

**BT403**

**AGRICULTURAL BIOTECHNOLOGY**

**HANDOUTS**

**VIRTUAL UNIVERSITY OF PAKISTAN**

## **1. Agriculture Biotechnology Introduction**

Biotechnology: it is use of biological processes, organisms, or systems. It is defined as a set of tools that uses living organisms (or parts of organisms) to make or modify a product, improve plants, trees or animals, or develop microorganisms for specific uses.

### **Benefits of Biotechnology**

It is used for all these three purposes

#### **HEAL THE WORLD**

Biotechnology heals the world by creating more precise tools for disease detection and by reducing rates of infectious disease.

#### **FUEL THE WORLD**

It fuels the world by using biofuels, improving manufacturing process efficiency and by reducing the use of and reliance on petrochemicals

#### **FEED THE WORLD**

It feeds the world by generating higher crop yields, using biotech crops and by improving food and crop oil content.

**Agriculture Biotechnology** is collection of scientific techniques to improve plants, animals and microorganisms, to manipulate the genetic makeup, for the production or processing of

agricultural products, to increase agricultural productivity, to enhance breeders' ability to make improvements in crops and livestock and to enable improvements that are not possible with traditional crossing of related species alone.

Agricultural biotechnology is the term used in crop and livestock improvement through biotechnology tools. This monograph will focus only on agricultural crop biotechnology. Biotechnology encompasses a number of tools and elements of conventional breeding techniques, bioinformatics, microbiology, molecular genetics, biochemistry, plant physiology, and molecular biology.

## 2. History of agriculture biotechnology

### A brief history

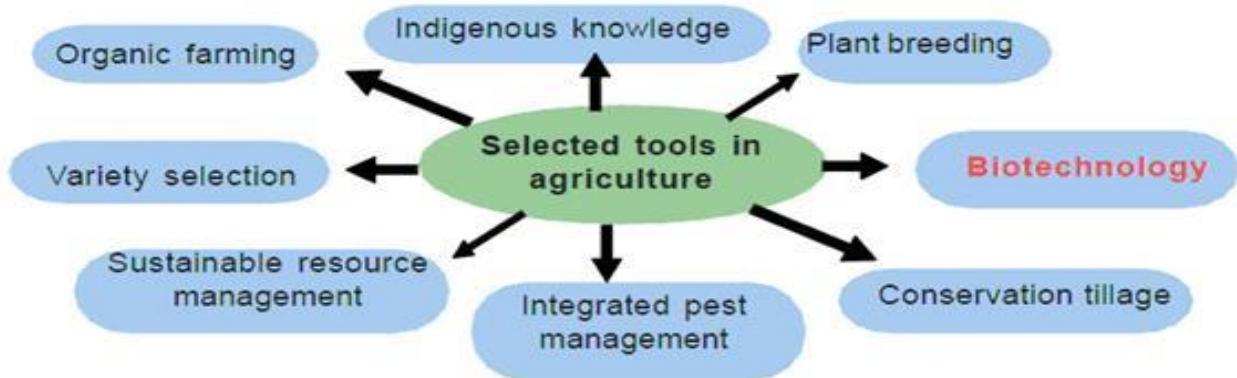
- It is practiced for so long about 8-10,000 years ago to improve organisms by: Selection and breeding
- **Selection:** it is selection and saving of the best looking plants and seeds, the selection features are: Faster growth, higher yields, pest and disease resistance, larger seeds, or sweeter fruits
- **Breeding:** it is done by artificially mating, cross-pollination, and desirable characteristics from different parent plants could be combined in the offspring. Superior plants are selected and breed them to create new and improved varieties of different crops.
- In 1990 -The first food product of biotechnology (an enzyme used in cheese production and a yeast used for baking) appeared on the market.
- In 1995, farmers have been growing genetically engineered (GE) crops.
- In 2003, 7 million farmers in 18 countries more than 85 percent of them resource-poor farmers in the developing world were planting biotech crops.

### Why is agricultural biotechnology important?

In a world where 7 Billion people, living mostly in rural areas, go hungry every day, Food demand is set to double in the next thirty years and arable land is limited, Advances in agriculture are critical if we are to reduce hunger and promote growth and development in a socially acceptable and environmentally sustainable way.

## **Benefits of agriculture biotechnology**

Agricultural biotechnology has been used to protect crops from disastrous diseases. Biotech crops can make farming more profitable by increasing crop quality and may in some cases increase yields. Biotech crops may provide enhanced quality traits such as increased levels of beta-carotene in rice to aid in reducing vitamin A deficiencies. Agriculture biotechnology produces herbicide-tolerant crops. The tools of agricultural biotechnology have been invaluable for researchers in helping to understand the basic biology of living organisms. Biotechnology has helped to make both insect pest control and weed management safer and easier while safeguarding crops against diseases.

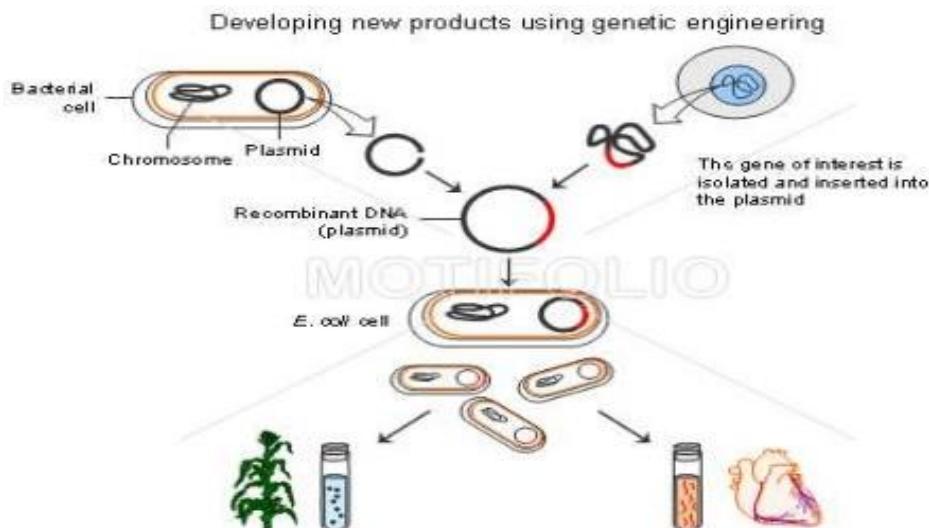


## **3. Applications of agriculture biotechnology I**

### **Genetic engineering:**

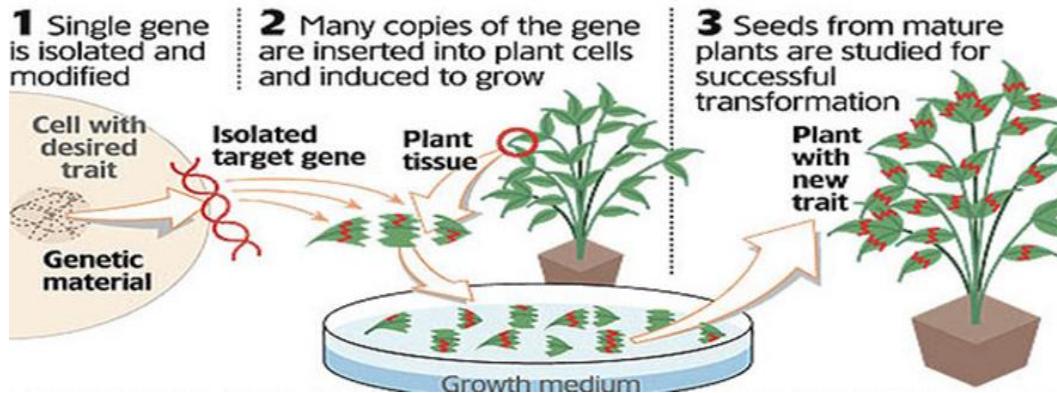
Genetic engineering is also called genetic modification, is the direct manipulation of an organism's genes using biotechnology. All crops improved with transferred DNA to date have been developed to aid farmers to increase productivity by reducing crop damage from weeds, diseases or insects. It inserts fragments of DNA into chromosomes of cells, uses tissue culture to regenerate the cells into a whole organism with Different genetic

composition from the original cells. This is also known as rDNA technology that produces transgenic organisms.



