TISSUE CULTURE TECHNIQUES

Tissue culture is one of the powerful tools in the plant system, in view of the totipotency of plant cell, which enables theoretically, any cell to regenerate into a whole plant in proper medium. However, there are certain tissues or seeds which are again rather difficult to grow in culture or regenerate. Such seeds or tissues are called recalcitrant, in view of the difficulty in regeneration. However, in several cases, the recalcitrance can be overcome by manipulation of the media and culture set up. The importance of tissue culture lies principally in its capacity to secure rapid propagation of a large number of plants, originally derived from a small amount of tissue. In addition to securing mass propagation, artificial culturing of tissues has manifold advantages.

Embryo Culture : The supply of proper nutrients in artificial medium under cultural set up, can promote growth of hybrid cells and mutants which being unstable in the beginning, require proper nursing in artificial medium. In fact, the embryos in culture have been utilized for overcoming compatibility barrier.

Haploid Culture : Over and above the diploid tissues, the pollen can also be cultured for the production of haploid plants as initially done by Nitsch and Nitsch in France and Maheswari and Mukherjee in India. Such haploid plants are very useful for transferring foreign gene in the cells. This is because of the fact that, single set of genes of haploid tissue does not pose any complex problem for the expression of a foreign gene. If the tissue would have been diploid, the problem of gene interaction as well as dominance and recessive expression might stand in the functioning of the foreign gene. Further, the haploid plant also can be cultured for production of homozygous diploid through doubling of chromosomes by colchicine treatment. In all cases, the totipotency of plant cell, that is the capacity of regeneration of the whole plant from a single cell is the basic issue for any programme on tissue culture.

Protoplast Culture : For foreign gene transfer, however, one of the essential steps in tissue culture is the use of protoplast, devoid of any cell wall. The protoplast culture technique, where the cell walls are digested through enzymes – cellulases and macerozymes, leaving the naked protoplast with the nucleus, provides the ideal medium for incorporation of foreign genes in the cell.

Protoplast Fusion: An offshoot of protoplast culture technique utilized in biotechnological procedure is cell fusion. This method permits the fusion of cells of widely different species to undergo fusion. This is mediated by certain agents, such as polyethylene glycol (PEG), leading to the production of hybrid cells through fusion of two nuclei. Such cells are also known as somatic hybrid cell as they contain diploid somatic nuclei of two different species. In such cases, chromosome number also become doubly diploids due to fusion of two diploids. However, if the protoplast of two haploid plants are used in fusion, the regenerated plant can be diploid.

The cell fusion method which is now widely used, was achieved initially in species of Petunia by Cocking, Nicotiana by Carlson, as well as Solanum by Melchers. The hybrid regenerants of tomato and potato were termed as "Pomato" raised by Melchers in Germany.

Suspension Culture : Moreover, just as pieces of tissues, explants of different organs can be cultured, cell suspensions following softening and suspension through specific enzymes and media, are used to yield single cell in suspension which can conveniently be cultured for regeneration. Production of secondary metabolites and biotransformation are the important applications. Cell plating is done to isolate the mutant lines through single cell culture.

Micropropagation : One of the important uses of tissue culture is to utilize mass propagation in vitro for conservation of endangered species, as well as species of economic and medicinal value. In view of rapid denudation of forests and other human practices in relation to industry, agriculture and excessive land use, several valuable species are on the verge of extinction. The propagation in vitro through organogenesis or embryogenesis which utilizes only small amount of tissue, has become a

powerful tool for increase of individuals. The seeds of endangered as well as other economically valuable plants can also be maintained for a long period without the loss of viability through preservation in ultracool temperature, otherwise termed as cryopreservation.

Artificial Seed : Somatic embryoids may develop in vitro through embryogenesis. These can be preserved through the preparation of artificial seeds. The artificial seeds in which the cultured embryos are preserved by coating with sodium alginate. It can maintain the viability for a long period before being attempted for regeneration.

Conservation : Cryopreservation and artificial seeding have now become extremely important strategies for conservation. The techniques for cryopreservation of both seeds and plant organs in culture form important components of germplasm bank and seed bank as well. However, germplasm bank includes both in situ and ex situ conservation in different Biosphere Reserves and National Parks.

Somaclonal Variation : In addition to these advantages, the culturing in artificial medium may lead occasionally to the origin of abnormal cells termed as 'somatic variants'. Such somatic variants if successfully cultured and regenerated, somatic mutants may arise. Therefore, culturing of tissues is designed to secure mass propagation of identical individuals and also occasionally, the variants. The origin of variants thus contributes to enrichment of genetic diversity.

