## Applications and achievements

Tissue culture consists of growing plants cells as relatively on organized masses of cells on an agar medium (callus culture) or as a suspension of free cells and small cell masses in a liquid medium (suspension culture). Tissue culture is used for vegetative multiplication of many species and in some cases for recovery of virus-free plants. It has potential application in production of somatic hybrids, organelle and cytoplasm transfer, genetic transformation and germplasm storage through freeze-preservation. Having the right plant material the right media and the right working environment crop improvement through tissue culture becomes less difficult. Crops which have gone the process of tissue culture have several advantages.

The various applications of plant tissue and cells culture techniques are as below:

**Micropropagation /clonal propagation**

Clonal propagation refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. The vegetative propagation of plants is labour-intensive, low in productivity and seasonal. The tissue culture methods of plant propagation, known as 'micropropagation' utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium. The regeneration of plantlets in cultured tissue was described by Murashige in 1974. Fossard (1987) gave a detailed account of stages of micropropagation.

The micropropagation is rapid and has been adopted for commercialization of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants. The micropropagation techniques are preferred over the conventional asexual propagation methods because of the following reasons:

- In the micropropagation method, only a small amount of tissue is required to regenerate millions of clonal plants in a year.
- Micropropagation is also used as a method to develop resistance in many species.
- *In vitro* stock can be quickly proliferated as it is season independent.
- Long term storage of valuable germplasm possible.

The factors that affect micropropagation are:

- Genotype and the physiological status of the plant e.g. plants with vigorous germination are more suitable for micropropagation.

- The culture medium and the culture environment like light, temperature etc. For example an illumination of 16 hours a day and 8 hours night is satisfactory for shoot proliferation and a temperature of $25^0$C is optimal for the growth.

The benefits of micropropagation are:

- Rapid multiplication of superior clones can be carried out throughout the year, irrespective of seasonal variations.
- Multiplication of disease free plants e.g. virus free plants of sweet potato (*Ipomea batatus*), cassava (*Manihot esculenta*)
- Multiplication of sexually derived sterile hybrids.
- It is a cost effective process as it requires minimum growing space.

**Production of virus free plants**

The viral diseases in plants transfer easily and lower the quality and yield of the plants. It is very difficult to treat and cure the virus infected plants therefore the plant breeders are always interested in developing and growing virus free plants.

In some crops like ornamental plants, it has become possible to produce virus free plants through tissue culture at the commercial level. This is done by regenerating plants from cultured tissues derived from

- Virus free plants,
- Meristems that are generally free of infection

  In the elimination of the virus, the size of the meristem used in cultures play a very critical role because most of the viruses exist by establishing a gradient in plant tissues. The regeneration of virus-free plants through cultures is inversely proportional to the size of the meristem used.
- Meristems treated with heat shock (34-$36^0$C) to inactivate the virus
- Callus, which is usually virus free like meristems.
- Chemical treatment of the media- attempts have been made to eradicate the viruses from infected plants by treating the culture medium with chemicals e.g. addition of cytokinins suppressed the multiplication of certain viruses.

Among the culture techniques, meristem-tip culture is the most reliable method for virus and other pathogen elimination. Viruses have been eliminated from a number of economically

important plant species, which has resulted in a significant increase in the yield and production e.g. potato virus X from potato, mosaic virus from cassava etc.

**Rejuvenation plant materials**

Plant tissues from an old plant can be rejuvenated through tissue culture and able to grow again as new. e.g old cassava material have been rejuvenated to produce young plantlets through tissue culture.

**Somaclonal variation**

Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits; such a variation is known as somaclonal variation. Somalconal variation has been described in sugarcane, potato, tomato etc. Some variants are obtained in homozygous condition in the plants regenerated from the cells *in vitro* ($R_0$ generation), but most variants are recovered in the selfed progeny of the tissue culture-regenerated plants ($R_1$ generation). Somaclonal variation most likely arises as a result of chromosome structural changes, e.g., small deletions and duplications, gene mutations, plasma gene mutations, mitotic crossing over and possibly, transposons. Somaclonal variation may be profitably utilized in crop improvement since it reduces the time required for releasing the new variety by at least two years as compared to mutation breeding and by three years in comparison to back cross method of gene transfer. A majority of the variants obtained and described so far is considered as boon to the crop improvement and some of the systems are explained below.