## Genetic engineering

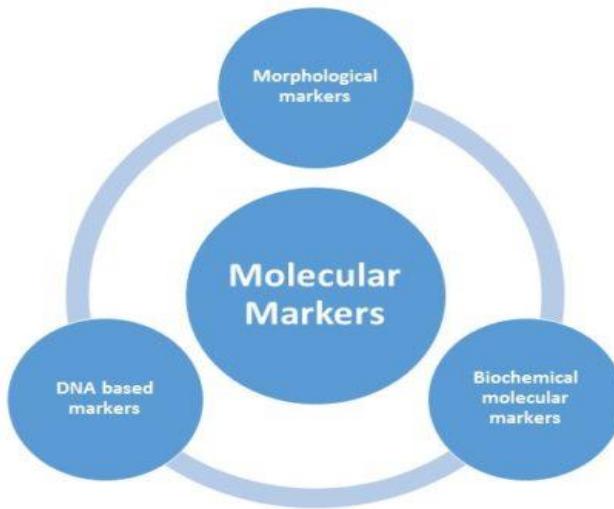
Researchers isolate a gene from an organism that has the trait they want to impart to a plant.



**Molecular markers:** Scientists can use molecular markers to select plants or animals that possess a desirable gene, even in the absence of a visible trait. Thus, breeding is more precise and efficient.

### Types of Molecular markers:

There are three types of molecular markers:



### **Morphological markers:**

Morphological markers are those traits that are scored visually, or morphological markers are those genetic markers whose inheritance can be followed with the naked eye.



**Biochemical molecular markers:** A molecular marker is a molecule contained within a sample taken from an organism (Biochemical molecules). The first biochemical molecular markers used were the protein based markers. One of the earliest protein based markers to be used was Isozyme. These are different forms of an enzyme exhibiting the same catalytic activity but differing in charge and electrophoretic mobility.

### **DNA based markers:**

The sequence of nucleotides in DNA of an individual is unique and thus determines its identity. The ultimate difference between individuals lies in the nucleotide sequence of their DNA. These can be used to diagnose the presence of the gene without having to wait for gene effect to be seen.

## **4. Applications of agriculture biotechnology II**

**Molecular diagnostics:** Molecular diagnostics are methods to detect genes or gene products that are very precise and specific. Molecular diagnostics are used in agriculture to more accurately diagnose crop/livestock diseases.

### **Vaccines;**

Biotechnology-derived vaccines are used in livestock and humans. They may be cheaper, better and/or safer than traditional vaccines. They are also stable at room temperature, and do not need refrigerated storage. Biotechnology-derived vaccines are used in livestock and humans. They are cheaper, better, safer than traditional vaccines, stable at room temperature and do not need refrigerated storage

### **Tissue culture**

Tissue culture is the regeneration of plants in the laboratory from disease-free plant parts. This technique allows for the reproduction of disease-free planting material for crops. Examples of crops produced using tissue culture include citrus, pineapples, avocados, mangoes, bananas, coffee and papaya.

### **Flowers**

Gene identification and transfer techniques used to improve the color, smell, size and other features of flowers. It is used to make improvements to other common ornamental plants, in particular, shrubs and trees. Some of these changes are similar to those made to crops, such

as enhancing the cold resistance of a breed of tropical plant, so it can be grown in northern gardens.

## **Nutrient Supplementation**

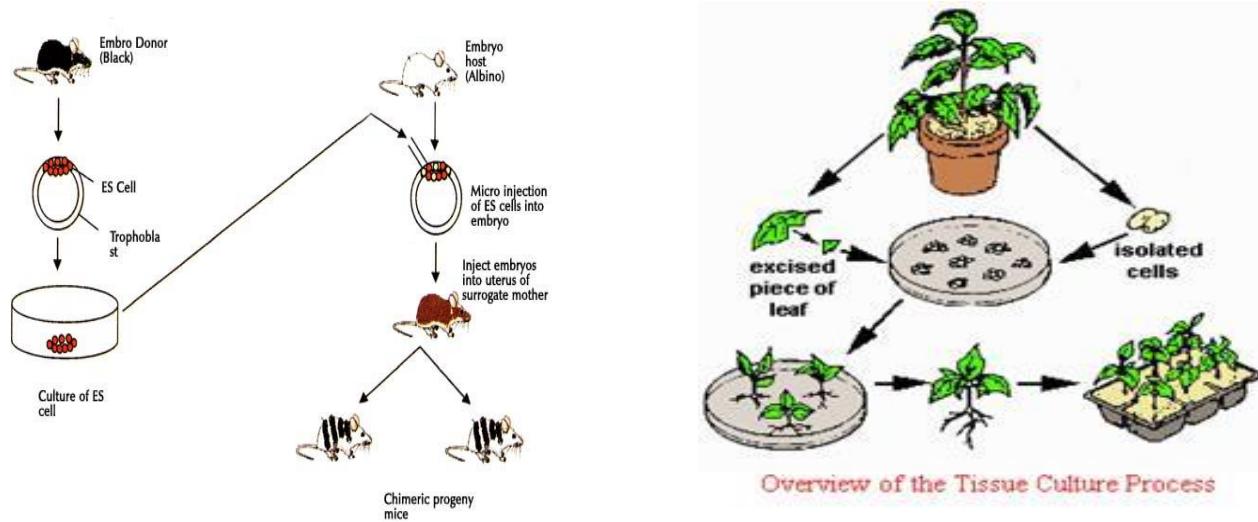
In an effort to improve human health, nutrient supplementation is needed, particularly in underdeveloped countries, scientists are creating genetically altered foods that contain nutrients known to help fight disease or malnourishment. An example of this is *Golden Rice*, which contains beta-carotene, the precursor for Vitamin A production in our bodies.

## **Pest resistant crops**

Pest resistant GM crops (primarily cotton and maize) are genetically modified so they are toxic to certain insects. They are often called Bt crops. The introduced genes were originally identified in a bacterial species called *Bacillus thuringiensis*.

## **5. Tissue culture**

**Introduction:** Tissue culture is the term used for “the process of growing cells artificially in the laboratory” Tissue culture involves both plant and animal cells. Tissue culture produces clones, in which all product cells have the same genotype (unless affected by mutation during culture).



**Brief history:** Tissue culture had its origins at the beginning of the 20th century with the work of 1- Gottleib Haberlandt (plants) and 2-Alexis Carrel (animals).

The first commercial use of plant clonal propagation on artificial media was in the germination and growth of orchid plants, in the 1920's

In the 1950's and 60's there was a great deal of research, but it was only after the development of a reliable artificial medium (Murashige & Skoog, 1962) that plant tissue culture really 'took off' commercially

### Critical requirements:

Tissue culture of both plant and animal has several critical requirements.

1. Appropriate tissue (some tissues culture better than others)
2. A suitable growth medium containing energy sources and inorganic salts to supply cell growth needs.
3. This can be liquid or semisolid
4. Aseptic (sterile) conditions, as microorganisms grow much more quickly than plant and animal tissue and can over run a culture
5. Growth regulators - in plants, both auxins & cytokinins. In animals, this is not as well defined and the growth substances are provided in serum from the cell types of interest

6. Frequent subculturing to ensure adequate nutrition and to avoid the build up of waste metabolites

## Steps of tissue culturing

### 1. selection of explant:

Explants are small pieces of plant parts or tissues that are aseptically cut and used to initiate a culture in a nutrient medium. Explants can be taken from different parts of a plant such as shoots, leaves, stems, flowers, roots, and from many types of mature cells provided they are able to de-differentiate into totipotent cells.



### 2. Establishment of the explant

Establishment of the explant in a culture medium. The medium sustains the plant cells and encourages cell division. It can be solid or liquid



### 3. Multiplication

The explant gives rise to a callus (a mass of loosely arranged cells) which is manipulated by varying sugar concentrations and the auxin (low): cytokinin (high) ratios to form multiple shoots. The callus may be subdivided a number of times. Dividing shoots. Warmth and good light are essential



#### **4. Root formation**

The shoots are transferred to a growth medium with relatively higher auxin: cytokinin ratios. Promote root formation. Ready to transfer to soil



## **6. Growth Regulators**

### **Growth**

Growth is an irreversible change in Mass, i.e. increase in size, volume and weight of any part of plant's body. It means quantitative increase in plant body e.g. Cell division Cell enlargement.

### **Development**

Development is an irreversible change in state. It means the qualitative change in plant body e.g. Seed Seedling Vegetative maturation Flowering. Growth is a continuous process Development is phase to phase process.

