Introduction

The conventional breeding methods are the most widely used for crop improvement. But in certain situations, these methods have to be supplemented with plant tissue culture techniques either to increase their efficiency or to be able to achieve the objective, which is not possible through the conventional methods. One example of each situation would illustrate the point. Production of pure lines or in breeds involve six to seven generations of selfing. Production of haploids through distant crosses or using pollens, anther or ovary culture, followed by chromosome doubling, reduces this time to two generations. This represents a saving of four to six years. The other example is the transfer of a useful bacterial gene say, cry (crystal protein) gene from *Bacillus thuringiensis*, into a plant cell and ultimately regeneration of whole plants containing and expressing this gene.(transgenic plants). This can be achieved by a combination of tissue culture and genetic engineering, none of the conventional breeding approaches can ever produce such a plant.

The term tissue culture is commonly used in a very wide sense to include in vitro culture of plant cells, tissue as well as organs. But in a strict sense, tissue culture denotes the in-vitro cultivation of plant cells in an unorganized mass e.g., callus cultures. Another term, cell culture is used for in-vitro culture of single or relatively small groups of plant cells e.g., suspension cultures. But in general, the term tissue culture is applied to both callus and suspension cultures and cell culture is often used for callus culture as well. When organized structures like root tips, shoot tips, embryos, etc are cultured in-vitro to obtain their development as organized.

Plant Cell Totipotency: Totipotency is the genetic potential of a plant cell to produce the entire plant. In other words, totipotency is the cell characteristic in which the potential for forming all the cell types in the adult organism is retained. The basis of tissue culture is to grow large number of cells in a sterile controlled environment. The cells are obtained from stem, root or other plant parts and are allowed to grow in culture medium containing mineral nutrients, vitamins and hormones to encourage cell division and growth. As a result, the cells in culture will produce an un-organised proliferative mass of cells which is known as callus tissue.

The cells that comprise the callus mass are totipotent. Thus a callus tissue may be in a broader sense totipotent, i.e., it may be able to regenerated back to normal plant given certain manipulations of the medium and the cultural environment. Truly speaking, totipotency of the cell is manifested through the process of differentiation and the hormones in this process play the major role than any other manipulations.

MS Media:

The MS medium of Murashige and Skoog (1962) salt composition is very widely used in different culture systems. In the development of this medium and also other media, it was demonstrated that not only the presence of necessary nutrients but also the actual and relative concentration of various inorganic nutrients are of crucial significance. The B5 medium of Gamborg et.al (1968) or its derivatives have been useful for cell and protoplast culture. This medium was originally designed for callus and suspension culture but it has also been effectively used for plant regeneration. It differ from MS medium in having much lower amounts of nitrates in the form of ammonium. The N_6 medium was developed for cereal anther culture and is used successfully with other types of cereal tissue culture (Chu,1978).Any success with a medium is in all probability due to the fact that the ratios as well as concentrations most nearly match the optimum requirements for the cells or tissues for growth and/or differentiation. It generally consists of inorganic nutrients, carbon and energy sources, vitamins, phytohormones and organic supplements which include organic nitrogen, acids and complex substances.

1. **Inorganic nutrients.** In addition to C,H and O, all nutrient media provide 12 elements essential for growth , viz., N, P, K, Ca, S, Mg (these six are called macronutrients, and are needed in concentration >0.5 m mol l^{-l} or 0.5 mM), Fe, Zn, Mn, Cu, B and (these six are known as micronutrients, and required in concentration <0.5 m mol^{-l}). The different tissue culture media provide different concentrations of the inorganic nutrients, the whites' medium being rather poor, and B5 and MS media being quite rich, especially for K and N. The media also provide Na and Cl but their necessity is not established. Generally, iron is provided as iron EDTA complex to keep it available at high (>5.8) pH. Nitrate is superior to ammonium as the sole N source, but use of NH^{+} checks the drift of pH towards alkalinity.

2. Vitamins: For optimum callus growth, the following vitamins are required, inositol, thiamine, pyridoxine and nicotinic acid of which thiamine is essential and the rest are promotary. Pantothenic acid is also known to be promotary, but is not included in most of the recipes.

Carbon source: Sucrose (20-50 g/l) is the most commonly used carbon source for all cultured plant materials, including even green shoots. Autoclaving hydrolyses sucrose, which enhance its availability to plant cells. In some systems, e.g., monocots, glucose may be superior to sucrose. Plant tissues can utilize other sugars like maltose, galactose, lactose, mannose and even starch, but these are rarely used.

Complex organic additives. In earlier studies, complex additives like yeast extract, coconut milk, casein hydrolysate, corn milk, malt extract and tomato juice were used to support plant tissue growth. But such additives are no more preferred. In many cases, their effects could be reproduced by a single amino acid, e.g., L-asparagine or L- glutamine. Such additives should be used only when synthetic media fail.

Role of plant growth regulators in plant tissue culture

The following growth regulators are used in plant tissue culture.

Auxin, e.g., IAA (indole -3-acetic acid), IBA (indole-3-butyric acid), NAA (naphthalene acetic acid), NOA (napthoxy acetic acid), 2,4-D (2,4-dichlorophenoxy acetic acid) etc., are commonly used to support cell division and callus growth (especially 2,4-D), somatic embryo induction, rooting etc.

Cytokinin like kinetin (furfurylamino purine), BAP (benzylamino purine), zeatin, 2-ip (isopentenyl adenine), TDZ (thidiazuron , a compound having cytokine activity) are employed to promote cell division, regeneration of shoots, often somatic embryo induction and to enhance proliferation and growth of axillary buds.

Abscisic acid promotes somatic embryo and shoot bud regeneration in many species and markedly improves somatic embryo maturation.

Gibberellins: Of the over 20 gibberellins, GA3 is almost exclusively used, it promotes shoot elongation and somatic embryo germination. The common range of concentration used are as follows: auxins,0.1-3 mg/l; cytokinin , 0.1-3 mg/l ; ABA, upto 0.2 mg/l and GA_3 0.1-1 mg/l.

Callus and suspension cultures.

When explants are cultured on a suitable growth regulator combination, many of its cells undergo division. Even mature and certain differentiated, e.g., parenchyma and often colenchyma, cells undergo changes to become meristematic, this is called dedifferentiation. Dedifferentiation involves, among other things, renewed and enhanced RNA and protein synthesis leading to the formation of new cellular components needed for meristematic activity. Initially, cell division are confined to the cut ends, but subsequently it covers the entire explants. The resulting cell mass is ordinarily unorganized but it often consists of several cell types including fibers, and vascular elements.

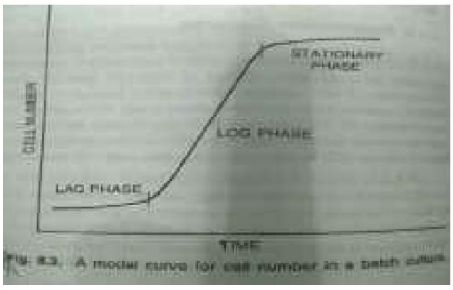
Callus cultures: Tissues and cells cultured on an agar-gelled medium form an unorganized mass of cells called callus. Callus culture need to be sub-cultured every 3-5 weeks in view of cell growth, nutrient depletion and medium drying. Therefore, calluses are easy to maintain and are the most widely used.

Suspension cultures: Tissue and cells cultured in a liquid medium produce a suspension of single cells and cells clumps of few to many cells, these are called suspension cultures. Liquid cultures must be constantly agitated, generally by a gyratory shaker at 100-250rpm, to facilitate aeration dissociation of cell clumps into smaller pieces. Suspension cultures grow much faster than callus culture , need to be sub-cultured about every week, allow a more accurate determination of the nutritional requirements of cells and are the only system amenable to scaling up for large scale production of cells and even somatic embryos. The suspension cultures are grouped as follows ;

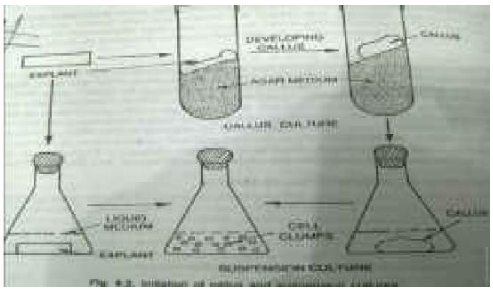
1.Batch cultures

2.Continuous cultures

3.Immobilized cell cultures



A model curve for cell number In a batch culture



Initiation of callus and suspension culture.

1. **Batch cultures:** In batch culture, the same medium and the cells produced are retained in the culture vessel, e.g., culture flasks (100-250 ml), fermenters (variable size) etc. The cell number or biomass of a batch culture exhibits a typical sigmoidal curve, having a lag phase during which the cell number or biomass remains unchanged, followed by a logarithmic (log) phase when there is a rapid increase in cell number and, finally, ending in a stationary phase during which cell number does not change. The lag phase duration depends mainly on inoculum size and growth phase of the culture from which the inoculum is taken. The log phase lasts about 3-4 cell generations, and the duration of a cell generation may vary from 22-48 hr, depending mainly on the plant species. The stationary phase is forced on the culture by a depletion of nutrients and possibly due to an accumulation of cellular waste. If the culture is kept in stationary phase for a prolonged period, the cells may die.

