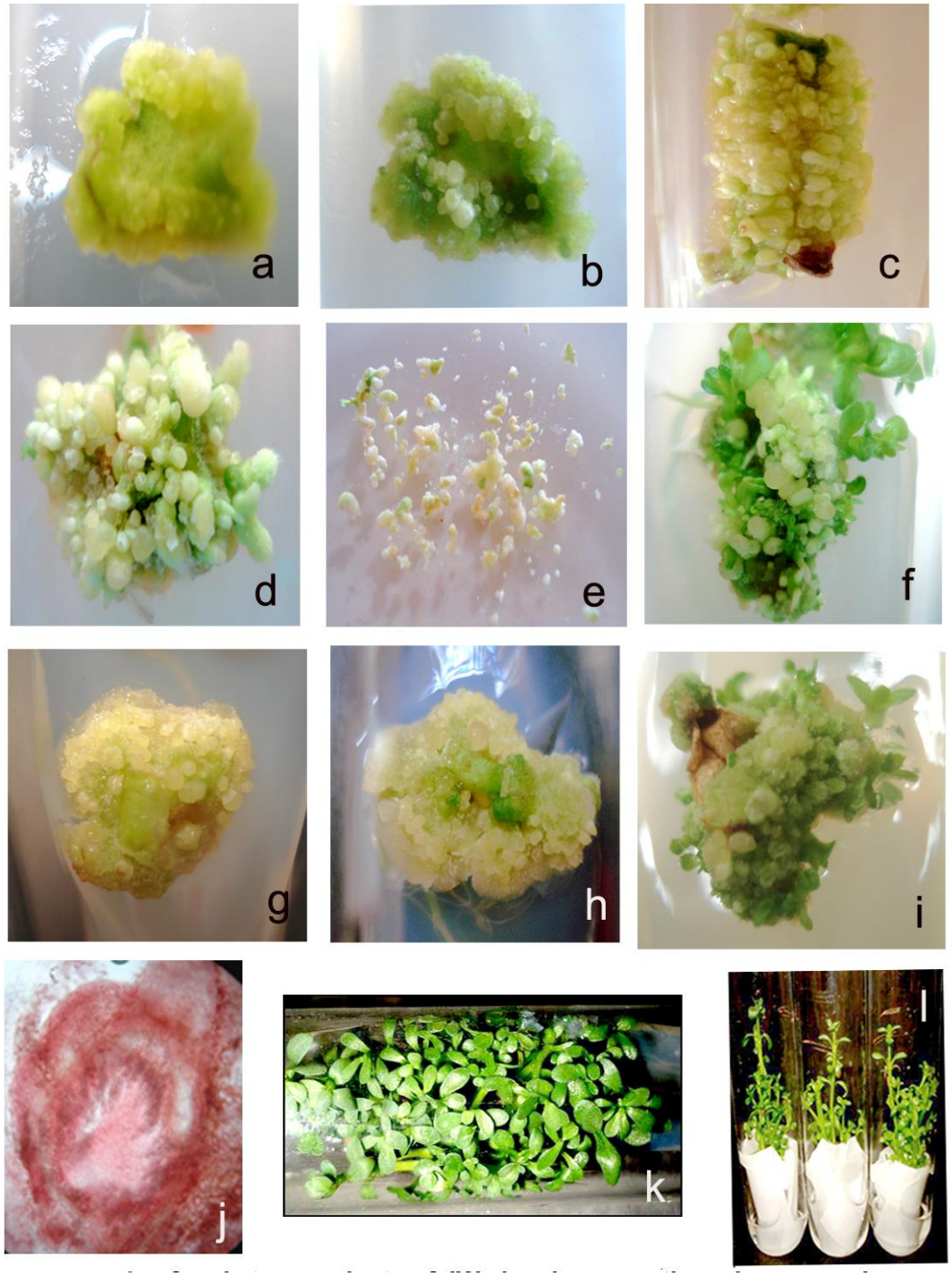


Fig. 19: Somatic embryogenesis from leaf and stem explants of *B. monnieri* a: Small protuberance emerge from cut ends of leaf explants. b-c: Globular somatic embryos developed all over the surface of the explants. d: Maturation of embryos acquiring different shapes. e: Germination of embryos. f- Scattered somatic embryos. g- Small protuberances emerge from cut ends of stem explants. h- developing embryos. i- Embryos maturation and germination. j: Cross section of an embryo. k-l: Multiplication of plantlets.

Jain M., Tiwari S., Guruprasad K.N. and G.P. Pandey (2010) Influence of Different Media on Somatic Embryogenesis of *Bacopa monnieri.* J. Trop. Med. Plants. Vol. 11(2) 163-167.

Precautions: Appendix-1

Chapter 7

Clonal propagation of *Bacopa monnieri* (*Brahmi*) from axillary buds.

Theory:

The growing points of shoots (apical and axillary buds) on culturing, give rise to small organised shoots which can then be rooted. Generally, this occurs without an interfering callus phase. The plantlets thus obtained are hardened and transplanted into soil. This process is termed as micropropagation and is used to propagate plants true-to-type that is clones. The main application of this technique is in horticulture for mass propagation of clones for the commercial market, and also in the production of virus-free plants.
The process involves following steps:
Stage 0- Preparative stage (Preparation and maintenance of mother plants).
Stage I - initiation of cultures (Explant surface sterilization and establishment of culture).
Stage II - Multiplication (adventitious bud formation and enhance axillary branching).
Stage III - Rooting of shoots.
Stage IV -Transplantation (Acclimatization).

Materials Required:

Experimental material: Brahmi (*Bacopa monnieri*) nodal sections containing axillary buds.
Apparatus: Flasks and beakers, forceps (8 inches), Scalpel or blade holder (no.4) with surgical blade (24 no.), petri dishes, distilled water **all should be sterilized**.
Medium:

- Gamborg's B5 medium without growth regulators (as control for comparing the effects of growth regulators. *It can also be used to obtain rooting from the isolated shoots.
- Gamborg's B5 medium with growth regulators BAP (1.0 $mg.l^{-1}$).

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$
Gelling agent: Agar (0.8%)

Preparation of B5 medium:

Refer Chapter 4 with following modifications: Replace 2,4-D with the given concentration of BAP; keeping the rest of the procedure same.

Selection of the explants (Brahmi shoots):

- Collect explants from actively growing shoots.
- The explants should be collected from plants neither too young nor too old.
- Detach the leaves in such a way that their petiole remains intact with the shoots.
- Wash the explants with tap water followed by distilled water.
- Cut short the stems if they are too lengthy and collect it in a beaker.

Check list: (Before starting the inoculation)

The hood/transfer area should contain the following before putting on the UV light

- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.
- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps and blade holder/scalpel dipped in a beaker of ethanol.
- Surgical blades.
- Sterile petri dishes which is used for preparation of the explants.
- Media tubes.