## **Plant growth regulating compounds**

- Plant growth regulating compounds are:
- Natural and Synthetic
- Natural- found naturally in plants
- Synthetic- human made
- Both groups regulate or influence:
- Cell division
- Cell differentiation
- Root and shoot growth
- Senescence (plant aging)

## **Plant growth regulators**

Plant Growth regulators (PGR) refers to natural or synthetic substances influence the growth and development.

### **Classification of PGR On the Basis of Origin**

**Natural hormone:** natural hormones are produced by some tissues in the plant they are also called endogenous hormones. e.g. IAA (Indol acetic acid)

**Synthetic hormone:** synthetic hormones are produced artificially and similar to natural hormone in physiological activity. They are also also called Exogenous hormones. e.g. 2,4- D, NAA (Naphthalene acetic acid).

**Growth promoting hormones/Growth promoter:** they increase the growth of plant. e.g. Auxins. Gibberellins, Cytokinins etc.

**Growth inhibiting hormones/Growth retardant:** they inhibit the growth of plant. e.g. ABA, Ethylene.

### **Auxins**

It is derived from the Greek word "auxein" means- "to grow/increase". Auxins may be defined as growth promoting substances which promote growth along the vertical axis when applied in low concentration to the shoot of the plant. Auxins are synthesized in the stem and root

apices and transported through the plant axis. They occur universally in all plants as Active growth = Auxin production.

**Role of Auxins:** Auxins stimulate cell elongation and influence a host of other developmental responses, they are involved in root initiation, vascular differentiation, tropic responses, apical dominance and in development of auxiliary buds, flowers and fruits

Auxins in plant tissue culture are used to induce callus from explants , and cause root and shoot morphogenesis and parthenocarpy

**Gibberellin:** They have a regulatory function and are produced in the shoot apex primarily in the leaf primordial (leaf bud) and root system.

**Role of gibberellins:** They are used to stimulates stem growth dramatically, they also stimulate cell division, Cell elongation (or both) and controls enzyme secretions, they are involved in overcoming dormancy in seeds and buds also used commercially in increasing fruit size of seedless grapes and Stimulating seed germination & seedling growth

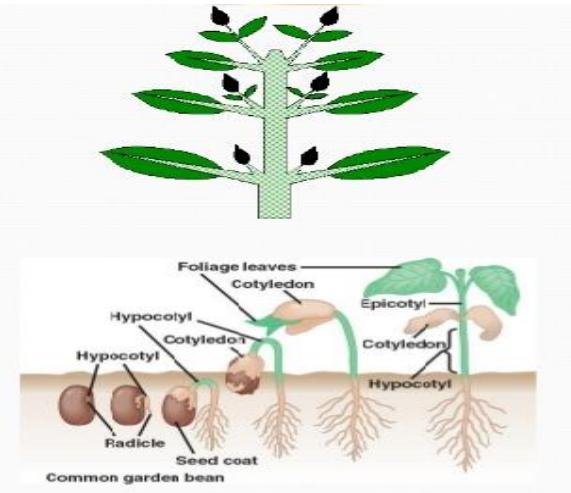
### **Cytokinins :**

Roles of cytokinins are : Promotion of cell division also found in all tissues with considerable cell division. Ex: embryos (seeds) and germinating seeds, young developing fruits roots supply cytokinins upward to the shoots. They also interact with auxins to influence differentiation of tissues (may be used to stimulate bud formation).

### **Effect of cytokinins**

## Cytokinins

- Cytokinins also modify apical dominance in stems and promote lateral bud growth.
- They also stimulate seed germination.



## Ethylene

**It is a growth retardant.** Ethylene promotes ripening , it is gaseous hormone, it is produced in the actively growing meristems of the plant, in senescing ripening or ageing fruits, in senescing (ageing or dying) flowers, in germinating seeds and in certain plant tissues as a response to bending, wounding or bruising. Ethylene as a gas, diffuses readily throughout the plant. It may promote leaf senescing and abscission (leaf fall). Increases female flowers in cucumbers (economically - will increase fruit production). It is also responsible for de greening of oranges, lemons and grapefruit – ethylene gas breaks down chlorophyll and lets colors show through.

## Abscisic acid

**It is also a growth retardant** which induces stomata closing, also involved in inhibition of bud growth and shoot formation. It is widespread in plant body – moves readily through plant. ABA appears to be synthesized by the leaves, it interacts with other hormones in the plant, counteracting the growth - promoting the effects of auxins & gibberellins. It is also involved with leaf and fruit abscission (fall), onset of dormancy in seeds and onset of dormancy (rest period) in perennial flowers and shrubs. ABA is effective in inducing closure of stomata in leaves, indicating a role in the stress physiology in plants. (ex: increases in ABA following water, heat and high salinity stress to the plant)

## 7. Sterile Techniques

Sterile techniques are used to clean equipment and for surface sterilization of explants.

### Clean Equipment

It is used for successful tissue culture requires the maintenance of a sterile environment. All tissue culture work is done in a laminar flow hood. The laminar flow hood filters air with a dust filter and a high-efficiency particulate air (HEPA) filter. It is important to keep the hood clean, which can be done by wiping it with 70% alcohol. The instruments used should also be dipped in 70% ethanol and sterilized using flame or glass beads. Hands should be disinfected with ethanol before handling cultures in order to avoid contamination.

It is imperative to maintain axenic conditions throughout the life of cultures: from explant to the production of whole plants. Entire experiments have been lost because of an episode of fungal or bacterial contamination at any stage of culture. Especially problematic are fungal contaminants that are propagated by spores that might blow into a hood from an environmental source. Therefore, it is important to work away from the unsterile edge of a laminar flow hood.

### **Surface Sterilization of Explants**

Plant tissues inherently have various bacteria and fungi on their surfaces. It is important that the explant be devoid of any surface contaminants prior to tissue culture since contaminants can grow in the culture medium, rendering the culture non sterile. In addition, they compete with the plant tissue for nutrition, thus depriving the plant tissue of nutrients. Bacteria and especially fungi can rapidly overtake plant tissues and kill them. The surface sterilants are chosen for an experiment typically depend on the type of explant and also plant species. Explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field-grown tissues. The time of sterilization is dependent on the type of tissue; for example, leaf tissue will require a shorter sterilization time than will seeds with a tough seed coat. Wetting agents such as Tween added to the sterilant can improve surface contact with the tissue.

Although surface contamination can be eliminated by sterilization, it is very difficult to remove contaminants that are present inside the explant that may show up at a later stage in culture. This internal contamination can be controlled to a certain extent by frequent transfer to fresh medium or by the use of a low concentration of antibiotics in the medium. Overexposing tissues to decontaminating chemicals can also kill tissues, so there is a balancing act between sterilizing explants and killing the explants themselves.

### **Culture Conditions and Vessels**

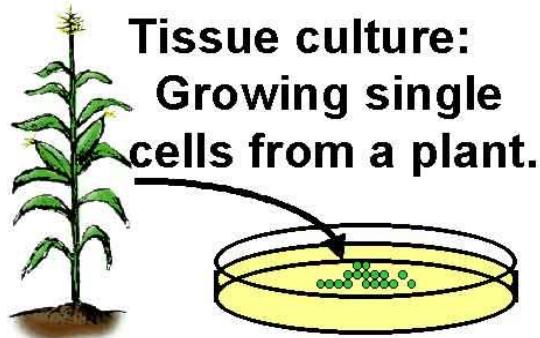
Cultures are grown in walk-in growth rooms or growth chambers. Humidity, light, and temperature have to be controlled for proper growth of cultures. A 16-h light photoperiod is optimal for tissue cultures, and a temperature of 22–25°C is used in most laboratories. A light intensity of 25–50  $\mu\text{mol.m}^{-2}\text{s}^{-1}$  PAR (photosynthetically active radiation) is typical for

tissue cultures and is supplied by cool white fluorescent lamps. A relative humidity of 50–60% is maintained in the growth chambers. Some cultures are also incubated in the dark. Cultures can be grown in various kinds of vessels such as petri plates, test tubes, “Magenta boxes,” bottles, and flasks

## 8. Basic Steps of plant tissue culture

### Plant tissue culture

- Plant tissue culture is a technique of growing plant cells, tissues, organs, seeds or other plant parts in a sterile environment on a nutrient medium



### Basic techniques of plant tissue culture are

- Culture vessels
- Culture medium
- Sterilization
- Inoculation
- Incubation
- Induction of callus
- Morphogenesis = Organogenesis   Embryogenesis
- Hardening

**Culture vessels:** Cultures can be grown in various kinds of vessels such as petri plates, test tubes, “Magenta boxes,” bottles, and flasks.



