# AN INTRODUCTION TO THE THEORY AND PRACTICE OF PLANT TISSUE CULTURE

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A major role of modern science and technology is to provide for the increasing requirements of food, medicine, clothing etc. of the expanding human population on earth. Plant tissue culture can contribute to the increased production of food, fibre, medicinal and other useful plants. This technology has been already employed to increase the yield, pest resistance and tolerance to environmental stresses of several agricultural crops. Therefore, an understanding about tissue culture and its potential use is important.

## History of Tissue Culture

Before a detailed discussion of tissue culture technology, a brief historical sketch is given here. Gottlieb Herbaland, a German Botanist is considered as the father of Tissue culture. He was the first person to grow parts of plants in an artificial culture medium. These experiments conducted in 1898 involved several plant species. For example, he used cells from the palisade parenchyma of the species *Eichhornia crassipes* and *Labium puerperium* and also the leaf epidermal cells of *Ornithogalum* sp. for artificial culture. The culture medium employed here was Knop's salt solution enriched with sucrose.

After several days of culture in this solution, Herbeland observed a significant growth in size of the palisade cells. Although, there was no cell division in this medium, Herbeland could describe the method of culture of cells isolated from a parent plant in an artificial medium. He could also propose several hypotheses in this field of science which were proved to be correct by the subsequent work of other scientists. The concept of growth control substances proposed by Herbeland is one of his major contributions. He named these substances as growth enzymes and he believed that they are produced by a specialized group of cells. The secretions of these cells were believed to affect the growth of other cells.

Since the work of Herbeland, many other scientists have contributed to improve the plant tissue culture technology to its present advanced status. Among them, the contributions of Philip White, Morel, Skoog, Murashige, Miller, De Fossard etc. are worth of special mention. These and other scientists identified the factors that are responsible for the failures at different stages of tissue culture and found methods of overcoming them. Thanks to these findings, plant tissue culture has attained so much success that it is now being applied in a multitude of food, ornamental and medicinal plants for their improvement.

## Physiological basis in tissue culture

### Cell totipotency

Even a huge tree has been formed from a single cell called the zygote which is capable of division and subdivision giving rise to an intact plant. This ability of a cell is called totipotency. *In vitro* culture techniques are based on this concept and according to that appropriate conditions should be provided for the initiation and regulation of the cell division and subsequent cell differentiation and regeneration of plantlets (small plants).

In tissue culture procedures, usually a normal differentiated tissue with non dividing, quiescent cells are plated on a culture medium. These cells first undergo certain physiological changes due to interaction with the exogenous growth regulators. As a result of this they acquire a meristematic state. This phenomenon is called dedifferentiation and usually results in the callus formation. In further interaction with some factors in the culture medium those dedifferentiated cells in a callus tissue could again become capable of

forming an intact plant or plant organs. This phenomenon is termed redifferentiation. Thus, for a non-dividing, mature, differentiated cell to express its totipotency it should become dedifferentiated and only then to be redifferentiated. On the other hand the redifferentiation can take place directly from dedifferentiated cells avoiding the callus formation.

**Basic Requirements for Plant Tissue Culture**

Tissue culture is the growing of a part of a tissue of a plant (or an animal) or a part of it in a medium containing nutrients under aseptic conditions in a closed environment *(in vitro)*. For example, a part of the terminal bud of a plant can be made to grow in a medium containing all necessary nutrients solidified using agar. This medium should be contained in a culture tube or a flask. Moreover, the explant (part of the plant used for tissue culture), the medium etc. should be free from micro-organisms and therefore should be disinfected and raised under closed conditions.

**Culture medium**

For cell division and subsequent differentiation, a plant cell *in vivo* requires numerous nutrients, growth regulators etc. Similarly an artificial medium (culture medium) should also provide all those vital constituents to carry out the normal cell functions *in vitro*. Usually a culture medium consists mainly of macro elements (elements required in greater concentrations than 0.5m. mol per liter), micro elements (elements required in lesser amounts than 0.5m. mol per liter), vitamins, plant hormones, sugar etc.

**Macro and micro elements**

Besides carbon, hydrogen and oxygen there are about 12 elements required for normal plant growth. They are nitrogen, phosphorus, sulphur, calcium, potassium, magnesium, iron, manganese, copper, zinc, boron and molybdenum. Among these elements the first six are macro elements while the rest is considered as micro elements. Both macro and micro elements should be added in proper concentration recommended for particular medium and lack of elements may affect the *in vitro* plants.

