Plant tissue culture: is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation.

Applications

Plant tissue culture is used widely in the plant sciences, forestry, and in horticulture. Applications include:

-The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.

-To conserve rare or endangered plant species.

-A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.

-Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.[7]

-To cross distantly related species by protoplast fusion

-To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example in vitro selection for stress tolerant plants.[8]

-To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (Embryo Rescue).

-For chromosome doubling and induction of polyploidy.

As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.

-Certain techniques such as meristem tip culture can be used to produce clean plant material from virused stock, such as potatoes and many species of soft fruit.

-Production of identical sterile hybrid species can be obtained.

-The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.

-To quickly produce mature plants.

-The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.

-The regeneration of whole plants from plant cells that have been genetically modified.

-The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.

-The production of plants from seeds that otherwise have very low chances of germinating and growing..

-To clear particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

-micropropagation using meristem and shoot culture to produce large numbers of identical individuals

-screening programmes of cells, rather than plants for advantageous characters

-large-scale growth of plant cells in liquid culture as a source of secondary products

-crossing distantly related species by protoplast fusion and regeneration of the novel hybrid

-production of dihaploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes

-as a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants

-removal of viruses by propagation from meristematic tissues

History of Plant Tissue Culture and Biotechnology: Biotechnology is name given to the methods and techniques that involve the use of living organisms like bacteria, yeast, plant cells etc. or their parts or products as tools (for example, genes and enzymes). They are used in a number of fields: food processing, agriculture, pharmaceutics, and medicine, among others. Plant tissue culture can be defined as culture of plant

seeds, organs, explants, tissues, cells, or protoplasts on nutrient media under sterile conditions.In 1902, a German physiologist, Gottlieb Haberlandt developed the concept of in vitro cell culture. He isolated single fully differentiated individual plant cells from different plant species like palisade cells from leaves of Laminum purpureum, glandular hair of Pulmonaria and pith cells from petioles of Eicchornia crassiples etc and was first to culture them in Knop's salt solution enriched with glucose. In his cultures, cells increased in size, accumulated starch but failed to divide. Therefore, Haberlandt's prediction failed that the cultured plant cells could grow, divide and develop into embryo and then to whole plant. This potential of a cell is known as totipotency, a term coined by Steward in 1968. Despite lack of success, Haberlandt made several predictions about the requirements in media in experimental conditions which could possibly induce cell division, proliferation and embryo induction. G Haberlandt is thus regarded as father of tissue culture. Taking cue from Haberlandt's failure, Hannig (1904) chose embryogenic tissue to culture. He excised nearly mature embryos from seeds of several species of crucifers and successfully grew them to maturity on mineral salts and sugar solution. In 1908, Simon regenerated callus, buds and roots from Poplar stem segments and established the basis for callus culture. For about next 30 years (upto 1934), there was very little further progress in cell culture research. Within this period, an innovative approach to tissue culture using meristematic cells like root and stem tips was reported by Kolte (1922) and Robbins (1922) working independently. All these research attempts involving culture of isolated cells, root tips or stem tips ended in development of calluses. There were two objectives to be achieved before putting Haberlandt's prediction to fruition. First, to make the callus obtained from the explants to proliferate endlessly and second to induce these regenerated calluses to undergo organogenesis and form whole plants. It was in 1930s, when progress in plant tissue culture accelerated rapidly owing to an important discovery that vitamin B and natural auxins were necessary for the growth of isolated tissues containing meristems. This breakthrough came from White (1934) who reported that not only could cultured tomato root tips grow but could be repeatedly subcultured to fresh medium of inorganic salts supplemented with yeast extract. He later (1937) replaced YE by vitamin B namely pyridoxine, thiamine and proved their growth promoting effect. In 1926, Fritz Went discovered first plant growth regulator (PGR), indoleacetic acid (IAA). IAA is a naturally occurring

member of a class of PGRs termed ‘auxins’. Roger J Gautheret (1934) reported the successful culture of cambium cells of several tree species to produce callus and 2 addition of auxin enhanced the proliferation of his cambial cultures. Further research by Nobecourt (1937), who could successful grow continuous callus cultures of carrot slices and White (1939) who obtained similar results from tumour tissues of hybrid Nicotiana glauca x N langsdorffii. Thus, the possibility of cultivating plant tissues for an unlimited period was independently endorsed by Gautheret, White and Nobecourt in 1939. Adding to the ongoing improvements in the culture media, Johannes Van Overbeek (1941) reported growth of seedlings from heart shaped embryos by enriching culture media with coconut milk besides the usual salts, vitamins and other nutrients. This provided tremendous impetus for further work in embryo culture. Stem tip cultures yielded success when Ernest Ball (1946) devised a method to identify the exact part of shoot meristem that gives rise to whole plant. After 1950, there was an immense advancement in knowledge of effect of PGRs on plant development. The fact that coconut milk (embryo sac fluid) is nutritional requirement for tobacco callus besides auxin, indicated the non auxinic nature of milk. This prompted further research and so other classes of PGRs were recognized. Skoog and Tsui (1957) demonstrated induction of cell division and bud formation in tobacco by adenine. This led to further investigations by Skoog and Miller (1955) who isolated ‘kinetin’- a derivative of adenine (6- furyl aminopurine). Kinetin and many such other compounds which show bud promoting activities are collectively called cytokinins, a cell division promoter in cells of highly mature and differentiated tissues. Skoog and Miller worked further to propose the concept of hormonal control of organ formation (1957). Their experiment on tobacco pith cultures showed that high concentration of auxin prmoted rooting and high kinetin induces bud formation or shooting. However, now the concept is altered to multiple factors like source of plant tissue, environmental factors, composition of media, polarity, growth substances being responsible for determination of organogenesis. Besides PGRs, scientists tried to improve culture media by differing essentially in mineral content. In this direction, Murashige and Skoog (1962) prepared a medium by increasing the concentration of salts twenty-five times higher than Knops. This media enhanced the growth of tobacco tissues by five times. Even today MS medium has immense commercial application in tissue culture. Having achieved success and expertise in growth of callus cultures from explants under

in vitro conditions, focus now shifted to preparation of single cell cultures. Muir (1953-54) demonstrated that when callus tissues were transferred to liquid medium and subjected to shaking, callus tissues broke into single cells. Bergmann (1960) developed a technique for cloning of these single cells by filtering suspension cultures. This technique called Plating technique is widely used for cloning isolated single protoplasts. Next step for realization of Haberlandt's objectives was development of whole plant from the proliferated tissue of these cells. Vasil and Hilderbrandt were first to regenerate plantlets from colonies of isolated cells of hybrid Nicotiana glutinosa x N tabacum. In 1966, the classical work of Steward on induction of somatic embryos from free cells in carrot suspension cultures brought an important breakthrough by finally demonstrating totipotency of somatic cells, thereby validating the ideas of Haberlandt. This ability of regenerating plants from single somatic cells through normal developmental process had great applications in both plant propagation and also 3 genetic engineering. For e.g. micropropagation where small amounts of tissue can be used to continuously raise thousand more plants. Morel utilized this application for rapid propagation of orchids and Dahlias. He was also the first scientist to free the orchid and Dahlia plants from virus by cultivating shoot meristem of infected plants. The role of tissue culture in plant genetic engineering was first exemplified by Kanta and Maheshwari (1962). They developed a technique of test tube fertlization which involved growing of excised ovules and pollen grains in the same medium thus overcoming the incompatibility barriers at sexual level. In 1966, Guha and Maheshwari cultured anthers of Datura and raised embryos which developed into haploid plants initiating androgenesis. This discovery received significant attention since plants recovered from doubled haploid cells are homozygous and express all recessive genes thus making them ideal for pure breeding lines. Next breakthrough in application of tissue culture came with isolation and regeneration of protoplasts first demonstrated by Prof. Edward C Cocking in 1960. Plant protoplasts are naked cells from which cell wall has been removed. Cocking produced large quantities of protoplasts by using cell wall degrading enzymes. After success in regeneration of protoplasts, Carlson (1972) isolated protoplasts from Nicotiana glauca x N. langsdorfii and fused them to produce first somatic hybrid. Since then many divergent somatic hybrids have been produced. With the advent of restriction enzymes in early 1970s, tissue culture headed towards a new research area. The totipotent plant cells could now be

altered by insertion of specific foreign genes giving rise to genetically modified crops. In 1970, Smith and Nathans isolated first restriction enzyme from Haemophillus influenzae which was later purified and named Hind III. Same year witnessed other nobel prize winning discovery by Baltimore who isolated Reverse transcriptase from RNA tumor viruses. This is a useful enzyme in genetic engineering which functions to convert RNA to DNA and hence useful in construction of complementary DNA from messenger RNA. Another pathbreaking discovery establishing potential of genetic engineering came in 1972 when Paul Berg working at Stanford University produced first recombinant DNA in vitro by combining DNA from SV40 virus with that of lambda virus. This led to construction of first recombinant organism by Cohen and Boyer in 1973. Genetic engineering's potential was first exploited when a man made insulin gene was used to manufacture a human protein in bacteria. Agrobacterium tumefaciens plays a crucial role in plant genetic engineering. The involvement of this bacterium in crown gall disease in plants was recognized as early as 1907 by Smith and Townsend. However, it was in 1974, that Zaenen et al discovered that Ti plasmid is the tumor inducing principle of Agrobacterium. This was followed by its successful integration in plants by Chilton et al in 1977. Zambryski et al in 1980 isolated and studied the detailed structure of TDNA and its border sequences. Soon thereafter in 1984, transformation of tobacco with Agrobacterium was accomplished to develop transgenic plants. Simultaneously, there was an upsurge in development of techniques of genetic engineering in mid 1970s. Sanger et al(1977) and Maxam and Gilbert (1977) reported techniques for large scale DNA sequencing. This was followed by complete genome sequencing projects on many prokaryotes and eukaryotes like Haemophilus influenzae in 1995, E coli in 1997. Human genome was sequenced successfully in 2001, thus laying foundation of genomics which is the main focus of present day biotechnology.

Modes of Culture: The plant cells if cultured on a solid surface will grow as friable, pale brown, unorganized mass of cells called callus. Tissues and cells of plant cultured in a liquid medium aerated by agitation grow as suspension of single cells and cell clumps. For growth, the cells need to divide, whereas, the cells breaking up from explant are mature, non-dividing. Therefore, the differentiated tissue undergoes modifications to become meristematic. This phenomenon of a mature cell reverting back to meristematic state to form undifferentiated callus tissue is called dedifferentiation.

(i) Callus culture: The culture of undifferentiated mass of cell on agar media produced from an explant of a seedling or other plant part is called callus culture. For callus formation, auxin and cytokinins, both are required. Callus can be subcultured indefinitely by transferring a small piece of the same to fresh agar medium. Subculturing needs to be done every 3-5 weeks in view of cell growth, nutrient depletion and medium drying. The rate of growth of callus grown on solid agar medium is relatively slow. The new cells are formed on the periphery of existing callus mass. Consequently, callus consists of cells which vary considerably in age. Since nutrients are gradually depleted from the agar, a vertical nutrient gradient is formed. Because of low degree of uniformity among cells in callus, slower growth rate and development of nutrient gradients, the usefulness of callus in experimental system is limited. The main use of callus culture is for purposes of maintaining cell lines and for morphogenesis.