1. **Sugarcane:** Through tissue culture, variants with resistance to eye spot disease *(Helminthosporium sacchari)* Fiji disease (Virus) and downy mildew (*Sclerospora sacchari*) were isolated. The variants showed higher resistance to Fiji disease and downy mildew than their parent clones. Even resistant lines exhibited a shift towards higher resistance.

2. **Potato:** The protoplast culture in the potato cultivar Russet Burbank, an important cultivar excluded from potato improvement because of its sterility, produced total of 1,700 somaclones. From this huge population, 15 stable somaclones were identified, thus providing enough variability for potato improvement. In the same way, somaclone having resistance to late blight (*Phytophthora infestans*) and early blight (*Alternaria solani*) were identified.

3. **Maize:** In maize, the plants with T cytoplasm are male sterile and *Drechslera maydis* T. toxin susceptible. When these plants were subject to *in vitro* culture, somaclones were produced with the characters of male fertility and toxin resistance. The result was due to alterations in mtDNA which is responsible for toxin tolerance.

4. **Rice**: Somaclones were observed in the dihaploids of cultivar Norin l0 for chlorophyll development, plant height, heading date, maturity and grain yield. In the same way, doubled haploid regenerants from the cultivar Calrose 76 showed variation for height, seed number and size, panicle size and leaf morphology, tiller number and height.

5. **Wheat**: The embryo culture technique adopted in wheat has thrown out some 200 plants from a single immature embryo. The initial somaclonal regenerants displayed phenotypic variations. The analysis of regenerants obtained from the cultivar Yaqui 50E showed variations for the characters like plant height, maturity, tiller number, presence of awns, glume colour, grain colour, etc. The existence of somaclonal variation was also supported by the appearance and disappearance of some specific bands of gliadin protein.

Somaclonal variation in agronomically important plant species

| Species | Explant | Variant characters | Transmission |
|---|---|---|---|
| *Avena sativa* | Immatue embryo, apical meristem | Plant height, heading date, leaf striping, awns | Sexual |
| *Triticuam aestivum* | Immature embryo | Plant height, spike shape, awns, maturity, tillering, leaf wax, gliadine, amylase | Sexual |
| *Oryza sativa* | Seed embryo | Number of tillers, panicle size, seed fertility, flowering date, plant height | Sexual |
| *Saccharum officinarum* | Various | Eyespot , Fiji virus, downy mildew, caulm colour, spot disease, auricle length, esterase isozyme, sugar yield | **Asexual** |
| *Zea mays* | Immature embryo | Endosperm and seedling mutants, *D. maydis* race T toxin resistance mtDNA sequence rearrangement | Sexual |
| *Solanum tuberosum* | Protoplast, leaf callus | Tuber shape, yield, maturity date, plant habit, stem, leaf and flower morphology, early and late blight resistance | Asexual |
| *Lycopersicon esculentum* | Leaf | Male sterilty, jointless pedicel, fruit colour, indeterminate type | Sexual |
| *Nicotaina species* | Anthers, protoplasts, leaf callus | Plant height, leaf size, yield grade index, alkaloids, reducing sugars, specific leaf chlorophyll loci | Sexual |

| | | | |
|---|---|---|---|
| *Medicago sativa* | Immature ovaries | Multifoliate leaves, petiole length, plant habit, plant height, dry matter yield | Asexual |
| *Brassica species* | Anthers, embryos, meristems | Flowering time, growth habit, waxiness, glucosinolates, *Phoma lingam* tolerance | Sexual |

**Applications of somaclonal variations**

- Methodology of introducing somaclonal variations is simpler and easier as compared to recombinant DNA technology.
- Development and production of plants with disease resistance e.g. rice, wheat, apple, tomato etc.
- Development of biochemical mutants with abiotic stress resistance e.g. aluminium tolerance in carrot, salt tolerance in tobacco and maize.
- Development of somaclonal variants with herbicide resistance e.g. tobacco resistant to sulfonylurea.
- Development of seeds with improved quality e.g. a new variety of Lathyrus sativa seeds (Lathyrus Bio L 212) with low content of neurotoxin.
- Central Institute for Medicinal and Aromatic Plants (CIMAP), Lucknow, India has released bio-13 – a somaclonal variant of Citronella java (with 37% more oil and 39% more citronellon), a medicinal plant as Bio-13 for commercial cultivation.
- Super tomatoes- Heinz Co. and DNA plant Technology Laboratories (USA) developed Super tomatoes with high solid component by screening somaclones that helped in reducing the shipping and processing costs.

