PLANT TISSUE CULTURE

Plant tissue culture is the aseptic culture of any plant part in vitro. Micro-propagation is the rapid vegetative propagation of plants via tissue culture techniques. Micro-propagation permits the manipulation of physical and chemical conditions in the production of large numbers of high quality plant material within a short period of time.

Lab Requirements for Plant Tissue Culture

For a standard tissue culture laboratory, the following minimum facilities are necessary:

1. a **washing area** where glassware and plastic ware are washed, stored

2. a **media preparation room** used for the preparation storage and sterilization of culture media; this room should be equipped with working benches, a deepfreeze, a refrigerator, weighing balance, a hot plate-cum-magnetic stirrer, a pH meter, a vacuum pump, and an autoclave

3. an **aseptic transfer area** (initiation and sub-culturing of plantlets)

4. an **incubator or a culture room** where controlled temperature, diurnal illumination, and humidity are maintained and cultures are grown; the room should have racks for placing culture tubes, flasks, etc. and also a shaking machine for growing suspension cultures

Adantages of Propagation by Tissue Culture

- Elimination of diseases and the production of disease free plantlets
- Rapid production of large numbers of genetically identical plantlets
- Introduction of new varieties and or genotypes
- Preservation of germplasm
- Production of haploid plants which can be used for plant breeding
- Production of plantlets from species in which plant development from seed is difficult

Media Composition and Media Preparation

Plants grown *in vitro* require similar nutrients to the plants grown in the soil. The basic components of any cultural medium are:

1. **Inorganic Nutrients** – These are the mineral elements required for growth and include both macronutrients and micronutrients. Macronutrients are N, P, K, Ca, Mg and S. Commonly used micronutrients are Fe, Mn, B, Cu and Co.

2. **Carbon and energy source** – Sucrose is the most preferred carbon source, though glucose is equally good.

3. **Organic supplements –** These include vitamins like thiamine (vit. B1), nicotinic acid (B3), pyridoxine (B6), calcium pantothenate (B5) and myoinositol. Sometimes aminoacids like casein hydrolysate, L-glutamin, L-glycerine, L-arginine etc are added. Other organic supplements include coconut milk, yeast and malt extract, ground banan etc. Activated charcoal and some antibiotics are added to the media for the culture of some plants.

4. **Growth Regulators –** Plant growth regulators auxins, cytokinins, gibberellins and abscisic acid are important in tissue culture

5. **Solidifying agent** – For semi-solid or solid media, agar or gelatine is used as gelling agent

Media Preparation

While preparing media, stock solutions (concentrated Solutions) are prepared, which when mixed together constitutes basal mediumstock solutions are prepared for macronutrients, micronutirnts, iron EDTA and organic nutrients. Separate stocks are prepared for each growth regulators as well as other additives. After mixing all components, pH of the medium is adjusted to 5.7 – 5.8. the medium is then sterilized by autoclaving and used.

Sterilization Techniques Used in Tissue Culture (Aseptic Techniques)

The most important and difficult aspect of in vito technique is the requirement to carry out various operations under aseptic conditions. Bacteria and fungi are the most common contaminants in cultures. Culture media provides optimal growth conditions for these contaminants and plant tissue is finally killed by contamination. Hence, a completely aseptic environment inside the culture vessel is absolutely essential.

The following are the common techniques used for sterilization:

1. **Dry heat (l60-180°C for 3 hr)** – Used for sterilization of empty glassware (culture vessels, pipettes, etc.), and certain Plastic ware (Teflon FEP); instruments like scalpels, forceps, needles, etc.

2. **Flame sterilization** – For instruments like scalpels, forceps, etc.; mouths of culture vessels

3. **Autoclaving (121°C at 15psi. for 15-40 min)** - Media, culture vessels glass and plasticware, contaminated cultures etc. are autoclaved

4. **Filter sterilization**

    a. Liquid (membrane filter of 0.45µm or smaller pore size) - Heat labile compounds like GA3, ABA, zeatin, urea, enzymes, etc.

    b. Air (HEPA filter) - Air blown through laminar flow cabinets

5. **Wiping with 70% Ethanol** - Platform of laminar flow cabinets, hands of the operator, etc.

6. **Surface sterilization** - All plant materials to be cultured are surface sterilized using sodium or calcium hypochlorite solution (0.3 - 0.6% for 15 -30 min.) or mercuric chloride (0.1-1% for 2 – 10min.) or bromine water (1-2% for 2 -10min.).