Callus culture

Seed germination

There are many chemical material used to sterilize surface of seeds such as alcohol, hypochloride (sodium or calcium, silver nitrate ,mercuric choride.Time and type of sterilized material differ with differing of plant seeds.

Media of growing seedlings

Arnon and Hogland media (1944) is the best media to grow the seeds which is composed macro and micro nutrients and ferrous citrate ,Also can used MS medium to

Composion of Arnon and Hogland medium

Sterilizing seeds incubated in incubation room in the dark .After root rising,seeds tranclated to the proper light conditions of 16 hours photoperiod and 8 hours dark period

Initiation of callus

There are three stages to initiate callus

1-Induction of growth

Fresh medium induces quiescent cells (stationary phase) to enter the cell cycle. DNA,RNA synthesis and protein synthesis increase in the cells(which is necessary in cell division).Number of cells and their size remain stable .Period of this stage depend on tissue of explant.

Media preparation

The plant tissue culture media most commonly used are available in the market as dry powders. The simplest methods of preparing media is to dissolve these powders (containing inorganic and organic nutrients in some quantity of distilled water after the contents have been mixed in water, sugar, agar and other organic supplements are added finally, the volume is made up to one liter. The pH is adiusted and the medium is autoclaved. Another convenient procedure is to prepare stock solutions when mixed together in appropriate quantities, constitute a basal media.

The composition of White,Murasigh and Skoog (MS) and B_5 media

Types of media

1- Solid media
2- Liquid media

Subculture

After a period of time, it become necessary, chiefly due to nutrient depletion and medium drying , to transfer organs and tissues to fresh medium. This is known as subculture.Callus culture are subculture every 4-5 weeks, depended on species of plant and on type of medium.

(ii) Cell suspension culture: The culture of tissues and cells cultured in a liquid nutrient

culture. A callus mass friable in texture is transferred to liquid medium and vessel is incubated on shaker to facilitate aeration and dissociation of cell clumps into smaller pieces. Gradually, over several weeks by subculturing, cells of callus dissociate and a liquid suspension culture is obtained. Cell suspensions are also maintained by subculturing of cells in early stationary phase to a fresh medium. Their growth is much faster than callus cultures and hence need to be subcultured more frequently (3-14days). Cell suspension cultures when fully established consist of a nearly homogeneous population. This system has an advantage that the nutrients can be continually adjusted and hence it is the only system which can be scaled up for large scale production of cells and even somatic embryos. The initiation of a cell suspension culture requires a relatively large amount of callus to serve as the inoculum, for example, approximately 3 2-3 g for 100 cm (Helgeson, 1979). When the plant material is first placed in the medium, there is an initial lag period prior to any sign of cell division (Fig. 9.1). This is

followed by an exponential rise in cell number, and a linear increase in the cell population. There is a gradual deceleration in the division rate. Finally, the cells enter a stationary or nondividing stage. In order to maintain the viability of the culture, the cells should be subcultured early during this stationary phase. Fig. 9.1. Growth curve of a cell suspension grown under batch conditions relating total cell number per unit volume to time.

Stationary / Progressive deceleration ,.t I E Linear C" I U It Lag Exponential Time 106 Experiments in plant tissue culture Because cells from different plant material vary in the length of time they remain viable during the stationary phase, it may be prudent tc subculture during the period of progressive deceleration. Passage time can be learned only from experience, and a given suspension culture should be subcultured at a time approximating the maximum cell density. For many suspension cultures the maximum cell density is reached within about 18 to 25 days, although the passage time for some extremely active cultures may be as short as 6 to 9 days (Street, 1977). At the time of the first subculture it will be necessary to filter the culture through a nylon net or stainless steel filter to remove the larger cell aggregates and residual inoculum that would clog the orifice of a pipette. A small sample should be withdrawn, and the cell density determined before subculturing. There is a critical cell density below which the culture will not grow; for example, this value is 9-15 X 10' cells/cm for a clone of sycamore cells (Acer pseuoplatanus) (Street, 1977)

The suspension cultures are broadly classified as:

Batch culture: The culture medium and the cells produced are retained in the culture flask. These cultures are maintained continuously by subculturing i.e. by transferring a small aliquot of inoculum from the grown culture to fresh medium at regular intervals. The biomass or cell number of a batch culture follows a typical sigmoidal curve, where to start with the culture passes through lag phase during which cell number is constant, followed by brief exponential or log phase where there is a rapid increase in cell number because of culture cell division. Finally, the growth decreases after 3-4 generations which is the doubling time (time taken for doubling of cell number) and culture enters stationary phase during which cell number again becomes static. The cells stop dividing due to depletion of nutrients and accumulation of cellular wastes. Batch cultures undergo a constant change in 11 cell density and metabolism and hence, not used for studies related to aspects of cell behavior. But batch cultures are convenient to

maintain, hence used for initiation of cell suspension and scaling up for continuous cultures.

Continuous culture: Here steady state of cell density is maintained by regularly replacing a portion of the used up medium with fresh medium. Continuous culture are further classified into two types:

1- Closed 2-Open

In closed type, the used medium is replaced with fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

In open type, both cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate. There are further two types of open continuous suspension culture: turbidostat and chemostat. In turbidostat, cells are allowed to grow upto a certain turbidity (decided on the basis of optical density) when a predetermined volume of the culture is replaced by fresh culture. On the other hand, in chemostat, the fresh culture medium to be added has one nutrient kept at a concentration so that it is depleted rapidly and becomes growth limiting while other nutrients are still in concentration higher than required. Increase or decrease in the concentration of growth limiting factor is correspondingly expressed by increase or decrease in growth rate of cells. Thus, the desired rate of cell growth can be maintained by adjusting the level of concentration of growth limiting factor with respect to that of other constituents. Chemostats are useful for the determination of effects of individual nutrients on cell growth and metabolism.

Types of Suspension Cultures

There are two types of suspension cultures,

A) Batch Culture:

a. Slowly rotating culture
b. Shake culture
c. Spinning culture
d. Stirred culture

B) Continuous Culture:

Organ culture

Different types of organs (e.g. roots, ovary, ovule, endosperm, anther) are excised from the plants. Then these are separately put over the surface of solidified gelled medium. The inoculated cultures are incubated in controlled growth chamber.

The cultures are named on the basis of organs used such as root culture, ovary culture, ovule culture, endosperm culture and another culture.

Micropropagation: In vitro propagation of plants vegetatively by tissue culture to produce genetically similar copies of a cultivar is referred to as micropropagation or clonal propagation. **Micro-propagation Methods**: The ability of mature cell to dedifferentiate into callus tissue and the technique of cloning isolated single cell in vitro discussed earlier in this chapter have demonstrated that the somatic cells can differentiate to a whole plant under particular conditions. This potential of cell to divide and develop into multicellular plant is termed as cellular totipotency. To express totipotency, after dedifferentiation, the cell has to undergo redifferentiation or

regeneration which is the ability of dedifferentiated cell to form plant or plant organs. This may occur through either of two processes: Organogenesis or Embryogenesis

Micropropagation involves following major stages:

Stage 0 Selection and maintenance of stock plants for culture initiation (3 months)

Stage I Preparation and establishment of explant on suitable culture medium (3-24 months) (usually shoot tips and axillary buds used)

Stage II Regeneration: multiplication of shoots or somatic embryos on defined (10-36 months) culture medium

Stage III Rooting of regenerated shoots/ somatic embryo in vitro (1-6 weeks)

Stage IV Transfer of plantlets to sterilized soil for hardening under greenhouse environment

(Stage III can be skipped for in vivo rooting of stage II regenerated shoots)

Advantages of micropropagation over conventional propagation methods

• Genotype constitution maintained as there is lesser variation in somatic embryo

• Easier transport and storage is facilitated by small size propagules and their ability to grow in soil less medium.

• Control over growing conditions as the production of planting material is completely under artificial control in vitro

• Reduced growth cycle and rapid multiplication as shoot multiplication has short cycle and each cycle results in exponential increase in number of shoots

• Selective multiplication can be done for e.g. auxotrophs, aneuploids, selected sex in dioecious species

• Virus free plants can be raised and maintained through meristem culture which is the only method available for this

Disadvantages:

• Involves high cost

• Somaclonal variation- any variation if occurs during multiplication may go unnoticed

• Recalcitrancy of species/ genotype- many tree sp like mango etc do not respond to in vitro growth

Application of micropropagation:

1. Commercial production of secondary metabolites

2. Production of synthetic seeds: Synthetic seed is a bead of gel containing somatic embryo or shoot bud with growth regulator, nutrients, fungicides, pesticides etc needed for development of complete plantlet.

3. Raising somaclonal variants: The genetic variability occurring in somatic cells, plants produced in vitro by tissue culture are referred to as somaclonal. When these variations involve traits of economic importance, these are raised and maintained by micropropagation.

4. Production of disease free plants: Most of the horticultural fruit and ornamental crops are infected by fungal, viral, bacterial diseases. Micropropagation provides a rapid method for production of pathogen free plants. In case of viral diseases especially, the apical meristems of infected plants are free or carry very low concentrations of viruses. Thus culturing meristem tips provides disease free plants.

(i)Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds. These shoot buds are monopolar structures which in turn give rise to leaf primordial and the apical meristem. The buds have procambial strands connected with preexisting vascular tissue present in the explant or callus. The stimulation of shoot bud differentiation in plants depends on many factors which differ for different plant species. In general, it is promoted by cytokinin and inhibited by auxins. The classical studies of Skoog and 15 Miller (1957) demonstrated that the relative ratio of CK and auxin is important in determining nature of organogenesis in tobacco pith tissue. In tobacco, high level of CK initiates bud formation while high concentration of auxin favours rooting. But there have been studies in other plant species which donot follow this concept of auxin/CK ratio. In most cereals, callus tissue exhibits organogenesis when it is subcultured from a medium containing 2,4-D to a medium whre 2,4-D is replaced by IAA or NAA. GA3, which in general has inhibitory effect on shoot buds whereas many

species show enhanced shoot regeneration due to abscissic acid. The variable responses of different plant species to the growth regulators is because the requirement of exogenous GRs depends on their endogenous levels which might differ in different plant species and also in different plant materials.

Other factors affecting organnogenesis are size and souce of the explant. The larger the explant (containing parenchyma, cambium and vascular tissue), more is likelihood of shoot bud formation. Also, genotype of explant affects shoot regeneration as explant taken from different plant varieties of same species show different frequencies of shoot bud differentiation. Light has been shown to have inhibitory effect. Even the quality of light has effect as blue light has been shown to induce shoot formation and root by red light in tobacco. The optimum temperature required may vary with plant species.