Treatment inside the hood

Surface sterilization of the explants: Replace leaves/stem with shoots and rest of the procedure remains same as mentioned in Chapter 4.

Inoculation:

1. Flame sterilize the forceps.
2. Fix the surgical blade tightly on to the blade holder (do not touch it with hands).
3. Cut the petioles carefully from the nodal portion of the shoots. Leave some portion of the petioles attached to the node to avoid any damage to the buds present in the axils.
4. Apply two cuts; one at the bottom and another above the node portion in such a way that approx. 1cm sized nodal section is obtained with the axillary buds intact.
5. Cut the shoot into 2 or 3 node segments each.
6. Hold the nodal section from the top and inoculate it vertically in the media with the help of forceps including the control tubes.
7. Label the test tubes with name and type of the explant, date of inoculation and the type of media.
8. *To obtain complete plantlets, isolate the shoots from the clump, remove the adhered media and subculture them to basal Gamborg's B5 medium (without growth regulators) until rooting is obtained.

Culture Conditions

The culture tubes are kept at 25±2°C in light conditions ($50\mu Em^2s^{-l}$) under 16/8 hrs of photoperiod (16 hrs light and 8 hrs dark).

Observations

Stages	Number of explants	Days of Culture	Details (write your observations including the mentioned characteristics comparing with control)
Shoot Induction	• Inoculated • Got contaminated		• Number of shoot buds emerging other than the axillary buds.
Shoot Multiplication			• Elongation of shoots (size in mm.) and number of shoots multiplied.

Rooting in Basal B5 medium	• Remained • Showing growth symptoms		• Rooting of the shoots (number of roots/shoot) and number of complete plantlets obtained.

Results:

……..number of plantlets from……..number of explants is obtained in ……..days of culture.

Fig. 20: *In vitro* shoot regeneration from nodal sections containing axillary buds

Jain M., Tiwari S., Guruprasad, K.N. and Pandey G.P. (2011) Micropropagation and encapsulation of two accessions of *Bacopa monnieri*: an endangered medicinal plant. International Journal of Advanced Biotechnology Research. Volume 1 (1):11-19 .

Precautions: Appendix-1

Chapter 8

Preparation of synthetic seeds from somatic embryos/axillary buds of *Bacopa monnieri* (*Brahmi*)

Theory:

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant and that retain this potential also after storage.
There are two types of synthetic seeds: desiccated and hydrated.

1) **Desiccated synthetic seeds**: are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyox) followed by their desiccation. Such types of synseeds are produced only in plant species whose somatic embryos are desiccation tolerant.
2) **Hydrated synthetic seeds**: are commonly produced seeds for those plant species where the somatic embryos are recalcitrant and sensitive to desiccation.

Hydrated synthetic seeds are produced by mixing the somatic embryos/buds with sodium alginate and dropped into complexing bath containing calcium chloride using a dropper or pipette. Each drop containing an embryo/bud at the center will form into a calcium alginate capsule or bead around them. The formation of the bead is due to complexing of sodium alginate with di or trivalent metal cations (calcium) to form calcium alginate. The complexing occurs via formation of ionic bonds between carboxylic acid groups of the guluronic acid molecules of the alginate. Sodium alginate is commonly used as hydragel because of its gelatin properties, ease of use, less viscosity and low cost.

Materials Required:

Experimental material: Brahmi (*Bacopa monnieri*) somatic embryos/nodal sections containing axillary buds.
Apparatus: Flasks and beakers, forceps (8 inches), Scalpel or blade holder (no.4) with surgical blade (24 no.), wide mouthed pipette, rubber bulb, petri dishes, distilled water **all should be sterilized**.
Medium:

- Gamborg's B5 medium without growth regulators (as control for comparing the effects of growth regulators).
- Gamborg's B5 medium with growth regulators BAP (0.5 $mg.l^{-1}$).

Chemicals: Ethanol (pure and 70%), 0.1% $HgCl_2$.
Gelling agent: Agar (0.8%)

Preparation of B5 medium:

Refer Chapter 4 with following modifications: Replace 2,4-D with the given concentration of BAP; keeping the rest of the procedure same.

Preparation of 3%w/v solution of Na-alginate (125 ml):

Weigh the 3.75 gms of Na-alg. Place 125 ml of distilled water in a flask on magnetic stirrer. Now add little amount of pre weighed Na-alg in water. Allow it to dissolve. Now again add little amount of Na-alg. Keep on doing this until a viscous gel like solution is prepared from 2gms of Na-alg. Now add growth regulator BAP (0.1 mg). Mix thoroughly and set the pH at 5.8 with the help of 0.1N NaOH/HCl. Put cotton plug and wrap it with paper. Autoclave it along with the media.

Preparation of 75 mM solution of $CaCl_2$ (125ml):

Weigh 2.75 gms of $CaCl_2$ and dissolve it in 125 ml of distilled water. Put cotton plug and wrap it with paper. Autoclave it along with the media.

Selection of the explants (Brahmi shoots):

- Collect explants from actively growing shoots.
- The explants should be collected from plants neither too young nor too old.
- Detach the leaves in such a way that their petiole remains intact with the shoots.
- Wash the explants with tap water followed by distilled water.
- Cut short the stems if they are too lengthy and collect it in a beaker.
- If somatic embryos are selected, then directly go for synthetic seed preparation skipping the surface sterilization procedures.

Check list: (Before starting the inoculation)

The hood/transfer area should contain the following before putting on the UV light

- Spirit lamp/burner
- A large jar (which can be used as a "sink" for discard).
- Flask containing 0.1% $HgCl_2$.
- Flask containing 70% ethanol.
- Flask containing sterile distilled water (SDW for rinsing).
- Sterile forceps and blade holder/scalpel dipped in a beaker of ethanol.
- Surgical blades.
- Sterile petri dishes which is used for preparation of the explants.
- Media tubes.

Treatment inside the hood

Surface sterilization of the explants: Replace leaves/stem with shoots and rest of the procedure remains same as mentioned in Chapter 4.

Preparation of synthetic seeds and inoculation of seeds in media:

1. Flame sterilize the forceps.
2. Fix the surgical blade tightly on to the blade holder (do not touch it with hands).
3. Cut the petioles carefully from the nodal portion of the shoots. Leave some portion of the petioles attached to the node to avoid any damage to the buds present in the axils.
4. Apply two cuts; one at the bottom and another above the node portion in such a way that approx. 1cm sized nodal section is obtained with the axillary buds intact.

5. Cut the shoot into 2 or 3 node segments each.
6. Put the nodal sections in Na-Alginate sol.
7. Mix thoroughly.
8. Now with the help of pipette lift a nodal section along with little Na-Alginate.
9. Drop this in $CaCl_2$ solution in one shot so that trail formation is avoided.
10. Repeat the process with each node section one by one.
11. Keep them embedded in $CaCl_2$ solution for 20-30 minutes.
12. Now take out the beads and rinse them three times with sterile distilled water.
13. Without damaging the seeds, hold them with the help of forceps and inoculate them one at a time in each media tube including the control tubes.
14. Label the test tubes with date of inoculation and the type of medium.

