General Techniques of Plant Tissue Culture

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1. PLANT TISSUE CULTURE

1.1.Introduction

Plant tissue culture is an essential component of plant biotechnology. Apart from mass multiplication of elites, it also provides the means to multiply and regenerate novel plants from genetically engineered cells. The promising plant thus produced may be readily cloned in cultures under aseptic conditions. Tissue Culture is widely used in –

- Obtaining disease free plants.
- Rapid propagation of plants those are difficult to propagate.
- Somatic hybridization.
- Genetics improvement of commercial plants.
- Obtaining androgenic and gynogenic haploid plants for breeding programmes.

Tissue Culture is becoming as an alternative means to vegetative propagation of plants. In vitro growing plants are usually free from bacterial and fungal diseases. Virus eradication and maintenance of plants in virus free stage can also be rapidly achieved in cultures. Three main methods generally used in tissue culture are –

- Micro propagation through the enhanced multiplication of axillary bud.
- Organogenesis.
- Somatic embryogenesis.

At present the most successful and commonly used method is enhanced shoot multiplication from axillary bud. Axillary buds are present in the axis of leaves. In tissue culture, by using optimum concentration of cytokinin or combination of cytokinin and Auxin the dormancy of the axillary buds can be broken. Once the dormancy is broken, they develop into shoots. By using media containing optimum concentrations of plant growth regulators, they can be made to multiply very rapidly. Modern plant tissue culture is performed under aseptic conditions under filtered air. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol) is required. Mercuric chloride is used as a plant sterilizing agent today, as it is dangerous to use, and is difficult to dispose off. Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed

directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar. The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will often produce an unorganized growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As culture grows, pieces are typically sliced off and transferred to new media (subcultures) to allow for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard. As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants.

1.2.History of tissue culture

1838- Cell theory, indicating towards totipotentiality of cells by **Schleiden** and **Schwann**.

1902- First but unsuccessful attempt of tissue culture using monocots by **Haberlandt.**He also explained the concept of cell totipotency**.**

1904- First attempt in embryo culture of selected Crucifers by **Hannig.**

1922- A symbiotic germination of orchid seeds by **Knudson.**

1922- *In vitro* culture of root tips by **Robbins.**

1924- Callus formation on carrot root explants by use of lactic acid by **Meyer**.

1934- *In vitro* culture of cambial tissues of different trees and shrubs failed by **Guatheret.**

1934- Identification of the first plant hormone, IAA, leading to cell enlargement by **Kogl.**

1941- Coconut Milk used for growth and development of very young Datura embryos by **Overbeek.**

1942- Observation of secondary metabolites in plant callus cultures by **Gautheret**.

1943- Tumor-inducing principle of crown gall tumors identified by **Braun**.

1944- First *In vitro* culture of tobacco used to study adventitious shoot formation by **Skoog.**

1946- First whole plants of *Lupines* and *Tropaeolum* from shoot tips by **Ball.**
1948- Formation of adventitious shoots and roots in tobacco by **Skoog**.
1957- Discovery that root or shoot formation in culture depends on auxin: cytokinins ratio by **Skoog** and **Miller.**

1958- *In vitro* culture of excised ovules of *Papaver somniferum* by **Maheshwari**.
1958- Regeneration of somatic embryos from nucleus of Citrus ovules by **Maheshwari and Rangaswamy**.
1962- Development of MS medium by **Murashige and Skoog.**
1964- First haploid plants from Datura androgenesis by **Guha and Maheshwari.**
1973- Cytokinins found to be capable of breaking dormancy in *Gerberas* by **Pierik**
1978- Somatic hybridization of tomato and potato resulting pomato by **Melchers.**
1978- Industrial scale fermentation of plant cells for production of shikonin. (Selection of cell lines with higher yield of secondary products) by **Tabata.**
1981- Introduction of the term somaclonal variation by **Larkin**.
1981- Isolation of auxotroph by cell colony screening in haploid protoplasts of *Nicotiana plumbaginifolia* treated with mutagens by **Sidorov.**
1985- Infection and transformation of leaf discs with *Agrobacterium tumefaciens* and regeneration of transformed plants by **Horsch.**
1985- Development of disarmed Ti-plasmid vector system for plant transformation by **Fraley**.
1985- Development of binary vector system for plant transformation.
1985- Gene transfer in protoplasts of Dicot and Monocot plants by electroporation.
1993- *In vitro* fertilization with isolated single gametes resulting in zygotic embryogenesis and recovery of fertile maize plants by **Kranz.**
1993- "Green Hairy roots" showing photoautotrophy due to development of photosynthetic ability by **Flores.**

1996- Development of ‘agrolistic’ method of plant transformation by **Hansen**.

1.3.Importance of tissue culture

- In a relatively short time and space a large number of plantlets can be produced starting from the single explant.
- Taking an explant does not usually destroy the mother plant, so rare and endangered plants can be cloned safely.
- It is easy to select desirable traits directly from the culture setup (*in vitro)* thereby decreasing the amount of space required, for field trials.
- Once established, a plant tissue culture line can give a continuous supply of young plants throughout the year.
- The time required is much shortened, no need to wait for the whole life cycle of seed development. For species that have long generation time, low level of seed production, or seeds that readily do not germinate, rapid propagation is possible.
- *In vitro* growing plants usually free, from the bacterial and fungal diseases. Virus eradication and maintenance of plants in virus free state. This facilitates movement of plant across international boundaries.
- Plant tissue banks can be frozen and then regenerated through tissue culture. It preserves the pollen and cell collections from which plants may be propagated.