(ii)Somatic embryogenesis: is a process involving redifferentiation of meristematic cells into nonzygotic somatic embryo which are capable of germinating to form complete plants. Somatic embryos are bipolar structures with radical and plumule is contrast to monopolar shoot bud with only plumular end in organogenesis. While developing into somatic embryo, the mersitematic cells break any cytoplasmic or vascular connections with other cells around it and become isolated. Therefore, unlike shoot bud, the somatic embryos are easily separable from explants.

Somatic embryogenesis involves three distinct steps which are absent in organogenesis:

• Induction: is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called embryogenic clumps (ECs). These ECs develop into initial stages of somatic embryo i.e. globular stage.

• Maturation: In this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped, torpedo to cotyledonary stages. The mature embryo here undergoes biochemical changes to acquire hardiness.

• Conversion: Embryos germinate to produce seedlings. Somatic embryogenesis is influenced by following factors:

• Growth regulators: The presence of auxin (generally 2,4-D) in the medium is essential for induction phase. 2,4-D induces dedifferentiation of explant cells to form ECs. When auxin is removed or its concentration is reduced, ECs

convert to somatic embryos. Once induced, cells don't need PGRs. Still some doses of CK at maturation and conversion make better plants. Maturation is achieved by culturing somatic embryos on high sucrose medium. Also, ABA is added as it gives hardening due to water loss which is important for embryo maturation.

Ethylene inhibits both somatic embryogenesis and organogenesis. Therefore, silver nitrate is added to the medium as inhibitor of ethylene for plant regeneration.

• Nitrogen source: NH_4^+ form of nitrogen is essential for induction of somatic embryogenesis while NO_3^- form is required during maturation phase.

• Other factors: Like shoot bud differentiation, explant genotype has influence on somatic embryogenesis also. In cereals, use of maltose as carbohydrate source promotes both somatic embryo induction and maturation.

In vitro culture Applications

• Protoplast

• Clonal plant propagation

• Virus-free plants

• Genetic modified plants

• Germoplasm bank

• Somatic fusion

• Synthetic seeds

Protoplast Fusion and Somatic Hybridization: Purified protoplasts once obtained from any two different sources (can be different tissues, different plants or species or different genera), they can be fused together to form somatic hybrids. This non-conventional method of genetic recombination involving protoplast fusion under in vitro conditions and subsequent development of their product to a hybrid plant is known as somatic hybridization. First, somatic hybrid plant of Nicotiana glauca (+) N. langsdorfii was reported by Carlson in 1972. Protoplasts can be induced to fuse by variety of fusogens or electrical manipulations which induce membrane instability. Most commonly reported fusion inducing agents are sodium nitrate (used by Carlson), high pH/Ca2+ concentration and Polyethylene glycol (PEG) treatment. Sodium nitrate treatment results in low frequency of heterokaryon formation, high pH and high Ca2+ concentration suits few plant species whereas PEG is the most favoured fusogen for its reproducible high frequency of heterokaryon formation and low toxicity. However, treatment with PEG in presence of high pH/ Ca2+ is reported to be most effective in enhancing heterokaryon formation and their survivability. A more selective, simpler, quick and non toxic approach is electrofusion which utilizes electric shock or short pulse of high voltage to promote membrane fusion between two cells.

Application of Somatic Hybridization:

• Genetic recombination in asexual or sterile plants: Protoplast fusion has overcome the impediment of reproduction in haploid, triploid and aneuploid plants. Also, genomes of asexually reproducing plants can be recombined using this approach viz.protoplasts isolated from dihaploid potato clones have been fused with protoplasts of S. brevidens to produce hybrids of practical breeding value.

• Genetic recombination between sexually incompatible species: The incompatibility barriers in sexual recombination at interspecific or intergeneric levels are also overcome by somatic hybrisation. Generally, somatic hybrids are used for transfer of useful genes such as disease resistance, abiotic stress resistance or genes of industrial use for e.g. Datura hybrids (D. innoxia + D discolor, D. innoxia + D stramonium) show heterosis for scopolamine (alkaloid) content which is 20-25% higher than in parent species and therefore has industrial application.

• Cytoplasm transfer: Somatic hybridization minimizes the time taken for cytoplasm transfer to one year from 6-7 years required in back cross method. Also, this method allows cytoplasm transfer between sexually incompatible species. Cybrids have cytoplasm from both parents but nucleus of only one. Nucleus of other parent is irradiated. This approach has been potentially used to transfer two desirable traits – cytoplasmic male sterility (CMS) and resistance to atrazine herbicide, both coded by cytoplasmic genes in Brassica to different crops like tobacco, rice etc.

Anther Culture Culturing anther on a suitable media to regenerate into haploid plants is called anther culture. First time, haploid plants were discovered in Datura stramonium by A.D. Bergner in 1921. Guha and Maheshwari (1964) pioneered the formation of embryos from anthers of Datura innoxia grown in vitro. After this, haploid plants have been produced via anther culture in more than 170 species. The anther culture technique is useful in haploid production. Haploid production: Haploid plant is defined as a sporophyte with gametophytic chromosome number. The in vitro production of haploid plants can be achieved by many techniques like:

• Delayed pollination which may not result in fertilization and hence only female genome grows up to form a haploid plant.

• Temperature shock – Extremes of temperature (both high and low) are used to suppress syngamy or make pollen inactive, thus leading to induction of haploidy.

• Irradiation effect - X rays, UV rays induce chromosomal breakage in pollen cells thus making them sterile which in turn results in haploid production.

• Chemical treatment – Treatment with colchicines, maleic hydrazide and toluene blue etc also induces chromosomal elimination.

• Genome elimination by distant hybridization – In case of distant crosses like inter-generic and inter-specific crosses where during the developmental process, one of the parental genomes is selectively eliminated subsequently leading to formation of haploid plants. Therefore, production of a haploid plant where egg cell is inactivated and only male genome is present is called androgenesis. Similarly, production of haploid by development of unfertilized egg cell due to inactivation of pollen is called gynogenesis. Among all the methods illustrated above, anther culture is the most popular and successful for haploid production.

Anther culture procedure:

Step1 Experimental material: Young healthy plants grown under controlled conditions are used as experimental material from which flower bud of right stage (varies with species) is excised.

Step2 Disinfestation, excision and culture of anther: Flower buds are surface sterilized in laminar flow chamber followed by excision of anther from the bud. Stage of pollen 17 development is determined by squashing an anther in acetocarmine and observing it under microscope. While excising anthers from flower buds, care is taken that anthers are not injured as injury leads to callusing hence giving mixture of diploids, haploids and aneuploids.

Step3 Culture medium conditions: The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period. Medium should have sucrose for induction of embryogenesis.

Step 4 Haploid plants: In species following direct androgenesis i.e. which develop through embryoid formation, small plants emerge in 3-8 weeks after culturing which are then transferred on to a rooting medium with low salt and small amount of auxin. Those species undergoing indirect androgenesis involving callus formation, callus is removed from the anther and placed onto

a regeneration medium with suitable ratio of cytokinin to auxin. The haploid plants thus produced in both cases are transplanted to soil in small pots and maintained under controlled conditions in greenhouse

Diploidisation of haploid plants: Haploid plants produced from anther culture maintained in vitro can grow till the flowering stage but cannot be perpetuated. Since these plants are haploid and have only one set of homologous chromosomes of the diploid species, they cannot form viable gametes and hence no seed setting takes place for further perpetuation. Therefore, it is necessary to double the chromosome number of haploids to obtain homozygous diploids or dihaploid plants followed by their transfer to culture medium for further growth. Application of haploid production: Diploidisation of haploid plants result in rapid achievement of homozygous traits in doubled haplouds, hence these anther derived haploid plants have been used in breeding and improvement of crop species.

1. Production of homozygous lines: The most important use of haploids is the production of homozygous lines which may be used directly as cultivars or may be used in breeding programme. For e.g. doubled haploids have been used for rapid development of inbred lines in hybrid maize programme. The anthers from F1 hybrids of selected or desirable cross are excellent breeding material for raising anther derived homozygous plants or doubled haploids in which complementary parental characteristics are combined in one generation. The doubled haploid plants are subjected to selection for superior plants (Fig.2). This approach is described as hybrid sorting where recombinant superior gametes are virtually being selected since the heterozygous gene combination in the F1 hybrid is transformed into homozygous combinations. Hybrid sorting reduces the time required for haploid breeding by 4-5 years as in conventional breeding by pedigree/ bulk method, the same requires ten years. Also, selection among DH lines reduces the size of breeding population. 18 Year 1 Parent A X Parent B Year 2 F1 Anther culture Haploid plants (greenhouse) Year 3 Doubled haploid plants Selection Year 4-6 Superior progenies Selection Superior progeny released as variety Fig. 2: Anther culture derived haploid plants for hybrid sorting

2. Gametoclonal variation: The variation observed among haploid plants having gametic chromosome number developing from anther culture is called gametoclonal variation. Such variations resulting in desirable traits are subjected to selection at haploid level followed by diploidisation to get homozygous plants which can be released as new varieties.

3. Selection of desirable mutants: Haploids offer a system where even recessive mutations are expressed unlike diploids where they express only in segregrating single plant progeny in M2 generation. Therefore, in several crops desirable mutants including traits like resistance to diseases, antibiotics, salts etc have been isolated from haploids derived from anther culture. For e.g. tobacco mutants resistant to black shank disease and wheat lines resistant to scab (Fusarium graminearum) have been selected and used as improved cultivars.

Problems associated with haploid plants:

• Many species are not yet amenable for haploid production

• Deleterious mutations may be induced during in vitro phase.

• Plants having more or lesser than gametic chromosome number is also obtained which necessitates cytological analysis first.

• Occurrence of gametoclonal variation limits the use of anther derived embryos for genetic transformation The major advantages of cell culture systems over the conventional cultivation of whole plants are:

• Higher and quicker yields of product from very small amount of plant material needed to initiate the culture in contrast to large amounts of mature plant tissues processed to achieve 19 low yields of final product, for e.g. the dry weight of shikonin produced from cell culture is 20% more than from plants.

• In case of plant material facing threat of extinction or are limited in supply like L erythrorhizon, in vitro production of secondary metabolites is saving option.

• Controlled environmental conditions in cell culture ensure continuous supply of metabolites. In conventional system, source plant may be seasonal, location specific and also subject to environmental degradation. Also, in vitro culture of cells is more economical for those plants which take long to achieve maturity.

• Bioconversion: Low cost precursors are supplied as substrates to cell cultures for conversion to the high cost final product, thus minimizing labor, cost and time. Also, specific substrates can be biotransformed to more valuable product by single step reaction in vitro.

• Production of novel compounds: Mutants cell lines can be utilized to produce novel compounds which were not previously found naturally in plants viz.cell suspension cultures of Rauwolfia serpentina produce novel glucosides of ajmaline (alkaloid)

Major causes of Somaclonal Variation:

• Physiological: Variations induced by physiological factors in culture medium for e.g. prolonged exposure to PGRs (2,4-D; 2,4,5-T) results in variability among the regenerants. Often such variations are epigenetic and hence donot follow Mendelian inheritance.