**Culture medium:** The important media used for all purpose experiment are Murashige and Skoog medium (MS medium). The culture medium is closed with cotton plug/ or aluminium foil sheet. The pH of the medium is adjusted to 5.8 (acidic range).

#### **Composition of culture medium:**

- Nutrient Medium
- Medium depends upon the type of plant tissue or cell used for culture
- Generally nutrient consist of
- inorganic salts (both micro & macro elements) a carbon source (usually sucrose)
- Vitamins (eg. nicotinic acid, thiamine, pyridoxine)
- Amino acids (eg. arginine)
- Growth regulators (eg. auxins)
- An optimum pH (5.7) is also very important

#### **Sterilization**

Sterilization Methods are used in Tissue Culture Laboratory. All the materials, e.g., vessels, instruments, medium, plant material, etc., used in culture work must be freed from microbes therefore, they are sterilized.

#### **Sterilization techniques**

- sterilization is achieved by one of the following approaches:
- dry heat treatment
- flame sterilization
- autoclaving
- filter sterilization
- wiping with 70% ethanol
- surface sterilization.

#### **Inoculation**

Transfer of explant (root, stem, leaf, etc.) on to a culture medium is called inoculation. The inoculation is carried out under aseptic condition for which an apparatus called laminar air flow chamber is used. Flamed and cooled forceps are used for transfer of plant materials to different culture media kept in glasswares.

#### **Incubation**

The culture medium with the inoculum is incubated at 26 - 28°C with the light intensity at 2000 to 4000 lux (unit of intensity of light) and allowing photoperiod of 16 hour of light and 8 hours of darkness.

**Induction of callus:** Due to activity of auxins and cytokinins, the explant is induced to form callus. The callus is an unorganized mass of undifferentiated tissue. The mechanism of callus formation is that auxin induces cell elongation and cytokinin induces cell division as a result of which masses of cells are formed.

➤ **Morphogenesis**

Formation of new organs from the callus under the influence of auxin and cytokinin is called morphogenesis.

Roots and shoots are differentiated from the callus.

Such embryos are called somatic embryos resulting in the formation of young plantlet.

➤ **Types of morphogenesis:**

Organogenesis

Embryogenesis

➤ **Organogenesis:** it is formation of new organs such as shoot and root is known as organogenesis. The development of shoot from the callus is called caulogenesis and formation of root is called rhizogenesis respectively.

➤ **Embryogenesis**

Formation of embryos (ie. bipolar structure having shoot and root) from the callus is called embryogenesis.

These embryos arise from somatic callus tissue and are called somatic embryos or embryoids or somaclonal embryos.

➤ **Hardening**

Exposing the plantlets to the natural environment in a stepwise manner is known as hardening.

Finally the plantlets are gradually transferred to the soil.

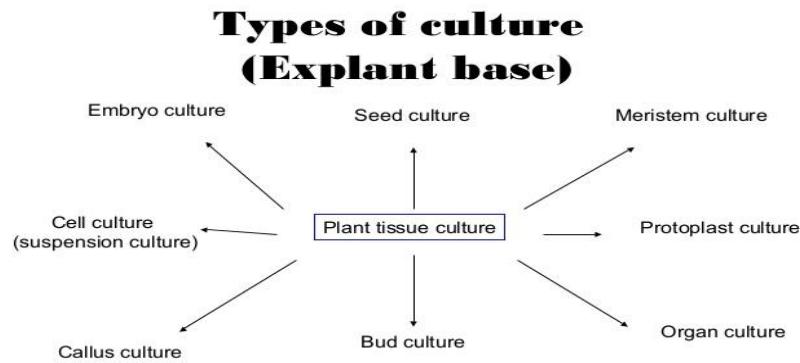
## 9. Types of tissue culture, callus culture

### Types of tissue culture

- These are the types of in vitro cultures
- Callus Cultures
- Cell Suspension Culture

- Anther/Microspore Culture
- Protoplast Culture
- Embryo Culture
- Meristem Culture

## Types of in vitro cultures



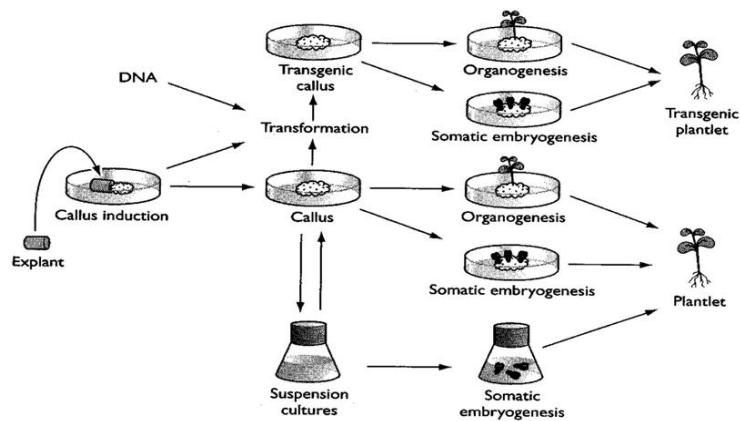
### Callus Culture

It is an unorganized mass of thin-walled parenchyma cells which is involved in formation of wound callus is observed in all groups of living organisms. In Plant tissue culture, callus is produced by cultivation of plant tissue on nutrient media under in vitro conditions. Presence of growth hormones in the culture media promotes callus formation and proliferation

### Role of Callus

In vitro callus provides totipotent cells for plant regeneration via organogenesis or somatic embryogenesis. Callus is used as a target tissue for genetic transformation. Callus formation is initiated for plant regeneration of other transformed tissues

Dispersal of friable callus into single cells is used for the initiating cell suspension cultures



*Alternative applications of callus in plant tissue culture and genetic transformation.*

### Initiation and maintenance of callus cultures

- (1) Selection of suitable parent material
- (2) Choice of explant and method of isolation
- (3) Culture medium and conditions required
- (4) Optimization of culture conditions

#### Selection of suitable parent material

Parent plant must be healthy and free from decay or disease

Mother plant be actively growing and not be about to enter a period of dormancy

#### Choice of explant and method of isolation

- Any part of mother plant; Plant organs or specific plant tissue or plant cells
- Explant must contain living cells
- Younger tissue is more callus responsive due to the presence of large no. of actively dividing cells
- Explants isolated in sterile conditions
- Ensure proper sterilization methods for a particular explant

#### Culture medium and conditions required

- Culture the sterilized explants on suitable autoclaved culture medium
- Incubate cultures at  $22-24 \pm 2^\circ\text{C}$  in light or dark
- Most callus will be initiated from the cut surfaces within 3-8 wks.

#### Optimization of culture conditions

- Consult literature to know previous callus initiation attempts for species under consideration

- For pioneering callus culture attempt, modify medium previously used for a related species
- Start with one of the defined media and manipulate hormone concentrations
- Set up a growth Latin Square of 25 culture plates with 5 each of auxin and cytokinin conc.
- Callus for this growth trial should be uniform and of large (20 mg) size.

#### **Callus growth measurement**

- Subculture vigorous growing callus (2-5 mm dia.)
- Slow growing callus plus explant transferred to fresh medium
- Callus growth assessment via fresh and dry wets.
- Plot a growth curve.