1.4.Types of tissue culture

Callus culture: Callus culture may be defined as production and maintenance of an unorganized mass of proliferative cell from isolated plant cell, tissue or organ by growing them on artificial nutrient medium in glass vials under controlled aseptic conditions.

Organ culture: That may allow differentiation and preservation of the architecture. The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordial or whole or part of an organ in way and function.

Single cell culture: Single cell culture is a method of growing isolated single cell aseptically on nutrient medium under controlled condition.

Suspension culture: Suspension culture is a type of culture in which single cell or small aggregates of cell multiply while suspended in agitated liquid medium. Suspension cultures are used in induction of somatic embryos and shoots, production of secondary metabolites, *in vitro* mutagenesis, selection of mutants and genetic transformation studies.

Embryo culture: Embryo culture may be defined as aseptic isolation of embryo (of different developmental stages) from the bulk of maternal tissue of mature seed or capsule and *in vitro* culture under aseptic and controlled physical condition in glass vials containing nutrient semisolid or liquid medium to grow directly into plantlet.

Anther culture: Androgenesis is the *in vitro* development of haploid plants originating from potent pollen grains through a series of cell division and differentiation.

Pollen culture: Pollen culture is the *in vitro* technique by which the pollen-grains (preferably at the microscope stages) are squeezed from the intact anther and then cultured on nutrient medium where the microspores without producing male gametes.

Somatic Embryogenesis: Somatic embryogenesis is the process of a single or group of cells initiating the development pathway that leads to reproducible regeneration of non zygotic embryos capable of germinating to form complete plants.

Protoplast Culture: It is the culture of isolated protoplasts which are naked plant cells surrounded by plasma membrane which is potentially capable of

cell wall regeneration, cell division, growth and plant regeneration on suitable medium under aseptic condition

Shoot tip and Meristem culture: The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro* producing clumps of shoots from either axillary or adventitious buds. This method can, be used for clonal propagation.

Explant Culture: There are variety of forms of seed plants viz., trees, herbs, grasses, which exhibit the basic morphological units i.e. root, stem and leaves. Parenchyma is the most versatile of all types of tissues. They are capable of division and growth.

1.5.Plant *in vitro* culture techniques

The promise of plant *in vitro* technologies in three major areas, namely micro propagation, somatic cell genetics and generation of transgenic plant.

Micropropagation – Propagation in tissue culture (micropropagation) is, used to develop high-quality clonal plants (Smith 1990). The main advantages are attributed to the potential of rapid, large scale propagation of new genotypes, the use of small amount of original germplasm.

Somatic cell genetics- Contribution of *in vitro* methods to plant breeding i.e. somatic cell genetics is most significant, mostly in terms of haploid production and somatic hybridization.

Transgenic plants- Expression of mammalian genes or other plant gene is becoming routine for several plant species. One of the successful approaches has been engineered for resistance against insects, virus and other pathogens as well as herbicide.

Biodiversity and strategies to preserve biodiversity- Biodiversity in common place is defining as species richness in given habitat. Biodiversity exits as three major levels. Genetic biodiversity, species biodiversity and ecosystem biodiversity (Daniels, 1997)

Advantages-

- In a relatively short time and space a large number of plantlets can be produced starting from the single explants.
- In the living plant the behavior of each part of tissue is strongly influenced by correlative controls imposed by the rest of the plant by isolating it *in vitro*, the nature of some of these correlative controls can be determined.
- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and nepenthes.

Applications-

- Micro propagation is widely used in forestry and in floriculture. Micro propagation can also be used to conserve rare or endangered plant species.
- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.

- Large-scale growth of plant cells in liquid culture inside bioreactors as a source of secondary products, like recombinant proteins used as bio-pharmaceuticals.
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- To cross-pollinate distantly related species and then tissue culture the resulting embryo this would otherwise normally die (Embryo Rescue).

For production of doubled monoploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programs, usually by treatment with colchicines which causes doubling of the chromosome number.

2. MICROPROPAGATION

2.1.Stages of Micropropagation

Micropropagation is one of the most popular techniques of tissue culture. It is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micro propagation is used to multiply novel plants, such as those that have been genetically modified or breed through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction. Generally interest in the use of this technique for clonal propagation of crop plants originated from the success in this area with orchids, the credit for which goes to French botanist G. Morel (1960). During last three decades progress in this field has been such that multiplication of many ornamental and fruit cultivars is being practiced on commercial feasible method of clonal propagation. Micropropagation can be defined as a technique in which any vegetative (meristmatic) part of plant such as shoot tip, shoot bud etc is excised aseptically and cultured on sterile media under controlled conditions to give rise to plantlet which is exact copy of its donor plant. In Simple words, it can be defined as clonal propagation *in vitro*.

The word ‘clone’ was first used by Webber for cultivated plants that were propagated vegetatively. The word derived from Greek (clone= twig, spray or a slip ,like those broken off as propagules for multiplication).It signifies that plants grown from such vegetative parts are not individuals in the

ordinary sense, but are simply transplanted parts of the same individual and such plants are identical. Thus, Clonal propagation is the multiplication of genetically identical individuals by asexual reproduction.