Culture Conditions

The culture tubes are kept at 25±2°C in light conditions (50µEm^2s^{-l}) under 16/8 hrs of photoperiod (16 hrs light and 8 hrs dark).

Observations

Number of seeds inoculated (SI)	Number of seeds Germinated (SG)	% seed germination $\frac{SI}{SG}$ X100

Result: The seeds show……% germination after……days of culture.

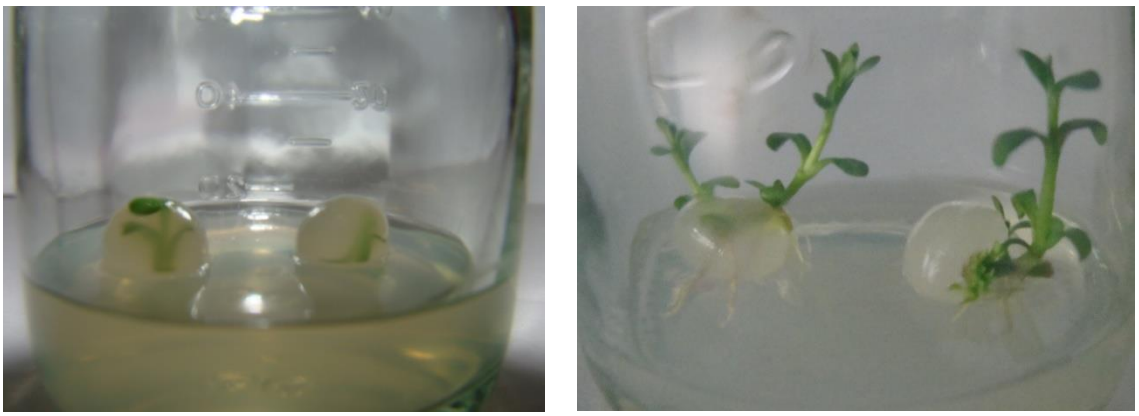


Fig. 21: Synthetic seeds: a) Encapsulated Axillary buds b) Syn seeds showing bipolar germination

Precautions: Appendix-1

Chapter 9

Acclimatization of *in vitro* regenerated plants of *Bacopa monnieri* (*Brahmi*)

Theory:

The two main deficiencies of *in vitro* grown plants are: (i) poor control of water loss, and (ii) heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. For the initial 10-15 days, it is essential to maintain high humidity (90-100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to the ambient level over a period of 2-4 weeks. Thus, in the first phase of acclimatization the main environmental stress to the plants is the change from a substrate rich in organic nutrients to one providing only inorganic nutrients. This probably induces the photosynthetic machinery of the plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions. Gradually the plants get fully acclimatized and are removed to a nethouse or field for better growth.

Materials Required:

Soil mix (sand and sandy soil in the ratio 1:3, autoclaved), small pots, transparent plastic bags, rubber bands, punching machine, water.

Procedure:

1. Take out the plants from the test tubes with the help of forceps.
2. Wash the lower part of the plants gently to remove the medium sticking to them.
3. Transfer the plantlets to pots containing autoclaved soil mix and cover the plant with clean, transparent plastic bags with the help of a rubber band.
4. Make small holes with a punching machine for air circulation.
5. Keep the pots in lab conditions.
6. The holes are gradually enlarged over a period of time.
7. Gradually increase the light intensity for the plants also.
8. Open the bag after 8-10 days and after another 8-10 days grow the plant under regular greenhouse conditions.
9. During the acclimatization phase in green house conditions, the plants need to be fed regularly, by drenching of the potting medium, with a liquid fertilizer.

Observations

Number of Plants transferred to soil conditions (Pt)	**Number of plants survived in soil conditions (Ps)**	**% Survival in soil** $\frac{Ps}{Pt} \times 100$

Results:

The plants show…….% survival rate in soil conditions.

Fig. 22: Acclimatized *in vitro* regenerated plants of *B. monnieri* in soil conditions

Precautions: Appendix-1

Chapter 10

Low cost strategies in plant tissue culture

Low cost options should lower the cost of production without compromising the quality of the micropropagules and plants. Cost reduction is achieved by improving process efficiency and better utilization of resources. Reducing the cost should not result in high contamination of cultures or produce plants with poor field performance.

Instruments:

Autoclave: Certain spores from fungi and bacteria are killed only at 121°C and 1.05kg/sq.cm (15 pounds per sq. inch) pressure. Self-generating steam autoclaves are more dependable. Use of pressure cooker can be a good alternative for autoclaves:

a. Place items on a rack inside the pressure cooker.
b. Add water to a level below the rack.
c. Close the pressure cooker, making sure that the seal is well seated.
d. Place the pressure cooker on high heat.
e. Begin timing when a steady stream of steam emerges from the vent.
f. After 15 minutes, turn the heat off and let cool before opening the pressure cooker.

Precautions:

- Check the pressure cooker seal. If stiff or damaged, do not use the pressure cooker.
- Opening a pressure cooker while still warm is very dangerous.
- Place forceps and scalpels in beakers with business end down, and cover with a doubled piece of aluminium foil/paper. If foil is used then snap a rubber band around the foil immediately as soon as the pressure cooker is cool enough to open.

Laminar Air Flow: LAF is one of the costliest instruments used for plant tissue culture which creates aseptic environment for transfer of cultures. The environment can be created to some extent by performing the procedure on a clean platform. Swab it with 70% alcohol. Swab your hands and media tubes with 70% alcohol. Place two burners in a straight vertical line on the platform keeping the working space in between the two burners. Allow the alcohol to evaporate and then put on the flames of both the burners and perform all the procedures between the two flames. This method cannot totally replace the LAF and its efficiency but can be utilized for non research based practicals for students.

Lights: Light intensity from four tube lights (40 Watts) in a shelf can be managed with only two tube lights and covering the shelf with shining gift wrap paper.

Glass bead sterilizers: Used to sterilize forceps and scalpels can be replaced with conventional flame sterilization using spirit lamps or gas burners.

Glasswares: Instead of Petri dishes for preparation of explants, stainless steel plates, ceramic tiles or brown wrapping paper can be used; all of these can be autoclaved. Beakers can be replaced by autoclavable plastic jars/containers available in market.

Chemicals: The maximum cost of media for plant tissue culture is contributed by the carbohydrate source and gelling agent.

Carbohydrate source: Sucrose is the most commonly used carbon source in the micropropagation of plants. It adds significantly to the media cost. Household sugar and other sugar sources can be used to reduce the cost of the medium. Use of common sugar reduces the cost of the medium between 78 to 87%.

