CHAPTER 2

PLANT CELL CULTURE AND APPLICATIONS

6.2.1. Introduction

The plant cell culture is based on a unique property of the cell, i.e., totipotency which may be defined as the ability of a plant cell to regenerate into whole plant on a defined artificial nutrient medium under the suitable physical conditions in the laboratory. In fact, it was Schwann who first drew attention to the fact that a single cell has the capacity to both grow and divide in a self regulatory fashion and that a single cell is also totipotent. This was stated in the famous 'cell theory' which was proposed in the beginning of 19th century by Schleiden and Schwann. **Gottlieb Haberlandt,** who is regarded as **'Father of Plant Tissue Culture',** first attempted in 1902 to cultivate the mechanically isolated plant leaf cells on a simple nutrient medium. Though unsuccessful in achieving the growth and differentiation of the cultured cells, he has made several predictions in plant tissue culture like the concept of growth hormones, the use of embryo sac fluids, the cultivation of artificial embryos from somatic cells, etc. and indeed all of his predictions were found to be true as demonstrated by later researchers. In the first phase during the period 1902 - 1930s, attempts were made by several scientists to culture the isolated plant organs such as roots and shoot apices (organ culture). In the second phase during 1940s - 1970s, the extensive studies were undertaken to develop the suitable nutrient media to culture plant tissues, embryos, anthers, pollen, cells and protoplasts, and the regeneration of complete plants (in vitro morphogenesis) from cultured tissues and cells. Since 1980s, a new era has started involving the introduction of foreign genes into crop plants using cell and tissue culture systems to develop genetically modified (GM) or transgenic crops with improved characteristics, which may be responsible for the 'second green revolution.

6.2.2. Cell and Tissue Culture Techniques

Basic technique

The whole plants can be regenerated virtually from any plant part (referred to as explant) or **cells**. The basic technique of plant tissue culture **(Fig. 1)** involves the following steps:

1. Selection of suitable explants like shoot tip, leaf, cotyledon and hypocotyls.
2. Surface sterilization of the explants by disinfectants (e.g. sodium hypochlorite) and then washing the explants with sterile distilled water.
3. Inoculation (transfer) of the explants onto the suitable nutrient medium (shoot regeneration medium, which is sterilized by autoclaving or filter-sterilized to avoid

microbial contamination) in culture vessels under sterile conditions (i.e., in laminar flow cabinet).

4. Growing the cultures in the growth chamber or plant tissue culture room **(Fig. 2A)**, having the appropriate physical conditions [i.e., artificial light (16 h photoperiod), temperature (~26°C) and relative humidity (50-60%)].
5. Regeneration of shoots from cultured plant tissues and their elongation.
6. Rooting of regenerated shoots on rooting medium.
7. Transfer of plants to the transgenic green-house **(Fig. 2B)** or field conditions following the acclimatization (tissue hardening) of the regenerated plants.

Nutrient media

The *in vitro* culture of plant parts or cells require a variety of nutrients and suitable physical conditions, unlike the intact plants which can synthesize their own food and many other essential compounds needed for their growth and development using light, CO_2, water and minerals. The composition of plant tissue culture medium can vary, depending upon the type of plant tissues or cells that are used for culture.

The typical plant tissue culture nutrient medium consists of inorganic salts (both micro- and macro-elements), a carbon source (usually sucrose), vitamins (e.g. nicotinic acid, thiamine, pyridoxine and myo-inositol), amino acids (e.g., arginine) and growth regulators (e.g. auxins, cytokinins and gibbrellins). Other compounds like casein hydrolysate, coconut milk, malt extract, yeast extract, tomato juice, etc. may be added

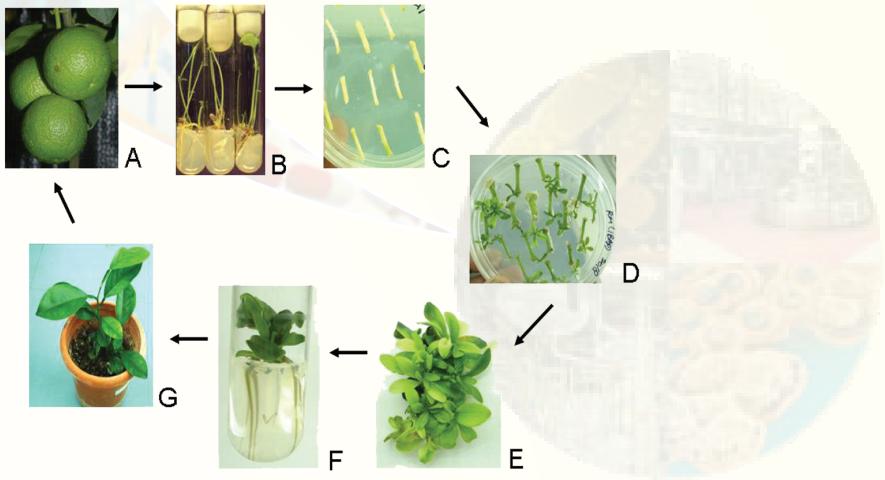


Fig. 1. Various steps involved in the regeneration of whole plants using tissue culture techniques. (A) Citrus plant with fruits, (B) Seedlings raised by germinating Citrus seeds on nutrient medium, (C) Transfer of epicotyl explants onto shoot regeneration medium, (D) Induction of shoots from explants, (E) Elongation of shoots, (F) Rooting of *in vitro* shoot, (G) Regenerated plant in a pot.

Fig. 2. Plant tissue culture room (A) and transgenic green-house (B).

for specific purposes. Plant hormones play a pivotal role in growth and differentiation of cultured cells and tissues. An optimum pH (usually 5.7) is also very important.

There are two kinds of media - **liquid and solid media**, and in the later case a gelling agent such as **agar** (a polysaccharide obtained from a red algae, *Gelidium amansii)* is added for solidification of the medium. There are several types of media like MS, LS, B5 and Nitsch's medium, and the choice of media is dictated by the plant species, variety or plant part. However, the most extensively used nutrient medium is MS medium, which was developed by Murashige and Skoog in 1962. The success of plant regeneration from cultured cells and tissues are largely governed by the composition of the culture medium. The sterilization of nutrient media by autoclaving or membrane filter-sterilization is utmost important to avoid microbial contamination.

Types of cultures

Organ culture: It deals with the culture of the isolated organs (like roots) under laboratory conditions *(in vitro)*, and different names are given depending upon the organ utilized for the culture. For instance, the culture of roots, endosperm, ovary, ovule and anther are called as i) Root culture, ii) Endosperm culture, iii) Ovary culture, iv) Ovule culture and v) Anther culture respectively.

Explant culture: The culture of plant parts **(explants)** is known as explant culture. The explants can be any part of the plant like the piece of stem, leaf, cotyledon, hypocotyls, etc. The explant cultures are generally used to induce callus or plant regeneration.

Callus culture: Callus refers to an unorganised mass of cells, which are generally parenchymatous in nature **(Fig. 3A)**. A variety of plant parts can be induced to show callusing response, but the response varies with the composition of culture media. Generally, auxins are added to culture medium for callus induction but the nature and quantity of auxin added depends on the nature and source of explant and its genotype besides other factors. Callus cultures can be maintained for prolonged period of time by repeated sub-culture. Callus cultures are used for

- plant regeneration.
- preparation of the single cell suspensions and protoplasts.
- genetic transformation studies.

Cell suspension cultures: Single cells can be isolated from either callus or any other part of the plant (e.g. leaf) and cultured in liquid medium. Both mechanical and enzymatic methods can be used for isolation of plant cells. Mechanical method involves grinding of the tissue to a fine suspension in a buffered medium followed by filtration/centrifugation to get rid of cell debris. The enzymatic method is based on the usage of enzymes (pectinase/macerozyme), which dissolve the middle lamella between the cells, i.e., the inter-cellular cement, to release single cells. Once the cells have been isolated, they may be cultured by **batch cultures** or **continuous cultures**, which have been discussed in detail (see the previous chapter). The cell suspension cultures can be used for:

- induction of somatic embryos/shoots.
- *in vitro* mutagenesis and mutant selection.
- genetic transformation.
- production of secondary metabolites.