Plant regeneration can be achieved by culturing tissue sections either lacking a preformed meristem (adventitious origin) e.g. Axillary Bud Proliferation approach or from Callus and Cell cultures (De Novo Origin). It is the stimulation of Axillary buds, which are usually present in the axil of each leaf to develop into a shoot. This technique exploits the normal ontogenic route for branch development by lateral (Axillary) meristem. In nature these buds remain dormant for various periods depending upon the growth pattern of plant. In some species, removal of terminal bud is essential to break the apical dominance and stimulate the Axillary bud to grow into shoot. Due to continuous application of cytokine in cultured medium the shoot formed by the bud, which is present on explants (nodal segment/shoot tip cutting) develops Axillary buds. The shoot is then separated and rooted to produce plants or shoots are used as propagules for propagation. The merit of using Axillary bud proliferation from meristem, shoot tip or bud culture as a mean of regeneration is that the incipient shoot has already differentiated *in vivo*. Thus, only elongation and root differentiation are require to establish a complete plant.

Another, major advantage of this technique is that, it preserves the precise arrangement of all layers necessary if a chimeral plant genetic section is to be maintained. In a typical chimera, the surface layers of developed meristem are of differing genetic background and it is their contribution in particular arrangement to the plant organ that produces the desisted characteristics.

As long as the integrity of the meristem remains intact and development is normal *in vitro*, then the chimeral pattern will be preserved. If however, callus tissue were allowed to form and shoot proliferation subsequently was from adventitious origin, then there would be a risk that the chimeral layers of original explants may not all be represented in the specially require from in the adventitious shoots.

Micropropagation Methods

Among the various applications of plant tissue culture, micropropagation of plant species has attained the status of large plant based study. The development in the study of various aspects of plant growth and

differentiation were rapid during 1960s and 70s. The technique of culturing plants becomes a wide subject embracing morphology, physiology, biochemistry, molecular biology and genetic engineering.

1. Somatic Embryogenesis
2. Axillary Bud
3. Adventitious Budding

General Technique of Micropropagation

The process of plant micropropagation aims to produce clones (true copies of a plant in large numbers). The process is usually divided into the following stages:

Stage 0: Pre-propagation Stage

The pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels and net covered tunnels, provide high quality explant source plants with minimal infection. Collection of explants for clonal propagation should be done after appropriate pre-treatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. This improves growth and multiplication rates of *in vitro* cultures. The control of contamination begins with the pretreatment of the donor plants.

The choice of explant depends on the methods of shoot multiplication to be followed. All plant organs viz. nodal segment, inter-nodal segments, shoot tip, root tip. For axillary bud induction, callus culture, somatic embryogenesis explants nodal segments, internodes and leaves are collected.

Stage 1: Initiation of Aseptic Culture:

- In this stage sterilization of explants and establishment of explants were done. The plant organ used to initiate a culture is called explant. The choice of explant depends on the method of shoot multiplication to be followed.
- For micropropagation work the explant of choice is nodes
- For callus culture work the explant of choice is internodes and leaves.
- For somatic embryogenesis the explant is internodes and leaves.

Stage 2: Multiplication of Culture:

This is the most important stage and the rate of multiplication determines the largely success of micropropagation system this can be achieved by-

Enhanced axillary branching

- Adventitious bud formation
- Through callusing

Enhanced axillary branching:

The axillary bud present in the axil of each leaf either develops into a single shoot or form a cluster of shoots in the presence of cytokinins (BAP 1.0mg/l) in the medium.

Adventitious Bud Formation:

Buds arising from any part other than the leaf axils or shoot apex are called adventitious buds. It is a standard horticulture practice.

Through Callusing:

Plant cells are totipotent. In tissue culture, the mass of differentiated cells commonly known as callus. This either gives rise to shoot bud or bipolar structure resembling embryo (somatic embryo). This method is used when aim is to induce variability especially in self-pollinating species with narrow genetic base.

Stage 3: *In Vitro* Rooting of Shoots

In-vitro grown shoots lack root system. For induction of roots they were transferred to rooting medium. For rooting half strength MS medium supplemented with 1.0mg/l auxin was used.

Stage 4: Hardening and Acclimatization of Tissue Culture Plantlets

This is the final stage and requires careful handling of plants. The transplantation from completely controlled conditions should be gradual. This process of gradually preparing the plants to survive in the field conditions is called acclimatization. The plants produced in tissue culture, although green in color; do not prepare sufficient food for their own survival. Also inside the culture vessels humidity is very high and thus the natural protective covering of cuticle is not fully developed. Therefore immediately

after transfer plants were maintained under high humidity. Optimum conditions were provided to plants in green house.

2.2.Advantages of Micropropagation

Micropropagation has a number of advantages over traditional plant propagation techniques:

- The main advantage of micropropagation is the production of many plants that are clones of each other.
- Micropropagation can be used to produce disease-free plants.
- Micropropagation produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.
- It can have an extraordinarily high frequency rate, producing thousands of propagules while conventional techniques might only produce a fraction of this a number.
- It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.
- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored.
- Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
- Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
- A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

2.3.Disadvantages of Micropropagation

Micropropagation is not always the perfect means of multiplying plants, conditions that limits its use include:

- It is very expensive, and can have a labour cost of more than 70%.

- A monoculture is produced after micropropagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same infections.
- An infected plant sample can produce infected progeny. This is uncommon if the stock plants are carefully screened and vetted to prevent culturing plants infected with virus or fungus.

Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produce secondary metabolic chemicals that stunt or kill the explant.

2.4.Different tasks

Sometimes plants or cultivars do not come true to type after being tissue cultured; this is often dependent on the type of explant material utilized during the initiation phase or the result of the age of the cell or propagule line.