Callus Culture

Explants, when cultured on the appropriate medium, usually with both an auxin and a cytokinin, can give rise to an unorganised, growing and dividing mass of cells. This undifferentiated mass of dividing cells is called **callus**. Any plant tissue can be used as an explant for callus culture. A medium containing appropriate combination of growth regulators induces callus which is sub-cultured on to fresh medium periodically. During callus formation there is some degree of dedifferentiation both in morphology (callus is usually composed of unspecialised parenchyma cells) and metabolism. Callus cultures fall into one of two categories: compact or friable. In compact callus the cells are densely aggregated, whereas in friable callus the cells are only loosely associated with each other and the callus become soft and breaks apart easily.

Callus culture is often performed in the dark as light can encourage differentiation of the callus. During long-term culture, the culture may lose the requirement for auxin and/or cytokinin. This process, known as 'habituation', is common in callus cultures from some plant species.

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies. Callus cultures are slow growing systems on a static medium and hence offer a unique system to study various aspects of plant metabolism and differentiation.

Plant Regeneration

Regeneration refers to the development of organised structures like roots, shoots, flower buds, somatic embryos (SEs), etc. from cultured cells/tissues. The term **organogenesis** is also used to describe these events. Organogenesis relies on the production of organs, either directly from an explant or from a callus culture.

Shoot Regeneration - Cultured cells of many plant species show shoot regeneration under appropriate conditions. When callus is transferred onto medium favouring shoot regeneration, clusters of meristematic cells, called nodules or meristemoids appear. Shoot buds usually arise from meristemoids, which give rise to leaf primordia and the apical meristem. The

developing buds develop procambial strands, which become connected with the pre-existing vascular tissue present in the explant/callus. In general, organ formation depended on the auxin/ cytokinin ratio and not on their absolute concentrations. In tobacco a higher ratio of cytokinin led to shoot regeneration, while a higher ratio of auxin promoted root regeneration.

Somatic embryogenesis

A somatic embryo (SE) is an embryo derived from a somatic cell, other than zygote, usually on culture in vitro, and the process is known as somatic embryogenesis. In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus.

Somatic embryos generally originate from single cells, which divide to form a group of meristematic cells. Usually, this multicellular group becomes isolated by breaking cytoplasmic connections with the other cells around it and subsequently by cutinization of the outer walls of this differentiating cell mass.

The cells of meristematic mass continue to divide to give rise to globular (round ball-shaped), heart-shaped, torpedo and cotyeledonary stages. In general, the essential features of SE development, especially after the globular stage, are comparable to those of zygotic embryos.

Somatic embryos are bipolar structures in that they have a radicle and a plumule. The radicular end is always oriented toward the centre of callus or cell mass, while the plumular end always sticks out from the cell mass.

Anther Culture and Haploid Production

Haploid plants may be obtained from pollen grains by placing anthers or isolated pollen grains on a suitable culture medium; this constitutes anther and pollen culture,

respectively. Flower buds of the appropriate developmental stage are collected, surface sterilized, and their anthers are excised and placed horizontally on culture medium. Care should be taken to avoid injury to anthers since it may induce callus formation from anther walls. Alternatively, pollen grains may be separated from anthers and cultured on a suitable medium.

The haploid plants derived from anther culture are sterile. By doubling their chromosome number these plants can be made fertile and resultant plants will be homozygous diploid. They can be used as pure lines in breeding programme.

Significance

1. This is the best, convenient and rapid method for the production of large number of haploids.
2. Haploids are very important in the study of fundamental genetics, mutation studies and cytogenetic studies.
3. Haploids are very useful in plant breeding programme
4. Haploids are used for the production of monosomics, nullisomics and other aneuploids.

Somaclonal Variation

Cell or tissue cultures undergo frequent genetic changes. Such genetic variations occurring in cell or tissue cultures are known as somaclonal variations. The cause of variation may be changes in the chromosome number and structure or gene mutations. Polyploidy, aneuploidy, translocations, inversions and deletions have been reported in several cases. Meiotic crossing over involving symmetric and asymmetric recombination could also be responsible for the variation observed in the regenerated plants. Methylation at the DNA level in tissue culture has also been considered as a possible reason for ‘somaclonal variation’. Genotype, explant, duration of culture and various growth hormones influence the frequency of somaclonal variation.

Somaclonal variation has actually been used in plant breeding programmes. The usefulness of this variability in crop improvement programmes, was first demonstrated through the recovery of disease resistant plants in potato (resistance against late blight and early blight) and sugarcane (resistance against eyespot disease disease, Fiji disease, and downy mildew).