## **10. Cell Suspension Cultures**

Cell suspension cultures are rapidly dividing suspensions of cells grown in liquid medium; they grow more rapidly than callus cultures and more ready to culture manipulations. They are comprised of cell aggregates and dispersed single cells

#### **Techniques of cell culture**

##### **Initiation of cell suspension culture**

Fragments of undifferentiated callus      2 - 3 g / 100 mL



Liquid medium



↓      **aeration** -----



Subculture



↓      **agitation** -----



Suspension cell cultures

## **Initiation of Cell Suspensions:**

Initiation and establishment time depends on plant species and growth medium. Dicots are easier to establish in suspensions than monocot. It is initiated by agitating a fragment of callus in a vol. of liquid medium on a shaker, there are three procedures used for cell dispersion:

1. Initiate from friable callus
2. non-friable callus
3. Callus treated with cell wall degrading enzymes

### **Initiation from Friable Callus**

It is most commonly used starting material, easily fragmented during agitation in liquid medium, achieved by:

- Callus passaged on a 7d cycle for 2-3 wks.
- Ratio of auxin/cytokinins altered, increase auxin concentration. Use 2-3 g of friable callus per 100 ml of liquid medium.
- Low levels of callus tissue fail to replicate.
- Subculture the cells to fresh medium at a ratio of 1:1. Filter the actively dividing cell suspensions to remove large callus aggregates to get fine suspensions

### **Initiation from non-friable callus**

First repeatedly subculture or transfer callus fragments to semi-solid medium until friable, initiate and establish cell suspensions

- **Initiation from callus treated with enzymes**

- Pectinase
- Cellulase

(breaks down middle lamella of cell wall and separates plant cells)

## **Maintenance of Cell Suspensions**

- Varieties of culture vessels are available, suitable ones allow large surface area to maximize gas exchange. E.g. 20 ml culture medium + 100 ml flask, 50-100 ml in a 250 ml flask, flasks enclosed with sterilized/dried aluminium foil caps. Shaker speed should be 100-120 rpm with optimum incubation conditions

### **Small batch culture**

- Culture volume : in a small fixed volume : <100 ML

## **Growth Characteristics of Cell Suspensions**

- Plant cell suspensions consist of cells with diverse morphology and state of aggregation. Two morphological types of cells can be distinguished:
  - Cell aggregates made up of small cells
  - Large and elongated single cells
- Proportion of the cell type depends on the passage of culture and nature of auxin

## **Monitoring the Growth of Culture**

**To monitor the growth following should be checked:**

- Cell number
- PCV
- Fresh wt. and Dry wt.
- Cell Viability
- Medium conductivity & pH
- Use two methods simultaneously till stationary phase

## **Growth Curve of Suspension Cultures**

- Plotted for fresh, dry wts. and PCV.
- Gives the length of culture cycle
- Used to decide the subculture interval of suspensions
- Lag Phase
- Exponential Phase
- Linear Phase
- Decelerating Phase
- Stationary Phase

## **Uses of Cell Suspensions**

You can study various factors and compounds affecting growth and differentiation, cell division, rapid preparation of protoplasts, large scale production of commercial plants via somatic embryogenesis and commercial production of secondary metabolites.

## **11. Anther/Microspore Culture**

### **Introduction**

Anthers or pollens can be cultured on a suitable medium containing sucrose (usually 2%), iron, vitamins, hormones etc. The hormonal component of the medium is important for initiation of growth. Usually to the culture medium auxin, cytokinin etc. are added either singly or in various combinations. Low concentration of auxin stimulates callus formation. In a medium supplemented with auxin embryoid formation usually occurs at a faster rate as observed in

anther culture of *Datura*. Anthers cultured on a medium containing coconut milk or kinetin develop embryoids which later form haploid plantlets. Callus is formed from pollen grains on a medium supplemented with yeast extract or casein hydrolysate.

### **Factors Affecting Anther and Pollen Culture:**

#### **1. Activated charcoal:**

It has a stimulatory effect on embryogenesis and this has been observed in anther cultures of potato, rye, tobacco, etc. This may be due to removal of inhibitory substances from agar by activated charcoal. Charcoal may absorb the degradation product (5-2-furfural) of sucrose. Anther cultures of *Petunia* and *Nicotiana* indicate that activated charcoal removes both exogenous and endogenous growth hormones from culture medium.

#### **Temperature:**

Temperature has significant effect on pollen embryoid development. In *Datura* embryoids are not formed if cultures are maintained at 20°C or below. In *Nicotiana tabacum* optimal temperature for embryoid growth is 25°C.

Pre-treatment of anthers at 3—10°C for 2—30 days stimulates embryogenesis. Wenzel ('77) observed that buds of *Secale cereale* pretreated at 6°C for 6—10 days develop embryoids. In *N. tabacum* if the buds are pre-treated at 5°C for 72 hours than 58% anthers produce embryoids. Sometimes pre-treatment at high temperature helps embryoid formation. In *Brassica campestris* pre-treatment of anthers at 35°C for 24 hours helps embryoid formation.

Centrifugation of the anthers at 3—5°C for approximately 30 minutes helps embryoid formation

#### **Stage of the anther:**

- Particular stage of the anther at the time of culture is important. Usually anthers just before or immediately after pollen mitosis are most suitable for culture.
- Suitable stages of anthers for culture are pre-mitotic, mitotic and post-mitotic.

##### **a. Pre-mitotic stage:**

Anthers at this stage have microspores which have just completed the first meiotic division and the pollens are immature, uninucleate and starch-free.

Anthers of *Hordeum vulgare* and *Hyoscyamus* at this stage are suitable for culture. According to Nitsch ('72) and Sunderland ('71) anthers with uninucleate pollens are suitable for culture.

##### **b. Mitotic stage:**

In some plants, anthers at first pollen division stage are most suitable for culture, as observed in *Nicotiana tabacum* and *Datura innoxia*.

**c. Post-mitotic stage:**

Early bi-cellular stage of pollen development is most suitable for culture in *Atropa belladonna* and *Nicotiana* sp. etc. Mature anthers are usually unsuitable for culture, but in *Brassica oleracea* mature anthers are the proper stage for anther culture. Anthers of proper stage are chosen by selecting flower buds of definite length under fixed environmental conditions.

**Photoperiod and light intensity:**

Higher number of embryoids are formed when anthers are taken from plant grown under short days and high light intensities.

**Flowering time:**

Anthers taken from flowers at the beginning of the flowering period of the plant are most suitable for culture.

**Endogenous auxin:**

Embryogenic pollens are found near the tapetum within the anthers. The tapetum may release some substance which initiates embryogenic development in pollens. This is observed in *Hyoscyamus niger* by Raghavan ('78).

**Age of the plant:**

Usually anthers from younger plants are more suitable for culture.

**Methods of Anther culture**

1. Selected plants are cultivated until they reach flower bud stage.
2. In some cases flower buds are chilled few days prior to culture.
3. Flower buds of proper size and developmental stage are taken and surfaces sterilized with alcohol or hypochlorite solution for 10—20 minutes. Buds are rinsed several times in sterile double distilled water.
4. The anthers are carefully excised from flower buds using force and dissecting needle. Filaments must be removed prior to culture; otherwise callus may be formed at the cut ends.
5. Anthers may be cultured either on agar-solidified culture medium or placed on a filter paper bridge over a liquid medium.

6. Anthers are cultured at 25°C in presence or absence of light. Light is essential after plantlets are formed. Continuous illumination from cool white fluorescent lamp of 300 lux is satisfactory.

7. After a period of 4—5 weeks in culture plantlets are formed. From a single anther many plantlets are formed

8. These plantlets are carefully separated quite early and cultured on a fresh root-inducing medium containing 0.5% agar and all other components in half-strength to that of the anther culture medium.

9. After formation of proper root system they are transplanted to pots. These pots are preferably kept in humid condition for few days

## 12. Protoplast Culture

### Protoplasts

Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components. They represent the functional plant cells but for the lack of the barrier, cell wall. Protoplasts of different species can be fused to generate a hybrid and this process is referred to as somatic hybridization (or protoplast fusion). Cybridization is the phenomenon of fusion of a normal protoplast with an enucleated (without nucleus) protoplast that results in the formation of a cybrid or cytoplasm (cytoplasmic hybrids).