3. MICROPROPAGATION OF BAMBOO

3.1.Introduction to Explant Material

The bamboos are a group of woody perennial evergreen (except for certain temperate species) plants in the true grass family Poaceae, subfamily Bambusoideae, tribe Bambuseae. Some are giant bamboos, the largest members of the grass family. Bamboos are the fastest growing woody plants in the world. They are of economic and high cultural significance in East Asia and South East Asia where they are used extensively in gardens, as a building material, and as a food source.

Diversity: Around 92 genera and 5,000 species.

Mass flowering

Although some bamboos flower every year, most species flower infrequently. In fact, many bamboos only flower at intervals as long as 60 or 120 years. These taxa exhibit mass flowering (or gregarious flowering), with all plants in the population flowering simultaneously. The longest mass flowering interval known is 130 years, and is found for all the species *Phyllostachys bambusoides* (Sieb. & Zucc.). In this species, all plants of the

same stock flower at the same time, regardless of differences in geographic locations or climatic conditions, then the bamboo dies.

Medicine

Bamboo is used in Chinese medicine for treating infections and healing. It is a low-calorie source of potassium. It is known for its sweet taste and as a good source of nutrients and protein.

In Ayurveda, the Indian system of traditional medicine, the silicious concentration found in the culms of the bamboo stem is called *banslochan*. It is known as *tabashir* or *tawashir* in *Unani-Tibb* the Indo-Persian system of medicine. In English it is called "bamboo manna". This concretion is said to be a tonic for the respiratory diseases. It was earlier obtained from *Melocanna bambusoides* and is very hard to get; it has been largely replaced by synthetic silicic acid. In most Indian literature, *Bambusa arundinacea* is described as the source of bamboo manna.

The fiber of bamboo has been used to make paper in China since early times. A high quality hand-made paper is still produced in small quantities. Coarse bamboo paper is still used to make spirit money in many Chinese communities.

3.2.Classification

Dendrocalamus strictus

Vernacular Names- Bengal - *Karali*, Gujarat - *Nakur bans; Kiri bidiru*; Maharashtra - *Male bamboo, narvel*; Orissa - *Salia*; Tamilnadu - *Kalmungil*; Andhra - *Sadanapa Veduru*; Tripura - *Lathi bans*; Kerala – *Kallumula*

Classification of Bamboo

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Liliopsida*

Order: *Poales*

Genus: *Dendrocalamus*

Family: *Poaceae*(alt. *Gramineae*)

Subfamily: *Bambusoideae*

Tribes: *Bambuseae.*

Soort *Dendrocalamus strictus* (Roxb.) Nees

Common names:

Bambú-grande ,Bans ,Calcutta bamboo ,Male bamboo ,Solid bamboo

Economic importance:

- Human food: Cereal; Vegetable
- Materials: Cane; Fiber (used for rafters, baskets, sticks, etc.)

Clonal propagation of bamboo (*Dendrocalamus strictus*)

Bamboo grows naturally in many types of forests. About 50% of the annual production of bamboo in our country is used by various industries like pulp, paper, rayon, mat boards, besides agricultural implements. It is also used for making baskets, bridges, coffins, beds, toys and weapons. A grove of bamboo at ground zero in the area that destroyed Hiroshima in 1945 sprouted new shoots within 1 month. The distribution of bamboo in India is largely governed by rainfall, temperature, and altitude and soil types. In recent years bamboos are in great demand; but no availability of sufficient quantity of saplings and seeds are the major problem. Commonly called as solid or lathy bamboo. The species is widely distributed in dry deciduous forests and grows rapidly in all climatic conditions. It grows better in the drier parts and on sandstone, granite and coarse grained soils with low moisture-retaining capacity and soils with pH 5.5–7.6. It grows more than 8 feet in 6 months. The pulp is used for making quality paper and the clumps, being strong and elastic, are used for lathies,shafts, axe handles, walking sticks, agricultural, and industrial implements and therefore it is rightly called as ‘poorman’s timber'. of *D. strictus* is the erratic flowering and non-availability of seeds on regular basis, besides low viability of seeds. Moreover the seeds have to be stored in 3 to 5°C after reducing the moisture (8%) or stored in a desiccator with anhydrous Calcium chloride.

4. MATERIAL AND METHODS

4.1.Lab requirements

The present investigation was carried out at Arid Forest Research Institute, Jodhpur (Rajasthan). The experimental material for the present investigation on Micropropagation of *Dendrocalamus stritus*

APPARATUS-

1. pH Meter
2. Microwave oven
3. Electronic Weighing Balance
4. Refrigerator
5. Defreeze
6. Oven
7. Autoclave
8. Laminar Air Flow
9. Incubator

CHEMICALS-

1. Ammonium Nitrate
2. Potassium Nitrate
3. Boric Acid
4. Potassium Di Hydrogen ortho Phosphate
5. Potassium Iodide
6. Sodium Molybdate di hydrate
7. Cobaltous Chloride
8. Calcium Chloride
9. Magnesium Sulphate
10. Manganese Sulphate