   Batch cultures are maintained by sub-culturing. They are used for initiation of cell suspensions, which may be used for cloning, cell selection or as seed cultures for scaling up or continuous cultures. They are, however, unsuitable for studies on cell growth and metabolism because there is a constant change in cell density and nutritional status of the medium. But batch cultures are much more convenient than continuous cultures and, as a result, are routinely used.

2. **Continuous cultures:** In a continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the used or spent medium by fresh medium. Such culture systems are of either (1) closed or (2) open type. In a closed continuous culture, cells are separated from the used medium taken out for

replacement, and added back to the culture so that cell biomass keeps on increasing. In contrast, both cells and the used medium are taken out from open continuous cultures and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at sub maximal growth indefinitely.

The open cultures are of either turbidostat or chemostat types. In a turbidostat, cells are allowed to grow up to a pre selected turbidity (usually, measured as OD) when a predetermined volume of the culture is replaced by fresh normal culture medium. But in chemostat, a chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while other nutrients are still in concentrations higher than required. In such a situation, any addition of growth-limiting nutrients is reflected in cell growth. Chemostats are ideal for the determination of effects of individual nutrients on cell growth and metabolism.

3. **Immobilized Cell Culture:** Plant cells and cell groups may be encapsulated in a suitable material, e.g., agarose and calcium alginate gels, or entrapped in membranes or stainless steel screens. The gel beads containing cells may be packed in a suitable column or, alternatively, cells may be packed in a column of a membrane or wire cloth. Liquid medium is continuously run through the column to provide nutrients and aeration to cells. Immobilization of cells changes their cellular physiology in comparison to suspension culture cells; this offer several advantages for their use in biochemical production, but they are usually not used for studies.

Subculture:

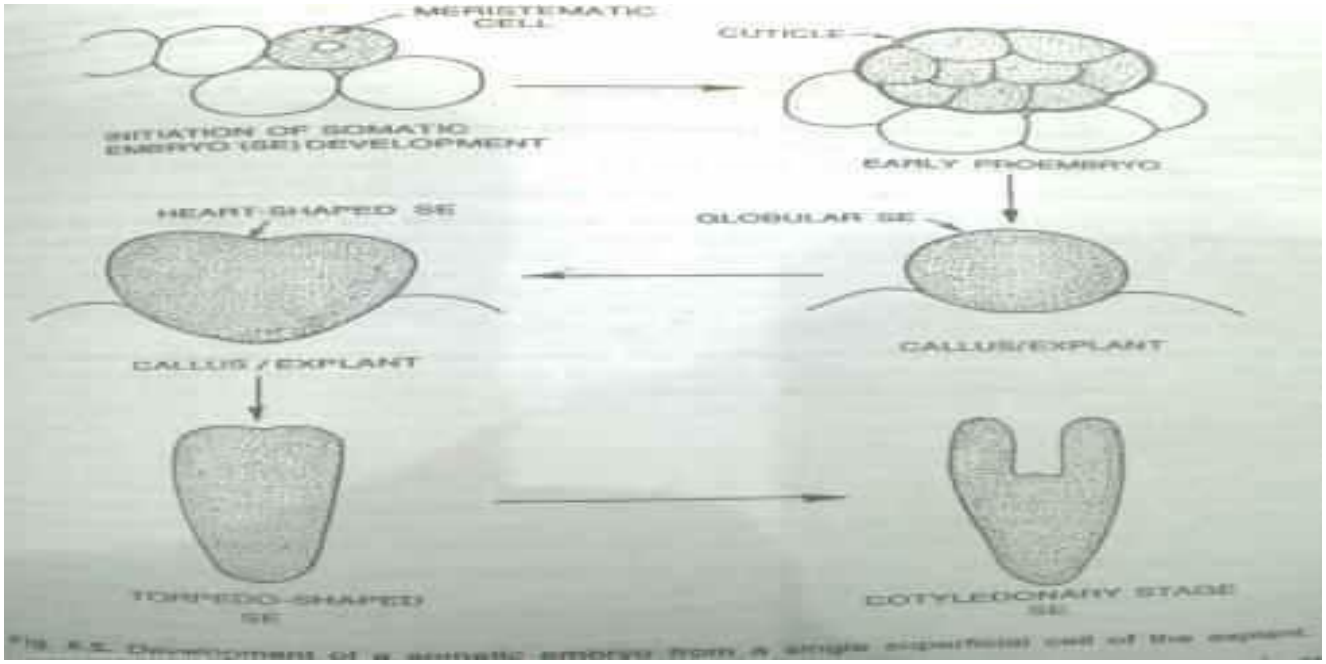
After a period of time, it becomes necessary to transfer organs or tissues to fresh media chiefly due to nutrient depletion and medium drying. This is particularly true of tissue and cell cultures where a portion of tissue is used to inoculate new culture tubes or flasks, this is known as subculturing. In general, callus culture are sub cultured every 4-6 weeks, while suspension culture need to be sub-cultured every 3-14 days. Plant cell and tissue cultures may be maintained indefinitely by serial sub-culturing.

Somatic embryogenesis

A somatic embryo is an embryo derived from a somatic cell, other than zygote, and obtained usually on culture of the somatic cells in vitro, this process is known as somatic embryogenesis. In contrast, embryos developing from zygotes are called zygotic or often simple embryos, while those derived from pollen are known as pollen embryos or androgenetic embryos. By 1978, somatic embryogenesis was reported from 80 species belonging to 33 families, the list has expanded considerably since then(over 100 species by 1993).

Developmental pattern of somatic embryogenesis. Somatic embryos generally originate from single cells, which divide to form a group of meristematic cells. Usually, this multicellular group becomes isolated by breaking cytoplasmic connections with the other cells around it and subsequently by cutinization of the outer wall of this differentiating cell mass. The cells of this meristematic mass continue to divide to give rise to globular (round ball shaped), heart shaped, torpedo and cotyeledonary stages of somatic embryos. In general , the essential features of SE development, especially after the globular stage, are comparable to those of zygotic embryos.

Somatic embryos are bipolar structures in that they have a radicle and a plumule. The radicular end is always oriented towards the centre of callus or cell mass, while the plumular end always sticks out from the cell mass. In contrast , a shoot bud is monopolar as it does not have a radicular end. In many somatic embryos, radicle is suppressed so that they often do not produce roots ,in such cases, roots have to be generated from the shoot produced by germinating somatic embryos.



Development of a somatic embryo from a single superficial cells of the plant.

SEs often show abnormal developmental features, e.g., three or more cotyledons, bell shaped cotyledons, large embryo size , etc, these problems can often overcome by the presence of ABA or a suitable concentration of mannitol. In some species, normal looking somatic embryos are formed, but they fail to germinate, at least some SEs do not germinate in most species.

The SEs regenerating from an explants or a callus are termed as primary somatic embryos. In many cases, SEs regenerate from the tissues of other SEs or the parts of germinating SEs, such SEs are called secondary somatic embryos. Ordinarily SEs originate from cells at the surface of callus explants, e.g., from epidermal cells of Ranunculus stem.

SE encompasses various stages from callus initiation to embryo development and maturation and subsequently plantlet formation. Equally important is the sequence of media and especially the growth regulators. For many species, one media is used for initial callusing and for the maintenance of callus, a second medium is used for somatic embryo maturation, and a third to allow their growth into plants. An elaborate sequence of media is essential where somatic embryogenesis is lacking or difficult.

Somatic embryos have been grown on a range of media from the dilute whites' medium to very high salt MS medium, but the latter has been extensively employed. The addition of reduced nitrogen in the medium helps in both embryo initiation and maturation. Of all the amino acids, L-glutamine seems to play a special role. Another factor is chelated form of iron in the media. In the absence of iron, embryo development fails to pass from the globular to the heart-shaped stage.

Growth regulators:

Growth regulators in the medium , especially auxin in combination with cytokinin appear essential for the onset of growth and the induction of embryogenesis. Of all the auxins, 2,4-D followed by NAA have proven to be extremely useful. Effective concentration ranges are 0.5-27.6 µm for 2,4-D AND 0.5-10.7 µm for NAA. The auxin for the primary (callus initiation) and secondary media (embryo development) may be same or different. One auxin or several may be used in the same medium. Cytokinins have been important in a number of species. Cytokinins have been used in the primary medium invariably during embryogenesis of crop plants. The effective conc. range for kinetin is 0.5-5.0 µM. Cytokinins are important in fostering somatic embryo maturation and especially cotyledon development. Cytokinins are sometimes required for growth of embryos into plantlets. Gibberellin are rarely incorporated in primary culture media. However, they have proven useful in fostering embryo maturation or in stimulation of rooting and subsequent growth of plants in a number of cases. The role of growth inhibitor ABA in somatic embryogenesis has emerged when added at non inhibitory level (0.1-1.0 µM).ABA promotes somatic embryo development and maturation and at the same time inhibits abnormal proliferation and initiation of accessory/adventitious embryos. ABA, anti-auxins (5-hydroxynitrobenzyl bromide,7-azaindole,2,4,6-trichlorophenoxy acetic acid, p-chlorophenoisobutyric acid) and other growth inhibitors may serve to promote somatic embryo maturation by countering the effects of growth promoters. Their addition to culture media may permit somatic maturation to proceed under conditions when it normally would not occur.

The addition of activated charcoal to the medium has proved to be useful for somatic

embryo development. Charcoal media shows lower level of phenylacetic acid and p-OH benzoic acid compounds. Also, it absorbs 5-hydroxymethy furfural , an inhibitor formed by sucrose degradation during autoclaving.