**Protoplast isolation:** It refers to the separation of protoplast from plant tissue, it is important to isolate viable and uninjured protoplast as gently and as quickly as possible, it involves two methods:

- a. Mechanical
- b. Enzymatic

### Mechanical method:

Tissue is immersed in 1.0 M sucrose until protoplasm shrunk away from their enclosing cell wall (Plasmolysis). Plasmolysed tissue is cut with a sharp knife at such a thickness that only cell walls are cut. Plasmolysed cell. Undamaged protoplast in strips are released by osmotic swelling when placed in a low concentration of sucrose solution. Problem encountered: some cells release uncut complete protoplast while the rest produces broken dead protoplasts

### **Enzymatic method:**

it refers to the use of enzymes to dissolve the cell wall for releasing protoplasts. It involves two methods:

- I. Direct method (One step only)
- II. Sequential method (Two step method)

- **Direct method :**
- Incubation of leaf segments overnight in enzyme solution
- Mixture is filtered and centrifuged
- Protoplast forms pellet
- Then washed with sorbitol and re-centrifuged
- Clean protoplasts float
- They are pipetted out

### **Sequential method**

Two enzyme mixtures(mixture A and mixture B) are used one after the other. Leaf segments with mixture A (Macerozyme in manifold at pH 5.8) are vacuumed infiltrated for 5 mins, transferred to a water bath at 25°C and subjected to slow shaking. The enzyme mixture is then replaced by fresh ‘enzyme mixture A’ and leaf segments are incubated for another hour. The mixture is filtered using nylon mesh and centrifuged for 1 min, washed 3 times with 13% mannitol . Cells are then incubated with ‘enzyme mixture B’ (Cellulase in mannitol solution

at pH 5.4) for above 90 mins at 30°C. The mixture is centrifuged for 1 min so that protoplast form a pellet and clean 3 times with sorbitol

### **Purification of protoplast**

Protoplasts are purified by removing: Undigested material (debris), Bursts protoplasts , and enzymes. Debris are removed by filtering the preparation through a nylon mesh . Enzymes are removed by centrifugation whereby the protoplasts settle to the bottom of the tube and the supernatant removed with the help of a pipette. Intact protoplasts are separated from broken protoplasts through centrifugation and removed by a pipette as they are collected at the top of tube

### **Protoplast Culture**

Isolated protoplast can be cultured in an appropriate medium to reform cell wall and generate callus. Optimal culture conditions are:

- Optimal density to the culture.
- Optimal auxin to cytokinin ratio, glucose and sucrose.
- Maintain osmoprotectant in the medium
- Temperature: 20-28°C pH: 5.5-5.9 0.25% Casein hydrolysate BAP and NAA

### **Culture of protoplasts**

Protoplasts cultured in suitable nutrient media first generate a new cell wall. The formation of a complete cell with a wall is followed by an increase in size, number of cell organelles, and induction of cell division. The first cell division may occur within 2 to 7 days of culture. It results in small clumps of cell, also known as micro colony, within 1 to 3 weeks. From such clumps, there are two routes to generate a complete plant (depending on the species). Plants are regenerated through organogenesis from callus masses. The micro calli can be made to develop into somatic embryos, which are then converted into whole plant through germination

## **Importance of Protoplast Culture**

The protoplast in culture can be regenerated into a whole plant. Hybrids can be developed from protoplast fusion. It is easy to perform single cell cloning with protoplasts. Genetic transformations can be achieved through genetic engineering of protoplast DNA. Protoplasts are excellent materials for ultra-structural studies.

Isolation of cell organelles and chromosomes is easy from protoplasts. Protoplasts are useful for membrane studies (transport and uptake processes). Isolation of mutants from protoplast cultures is easy.

## **13.      Embryo Culture**

### **What is embryo?**

A seed plant embryo is part of a seed, consisting of precursor tissues for the leaves, stem and root as well as one or more cotyledons. The young sporophyte of a seed plant usually comprising a rudimentary plant with plumule, radicle, and cotyledons

### **What is Embryo Culture?**

The embryo of different developmental stages, formed within the female gametophyte through sexual process, can be isolated aseptically from the bulk of maternal tissues of ovule, seed or capsule and cultured in vitro under aseptic and controlled physical conditions in glass vials containing nutrient solid or liquid medium to grow directly into plantlet

### **Culturing method**

The general method of embryo culture follows the following steps.

1. Pluck healthy and mature fruits from the field and wash thoroughly in running water for about an hour.
2. Surface sterilize with 0.01% Tween-20 for 15 min, rinse seeds several times with distilled water and finally treat with 0.01% HgCl<sub>2</sub> solution for 10-15 min.
3. Finally rinse it for six times with sterile distilled water.

4. Incubate the cultures at 22-25°C under a 16 h photoperiod of 2000 lux luminous intensity.
5. After two weeks of inoculation the embryo begins to swell on callus proliferation medium. Distinct callus growth is observed after 4 weeks.
6. After 8 weeks of inoculation transfer the callus on shoot regeneration medium. Within 4 weeks of transfer into second medium the callus turns green and produces soft spongy tissue. Some of these tissues are differentiated into embryoids.
7. The embryoids produce cluster of budlets when subcultured onto shoot regeneration medium.
8. The budlets grow into shoots and produce 2-3 leaf appendages within 12 weeks.
9. Thereafter, they are separated into individual shoots and then subcultured into a fresh medium of the same composition until shoots develop.

## **Types of Embryo Culture**

**There are two types of embryo culture:**

- 1. Mature Embryo Culture**

Mature embryos are isolated from ripe seeds and cultured in vitro. Mature embryo cultures are carried out in the following conditions

## **Conditions**

1. When the embryos remain dormant for long periods.
2. Low survival of embryos in vivo.
3. To avoid inhibition in the seed for germination.
4. For converting sterile seeds to viable seedlings.

## **2. Embryo Rescue**

Embryo rescue involves the culture of immature embryos to rescue them from unripe or hybrid seeds which fail to germinate. This approach is very useful to avoid embryo abortion and produce a viable plant. Wild hybridization involving crossing of two different species of plants from the same genus or different genera often results in failure. This is mainly because the normal development of zygote and seed is hindered due to genetic barriers.

### **Applications of Embryo Culture:**

#### **Embryo culture can be used in:**

- Prevention of Embryo Abortion
- Overcoming Seed Dormancy
- Shortening of Breeding Cycle
- Production of Haploids
- Overcoming Seed Sterility
- Clonal Propagation

## **14. Meristem/shoot tip culture**

### **Meristem**

A meristem is the tissue in most plants containing undifferentiated cells (meristematic cells), found in zones of the plant where growth can take place

### **What is Meristem Culture?**

Meristem culture is the in vitro culture of a generally shiny special dome-like structure measuring less than 0.1 mm in length and only one or two pairs of the youngest leaf primordia, most often excised from the shoot apex.

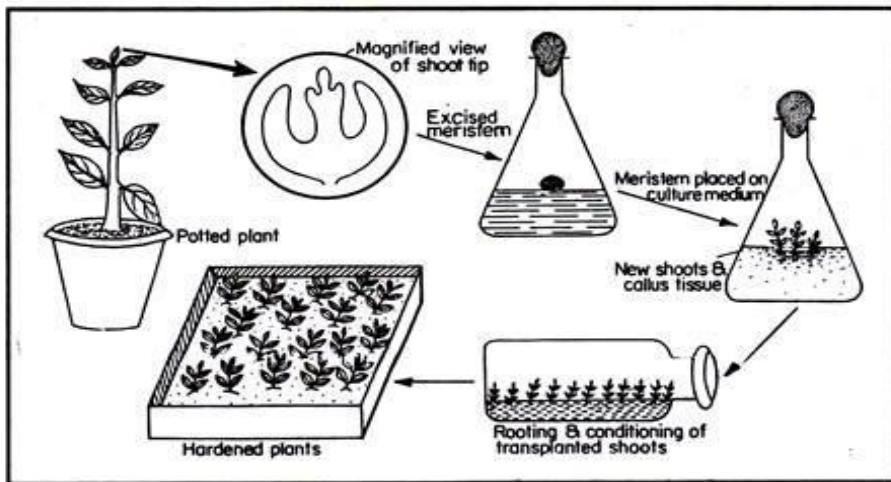
### **Principle**

The excised shoot tips and meristem can be cultured aseptically on agar solidified simple nutrient medium or on paper bridges dipping into liquid medium and under the appropriate

condition will grow out directly into a small leafy shoot or multiple shoots. Alternatively the meristem may form a small callus at its cut case on which a large number of shoot primordia will develop. These shoot primordia grow out into multiple shoots. Once the shoots have been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves separating the shoot into small segments each containing one node. The axillary bud on each segment will grow out in culture to form yet another shoot.

### Protocol

- Remove the young twigs from a healthy plant. Cut the tip (1 cm) portion of the twig.
- Surface sterilize the shoot apices by incubation in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The ex-plants are thoroughly rinsed 4 times in sterile distilled water.
- Transfer each explants to a sterilized petri dish.
- Remove the outer leaves from each shoot apices with a pair of jeweler's forceps. This lessens the possibility of cutting into the softer underlying tissues.
- After the removal of all outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of filter-paper Bridge. Flame the neck of the culture tube before and after the transfer of the excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.
- Incubate the culture under 16hrs light at 25°C
- As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, develop root, transfer them to hormone free medium
- The plantlets formed by this way are later transferred to pots containing compost and kept under greenhouse conditions



□ Fig 2.3

**Flow diagram illustrating the technique of shoot tip or meristem culture**

## **Importance of Shoot Tip/Meristem Culture**

The uses of shoot tips and meristem in tissue culture are very varied and include mainly:

1. Virus eradication,
2. Micro-propagation and
3. Storage of genetic resources.

### **Virus Eradication**

This technique is also valuable for the maintenance of carefully defined stocks of specific varieties and cultivars in disease Free State. The size of the meristem explant is critical for virus eradication.