• Genetic: All the alterations at chromosomal level are grouped under genetic cause of variation observed in regenerants. Chromosomal rearrangements such as deletion, duplication, translocation, inversion polyploidy, aneuploidy, have been reported to be the chief source of somaclonal variation. Meiotic crossing over involving symmetric and asymmetric recombination could also be responsible for variation observed among somaclones. Transposable elements like Ac-Ds in maize have been shown to get activated in in vitro culture. In maize (Zea mays L.) and broad beans (Vicia faba L), late replicating heterochromatin is the main cause of somaclonal variation. Single gene

mutations in cultures also give rise to variations which are not detected in plants regenerated in vitro from any cell or tissue (R0 plants) but express in R1 plants (after selfing R0 plants).

• Biochemical: The most common kind of biochemical variation is change in carbon metabolism leading to failure of photosynthesis viz. albinos is rice. Any variation in other cell processes like starch biosynthesis, carotenoid pathway, nitrogen metabolism, antibiotic resistance etc also lead to somaclonal varaiation.

Application in Crop Improvement: Somaclonal variation represent useful source for introduction of valuable variations to plant breeders. Cell culture systems are well defined controlled environments, away from limitations of availability of space, time and variations due to environmental effects which are major bottlenecks in conventional mutation breeding. Somaclonal variation occurs at much higher frequencies than induced mutants which are associated with undesirable features. Cell culture systems allow plant breeder to have greater control on selection process as here they have the option to select from large amount of genetically uniform material. Therefore, this is the only approach for genetic improvement in perennial species limited by narrow germplasm and long regeneration cycle, asexually propagated plants like bananas, for isolation of biochemical mutants like auxotrophs. Somaclonal variants have been isolated for variety of valuable traits like disease resistance, stress (salt, low temperature) resistance, improved yield and efficient nutrient uptake etc. Bio-13 is a somaclonal variant of Citronella java, a medicinal plant which yields 37% more oil and 39% more citronellol than the control varieties. Pusa Jai Kisan, with bolder seeds and higher yield developed at Indian Agricultural Research Institute is another successful application of somaclonal variation of Brassica juncea variety 'Varuna'. Somaclonal variants in Lathyrus with low toxin level have also been developed at Indian Agricultural Research Institute.

Germplasm Conservation The genetic material especially its molecular and chemical constitution that is inherited and transmitted from one generation to other is referred to as germplasm. In other words, the sum total of all the genes present in a crop and its related species constitutes its germplasm. It is 25 generally represented by a collection of various strains and species. Germplasm is valuable because it contains diversity of genotypes that is needed to develop new and improved genetic stocks, varieties and hybrids. Therefore, germplasm is the basic indispensable ingredient of all breeding programmes and great emphasis is placed on collection, evaluation and

conservation of germplasm. The continuing search for high yielding varieties of crop plants with resistance to biotic and abiotic stresses necessitates the conservation of germplasm of different crops and their wild and weedy relatives.

A) In- situ conservation: In situ (on-site) conservation refers to the maintenance and use of wild plant populations in the habitats where they naturally occur and have evolved without the help of human beings. The wild populations regenerate naturally and are also dispersed naturally by wild animals, winds and in water courses. There exists an intricate relationship, often interdependence, between the different species and other components of the environment (such as their pests and diseases) in which they occur. The evolution is purely driven by environmental pressures and any changes in one component affect the other. Provided that changes are not too drastic, this dynamic co-evolution leads to greater diversity and better adapted germplasm. The conservation of the forests and other wild plant species is often carried out through protected areas such as national parks, gene sanctuary and nature reserves. However, this mode of conservation has certain limitations such as there is risk of loss of material due to environmental hazards.

(B) Ex-situ conservation: Ex situ (off-site) conservation of germplasm takes place outside the natural habitat or outside the production system, in facilities specifically created for this purpose. This is the chief mode of preservation of genetic resources for both cultivated and wild material. The most convenient method of ex-situ germplasm conservation is in the form of seeds. Thus, majority of field crops and vegetables which produce orthodox (dessication tolerant) seeds are conserved in gene banks by reducing their moisture content (3-7%) and storing under low humidity and low temperature. In case of crops with dessication sensitive or recalcitrant seeds (which lose their viability after being dried below a critical limit) and also in vegetatively propagated crops,

in vitro methods are the most useful for germplasm conservation. This tissue culture based method has been mainly utilized for conservation of somaclonal and gametoclonal variations in cultures, plant material from endangered sp., plants of medicinal value, storage of pollen, storage of meristem culture for production of disease free plants and genetically engineered materials.

In vitro Germplasm conservation: Germplasm can be stored in vitro in variety of forms including isolated protoplasts, cells from suspension or callus

cultures, meristem tips, somatic embryos, shoot tips or propagules at various stages of development.

Methods for in vitro germplasm conservation are classified into two groups based on culture growth:-

1. Slow growth cultures: where limited growth of culture is allowed. This is a simple, effective and economic method and can be used in all species where shoot tip/ nodal explant are available. In thses techniques, growth is suspended by either cold storage or lowering oxygen concentration. Such methods require serial subculturing for periodic renewal of cultures. The storage of germplasm by repeated cultures has some disadvantages like during subculturing there is risk of contamination by pathogen, genetic changes may also occur.

2. Cryopreservation: Any growth in plant cell and tissue culture is brought to a halt still retaining its viability in this technique by storing at ultra low temperature (-196°C) using liquid nitrogen. This method, also called freezepreservation, is most popular and effective for indefinite storage. Cryopreservation for germplasm purposes utilizes shoot tips and buds only but protoplasts, cells, tissues and somatic embryos are also cryopreserved for other tissue culture processes.

Factors affecting viability of cells frozen for cryopreservation:

• Physiological state of material: Cells in the late lag or exponential phase are considered ideal for freeze preservation. After thawing, these cytoplasm rich cells are able to retain their viability and grow again from the actively dividing meristematic cell component. But in shoot tips, embryos etc, tissue is large with highly vacuolated cells which get damaged by freezing and are unable to recover back.

• Prefreezing treatment: Conditioning treatment given to cells before freezing results in their hardening and increased survival rates. Such hardening treatments include growing culture in presence of cryoprotectant or growing at low temperature (4°C) (for cold dormant sp) or in presence of osmotic agents like sucrose. These treatments function by either changing the cell water content, metabolite content or membrane permeabilites.

• Cryoprotectants: are chemicals imparting protection to withstand low temperature. For plants, most frequently used cryoprotectant is Dimethyl sulphoxide (DMSO). About 5-10% of DMSO is prepared and added gradually

to prevent plasmolysis of the cells. Other commonly used cryoprotectants include glycerol, polyvinyl pyrollidone, polyethylene glycol (PEG) etc.

• Thawing rate and reculture: For better survival of preserved samples, rapid thawing from - 196°C to about 22°C is recommended. By thawing rapidly, the damaging effects of ice crystal formation (crystallization of cell water while freezing) are minimized. These thawed samples during reculturing require special growth conditions, for enhanced recovery rates like dim light, high osmoticum, gibbrellic acid, and activated charcoal in the medium. Methods of Cryopreservation: The sensitivity of cells to low temperature varies with the species. However, usually the sample to be preserved are treated with suitable cryoprotectant and then frozen by any one of the following methods:

• Rapid freezing: The vials with plant materials are directly dipped in liquid nitrogen. The temperature lowers very fast at the rate of 200°C/minute. It is a very hard treatment and hence survival rate is low. However, this method has been successful for germplasm conservation of large number of species where plant material with small size and low water content has been chosen.

• Controlled freezing: The plant material is cooled stepwise from room temperature to intermediate temperature (-20°C) maintained at that temperature for thirty minutes followed with rapid freezing by dipping into liquid nitrogen. This is a reliable method and is applicable to wide range of plant materials including shoot apices, buds and suspension cultures.

Advantages of Cryopreservation:

• Indefinite preservation as metabolism comes to halt

• Low maintenance as only liquid nitrogen needs to be replenished

• No contamination

• Applicable to all species amenable to tissue culture

Limitations:

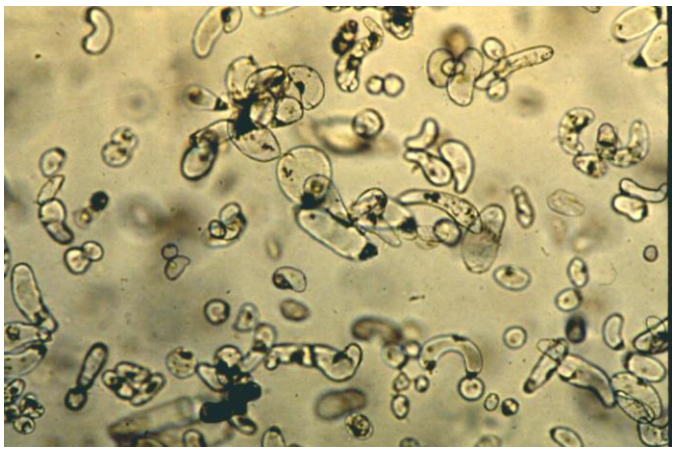
• Sophisticated equipment and facilities required

• Expertise needed

• Cells/tissues get damaged due to ice crystal formation or high solute concentration during dessication.

Cell Suspension Culture

Suspension culture is a type of culture in which single cells or small aggregates of cells multiply while suspended in liquid medium. It is also referred to as cell culture or cell suspension culture. Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Such systems contribute to our understanding of the interrelationships and complementary influences of cells in multicellular organisms. Many plant biotechnologists recognized the merits of applying cell cultures over an intact organ or whole plant cultures to synthesize natural products.



Brief history

- The attempts by **Haberlandt** failed to achieve divisons in free cells, but his detailed paper in 1902 stimulated further studies in this area.
- **H. Muir** (1953)– First reported that the fragments of callus ***Nicotiana tabacum*** could be cultured in the form of cell suspension
- **C. Steward** and **E. M. Shantz** (1956)– reported the suspension cultures from carrot root explants and obtained very large number of plantlets from the culture.

Principles of cell suspension culture

1. To achieve an ideal cell suspension most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses.
2. After eliminating the large cellular pieces, only single cells and small cell aggregates are again transferred to fresh medium and after 2 or 3 weeks a suspension of actively growing cells are produced.

3. In culture, the single cells divide to form a callus tissue. Such callus tissue also retains the capacity to regenerate the plantlets through organogenesis and embryogenesis.

Isolation of single cells

From plant organs

The most suitable material for the isolation of single cells is the **leaf tissue**, since a more or less homogenous population of cells in the leaves offer good material for raising defined and controlled large scale cell cultures.

Two important methods to isolate single cells from leaf are:

1. **Mechanical Method**
2. **Enzymatic Method**

From cultured tissues

The most widely applied approach is to obtain a single cell system from cultured tissues.