11. Zink Sulphate
12. Cupric Sulphate
13. Sodium Ethylene di amine tetra acetic acid
14. Ferrous Sulphate
15. Thiamine HCl
16. Nicotinic Acid
17. Pyridoxine
18. Glycine
19. Myoinositole
20. Sodium Hydroxide
21. Hydro Chloric Acid
22. Sucrose
23. Agar-Agar
24. Citric Acid
25. Glutamic Acid
26. Adenine Sulphate Di hydrate
27. Asparagine
28. Arginine
29. Mercuric Chloride
30. Ascorbic Acid
31. Sodium Hypochlorite
32. Banzyl Amino Purine(BAP)
33. Indole Acetic Acid(IAA)
34. α-Naphthalene Acetic Acid(NAA)

35.2,4-Di chlorophenoxyacetic acid(2,4-D)

Tissue culture media:-

Generally all culture media are made up of:

- Macronutrients
- Micronutrients
- Vitamins
- Growth regulations
- Carbohydrates (Sucrose)

Formulation designed by Murashige and Skoog (1962), revised by Linsmair and Skoog (1965) can be regarded as standard. Special plant groups like conifers have nutritional requirements, which appear, not to meet by standard media, and then some additional nutrients are required in media.

MEDIA CONSTITUENTS:-

Inorganic Nutrients:-

- Mineral elements are very important in the life of a plant.
- Mg is a part of chlorophyll molecules
- Ca is a component of cell wall
- **N** is an essential part of amino acids, vitamins, proteins and nucleic acid.
- Fe, Zn, and Mo are part of certain enzymes.
- Besides C, H, and O there are 12 elements, known to be essential for plant growth viz. N, P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, B and Mo.

Macro elements:-

- **C**- Carbon forms the backbone of many plants Bio-molecules, including starches and cellulose. It is fixed through photosynthesis from the carbon synthesis in the air and is a part of the carbohydrates that store energy in the plant.

- **H**- Hydrogen also is necessary for building the plant and it is obtained almost entirely from water.
- **O**- Oxygen is necessary for cellular respiration. Cellular respiration is the process of generating energy rich adenosine tri phosphate (ATP) via the consumption of sugars made in photosynthesis. Plants produce oxygen gas during photosynthesis to produce glucose but then require oxygen to undergo aerobic cellular respiration and break down this glucose and produce ATP.
- **N**- Nitrogen is an essential component of all proteins. Nitrogen deficiency most often results in stunted growth.
- **P**- Phosphorus is important in plant bioenergetics as a component of ATP. It is needed for the conversion of light energy to chemical energy (ATP) during photosynthesis. Phosphorus can also be used to modify the activity of various enzymes by phosphorylation and can be used for cell signaling. Since ATP can be used for the biosynthesis of many plant bio molecules, it is important for plant growth and flower/seed formation.
- **K**- Potassium regulates the opening and closing of the stomata by a potassium ion pump. Since stomata are important in water regulation, potassium reduces water loss from the leaves and increases drought tolerances. Potassium deficiency may cause necrosis or intervenial chlorosis.
- **Ca**- Calcium regulates transport of other nutrients into the plant. It is also involved in the activation of certain plant enzymes. Calcium deficiency results in stunting.
- **Mg**- Magnesium is an important part of chlorophyll, a critical plant pigment important in photosynthesis. It is important in the production of ATP through its role as an enzyme cofactor. There are many other biological roles for magnesium in biological system for more information. Magnesium deficiency can result in intervenial chlorosis.
- **S-** Sulphur is a structural component of some amino acids and vitamins. It is essential in the manufacturing of chloroplasts.

Microelements: - These are essential as catalysts for many biochemical reactions; microelement deficiency symptoms include Leaf chlorosis (Fe, Zn, and Mn) Shoot tip necrosis (B, Co, Ni) inhibits ethylene synthesis.

- **Fe-** Iron is necessary for photosynthesis and is present as an enzyme cofactor in plants. Iron deficiency can result in intervenial chlorosis and necrosis.

- **Zn-** Zinc is required in a large number of enzymes and plays an essential role in DNA transcription. A typical symptom of zinc deficiency is the stunted growth of leaves, commonly known as "little leaf" and is caused by the oxidative degradation of the growth hormone auxin.

- **Mn-** Manganese is necessary for building the chloroplasts. Manganese deficiency may result in coloration abnormalities, such as discolored spots on the foliage.

- **B-** Boron is important for binding of pectin in the RG II region of primary cell wall; secondary roles may be in sugar transport, cell division and synthesizing certain enzymes. Boron deficiency causes necrosis in young leaves and stunting.

- **Co-** Cobalt has proved to be beneficial to at least some plants, but is essential in others, such as legumes where it is required for nitrogen fixation.

- **Ni-** In higher plants, Nickel is essential for activation of ureases, an enzyme involved with nitrogen metabolism that is required to process urea. Without Nickel, toxic leaves of urea accumulate, leading to the formation of necrotic lesions. In lower plants, Nickel activates several enzymes involved in a variety of processes and can substitute for Zinc and iron as a cofactor in some enzymes.

- **Si-** Silicon deposited in the cell walls and contributes to its mechanical properties including rigidity and elasticity.

- **Na-** Sodium involved in the regeneration of phosphoenolpyruvate in CAM and C_4 plants. It is also substitute for potassium in some circumstances.

- **V**- Vanadium may be required by some plants, but at very low concentrations. It may also substitute for Molybdenum.

- **Se**- Selenium and Sodium may also be beneficial. Sodium can replace potassium's regulation of stomatal opening and closing.

Organic Nutrients :-

Vitamins: Plants can produce their requirements of vitamins. However, plant cell cultures need to be supplemented with certain vitamins like Thiamine (vit B_1), Niacin (vit B_3), Pyridoxine (vit B_6), and Myo-inositol (Member of the vit. B complex).