Mass cell culture: It involves the large-scale culture of cells in specially designed 'plant bioreactors', which essentially do not have a stirrer (as plant cells are shear sensitive). In place of stirrer, gas is gently bubbled, which provides stirring as well as meet the demand of a higher oxygen supply. Mass cell cultures are ideal for producing pharmaceutically important secondary metabolites.

Protoplast culture: Protoplasts are plant cells without cell wall **(Fig. 3B)** and can be isolated from a variety of plant tissues (usually leaves, callus pieces, single cells or pollen grains) by enzymatic method using cell wall digesting enzymes (cellulases, hemicellulases and pectinases). Protoplasts are usually cultured by suspension culture in petri plates. As the protoplasts lack cell wall, they can be utilized for many purposes such as:

- Various biochemical and metabolic studies.
- Fusion of two somatic cells to produce somatic hybrids.
- Fusion of enucleated and nucleated protoplasts to produce cytoplasmic hybrids (Cybrids).
- Genetic transformation.

Protoplast fusion: The protoplasts from two different plant genotypes can be fused in the presence of fusogenic agents like polyethylene glycol (PEG - most widely used and most successful method for protoplast fusion) or by electro-fusion. The hybrid cells **(heterokaryons)** can be selected by various methods such as the use of different antibiotic markers or fluorescent dyes for two different protoplasts. Then, regenerated somatic hybrids from hybrid cells are characterized by morphology and molecular analysis (e.g. RAPD). This technique offers a unique method to raise new genotypes (i.e. somatic hybrids between distantly related plants or cybrids) with useful agronomic traits.

Plant regeneration pathways

The plants can be regenerated by either **organogenesis** and **somatic embryogenesis**.

Organogenesis means formation of organs like shoots from the cultured explants **(Fig. 3C)**. Miller and Skoog experimentally proved that formation of shoot or root first on the cultured tissue depends on the relative concentration of auxin and cytokinin. If auxins are high in the medium, then it promotes rooting while if cytokinins are high, shoot formation is promoted.

In **somatic embryogenesis**, the totipotent cells may undergo embryogenic pathway to form somatic embryos **(Fig. 3D-G)**, which can be grown to regenerate into complete plants. Generally, somatic embryos resemble the zygotic embryos (seed embryos) except in their place of origin and larger size. For the first time, Steward in 1958 and Reinert in 1959 independently reported the somatic embryogenesis from carrot cultures.

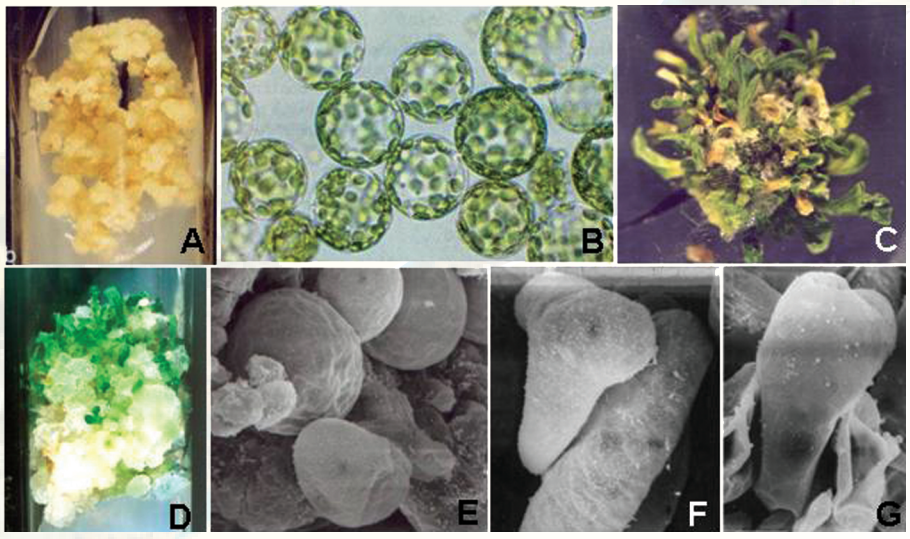


Fig. 3. Callus culture of indica rice (A), protoplasts isolated from leaf mesophyll cells of tobacco (B), multiple shoot regeneration from leaf explants of brinjal (C), regeneration of somatic embryos from leaf explants of brinjal (D) and scanning electron microscopy photographs of the various stages of somatic embryogenesis - globular (E), heart-shape (F) and torpedo (G) stage embryos from the brinjal culture (D).

6.2.3. Applications of Cell and Tissue Culture

The plant cell culture offers many potential applications in agriculture and health-care. Some of the important applications of plant cell and tissue culture are given below.

Micropropagation

Vegetative propagation of plants is of considerable importance in agriculture, horticulture and forestry as it provides the multiplication of uniform material for crop planting **(Clones)**. Traditionally, it is done by using cuttings, budding, grafting, corms, tubers and other vegetative propagules. The main problem with this method is that it is labour-intensive, low productivity and seasonal. Thus, tissue culture method of plant propagation, known as '**micropropagation**' can be used to overcome the problems mentioned above. This technique utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium, and has been adopted for commercialisation of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants.

There are four defined steps in micropropagation method. These are:

- **Initiation of culture -** from an explant like shoot tip on a suitable nutrient medium.
- **Shoot multiplication -** multiple shoots formation **(Fig. 4A)** from the cultured explant.
- **Rooting of shoots -** rooting of *in vitro* developed shoots on rooting medium.
- **Transplantation -** the hardening of tissue culture raised plants **(Fig. 4B)** as they are tender and subsequent transplantation to the green-houses or field.

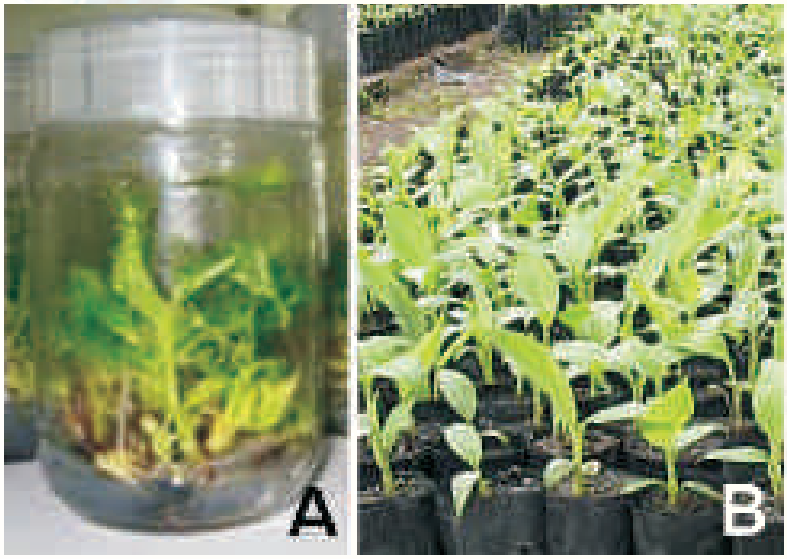


Fig. 4. Micropropagation of banana using tissue culture techniques. Multiple shoot regeneration (A) and acclimatized regenerated banana plants in green-house (B).

In recent years, much effort has been devoted to develop automated, robotised and more efficient transplant production methods. In India, the Department of Biotechnology (DBT) initiated a major network programme in 1989, and so far propagation protocols have been developed for more than 60 elite plant species.