**Carbon source**

Carbon can be considered as the most important element in the organic processes. Therefore, it is essential to provide a carbon source to the culture medium. Sucrose, glucose, fructose are used for this purpose and among them sucrose is the most preferred. Sugars such as moltose, galactose and lactose are rarely utilized.

**Vitamins**

Vitamins also play a major role in many biological processes of *in vitro* cultures as well as *in vivo* plants. Some vitamins such as vitamin $B_1$ (Thiamin), vitamin $B_6$ (Pyridoxin), vitamin $B_2$ (Riboflavin), vitamin C (Ascorbic acid), vitamin B with vitamin M (Folic acid), vitamin H (Biotin) and Nicotinic acid are the more important in tissue culture. Moreover, a carbohydrate called myoinositol (Inositol) is also used as a constituent of vitamin B complex. Vitamins function as coenzymes (as a part of enzymes) and take part in various important reactions of plant metabolism. However, it is advisable to use the vitamins in appropriate concentrations recommended for the particular medium.

**Plant hormones**

The objective of plant cell, tissue or organ culture may be to obtain either a new form of a plant or a callus. A callus is a mass of unorganized cells formed as a result of division of a cell/cells. By manipulating tissue culture media and conditions it is possible to regulate the development path-way of cells of *in vitro* cultures (see fig. 1).

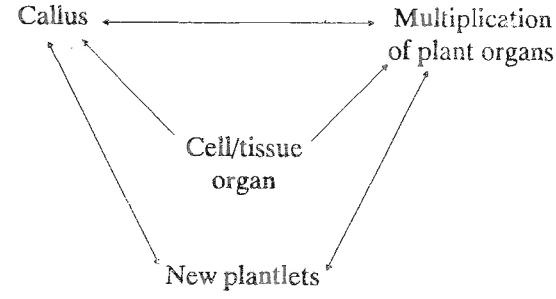


**Figure 1 : Several stages of plant tissue culture and their inter-relationships**

There are five main groups of plant hormones viz auxins, cytokinins, gibberallines, abscisic acid and ethylene. Among these hormones only auxins and cytokinins are frequently used in tissue culture media. Gibberellin and abscisic acid are rarely used. Usually the ratio of the concentrations of auxins and cytokinins used in culture media determine whether the cultures would give rise to roots, shoots or callus tissues.

**Auxins**

In 1926 F.W. Went Coined the term auxin which was derived formed from a Greek word *auxin* (to increase). In nature, plants contain auxins in very small quantities and it is synthesized in meristem tissues of shoots and root-tips, developing leaves, flowers and fruits. This group of hormones is involved in the regulation of *in vivo* processes like elongation of stem and internodes, tropisms, apical dominance, abscission, rooting etc. In tissue culture auxins are used to induce cell division (for example to obtain callus tissues) and root differentiation. There are several auxins like indole-3-acetic acid (3-IAA), 3-indolebutric acid (3-IBA), $\alpha$-naphthalene acetic acid ($\alpha$ -NAA), 2-4-dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) which are used in the preparation of culture media.

Each member of this group behaves in a unique manner in culture media *in vitro* and the effect of different hormone on the same tissue under similar conditions may be different.

For example, 3-IBA and $\alpha$ -NAA are useful in inducing root differentiation of cuttings while 2-4-D and 2,4,5-T are very effective for the induction and growth of callus. Moreover, 3-IBA and $\alpha$ -NAA in interaction with cytokinins can be used for shoot proliferation.

**Cytokinins**

The word cytokinin in latin means cell division. In nature, cytokinins are found in different tissues of plants such as root-tips, ripening fruits, germinating seeds, xylem sap etc. and are important in such processes like cell division, modification, apical dominance and shoot differentiation. In tissue culture, cytokinins are used for cell division and differentiation of adventitious shoots from callus and organs. Among frequently used cytokinins 6-benzylaminopurine (6-BAP), 2-isopentenyl adenine (2ip) and kinetin can be considered as the most effective.

**Other growth stimulants**

While vital constituents such as macro and micro elements, vitamins and hormones are essential in the medium, other substance such as potato extract, yeast extract and coconut milk (coconut water) may enhance the growth processes on *in vitro* cultures. However, they should be added only if necessary as their composition can vary from one sample to another.