Thiamine – Involved in the direct biosynthesis of certain amino acids and essential co-factor of carbohydrates metabolism.

Vit E – Antioxidants.

Vit C- To prevent blacking during explant isolation.

Vit D- Growth regulatory effect

Amino Acids – Glycine- has little benefit in the growth of plant. They may be directly utilized by plant own be provided as N_2 source.

Carbon Sources- Sucrose (is most commonly used carbon source) at a concentration of 3%, glucose and fructose also known to support plant growth. Sucrose in the medium is necessary for various metabolic activities.

Growth Regulators :

Auxin – Auxin are involved in cell division and elongation and in cell wall synthesis. IAA, IBA, NAA, 2, 4-D are the most frequently used auxin in plant tissue culture. The principal naturally occurring auxin, the IAA is not often used in the tissue culture, because it is unstable. IBA is slightly more potent than IAA and is not easily broken down. Hormones of this group are involved with elongation of stems and inter nodes, tropism, apical dominance abscission, rooting etc.

Cytokinin –These hormones, are concerned with cell division, modification of apical dominance, shoot differentiation etc. Most commonly used cytokinins are BAP, BA, Kinetin, 2 ip and Zeatin. They usually promote cell

division if added together with an auxin. Of these, BAP is the most effective cytokinins for stimulating axillary shoot proliferation.

Gibberellins – There are over 20 known gibberellins. Of these, generally, GA_3 is used. They are rarely used and reported to stimulate normal development of plantlets from *in vitro* formed adventives embryos.

Others – Abscisic acid is most often required for normal growth and development of somatic embryos and only in its presence they resemble zygotic embryos.

Gelling Agent :-

In static cultures if liquid medium is used the tissue would get submerged and die due to lack of oxygen. A gelling agent is generally used to circumvent this problem. The most desirable property of a gelling agent is that it should with stand sterilization by autoclaving and the medium should be liquid when hot but form a semisolid gel when cool. Some important gelling agents are – Agar, Agarose ,Gelrite

Agar – This is obtained from red algae, especially *Gelidium amansii*. Complex mixture of related polysaccharides built up from the sugar, galactose. These include the natural polymer fractions, agarose, which gives strength to the gel and the highly charged anionic polysaccharides agaropectins which give agar its viscosity. Agar is used at varying concentration from 0.8 to 1%.

Agarose- Is commonly preferred over agar for protoplast culture.

Gelrite- Is a good alternative to agar not only because of its lower cost per liter of medium (0.1-0.2% is sufficient) but also for the many advantage it offers.

Preparation of the stocks solutions:-

It is difficult to weigh and mix all the constituents just before preparation of medium. It is time consuming and a tedious job. Again if 100 ml medium is to be prepared, then it is very difficult to weigh some constituents that are used in very small quantity for 1 liter medium. So it is convenient to prepare concentrated stock solution of macro salts, micro salts, Vitamins, amino acids, hormones etc. and all stocks solution should be stored in a refrigerator

and should be checked visually for contamination with microorganism or precipitation of ingredients.

Major types of media

1. White's medium - is one of the earliest plant tissue culture media
2. MS medium - The most extensively used nutrient medium is MS medium (developed by Murashige and Skoog in 1962)
3. B_5 medium - developed by Gamborg for cell suspension and callus culture and at present its modified form used for protoplast culture.
4. N_6 medium - formulated by Chu and used for cereal anther culture.
5. Nitsch's medium- developed by Nitsch and used for anther culture.

Table:-Composition of Plant Tissue Culture Media Formulated By Murashige and Skoog (1962)

Stock	Ingredients	Composition of Stock Solution	Volume of Stock in Final Media
Inorganic nutrients			
A	NH_4NO_3	33.0gm/400ml D.W.	20ml/l
B	KNO_3	38.0gm/400ml D.W.	20ml/l
C	H_3BO_3 KH_2PO_4 KI	0.248gm 6.8gm 0.33gm/200ml D.W.	5ml/l
D	$NaMoO_4.2H_2O$ $CoCl_2.2H_2O$	0.05gm 0.005gm/200mlD.W.	1ml/l
E	$CaCl_2.2H_2O$	17.6gm/200ml D.W.	5ml/l
F	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$	14.8gm O.892gm 0.344gm/200mlD.W	5ml/l
G	$CuSO_4.5H_2O$	0.005gm/200mlD.W	1ml/l
H	Na_2EDTA $FeSO_4.7H_2O$	1.49gm 1.114gm/200mlD.W	5ml/l

Organic nutrients			
I	Thiamine HCL Nicotinic acid Pyridoxine HCL Glycine	0.01gm 0.05gm 0.05gm 0.002gm/100ml D.W	1ml/l
J	Myo-inositol	2gm/200ml D.W.	10ml/l

[pH =5.8, sucrose- 3%, agar 0.8 %(w/v)

4.2.Sterilization

It is very important to maintain aseptic environment during the **in vitro** culture of plant cells and tissues. Following are some of the methods adopted for sterilization:

(a) Sterilization of Glassware- The glassware can be sterilized in a hot air oven at 160-180^{0}C for 2-4 hours.

(b) Sterilization of instruments- The metallic instruments are incinerated by dipping them in 75% ethanol followed by flaming and cooling.

