Plant tissue Culture

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Introduction

A plant cell, which is specialized to carry out a function or a group of functions could be made to revert back to an embryonic state (de-differentiate) of cell division to give rise to undifferentiated cells. These could be again made to re-differentiate into various types of cells or even to an entire plant under suitable conditions. This remarkable feature in plant cells termed totipotency is the most fundamental feature that distinguishes them from animal cells. A plant unlike an animal must perform all activities while virtually tied to one place throughout its life. It is unable to run away to safety when faced with challenging situations. It must rapidly change and adapt to the new conditions or perish. This has rendered a high degree of plasticity to plant cells during the course of their evolution. Totipotency is a manifestation of this plasticity. It is due to this feature that the different parts of a plant can change and grow in different directions to get the best advantage in a changing environment.

In plant tissue culture totipotency is exploited to the maximum. Various parts of a plant, even a cell, tissue, organ or a whole plant is made to grow in an artificial environment under aseptic conditions. The cells of the explant (the part which is cultured) are made to divide and differentiate as desired. Techniques of plant tissue culture have been developed where tens of thousand entire plants can be produced from a small group of cells isolated from a selected plant. This technique known as micropropagation is only one of the many uses of tissue culture.

For normal growth, a plant absorbs water and mineral nutrients from the soil and CO_2 from the atmosphere. With the use of energy from sunlight it photosynthesizes carbohydrates that are used for growth and metabolism. All other complex molecules required by the plant are synthesized using these carbohydrates.

When a plant or a part of it is grown in tissue culture, these basic requirements have to be provided. A tissue culture medium contains all essential mineral nutrients required in macro or micro quantities (Annexure 1). Under the artificial conditions provided, even in the presence of light, photosynthesis as well as the synthesis of other complex molecules such as certain vitamins and amino acids does not take place efficiently. Therefore a source of carbohydrate, mainly sucrose, vitamins and amino acid are incorporated into the medium. The types and quantity to be incorporated in the medium depends on the species and the type of tissue that is cultured. Normal growth of a plant in nature is under the control of endogenous plant hormones or growth regulators (Annexure 2). These too have to be incorporated to the medium to control the growth of the cells as desired. By manipulating the constituents in the culture

medium, growth of cells or tissues may be controlled as desired. This makes it possible to use tissue culture in the study of basic biological processes in plants. Apart from this many applications of tissue culture are used in the improvement and commercial propagation of useful species as shown below.

Applications of plant tissue culture

Embryo culture

This is the sterile isolation and growth of mature or immature zygotic embryos in a culture medium to obtain a viable plant. This technique has various applications.

Embryo rescue: In enforced self fertilization in cross pollinated species or in wide crosses where two distantly related species have been crossed artificially, the development of the zygote into an embryo may not take place within the seed due to barriers that prevent its development. Early rescue of the embryo and growth in an artificial medium could help in the development of a viable plant.

Breaking the dormancy of seeds: Certain species have seeds with long dormancy periods, which prevent their natural germination. Embryo culture in a suitable medium could break this dormancy. This is important in breeding programmes involving species with dormant seeds that lengthen the life cycle.

Germplasm exchange: Seeds of many tree species rapidly lose their viability on dehydration and cannot be stored under conditions that are normally used in the storage of most seeds. Thus it is not possible to transport them over long distances, as they would lose their viability on arrival at the destination. Shipping embryo cultures could overcome this problem. It has also the advantage of having a lower bulk for shipment and guarantees quarantine requirements due to the absence of microorganism.

Meristem / shoot tip culture:

Meristem or shoot tip culture is the sterile isolation and growth of the apical meristem or the shoot tip. This has many applications.

Micropropagation: The apical meristem or the shoot tip can be induced to develop a large number of axillary shoots rapidly. Its main application is in micropropagation for the rapid production of propagules. The shoots can be separated and continuously subcultured. This results in the mass production of plants when the shoots are rooted to develop an entire plant e.g. (Banana, Potato, Eucalyptus, Strawberry, Orchids, etc). This is of commercial use in plant propagation in the horticultural sector.

Induction of flowering: The apical meristem is able to grow into a vegetative shoot or a reproductive shoot such as a flower or an inflorescence during the life cycle of a plant. In most

plants the transition of the meristem into a floral state depends on the maturity of the plant and environmental conditions. The cues that bring about flowering in some species of bamboo and *Strobilanthus* is not known. They exhibit the phenomenon of mast flowering once in their lifetime after many years of vegetative growth. Meristem or shoot tip culture in such species is used to induce flowering and study its causes. It is also useful in seed production in rarely flowering species and for hybridization in their improvement.