**Agar as a gelling agent**

In tissue culture, nutrient media can be in liquid state as well as in the solidified form depending on the purpose of the culture. These two states of media have their own advantages and disadvantages. For example, in liquid media, tissues are submerged and this could lead to the death of tissues due to lack of oxygen, unless proper measures for aeration are taken.

Therefore, sometimes media have to be solidified with a gelling agent like agar, a polysaccharide obtained from the extracts of seaweeds (a red algae). Agar promotes gas exchange and the diffusion of nutrients. The optimal concentration of agar is about 0.8%. Besides agar, there is another gelling agent called Gelrite available commercially. It is produced from bacteria (*Pseudomonas* spp.).

**pH value of the medium**

The pH value of the medium is an important factor and therefore its maintenance on the optimum is very essential. For example, pH value afects the gel strength of the medium. Usually the lower the pH value the softer is the medium. With the increase of pH the hardness of the medium will increase. Therefore, deviation from the optimum pH value could be harmful to the plant material.

There are numerous tissue culture media used for different culture purposes. Among these, media of Murashige and Skoog, (MS), $N_6$, Gamborg ($B_5$), E euwens ($Y_3$), White and Blaydes are widely used. For example, we have used three media namely MS, $N_6$ and Blaydes for rice anther culture experiments and $N_6$

was the most suitable medium for many varieties. Potato extract enhanced the callus induction of those varieties.

### Explant

Tissue culture techniques are being used in several specified sections whose names have been formed according to the explant, a plant part or part of the organ used to initiate the culture. Due to numerous applications of tissue culture many parts of plants have been used as the initial material and based on these parts (explants) different sections of tissue culture, can be now identified (see table 1).

**Table 1: Main sections in plant tissue culture**

| Nomenclature | | Examples |
|---|---|---|
| 1. | Leaf culture | Anthurium, Coffee, Cauliflower |
| 2. | Embryo culture | Coconut, Carrot, Almond |
| 3. | Shoot-tip culture | Onion, Cabbage, Potato, Sweet potato, Cassava (Manihot) |
| 4. | Root-tip culture | Oil palm, Cauliflower |
| 5. | Anther culture | Rice, Tobacco, Barley |
| 6. | Cell culture | Tomato, Potato |
| 7. | Axillary bud culture | Pineapple, Ginger, Sweet potato Cucumber |
| 8. | Protoplast culture | Tomato, Carrot, Tobacco |

Microbes like bacteria and fungi are capable of fast growth in culture media and thus form metabolic wastes which may be toxic to plant tissues. Therefore, almost everything used in tissue culture including culture vessels, culture media, plant material, equipment, culture - transferring area etc. should be sterilized before the culture procedure.

Laminar air flow (LAF) cabinet allows a sterile and particle free (microbe-free) environment, providing a micro-filtered ultraclean air flow in which the tissue culturist can perform the culture procedure. If a LAF cabinet is not available, a special room may be modified for the culture operations. An ultra-violet (UV) sterilizing lamp promotes to avoid microbial contaminations. But UV lamp should be handled carefully as its rays can be harmful for skin.

Large number of microorganisms are found on the surfaces of plant tissues. Therefore, surfaces of plant tissues should be sterilized thoroughly before culture. There are several sterilizing agents used to decontaminate plant tissues. Calcium hypochlorite, Sodium hypochlorite, Bromine water, Hydrogen peroxide etc. are successfully used in many tissue culture procedures. However, the sterilizing agent to be used should not be toxic to the given plant tissues. Optimal concentration of sterilants and the adequate duration of their treatment should be chosen for an effective sterilization.

All instruments used in tissue culture technology like forceps, knives, blades etc. should be dipped in 96% ethyl alcohol and sterilized in a spirit lamp-flame.

Autoclave serves to sterilize media vessels and other required items by steam under a certain pressure and temperature. However, a household pressure cooker can also be for the same purpose if an autoclave is not available. A great care should be taken not to exceed the required duration of autoclaving since it affects the culture medium, destroying some vital components in the medium.

### Culture room

A culture room should provide appropriate and adequate conditions to store cultures under optimal conditions (i.e. it should provide optimal temperature, light intensity, photo period and if possible humidity) / and to observe them. It should possess a microbe-free environment at least to a certain extent. Most culture rooms are equipped with fluorescent lamps, air conditioned environment with humidities.