Virus-free plants

Most of the crops plants, particularly vegetatively propagated plants are systemically infected with viruses. If the stock of a cultivar (vegetative propagules) are infected with a virus, the entire clonal population raised with such stock will also be infected and reduce the yield and quality significantly. Therefore, the production of virus-free plants is important to increase yield and quality. Interestingly, the distribution of viruses in plants is uneven, and the apical or axillary meristems are generally free from viral particles. This has enabled scientists to produce virus-free plants by culturing small meristems (usually less than 1 mm long) collected from virus-infected plants. Meristem culture technique is now successfully used to produce virus-free plants (but not virus-resistant plants) in a number of important clonal plants like potato, sugarcane, banana and apple. The scheme used for the production of virus-free plants by meristem culture is essentially same as micropropagation technique, except that the starting material (explant) is meristem which is dissected out from shoot tips of infected plants.

Artificial seeds

The artificial seeds (also called as synthetic seeds or somatic seeds) can be utilized for the rapid and mass propagation of elite plant species as well as hybrid varieties. Artificial seeds are produced either by encapsulating the somatic embryos in a protective coating, i.e., calcium alginate beads or by desiccating the somatic embryos with or without coating. A typical artificial seed is shown in **Fig. 5**.

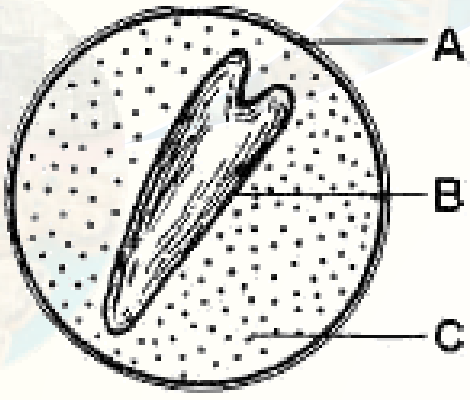


Fig. 5. The diagrammatic representation of an artificial seed. The artificial seed coat (A), somatic embryo at torpedo stage (B) and artificial endosperm (C).

Embryo rescue

It is very difficult to produce hybrids in case of inter-specific and inter-generic crosses (crosses between distantly related plants) because of abnormal development of the endosperm, which causes premature death of the hybrid embryo and leads to sterile seeds. The embryo from such sterile hybrid seeds can be excised at an appropriate time and cultured on a suitable nutrient medium to produce novel hybrids which is otherwise not possible. This is known as embryo rescue. Several useful hybrids were produced in a variety of crops using this technique.

Haploids and triploids

The technique of haploid production through anther and pollen culture as well ovary culture is of immense use in plant breeding to improve crop plants. It enables raising plants expressing traits that are otherwise recessive. The genetically homozygous diploid plants, which serve as parents in cross breeding can also be produced by diploidisation of haploid plants using colchicine chemical.

The endosperm is a triploid tissue and an excellent material to produce triploid plants by culturing endosperm tissue. Triploid plants usually show seed sterility or seedlessness, which is desirable in crops like citrus, apple and pear.

Somatic hybrids and cybrids

For many years, sexual hybridisation has been successfully used for crop improvement, however this method is limited in most cases, especially inter-specific and inter-generic crosses. The **somatic cell hybridisation** (also known as **parasexual hybridisation**) offers an excellent alternative for obtaining distant hybrids with useful agronomic traits (known as **somatic hybrids** or **parasexual hybrids**), which would never be formed in nature through sexual fertilization. Essentially, the protoplasts are isolated from the two different plants and are allowed to fuse with each other and the required fusion products (hybrid cells) are selected as discussed earlier in this chapter (see protoplast isolation and fusion).

The first inter-specific somatic hybrid between *Nicotiana glauca* and *N. langsdorffii* was produced by Carlson and his associates in 1972. Later, Melchers and his team in 1978 developed the first inter-genetic somatic hybrids between *Solanum tuberosum* (potato) and *Lycopersicon esculentum* (tomato) and the hybrids are known as '**Pomatoes** or **Topatoes**'. Several inter-specific somatic hybrids have been produced in number of crops like brinjal **(Fig. 6)**.

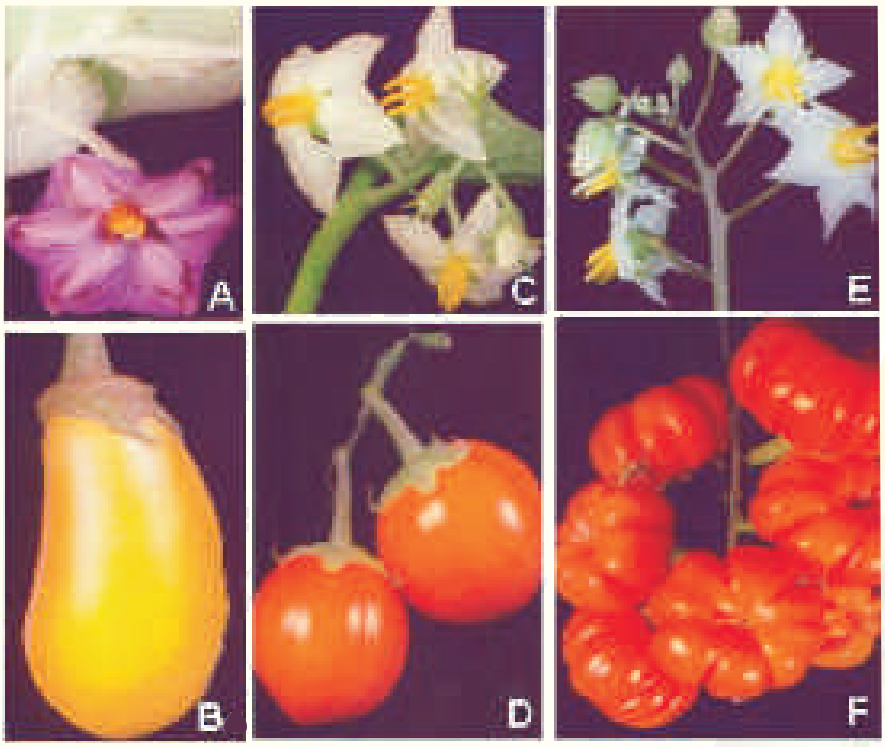


Fig. 6. Development of somatic hybrids in brinjal for transferring disease resistance from wild species to the cultivated variety. Flowers and fruits of the cultivated brinjal **(A & B)**, somatic hybrids **(C & D)** and wild-type brinjal **(E & F)**.

It is also possible to produce cytoplasmic hybrids **(cybrids)** through protoplast fusion in which the genomes of one of the partners is lost. Alternatively, the isolated and purified organelles - chloroplasts or mitochondria from one species can be fused with the recipient protoplasts from a different plant species (known as **organelle transfer** or **organelle uptake**) to transfer useful cytoplasmic traits like herbicide tolerance and cytoplasmic male sterility.

Production of secondary metabolites

Plants produce throusands of sophisticated chemical molecules. These include the chemicals required for a plant's basic metabolic processes such as sugars, lipids, amino acids and nucleic acids **(primary metabolites)** and also some other additional products such as alkaloids, resins, tannins, latex, etc. **(secondary metabolites)**.

The function of secondary metabolites in plants is not clearly understood, although they have been implicated in defence mechanism of the plant against pests and pathogens as well as

feeding by animals. However, many of the secondary products, especially various alkaloids are of immense use in medicine **(Table 1)**. Such compounds are produced in plants only in small amounts, and therefore, they are quite expensive.

Table 1. Few examples of industrially important plant secondary metabolites produced through cell and tissue cultures

Product	Plant source	Uses
Artemisin	*Artemisia* spp.	Antimalarial
Azadirachtin	*Azadirachta indica* (Neem)	Insecticidal
Berberine	*Coptis japonica*	Antibacterial, Antiinflammatory
Capsaicin	*Capsicum annuum* (chilli)	Reumatic pain treatment
Codeine	*Papaver* spp.	Analgesic
Digoxin	*Digitalis lanata*	Cardiac tonic
Diosgenin	*Dioscorea deltoidea*	Antifertility
Scopolamine	*Datura stramonium*	Antihypertensive
Quinine	*Cinchona officinalis*	Antimalarial
Shikonin	*Lithospermum erythrorhizon*	Antimicrobial; Red pigment used in lipstics & dye for silk
Taxol	*Taxus* spp.	Anticarcinogenic
Vincristine	*Cathranthus roseus*	Anticarcinogenic

The indiscriminative use of such valuable medicinal and other plants has brought them to near extinction. A possible solution in this direction is provided by cell and root cultures. Certain plant products such as shikonin are being produced commercially in large-scale using bioreactors.