Virus free plants: The rapidly dividing cells of the meristem, of a plant infected with a viral disease, often do not have viral particles within its cells. The sterile isolation of the meristem and culture in a suitable medium will give rise to the development of virus free plants. Sometimes it may be necessary to give a heat treatment to destroy virus particles before a meristem is cultured.

Germplasm storage: Meristems or shoot tips may be cultured in a medium that arrests cell division but keeps them viable. They may be stored at low temperatures or even in liquid nitrogen for long-term storage, after pretreatments that minimize cell damage during exposure to low temperature. This is applied mainly in the storage of germplasm in plants that do not produce seeds (yams, banana) or in those that have recalcitrant seeds (mango, avocado). When required, the stored shoot tips or the meristems are rapidly thawed and cultured in a medium that makes them grow into normal plants.

Callus and cell culture

A callus is a mass of rapidly dividing cells that are initially not organized. Cells can be made to rapidly divide and form callus from various plant parts such as leaves, stems, roots, meristems, buds, seeds or excised embryos. It is easier to induce callus from cells that are dividing or are relatively undifferentiated. The appearance of callus may differ in colour or texture (hard, soft, friable) depending on the species or the explant from which it developed. Some types of calli, specially soft or friable ones can be used to form suspensions in liquid media. The suspended cells may separate into single cells and small aggregates that undergo cell division to proliferate. Once a callus or a suspension culture is initiated, repeated subculture results in an increase in cell quantity. These may be used in other applications.

Somatic embryogenesis / regeneration of plants:

The callus or cells in a liquid cell suspension could be induced to form organized structures such as roots shoots or even embryos. In 1960 Stewart was the first to discover that carrot cells in suspension developed into embryos that germinated into plantlets. These embryos are termed somatic embryos as they differentiated from the somatic cells of a plant. These are similar to the zygotic embryo that forms

as a result of pollination and fertilization. Complete plants can be germinated from mature somatic embryos. The callus may also differentiate into root or shoot meristems that further develop into roots or shoots. These shoots may be rooted to form entire plants. Therefore this technique is also used in the rapid development of prpagules.

Artificial seeds:

Somatic embryos may be encapsulated in a suitable polymer such as calcium alginate to form an artificial seed. Plant propagules in the form of encapsulated somatic embryos have been developed in certain species as an alternative to seeds. Even shoot tips can be encapsulated and used as plant propagules.

Somaclonal variations:

Plants regenerated from callus or cell suspensions are expected to be similar to each other. But sometimes they show variations caused by changes in the chromosomes or genes during to rapid cell division. This is termed somaclonal variation. These variations could be minimized by following certain protocols. Although somaclonal variation is a disadvantage in mass cloning of plants, it has certain advantages. Mutants with desirable characters may be selected among these variants to develop new genotypes that differ in only a few characters from the stable lines. However deleterious changes are more common than favorable ones.

Transgenic plants:

Recombinant DNA technology discovered by Cohen and Boyer in 1973 has become an indispensable tool in modern agriculture and medicine. This is applied in developing transgenic plants where the genetic material has been altered by the direct introduction of a DNA sequence (gene) from a donor plant, animal or microorganism. It requires a selected DNA sequence (gene) from a donor and a vector such as a virus, plasmid or an artificial carrier to introduce the DNA sequence into the recipient host cells. Once the transformed cells are formed, tissue culture techniques are required to regenerate viable plants.

Callus or cell suspensions could be used as recipient cells. They are co-cultured with a vector such as *Agrobacterium tumifaciens* into which the DNA sequence has been introduced. During co-culture, the selected DNA may become successfully incorporated into the genome of the recipient plant cells. Transgenic plants could then be differentiated from the callus or cell suspension.