Shoot tip culture

Morel and Martin (1952) developed the technique of meristem culture for in vivo virus eradication of Dahlia. George Morel(1965) was pioneer in applying shoot tip culture for micropropagation of orchid Cymbidium .In vitro clonal propagation gained momentum in the 1970s when Murashige (1974) gave the concept of developmental stages by defining establishment, proliferation and rooting and hardening stages. The concept stimulated awareness that a single medium is not sufficient for in vitro multiplication, but the propugale needs a series of transfers in specially designed physical and chemical environments to reach regeneration. This method has been more successful in herbaceous plants because of weak apical dominance and strong root regenerating capacities as compared to woody species. Many crops are being currently propagated commercially using these in vitro procedures.

Shoots of all angiosperm and gymnosperm grow by virtue of their apical meristems. The apical meristem is usually a dome of tissue located at the extreme tip of a shoot and measure 0.1 mm in diameter and 0.25 to 0.3 mm in length. The apical meristem together with one to three young leaf primordial measuring 0.1 to 0.5mm constitutes the shoot apex. The explants of meristem tip culture may either be the apical meristematic dome or the apical dome with few subjacent leaf primordial. Meristem or shoot tip is cut or isolated from stem by applying a V-shaped cut with a sterilized knife. For meristem tip culture, the cut is applied 0.3 to 0.5mm below the tip of the dome and the excised tissue(explants) is removed along with portions of procambial tissue and is immediately planted on the media. Size of the tip explants is decisive factor which governs the success of culture. When very small explants are used, the presence of leaf primordial appears to determine the capability of an explants to develop. In general, the larger the explants, the better are the chances for survival. It is generally agreed that large explants such as shoot tips measuring upto 2cm in length should be selected for in vitro micropropagation. However, when disease free plants are the objective, meristem tips should

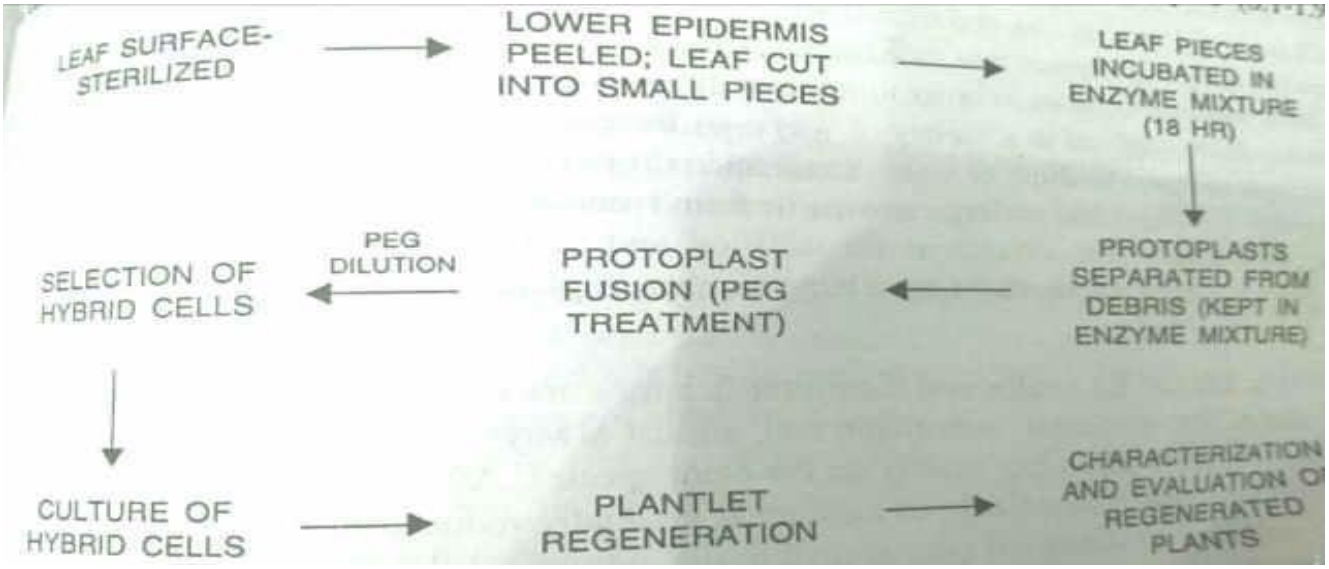
be cultured. Meristem tip between 0.2to 0.5 mm most frequently produce virus free plants and this method is referred to as meristem tip culture. Like all asexual propagation methods, success of meristem and shoot tip culture is affected by the season during which the explants are obtained. For plant species with a dormant period, best results may be expected when the explants are dissected at the end of their dormancy period. Further, actively growing shoot tips are recommended for their culture because of their strong growth potential and low virus concentration.

MS medium salts have been very satisfactory for such cultures though Whites' medium and Gautherets (1959) were the most widely used media during the early days of meristem culture. The growth additives vary from species to species and in some woody species culture one needs to add certain polyphenol oxidative compounds.

Somatic hybridization

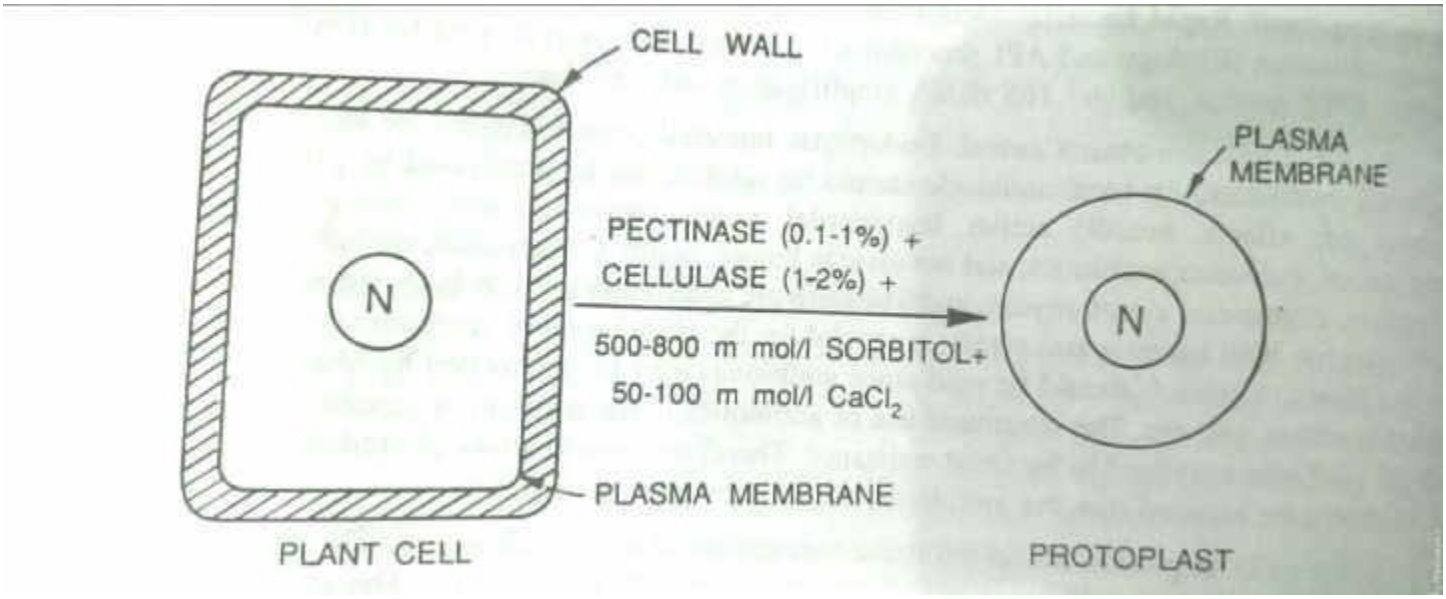
Production of hybrid plants through the fusion of protoplasts of two different plant species/varieties is called somatic hybridization, and such hybrids are known as somatic hybrids. The technique of somatic hybridization involves the following steps(i) isolation of protoplasts, (ii) fusion of the protoplasts of desired species/varieties, (iii) selection of hybrid cells, and (iv) culture of the hybrid cells and regeneration of hybrid plants from them. A brief , elementary consideration of these steps is presented below

Protoplast isolation. Isolation of protoplasts is readily achieved by treating cells/tissues with a suitable mixture of cell wall degrading enzymes. Usually, a mixture of pectinase or macerozyme (0.1-1.9%) and cellulose (1-2%) is appropriate for most plant materials. Hemicellulase may be necessary for some tissues. Generally, crude commercial preparations of enzymes are used. The pH of enzyme solution is adjusted between 4.7 and 8.0 and the temperature is kept at 25-30^{O}C. The osmotic concentration of enzyme mixture and of subsequent media is elevated (usually, by adding 500-800 m mol l^{-l} sorbitol or mannitol) is added to the osmoticum as it improves plasma membrane stability. The cells and tissues are incubated in the enzyme mixture for few to several (generally, 16-18) hours, naked protoplasts devoid of cell wall are gradually released in the enzyme mixture.