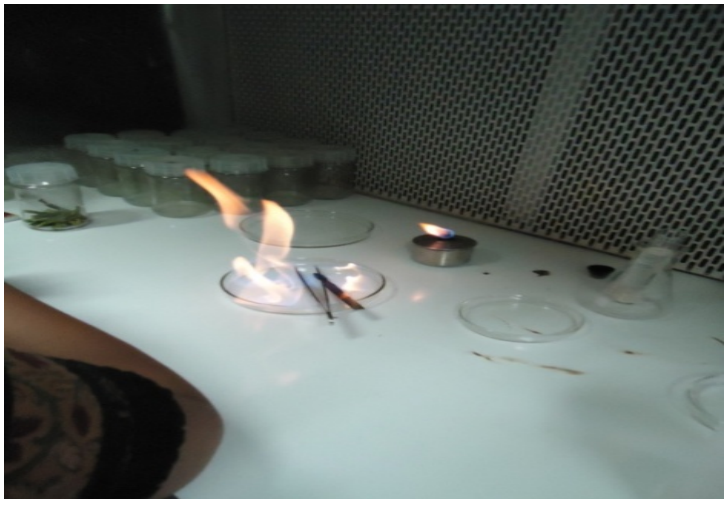


Figure- Sterilization

(c) Sterilization of nutrient media- The culture media are transferred into glass container, plugged with cotton or sealed with plastic closures and sterilized by autoclaving at 15 psi for 30 min. The autoclaving denatures the vitamins, plant extracts, amino acids and hormones therefore the solution of these compounds are sterilized by using Millipore filter paper with pore size of 0.2 micrometer diameter. Surface sterilization of juvenile material is generally not difficult. However, if older trees are used, as it is the initial and basic material for tree breeders when selection is done contamination of explants is sometimes a serious problem, unless the tree produce juvenile sprouts.

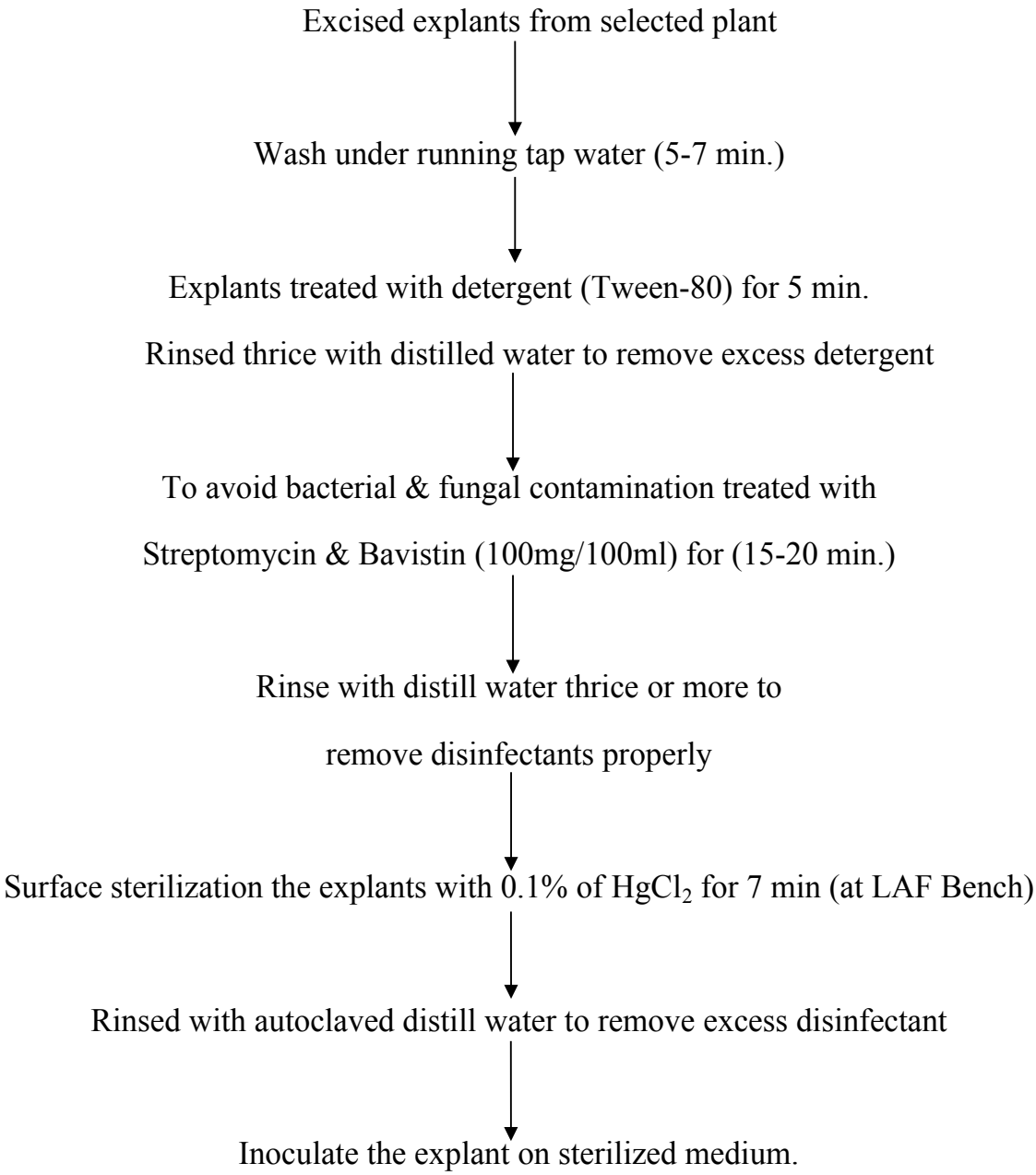
In some cases spores are deposited on field grown trees by insects. This contamination can be reduced by spraying these trees with insecticides and fungicides and by subsequently protecting expending shoot against insects by enclosing them in bags made of transparent film before collecting the explants. Surface of plant part carries a wide range of microbial contaminants. To avoid this source of infection the tissue must be thoroughly surface sterilized before planting it on the nutrient medium. To disinfect plant tissues various sterilizing agents have been used. Hypochlorite solutions have proved too effective in most cases. Ethyl and isopropyl alcohol have also been used to surface sterilize of plant tissues. Explants were washed in distilled water to remove dust particles, then they were washed in detergent solution and surface sterilized in 0.1% solution of $HgCl_2$, NaCl for 5 minutes. To remove the sterilant nodal segments were again washed with sterile distilled water.