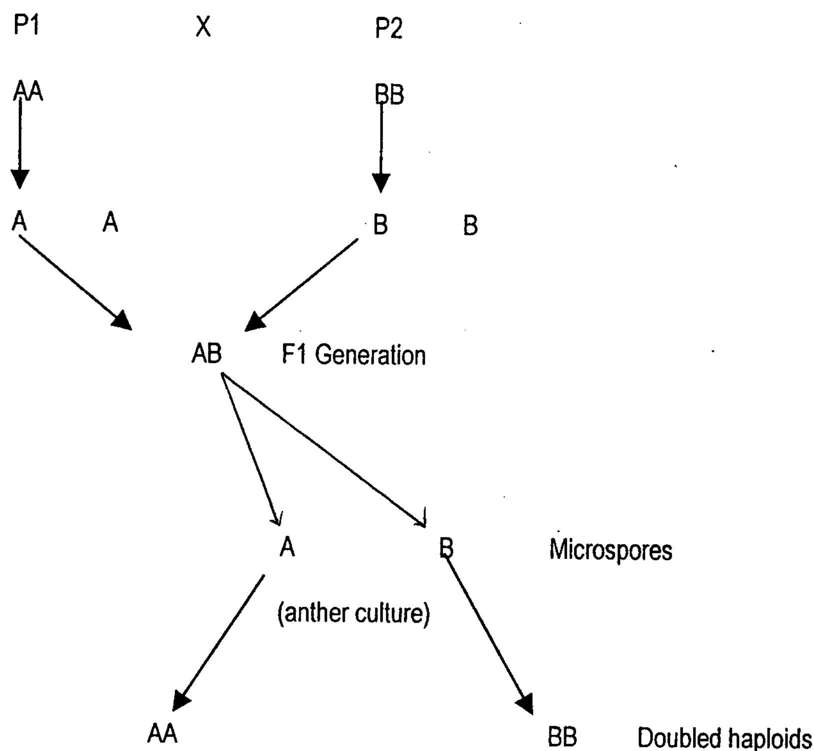
Production of secondary metabolites:

Suspension cultures are used in the production of useful secondary metabolites such as alkaloids, colourings, flavours, fragrances, terpenes etc, for the pharmaceutical, cosmetic and food industry. Large volumes of cell suspensions are cultured in bioreactors under specific conditions, to enhance the formation of high contents of the products. Shikonin, vinblastin, berberine and rosemarinic acid are some of these secondary metabolites that are produced by suspension culture on an industrial scale.

Anther culture

Anther culture is the sterile isolation of anthers and development of haploid or doubled haploid callus cultures from microspores. Plantlets could be differentiated from this callus. These plantlets have the haploid chromosome number or are often doubled haploids as there is a spontaneous doubling in the chromosome number. They are useful in accelerating plant breeding programmes. Conventional breeding programmes require over 8 generations after hybridization to produce homozygous lines. Homozygous lines would be available directly from F1 plants by culturing their anthers therby shortening the breeding cycle.

Anther culture is also useful in mutation breeding as it enables the expression of recessive mutants in the first generation after mutagen treatment.



Protoplast culture

Protoplast culture is the sterile isolation of cell protoplasts by dissolving the cell wall with enzymes to release the protoplasts. Protoplasts are also useful in the development of transganic plants and in somatic hybridization

Transgenic plants from protoplasts

DNA is directly injected into the protoplast by artificial vectors (electroporation, micro-injection and microporjectile bombardment).

The transformed protoplasts are next cultured in a suitable medium where they develop cell walls, divide and form callus, which are then induced to differentiate into plants with the foreign gene.

Somatic hybridization:

Wild relatives of crops have genes that make them resistant to disease or adverse environmental conditions. These genes could be incorporated to the genome of crops to improve their resistance to disease, drought conditions etc. This could be carried out by directly inserting the desired gene into the plant by genetic engineering. Another method is to cross the two species. Often wide crosses fail due to barriers to cross-pollination. Fusion of isolated protoplasts of the two species is another method of incorporating these genes.

Aids to study tissue culture

Literature Survey

Invaluable help for *in vitro* culture of higher plants can be obtained from handbooks, congress and symposium abstracts and reports and articles in journals.

Tissue culture laboratory

A specially equipped and maintained laboratory is needed.

Tissue culture involves four main steps.

- Preparation of culture media
- Sterilization of culture media, and vessels
- Surface sterilization of explants and their inoculation in culture media
- Incubation of cultured explants

It is necessary to carry out these steps in separate but adjoining rooms. It is very essential to maintain cleanliness in all these rooms in order to reduce contamination of cultures by microorganisms.

Equipment in a tissue culture laboratory

Media preparation room:

Balances – Analytical balance
Top loading balance
Hot plates
Stirrers
PH meter
Racks and cupboards for storage of chemicals and glassware
Refrigerators and freezers for storage of chemicals and stock solution
Water distillation and deionisation apparatus

Culture incubation room:

Culture Racks
Incubators
Air conditioner

Other equipment such as laminar hoods and microscopes may be kept in a separate room or in the culture room according to space available.

Annexure 1

Composition of a commonly used basal tissue culture medium - MS medium (Murashige and Skoog, 1962).