### Transplanting of healthy materials into soil (Hardening)

Since *in vitro* plantlets are grown on culture media under special conditions their anatomical, morphological and physiological characteristics are different from those grown under natural conditions. *In vitro* plantlets are grown in a closed space with high relative humidity. Therefore they exhibit several abnormalities in water relations and have reduced photosynthetic capacity. For example, reduced

deposition of cellulose and lignin causes the reduction of cell wall pressure leading to increased water uptake by cells and a glassy turgescence of leaves and stem. Furthermore, development of epicuticular wax on the leaves of *in vitro* plantlets is poor. The stomata are usually characterized by their malfunctioning, failing to close when needed.

All these changes result in the rapid wilting, slow growing and also high rates of mortality of the plantlets upon their transfer to *ex vitro* conditions. These drawbacks should be minimized to improve the survival or *ex vitro* plantlets. Plantlets have to be removed from culture vessels and washed thoroughly and transferred to the soil where microbial organisms are minimized. Usually the plantlets are acclimatized in a humid environment *ex vitro* and transferred to grow under normal greenhouse conditions after a certain period. This procedure is called hardening.

**Some practical applications of plant tissue culture**

Although for developing countries like Sri Lanka tissue culture technology is still economically not beneficial, it may be a very profitable field in the near future. Nowadays, tissue culture technology is being applied jn many areas. Some applications that are currently in use or will be useful in the near future for Sri Lankan are discussed below.

***In vitro* propagation**

By *in vitro* propagation, we mean the propagation of plants in a controlled, artificial environment using culture vessels aseptic techniques and a defined culture medium. For this purpose usually a small part of plants (e.g. -shoots) is cultured. Some explants give rise only to shoots while others can form both shoots and roots. In case of only shoot formation those explants should be transferred to another medium for rooting.

*In vitro* method can be used to multiply pathogen-free plants by using meristem-tip or shoot-tip ex plants. This is possible because the growing point of the shoot is free from pathogens even in infected plants. Another, advantage of *in vitro* propagation is that the cultures can be sotred under low temperatures to stop their growth, when there is no need for planting material. They can be made to grow again when needed. However, the greatest advantage of *in vitro* propagation is the posibiity of multiplying an elite genotype in large numbers to obtain genetically uniform plants.

***In vitro* Orchid production**

The commercial importance of orchids makes to necessary to improve and propagate them in large scale. Presently in Sri Lanka besides pot culture, husk culture, bedding etc. tissue culture methods are also practiced for propagation of orchids. *In vitro* methods for micropropagation consist of four main steps:

1. *In vitro* culture for pathogen-free plant material
2. Shoot multiplication
3. Root formation
4. Hardening

There are many specified media used for orchid propagation. Some of the widely used media are Krudson C orchid medium, Lindemann orchid medium and Vacin and Went medium. Usually shoot-tips are used as explants and they should be cultured aseptically.

Shoot-tips usually form round, glistening structures called protocorms, which may be multiplied indefinitely or induced to regenerate a whole plant. This protocorm mass should be chopped into several pieces and subcultured on a fresh medium to obtain more structures. This procedure can be repeated according to the requirements. Culture of chopped protocorm pieces on a regeneration medium promotes shoot formation. Thus, *in vitro* multiplication of orchids can produce several millions of plants as against a few hundred by conventional propagation techniques.

***In vitro* embryo culture**

Embryo culture is very essential in difficult crossing programmes in plant breeding. For example, embryo rescuer technique has helped to overcome the difficulties in several interspecific crossings of important crops like rice, barley, bean, pepper, papaw, tomato etc. Moreover, embryo culture *ex-ovule* (out side the environment of the ovule) can be used to study the nutrient requirements of the embryo during its development. Seed germination of some species possessing long periods of dormancy (deep dormancy) is

determined by their morphological, physiological and biochemical factors. *In vitro* embryo culture may help to overcome deep dormancy and initiate germination because in most cases the factors influencing the dormancy are located in the seed, outside the embryo. For example embryos of some coconut varieties like "Dikiri pol" and Makapuno do not germinate within its own endosperm. Excised embryo culture promotes their growth *in vitro*. Furthermore, excised immature embryo culture not only bypasses the stage of dormancy but also tends to undergo the embryogenic mode of development.

**Haploid plant production**

Haploid plants possess a single copy of the chromosomes characteristic for the given species. The success in haploid plant production from anthes and pollen grains has helped to obtain homozygous lines within one or two generations in more than 200 species. Since anther culture techniques offer the possibility to obtain genetically divers haploids, by doubling their chromosomes the plant breeding process can be expedited. Usually colchicine is used for chromosome duplication, although spontaneous diploidization has also been found in some species.