From plant organs

1. **Mechanical Method**

Gnanam and Kulandaivelu (1969) developed a procedure which has since been successfully used to isolate mesophyll cells active in photosynthesis and respiration, from mature leaves of several species of dicots and monocots including the grasses.

The procedure involves:

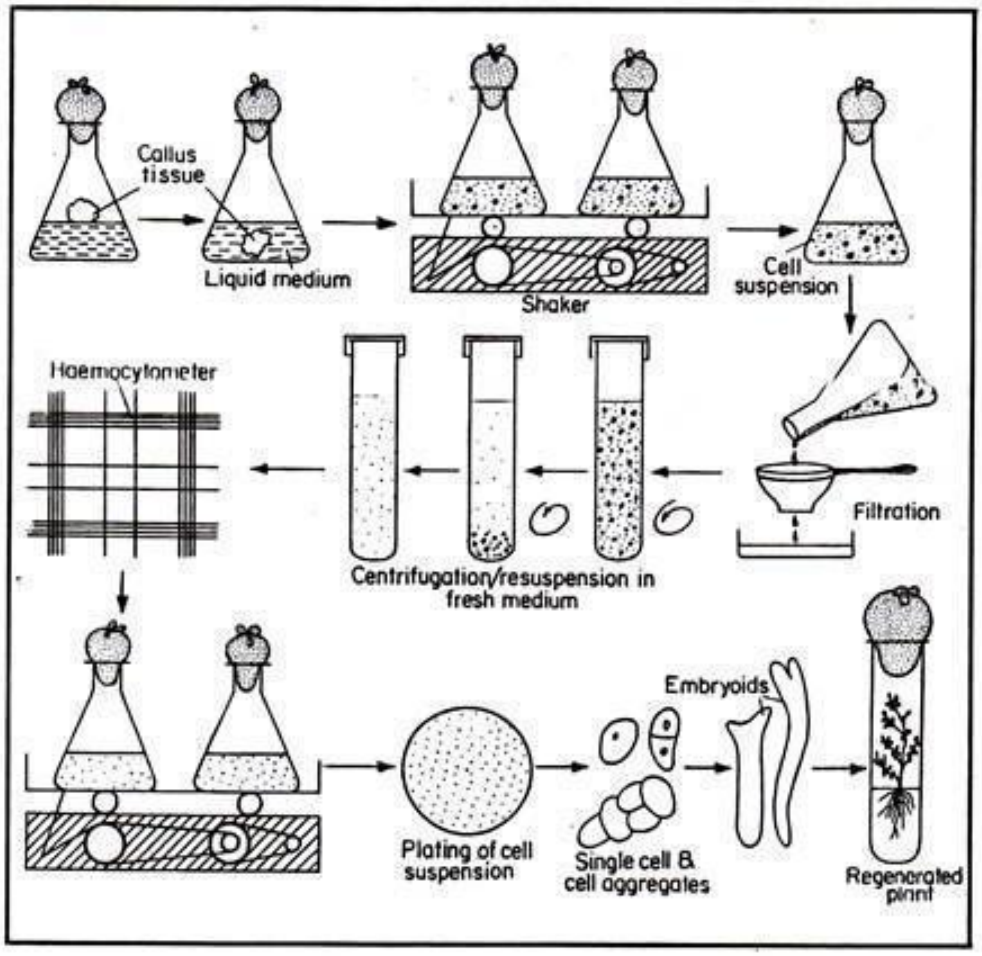
1. Mild maceration of 10g leaves in 40ml of the grinding medium (20µ mol. Sucrose, 10µ mol $MgCl_2$, 20µ mol tris HCl buffer, pH 7.8) with a mortor and pestle.
2. The homogenate obtained is passed through two layers of muslin cloth and the cells thus released are washed by centrifugation at low speed using the medium.

2. **Enzymatic method**

In 1968 Takabe et al treated tobacco leaf tissue with the enzyme pectinase and obtained a large number of metabolically active cells. **Isolation of single cells by the enzymatic method has been found convenient as it is possible to obtain high yields from preparations of spongy parenchyma with minimum damage or injury to the cells.** This can be accomplished by providing osmotic protection to the cells while providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of the parenchymatous tissue.

From cultured tissues

1. Raise sterile tissue culture plants and obtain callus from them.
2. The callus is separated from an explant and transferred to a fresh medium of the same composition.
3. Repeated subculture on an agar medium improves the friablity of a callus.
4. The pieces of undifferentiated and friable callus are transferred in a continuously agitated liquid medium dispersed in autoclaved flasks or other suitable vials.
5. Agitation is done by placing the flasks on shaker or suitable device.
6. Movement of the culture medium mild pressure on small pieces of tissues breaking them into free cells and small aggregates. Further it augments the gaseous exchange between the culture medium and the culture air and also ensures uniform distribution of cells in the medium.
7. The concentration of auxins and cytokinins is often critical for the growth of cell suspension and the concentration of auxin and cytokinins used for callus culture is generally reduced for suspension culture.
8. The cells in the cell suspension may vary in shapes and sizes. They may be oval, round, elongated or coiled.



❒ Fig 4.1

Flow diagram illustrating the method of cell suspension culture and regeneration of plant through embryogenesis

Types of cell suspension culture:

There are two types of suspension cultures:

1. **Batch culture**
2. **Continuous culture**

A) Batch culture:

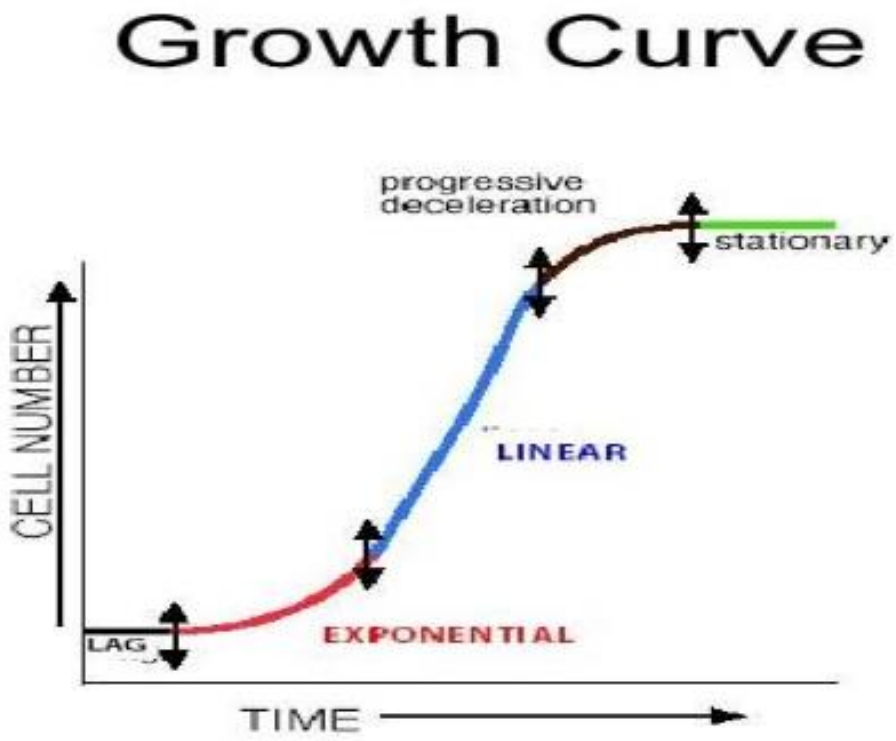
Here the cell material grows in a finite volume of agitate liquid medium. For instance, cell material in 20 ml or 40 ml or 60 ml liquid medium in each passage constitute a batch culture.

Batch suspension cultures are most commonly maintained in conical flasks incubated on orbital platform shakers at the speed of 80 – 120 rpm.

The biomass or cell number of a batch culture follows a typical curve

- The cells in culture exhibit the following five phases of a growth cycle
- 1. Lag phase : where cells prepare to divide.
- 2. Exponential phase : where the rate of cell division is highest.
- 3. Linear phase :where cell division slows but the rate of cells expansion increases.
- 4. Deceleration phase : where the rates of cell division and elogation decreases.
- 5. Stationary phase : where the number and size of cells remain constant.

Growth curve in batch culture



Different types of batch culture are

1. Slowly rotating cultures
2. Shake cultures
3. Spinning cultures
4. Stirred culture

1) Slowly rotating cultures:

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple-like projections. The capacity of each flask is 250 ml. Ten flasks are loaded in a circular manner on a large flat disc of a vertical shaker. When the flat disc rotates at the speed of 1-2 rp, the cell within each nipple of the flask are alternatively bathed in a culture medium and exposed to air.

2) Shake culture:

It is very simple and effective system of suspension culture. In this method, single cells and cell aggregates in fixed volume of liquid medium are placed in conical flask. Conical flasks are mounted with the help of clip on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at 60-180 rpm.

3) Spinning culture:

Large volume of cell suspension may be cultured in 10L,bottles which are rotated in a culture spinner at 120 rpm at an angle of 45 0.

4) Stirred culture:

This system is also used for large scale batch culture. In this method, the large culture is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through culture medium.

Continuous culture

The large culture vessel is kept dispersed continuously by bubbling sterile air through culture medium and the old liquid medium is continuously replaced by the fresh liquid medium (on depletion of some nutrients in the medium) to stabilize the physiological states of the growing cells.

Here nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase.

There are two types of continuous culture system

1. Chemostates
2. Turbidostates

Importance of cell suspension culture

1. To obtain single cell clones.
2. To study the morphological and biochemical changes during their growth and development phases.
3. To understand the pathways of cellular metabolism.
4. Single cell systems have a great potential for crop improvement.
5. Cells which are in a population of cultured cells invariably show cytogenetical and metabolic variations depending on the stage of the growth cycle and culture
6. To produce high yielding cultures as well as plants with superior agronomic traits.
7. Single cells derived from medicinally important plants can be studied for the production of secondary metabolites like alkaloids, glycosides.
8. For mutagenesis study. The mutagens can be added directly in the liquid medium. After the mutagen treatment, cells are plated on agar medium for the selection of mutant cell clones.

Cell viability test

The objective of cell suspension culture is to achieve rapid growth rates and uniform cells with all cells being viable. The viability of cells can be determined by following methods:

- ✓ Microscopy test: live cells having a well-defined healthy nucleus and streaming cytoplasm are easily observed under microscope.
- ✓ Reduction of tetrazolium salts: when cell masses are stained with 1-2% solution of 2,3,5- triphenyl tetrazolium chloride (TTC). The living cells reduce.
- ✓ Fluorescein diacetate (FDA): esterase present in live cells cleaves FDA to produce fluorescein which fluoresces under UV so that live cells appear green under UV.
- ✓ Evan's blue staining: this is the only dye which is taken up by dead cells.

Determination the single cell culture growth

There are different methods for determination the single cell culture growth are:

Cells number, cells volume, fresh weight, dry weight, protein content, DNA and RNA content.