Compound	Quantity	
	Mm l^{-1}	mg l^{-1}
Macro nutrients		
NH_4NO_3	20.6	1650
KNO_3	18.8	1900
$CaCl_2.2H_2O$	3.0	440
$MgSO_4.4H_2O$	1.5	370
KH_2PO_4	1.2	170
Micro Nutrients		
$MnSO_4.4H_2O$	100	22.3
$ZnSO_4.7H_2O$	30	8.6
H_3BO_3	100	6.2
KI	5	0.83
$Na_2MoO_4.2H_2O$	1.0	0.25
$CuSO_4.5H_2O$	0.1	0.025
$CoCl_2.6H_2O$	0.1	0.025
Na_2EDTA	100	37.3
$FeSO_4.7H_20$	100	27.3
Organic compounds		
Thiamine.HCl	0.3	0.1
Nicotinic acid	4.0	0.5
Pyridoxine	2.4	0.5
Glycine	26.6	2.0
Sucrose	876Mm	30,000

Annexure 2

Plant Growth Regulators:

Certain compounds are present within plant tissues which serve a regulatory rather than a nutritional role in growth and development. These compounds are active in very low concentrations and are termed plant hormones or plant growth regulators. Plant cells have the ability to synthesize growth regulators and the site where they are synthesized is remote from the site of action. They are synthesized in actively growing tissues where there is cell division. E.g. shoot and root tips, young leaves etc. The basic mechanism of regulation in cells is not fully understood. The type of plant hormone and the amount that is naturally present in a tissue may vary with the seasons, developmental stage of the tissue or its position in the plant. When tissues are cultured, growth and differentiation of cells are brought about by a balance between externally applied growth substances and those synthesized in the cultured tissues. Therefore the choice of the substance to be added to the culture medium and the amount needs to be experimentally determined. Synthetic substances with similar physiological activities have also been identified. These are also used in tissue culture media to control growth as desired.

There are several classes of plant growth regulators grouped as follows:
Auxins
Cytokinins
Gibberellins
Ethylene and Abscicins (or dormins)
The first two classes are the most important in regulating growth and morphogenesis in plant tissue cultures.

***Auxins**: Various naturally occurring auxins are known, namely, Indole acetic acid (IAA), Indole-3-butyric acid (IBA) etc. and conjugâtes of these. In addition many chemical analogues have been synthesized, Some of them are regularly used. E.g 2,4-dichlorophenoxyacetic acid (2,4-D), alpha naphthaleneacetic acid (NAA), 4-CPA Dicamba etc.*

Auxins were discovered some 70 years ago by the Dutch plant physiologist F. W. Went. He observed that auxins produced in the tip of Avena sativa coleoptile influence the curvature of the coleoptile just belw the tip. Shortly after this the root inducing capability of IAA was discovered. Later NAA and IBA were chemically synthesized. With the use of talc as a carrier for auxins rooting powder was commercially produced sixty years ago. The role of auxin in tissue culture was established by Skoog and Miller (1957). They found that the pith tissues of Tobacco stems formed shoots at high cytokinin and low auxin concentrations while they formed roots at low cytokinin and high auxin concentrations or formed callus at intermediate concentrations. A few years later the formation of somatic embryos by treatment of tissues with 2,4-D was observed.

***Cytokinins**: These are a complex class of plany growth regulators. The naturally occurring cytokinins, Zeatin, 2iP and their ribosides. For a long time 6-Benzylaminopurine has been considered to be a synthetic cytokinin, but has recently been shown to occur naturally too. All these are of the purine type buut in addition nonpurine cytokinins have been discovered e.g. thidiazuron (TDZ), CPPU.*

The discovery of cytokinins is closely linked to tissue culture. During the period tissue culture was discovered it was observed that malt coconut water and yeast extract promoted initiation and growth of buds in vitro. It was later found that this was due to the formation of purins from the nucleic acids that were present, during autoclaving. The active compound that formed was identified as kinetin.

Cytokinins promote cell division and are required in callus formation. They promote axillary branching and are thus useful in micropropagation. They also promote adventitious shoot formation, prevent senescence, inhibit root formation.

***Gibberellins:** These are not frequently used in tissue culture. They are reported to promote flowering, break dormancy, and cause stem elongation. There are about 90 known gibberellins. Of these the most commonly used is GA3.*

Absisc acid: Is also not used frequently in tissue culture. It plays a role in the development of dormancy.In tissue culture it prevents shoot growth and germination of embryos. It is formed in tissues during stress conditions.

Ethylene:
In contrast to other hormones this is a gas. Ethylene is synthesized during fruit ripening and senescence. In tissue cultures wounding and auxins bring about the formation of ethylene and is undesirable.