Agar: A semi-solid medium ensures adequate contact and better diffusion of medium constituents, between the plant tissue and the medium. It can also be easily removed from plantlets before their transfer to *in vivo* conditions. For these reasons, a semi-solid medium is often preferred over solid medium. Media solidified with gelling agents like agar increase the time to clean the culture containers and above all contribute to 70% of the costs of the medium.

A) Replacing agar, a sand supported protocol is suggested for culture.

1. Boil sand in water on gas stove.
2. Store the clean sand in a plastic bag.
3. Put ½ inch of sand in test tubes.
4. Sterilize the test tubes in autoclave/pressure cooker.
5. In Laminar air flow add enough sterile media solution to just saturate, but not cover, the sand.
7. Close the tubes with cotton plugs.

Precautions:

1. Do not microwave the sand, it will splatter all over.
2. While transferring cultures, make sure that they are in good contact with the sand but not "swimming" in the media.

B) Agar can also be replaced by using filter paper dipped in liquid media. This also reduces the costs of multiplication:

1) Prepare filter paper strips and fold it in the shape of letter
2) Pierce a minute hole in the centre of the V bend of the folded paper.
3) Insert this folded paper in the test tubes.
4) Fill the liquid media up to the V bend of the folded paper.
5) Autoclave the test tubes.
6) While inoculations insert the node in the minute hole of the paper such that the lower portion of the node is in contact of the liquid media and the axillary buds remain above the paper

Fig. 23: Filter paper technique

Source of water: Distilled water produced through electrical distillation is expensive. In some cases, alternative water sources can be used to lower the cost of the medium. If tap water is free from heavy metals and contaminants, it can be substituted for distilled water. In rural areas, rainwater can be collected in clean glass jars and used for tissue culture.

Proper choice of media vessels: Transparent plastic containers, that withstand autoclaving and washing, are extensively used for micropropagation in many developed countries. Plastic bags (approximately 10x15cm) have also been used for large-scale micropropagation and are very cost effective. Disposable plastic bags eliminate the cost of washing and of lids. Good quality plastic bags are sterile due to high temperature during manufacture. After pouring pre sterilized medium under the laminar flow, the top 2 to 5 cm of the bags is folded and several bags are held together with either large paper clips or plastic cloth-hanging pegs. After transfer of the explants and cuttings, the bags are closed with a heat-sealing machine or by knotting if the bags are 18-20 cm long. Plastic bags being lightweight can also be hung by thread and do not need elaborate shelving.
For minute seeds, germination can be achieved in eppendorfs instead of test tubes, this cut the cost considerably.

Vented closures: Cotton plugs prepared by stuffing cotton in cheese cloth tightly, reduces the cost of using microfilters. The cotton plugs can be reused two to three times.

Appendix 1

Precautions

- Pressure of the autoclave should not be manually released.
- UV light in the chamber should be kept on for 15-20 min.
- The plant material should not be kept inside the hood while UV light is on.
- During surface sterilization of the explants check that the explants get totally immersed in the sterilizants during the process.
- Flame the mouth of the test tubes while inoculating the seeds and again while capping the test tubes.
- Do not touch the plant material with your hands inside the hood.
- Flame sterilize the forceps/blade each time to transfer explants to avoid cross-contamination.
- Put one explant into each media tube (if the explants is contaminated it cannot spread to the others).
- While counting the embryos do not damage them as they are sensitive.
- The time duration of the surface sterilization process should be strictly monitored.
- While counting the shoots do not damage the young buds.
- Take care that the synthetic seeds do not get damaged while holding them with forceps.
- Do not damage the roots while removing the media sticking to the roots prior to planting them in soil.
- The soil in the pots should be soft and does not contain stones.
- If the symptoms of desiccation are observed during first step of acclimatization, spray water on the foliage.

Appendix 2

Glossary

Abscisic Acid - ABA. A plant growth regulator involved in abscission, dormancy, stomatal opening/closure, and inhibition of seed germination. It also affects the regulation of somatic cell embryogenesis in some plant species.

Activated Charcoal - Charcoal which has been treated to remove hydrocarbons and to increase its adsorptive properties.

Adventitious Bud - Buds that develop from unusual points of origin, in places other than at the end of a twig or in leaf axils, that is from callus or embryos, from sources other than zygotes. They appear when wounding stimulates their development.

Agar - A polysaccharide solidifying agent used in nutrient media preparations and obtained from certain types of red algae (Rhodophyta). Both the type of agar and its concentration can affect the growth and appearance of cultured explants. Agar is generally used at a concentration of 6-12 g/liter.

Androgenesis- Development of plants from male gametophytes.

Aneuploidy- The situation which exists when the nucleus of a cell does not contain an exact multiple of the haploid number of chromosomes; one or more chromosomes being present in greater or lesser number than the rest.

Antibiotic - Any chemical or biological agent that harms the growth of micro-organisms.

Apical- Pertaining to tip or apex.

Apical dominance- The phenomenon of suppression of growth of an axillary bud in the presence of the terminal bud on the branch.

Apical meristem- The meristem located at the apices of main and lateral shoots.

Apomixis - Replacement of the normal sexual reproduction by asexual reproduction, without fertilization. Thus "normal asexual reproduction" of plants, such as propagation from cuttings or leaves, has never been considered to be apomixis, but replacement of the seed by a plantlet, or replacement of the flower by bulbils are types of apomixis. Apomictically produced offspring are genetically identical to the parent plant.

Aseptic - Raising cultures from a tissue or an organ after freeing it of bacteria, fungi, and other micro-organisms, in an environment free of these microorganisms.

Autoclave - 1. An enclosed chamber in which substances are heated under pressure to sterilize utensils, liquids, glassware, etc., using steam. The routine method uses steam pressure of 1.05Kg/sq.cm at 121°C for 15 minutes, or longer to allow large volumes to reach the critical temperature. 2. A pressure cooker is a type of autoclave used to sterilize growth medium and instruments for tissue culture work.

Auxin - A group of plant growth regulators (natural or synthetic) which stimulate cell division, enlargement, apical dominance, root initiation, and flowering. Endogenous auxins are auxins that occur naturally. Indole-3-acetic (IAA) is a naturally occurring auxin. Exogenous auxins are auxins that are man-made or synthetic. Examples of exogenous auxins included 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-Butyric acid (IBA), α-Naphthaleneacetic acid (NAA), and 4-Chlorophenoxyacetic acid (CPA).

Axillary bud - when located in the axil of a leaf.

Axenic culture - A culture without foreign or undesired life forms. An axenic culture may include the purposeful cocultivation of different types of cells, tissues or organism.

Batch culture - A suspension culture in which cells grow in a finite volume of nutrient medium and follow a sigmoid pattern of growth.