A schematic representation of the various steps in somatic hybridization

Protoplasts have been isolated from virtually all plant parts, but leaf mesophyll is the most preferred tissue, at least in case of dicots, for this purpose. In general, fully expanded leaves are surface-sterilized, their lower epidermis is peeled off with a pair of forceps and the peeled areas are cut into small pieces with a scalpel and suspended in the enzyme mixture. When epidermis can not be peeled, leaf may be cut into pieces and treated with the enzyme mixture, vacuum infiltration may be used to facilitate the entry of enzymes into the tissues. After the period of incubation, protoplasts are washed with a suitable washing medium in order to remove the enzymes and the debris. The protoplasts may be cultured in a suitable medium in a variety of ways (i) Bergmann's plating technique (in agar medium), (ii) in a thin layer of liquid medium or (iii) in small microdrops of 50-100ul. Protoplasts readily regenerate cell wall (within 2-4 days) and undergo mitosis to form macroscopic colonies, which can be induced to regenerate whole plants. the conditions for isolation and culture of protoplasts and regeneration of complete plants has been standardized for a large number of plant species, but cereals still present some problems.



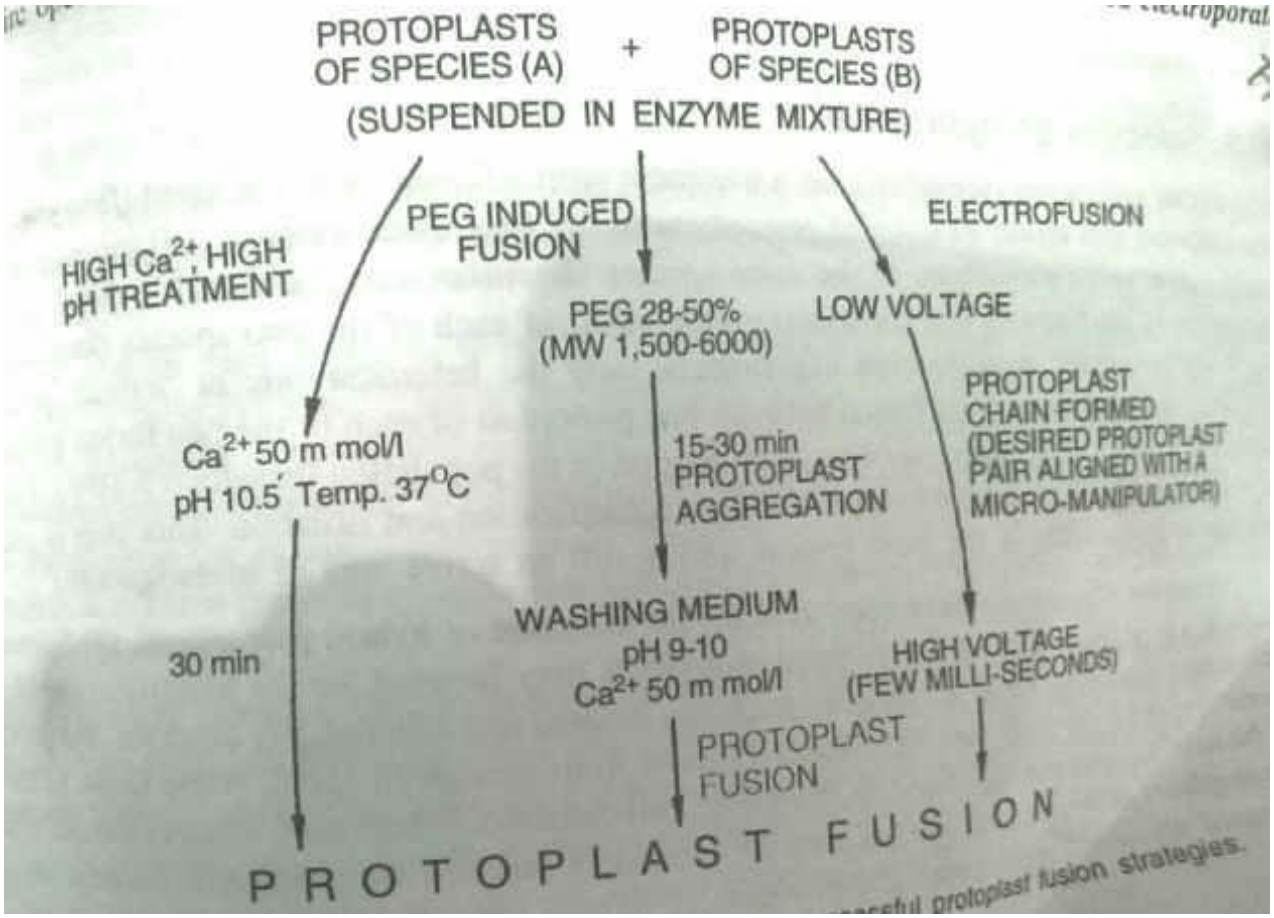
Generally, MS and B5 media, and their modifications are used for protoplast culture. The media are supplemented with a suitable osmoticum and, almost always, with an auxin and a cytokinin, their types and concentration depending mainly on the plant species. After 7-10 days of culture, protoplasts regenerate cell wall, and the osmolarity of medium is gradually reduced to that of normal medium. The macroscopic colonies are transferred onto normal tissue culture media. Protoplasts are very sensitive to light, therefore, they are cultured in diffuse light or dark for the first 4-7 days.

Protoplast fusion

The techniques for protoplast fusion are pretty well defined and highly effective for almost all the systems. A number of strategies have been used to induce fusion between protoplasts of different strains/species, of these the following three have been relatively more successful. Protoplasts of desired strains/ species are mixed in almost equal proportion, generally, they are mixed while still suspended in the enzyme mixture. The protoplast mixture is then subjected to high pH (10.5) and high Ca^{2+} concentration (50 m mol/l) at 37 ^{0}C for about 30 min(high pH-high Ca treatment).This technique is quite suitable for some species, while for some others it may be toxic.

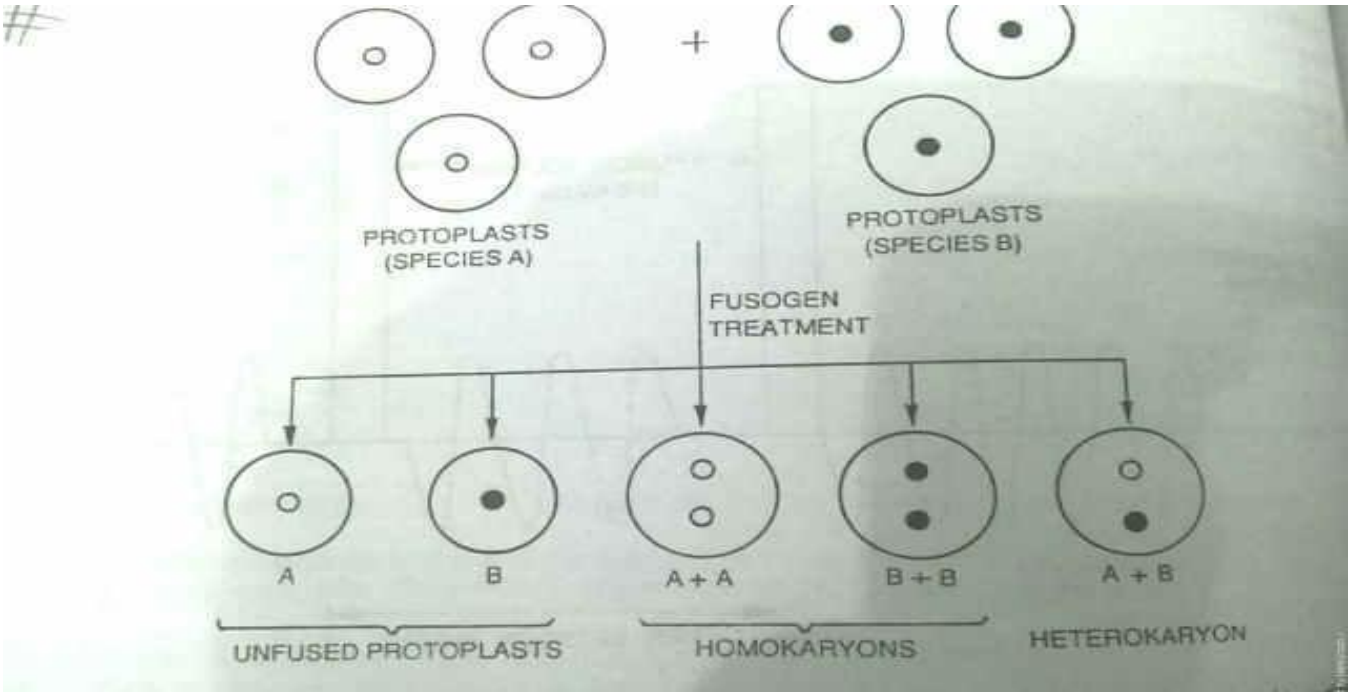
Polyethylene glycol (PEG) induced protoplast fusion is the most commonly used as it induces reproducible high frequency fusion accompanied with low toxicity to most cell types. The protoplast mixture is treated with 28-50% PEG for 15-30min, followed by gradual washing of the protoplasts to remove PEG, protoplast fusion occurs during the washing. The washing medium may be alkaline (pH 9-10) and contain a high Ca ion concentration (50 m mol/l), this approach is a combination of PEG and high pH-high Ca treatments and is usually more effective than either treatment alone. Peg is negatively charged and may bind to cation like Ca, which in turn, may bind to the negatively charged molecules present in plasma lemma, they can also bind to cationic molecules of plasma membrane. During the washing process, PEG molecule may pull out the plasma lemma components bound to them. This would disturb plasma lemma organization and may lead to the fusion of protoplasts located close to each other.