However, somaclonal variations are unwanted when the objective is mricropropagation of elite genotypes or genetic transformation that partly involved tissue culture. Under such circumstances, prevention or at least minimization of variation is of utmost importance. To achieve this, the frequency, nature and magnitude of somaclonal variation in relation to manipulation of media components, explant source, culture conditions etc. should essentially be understood.

Synthetic Seeds / Artificial Seeds

Synthetic seed or Artificial seed is a bead of gel containing a somatic embryo (or shoot bud), and the nutrients, growth regulators, pesticides, antibiotics, etc. needed for the development of a complete plantlet from the enclosed somatic embryo /shoot bud. They can be used for sowing as a seed and possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions, and also retain this potential even after storage.

Two types of synthetic seeds are known: *desiccated* and *hydrated*. The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri dishes and leaving them on the bench overnight to dry. Such types of synthetic seeds are produced only in plant species whose somatic embryos are desiccation tolerant.

Hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules. Of the many gels evaluated, calcium alginate is the most suitable. Artificial seeds can be easily made as follows: A 2% solution of sodium alginate is filled in a burette and allowed to drip drop by drop into a

100mM $CaCl_2$ solution. As the sodium alginate bead or drop forms at the tip of the burette, a somatic embryo is inserted into it with the help of a spatula before the drop falls into the CaCl solution. The beads become hardened as calcium alginate is formed; after about 20-30 min the artificial seeds are removed, washed with water and used for planting. Hydrated artificial seeds are sticky and difficult to handle on a large scale, and dry rapidly in the open air. These problems can be resolved by providing a waxy coating over the beads. Hydrated artificial seeds have to be planted soon after they are produced.

The synthetic seed technology offers tremendous potential in micropropagation and germplasm conservation. But, practical implementation of the synthetic seed technology is constrained due to the following main reasons:

1. Limited production of viable micropropagules useful in synthetic seed production.
2. Anomalous and asynchronous development of somatic embryos.
3. Improper maturation of the somatic embryos that makes them inefficient for germination and conversion into normal plants.
4. Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds.
5. Poor conversion of even apparently normally matured somatic embryos and other micropropagules into plantlets.

Protoplast Culture

Protoplasts are plant cells with the cell wall removed. The culture methods for isolated protoplasts are similar to those for single cells. Both semi-solid medium and liquid medium can be used, although the liquid medium is preferred. Protoplasts are fragile and easily damaged, and therefore must be cultured carefully. Liquid medium is not agitated and a high osmotic potential is maintained, at least in the initial stages. The liquid medium must be shallow enough to allow aeration in the absence of agitation. Protoplasts can be plated out on to solid medium and callus produced. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus.

Cultured protoplasts can be used not only for somatic cell fusions, but also for taking up foreign DNA, cell organelles, bacteria and virus particles. In view of this, the isolation and culture of protoplasts has become a very important area of research, within the realm of plant biotechnology.

Somatic Hybridization

Production of hybrid plants through the fusion of protoplasts of two different species/ genera is called somatic hybridization and such hybrids are called somatic hybrids. The technique of somatic hybridization involves the following four steps:

1. Isolation of protoplast
2. Fusion of protoplast
3. Selection of hybrid cells and
4. Culture of hybrid cells and regeneration of hybrid plants from them.

1. Protoplast Isolation

Protoplasts are plant cells with the cell wall removed. Two general approaches to removing the cell wall can be taken—mechanical or enzymatic isolation.

a. Mechanical Method of Isolation of Protoplast - In mechanical method, cells are kept in a suitable plasmolyticum and cut with a fine knife, so that protoplasts are released from cells cut through the cell wall, when the tissue is again deplasmolysed. This method is suitable for isolation of protoplasts from vacuolated cells. However, this method gives poor yield of protoplasts and is not suitable for isolating protoplst from meristematic and less vacuolated cells.

b. Enzymatic Method of Isolation of Protoplast - The enzymatic method is more efficient, since it gives large quantities of protoplasts, where cells are not broken and osmotic shrinkage is minimum. Enzymatic isolation can be done by using anyone of the two methods:

(i) direct (one step) method, in which treatment with macerozyme (or pectinase) and cellulase is done simultaneously, or

(ii) sequential (two step) method, in which cells are first isolated using macerozyme and cells are then treated with cellulase to isolate protoplasts.

13% sorbitol or mannitol is added as osmoticum and pH kept at 5.4.