Somaclonal variation

It has been observed that the long-term callus and cell suspension culture and plants regenerated from such cultures are often associated with chromosomal variations **(somaclonal variation)**. It is this property of cultured cells that finds potential application in the crop improvement and in the production of mutants (e.g., disease resistance in potato).

Larkin and Scowcroft (1981) proposed the term **'somaclones'** for plant variants obtained from tissue cultures of somatic tissues. Similarly, if the tissue from which the variants have been obtained is having gametophytic origin such as pollen or egg cell, it is known as **'gametoclonal variation.**

In vitro plant germplasm conservation

Germplasm refers to the sum total of all the genes present in a crop and its related species. The availability of a wide diversity (which includes land races, cultivars and primitive wild species of crop plants) and its conservation is utmost important as they are invaluable for future breeding programmes. However, the existence of primitive cultivars and wild relatives of crop plants has been endangered by the extensive use of newly introduced cultivars and hybrids, and this has posed a great concern to avoid the erosion of plant genetic resources. Therefore, attempts have been made to preserve the genetic resources by conventional methods (e.g. seeds, vegetative propagules, etc. and it is known as ***in vivo* gene banks**) as well as non-conventional methods, i.e. cell and tissue culture methods (known as ***in vitro* gene banks**). This will ensure the availability of valuable germplasm to breeder, to develop new and improved varieties.

The germplasm conservation through the conventional methods has several limitations such as, the short-lived seeds, seed dormancy, seed-borne diseases and high inputs of cost and labour. On the other hand, the biotechnological approaches for the conservation of plant germplasm can surpass many of these problems, and it can be done by the following approaches.

- **Freezing storage or Cryopreservation** - this utilizes the long-term preservation of cells and tissues (e.g. shoot tips, axillary buds, meristems, somatic embryos, etc.) at ultra-low temperature (-196°C, i.e. in liquid nitrogen) for indefinite time by using cryoprotectants (e.g. dimethylsulfoxide, glycerol, proline and mannitol). The cells and tissues can be recovered after thawing, and can be used for regeneration of plants.
- **Cold storage** - this uses the short- or medium-term storage of germplasm by using shoot tip, nodal or meristem explant cultures. The storage in done under conditions that impose slow growth such as low temperature (4°C or 15°C), nutrient limitation or the addition of growth retardants (e.g. absisic acid) in medium.

6.2.4. Gene transfer methods in plants

For achieving genetic transformation in plants, the basic pre-requisite is the construction of a vector (genetic vehicle) which carrys the genes of interest flanked by the necessary controlling sequences, i.e. promoter and terminator, and deliver the genes into the host plant. There are two kinds of gene transfer methods in plants as discussed below.

Vector-mediated or indirect gene transfer

Among the various vectors used in plant transformation, the Ti-plasmid of *Agrobacterium tumefaciens* has been used extensively. This bacterium contains large size plasmid, known as Ti-plasmid (tumour-inducing plasmid) and portion of this plasmid referred as **T-DNA** (transferred DNA) is transferred to plant genome in the infected cells and cause plant tumours (**crown galls - Fig. 7A**). This means that *A. tumefaciens* has natural ability to transfer T-DNA of its plasmid into plant genome. (plant chromosomes) upon infection of cells at the wound site, and therefore this bacterium is known as '**natural genetic engineer of plants**'. Because of this unique property, Ti-plasmid can be used as gene vectors for delivering useful foreign genes into plant cells and tissues. The foreign genes (**transgenes**), i.e., the gene of interest (e.g. Bt gene for insect resistance) and plant selection marker gene, usually an antibiotic gene like *nptII* which confer resistance to kanamycin are cloned in the T-DNA region of Ti-plasmid in place of unwanted DNA sequences **(Fig. 7B)**.

The general strategy for transforming plants is to collect leaf discs (in case of dicots) or embryogenic callus (in case of monocots like cereals) and then infect the tissue with Agrobacterium carrying recombinant disarmed Ti-plasmid vector **(Fig. 7B)**. The infected tissue can then be cultured (**co-cultivation**) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. Later, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic (e.g. kanamycin) to selectively eliminate non-transformed tissues. This **selection medium** also contain a bacteriostatic agent, i.e. an antibiotic like cefotaxime which suppresses or kills the Agrobacterium present with the transformed tissues and is no longer needed as the transfer of foreign genes has already taken place during co-cultivation. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root-inducing medium, and after another 3-4 weeks, complete plants can be transferred to soil following the hardening (acclimatisation) of regenerated plants. The presence of foreign genes in the putative transgenic plants can be confirmed by molecular techniques like PCR and Southern blot hybridisation.

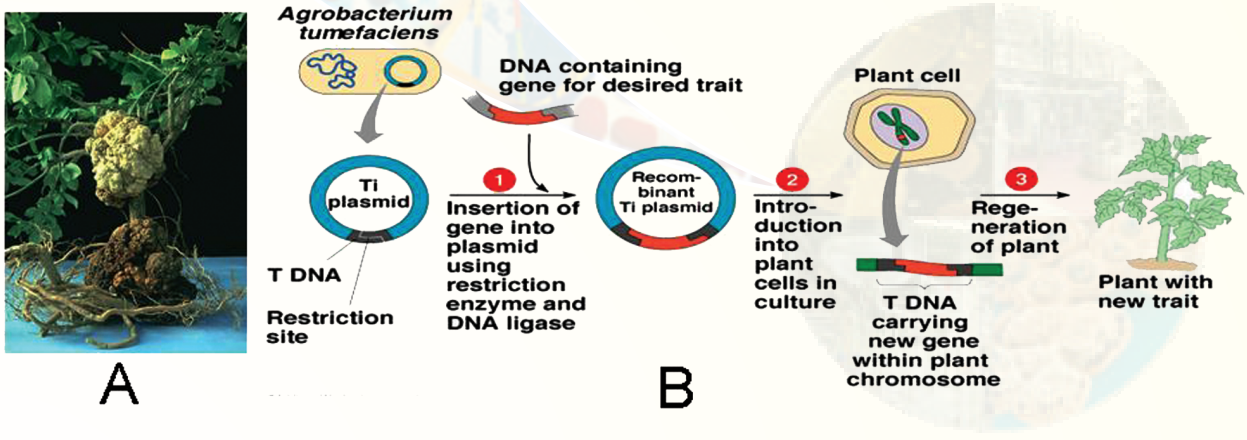


Fig. 7. Induction of crown gall on the wounded stem induced by wild-type virulent *Agrobacterium tumefaciens* (A), and the schematic diagram showing the cloning of the gene of interest in Ti-plasmid of Agrobacterium and its transfer to plant cells in culture to produce transgenic plants with desirable traits (B).

Vectorless or direct gene transfer

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The following are some of the common methods of direct gene transfer in plants.

Chemical mediated gene transfer: Certain chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts.

Microinjection: Here, the DNA is directly injected into plant protoplasts (specifically into the nucleus) using fine tipped (0.5-10 micrometer diameter) glass needle or micropipette to transform plant cells. The protoplasts are immobilized on a solid support (e.g. agarose on a microscopic slide) or held with a holding pipette under suction, and the DNA is injected into the protoplasts with the help of a fine microscope.

Electroporation: In this case, a pulse of high voltage is applied to protoplasts/cells/tissues, which makes transient (temporary) pores in the plasma-membrane which facilitates the uptake of foreign DNA. The DNA is mixed with the plant protoplasts in a specially made cuvettes before the application of the electric field.