(d) Sterilization method of Explants:-

Table: - Some of the agents used for surface sterilization of explants

Disinfectant	Concentration	Duration of treatment
Ethyl alcohol	75-95%	1-10min
Mercuric chloride	0.1-1.0%	2-10min
Sodium Hypochlorite	0.5-5%	5-30min

Flow chart showing complete explants sterilization process:



4.3. Tissue culture media

To prepare the medium, many researchers mix the stock solutions which were made previously since the medium compositions are generally complicated. For example, MS medium is prepared as follows:

The present studies pertained to the Micropropagation of *Dendrocalamus strictus.*This aspect deals with following studies for the Micropropagation of the bamboos:

1. Selection of explants.

2. Standardization of sterilization technique.
3. Establishment of explant.
4. To get maximum rate of shoot proliferation.

- **SELECTION OF EXPLANT**

Micropropagation is preferred because of genetic stability. For this experiment axillary bud and nodal explants were collected from 2-3 years old clumps of Bamboo plant. Explants were collected from soft; rapidly growing shoots provided the box material for initiation of culture.

- **STERILIZATION OF EXPLANT**

It was observed that explants were very susceptible to $HgCl_2$ and majority of them turned black and died. Surface sterilization of nodal explants with 0.1% $HgCl_2$ for 12-14 minutes gave up to 80% response, which was to be best among all the treatments for surface of *D. Strictus*.It was observed that explants very susceptible to time of $HgCl_2$ if time increased more than or less than 12 min. then the survival of explants effects.

- **AXILLARY BUD BREAK (SHOOT INDUCTION)**

Bud break was achieved in 20-25 days in different media compositions. Maximum percentage of bud break was achieved on MS medium supplemented with BAP (3 mg/l) in case of *Dendrocalamus strictus.* Multiple shoots were separated and regular sub culturing was done on pre-established cultures on MS medium supplemented 3.0 mg/l BAP.

Table: Effect of plant growth regulators (BAP) on number of shoot buds per explants formed in *Dendrocalamus strictus* after one week.

S. No.	MS Medium + BAP(mg/l)	No. of Shoots
1.	2.5	2-3
2.	5.0	3-6

SUMMARY

Plant biotechnology has an important role to play in solving problems related to improvement of forest and fruit trees. *In vitro* techniques are being increasingly applied to supplemented the conventional methods of vegetative

propagation and improvement of plants. *In vitro* technique of Micropropagation have the following distinct advantages over conventional methods of vegetative propagation; small spaces required, high multiplication rate, freedom from seasonal depending, controlled culture condition, freedom from microbes.

5. REFERENCES

1) Bonga, J.M. and Aderkas, P. V. 1992. *In vitro* culture of trees. Khmer forestry publishers, Netherlands.
2) Bonga, J.M. and Durzan, D.J (eds). 1987.Cell and Tissue Culture in Forestry Vol. 1,2,3 Martinus, Nijhoff Publishers, Dordrecht.
3) Bist,P., Kapoor, M.L., Sharma, S.K. and Chauhan, J.M.S. 1977.*In vitro* clonal propagation of juvenile *Eucalyptus* hybrid (*E. teriticornis* &*E. camaldulensis*) Indian Journal of Forestry. Vol .20 (1): 93-97.
4) Das. T. and Mitra, G.C. 1990. Micropropagation of *Eucalyptus tereticornis*, Smith, Plant Cell, Tissue and Organ Culture. 22:95 – 103
5) Durzan, D.J. 1988. Application of cell and tissue culture in Tree Improvement In: Application of Plant Tissue Culture. Bock, G and Marsh, J. John Wiley and Sons, New York
6) George,E.F.,P.DShcrrington,1984.Plant propagation by tissue culture.
7) Gupta, P.K. and Mascarenhas, A.F. Cell and Tissue Culture in Foresty, Vol. 3
8) Bonga J.M., Durzan, D.J.,Hussain, M., Somasundaram, T. R. and Subramanian, K.N. 1974. A recent advance in *Pongamia* culture. Indian For. 102:531-532.
9) Lahiri, A.K. 1974. Preliminary study on rooting green wood cutting of Pongamia. Indian for 100: 559-560.
10) Murashige T and Skoog F. 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum.15:473-497.
11) Sommer, H. E. and Brown, C. L. 1979. Application of tissue culture &. forest tree improvement. In W.R. Sharp, P.O. Larsen, E.F. Paddock, V. Raghavan, eds, Plant Cell and Tissue Culture, Principle and Applications, Ohio State Univ. Press, Colitinbits, 461-491.