The above fusion techniques are nonselective in that they induce fusion between any two or more protoplasts. A more selective and less drastic approach is the electrofusion technique, which utilize low voltage non uniform alternating electric current pulses to bring the protoplasts in close contact. Fusion of protoplast is brought about by a short pulse of high voltage. The duration of high voltage is a few microseconds and the voltages ranges from 500 to 1000 v/cm. The high voltage creates transient disturbances in the organization of plasma lemma, which leads to the fusion of neighboring protoplasts. The entire operation is carried out manually in a specially designed equipment, called electroporator.



Selection of hybrid cells

The protoplast suspension recovered after a treatment with a fusion including agent (fusogen) consists of the following cell types (i) unfused protoplasts of the two species/stains,(ii) products of fusion between two or more protoplasts of the same species(homokaryons), (iii) hybrid protoplasts produced by fusion between one (or more) protoplast of each of the two species (heterokaryons).In somatic hybridization experiments, only the heterokaryotic or hybrid protoplasts, particularly those resulting from fusion between one protoplast of each of the two fusion partners, are in interest. However, they form only a small proportion of the population (usually 0.5-10%).Therefore, an effective strategy has to be employed for their identification and isolation. This step is called the selection of hybrid cells, is the most critical, and is still an active area of investigation.



Regeneration of hybrid plants:

Once hybrid calli are obtained, plants are induced to regenerate from them since this is a prerequisite for their exploitation in crop improvement. Further, the hybrid plants must be at least partially fertile, in addition to have some useful property, to be of any use in breeding schemes. The culture techniques have been refined to a state where plant regeneration has been obtained in a number of somatic hybrids. But even today, it has not been possible to recover hybrid plants and/or calli from a number of somatic combinations, this phenomenon is called somatic incompatibility. The reasons for somatic incompatibility are not clearly understood. The somatic hybrids are of the following two types.

1. Symmetric hybrids. Some somatic hybrid plants retain the full or nearly full somatic complements of the two parental species, these are called symmetric hybrids. Such hybrids provide unique opportunities for synthesizing novel species, which may be of theoretical and/or practical interest e.g., Datura innoxia+Atropa belladonna.

2. Asymmetric hybrids. Many somatic hybrids exhibit the full somatic complement of one

parental species, while all or nearly all chromosomes of the other parental species are lost during the preceding mitotic divisions, such hybrids are referred to as asymmetric hybrids e.g., Daucus carota + Aegopodium podagraria.

Cybrids

Cybrids or cytoplasmic hybrids are cells or plants containing nucleus of one species but cytoplasm from both the parental species .They are produced in variable frequencies in normal protoplast fusion experiments due to one of the following (i) fusion of a normal protoplast of one species with an enucleate protoplast or a protoplast having an inactivated nucleus of the other species,(ii) elimination of the nucleus of one species from a normal heterokaryon, or (iii) gradual elimination of the chromosomes of one species from a hybrid cell during the subsequent mitotic divisions. Cybrids may be produced in relatively high frequency by (i) irradiating (with X-rays or gamma-rays) the protoplasts of one species prior to fusion in order to activate their nuclei, or (ii) by preparing enucleate protoplasts (cytoplasts) of one species and fusing them with normal protoplasts of the other species.

The objective of cybrid production is to combine the cytoplasmic genes of one species with the nuclear and cytoplasmic genes of another species. But the mitotic segregation of plasmagenes, as evidenced by the distribution of chloroplasts, leads to the recovery of plants having plasmagenes of one or the other species only, only a small proportion of the plants remain cybrid, which would further segregate into the two parental types.

Cryopreservation

Plant tissues and organs can be frozen and stored in liquid nitrogen at -196 degree Celsius for long term storage of germplasm. This would be of great value in the conservation of germplasm of those crops, which normally do not produce seeds e.g root and tuber crops, produce recalcitrant seeds or where it may not be desirable to store seed. In addition, roots and tubers loose viability rapidly and their storage requires large space, low temperature and is expensive. In addition, material modified by genetic engineering may sometimes be unstable, and hence may need to be conserved intact for the future use. In such cases, the

following approaches of germplasm conservation may be applied.(i) Freeze preservations (ii) Slow growth cultures (iii) Desiccated somatic embryos/artificial seeds and (iv) DNA clones.

The preservation of cells, tissues or organs in liquid nitrogen is called cryopreservation, and the science pertaining to this activity is known as cryobiology. Many studies have been carried out on cryopreservation of plant cells and organs, and the approach appears to have considerable promise in germplasm conservation.

At -196 degree Celsius, since at this temperature, all metabolic processes and growth are suppressed and the occurance of genetic, karyotypic, morphological and biochemical changes are also prevented. Cryopreservation has proved to be the most reliable method for long term preservation of cell cultures, calli, cell suspensions, protoplast, pollen, shoot tips and embryos have all been successfully preserved. a serious drawback of the technique is that a general protocol applicable to all species and ex- plants is not available. In addition, survival tends to decline with storage period in most of the cases, most likely due to injuries sustained by cells during the freezing step.

General concept of Transgenic plants and their utility

The most potent biotechnological approach is the transfer of specifically constructed gene assemblies through various transformation techniques, this constitutes genetic engineering. The plants obtain through genetic engineering contains a gene or genes usually from an unrelated organisms, such genes are called transgenes, and the plants containing transgenes are known as transgenic plants. The first transgenic plant was produced in 1983, when a tobacco line expressing Kanamycin resistance was produced. Soon transgenic crop varieties resistant to herbicides, insects or viruses or expressing Kanamycin resistance was produced. Soon transgenic crop varieties resistant to herbicides, insects or viruses or expressing male sterility, delayed ripening or slow fruit softening were developed. Flavr-Savr tomato was the first transgenic variety to reach the market, fruits of this variety remain fresh for prolonged period. In 1996, the area under transgenic varieties was 3 million hectares, which had increased to over 34 million hectares by 1998 in merely two years.

Application of transgenic plants

Transgenic plants or genetically modified plants (GMP) have both basic and applied uses, which are briefly summarized below.

1.They have proved to be extremely valuable toll in studies on plant molecular biology, regulation of gene action, identification of regulatory/promotary sequences, etc. For example,

T-DNA and transposable element produce mutations by becoming inserted within genes and thereby act as molecular tags for gene identification and isolation. Transgenic plants also permit the analysis of metabolic pathways, studies of cis and trans acting (DNA binding proteins)factors in gene function, and elucidation of plant responses to environmental stresses, etc.

A new patented technology called 'biosource' permits rapid and effective elucidation of functions of unknown genes/DNA sequence. The gene/DNA sequence of interest is integrated into the genome of a virus, which is then used to infect a plant. The target gene is expressed in the infected plant, and the effect of its expression on phenotype is carefully monitored. Bio source technology thus facilitates rapid evaluation of function of unknown genes, it is being used by biotechnology industries to develop unique gene databases in an effort to identify new useful genes e.g., those that add value to agriculture products.

2. Specific genes have been transferred into plants to improve their agronomic and other features. Genes conferring resistance to abiotic stresses, e.g., herbicides, have been transferred in crop plants, which enables the use of biodegradable herbicides like glyphosate in otherwise susceptible crops.

3. Genes for resistance to various biotic stresses have been engineered to generate transgenic plants resistant to insects, viruses, etc.

4.Several gene transfer have been aimed at improving the produce quality, e.g., protein or lipid quality etc., of transgenic plants, the effort have met with variable degree of success.
Improved quality may also be achieved by either suppression of or overproduction by endogenous genes.

5. Transgenic plants are aimed at produced novel biochemicals like interferons, insulin,

immunoglobulins, etc., or useful biopolymers like polyhyroxy-butyrate, which are not produced by normal plants. These compounds are extracted from the plants, and can be used as pharmaceutical or industrial substrate. Cultivation of transgenic plants for the recovery of pharmaceutical compounds (medicine) is popularly known as 'pharming', this term has already found a place in modern dictionaries.

6. Transgenic plants have been produced that express a gene encoding an antigenic protein from a pathogen. When mice were fed on such a plant produce, they became immunized against the pathogen. Therefore, use of transgenic plants as vaccines for immunization against pathogen is fast emerging as an important objective.

Some authors have projected that ' in a few years from now' we may found farms without food crops, but growing transgenic plants to produce new products, e.g, plastic from peas or plant oils to manufacture hydraulic fluids or nylon.

Agrobacterium-Mediated Gene Transfer

Gene transfer through *Agrobacterium* is achieved in the following two ways: (i) co-culture with tissue explants and (ii) *in planta* transformation.

Co-culture with Tissue Explants: The appropriate gene construct is inserted within the T-region of a disarmed Ti plasmid; either a co-integrate or a binary vector is used. The recombinant DNA is placed in *Agrobacterium*, which is then co-cultured with the plant cells or tissues to be transformed for about 2 days. In case of many plant species, small(a few mm diameter) leaf discs are excised from surface sterilized leaves and used for co-cultivation, e.g., in tomato, tobacco, petunia, etc. In general, the transgenic construct includes a selectable reporter gene, e.g., the bacterial neo gene. The neo gene is linked with suitable regulatory sequences that are functional in plant cells, such a gene is called chimeric gene since it contains sequences from several different genes.