Techniques (methods) of single cell culture

There are five important methods which are widely used for culturing single cells:

1. The filter-paper raft nurse:

a. Single cell are isolated from suspension cultures or a friable callus with the help of a micropipette or microspatuala.
b. Few days before cell isolation, sterile filter paper at different size are placed on the upper surface of the actively growing callus tissue of the same or different species.
c. The filter paper will be wetted by soaking the water and nutrient from the callus tissue.
d. The isolated single cell is placed on the wet filter paper raft and the whole culture system is incubated.

e. The single cell divides and redivides to forms a small cell colony, then transferred to fresh medium where it gives rise to the callus tissue.

2. Petri Dish Plating:

The technique developed by Bergmann (1960) is the most popular one for plating of single cells. The techniques are as follows:

a. A suspension of single cell is prepared from the stock cell suspension culture by filtering and centrifugation.
b. The solid medium (1.6 % agar added is melted in water both).
c. In front of laminar air flow, the cover of petri dish is opened. With the help of sterilized Pasteur pipette, 1.5 ml of single cell suspension is put and equal amount of melted agar medium when it cools down at 35 ^{0}C is added in the single cell suspension.
d. The single cell divides and redivides and ultimately forms a small cell colony. When a cell colony reaches a suitable size, it is transferred to fresh medium where it gives rise to the callus tissue.
e. The cultures are incubated under 16 hrs. light.
f. The petri dishes are observed under the microscope to see whether the cells have divided or not.
g. After certain days of inoculation, when cells start to divide to form pin-head to counting the number of dividing cells.
h. Pin-head shaped colonies when they reach a suitable size are transferred to fresh medium for further growth.

3. **The microchamber growth**: With the help of paraffin oil and cover glass a micro-chamber is formed on a glass slide and droplet containing single cells in medium is placed inside this micro-chamber and incubated for division

4. **The micro-droplet technique:** In this technique the single cells are cultured in special kind of apparatus named Cuprak dishes which have two kinds of chambers, small outer chamber filled with water and large inner chamber carrying numerous wells each filled with micro-droplet of medium containing single cells.

5. The nurse callus technique

This method is actually a modification of petri-dish plating method and paper raft nurse culture method.

Applications of cell culture

- ✓ Mutant screening and selection: Induced mutagens produce more frequency of mutants than spontaneous ones and screening them at cellular level and selection.
- ✓ Production of secondary metabolites: Plants being important source of variety of chemicals used in pharmacy, medicine and industry, cell cultures are effectively for production of these chemicals on a commercial scale for enhanced yield and better production control.
- ✓ cell culture could be used successfully to obtain single cell clones.
- ✓ Plants could be regenerated from the callus tissue derived from the single cell clones.
- ✓ The occurrence of high degree of spontaneous variability in the cultured tissue and their exploitation through single cell culture are very important in relation to crop improvement programmes.
- ✓ Biotransformation

Protoplast Fusion Technology and Its Biotechnological Applications.

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Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells.Protoplast can be obtained by specific lytic enzymes to remove cell wall.Protoplast fusion is a physical phenomenon,during fusion two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents. By protoplast fusion it is possible to transfer some useful genes such as diesese resistance,nitrogen fixation ,rapid growth rate ,more product formation rate,protein quality,frost hardiness,drought resistance,herbicide resistance ,heat and cold resistance from one species to another. Protoplast fusion an important tools in strain improvement for bringing genetic recombinations and developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties.These are the powerful techniques for engineering of microbial strains for desirable industrial properties. Protoplast fusion would continued to be an existing area of research in modern biotechnology.This technique in the future will be one of the most frequently used research tools for tissue culturists, molecular biologists, biochemical engineers and biotechnologists..This review describes the protoplast fusion technology and its biotechnological applications.

Introduction:

The protoplast includes the plasmalemma and everything contained within ie.the entire cell without its inherent cellulosic cell wall.In protoplast technology ,Two genetically different protoplast isolated from the somatic cells and are experimentally fused to obtain parasexual hybrid protoplasts.The hybrid protoplast contained heteroplasoic cytoplasm and two fused parent nuclei.Fusion of protoplast is relatively a new versatile technique to induce or promote genetic recombination in a variety of prokaryotic and eukaryotic cells(Bhojwani S.S. et al 1977).Protoplast fusion may be used to produce interspecific or even intergeneric hybrids.Protoplast fusion becomes an important tool of gene manipulation because it breakdown the barriers to genetic exchange imposed by conventional mating systems.Protoplast fusion technique has a great potential for genetic analysis and for strain improvement.It is particularly useful for industrially useful microorganisms(Murlidhar R.V and Panda T. 2000).

Enzymes used for breaking of cell walls:

For protoplast fusion it is important that the cell wall of plant and microorganisms is degraded .So various enzymes used for this process.cellulase and pectinase or macerozyme acting on plant cell wall.Bacterial cell wall are degraded by the action of lysozyme.Fungal wall degraded by Novozyme -234 which includes glucanase and chitinase. *Streptomyces* cell wall degraded by action of lysozyme and achromopeptidase (Narayanswamy S 1994)(Jogdand S.N 2001).

Methods of protoplast fusion:

Protoplast fusion can be broadly classified into two categories:

Spontaneous fusion: Protoplast during isolation often fuse spontaneously and this phenomenon is called spontaneous fiusion .During the enzyme treatment,protoplast from adjoining cells fuse through their plasmodesmata to form multinucleate protoplasts.

Induced fusion:Fusion of freely isolated protoplasts from different sources with the help of fusion inducing chemicals agent s is known as induced fusion.Normally isolated protoplast do not fuse with each other because the surface of isolated protoplast carries negative charges (-10mV to -30mV) around the outside of the plasma membrane. And thus their is a strong tendency in the protoplast to repel each other due to their same charges .So this type of fusion needs a fusion inducing chemicals which actually reduce the electronegativity of the isolated protoplast and allow them to fuse with each others(Narayanswamy S 1994).

The isolated protoplast can be induced to fuse by three ways;

Mechanical fusion:In this process the isolated protoplast are brought into intimate physical contact mechanically under microscope using micromanipulator or perfusion micropipette.

Chemofusion:Several chemicals has been used to induce protoplast fusionsuch as sodium nitrate ,polyethylene glycol,Calcium ions(Ca^{++}). Chemical fusogens cause the isolated protoplast to adhere each other and leads to tight agglutination followed by fusion of protoplast (Pasha C.R et al 2007) (Jogdand S.N.2001).In order to convert cellulosic materials to ethanol by single step process , Srinivasan R and Panda T(1997) carried out chemofusion between protoplasts of *Trichoderma reesei* QM9414 and *Saccharomyces cerrevesei* NCIM 3288 .Observed successful fusion suggest that endoglucanase is the key enzyme in the success of fusion.Iwata M et al(1986) were obtained Tetracycline resistant (Tet^r),erythromycin resistant (Ery^r) fusants of *Lactobacillus fermentatum* 604 carrying a 10 megadalton Tet^r plasmid and *L.fermentatum* 605 carrying a 38 megadalton Ery^r plasmid by means of polyethylene glycol induced protoplast fusion.

Chemofusion is a non specific,inexpensive,can cause massive fusion product,can be cytotoxic and non selecetive and having less fusion frequency.

Electrofusion: Recently ,mild electric stimulation is being used to fuse protoplast .In this two glass capilliary microelectrode are placed in contact with the protoplast .An electric field of low strength ($10Kvm^{-1}$) gives rise to dielectrophoretic dipole generation within the protoplast suspensionThis leads to pearl chain arrangement of protoplasts. Subsequent application of high strength of electric fields (100 kvm^{-1}) for some microseconds results in

electric breakdown of membrane and subsequent fusion(Ushishima S.T et al 1991) (Jogdand S.N.2001).Groth DI(1987) et al carried out electofusion of *Penicillium* protoplasts,after diaelectrophoresis and foun viable fusion products.Dimitrov AP and Christov AM(1992) reported electrically induced protoplast fusion using pulse electric field for diaelectrophoresis and suggest the possibility of electrically indused protoplast fusion at cation concentration that prevents fusion when sine – wave fields are applied.Gaint protoplast of *Pleurotus cornucopiae* were fused using the glass microelectrode fusion technique.To induce fusion Ca^{++} was necessary .Polyethylene glycol 400 (PEG) promoted fusion but also increased the adhesion of protoplasts(Magae Y et al 1986).In order to regulate electrofusion Urano N et al(1998) studied electrofusion procedures for yeast breeding and reported that cell membrane fusion behaviour of respiration deficient yeasts(P^-) was remarkably different from the normal yeasts(P^+).Induction of cell membrane fusion in P^+ protoplast appeared under the pulse conditions (height 2.5-5.5 $kVcm^{-1}$and duration 25-430μs) and the time interval of morphological change from the long to short state was 3–11 s. On the other hand, induction of cell membrane fusion in P^- protoplasts appeared under the higher pulse condition (height: 4.0–7.0 kV cm^{-1} and duration: 20–500 μs). The time interval of the morphological change from the long to short state was 110–170 s in cell membrane fusants of P^- protoplasts. The ξ-potential of P^+ protoplasts was −10 to−30 mV and that of the P^- protoplasts was −25–−60 mV. The surface charge of the P^- protoplasts was more negative than that of P^+ protoplasts; therefore, regulation of electrofusion among various kinds of yeast strains was possible by changing the surface charge of the protoplasts using mitochondrial mutations.

Electrofusion is easy to control having fusion frequency upto 100%.gives reproducibility.less cytotoxic.But equipment is sophisticated and expensive.

Mechanism of protoplast fusion:

The mechanism of protoplast fusion is not fully known .Several explanations have been put forward to understand the mechanism of protoplast fusion.Some are explained here:When the protoplasts are brought into close proximity ,this is followed an induction phase thereby changes induced in electrostatic potential of the membrane results in fusion .After the fusion,The membranes stabilizes and the surface potential returns to their former state.Other literature showed when the protoplasts are closely adhered ,the external fusogens cause disturbance in the intramembranous proteins and glycoproteines.This increases membrane fluidity and creates a region where lipid molecule intermix,allowing coalescence of adjacent membranes.The negative charge carried by protoplast is mainly due to intramembranous phosphate groups .The addition of Ca^{++} ions causes reduction in the zeta potential of plasma membrane and under this situation protoplasts are fused(Peberdy J.F 1980).The high molecular weight polymer (1000-6000) of PEG acts as a molecular bridges connecting the protoplasts. calcium ions linked the negatively charged PEG and membrane surface .On elution of the PEG ,the surface potential are disturbed ,leading to intramembrane contact and subsequent fusion, Besides this ,the strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity,thus inducing fusion.Protoplast fusion

takes place when the molecular distance between the protoplasts is 10A or less .This indicates that protoplast fusion is highly a traumatic events.(Jogdand S.N,2001)(Narayanswamy S 1994)

Protoplast fusion in fungi:

Production and regeneration of protoplasts is a useful technique for fungal transformations.Commercial preparation of enzymes which contain mixture of products to digest fungal cellwall used .Novozyme 234 includes (glucanase and chitinase) enzyme mixture is added to rapidly growing fungal tissue suspensed in an osmotic buffer (e.g.0.6 mol^{-1},KCl,1.2 mol^{-1},Sorbitol or 1.2 $mol^{-1}MgSO_4$).The protoplasts and DNA are mixed in presence of 15%(w/V)PEG 6000 and pH buffer (TRIS HCl).10 mml^{-1}.PEG causes clump formation in protoplasts. At 37^0C ,grow mycelium on cellophone placed on agar overnight.Incubte with enzyme at 30^0C for 1.5 hours in empty petridish having KCl,than filter protoplasts,wash protoplast in KCl (Centrifuge and resuspended the pellets).Protoplast fusion frequency in fungi is 0.2 -2%(Srinivas R.T and Panda T 1997)(Jogdand S.N 2001)

Protoplast technology for *Streptomyces* species:

Streptomyces spp also do not have natural means of mating .For obtaining protoplasts from *Streptomyces* lysozyme is used which breakes glycan portion of peptidoglycan wall.Cultures from spore suspension (2 days in shaker at 30^0C) harvest by centrifugation ,Resuspended in 0.03 mol^{-1} sucrose, washed and reharvest,Than resuspended in lysozyme solution in protoplasting medium(30 min – 2 hr at 30^0C) (Kohlar J and Darland G et al 1988) (Tehrani J.L et al 1992).