**Mutant selection**

An important use of cell cultures is in mutant selection in relation to crop improvement. Biochemical mutants are far more easily isolated from cell cultures than from whole plant populations. This is because a large number of cells, $10^{6}$-$10^{9}$, can be easily and effectively screened for biochemical mutant cells. The frequency of mutations can be increased several fold through mutagenic treatments and millions of cells can be screened. A large number of reports are available where mutants have been selected at cellular level. The cells are often selected directly by adding the toxic substance against which resistance is sought in the mutant cells. Using this method, cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins etc have actually been isolated. Biochemical mutants could be selected for disease resistance, improvement of nutritional quality, adaptation of plants to

stress conditions, e.g. saline soils, and to increase the biosynthesis of plant products used for medicinal or industrial purposes.

**Amino acid analogue resistant mutants**

Cereal grains are deficient in lysine; maize (*Zea maize*) is also deficient in tryptophan, while wheat (*T.aestivum*) and rice (*O.sativa*) are deficient in threonine. Pulses are deficient in methionine and tryptophan. Amino acid analogue-resistant cells may be expected to show a relatively higher concentration of that particular amino acid. For e.g., carrot (*D.carota*) and tobacco (*N.tabacum*) cell lines resistant to tryptophan analogue 5-methyl tryptophan show a 10-27-fold increase in the level of tryptophan. Similarly, rice cells resistant to lysine analogue 5-(*B-aminoethyl*)-cysteine, show much higher levels of lysine. This technique may prove useful in the development of crop varieties with better-balanced amino acid content.

**Disease resistant mutants**

Many pathogenic bacteria produce toxins that are toxic to plant cells. Plant cell cultures may be exposed to lethal concentrations of these toxins and resistant clones isolated. Plants regenerated from these resistant clones would be resistant to the disease producing pathogen. This technique should be applicable to all the pathogens, which produce the disease through the action of toxin. The technique can be applied to those cases only where the disease is the result of a toxin produced by the pathogen. But many of the pathogens do not seem to produce a toxin, or the toxin does not appear to be the primary cause of the disease.

**Stress resistant and other mutants**

Plant cells resistant to 4-5 times the normally toxic salt (NaCl) concentration have been isolated. Attempts to isolate such cells are being made. Similarly, attempts are being made to isolate clones that would produce more substances of medicinal or industrial value.

**Production of somatic hybrids and cybrids**

The Somatic cell hybridization/ parasexual hybridization or Protoplast fusion offers an alternative method for obtaining distant hybrids with desirable traits significantly between species or genera, which cannot be made to cross by conventional method of sexual hybridization. The applications of somatic hybridization are as follow:

a) **Creation of hybrids with disease resistance** - Many disease resistance genes (e.g. tobacco mosaic virus, potato virus X, club rot disease) could be successfully transferred

from one species to another. E.g resistance has been introduced in tomato against diseases such as TMV, spotted wilt virus and insect pests.

b) **Environmental tolerance** - using somatic hybridization the genes conferring tolerance for cold, frost and salt were introduced in e.g. in tomato.

c) **Cytoplasmic male sterility** - using cybridization method, it was possible to transfer cytoplasmic male sterility.

d) **Quality characters** - somatic hybrids with selective characteristics have been developed e.g. the production of high nicotine content.

**Somatic hybridization**

Protoplasts can be isolated from almost every plant species and cultured to produce callus. Protoplasts of two different species may be fused with the help of polyethylene glycol.

**Gene transformation**

Important crops can be greatly improved by genetic engineering by isolating a specific gene and then transferring it to selected crops. This raises the possibility of genetic modification of plant cells with the help of both homologous (from the same species) and heterologous (from a different species) DNA. It is also proposed that DNA plant viruses, such as cauliflower (*B.oleracea*) mosaic virus and potato leaf roll virus, plasmids (e.g., *Ti plasmid of Agrobacterium*) and transposons, may be used as the carriers of genes for genetic modification of plant cells.

***In vitro* plant germplasm conservation**

Germplasm refers to the sum total of all the genes present in a crop and its related species. The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock for its use at any time in future. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the existing and primitive plants will be lost. The germplasm is preserved by the following two ways:

(a) **In-situ conservation**- The germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in a near natural habitat along with several wild types.

(b) **Ex-situ conservation**- This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or *in vitro* cultures are preserved and stored as gene banks for long-term use.

***In vivo*** gene banks have been made to preserve the genetic resources by conventional methods e.g. seeds, vegetative propagules, etc. ***In vitro*** gene banks have been made to

preserve the genetic resources by non - conventional methods such as cell and tissue culture methods. This will ensure the availability of valuable germplasm to breeder to develop new and improved varieties.