Particle gun: A popular and widely used direct gene transfer method for delivering foreign genes into virtually any tissues and cells or even intact seedlings. In this method, the foreign DNA (containing the genes of interest) is coated (precipitated) onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded (shot) onto the target tissue or cells using a particle gun (also called as gene gun/shotgun/microprojectile gun). Then, the bombarded cells or tissues are cultured on selection medium to regenerate plants from the transformed cells **(Fig. 8)**.

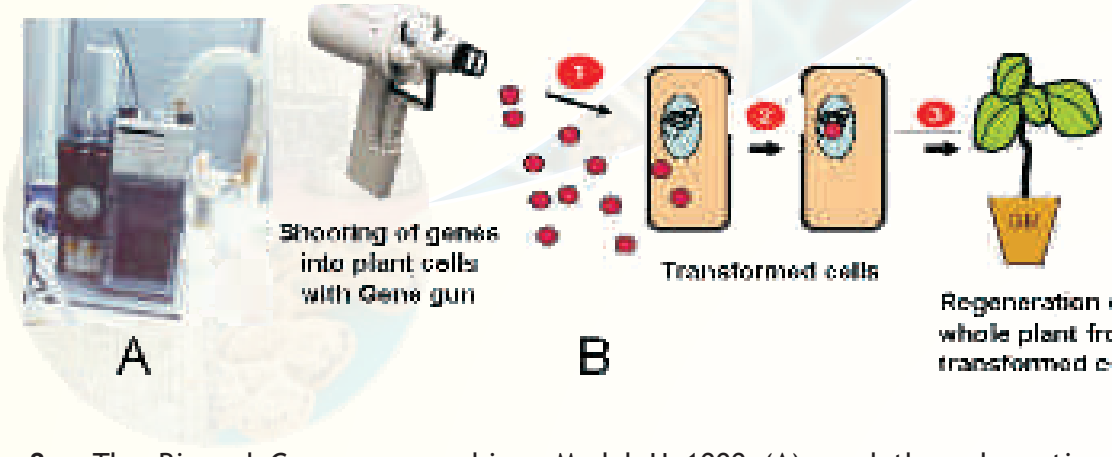


Fig. 8. The Bio-rad Gene gun machine, Model He1000 (A), and the schematic diagram showing the bombardment of gold particles containing the DNA (gene of interest) on their surface onto the plant cells in culture to produce transgenic plants with desirable traits (B).

Transgene analysis

The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For selection, the gene of interest is transferred along with a genetic marker gene (usually antibiotic resistant genes such as *npt*II that confer resistance to kanamycin), so that only the transformed cells can be selected and regenerated on the selection medium containing the selection agent (e.g. kanamycin). The untransformed cells are eliminated on the selection medium as they are susceptible to the antibiotic (due to the absence of antibiotic resistance gene). The segregation and stability of the transgene integration and expression in the subsequent generations can be studied by genetic and molecular analyses (PCR, Southern, Northern and Western blot analysis). (Refer to earlier chapters for details of these techniques).

6.2.5. Transgenic plants with beneficial traits

During the last couple of decades, considerable progress has been made to understand the function of genes, isolation of novel genes and promoters as well as the utilization of these genes for the development of transgenic or genetically modified (GM) crops with improved and new characters. There are many potential applications of plant genetic engineering and some important examples of transgenic crops, approved by the U.S Food and Drug administration are summarised in **Table 2.**

Table 2. Few Examples of transgenic crops approved by the U.S Food and Drug administration

Gene(s) introduced	New/Improved Character	Developer
Thioesterase	High laurate oil Weed	Calgene
EPSP synthase PAT & Barnase/	Control Weed Control &	Monsanto
Barstar	Hybrid production	AgrEvo
EPSP synthase	Weed control	Monsanto Monsanto,
Bt CrylA(b)	Insect resistance	Ciba-Geigy Northrup King
Bt CrylA(c)	Insect Resistance	Dekalb Genetics
Acetolactate synthase	Weed Control	DuPont
Nitrilase	Weed Control	Calgene
EPSP synthase	Weed Control	Monsanto
Bt CrylA(c)	Insect Control	Monsanto
Coat protein	Virus Resistance	Univ. Hawaii & Cornell Univ.
Bt CryIIIA & Coat protein	Insect & virus control	Monsanto
EPSP synthase	Weed control	Monsanto
Gmfad2-1	Improved Oil	DuPont
Bt CrylA(c)	Insect control	Monsanto Calgene
Antisense PG	Delayed ripening	

Stress tolerance

Crop plants are very productive under ideal cultural conditions, but ideal growing conditions rarely occur. Moreover, plants encounter both biotic (viral, bacterial, fungal pathogens, nematodes, insect pests and weeds) and abiotic (salinity, drought, extreme temperatures, nutrient deficiency, etc.) stresses and these stresses cause a colossal loss of crop yield and quality. The application of chemical and biological pesticides as well as the use of resistant varieties is only partial success and these have certain limitations. Therefore, newer and effective technologies are essential to meet the demand. In this context, biotechnological strategies can be used to create transgenic plants with increased resistance to diseases and pests as well as abiotic stresses.

Biotic stress tolerance

Herbicide tolerance: Weeds (plants growing where they are not wanted, e.g. Striga) decrease crop yields and quality primarily by competing with crop plants for light, water and nutrients. Farmers apply herbicides/weedicides (e.g. glyphosate) for the eradication of weeds in the fields, but the main problem with this is the development of herbicide tolerance by weeds. Newer techniques, based on biotechnological tools, have been developed which are quite effective for weed management as well as in increasing the yields and income. There are several biotechnological strategies for weed control, but the most commonly employed approach is the over-production of herbicide target enzyme (usually in the chloroplast) in the plant, so that it becomes insensitive to the herbicide. The popular example for such an approach is the introduction of a modified gene from an Agrobacterium species that encodes for a resistant form of the herbicide target enzyme into crop plants for tolerance against the most extensively used herbicide glyphosate (sold as Roundup) and is effective against many weeds. Roundup Ready GM crop plants such as canola **(Fig. 9A)**, soybean, corn and cotton tolerant to glyphosate has already been commercialised.

Pest resistance: All crop plants are affected by a variety of insects and nematodes, and significantly reduce their yield and quality. To minimize these losses (both food and money), farmers use the synthetic pesticides extensively which cause severe effects on human health and environment. The transgenic technology provides an alternative and innovative method to improve pest control management which are eco-friendly, effective, sustainable and beneficial in terms of yield. The first genes available for genetic engineering of crop plants for pest resistance were *Cry* genes (popularly known as Bt genes) from a bacterium *Bacillus thuringiensis*.

These are specific to particular group of insect pests, and are not harmful to useful insects like butter flies and silk warms. Transgenic crops (e.g. cotton, rice, maize, potato, tomato, brinjal, cauliflower, cabbage, etc.) with Bt genes have been developed for and such transgenic varieties proved effective in controlling the insect pests and it has been claimed worldwide that it has led to significant increase in yields, and dramatic reduction in pesticides' use. Bt crops have already been commercialised in several countries. The most notable example is Bt cotton (which contains *CrylAc* gene) that is resistant to a notorious insect pest Bollworm and in the year 2002, Bt cotton was adopted in India **(Fig. 9B)**.

Disease resistance: Pathogens (viruses, fungi and bacteria) infect crop plants and drastically reduce their yield and quality. Globally, several diseases have caused havoc at several points of time in several countries. For instance, the great Irish famine resulted in great damage of potato crop due to the late blight disease caused by fungal pathogen. India has also experienced a famine in the form of Bengal famine due to destruction of rice caused by fungal pathogen. Thus, there is a great concern about the control of plant diseases. Traditionally, farmers apply chemical pesticides or use resistant crop varieties developed by the breeders, but these methods have certain limitations.The alternative and useful strategy is the creation of disease resistant transgenic crop plants by transfer of resistance genes from varied sources.