Protoplast fusion in bacteria:

In bacteria protoplast can be obtained and fusion can be carried out with low frequency in some gram positive organisms.For gram negative bacteria it is possible to obtain protoplast but regeneration is difficult. The procedure is highly efficient and yields upto 80% transformants (Iwata M. et al 1986) (Jogdand S.N.2001).

Biotechnological applications of protoplast fusion:

Protoplasts contained all the intracellular organelles of cells and form a vital link in transfer of micromolecules between cyto organelles,currently most of the laboratories engaging in fungal genetics are using gene manipulation procedures based on protoplasts.Therefore to further improve the genetic properties of these strains using protoplast fusion are attempt to develop methods for preparation and regeneration of protoplasts.The process involves protoplast mutagenesis,transformation and protoplast fusion (Evans D.A.1983). The direct bioconversion of cellulosic materials to ethanol by the intergeneric fusants between *T.reesei* and *Saccharomyces cerevesie* appears to be are of the best technique for an alternative approaches for ethanol production Also this process is helpful in the production of a complete set of cellulases by the protoplast fusion of *T.reesei* and *A.niger* (one produced more amount of endo and exoglucanase and other produced more β- glucosidase(Ahmed M

yield of 0.459 g g^{-1} productivity of 0.67 g/l/h and fermentation efficiency of 90%.Electrically induced protoplast fusion employed for hybrid construction in ergosterol producing yeast strains.Some fusion products proved to be hybrid with respect to ergosterol content and to remain stable over several generations(Avram D et al 1992).Yari S et al (2002) studied the effects of protoplast fusion on δ- endotoxins production in *Bacillus thuringiensis* spp (H14) found that *Bacillus thuringiensis* fusants have 1.48 time more δ-endotoxins than wild type.According to US Patent 7241588 Fusants of *Penicillium chrysogenum* and *Cephalosporium acremonium* produced a novel lactam antibiotic .Kohlar J and Darland G(1988) investigated the protoplast fusion in *Streptomyces avermitillis* which involved in avermectin biosynthesis.resulting fusants showed improved properties in respect to ,rifampicin resistance and maintaing the ability to carry out the metylation of C-5 hydroxyl of the avermectin molecules.Arti Das and Anuradha Ghosh (1989) carried out protoplast fusion between two strains of *Aspergillus niger* 8-2 a fast growing strain and poor producer of glucoamylase and *Aspergillus niger* 8-7 ,a slow rowing strain and good producer of enzyme and the resulting fusant produced 68% more glucoamylase than parental strains.Bakthiari M.R.et al(2007) carried out protoplast fusion between different strains of *Tolypocladium inflatum* .One of the recombinants produced cyclosporine 2.8 times more than parental strain.

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Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 2656-2668

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Review Article https://doi.org/10.20546/ijcmas.2018.707.312

Methods of Plant Transformation- A Review

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Keywords

Transformation, Transgenic, Gene, *Agrobacterium*, Particle

Article Info

Accepted:
20 June 2018
Available Online:
10 July 2018

A B S T R A C T

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. However, the desire for higher transformation efficiency has stimulated work on not only improving various existing methods but also in inventing novel methods. The most published techniques for gene transfer into plant cells were dismissed as either disproven or impractical for use in routine production of transgenic plants. In many laboratories, virtually all the transformation work relies on particle bombardment with DNA coated microprojectiles or Agrobacterium mediated transformation for gene transfer to produce transgenic plants from a range of plant species.

Introduction

Plant genetic transformation permits direct introduction of agronomically useful genes into important crops and offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. The directed desirable gene transfer from one organism to another and the subsequent stable integration and expression of a foreign gene in the genome is referred to as 'Genetic Transformation'. The transferred gene is known as 'transgene' and the organisms that are developed after a successful gene transfer are known as 'transgenics' (Babaoglu *et al*., 2000).

Among the various r-DNA technologies, genetically modified plants expressing δ-endotoxin genes from *Bacillus thuringiensis* (*Bt*), protease inhibitors and plant lectins have been successfully developed, tested and demonstrated to be highly viable for pest management in different cropping systems during the last decade and a half (Gatehouse, 2008). Insect resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. Most of the plant derived genes produce chronic rather than toxic effects and many insect pests are less or not sensitive to most of these factors. Therefore, the genes for δ-endotoxins are expected to provide better solutions.

Advances in biotechnology have provided several unique opportunities that include access to various plant transformation techniques, novel and effective molecules, ability to change the levels of gene expression, capability to change the expression pattern of genes, and develop transgenics with different insecticidal genes. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert foreign genes that confer resistance to insects into the plant genome (Bennett, 1994). To sustain the crop yield potential and to meet the growing demand for food, crop productivity needs to be increased. However, in most crops it is believed that the genetic potential has been fully exploited for yield increase. As a result, any improvement in productivity has to revolve around the reduction of losses due to pests and diseases under optimal nutrition and abiotic factors. Recombinant DNA technology coupled with plant tissue culture has helped develop novel options for the economical management of various kinds of biotic stresses including insect pests. These technologies would be of immense value in reducing the losses caused by biotic stresses, including insect pests.

Transgenic plants display considerable potential to benefit both developed and developing countries. Transgenic plants expressing insecticidal *Bt* proteins alone or in conjunction with proteins providing tolerance to herbicide are revolutionizing agriculture (Shelton *et al*., 2002). The use of such crops with input traits for pest management, primarily insects and herbicide resistance, has risen dramatically since their first introduction in the mid 1990s.

India, the largest cotton growing country in the world has increased productivity by up to 50% while reducing the insecticide sprays by half, with environmental and health implications, besides increased income to cultivators after introduction of *Bt* cotton in 2002. Success achieved in cotton has served as an excellent model to emulate in many other crops such as rice, wheat, pulses and oilseeds that have the potential to make agriculture a viable profession for the peasants of India.

Transformation studies

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (DeBlock *et al*., 1984; Horsch *et al*., 1984; Paszkowski, 1984) has been extended to many plant species in at least 35 families.

Gene transfer successes include most major economic crops, vegetables and medicinal plants. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. The techniques have continued to evolve to over come a great variety of barriers experienced in the early phases of the development in the field of plant transformation.

Transformation methods

Gene delivery systems involve the use of several techniques for transfer of isolated genetic materials into a viable host cell. At present, there are two classes of delivery systems (Table 1): (a) Non-biological systems (which include chemical and physical methods) and (b) Biological systems. The desire for higher transformation efficiency has stimulated work on not only improving various existing methods but also in inventing novel methods.

Biological requirements for transformation

The essential requirements in a gene transfer system for production of transgenic plants are:

Availability of a target tissue including cells competent for plant regeneration.

A method to introduce DNA into those regenerable cells and

A procedure to select and regenerate transformed plants at a satisfactory frequency.

Practical requirements for transformation

Beyond the biological requirements to achieve transformation and the technical requirements for verification of reproducible transformation, desired characteristics to be considered in evaluating alternative techniques or developing new ones for cultivar improvement include:

(1) High efficiency, economy, and reproducibility, to readily produce many independent transformants for testing.

(2) Safety to operators, avoiding procedures, or substances requiring cumbersome precautions to avoid a high hazard to operators (e.g. potential carcinogenicity of Silicone carbide whiskers).

(3) Technical simplicity, involving a minimum of demanding or inherently variable manipulations, such as protoplast production and regeneration.

(4) Minimum time in tissue culture, to reduce associated costs and avoid undesirable somaclonal variation.

(5) Stable, uniform (nonchimeric) transformants for vegetatively propagated species, or fertile germline transformants for sexually propagated species.

(6) Simple integration patterns and low copy number of introduced genes, to minimize the probability of undesired gene disruption at insertion sites, or multicopy associated transgene silencing.

(7) Stable expression of introduced genes in the pattern expected from the chosen gene control sequences (DeBlock, 1993).

When tested against the above criteria, most published techniques for gene transfer into plant cells must be dismissed as either disproven or impractical for use in routine production of transgenic plants. As a result, in many laboratories, virtually all the transformation work relies on Particle bombardment with DNA coated microprojectiles or *Agrobacterium* mediated transformation for gene transfer to produce transgenic plants in a range of plant species (Birch, 1997).

Non-biological based transformation

Particle bombardment/Biolistics

Particle bombardment was first described as a method for the production of transgenic plants in 1987 (Sanford *et al*., 1987) as an alternative to protoplast transformation and especially for transformation of more recalcitrant cereals. Unique advantages of this methodology compared to alternative propulsion technologies are discussed elsewhere in terms of range of species and genotypes that have been engineered and the high transformation frequencies for major agronomic crops (McCabe and Christou, 1993).

In plant research, the major applications of biolistics include transient gene expression studies, production of transgenic plants and inoculation of plants with viral pathogens (Southgate *et al*., 1995; Sanford, 2000; Taylor and Fauquet, 2002).

Gene constructs for biolistics can be in the form of circular or linear plasmids or a linear expression cassette. Embryogenic cell cultures are likely the best explants to use for biolistic transformation because they can be spread out as uniform targets of cells and have high recovery capacity (Kikkert *et al*., 2004). Rice transformation has also been successfully achieved via the bombardment of embryogenic calli (Li *et al*., 1993; Sivamani *et al*., 1996; Cao *et al*., 1992; Zhang *et al*., 1996), in which transformation efficiency has been raised to 50% (Li *et al*., 1993). Particle bombardment has emerged as a reproducible method for wheat transformation (DeBlock *et al*., 1997; Bliffeld *et al*., 1999) and the first stable transformation in a commercially important conifer species (*Picea glauca*) was achieved via embryogenic callus tissue as explant (Ellis *et al*., 1993).