2.16.3 Types of cultures

Tissue culture is commonly used as a collective term to describe all kinds of *in vitro* plant cultures although strictly it should refer only to the cultures of unorganized aggregates of cells. Cultures are generally initiated from sterile pieces of a plant. These pieces are termed *explants*, and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm.

Callus cultures

Callus is an unorganized, undifferentiated mass of cells, formed when plant cells multiply in a disorganized way. The process where tissues and cells cultured on an agar media forms callus is known as *callus culture*. Callus can be initiated *in vitro* by placing small pieces of the whole plant (explants) onto a growth-supporting medium under sterile conditions. Under the stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, the metabolism of cells, which were in a quiescent state, is changed, and they begin active division. During this process, cell differentiation and specialization, which may have been occurring in the intact plant, are reversed, and the explant gives rise to new tissue, which is composed of meristematic and unspecialized cell types. The callus formed using on original explant is called *primary callus*. *Secondary callus* cultures are initiated from pieces of tissue dissected from primary callus.

Patterns of growth and differentiation

A typical unorganized plant callus, initiated from a new explant or a piece of a previously-established culture, has three stages of development, namely:

- The induction of cell division;
- A period of active cell division during which differentiated cells lose any specialized features they may have acquired and become dedifferentiated;
- A period when cell division slows down or ceases and when, within the callus, there is increasing cellular differentiation.

In many callus tissues, the relative levels of auxin and cytokinin control shoot and root formation. An undifferentiated callus can be regenerated into a whole plant by simply altering the concentration of growth regulators. Only the roots are formed when the auxin/cytokinin ratio is high and shoots are initiated when the cytokinin/auxin ratio is high. Intermediate levels of both hormones produce completely disorganized callus growth.

Protoplast cultures

A *protoplast* is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplasts can be induced to reform a cell wall and divide if placed in a suitable nutrient medium. A small cluster of cells eventually arises from each cell. Plants can be regenerated from such callus. At present isolated protoplasts are used chiefly in research into plant virus infections and for modifying the genetic information of the cell by inserting selected DNA fragments. Protoplasts may also be fused together to create plant cell hybrids.

Methods of protoplast preparation

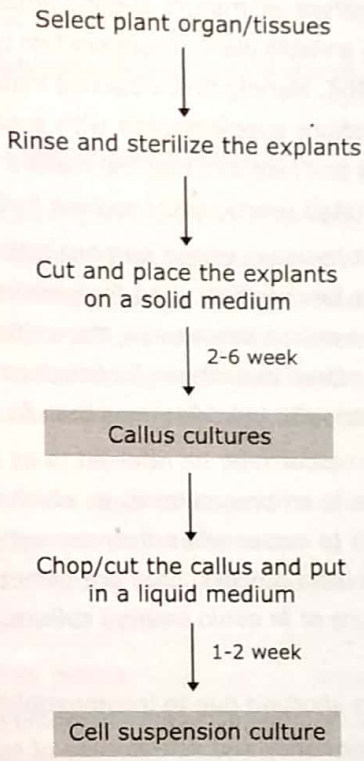
There are several methods by which protoplasts may be isolated:

- By mechanically cutting or breaking the cell wall;
- By digesting away the cell wall with enzymes;

  Protoplast isolation by enzymatic cell wall digestion method involves the use of enzymes to dissolve the cell wall for releasing protoplasts. The enzymatic method could be used as a one-step method (*direct method*), or as a two-step method (*sequential method*). In the one-step method, protoplasts are isolated directly from the tissue by using two enzymes, cellulase and pectinase, simultaneously. While, in the two-step method, cells are first isolated from callus or tissue by using pectinase and to this cell suspension cellulase is added to digest the cell wall and release protoplasts. Protoplasts can then be collected and purified using centrifugation to separate broken and damaged cells from intact protoplasts by taking advantage of their differing buoyant densities.
- By a combination of mechanical and enzymatic separation.

Cell–suspension cultures

Tissues and cells cultured in an agitated liquid medium produces a suspension of single cells and cells clumps of few to many cells; these are called *suspension cultures*. A friable callus can be inoculated in liquid medium, and through continuous shaking cell suspension cultures are obtained. Suspension cultures grow much faster than callus cultures. Many different methods of suspension cultures have been developed. They fall into two main types: *Batch cultures* in which cells are nurtured in a fixed volume of the medium until growth ceases, and *continuous cultures* in which cell growth is maintained by continuous replenishment of sterile nutrient media.