Bleach - A fluid, powder or other whitening (bleaching) or cleaning agent, usually with free chlorine ions. Commercial bleach contains calcium hypochlorite or sodium hypochlorite, and is a common disinfectant used for cleaning working surfaces, tools and plant materials in plant tissue culture and grafting.

Bleeding or Browning - Used to describe the occasional purplish-black coloration of media due to phenolic products given off by (usually fresh) transfers which may be phytotoxic.

Bud - An undeveloped or embryonic shoot and normally occurs in the axil of a leaf or at the tip of the stem. Once formed, a bud may remain for some time in a dormant condition, or it may form a shoot immediately.

Callus - A mass of unorganized, thin-walled, proliferate mass of undifferentiated plant cells, developed as the result of culture on nutrient media, a wound response.

Casein hydrolysate - A mixture rich in amino acids obtained by digestion (acid hydrolysis) of the milk protein, often used as a helpful supplement in tissue culture media.

Cell Differentiation - Continuous loss of physiological and cytological characters of young cells, resulting in getting the characters of adult cells. The unspecialized cells become modified and specialized for the performance of specific functions. Differentiation results from the controlled activation and de-activation of genes.

Cell line - A cell line arises from a primary culture at the time of the first successful subculture. The term "cell line" implies that cultures from it consist of lineages of cells originally present in the primary culture. The terms *finite* or *continuous* are used as prefixes if the status of the culture is

known. If not, the term *line* will suffice. The term *"continuous line" replaces the term "established line".* In any published description of a culture, one must make every attempt to publish the characterization or history of the culture. If such has already been published, a reference to the original publication must be made. In obtaining a culture, as originally named and described, must be maintained and any deviations in cultivation from the original must be reported in any publication.

Charcoal - See Activated Charcoal

Chemically Defined Medium - A nutritive solution for culturing cells in which each component is specifiable and ideally of known chemical structure.

Chimeras - Plants or plant tissues with cells of more than one genetic make-up.

Chlorosis - Reduced development or absence of green pigments leading to yellowing or whitening of green tissues in plant due to deficiency of light, magnesium, iron, or other factors.

Clone - Group of plants genetically identical in which all are derived from one selected individual by vegetative or in-vitro propagation, without the sexual process.

Closed continuous culture - A continuous culture in which inflow of fresh medium is balanced by outflow of corresponding volumes of spent medium. Cells are separated mechanically from out flowing medium and added back to the culture.

Contamination -Being infested with unwanted microorganisms such as bacteria or fungi.

Cryopreservation - Preservation and storage of cells, tissues and organs at temperatures around -196~ or by immersion into liquid nitrogen.

Cultivar - A category of plants that are, firstly, below the level of a sub-species taxonomically, and, secondly, found only in cultivation. It is an international term denoting certain cultivated plants that are clearly distinguishable from others by stated characteristics and that retain their distinguishing characters when reproduced under specific conditions.

Culture - Growing cells, tissues, plant organs, or whole plants in nutrient medium, under aseptic conditions, e.g. cell culture, embryo culture, shoot-tip culture, anther culture, etc.

Culture Medium - See Medium

Cutting - A detached plant part that under appropriate cultural conditions can regenerate the complete plant without a sexual process.

Cytokinin - Plant growth regulators (hormones) characterized as substances that induce cell division and cell differentiation. In tissue culture, these substances are associated with enhanced callus and shoot development. Endogenous cytokinins are cytokinins that occur naturally, include zeatin and 6-γ,γ-dimethylallylaminopurine (2iP). Exogenous cytokinins are cytokinins that are

man-made or synthetic; include 6-furfurylaminopurine (kinetin) and 6-benzylaminopurine (BA or BAP).

Dedifferentiation - Reversal of organized structures into an undifferentiated state.

Differentiation - A process in which unspecialized cells develop structures and functions typical of the cell type *in vivo* (characteristic of a particular type of cell). Development from one cell to many cells, accompanied by a modification of the new cells for the performance of particular functions. In tissue culture, the term is used to describe the formation of different cell types.

Distal - The side of an explant furthest from the point of attachment (i.e. the tip of a leaf).

Donor Plant - A plant used as a source of plant material for micro-propagation purposes.

Established Culture - An aseptic viable explant responding to primary culture conditions.

Ethanol - C_2H_6O, Commonly used to disinfest plant tissues, glassware utensils and working surfaces in tissue culture manipulations. The concentration used is 70% (v/v) for disinfecting and 95% (v/v) when flaming tools.

Euploidy - The situation which exists when the nucleus of a cell contains an exact multiple of the haploid number of chromosomes.

Excision - Cutting out and preparing a tissue, organ, etc., for culture.

Explant - Tissue asceptically obtained from its original site, prepared from the donor plant and transferred to an artificial medium for growth or maintenance.

Exponential phase - See Logarithmic phase

Ex Vitro - Organisms removed from tissue culture and transplanted; generally plants to soil or potting mixture.

Filter Sterilization - Process of sterilizing a liquid by passage through a filter with pores. Bacteria-proof filter membranes of pore size 0.45 µm or less are used.

Flaming - A technique for sterilizing instruments. The instrument is dipped in alcohol (usually 95% (v/v) ethanol) and then the alcohol on the instrument is ignited, thus heat-sterilizing the tool surface.

Formulation - See Medium

Friability - A term indicating the tendency for plant cells to separate from one another.

Fungicide - An agent, such as a chemical, that kills fungi (Bavistin etc).
Gelatin - A glutinous, proteinaceous gelling and solidifying agent. Occasionally used in place of agar in tissue culture.

Gene - One of a set of units of heredity, having a specific effect on the characteristics of an organism. Genes are composed of DNA and are arranged linearly on the chromosomes.

Genotype - The genetic make-up of an individual as determined by the set of genes carried in the chromosomes.

Gibberellins - Plant growth regulators involved in elongation, enhancement of flower, fruit and leaf size, germination, vernalization and other processes.

Haploid - Having single copy per cell of each chromosome characteristic of the species (= n).

Hardening - Adapting plants to outdoor conditions by gradually withholding water, lowering the temperature, increasing light intensity, or reducing the nutrient supply. The hardening-off process conditions plants for survival when transplanted outdoors. The term is also used for gradual acclimatization to *in vivo* conditions of plants grown *in vitro*, e.g., gradual decrease in humidity, acclimatization; autotrophic nutrient conditions.

HEPA Filter - (high efficiency particulate air filter) An enclosed work area that has sterile air moving across it. The air moves with uniform velocity along parallel flow lines. Room air is pulled into the unit and forced through a HEPA capable of screening out particles larger than 0.3 µm. HEPA filters are used in laminar air flow cabinets (hoods) for sterile transfer work.

Heterokaryon - A cell in which two or more nuclei of unlike genetic make-up are present, usually derived as a result of cell fusion.

Hormone - A specific organic product, produced in one part of a plant, and transported to another part where, at low concentrations, it promotes, inhibits or quantitatively modifies a biological process. Also called as growth regulator. May be natural or synthetic.