Virus resistance: There are several strategies for engineering plants for viral resistance, and these utilizes the genes from virus itself (e.g. the viral coat protein gene). The virus-derived resistance has given promising results in a number of crop plants such as tobacco, tomato, potato, alfalfa and papaya. Some viral resistance transgenic plants like papaya resistant to papaya ring spot virus **(Fig. 9C)** have been commercialised in some countries.

Fungi and bacteria: Plants respond to pathogens by inducing a variety of defence responses like pathogenesis-related proteins (PR proteins), enzymes that degrade/destroy fungal cell wall, antifungal proteins and compounds, phytoalexins, etc. Several transgenic crop plants showing increased resistance to fungal pathogens are being raised with genes coding for the different compounds mentioned above.

Some of these strategies, particularly the genes that encode for phytoalexins and cell wall degrading enzymes were also used for producing bacterial resistant transgenic plants.

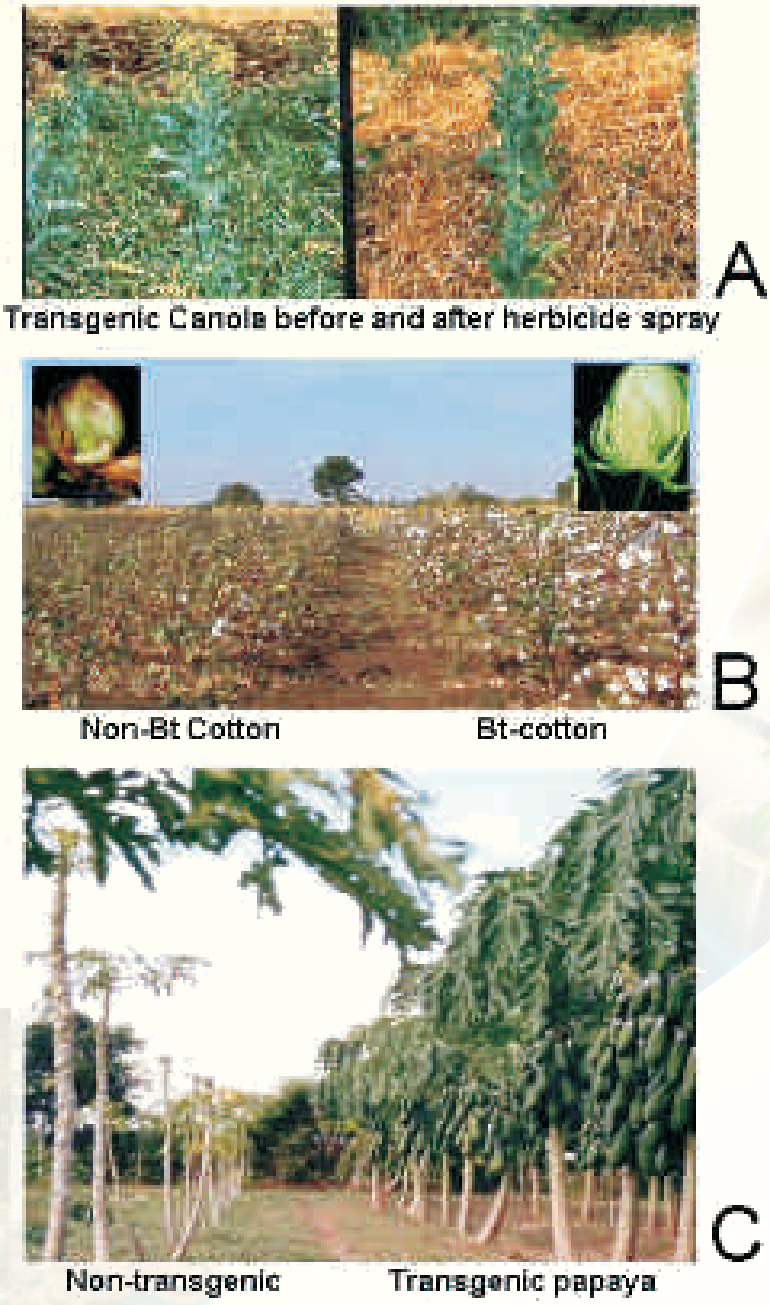


Fig.9. Genetically engineered crops with desirable traits. Transgenic canola for herbicide (glyphosate) tolerance before and after herbicide spray in the field (A); Bt cotton resistant to bollworm *(Helicoverpa armigera)* with healthy bolls (inserted photo) and non-Bt cotton with infested insect larvae (inserted photo) (B); Transgenic papaya resistant to papaya ringspot virus (c).

Abiotic stress tolerance

Plant growth and productivity are greatly affected by various environmental stresses/ abiotic stresses like high salinity and drought. Plant breeding efforts to produce abiotic stress tolerant plants while retaining high production is not very successful.

Plants have evolved many types of adaptations to cope with abiotic stress conditions like the production of the stress-related osmolytes like sugars (e.g. trihalose and fructans), sugar alcohols (e.g. mannitol) and amino acids (e.g. proline), glycine betaine, and certain proteins (e.g. antifreeze proteins). Transgenic plants have been developed which over-express the genes for one or more of the above mentioned compounds. Such plants have shown increased tolerance to environmental stresses.

Delayed fruit ripening

The gas hormone, ethylene is involved in the regulation of fruit ripening. Therefore, ripening can be slowed down by blocking or reducing ethylene production. This can be achieved by introducing ethylene forming gene(s) in a way that will suppress its own expression in the crop plant. Fruits from such plants ripen very slowly (however, they can be ripen by ethylene application) and are very important for export to longer distances without spoilage as they show longer-shelf life due to slow ripening. The notable example of this kind is the ***'Flavr Savr'*** transgenic tomatoes which were commercialized in U.S.

Male sterility

Male sterile plants are very important to prevent unnecessary pollination and to eliminate the process of emasculation during the production of hybrid plants. These are created by introducing a bacterial gene from *Bacillus amyloliquefaciens* that encode an enzyme **barnase**, which is an RNA hydrolyzing enzyme that inhibits pollen formation if, the expression of this gene specifically in the tapetal cells of anther using **tapetal-specific promoter** (e.g. TA29) to restrict its activity only to the cells involved in pollen production. Male fertility can be restored by introducing another gene from the same bacterium under the control of TA29, whose product **barstar** (protein) tightly bind with RNase, so that the normal pollen are formed. This **barnase/barstar** system was successfully utilized in producing male sterile/restorer lines in number of crops, particularly mustard for hybrid production.

Transgenic plants as bioreactors (Molecular farming)

Plants are amazing and cheap chemical factories that need only water, minerals, sun light and carbon dioxide to produce thousands of sophisticated chemical molecules with different structures. Given the right genes, plants can serve as bioreactors to modified or new compounds such as amino acids, proteins, vitamins, plastics, pharmaceuticals (peptides and proteins), drugs, enzymes for food industry and so on. Some of the potential and remarkable examples of this kind are described here.

Nutrient quality

Plants are the principle source of human nutrition. A small number of crop plants such as cereals, legumes, vegetables and root crops supply most of the energy and nutrients (in the form of fats, carbohydrates, proteins, vitamins and micronutrients) in the human diet. It is estimated that about 800 million people, mostly in developing countries are malnourished (particularly children) and suffering with several diseases due to the deficiency of nutrients. Therefore, the improvement of nutritional quality of crop plants is extremely important. Transgenic crops with improved nutritional quality have already been produced by introducing genes involved in the metabolism of vitamins, minerals and amino acids. Few examples of genetic modification of nutrition quality are described below.

Vitamin A: The source of vitamin A is either directly from animal food (e.g. milk, cheese and meat) or indirectly from green leafy vegetables (e.g. carrots, spinach, tomatoes, and chillies) and fruits (e.g. mango, melon and papaya), which contain carotenoids (beta-carotene - the provitamin A) that are converted to vitamin A in the body. Vitamin A deficiency can lead to night blindness, permanent blindness and skin disorder, among others. About 124 million children worldwide are deficient in vitamin A and a quarter of a million go blind each year due to vitamin A deficiency.