### **Micro Propagation**

A sexual or vegetative propagation of whole plants using tissue culture techniques is referred to as micro-propagation. Shoot tip or meristem culture of many plant species can successfully be used for micro-propagation.

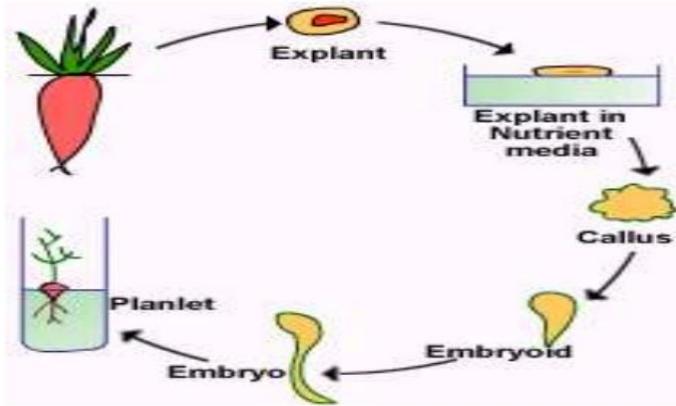
### **Storage of Genetic Resources**

Many plants produce seeds that are highly heterozygous in nature or that is recalcitrant. Such seeds are not accepted for storing genetic resources. So, the meristem from such plants can be stored in vitro.

## **15. Regeneration Methods of Plants in Culture**

### **Plant regeneration**

The process of growing an entire plant from a single cell or group of cells. Regeneration is possible because plant cells can be made totipotent using hormones. Differentiated tissue: stems, leaves, roots, etc. Undifferentiated (embryonic) cells are totipotent: can become a whole new plant by differentiating into a whole new plant.



### **Plant regeneration**

The main objective in plant cultures is to regenerate a plant or plant organ from the callus culture. The regeneration of plant or plant organs only taken place by the expression of cellular totipotency of the callus tissues. In agriculture biotechnology, tissue culture is most important for the regeneration of transgenic plants from single transformed cells

### **Organogenesis**

Organogenesis is the formation of organs: either shoot or root. In vitro organogenesis depends on the balance of auxin and cytokinin and the ability of the tissue to respond to phytohormones during culture. It takes place in three phases.

In the first phase the cells become competent;

Next, they dedifferentiate.

In the third phase, morphogenesis proceeds independently of the exogenous phytohormone.

### **Indirect Organogenesis**

Formation of organs indirectly via a callus phase is termed indirect organogenesis. Induction of plants using this technique does not ensure clonal fidelity, but it could be an ideal system for selecting somaclonal variants of desired characters and also for mass multiplication. Induction of plants via a callus phase has been used for the production of transgenic plants in which

the callus is transformed and plants regenerated or

the initial explant is transformed and callus and then shoots are developed from the explant.

### **Direct Organogenesis**

The production of direct buds or shoots from a tissue with no intervening callus stage is termed direct organogenesis. Plants have been propagated by direct organogenesis for improved multiplication rates, production of transgenic plants, and—most importantly—for clonal propagation. Typically, indirect organogenesis is more important for transgenic plant production.

## **16. Somatic Embryogenesis**

### **Introduction**

Somatic embryogenesis is an artificial process in which a plant or embryo is derived from a single somatic cell or group of somatic cells. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. ordinary plant tissue.

### **Somatic embryoid formation**

#### **It may be formed from:**

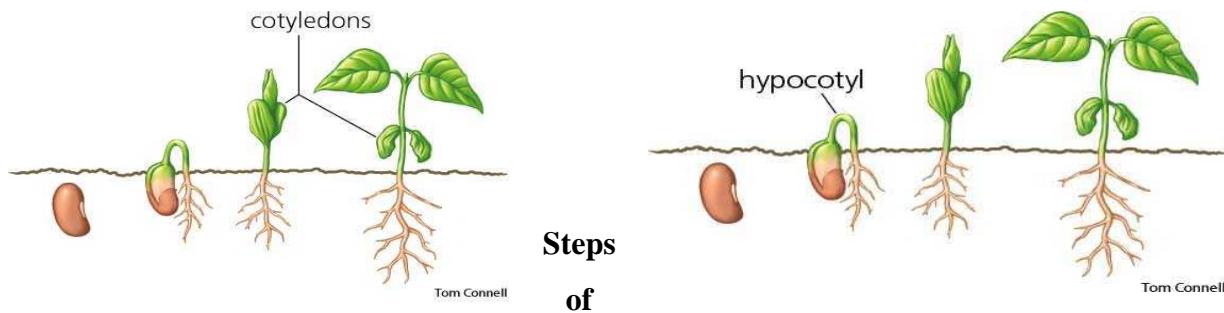
- Vegetative cells of a mature plant
- Reproductive cells other than zygote or
- Cotyledons, hypocotyl or young plantlets

### **Hypocotyl, Cotyledon**

The hypocotyl (short for "hypocotyledonous stem" meaning "below seed leaf") is the stem of a germinating seedling, found below the cotyledons (seed leaves) and above the radicle (root). It is the part of the stem of an embryo plant, beneath the stalks of the seed leaves or cotyledons

and directly above the root. An embryonic leaf in seed-bearing plants, one or more of which are the first leaves to appear from a germinating seed.

A cotyledon is a significant part of the embryo within the seed of a plant, and is defined as "the embryonic leaf in seed-bearing plants, one or more of which are the first to appear from a germinating seed."



### **somatic embryogenesis**

The steps which are involved in somatic embryogenesis are:

- Initiation of embryogenic culture
- Proliferation of embryogenic culture
- Pre-maturation of somatic embryos
- Maturation of somatic embryos
- Plant development

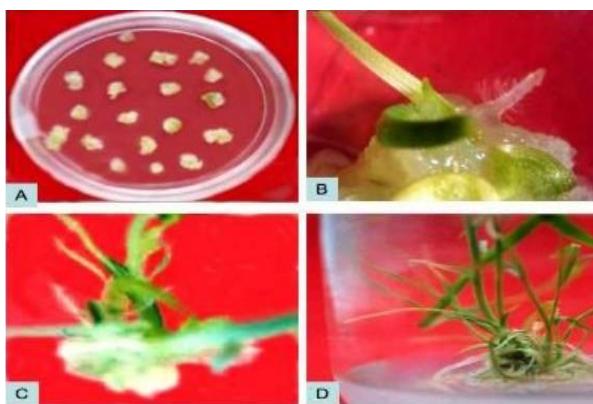
### **Types of Somatic Embryogenesis**

There are two types of somatic embryogenesis

- Direct somatic embryogenesis
- Indirect somatic embryogenesis

#### **Direct somatic embryogenesis**

In this type of embryogenesis, the embryos initiate directly from explants in the absence of callus formation.



### **Indirect Embryogenesis**

In this type of embrogenesis, the embryos are developed through cell proliferation i.e., callus formation. The cells from which embryos arise are called as ‘Induced embryogenic determined cells’ (IEDC). Here growth regulators with specific cultural conditions are required for initiation of callus and then redetermination of those cells into the embryo development.



### **Advantages:**

#### **Advantages of somatic embryogenesis are:**

- Higher propagation rate
- Suitable for suspension culture

- Artificial seed production
- Somaclonal variation
- Germplasm conservation
- Labour savings

## **Disadvantages**

- Disadvantages of somatic embryogenesis are:
- Low frequency embryo production
- Incomplete embryo production
- May create unwanted genetic variation
- Inability to generate large number of normal , free living plantlet
- Plantlets are weaker
- Respone tissue specific

## **Factors influencing somatic embryogenesis**

The factors that influence somatic hybridization are:

1. Auxin
2. Cytokinin
3. Nitrogen
4. Activated charcoal
5. Age of culture

### **1. Auxin**

In medium having relatively high concentration of auxin embryonal budding or embryonal clumps have been observed. For cell differentiation the medium should contain auxin and

reduced nitrogen. Subsequent development takes place in medium with no auxin or low concentration of auxin and reduced nitrogen. In some plants first and second stages occur in the first medium and plantlet development takes place in the second medium.