However, particle bombardment has some disadvantages. The transformation efficiency might be lower than with *Agrobacterium* mediated transformation and it is more costly, as well. Intracellular targets are random and DNA is not protected from damage. As a result, many researchers have avoided particle bombardment method because of the high frequency of complex integration patterns and multiple copy insertions that could cause gene silencing and variation of transgene expression (Dai *et al*., 2001; Darbani *et al*., 2008).

Biological gene transfer

Agrobacterium mediated transformation

The natural ability of the soil bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenus*, to transform host plants has been exploited in the development of transgenic plants. In 1970s the prospect of using *A. tumefaciens* for the rational gene transfer of exogenous DNA into crops was revolutionary. Genetic transformation of plants was viewed as a prospect. In retrospect, *Agrobacterium* was the logical and natural transformation candidate to consider since it naturally transfers DNA (T-DNA) located on the tumor inducing (Ti) plasmid into the nucleus of plant cells and stably incorporates the DNA into the plant genome (Chilton *et al*., 1977). Now forty five years later, this method has been the most widely used and powerful technique for the production of transgenic plants. However, there still remain many challenges for genotype independent transformation of many economically important crop species, as well as forest species (Stanton, 2003; De la Riva *et al*., 1998).

Despite the development of other non-biological methods of plant transformation (Shillioto *et al*., 1985; Uchimiya *et al*., 1986; Sanford, 1988; Arenchibia *et al*., 1992, 1995), *Agrobacterium* mediated transformation remains popular and is among the most effective. This is especially true among most dicotyledonous plants, where *Agrobacterium* is naturally infectious. *Agrobacterium* mediated gene transfer into monocotyledonous plants was thought to be not possible. However, reproducible and efficient methodologies have been established for rice (Hiei *et al*., 1994), banana (May *et al*., 1995, corn (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), sugarcane (Arencibia *et al*., 1998), forage grasses such as Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) (Bettany *et al*., 2003). Among the commercially important conifers, hybrid larch was the first to be stably transformed via co-cultivation of embryogenic tissue with *A. tumefaciens* (Levee *et al*., 1997). Subsequently, this method was successfully applied to several species of spruce (Klimaszewska *et al*., 2001; Charity *et al*., 2005; Grant *et al*., 2004).

Methods relative to transformation targets can be classified into two categories: (a) those requiring tissue culture and (b) *in planta* methods.

In tissue culture systems for plant transformation, the most important requirement is a large number of regenerable cells that are accessible to the gene transfer treatment and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation and selection treatments. A high multiplication ratio from a micropropagation system does not necessarily indicate a large number of regenerable cells accessible to gene transfer (Livingstone and Birch, 1995). Some time gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short lived (Ross *et al*., 1995). Further, tissue culture based methods can lead to unwanted somaclonal variations such as alterations in cytosine methylation, induction of point mutations and various chromosomal aberrations (Phillips *et al*., 1994; Singh, 2003; Clough, 2004). On the other hand, realization of whole plant transformants has been a problem in a large number of crop species as these plants have proven to be highly recalcitrant *in vitro*. As a result, other strategies are being evolved wherein the tissue culture component is obviated in the procedure and these are known as *in planta* methods.

Plant genetic transformation is of particular benefit to molecular genetic studies, crop improvement and production of pharmaceutical materials. *Agrobacterium*-based methods are usually superior for many species including dicots and monocots. The others are typically not done on a routine basis (Table 2). Biolistics is by far the most widely used direct transformation procedure both experimentally in research and commercially. So why have all these other methods emerged in the past 20-30 years, if we already have efficient transformation techniques in *Agrobacterium* and biolistics? There are two reasons. First of all, there is hope that a more efficient and less expensive method would be developed. The second and most important reason is the biolistics and *Agrobacterium* are patented.

In planta transformation

Although successful plant regeneration methods have been developed, the technology has not provided regeneration in several other crops for use in transformation protocols which is a serious limitation to the exploitation of gene transfer technology to its full potential. In the light of this major constraint, it becomes necessary to evolve transformation strategies that do not depend on tissue culture regeneration or those that substantially eliminate the intervening tissue culture steps. *In planta* transformation methods provide such an opportunity. Methods that involve delivery of transgenes in the form of naked DNA directly into the intact plants are called as *in planta* transformation methods. These methods exclude tissue culture steps, rely on simple protocols and required short time in order to obtain entire transformed individuals.

In many cases *in planta* methods have targeted meristems or other tissues with the assumption that at fertilization, the egg cell accepts the donation of an entire genome from the sperm cell that will ultimately give rise to zygotes (Chee and Slighton, 1995; Birch, 1997) and therefore is the right stage to integrate transgenes. For non-tissue culture based approaches of *in planta* transformation, *Agrobacterium* co-cultivation or microprojectile bombardment have been directed to transform cells in or around the apical meristems (Chee and Slighton, 1995;

Birch, 1997). Injection of naked DNA into ovaries has also been reported to produce transformed progeny (Zhou *et al.,* 1983).

Arabidopsis thaliana was the first plant that saw successful *in planta* transformation. Early stages of success in *Arabidopsis* transformation came from the work of Feldmann and Marks (1987). Transformation rates greatly improved when Bechtold *et al.* (1993) inoculated plants that were at the flowering stage. At present, there are very few species that can be routinely transformed in the absence of a tissue culture based regeneration system. *Arabidopsis* can be transformed by several *in planta* methods including vacuum infiltration (Clough and Bent, 1998), transformation of germinating seeds (Feldmann and Marks, 1987) and floral dipping (Clough and Bent, 1998). Other plants that were successfully subjected by vacuum infiltration include rapeseed, *Brassica campestris* and radish, *Raphanus sativus* (Ian and Hong, 2001; Desfeux *et al*., 2000). The labor intensive vacuum infiltration process was eliminated in favor of simple dipping of developing floral tissues (Clough and Bent, 1998). Also, the results indicate that the floral spray method of *Agrobacterium* can achieve high rates of *in planta* transformation comparable to the vacuum infiltration and floral dip methods (Chung *et al*., 2000).

Table.1 DNA delivery methods available to produce plant transformants

Plant transformation	
Non-biological based transformation (Direct method)	Biological gene transfer (Indirect method)
A) DNA transfer in protoplasts **1) Chemically stimulated DNA uptake by protoplast** **2) Electroporation** **3) Lipofection** **4) Microinjection** **5) Sonication**	**1) Agrobacterium mediated transformation** Primarily two methods **a) Co-cultivation with the explants tissue** **b) *In planta* transformation** **2) Transformation mediated by viral vector**
B) DNA transfer in plant tissues **1) Particle bombardment / Biolistics** **2) Silicon carbide fiber mediated gene transfer** **3) 3) Laser microbeam (UV) induced genetransfer**	

(Birch *et al*., 1997)

Table.2 Summary of gene delivery methods and their features

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Electroporation	Low to high	Unrestricted	With and without tissue culture phase	Protoplasts, meristems or pollen grains	Fast, simple and inexpensive in contrast with biolistics
Lipofection	Low	Recoverable species from protoplast	With tissue culture phase	Protoplast	High efficiency with combination of PEG based method, simple and non-toxic
Microinjection	High	Recoverable species from protoplast	With tissue culture phase	Protoplast	Very slow, precise, single cell targeting possibility, requires high skill, the chimeric nature of transgenic plants and ability of whole chromosome transformation
Sonication	Low	Unrestricted	With and without tissue culture	Protoplast cells, tissues and seedlings	Effective to transfect by virus particles and able to increase the *Agrobacterium* based transformation efficiency
Particlebombardment	High	Unrestricted	With and without tissue culture phase	Intact tissue or microspores	Efficient for viral infection, complex integration patterns, without specialized vectors and backbone free integration

(Darbani *et al.*, 2008)

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Silicon carbide mediate transformation	Low to high	Unrestricted	With tissue culture	Variety of cell types	Rapid, inexpensive and easy to set up
Laser beam mediated transformation	Low	Unrestricted	With tissue culture phase	Variety of cell types	Rapid and simple
Agrobacterium mediated method	High and stable	Many species, specially dicotyledonous plants	With and without tissue culture method	Different intact cells, tissues or whole plant	Possibility of *Agroinfection*, combination with sonication and biolistic methods and transgene size up to 150 kb
Virus based method	High and transient	Virus host specific limitation	With tissue culture	In planta inoculation	Rapid, inducible expression and with mosaic status

Utilizing naked DNA, cotton transformants were recovered following injection of DNA into the axil placenta about a day after self-pollination (Zhou *et al.*, 1983). Similarly, a mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers, or soybean seeds were imbibed with DNA (Trick and Finer, 1997; Langridge, 1992). These procedures, intriguing as they are, are impractical at present because of their low reproducibility.

Recent studies with *Agrobacterium* inoculation of germinating seeds of rice has provided transformation efficiencies higher than 40% (Supartana *et al.*, 2005), while providing 4.7 to 76% efficiency for the flower infiltration method and from 2.9 to 27.6% efficiency for the seedling infiltration method (Trieu *et al*., 2000).

Crop species that were successfully transformed by injuring the apical meristem of the differentiated embryo of the germinating seeds and then infecting with *Agrobacterium* include peanut, *Arachis hypogaea* L. (Rohini and Rao, 2000b & 2001), sunflower, *Helianthus annuus* L. (Rao and Rohini, 1999), safflower, *Carthamus tinctorius* L. (Rohini and Rao, 2000a), field bean, *Dolichos lablab* L. (Pavani, 2006), and cotton, *Gossypium* sp. (Keshamma *et al*., 2008). Maize, *Zea mays* L., was transformed by treating the silks with *Agrobacterium* and afterwards pollinated with the pollen of the same cultivar (Chumakov *et al*., 2006).

The above successes have in fact provided a great leverage for easy development of transgenic pants, as the methodology is simple, cost effective, does not call for high infrastructural requirement even to handle recalcitrant crops such as groundnut. Thus the technology of gene transfer for the development of recalcitrant crops has become a practical possibility for experimenting and producing viable transformants. However, the optimization of *Agrobacterium*-plant interaction is crucial for efficient transformation. Many factors including type of explant are important and they must be suitable to allow the recovery of whole transgenic plants (De la Ravi *et al*., 1998; Opabode 2006; Cheng, *et al*., 1997; Jones *et al*., 2005; Darbani *et al*., 2008).

Although, biotechnological advances, have provided many technologies for gene transfer into plant cells, virtually all the transformation work rely only on particle bombardment with DNA coated microprojectiles or Agrobacterium mediated transformation for gene transfer to produce transgenic plants. The review thus overwhelmingly emphasizes the importance of this method.

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How to cite this article:

Keshavareddy, G., A.R.V. Kumar and Vemanna S. Ramu. 2018. Methods of Plant Transformation- A Review *Int.J.Curr.Microbiol.App.Sci.* 7(07): 2656-2668.
doi: https://doi.org/10.20546/ijcmas.2018.707.312