2. Protoplast fusion

Protoplast fusion is a physical phenomenon. During fusion, 2 or more protoplasts come in contact and fuses with each other. This may be either spontaneous or can be induced.

a. Spontaneous Fusion - Protoplasts often fuse spontaneously by mere physical contact. This is often found among similar parental protoplasts

b. Induced Fusion – Isolated protoplasts from different plants can be induced to fuse by 3 methods:

i) Mechanical fusion – in this method, isolated protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulator and perfusion micropipette. Fusion will then take place spontaneously.

ii) Chemofusion – several chemicals have been used to induce protoplast fusion like sodium nitrate (NaNO3), Ca^{2+} ions, polyvinyl alcohol, polyethylene glycol (PEG) etc. They are commonly known as chemical fusogens. They cause isolated protoplasts to adhere to one another which lead to tight agglutination followed by fusion of protoplast.

PEG induced protoplast fusion is the most commonly used method as it induces reproducible high frequency fusion and is less toxic to most cell lines. In this process, protoplast mixture is treated with 28 -50% PEG for 15-30 min., followed by gradual washing of protoplasts to remove PEG.

iii) Electrofusion – in this technique, mild electrical simulation is being used to fuse protoplasts. An electrical field of low strength leads to pearl chain arrangement of protoplasts and subsequent application of high intensity electric impulse for some microseconds results in electric breakdown of membrane and subsequent fusion.

3. Hybrid Identification and Selection

After fusion treatments, the protoplast population consists of a heterogenous mixture of unfused parental types, products of fusion between two or more protoplasts of the same species (homokaryons) and 'hybrid' protoplasts produced by fusion between one (or more) protoplast(s) of each of the two species (heterokaryons). For the production of somatic hybrids, heterokaryons alone are neede and hence they should be identified and selected. An effective strategy has to be employed for their identification and isolation. This step is called the selection of hybrid cells.

A number of strategies have been used for the selection of hybrid protoplasts:

(i) Some visual markers, e.g., pigmentation, of the parental protoplasts may be used for the identification of hybrid cells under a microscope; these are then mechanically isolated and cultured. This approach is time consuming, and requires considerable skill and effort.

(ii) Second method is to exploit some properties (usually, deficiencies) of the parental species, which are not expressed in the hybrid cells due to complementarily between their genetic systems. These properties may be sensitivity to culture medium constituents, antimetabolites, inability to produce an essential biochemical (auxotrophic mutants), etc. These properties may be naturally present in the parental species or may be artificially induced through mutagenesis/genetic engineering. These strategies are simple, highly effective and the least demanding.

(iii) A more general and widely applicable strategy is to culture the entire protoplast population without applying any selection for the hybrid cells. All the types of protoplasts form calli; the hybrid calli are later identified on the basis of callus morphology, chromosome constitution, protein and enzyme banding patterns, etc. In some cases, the identification may be delayed till plants are regenerated.

4. Regeneration of Hybrid Plants

Once hybrid calli are obtained, plants are induced to regenerate from them since this is a prerequisite for their exploitation in crop improvement. Further, the hybrid plants must be at least partially fertile, in addition to having some useful property, to be of any use in breeding schemes.

Applications

1. Novel hybrid plants can be produced in sexually incompatible species
2. Heterozygous lines may be developed in species which are usually propagated by vegetative means.
3. Desired traits can be introduced by incorporating selective genome
4. Through the production of cytoplasmic hybrids cytoplasmic traits like cytoplasmic male sterility, enhanced photosynthetic efficiency etc. can be incorporated.

Cytoplasmic Hybrids or Cybrids - In sexual hybrids, the cytoplasm is derived from the maternal parent and in somatic hybrids, it is derived from both the parents. However, hybrids can be obtained, where nucleus is derived from one parent and cytoplasm is derived from bothparents. Such hybrids are called cytoplasmic hybrids or cybrids.

Cybrids can be obtained using anyone of the following methods: (i) fusion of normal protoplasts from one parent with enucleated protoplasts from the other parent; enucleated centrifugation, protoplasts can be obtained by high speed centrifugation (20,000 - 40,000g for 45-90 min) of protoplasts or by irradiation treatment, (ii) fusion of normal protoplasts from one parent and protoplasts containing non viable nuclei from the other; (iii) selective elimination of one of the nuclei from the heterokaryon or (iv) selective elimination of chromosomes of one parent at a later stage after fusion of the nuclei.

The technique of cybrid production has been utilized for transfer of cytoplasmic male sterility as has been successfully done in Nicotiana, Brassica and Petunia. Other characters like streptomycin resistance have also been transferred from N. tabacum to N. sylvestris, using this technique. CMS lines with 'Ogura cytoplasm', a herbicide (atrazine) resistant line and lines with increased nectar production have also been obtained using cybrids in crop brassicas.