During the leaf disc- Agrobacterium coculture, acetosyringone releases by plant cells induces the vir genes, which together bring about the transfer of recombinant T-DNA into many of the plant cells.The T-DNA would become integrated into the plant genome, and the transgene

would be expressed. As a result, the transformed plant cells would become resistant to kanamycin(due to the expression of neo gene).After 2 days, the leaf discs are transferred onto a regeneration medium containing appropriate concentration of kanamycin and carbenicillin. Kanamycin allows only transformed plant cells to divide and regenerate shoots in about 3-4 weeks, while carbenicillin kills Agrobacterium cells.The shoots are separated, rooted and finally transferred into soil.

The use of leaf disc for co-culture is better than that of protoplasts or cultured cells since the last two are likely to show somaclonal variation. Agrobacterium does infect some monocot plant species and forms crowngalls ,e.g., *Asparagus*, or induces swelling, e.g., in *Dioscorea bulbifera, Chlorophytum, Narcissus and Allium cepa*. In almost all of the cases, opine production by the gall/swelling tissues was observed, and integration of T-DNA into the genome of at least *D.bulbifera and Oryza sativa* have been demonstrated. However, the efficiency of transformation is rather low. Efficient transformation of monocot cells can be obtained by providing acetosyringone during the co culture of plant cells with Agrobacterium. Some plant species may secrete compounds, which may inhibit the induction of vir operons by acetosyringone. For example, some maize varieties secrete DIMBOA(2,4-dihydroxy-7-methoxy1,4-benzoxazin 3-one), which inhibits vir gene induction, this problem has been overcome by addition of excess acetosyringone. Using this approach, successful transformation has been obtained in barley, wheat, maize and rice.

2. **In Planta Transformation:**

Imbibition of Arabidopsis seeds in fresh culture of Agrobacerium leads to stable integration of DNA in Arabidopsis genome, the majority of about 14000 transformants screened represent independent and unique events. It appears that Agrobacterium cells enter the seedling duration are retained within the plants and when flowers develop they transform either the zygotes or the cells that give rise to zygote.

Alternatively, Arabidopsis plants about to flower are immersed in a fresh culture of Agrobacterium, and partial vacuum is created to facilitate the entry of bacterial cells into the plants. The plants are grown, selfed and the progeny so obtained are screened for the

identification of transformants.

The above two approaches for Agrobacterium mediated transformation eliminate the need for regeneration from tissue explants. Therefore, they are simple and easy and can be of general application if they are successful with other plant species. In addition, there is no risk of somaclonal variation, although mutations independent of stable T-DNA integration do occur due to abortive T-DNA insertion events.`

Golden rice

Vitamin A- deficiency causes blindness among children and may even lead to their death. Vit-A deficiency often occurs where rice is the staple food, since rice grain does not contain provitamin A, i.e., beta-carotene. Three transgenes providing phytoene synthase, phytoene disaturase, zeta-carotene desaturase and lycopene cyclase activities were transferred into rice by Agrobacterium mediated transformation. All the transgenes were introduced together in a single co-transformation experiment. The resulting trangenic rice popularly called "golden rice" transgenic line, the beta carotene content was as high as 85% of the total carotenoids present in the grain.

Iron deficiency anemia is the most common nutritional disorder in the world. Rice grain has the lowest iron contents among crops, it also has phytate, which reduces iron absorption in human intestine by up to 98%. In addition, iron absorption from a vegetarian diet is rather poor. Phytate is used for phosphate storage in seeds, and it is used during seed germination. The high iron rice was produced expressing in rice three transgenes, viz, ferretin encoding gene, metallothionein gene and a thermostable phytase encoding gene.

A high iron-high provitamin A rice line was produced by crossing 'Golden rice' and 'high iron' transgenic lines of rice. It is proposed to distribute this rice line free to subsistence farmers of the developing world with a view to alleviate vitamin A and iron deficiency.

Production of Haploid Plants

There are two approaches for the production of haploid plants. The two approaches are: (1) In Vivo Approach and (2) In Vitro Approach.

Haploid plants are characterized by possessing only a single set of chromosomes (gametophytic number of chromosomes i.e. n) in the sporophyte. This is in contrast to diploids which contain two sets (2n) of chromosomes. Haploid plants are of great significance for the production of homozygous lines (homozygous plants) and for the improvement of plants in plant breeding programmes.

In Vivo and in Vitro Approaches:

The importance of haploids in the field of plant breeding and genetics was realized long ago. Their practical application, however, has been restricted due to very a low frequency (< 0.001%) of their formation in nature.

The process of apomixes or parthenogenesis (development of embryo from an unfertilized egg) is responsible for the spontaneous natural production of haploids. Many attempts were made, both by in vivo and in vitro methods to develop haploids. The success was much higher by in vitro techniques.

In vivo techniques for haploid production:

There are several methods to induce haploid production in vivo. Some of them are listed below:

1. Androgenesis: Development of an egg cell containing male nucleus to a haploid is referred to as androgenesis. For a successful in vivo androgenesis, the egg nucleus has to be inactivated or eliminated before fertilization.
2. Gynogenesis: An unfertilized egg can be manipulated (by delayed pollination) to develop into a haploid plant.

3. Distant hybridization: Hybrids can be produced by elimination of one of the parental genomes as a result of distant (inter-specific or inter-generic crosses) hybridization.

4. Irradiation effects: Ultra violet rays or X-rays may be used to induce chromosomal breakage and their subsequent elimination to produce haploids.

5. Chemical treatment:

Certain chemicals (e.g., chloramphenicol, colchicine, nitrous oxide, maleic hydrazide) can induce chromosomal elimination in somatic cells which may result in haploids.

In vitro techniques for haploid production: In the plant biotechnology programmes, haploid production is achieved by two methods.

1. Androgenesis: Haploid production occurs through anther or pollen culture, and they are referred to as androgenic haploids.

2. Gynogenesis: Ovary or ovule culture that results in the production of haploids, known as gynogenic haploids.

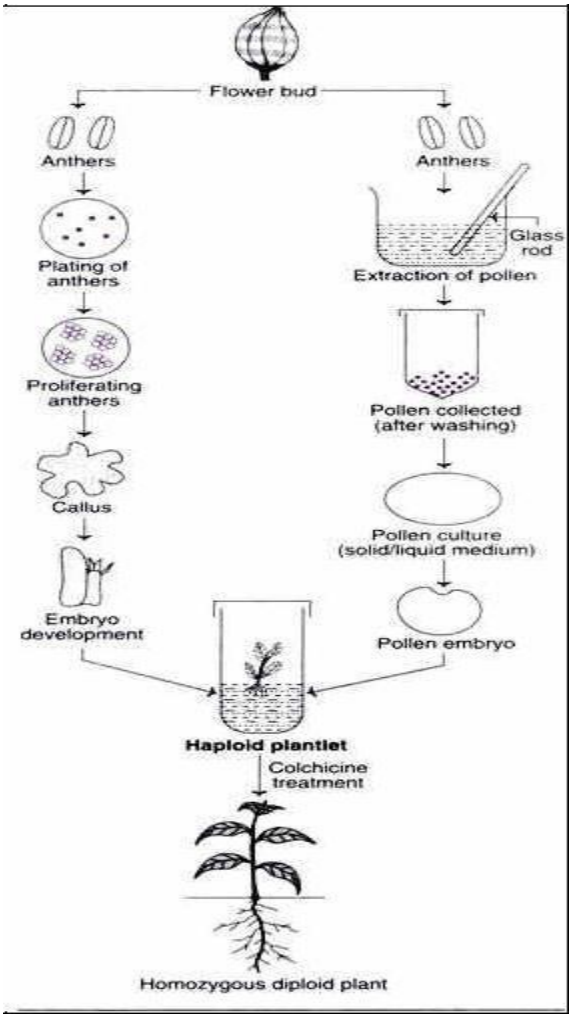
Androgenesis: In androgenesis, the male gametophyte (microspore or immature pollen) produces haploid plant. The basic principle is to stop the development of pollen cell into a gamete (sex cell) and force it to develop into a haploid plant. There are two approaches in androgenesis— anther culture and pollen (microspore) culture. Young plants, grown under optimal conditions of light, temperature and humidity, are suitable for androgenesis.

Anther Culture: The selected flower buds of young plants are surface-sterilized and anthers removed along with their filaments. The anthers are excised under aseptic conditions, and crushed in 1% acetocarmine to test the stage of pollen development.

If they are at the correct stage, each anther is gently separated (from the filament) and the intact anthers are inoculated on a nutrient medium. Injured anthers should not be used in cultures as they result in callusing of anther wall tissue.

The anther cultures are maintained in alternating periods of light (12-18 hr.) and darkness (6-12 hrs.) at 28°C. As the anthers proliferate, they produce callus which later forms an embryo

and then a haploid plant (Fig).



Pollen (Microspore) Culture: Haploid plants can be produced from immature pollen or microspores (male gametophytic cells). The pollen can be extracted by pressing and squeezing the anthers with a glass rod against the sides of a beaker. The pollen suspension is filtered to remove anther tissue debris.

Viable and large pollen (smaller pollen do not regenerate) are concentrated by filtration, washed and collected. These pollen are cultured on a solid or liquid medium. The callus/embryo formed is transferred to a suitable medium to finally produce a haploid plant (Fig. 45.1), and then a diploid plant (on colchicine treatment).