The staple food rice is extremely low in vitamin A, and therefore the improvement of vitamin A content is very important. In a remarkable example of genetic engineering, Prof. Ingo Potrykus and Dr. Peter Beyer developed genetically engineered rice (popularly known as '**Golden Rice**'), which is enriched in pro-vitamin A (beta-carotenoids) by introducing three genes involved in the biosynthetic pathway for carotenoid under the control of endosperm-specific promoter, so that gene products (enzymes) are synthesized in the rice endosperm. The seeds of Golden Rice are yellow in colour because of pro-vitamin A is produced in the entire grain **(Fig. 10A)**. Interestingly, they have also further engineered this Golden Rice by introducing three more genes from different organisms for iron source and its absorption. Similarly, transgenic crop plants are also being produced to raise the level of other vitamins, particularly vitamin E (which is an essential antioxidant in humans) and vitamin K (which is involved in bone formation).

Seed protein quality: The nutritional quality of cereals and legumes are limited because of deficiency of the essential amino acids, i.e. lysine in cereals, and methionine and tryptophan in pulses. Two genetic engineering approaches have been used to improve the seed protein quality. In the first case, a transgene (e.g. gene for protein containing sulphur rich amino acids was introduced into pea plant (which is deficient in methionine and cysteine, but rich in lysine) under the control of seed-specific promoter. In the second approach, the endogenous genes are modified, so as to increase the essential amino acids like lysine in the seed proteins of cereals.

Diagnostic and therapeutic proteins

Transgenic plants can also produce a variety of proteins used in diagnostics for detecting human diseases and therapeutics for curing human and animal diseases in large-scale with low cost. The monoclonal antibodies, blood plasma proteins, peptide hormones and cytokinins are being produced in transgenic plants and their parts such as tobacco (in leaves), potato (in tubers), sugarcane (in stems) and maize (in seed endosperm).

Edible vaccines

Crop plants offer a cost-effective bioreactor to express antigens which can be used as edible vaccines. Some of the antigenic determinants on the surface of viruses and bacteria are proteins made by the pathogen. The genes encoding these antigenic proteins can be isolated from the pathogens and expressed in plants, and such transgenic plants or their tissues producing antigens can be eaten for vaccination/immunisation **(edible vaccines)**. The expression of such antigenic proteins in crops like banana and tomato are useful for immunisation of humans since banana and tomato fruits can be eaten raw. In case of animals, such genes can be expressed in crops like alfalfa and other forage/fodder crops, which are suitable for vaccination. The edible vaccines that are produced in transgenic plants have great advantages like the alleviation of storage problems, easy delivery system by feeding and low cost as compared to the recombinant vaccines produced by bacterial fermentation. Vaccinating people against dreadful diseases like cholera and hepatitis B by feeding them banana/tomato, and vaccinating animals against important diseases such as foot and mouth disease by feeding them sugar beets could be a reality in the near future.

Biodegradable plastics

The biodegradable plastic, e.g. polyhydroxybutyrate (PHB) is being produced commercially by fermentation with the bacterium *Alcaligenes eutrophus*. The main drawback of bacterial PHB is its high production cost, making it substantially very expensive than synthetic plastics. Alternatively, transgenic plants can be used as factories to produce PHB. The genetically engineered Arabidopisis plants with the three genes involved in PHB synthesis from *A. eutrophus* produced PHB globules exclusively in their chloroplasts without effecting plant growth and development **(Fig. 10B)**. The large-scale production of PHB may be easily achieved in tree plants like populus, where PHB can be extracted from leaves. Industry has already begun to explore the production of biodegradable plastics from transgenic plants.

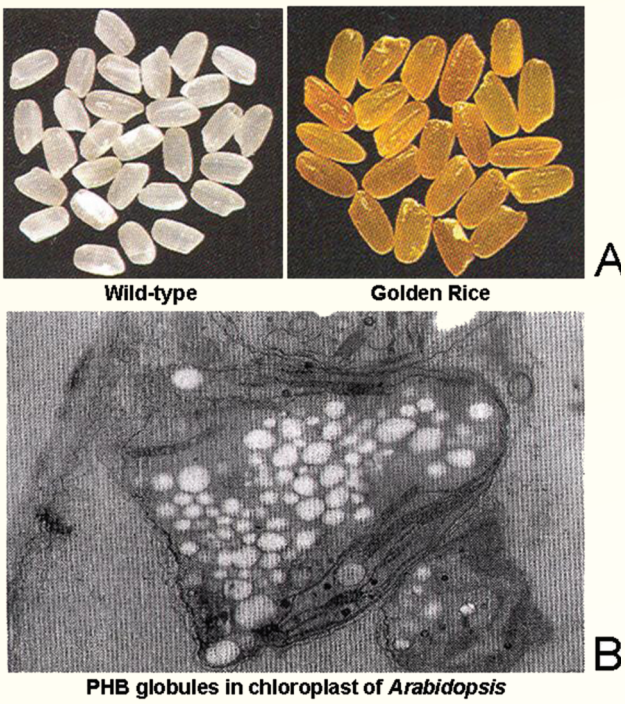


Fig. 10. Golden rice (A) and electron microscopic picture of chloroplast of transgenic Arabidopsis showing PHB globules (B).

Metabolic engineering and secondary products

Plant biotechnology will lead to improved plant sources for the production of valuable secondary metabolites mentioned in previous section on cell culture products. Biosynthetic pathway which lead to the production of such valuable products are being engineered for their over-production to reduce the cost. The over-expression of the gene, which encode for the first enzyme in a pathway generally results in higher levels of the desired end product, and this has been successfully done in the enhancement of taxol production from the transformed tissue cultures of *Taxus* sp. Another strategy involves the use of *Agrobacterium rhizogenes* to induce the excessive formation of secondary roots **(hairy roots)** in plants that normally produce useful secondary metabolites in this region.

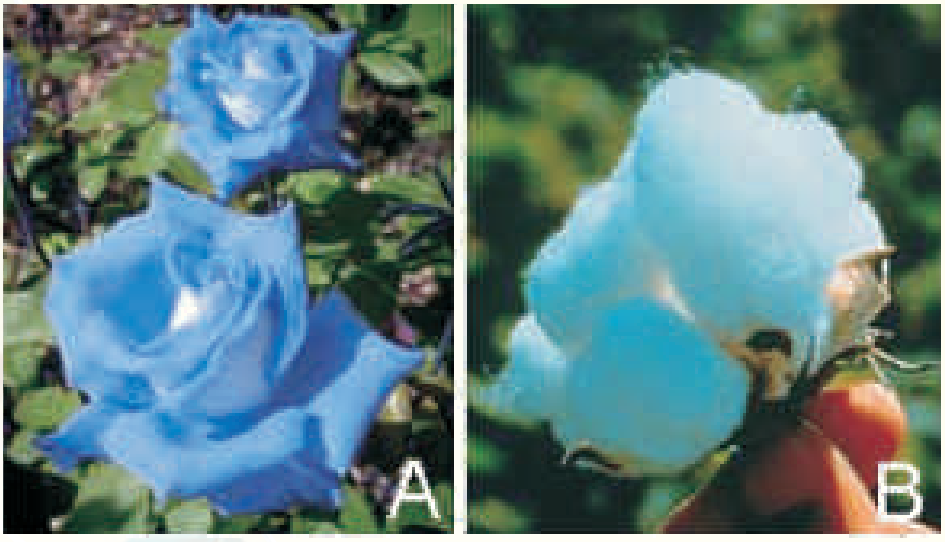
Other applications

There are many other applications of plant genetic engineering, which are listed below:

- Production of healthy oils with altered fatty acid profiles.
- Modification of starch properties for specific uses.
- Favourable change of grain storage products and their chemical composition to improve the processing of bread making with wheat flour, malting of barley and brewing of beer.