Hyperhydration - An abnormality shown by some shoots developed in culture, in which the shoots appear as overly succulent, crisp, water-logged, or glassy tissues. Earlier termed 'Vitrification'.

Hypochlorite - Generic term for aqueous solutions of sodium hypochlorite, potassium hypochlorite or calcium hypochlorite, which are oxidizing agents and used for disinfecting surfaces and surface-sterilizing tissues, and for bleaching.

Initiation - Early steps or stages of a tissue culture process (culture growth, organogenesis, embryogenesis)

Inoculum - A small piece of tissue cut from callus, or an explant from a tissue transferred into fresh medium for continued growth of the culture. Also aliquot of a suspension culture used for its subculture.

Inositol - C6H6(OH)6, A water-soluble nutrient frequently referred to as a "vitamin" in plant tissue culture.

In vitro - Literally 'in glass'; Living in test tubes, outside the organism or in an artificial environment, typically in glass vessels in which cultured cells, tissues, or whole plants may reside.

In vivo - Literally 'in life'; The natural conditions in which organisms reside. Refers to biological processes that take place within a living organism or cell under normal conditions.

Juvenile - A phase in the sexual cycle of a plant characterized by differences in appearance from the adult and which lacks the ability to respond to flower-inducing stimuli.

Kinetin - One of the cytokinins, a group of growth regulators that characteristically promote cell division in plants.

Kinin - A substance promoting cell division. In plant systems, the prefix cyto- has been added (cytokinin) to distinguish it from kinin in animal systems. See cytokinin.

Lag phase - The initial growth phase after inoculation, during which cell number remains relatively constant, prior to rapid growth.

Laminar Flow Hood - Cabinet for inoculation of cultures. The working area is kept sterile by a continuous, non-turbulent flow of sterilized air through a HEPA filter. See HEPA filter

Log phase - See Logarithmic phase

Logarithmic phase - The steepest slope of the growth curve; the phase of vigorous growth, during which cell number doubles every 20-30 minutes.

Longitudinal edge - The direction parallel to the ridge, following the greatest length of an area or object.

Macronutrient - For growth media: an essential element normally required in concentrations >0.5 millimole/l.

Medium - In plant tissue culture, a term for the liquid or solidified formulation upon which plant cells, tissues or organs develop.

Medium Formulation - Medium formulation In tissue culture, the particular formula for the culture medium. It commonly contains macro-elements and micro-elements, some vitamins (B vitamins, inositol), plant growth regulators (auxin, cytokinin and sometimes gibberellin), a carbohydrate source (usually sucrose or glucose) and often other substances, such as amino acids or complex growth factors. Media may be liquid or solidified with agar; the pH is adjusted (ca. 5-6) and the solution is sterilized (usually by filtration or autoclaving). Some formulations are very specific in the kind of explant or plant species that can be maintained; some are very general.

Meristem - Undifferentiated tissue, the cells of which are capable of active cell division and differentiation into specialized and permanent tissue such as shoots and roots. The main categories of meristems are: apical meristems (in root and shoot tips), lateral meristems (vascular and cork cambiums), and intercalary meristems (in the nodal region and at the base of certain leaves).

Meristem culture - In the present context, in vitro culture of a shiny, dome-like structure measuring less than 0.1 mm in length excised from the shoot apex.

Micronutrient - For growth media: An essential element normally required in concentrations < 0.5 millimole/litre.

Micropropagation - *In vitro* Clonal propagation of plants from shoot tips or nodal explants usually with an accelerated proliferation of shoots during subcultures.

Morphogenesis - The anatomical and physiological events involved in the growth and development of an organism resulting in the formation of its characteristic organs and structures, or in regeneration.

Myo Inositol - See Inositol

MS - Murashige and Skoog media

Nutrient medium - See Medium

Organ culture - Aseptic culture of organized structures, e.g. root tip, shoot tip, shoot segments, embryo, etc.

Organized tissue - Composed of regularly differentiated cells.

Organogenesis - Differentiation of organs from cultured cells or tissue.

pH - A measure of acidity and alkalinity. Equal to the log of the reciprocal of the hydrogen ion concentration of a solution, expressed in grams per litre. A reading of 7 is neutral (e.g., pure water), whereas below 7 is acid and above 7 is alkaline.

Phenolic Exudation - Many plant species contain phenolic compounds that blacken through exudation. The process is initiated after plants are wounded. Phenolic exudation may lead to growth inhibition or, in severe cases, to tissue necrosis and death. Antioxidants are incorporated into the sterilizing solution or isolation medium to prevent or reduce exudative browning (activated charcoal).

Photoperiod - The length of time plants are exposed to light in an alternating light-dark interval sequence.

Plagiotropic- Horizontal growth as opposed to vertical growth.

Plant Tissue Culture - The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.

Polyploidy - Containing three or more sets of chromosomes per nucleus (3n = triploid, 4n = tetraploid, 5n = pentaploid, etc.)

Population density - The number of cells per unit area or volume of a culture vessel. Also the number of cells per unit volume of medium in a suspension culture.

PPM - Plant Preservative Mixture - a broad-spectrum biocide/fungicide for plant tissue culture.

Primary culture - A culture started from cells, tissues or organs excised directly from organisms.

Primordia - Plural of primordium, plant organs in the earliest stages of differentiation.

Propagule - Any structure capable of giving rise to a new plant by asexual or sexual reproduction, including seeds, leaves, buds, etc.

Protocorm - In orchids, seed contains an unorganized embryo comprising only a few hundred cells. During seed germination the embryo first forms a tuberous structure called a protocorm, from which develops a complete plant. In cultures, vegetative parts of several orchids form round, glistening, protocorm-like structures which may be multiplied indefinitely or induced to regenerate a whole plant.

Proximal - The side of an explant closest to the point of attachment (i.e. the base of a leaf)

Radicle - That portion of the plant embryo which develops into the primary or seed root.

Regeneration - In tissue cultures, a morphogenetic response that results in the production of new organs, embryos or whole plants from cultured explants or calli derived from them.

Scarification - The chemical or physical treatment given to some seeds (where the seed coats are very hard or contain germination inhibitors) in order to break or weaken the seed coat sufficiently to permit germination.

Semi-solid - Gelled but not firmly so; small amounts of a gelling agent are used to obtain a semi-solid medium; called also semi-liquid.

Somaclonal Variation - Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.

Somatic- Referring to vegetative or non-sexual part or process.

Somatic embryogenesis - The process of embryo initiation and development from vegetative or non-gametic cells.

Somatic tissue - All the tissues that form the body of a plant.

Stationary phase - The plateau of the growth curve after log growth, during which cell number remains constant. New cells are produced at the same rate as older cells die.

Sterile - Medium or object with no perceptible or viable micro-organisms.

Sterilize - The process of elimination of micro-organisms, such as by chemicals, heat, irradiation or filtration.