The methods involved in the *in vitro* conservation of germplasm are:

**(a) Cryopreservation**- In cryopreservation (Greek-krayos-frost), the cells are preserved in the frozen state. The germplasm is stored at a very low temperature using solid carbon dioxide (at -79$^0$C), using low temperature deep freezers (at -80$^0$C), using vapour nitrogen (at- 150$^0$C) and liquid nitrogen (at-196$^0$C). The cells stay in completely inactive state and thus can be conserved for long periods. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Certain compounds like- DMSO (dimethyl sulfoxide), glycerol, ethylene, propylene, sucrose, mannose, glucose, praline, acetamide etc are added during the cryopreservation. These are called cryoprotectants and prevent the damage caused to cells (by freezing or thawing) by reducing the freezing point and super cooling point of water.

**(b) Cold storage**- Cold storage is a slow growth germplasm conservation method and conserves the germplasm at a low and non-freezing temperature (1-9$^0$C). The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation and thus prevents cryogenic injuries. Long-term cold storage is simple, cost effective and yields germplasm with good survival rate. Virus free strawberry plants could be preserved at 10$^0$C for about 6 years. Several grape plants have been stored for over 15 years by using a cold storage at temperature around 9$^0$C and transferring them in the fresh medium every year.

**(c) Low pressure and low oxygen storage**- In low- pressure storage, the atmospheric pressure surrounding the plant material is reduced and in the low oxygen storage, the oxygen concentration is reduced. The lowered partial pressure reduces the *in vitro* growth of plants. In the low-oxygen storage, the oxygen concentration is reduced and the partial pressure of oxygen below 50 mmHg reduces plant tissue growth. Due to the reduced availability of $O_2$, and reduced production of $CO_2$, the photosynthetic activity is reduced which inhibits the plant tissue growth and dimension. This method has also helped in increasing the shelf life of many fruits, vegetables and flowers.

The germplasm conservation through the conventional methods has several limitations such as short-lived seeds, seed dormancy, seed-borne diseases, and high inputs of cost and labour. The techniques of cryo-preservation (freezing cells and tissues at $-196^0$c) and using cold storages help us to overcome these problems.

**Production of synthetic seeds**

In synthetic seeds, the somatic embryos are encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides. These artificial seeds can be utilized for the rapid and mass propagation of desired plant species as well as hybrid varieties. The major benefits of synthetic seeds are:

- They can be stored up to a year without loss of viability
- Easy to handle and useful as units of delivery
- Can be directly sown in the soil like natural seeds and do not need acclimatization in green house.

**Production of secondary metabolites**

The most important chemicals produced using cell culture are secondary metabolites, which are defined as' those cell constituents which are not essential for survival'. These secondary metabolites include alkaloids, glycosides (steroids and phenolics), terpenoids, latex, tannins etc. It has been observed that as the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialize to produce secondary metabolites. The *in vitro* production of secondary metabolites is much higher from differentiated tissues when compared to non-differentiated tissues.

The cell cultures contribute in several ways to the production of natural products. These are:

- A new route of synthesis to establish products e.g. codeine, quinine, pyrethroids
- A route of synthesis to a novel product from plants difficult to grow or establish e.g. thebain from *Papaver bracteatum*
- A source of novel chemicals in their own right e.g. rutacultin from culture of *Ruta*
- As biotransformation systems either on their own or as part of a larger chemical process e.g. digoxin synthesis.

**The advantages of *in vitro* production of secondary metabolites**

- The cell cultures and cell growth are easily controlled in order to facilitate improved product formation.
- The recovery of the product is easy.

- As the cell culture systems are independent of environmental factors, seasonal variations, pest and microbial diseases, geographical location constraints, it is easy to increase the production of the required metabolite.
- Mutant cell lines can be developed for the production of novel and commercially useful compounds.
- Compounds are produced under controlled conditions as per the market demands.
- The production time is less and cost effective due to minimal labour involved.