Comparison between anther and pollen cultures:

Anther culture is easy, quick and practicable. Anther walls act as conditioning factors and promote culture growth. Thus, anther cultures are reasonably efficient for haploid production. The major limitation is that the plants not only originate from pollen but also from other parts of anther. This results in the population of plants at different ploidy levels (diploids, aneuploids). The disadvantages associated with anther culture can be overcome by pollen culture.

Many workers prefer pollen culture, even though the degree of success is low, as it offers the following advantages:

i. Undesirable effects of anther wall and associated tissues can be avoided.

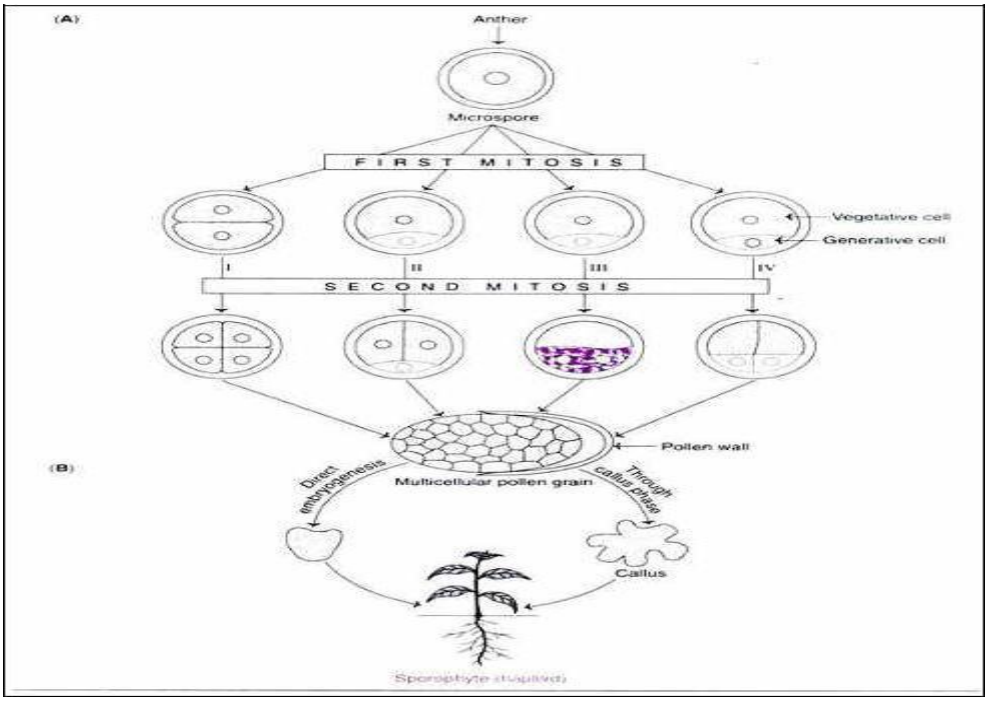
ii. Androgenesis, starting from a single cell, can be better regulated.

iii. Isolated microspores (pollen) are ideal for various genetic manipulations (transformation, mutagenesis).

iv. The yield of haploid plants is relatively higher.

Development of Androgenic Haploids:

The process of in vitro androgenesis for the ultimate production of haploid plants is depicted in Fig below.



The cultured microspores mainly follow four distinct pathways during the initial stages of in vitro androgenesis.

Pathway I:

The uninucleate microspore undergoes equal division to form two daughter cells of equal size e.g. Datura innoxia.

Pathway II:

In certain plants, the microspore divides unequally to give bigger vegetative cell and a smaller generative cell. It is the vegetative cell that undergoes further divisions to form callus or embryo. The generative cell, on the other hand, degenerates after one or two divisions—e.g., Nicotiana tabacum, Capsicum annuum.

Pathway III:

In this case, the microspore undergoes unequal division. The embryos are formed from the generative cell while the vegetative cell does not divide at all or undergoes limited number of divisions e.g. HyoScyamus niger.

Pathway IV:

The microspore divides unequally as in pathways I and II. However, in this case, both vegetative and generative cells can further divide and contribute to the development of haploid plant e.g. Datura metel, Atropa belladonna.

At the initial stages, the microspore may follow any one of the four pathways described above. As the cells divide, the pollen grain becomes multicellular and burst open. This multicellular mass may form a callus which later differentiates into a plant (through callus phase). Alternately, the multicellular mass may produce the plant through direct embryogenesis (Fig. 45.1).

Factors Affecting Androgenesis:

A good knowledge of the various factors that influence androgenesis will help to improve the production of androgenic haploids. Some of these factors are briefly described.

Genotype of donar plants: The success of anther or pollen culture largely depends on the genotype of the donor plant. It is therefore important to select only highly responsive genotypes. Some workers choose a breeding approach for improvement of genotype before they are used in androgenesis.

Stage of microspore or pollen: The selection of anthers at an ideal stage of microspore development is very critical for haploid production. In general, microspores ranging from tetrad to bi-nucleate stages are more responsive. Anthers at a very young stage (with microspore mother cells or tetrads) and late stage (with bi-nucleate microspores) are usually not suitable for androgenesis. However, for maximum production of androgenic haploids, the suitable stage of microspore development is dependent on the plant species, and has to be carefully selected.

Physiological status of a donor plant: The plants grown under best natural environmental conditions (light, temperature, nutrition, CO_2 etc.) with good anthers and healthy microspores

are most suitable as donor plants. Flowers obtained from young plants, at the beginning of the flowering season are highly responsive. The use of pesticides should be avoided at least 3-4 weeks preceding sampling.

Pretreatment of anthers: The basic principle of native androgenesis is to stop the conversion of pollen cell into a gamete, and force its development into a plant. This is in fact an abnormal pathway induced to achieve in vitro androgenesis. Appropriate treatment of anthers is required for good success of haploid production.

Treatment methods are variable and largely depend on the donor plant species:

1. Chemical treatment: Certain chemicals are known to induce parthenogenesis e.g. 2-chloroethylphosphonic acid (ethral). When plants are treated with ethral, multinucleated pollens are produced. These pollens when cultured may form embryos.

2. Temperature influence: In general, when the buds are treated with cold temperatures (3-6°C) for about 3 days, induction occurs to yield pollen embryos in some plants e.g. Datura, Nicotiana. Further, induction of androgenesis is better if anthers are stored at low temperature, prior to culture e.g. maize, rye. There are also reports that pretreatment of anthers of certain plants at higher temperatures (35°C) stimulates androgenesis e.g. some species of Brassica and Capsicum.

Effect of light: In general, the production of haploids is better in light. There are however, certain plants which can grow well in both light and dark. Isolated pollen (not the anther) appears to be sensitive to light. Thus, low intensity of light promotes development of embryos in pollen cultures e.g. tobacco.

Effect of culture medium: The success of anther culture and androgenesis is also dependent on the composition of the medium. There is, however, no single medium suitable for anther cultures of all plant species. the commonly used media for anther culture are MS, Whites, Nitsch and Nitsch, N6 and B5. These media in fact are the same as used in plant cell and tissue cultures. In recent years, some workers have developed specially designed media for anther

cultures of cereals.

Sucrose, nitrate, ammonium salts, amino acids and minerals are essential for androgenesis. In some species, growth regulators — auxin and/or cytokinin are required for optimal growth. In certain plant species, addition of glutathione and ascorbic acid promotes androgenesis. When the anther culture medium is supplemented with activated charcoal, enhanced androgenesis is observed. It is believed that the activated charcoal removes the inhibitors from the medium and facilitates haploid formation.

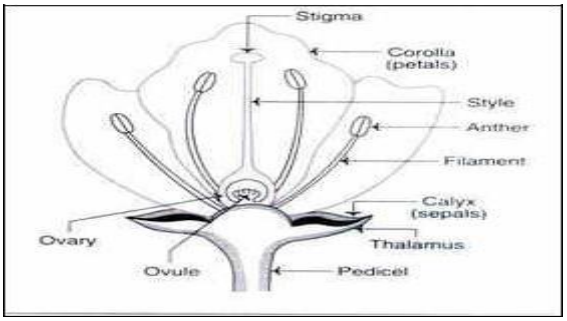
Gynogenesis:

Haploid plants can be developed from ovary or ovule cultures. It is possible to trigger female gametophytes (megaspores) of angiosperms to develop into a sporophyte. The plants so produced are referred to as gynogenic haploids.

Gynogenic haploids were first developed by San Noem (1976) from the ovary cultures of *Hordeum vulgare*. This technique was later applied for raising haploid plants of rice, wheat, maize, sunflower, sugar beet and tobacco.

In vitro culture of un-pollinated ovaries (or ovules) is usually employed when the anther cultures give .unsatisfactory results for the production of haploid plants. The procedure for gynogenic haploid production is briefly described.

The flower buds are excised 24-48 hr. prior to anthesis from un-pollinated ovaries. After removal of calyx, corolla and stamens, the ovaries (see Fig below) are subjected to surface sterilization. The ovary, with a cut end at the distal part of pedicel, is inserted in the solid culture medium.



Whenever a liquid medium is used, the ovaries are placed on a filter paper or allowed to float over the medium with pedicel inserted through filter paper. The commonly used media are MS, Whites', Nitschs, supplemented growth factors. Production of gynogenic haploids is particularly useful in plants with male sterile genotype. For such plant species, this technique is superior to another culture technique.