Stock plants - The plants from which cuttings or explants are taken for multiplication.

Sub-culture - Division and transfer of a portion or inoculum of a culture to fresh medium. Sometimes used to denote the adding of fresh liquid to a suspension culture.

Suspension culture - A type of culture in which cells or cell aggregates are cultured in liquid medium.

Synchronous culture - A culture in which a majority of cells are in the same phase of the cycle or the embryos are in the same stage of development.

Synthetic seeds - Somatic embryos encapsulated in hydrated or desiccated coating, which protects the embryo from mechanical damage during handling and allows their germination like sexual seeds. The capsule may also serve as an artificial endosperm.

Terminal bud - when located at the tip of a stem (apical is equivalent but rather reserved for the one at the top of the plant)

Tissue culture - A general term used to describe the culture of cells, tissues or organs in a nutrient medium under sterile conditions

Totipotency - Potentiality or property of a cell to produce a whole organism.

Transgenic plants - Plants which have been genetically engineered to modify certain characteristics by introducing one or a few selected genes.

Transverse - Across the width of the explant, the direction perpendicular to the ridge, the perpendicular side of the longitudinal side.

Undefined medium - A medium or substance added to medium in which not all of the constituents or their concentrations are chemically defined, such as media containing coconut milk, malt extract, casein hydrolysate, fish emulsion or other complex compounds.

Undifferentiated - Lacking the specialized or differential gene expression characteristic of specialized cells.

Vessel - A container, such as a Petri dish, jar, baby food jar or test tube, used for tissue culture.

Viability - The capability to live and develop normally.

Viable - Capable of germinating, living, growing and developing.

w/v - Weight per volume; the weight of a constituent in 100 cm3 of solution, expressed as a percentage.

Wild-type - The most frequently encountered phenotype in natural breeding populations.

Zone of elongation - The section of the young root or shoot just behind the apical meristem, in which the cells are enlarging and elongating rapidly.

Majority of glossary terms sourced from: http://www.fao.org.

List of Journals and e-links

Plant Tissue Culture

http://aggie.horticulture.tamu.edu/tisscult/pltissue/pltissue.html
Many dimensions of Plant Tissue Culture research

http://aggie-horticulture.tamu.edu/tisscult/biotech/biotech.html
Plant Tissue Culture - Biotechnology

http://aggie-horticulture.tamu.edu/tisscult/tcintro.html
Plant Tissue Culture Information exchange

http://indycc1.agri.huji.ac.il/~tzvika/iaptc/ip-home.htm
International association for Plant Tissue Culture

http://sbc.ucdavis.edu/
Plant biotechnology

http://www.access-excellence.org/l
Scitalk - Plant Tissue Culture

http://www.agbiotechnet.com/Directory/browse.asp
Agbiotechnet directory lists major organisations involved in Tissue Culture

http://www.agbiotechnet.com/plant_tissue_culture.asp
Plant Tissue Culture

http://www.biotech.cornell.edu/BiotechTiss.html
Plant Tissue Culture and Transformation facility

http://www.home.turbonet.com/kitchenculture/teinfo.htm
Plant Tissue Culture links

http://www.liv.ac.uk/~sd21/tisscult/introduction.htm
Plant Tissue Culture at Liverpool

http://www.teriin.org/division/bbdiv/ptc/mtp.jpg
Plant Tissue Culture at TERI - India

http://www.teriin.org/division/bbdiv/ptc/ptc.htm
Micropropagation Technological park

https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Aldrich/Bulletin/al_techbull_al228.pdf

https://www.quora.com/How-do-pH-probes-work

https://www.terrauniversal.com/blog/all-terra-blogs/horizontal-vs-vertical-laminar-flow-hoods/

Plant Genetic Engineering

http://photoscience.la.asu.edu/photosyn/courses/B10_343lecture/geneng.html
Plant genetic engineering - Methodology

http://www.agbionet.com/transgenic_plants.asp

http://www.cfsan.fda.gov/~lrd/biotechm.html
FDA - CFSAN Biotechnology

http://www.genengnews.com/top100.asp
Genetic engineering news top 100 sites

http://www.nal.usda.gov/pgdic
Plant genome data and information center

http://www.ncbe.reading.ac.uk/NCBE/GMFOOD/technology.html
National Center for Biotechnology Education Guide - Genetically

http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/PDF/TransSG.pdf
Transformer protocol - Student's guide

http://www.psci-com.ac.uk
Plant Genetic Engineering

http://www.scidev.net/gmcrops

http://www.teriin.org/gmp/links.htm
Genetically modified plants

http://www.thecampaign.org/News/novo1j.htm

http://www.wellcome.ac.uk/en/1/miseduresbio.html
A biotechnology resource for teachers of post - 16 biology

Genetic Engineering Applications

http://www.biotech-info.net/bt-trangenics.html

Insect resistance

http://www.biotech-info.net/disease.resistance.html
Disease resistance

http://www.biotech-info.net/herbicide-tolerance.html
Herbicide tolerance

http://www.biotech-info.net/new_era.html
New Era applications

http://www.biotech-info.net/other-apps.html
Biotechnology information - other applications

http://www.epa.gov/pesticides/biopesticides
EPA - Regulating Biopesticides

Biotechnology Resources

http://http://www2.lib.udel.edu/subj/biotech/db.htm
Databases for Biotechnology

http://http://www2.lib.udel.edu/subj/biotech/internet.htm
Internet resources for Biotechnology

http://abstracts.aspb.org/pb2003/public/P25/0303.html
Modern techniques in plant biotechnology

http://aggie.horticulture.tamu.edu/tisscult/biotechsites.html
Biotechnology websites

http://agnic.umd.edu
Agricultural Biotechnology

http://basepair.library.umc.edu/scopingthesciences/plantbiotech.htm
Plant Biotechnology - Scoping the sciences

http://bubl.ac.uk/link/b/biotechnology.htm
Biotechnology Internet Resources

http://www.accessexcellence.org/AB/GG
Graphic gallery - representing important processes of biotechnology

http://www.agbionet.com
Online service for Agricultural biotechnology information resource

http://www.biols.susx.ac.uk/home/Peter_Scott/PB/prac.html
Practical techniques

http://www.bioscope.org
European 'Ask a Scientist' site in Biotechnology

http://www.biospace.com/b2/industry/internetlinks.cfm
Biospace: Internet links

http://www.biotech-info.net/risks.html
Environmental impacts (Ecological risks)

http://www.biotech-info.net/sources.html
Biotechnology sources and links

http://www.biotechbasics.nsf/basics.html?openPage
Biotechnology basics

http://www.biotechknowledge.monsanto.com/biotech/bbasics.nsf/timeline.html
Biotech Timeline

http://www.cato.com/biotech
Biotechnology Information Directory Section - World Wide Web Virtual Library