Organ culture

Organ culture is used as a general term for those types of culture in which an organized form of growth can be continuously maintained. It includes the aseptic isolation of definite structures such as leaf primordia, immature flowers and fruits and their growth *in vitro*. Differentiated plant organs can usually be grown in culture without loss of integrity. They can be of two types:

Determinate organs, which are destined to have a defined size and shape (e.g. leaves, flowers and fruits);

Indeterminate organs, where growth is potentially unlimited (apical meristems of roots and non-flowering shoots).

Meristem culture

Meristematic cells retain the power of division long after embryogenesis is over. Based on its position in plant, meristems are classified into three types: Apical, intercalary and lateral meristems. Apical meristem is found at the tips of the roots, stems and branches. It plays a major role in increasing the length of plant. Culture of apical meristems, particularly of shoot apical meristem, is known as *meristem culture*.

The major advantages of meristem culture are that it provides clonal propagation with maximal genetic stability and production of virus-free plants. Apical meristems in plants are suitable parts for the production of virus-free plants since the infected plant's meristems typically are either nearly or totally virus-free. The reasons proposed for the escape of meristem from virus invasion are:

- Viruses readily move in a plant body through the vascular system which is absent in the meristem.
- High metabolic activity in the actively dividing meristem cells does not allow virus multiplication.
- The *virus inactivating systems* in the plant body, if any, has higher activity in the meristem than in any other region. Thus, the meristem is protected from infection.
- A high endogenous auxin level in shoot apices may inhibit virus multiplication.

Embryo culture

Embryo culture involves isolating an immature or mature zygotic embryo aseptically and growing on an aseptic nutrient medium with the goal of obtaining a viable plant. There are two types of embryo culture - Immature embryo culture and mature embryo culture. In 1904, Hannig first obtained viable plants from aseptically isolated mature embryos and grown on a mineral salt medium supplemented with sugar. In 1924, Dietrich cultured mature and immature embryos of various plant species and reported that the mature embryos grew immediately, circumventing dormancy. The immature embryos germinated precociously without further embryo development.

The term embryo rescue refers to *in vitro* techniques whose purpose is to promote the development of an immature or weak embryo into a viable plant. It has been widely used for growing plants in which failure of endosperm to develop causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryo rescue techniques also have been utilized to obtain progeny from intraspecific hybridizations that do not normally produce viable seed.

Depending on the organ cultured, embryo rescue may be referred to as embryo, ovule or ovary culture. The most commonly used embryo rescue procedure is embryo culture, in which embryos are excised and placed directly onto culture medium. Embryos are difficult to excise when they are very young or from small-seeded species. To prevent damaging embryos during the excision process, they are sometimes cultured while still inside the ovule. This technique is referred to as ovule culture or *in ovolo* embryo culture. In ovary or pod culture, the entire ovary is placed into the culture.

Application:
- Overcoming embryo abortion due to incompatibility barriers.
- Overcoming seed dormancy and self-sterility of seeds.
- Embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor.
- Shortening of breeding cycle.

Haploid culture

Haploid plants bear the gametic chromosome number of a species and are generally derived from gametophytic tissue that develops during the reproductive phase of plants. Gametophytes develop after meiosis both in anthers (microsporogenesis) and in ovules (megasporogenesis). Haploid microspores develop into pollen grains (male gametophytes), whereas haploid megaspores generate an eight-celled embryo sac (female gametophyte) bearing the egg cell. The union of a haploid sperm cell from the male gametophyte with a haploid egg cell from the female gametophyte upon fertilization produces a zygote that develops into an embryo, restoring the somatic chromosome number of a species. An interruption of normal sexual development, either natural or induced, causes the microspore or megaspore to undergo mitotic divisions without fertilization, eventually resulting in a haploid plant. Such extraordinary development of these gametophytic tissues can be induced in plant tissue culture, resulting in androgenesis or gynogenesis of higher plants.

Anther culture: Guha and Maheshwari first demonstrated the possibility of androgenesis by anther culture of *Datura innoxia*. Although anther culture has been the most successful means to obtain haploid plants, microspore (pollen) culture and ovule culture have also been successful, especially for plants where anther culture has failed. Anther culture (called **androgenesis**) is a technique by which the developing anthers from unopened flower bud are cultured on a nutrient medium where the microspores within the cultured anther develop into callus tissue or

embryoids that give rise to haploid plantlets (**androgenic haploid**) either through organogenesis or embryogenesis. Haploid plants develop from anther culture either directly or indirectly through a callus phase. Direct androgenesis mimics zygotic embryogenesis; however, neither a suspensor nor an endosperm is present. At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4 to 8 weeks, the cotyledons unfold and plantlets emerge from the anthers. During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenic and direct androgenic pathways. After the globular stage, irregular and asynchronous divisions occur and callus is formed. This callus must then undergo organogenesis for haploid plants to be recovered.