**Table showing plant species and secondary metabolites obtained from them using tissue culture techniques**

| **Product** | **Plant source** | **Uses** |
|---|---|---|
| Artemisin | *Artemisia spp.* | Antimalarial |
| Azadirachtin | *Azadirachta indica* | Insecticidal |
| Berberine | *Coptis japonica* | Antibacterial, anti inflammatory |
| Capsaicin | *Capsicum annum* | Cures Rheumatic pain |
| Codeine | *Papaver spp.* | Analgesic |
| Camptothecin | *Campatotheca accuminata* | Anticancer |
| Cephalotaxine | *Cephalotaxus harringtonia* | Antitumour |
| Digoxin | *Digitalis lanata* | Cardiac tonic |
| Pyrethrin | *Chrysanthemum cinerariaefolium* | Insecticide (for grain storage) |
| Morphine | *Papaver somniferum* | Analgesic, sedative |
| Quinine | *Cinchona officinalis* | Antimalarial |
| Taxol | *Taxus spp.* | Anticarcinogenic |
| Vincristine | *Cathranthus roseus* | Anticarcinogenic |
| Scopolamine | *Datura stramonium* | Antihypertensive |

**Anther culture**

Plants produced through anther culture are haploids. Doubling the chromosomes without going into series of backcrossing can produce homozygous plants. This technique has profound application to plant breeder and shortens the time of breeding by half.

**Embryo rescue**

Many important plants are difficult to propagate through seeds. They take a long time for seeds to germinate or the seeds do not germinate at all. This can be overcome through

embryo culture. The seeds are surface sterilized and split open in aseptic condition and the tiny embryo is excised and planted in a nutrient medium and then grows to a complete plant.

**Organelle transfer**

In some cases, it may be desirable to transfer only organelles or the cytoplasm into a new genetic background. This may be achieved through the use of plant protoplasts. Chloroplasts have been transferred, and other organelles including nucleus may be transferred.

**Achievements and future prospects**

Tissue culture techniques are being exploited to enhance crop production and to aid crop improvement efforts. Faster clonal multiplication is being exploited on commercial scale for many horticultural species e.g. oil palm, mentha, roses, carnation etc. Tissue cultured somatic tissues are now routinely being used for conservation of those species whose seeds are recalcitrant or ones which do not produce seed at all.

Embryo culture has helped in rescuing hybrid embryos enabling the recovery of many interspecific hybrids and haploid plants. Shoot tip (meristem) culture plays a vital which is of great importance in germplasm exchange, and the development of serological techniques for the detection of viruses in plant materials is a great help to the efforts in this direction.

**Questions**

1. The vegetative propagation of plants is ……

   a). Labour-intensive  b). Low in productivity

   c). Seasonal  **d). All the above**

2. Detailed account of stages of micropropagation was given by ……...

   **a). Fossard**  b). Murashige

   c). Skoog  d). None of the above

3. Detailed account of stages of micropropagation was given by ……...

   **a). Fossard**  b). Murashige

   c). Skoog  d). None of the above

4. The benefits of micropropagation are ……...

   a). Rapid multiplication of superior clones  b). Multiplication of disease free plants

   c). Cost effective process  **d). All the above**

5. Somaclonal variation arises as a result of chromosome structural changes like ……...

   a). Deletions and duplications  b). Gene mutations

   c). Transposons  **d). All the above**

6. The methods involved in the *in vitro* conservation of germplasm are ……...

   a). Cryopreservation  b). Cold storage

   c). Low pressure and low oxygen storage  **d). All the above**

7. Cryopreservation involves the usage of ……...

   a). Solid carbon dioxide (at $-79^0$C)  b). Vapour nitrogen (at- $150^0$C)

   c). Liquid nitrogen (at-$196^0$C)  **d). All the above**

8. The major benefits of synthetic seeds are ……...

   a). Easy to store without viability loss  b). Easy to handle

   c). Can be directly sown in soil  **d). All the above**

9. Tissue culture is used for ……

   a). Vegetative multiplication  b). Virus-free plants

**c). Both a and b** d). None of the above

10. Tissue culture has potential application in ……

a). Production of somatic hybrids b). Organelle and cytoplasm transfer

c). Genetic transformation **d). All the above**

11. Virus free plants in tissue culture are produced by ……..

**a). Meristem tip culture** b). Shoot tip culture

c). Nodal culture d). All the above

12. Tissue culture is used for …..

a). Production of virus free plants b). Rejuvenation of old plant materials

c). Hybrid production **d). All the above**

13. Cryo preservation of tissue culture materials in liquid nitrogen is at

**a). $-196^{0}$ C** b). $-190^{0}$ C

c). $-96^{0}$ C d). $-90^{0}$ C

14. Dimethylsufoxide is used as …… in tissue culture.

**a). Cryoprotectant** b). Growth regulator

c). Osmaticum d). None of the above

**Additional reading…**

http://www.biotechnology4u.com/plant_biotechnology_applications_cell_tissue_culture.html