http://www.ejbiotechnology.info/feedback/information.html
Information sources

http://www.ejbiotechnology/info/feedback/links.html
Biotechnology links

http://www.engin.umich.edu/dept/che/research/wang/pbiotech.html
Bioprocessing Engineering Laboratory - Plant Biotechnology techniques

http://www.fao.org/ag/guides/subject/b.htm
Biotechnology - Gateway

http://www.growmorebiotech.com/profile.htm
Growmore Biotechnology - India

http://www.i-bio.org
Information on U.K. Biotechnology

http://www.isaaa.org
International service for the acquisition of Agri-biotechnology applications

http://www.isb.vt.edu/2002menu/research_resources.cfm
Research resources

http://www.isb.vt.edu/cfdocs/indexlinks.cfm
Annotated list of sites related to Agricultural/Environmental Biotechnology

http://www.library.umc.edu/free-e_res.htm
Free Electronic Resources (Rowland Medical Library)

http://www.nal.usda.gov/bic
Biotechnology information resources

http://www.nal.usda.gov/bic/biotech_Patents
Agricultural Biotechnology patents and new technologies

http://www.nbiap.vt.edu
Information systems for Biotechnology

http://www.scienceboard.net/studies/studies.asp?studyId=64
Science Advisory Board - Tools and techniques of Plant Biotechnology

http://www.teriin.org/info/links/links.htm
Teri - Internet resources

http://www.biotekjournal.net/intro.htm
Research journal of Biotechnology

http://plant-tc.coafes.umn.edu/jan.htm
Plant Tissue Culture - Journals and newsletters

http://http://www2.lib.udel.edu/subj/biotech/ej.htm
Electronic Journals for Biotechnology

http://www.argosbiotech.de/500/plantbiotech.htm

http://www.bio.org/links/journals.htm
Links-Biotechnology Journals on the web

http://www.bioline.org.br/journals

http://www.biomedcentral.com
BMC Biotechnology

http://www.biotechniques.com
Article search

http://www.cabi-publishing.org/JOURNALS/IVP/Index.asp
In Vitro Cellular and Developmental Biology - Plant

http://www.dpw.wau.nl/links/Plant_Biotechnology/Journals
Plant Link Library - Plant Biotechnology Journals

http://www.ejbiotechnology.info/index.html
EJB - Electronic Journal of Biotechnology

http://www.jplantbiotech.com
Journal of Plant Biotechnology published by Korean Society of Plant Biotechnology

http://www.kluweronline.com/issn/o167-6857
Plant cell, Tissue and Organ culture: An International Journal on Biotechnology of Higher plants

http://www.nature.com/BIOENT
Bioentrepreneur

http://www.nature.com/nbt
Nature Biotechnology - Journal

http://www.springerlink.com

http://www.globalrph.com/aseptic.htm

Springer Journals

List of Journals and Newsletters

1. Acta Botanica Hungarica
2. Acta Botanica Gallica
3. Botanica Complutensis (at Universidad Complutense, Madrid)
4. Botanica Helvetica became Alpine Botany in 2011
5. Botanica Lithuanica
6. Botanica Macaronesica - Jardín Botánico Viera y Clavijo
7. Botanica Marina
8. Botanica Serbica
9. Botanical Bulletin of Academia Sinica (Taiwan) has become Botanical Studies
10. Botanical Electronic News
11. Botanical Journal of the Linnean Society
12. Botanical Journal of Scotland, The
13. Botanical Review
14. Botanical Studies - formerly Botanical Bulletin of Academia Sinica (Taiwan)
15. Botanika Steciana
16. Botanische Jahrbücher für Systematik Pflanzengeschichte und Pflanzengeographie (Now Plant Diversity and Evolution)
17. Botanische Rundbrief für den Bezirk Neubrandenburg
18. Botany, formerly Canadian Journal of Botany
19. Biotechniques
20. Biotechnology
21. Biotechnology Advances
22. Biotechnology and Bioengineering
23. Biotechnology, Agronomy, Society and Environment
24. Biotechnology and Development Monitor
25. Biotechnology Letters
26. Biotechnology Techniques (merged with Biotechnology Letters)
27. Critical Reviews in Biotechnology
28. Critical Reviews in Plant Sciences
29. Indian Journal of Biotechnology
30. International Journal of Plant Sciences

1. International Journal of Plant Sciences - 1992 to present at JSTOR
2. Botanical Gazette - 1876 to 1991 archive at JSTOR
3. Botanical Bulletin - 1875 to 1876 archive at JSTOR

31. In Vitro Cellular and Developmental Biology - Plant
32. Journal of Plant Biology [1958-2008] [2009-] (formerly Korean Journal of Botany)
33. Journal of Plant Biochemistry and Biotechnology
34. Journal of Plant Biotechnology (Korean Society of Plant Biotechnology)
35. Plant Biotechnology (1997-present; formerly "Plant Tissue Culture Letters")
36. Plant Biotechnology Journal
37. Plant Biotechnology Reports
38. Plant Cell, The
39. Plant, Cell & Environment
40. Plant Cell, Tissue and Organ Culture
41. Plant Cell Reports
42. Plant Tissue Culture
43. Plant Tissue Culture Letters (1984-1996) Currently "Plant Biotechnology")
44. Transgenic Plant Journal
45. Transgenic Research
46. Trends in Biotechnology
47. Trends in Plant Science

Glossaries

http://biotechterms.org/sourcebook/index_kc.phtml

http://www.fao.org fao research and technology

http://darwin.nmsu.edu/~molbio/bioABACUShome.htm
BioABACUS - Biotechnology Abbreviation & Acronym Uncovering Service

http://www.agen.ufl.edu/~foodsaf/wi008.html
Biotechnology and food glossary

http://www.biotechinfo.ie/content/content.asp?
Biotechnology - Ireland's biotechnology information source

http://www.cato.com/biotech/bio-info.html#standards
Virtual library Biotechnology - Data Standards and Glossaries

Societies

International association of plant biotechnology
http://www.iapb-stl.org/
Plant tissue culture association of India

http://www.world-food.net

Bangladesh association for plant tissue culture and biotechnology

http://www.baptcb.org/

Botanical Society of America
http://www.botany.org/newsite/education/

American Society of Plant Biologists
http://www.aspb.org/education/NEWK12.CFM

American Phytopathological Society
http://www.apsnet.org/edcenter/Pages/default.aspx

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- Belarmino, M.M. and Gabon. C.F. 1999 Low-cost micropropagation of chrysanthemum (Chrysanthemum morifolium L.) through tissue culture. Phillippine J. Science. 128.
- Biotechnology Procedures and experiments hand book (Ed.) S. Harisha Infinity science Press LLC Hingham, Massachusetts New Delhi, India.
- Drew, R.A. 1997. The Application of Biotechnology to the Conservation and Improvement of Tropical and Subtropical Fruit Species.
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