- Removal of undesirable toxic compounds in certain plants.
- Development of blue roses **(Fig. 11A)**, which is otherwise not possible by conventional plant breeding because of the absence of blue pigment in roses.
- Development of blue coloured cotton **(Fig. 11B)** and also with other colours.
- Development of tear-less onions, caffeine-free coffee and low nicotine tobacco.

Undoubtedly, there will be many more exciting applications of plant genetic engineering in the future.



Blue Rose **Blue Colore Cotton**

Fig. 12. Genetically modified blue rose (A) and blue coloured cotton (B).

6.2.6. Biosafety in Plant Genetic Engineering

The GM crops are fast becoming a part of agriculture throughout the world because of their contribution to the increased crop productivity and to global food, feed and fibre security, besides their use in health-care and industry. However, the constraints associated with public acceptance of transgenic crops continue to be important challenges facing the global community. The following are the major concerns about GM crops and GM foods:

- The safety of GM food for human and animal consumption (e.g. GM food may cause allergenicity).
- The effect of GM crops on biodiversity and environment.
- The effect of GM crops on non-target and beneficial insects/microbes.
- Transgenes may escape through pollen to related plant species (gene pollution) and may lead to the development of super weeds.

- The GM crops may change the fundamental vegetable nature of plants as the genes from animals (e.g. fish or mouse) are being introduced into crop plants.
- The antibiotic resistance marker genes used to produce transgenic crops may horizontally transfer into microbes and thus exacerbate problem of antibiotic resistance in human and animal pathogens (i.e. transgenes may move from plants to gut microflora of humans and animals).
- The GM crops may lead to the change in the evolutionary pattern.

Unfortunately, the public debate over the benefits and hazards of plant gene technology suffers from an astounding array of misinformation, misunderstanding and manipulation. In fact, with the continuing accumulation of evidence of safety and efficiency, and the complete absence of any evidence of harm to the humans and animals as well as the environment, more and more consumers are becoming comfortable because of the increased awareness about the potential of the plant genetic engineering. Further, the transgenic crops (e.g. cotton, tomato, corn and soybean) which have already entered the market place (which carry a label, i.e., GM crop or GM food in several countries were subjected for the extensive field trials for environmental safety related to wild species and competitive performance of transgenic and toxic effects as per the standard regulatory policies before they were approved for commercialisation. In fact, in 2009, a record of 14 million farmers from 25 countries cultivated 134 million hectares (330 million acres) with the crops that were genetically engineered for herbicide, insect resistance, delayed fruit ripening and improved oil quality. The global area of biotech crops planted from 1996 to 2009 is shown in **Fig. 12**.

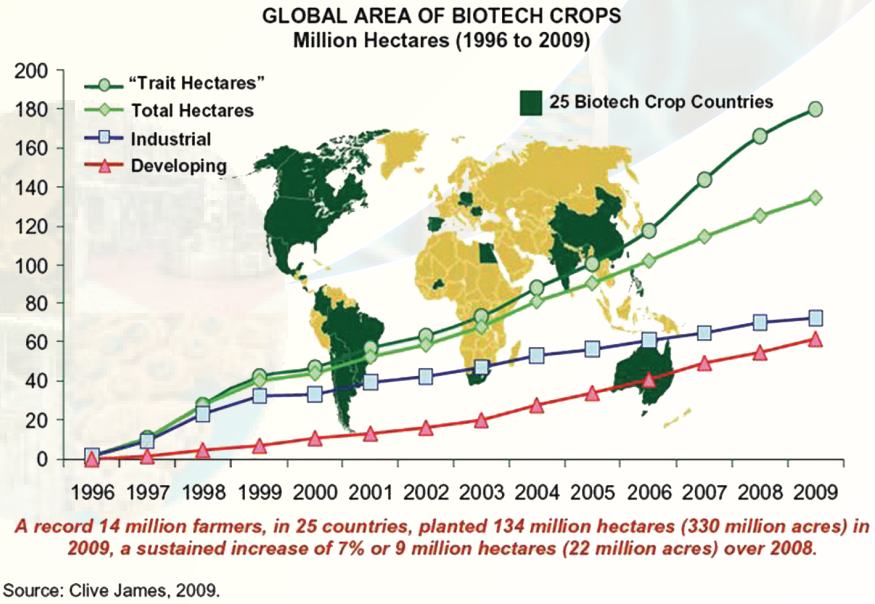


Fig. 12. Global area of biotech crops from 1996 to 2009 (in million hectares).

Nevertheless, the thorough assessment of the risks associated with transgenics for plants, animals and humans is important before they are released. Foods or food ingredients derived from GMOs must be shown to be as safe as or safer than their traditional counterpart before they can be recommended for public use. In general, many scientists believe that plant genetic engineering is the 'technology' that can solve various human problems, especially hunger and disease.

Review Questions

1. What is meant by plant tissue culture? List the various steps involved in this technique?
2. What is plant regeneration? Give the different pathways of plant regeneration.
3. Name a few useful secondary metabolites that can be produced through plant cell culture technology.
4. List the various applications of plant cell and tissue culture technology.
5. Differentiate:
   i) Cell culture and protoplast culture
   ii) Direct gene transfer and indirect gene transfer
6. Explain how embryo rescue can be used to produce novel hybrids.
7. Write short note on artificial seeds.
8. How are plant cells converted to protoplasts? Why are protoplasts more amenable to produce somatic cell hybrids and cybrids?
9. What is genetic engineering? Add a note on the various steps involved in this technology.
10. Why is *Agrobacterium* described as 'natural genetic engineer of plants'?
11. Explain how gene gun functions to deliver genes into plant cells?
12. What are the proposed benefits of genetic engineering in crop improvement?
13. What are the genetic engineering strategies to create the following traits in transgenic crops:
    a) Herbicide tolerance
    b) Abiotic stress tolerance
    c) Insect resistance
    d) Virus resistance
14. What is the common strategy to produce transgenic crops with delayed ripening and longer shelf life of fruits?

15. What is meant by 'Golden Rice'? In what way it is different from the normal rice?

16. Write briefly the benefits of biodegradable plastics that are produced from GM plants.

17. What are the transgenic crops that are commercialised globally?

18. Explain the social, economical and environmental implications of genetic engineering techniques.

19. Define:

Explant	Hairy roots
Clone	Roundup
Callus	PR proteins
Micropropagation	Bt genes
Encapsulation	Male sterility
Embryo rescue	*Flavr Savr*
Somatic hybrids	Molecular farming
Cybrids	PHB
In vitro gene bank	Edible vaccines
T-DNA	Metabolic engineering
Crown galls	

20. Fill in the blanks:

I) Rapid multiplication of plants by tissue culture techniques is referred to as _____.

ii) Gottlieb Haberlandt is known as father of _____.

iii) The most commonly employed gene transfer method in plants is _____.

References

1. Bhojwani SS & Razdan MK. 1996. Plant Tissue Culture: Theory and Practice (Revised Edition), Elsevier, Amsterdam.
2. Chrispeels MJ & Sadava DE. 2003. Plants, Genes and Crop Biotechnology (Second Edition), Jones and Bartlett Publishers, Inc., Boston.
3. Davey MR & Anthony P. 2010. Plant Cell Culture: Essential Methods, Wiley-Blackwell, Oxford.
4. Gamborg OL & Phillips GC.1996. Plant Cell, Tissue and Organ Culture, Narosa Publishing House, New Delhi.
5. Glick BR & Pasternak JJ. 1998. Molecular Biotechnology: Principles and Applications of Recombinant DNA (Second Edition), ASM Press, Washington, D.C.
6. Oksman-Caldentey K-M & Barz WH. 2002. Plant Biotechnology and Transgenic Plants, Marcel Dekker, Inc., New York.
7. Singh BD. 2006. Plant Biotechnology, Kalyani Publishers, Ludhiana.
8. Slater A, Scott N & Fowler M. 2003. Plant Biotechnology: The Genetic Manipulation of Plants, Oxford University Press, Oxford.