Limitations of Gynogenesis:

In practice, production of haploid plants by ovary/ ovule cultures is not used as frequently as anther/ pollen cultures in crop improvement programmes.

The major limitations of gynogenesis are listed:

1. The dissection of unfertilized ovaries and ovules is rather difficult.

2. The presence of only one ovary per flower is another disadvantage. In contrast, there are a large number of microspores in one another.

However, the future of gynogenesis may be more promising with improved and refined methods.

Application

Anther Culture: Anther culture is technique by which the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and are cultured on a nutrient medium where the microspore within the cultured anther develop into callus tissue or embryoids that give rise to haploid plantlets either through organogenesis or embryogenesis.

Pollen Culture: Pollen or microspore culture is an in vitro technique by which the pollen grains, preferably at the uninucleated stage, are squeezed out aseptically from the intact anther and then cultured on nutrient medium where the microscope, without producing male gametes, develop into haploid embryoids or callus tissues that give rise to haploid plantlets by

embryogenesis or organogenesis.

Ovule Culture: Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions

Vectorless gene transfer:

To achieve genetic transformation in plants, we need the construction of a vector (genetic vehicle) which transports the genes of interest, flanked by the necessary controlling sequences i.e. promoter and terminator, and deliver the genes into the host plant. Transfer of desired genes from one organism into another is an important aspect of genetic engineering. Gene transfer is achieved by two kinds of transfer methods: (i) Indirect method through vectors or carriers and (ii) Direct or vectorless transfer method. Vectorless Gene Transfer can also be transferred directly by the following methods:

Chemical mediated gene transfer e.g. chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts. Calcium phosphate is also used to transfer DNA into cultured cells.

Microinjection where the DNA is directly injected into plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5 - 1.0 micrometer diameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs, and the cells of early embryo.

Electroporation involves a pulse of high voltage applied to protoplasts/cells/ tissues to make transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA.

The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

Particle gun/Particle bombardment - In this method, the foreign DNA containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3

micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as gene gun/shot gun/microprojectile gun).The microprojectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Two types of plant tissue are commonly used for particle bombardment- Primary explants and the proliferating embryonic tissues.

Transformation - This method is used for introducing foreign DNA into bacterial cells e.g. E. Coli. The transformation frequency (the fraction of cell population that can be transferred) is very good in this method. E.g. the uptake of plasmid DNA by E. coli is carried out in ice cold $CaCl_2$ (0-50C) followed by heat shock treatment at 37-450C for about 90 sec. The transformation efficiency refers to the number of transformants per microgram of added DNA. The $CaCl_2$ breaks the cell wall at certain regions and binds the DNA to the cell surface.

Conjuction - It is a natural microbial recombination process and is used as a method for gene transfer. In conjuction, two live bacteria come together and the single stranded DNA is transferred via cytoplasmic bridges from the donor bacteria to the recipient bacteria.

Liposome mediated gene transfer or Lipofection - Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adher to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells.

Selection of transformed cells from untransformed cells

The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For this, a marker gene (e.g. for antibiotic resistance) is introduced into the plant along with the transgene followed by the selection of an appropriate selection medium (containing the antibiotic). The segregation and stability of the transgene integration and expression in the subsequent generations can be studied by genetic and molecular analyses (Northern, Southern, Western blot, PCR)

Bt cotton

Bt cotton is a genetically modified organism (GMO) cotton variety, which produces an insecticide to bollworm. It is produced by Monsanto. Strains of the bacterium *Bacillus thuringiensis* produce over 200 different Bt toxins, each harmful to different insects. Most notably, Bt toxins are insecticidal to the larvae of moths and butterflies, beetles, cotton bollworms and ghtu flies but are harmless to other forms of life. The gene coding for Bt toxin has been inserted into cotton as a transgene, causing it to produce this natural insecticide in its tissues. In many regions, the main pests in commercial cotton are lepidopteran larvae, which are killed by the Bt protein in the genetically modified cotton they eat. This eliminates the need to use large amounts of broad-spectrum insecticides to kill lepidopteran pests (some of which have developed pyrethroid resistance). This spares natural insect predators in the farm ecology and further contributes to non insecticide pest management.

Bt cotton is ineffective against many cotton pests such as plant bugs, stink bugs, and aphids; depending on circumstances it may be desirable to use insecticides in prevention. A 2006 study done by Cornell researchers, the Center for Chinese Agricultural Policy and the Chinese Academy of Science on Bt cotton farming in China found that after seven years these secondary pests that were normally controlled by pesticide had increased, necessitating the use of pesticides at similar levels to non-Bt cotton and causing less profit for farmers because of the extra expense of GM seeds.

Mechanism

Bt cotton was created through the addition of genes encoding toxin crystals in the Cry group of endotoxin. When insects attack and eat the cotton plant the Cry toxins are dissolved due to the high pH level of the insects stomach. The dissolved and activated Cry molecules bond to cadherin-like proteins on cells comprising the brush border molecules. The epithelium of the brush border membranes separates the body cavity from the gut whilst allowing access for nutrients. The Cry toxin molecules attach themselves to specific locations on the cadherin-like proteins present on the epithelial cells of the midge and ion channels are formed which allow

the flow of potassium. Regulation of potassium concentration is essential and, if left unchecked, causes death of cells. Due to the formation of Cry ion channels sufficient regulation of potassium ions is lost and results in the death of epithelial cells. The death of such cells creates gaps in the brush border membrane.

History

Bt cotton was first approved for field trials in the United States in 1993, and first approved commercial use in the United States in 1995. Bt cotton was approved by the Chinese government in 1997.

In 2002, a joint venture between Monsanto and Mahyco introduced Bt cotton to India. In 2011, India grew the largest GM cotton crop at 10.6 million hectares. The U.S. GM cotton crop was 4.0 million hectares, the second largest area in the world, followed by China with 3.9 million hectares and Pakistan with 2.6 million hectares. By 2014, 96% of cotton grown in the United States was genetically modified and 95% of cotton grown in India was GM. India is the largest producer of cotton, and GM cotton, as of 2014.

Advantages

Bt cotton has several advantages over non Bt cotton. The important advantages of Bt cotton are briefly :

- Increases yield of cotton due to effective control of three types of bollworms, viz. American, Spotted and Pink bollworms.
- Insects belonged to Lepidoptera (Bollworms) are sensitive to crystalline endotoxic protein produced by Bt gene which in turn protects cotton from bollworms.
- Reduction in pesticide use in the cultivation of Bt cotton in which bollworms are major pests.
- Reduction in the cost of cultivation and lower farming risks.
- Reduction in environmental pollution by the use of insecticides rarely.
- Bt cotton exhibit genetic resistance or inbuilt resistance which is a permanent type of

resistance and not affected by environmental factors. Thus protects crop from bollworms.

- Bt cotton is ecofriendly and does not have adverse effect on parasites, predators, beneficial insecticides and organisms present in soil.
- It promotes multiplication of parasites and predators which help in controlling the bollworms by feeding on larvae and eggs of bollworm.
- No health hazards due to rare use of insecticides (particularly who is engaged in spraying of insecticides).
- Bt cotton are early in maturing as compared to non Bt cotton.

The main selling points of Bt cotton are the reductions in pesticides to be sprayed on a crop and the ecological benefits which stem from that. China first planted Bt cotton in 1997 specifically in response to an outbreak of cotton bollworm, *Helicoverpa armigera*, that farmers were struggling to control with conventional pesticides. Similarly in India and the US, Bt cotton initially alleviated the issues with pests whilst increasing yields and delivering higher profits for farmers.

Studies showed that the lower levels of pesticide being sprayed on the cotton crops promoted biodiversity by allowing non-target species like ladybirds, lacewings and spiders to become more abundant. Likewise it was found that integrated pest management strategies (IPM) were becoming more effective due to the lower levels of pesticide encouraging the growth of natural enemy populations.

Issues with genetically modified plants.

1. The secondary pests will no longer be controlled in the absence of sprays for the major pests.

2. Cost of production of transgenic plant is very high.

3. Cost of transgenic seed sold to the farmers is very high with the result that there is increased burden on the farmer.

3. Cost of transgenic seed sold to the farmers is very high with the result that there is increased burden on the farmer.

4. Development of resistance in insect population may limit the usefulness of transgenic crops for pest management.

5. Evolution of new insect biotypes; Due to deployment of transgenic crops there is danger of evolution of new biotypes of insects.

6. Insect sensitivity. There are many species of insects that are not susceptible to currently available Bt proteins. There is need broaden the pool of genes, which can be effective against insects that are not sensitive to currently available genes.

7. Gene escapes into the environment.

8. Secondary pest problem. Most crops are not attacked by a single pest species, but a complex of insect pests attack on it. In the absence of competition from major pests, secondary pests may assume a major pest status. Effective and timely controlled measures should be adopted for the control of secondary pests on transgenic crops.

9.Effect on non-target organisms: One of the major concerns of transgenic crops is their effect on non-target organisms, about which little is known at the moment. Bt proteins are rapidly degraded by the stomach juice of vertebrates. Most Bt toxins are specific to insects as they are activated in the alkaline milieu of insect gut. However, Bt proteins can have harmful effect on the beneficial insects. Although , such effects are much less severe than those of the broad spectrum insecticides.