*In vitro* development of plants from male gametophytes (*in vitro* androgenesis) can be divided into the following.

1. Androgenesis of microspore - Here non-zygotic, embryo like vegetative structures called embryoids are formed in culture which are capable of developing into embryos and subsequently to whole plants. Haploid plants are developed from pollen grains in this case.

2. Androgenesis of callus - Haploid plantlets are derived from callus which is usually formed within an anther.
   Example - Rice *(Oryza)*, Barley *(Hordeum)*

3. Androgenesis of microspore and callus - Both processes are taking place but callus formation phase is so short making it difficult to study the organogenetical processes without special cytological approaches.

**Suspension culture**

The culture in which individual cells or aggregates of cells multiply while suspended in liquid medium is called suspension culture. Usually inoculation of friable callus tissues to a liquid medium and subsequent shaking by an orbital shaker can maintain a homogeneous population of cells. If calli are not available, other plant organs can be selected, surfaced-sterilized and used as the first inoculum. Agitation of the medim is very essential since it is capable of breaking cell aggregates into smaller clumps and single cells maintaining and uniform cell distribution in the medium.

Cell suspension cultures are used to study secondary metabolism in cells, enzyme induction and gene expression. They also serve as a source material for enzymes in industry.

***In vitro* fertilization**

This is also called test-tube fertilization. These techniques can be applied to overcome drawbacks like self incompatibility in some species and cross incompatibility appearing in interspecific and intergeneric hybridization. For example, an interspecific hybrid *Nicotiana tabacum* X *Nicotrana rosulata* had been produced by using this technique. This method could also be applied for self-pollination of such species which have mechanisms to prevent it. A fertilized zygote can be made to grow in an artificial medium without any rest. Therefore, pre-fertilization of pre-zygotic barriers and embryo-endosperm incompatibility and other barriers can be avoided by this technique. To fertilize the excised plated ovules surface sterlized pollen grains are to be placed.

To perform *in vitro* fertilization surface sterilized pollen grains are to be placed on the excised ownles plated on a suitable medium.

***In vitro* germ-plasm conservation**

Massive and indiscriminate deforestation has led not only to global weather changes resulting in drought and increasing green house effect, but also to the disappearance of some species from the flora. There-

fore, efforts should be taken to conserve the threatened flora. One of the remedies for this problem can be *in vitro* germ-plasm conservation since this technology make a it possible to safeguard the genetic material in a relatively little space in a viable form for future use. This technology also promotes to maintain pathogene-free germplasm collections. *In vitro* germplasm conservation can be performed by four basic methods,

1. Maintenance under normal growth
2. Growth limitation
3. Cold storage
4. Cryopreservation (freeze conservation)

Among them the last three approaches are most effective in contrast to the first one.

Selection for stress factor resistances by *in vitro* techniques

Cultured plant cells or tissues are unique objects for pure and applied research and are ideal material to produce varieties with improved disease or stress tolerance. This method is based on spontaneous or induced mutagenesis *in vitro* and after which cells will be selected. Plants are regenerated from showing resistance to the stress factor cells. For example, salt-tolerant varieties of several species like rice, alfalfa etc. have been obtained from callus tissue. Screening for drought-tolerant coconut forms is reported to have been successful by using *in vitro* embryo culture.

Selection for disease resistant varieties by *in vitro* techniques

One of the major objectives in plant breeding is to obtain varieties with improved disease resistance. Tissue culture methods can be used for the screening of disease resistant forms within a relatively short period. The methodological aspects of this technology are almost the same as those of screening for stress tolerance. The production of disease resistant plants by *in vitro* selection involves several steps.

1. Exposing cultures to certain concentrations of phytotoxin or compounds which are similar to phytotoxin produced by the pathogen.
2. subculture of survived cultures.
3. Regeneration of plants from the survived cultures.
4. Test for the resistance of plants to the particular pathogen.
5. Studying the inheritance resistance.

In spite of several drawbacks, *in vitro* technology in selection for disease resistance has many advantages. For example, this method allows to conduct the selection procedure in a relatively small space during a short period compared to conventional methods.

Presently, *in vitro* technology is an expensive methodology for developing countries like Sri Lanka. But, in the near future it will be one of the more effective and economically feasible techniques in plant breeding and propagation. However, a great care should be taken to avoid dangerous consequences that can result from improper handling.