Application:

- Production of haploid plants.
- Production of homozygous diploid lines through chromosome doubling, thus reducing the breeding cycle.
- Production of useful gametoclonal variations.

Ovule culture: An ovule is a megasporangium covered by integument. Ovule culture (**gynogenesis**) involves the development of haploid from unfertilized cells of embryo sac present in ovule. As with androgenesis, gynogenic haploids may develop directly or indirectly via regeneration from callus. Direct gynogenesis usually involves the egg cell, synergids, or antipodal cells with organized cell divisions leading first to the formation of proembryos and then to well-differentiated embryos. In indirect gynogenesis, callus may be formed directly from the egg cell, synergids, polar nuclei, or antipodal cells, or may develop from proembryos. Plants regenerated from callus may be haploid, diploid or mixoploid.

The relative scarcity of haploid cells within an ovule compared to the thousands generally found within anthers has made ovule culture, or gynogenesis, less attractive alternative to anther or microspore culture for developing haploid plants. However, for a few species including onion (*Allium sativum*), ovule culture has been successful. San Noeum first reported successful ovule culture of barley.

Application:

- Production of haploid plants.
- Recovery of hybrid embryos overcoming embryo abortion at very early stages of development of zygote due to incompatibility barriers.
- Achievement of *in vitro* fertilization.

Somatic embryogenesis

Embryos do not necessarily originate from zygotes. Instead, they can originate from a variety of somatic cells and are consequently referred to as *somatic embryogenesis*. It relies on plant regeneration through a process analogous to zygotic embryo formation.

Somatic embryogenesis is the process in which a single or a group of somatic cells initiate the developmental pathway that leads to the formation of non-zygotic embryos (**zygotic embryos** develop from fertilized eggs termed zygote whereas **non-zygotic embryos** develop from cells other than the zygote). Somatic embryos are *bipolar* structures in that they have a radicle and a plumule. These embryos have no connection with pre-existing vascular tissue within the maternal callus. Thus, somatic embryogenesis is a nonsexual developmental process that produces a bipolar embryo with a closed vascular system from somatic tissues of a plant.

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. Though some common tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis.

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.

2.16.6 Applications of cell and tissue culture

Clonal propagation

In nature, the methods of plant propagation may be either asexual (by multiplication of vegetative parts) or sexual (through generation of seeds). Multiplication of genetically identical copies of a cultivar by asexual reproduction is called *clonal propagation*. A population derived from a single individual by asexual reproduction constitutes a clone. Clonal propagation through tissue culture is popularly known as **micropropagation**. It involves *in vitro* propagation of selected genotype and the ultimate establishment of the plant in the field or a green house. Use of tissue culture for micropropagation was initiated by G. Morel (1960), who found this as the only commercially viable approach for orchid propagation. Plants obtained from tissue culture are called *microplants*. Microplants can be generated in three different ways:

- From pre-existing shoot buds or primordial buds (meristems) which are encouraged to grow and proliferate;
- Following shoot morphogenesis when new shoots are induced to form in unorganized tissues or directly upon explanted tissues of the mother plant;
- Through the formation of somatic embryos (called somatic embryogenesis).

There are five stages in micropropagation:

1. Preparation of explant
2. Formation of callus
3. Shoot development
4. Root formation
5. Transfer to a glasshouse

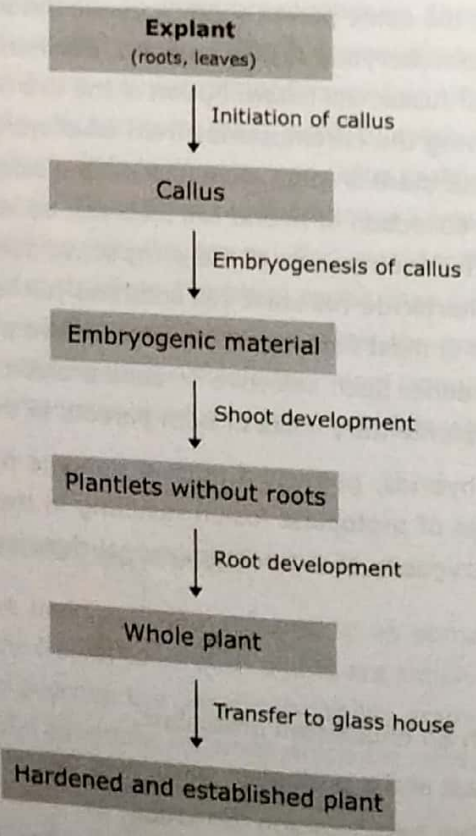


Figure 2.39 Process of micropropagation.