## **2. Cytokinin**

- The role of cytokinin in embryogenesis is not clear. Embryogenesis in carrot cell suspension is stimulated by addition of zeatin in medium lacking auxin but inhibited by the addition of kinetin. Inhibitory effect of exogenous cytokinin may be due to an increase in endogenous cytokinin in growing embryoids. The role of cytokinin in embryogenesis is not clear. Embryogenesis in carrot cell suspension is stimulated by addition of zeatin in medium lacking auxin but inhibited by the addition of kinetin. Inhibitory effect of exogenous cytokinin may be due to an increase in endogenous cytokinin in growing embryoids

## **3. Nitrogen**

The ratio of nitrogen to auxin is an important factor controlling embryogenesis. Embryo development can be initiated on White's medium with low nitrogen content only in absence of auxin. At low nitrogen concentration organic nitrogen is more suitable than inorganic nitrogen. Substances used as a source of nitrogen are potassium nitrate, ammonium chloride, glutamine, glutamic acid, alanine, urea etc.

## **4. Activated charcoal**

Presence of activated charcoal in the medium helps embryogenesis in several cases. Activated charcoal may adsorb the inhibitory substances present in the medium.

## **5. Age of the culture**

Embryogenesis usually occurs in short-term cultures. With older cultures this ability decreases and ultimately it is completely lost. This may be due to either the inability to synthesise some embryogenetic substances or changes in the ploidy level which may lead to loss of morphogenetic potential.

## **17.Application of plant cell Culture in crop improvement**

### **Introduction**

Plant tissue culture comprises a set of in vitro techniques, methods and strategies that are part of the group of technologies called plant biotechnology. Tissue culture has been exploited to create genetic variability from which crop plants can be improved, to improve the state of health of the planted material and to increase the number of desirable germplasms available to the plant breeder.

**Germplasm** are living genetic resources such as seeds or tissues that are maintained for the purpose of plant breeding, preservation, and other research uses. Tissue culture protocols are available for most crop species, although continued optimization is still required for many crops, especially cereals and woody plants. Tissue culture techniques, in combination with molecular techniques, have been successfully used to incorporate specific traits through gene transfer. In vitro techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in the breeding lines, often via haploid production.

**Haploid** is the term used when a cell has half the usual number of chromosomes. Cell culture has also produced somaclonal and gametoclonal variants with crop improvement potential.

**Somaclonal variation** is the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are an important source of this variation

**Gametoclonal variation** has been defined as the variation among plants regenerated from gametic cells in culture. The culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars. Large scale micro propagation laboratories are providing millions of plants for the commercial ornamental market and the agricultural, clonally propagated crop

market. With selected laboratory material typically taking one or two decades to reach the commercial market through plant breeding, this technology can be expected to have an ever increasing impact on crop improvement as we approach the new decade.

## **Applications**

- The applications of various tissue culture approaches to crop improvement are following:
- Breeding & biotechnology
- Wide hybridization
- Haploidy
- Somaclonal variation
- Micropropagation
- Synthetic seed
- Pathogen eradication
- Germplasm preservation

## **18. Plant Breeding and Biotechnology**

Plant breeding can be conveniently separated into two activities: manipulating genetic variability and plant evaluation. Historically, selection of plants was made by simply harvesting the seeds from those plants that performed best in the field. In spite of the general lack of integration of most plant biotechnology and plant breeding programs, field trials of transgenic plants have recently become much more common. More than 50 different plant species have already been genetically modified, either by vector dependent (e.g. Agrobacterium) or vector independent (e.g. biolistic, micro-injection and liposome) methods. In almost all cases, some type of tissue culture technology has been used to recover the modified cells or tissues. In fact, tissue culture techniques have played a major role in the development of plant genetic engineering. Tissue culture will continue to play a key role in the genetic engineering process for the predictable future, especially in efficient gene transfer and transgenic plant recovery.

## **19. Wide Hybridization**

### **Definition**

Hybridization between individuals from different species, belonging to same genus or different genera, is termed as distant or wide hybridization. A critical requirement for crop improvement is the introduction of new genetic material into the cultivated lines of interest, whether via single genes, through genetic engineering, or multiple genes, through conventional hybridization or tissue culture techniques. During fertilization in angiosperms, pollen grains must reach the stigma of the host plant, germinate and produce a pollen tube. The pollen tube must penetrate the stigma and style and reach the ovule. The discharge of sperm within the female gametophyte triggers syngamy and the two sperm nuclei must then fuse with their respective partners. The egg nucleus and fusion nucleus then form a developing embryo and the nutritional endosperm, respectively. This process can be blocked at any number of stages, resulting in a functional barrier to hybridization and the blockage of gene transfer between the two plants.

### **Barrier for crossing:**

There are two barriers for crossing

Pre-Zygotic

Post-Zygotic

### **Pre –zygotic Barriers**

Post- zygote barriers are those which occur prior to fertilization, they are due to the genic differences in different species. There is failure of pollen germination, slow growth of pollen tube, inability of the pollen to reach the ovary and arrest of pollen tube in style ovary and ovule.

### **Post- Zygotic barriers**

Post- zygote barriers are those which occur after fertilization, hybrid in viability and weakness leading to chromosome elimination, lethality and embryo abortion, hybrid sterility and hybrid breakdown with weak or sterile individuals in F2 owing to recombination of genes complements of the parental species.

### **Techniques to overcome isolation barriers**

Pre-zygotic barriers can be overcome by: In-vitro fertilization, Protoplast fusion, Embryo culture, Use of growth hormones (IAA, NAA) and adopting bridging species technique.

## **In vitro Fertilization**

IVF has been used to facilitate both interspecific and intergeneric crosses, to overcome physiological based self-incompatibility and to produce hybrids. A wide range of plant species has been recovered through IVF via pollination of pistils and self and cross-pollination of ovules. This range includes agricultural crops, such as tobacco, clover, corn, rice, cole, canola, poppy and cotton. The use of delayed pollination, distant hybridization, pollination with abortive or irradiated pollen, and physical and chemical treatment of the host ovary have been used to induce haploidy.

## **Protoplast Fusion**

Protoplast fusion has often been suggested as a means of developing unique hybrid plants which cannot be produced by conventional sexual hybridization. Protoplasts can be produced from many plants, including most crop species. However, while any two plant protoplasts can be fused by chemical or physical means, production of unique somatic hybrid plants is limited by the ability to regenerate the fused product and sterility in the interspecific hybrids rather than the production of protoplasts. Perhaps the best example of the use of protoplasts to improve crop production is that of Nicotiana, where the somatic hybrid products of a chemical fusion of protoplasts have been used to modify the alkaloid and disease-resistant traits of commercial tobacco cultivars.

### **Protoplast fusion should focus on four areas:**

Agriculturally important traits

Achieving combinations that can only be accomplished by protoplast fusion

A somatic hybrids integrated into a conventional breeding programme and

The extension of protoplast regeneration to a wider range of crop species

## **Embryo Culture**

The most common reason for post-zygotic failure of wide hybridization is embryo abortion due to poor endosperm development. Embryo culture has been successful in overcoming this major barrier as well as solving the problems of low seed set, seed dormancy, slow seed germination, inducing embryo

growth in the absence of a symbiotic partner, and the production of monoploids of barley. Interspecific and intergeneric hybrids of a number of agriculturally important crops have been successfully produced, including cotton, barley, tomato, rice, jute, *Hordeum X Secale*, *Triticum x Secale*, *Tripsacumx lea* and some Brassicas.

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## **Bridging species technique**

When direct cross between two species with the same or different ploidy levels are difficult to accomplish a third species (Bridge species) is used to make such crosses possible

## **Pre-zygotic barriers**

Pre- zygote barriers can be overcome by: Tissue culture techniques, Back crossing, Doubling of chromosomes and Embryo rescue.

### **Backcrossing**

**Backcrossing** is a **crossing** of a hybrid with one of its parents or an individual genetically similar to its parent, in order to achieve offspring with a genetic identity which is closer to that of the parent. It is used in horticulture, animal breeding and in production of gene knockout organisms.

### **Doubling of chromosomes**

Artificial production of **doubled** haploids is important in plant breeding. Haploid cells are produced from pollen or egg cells or from other cells of the gametophyte, then by induced or spontaneous **chromosome doubling**, a **doubled** haploid cell is produced, which can be grown into a **doubled** haploid plant.

## **Embryo rescue**

The term “**embryo rescue**” refers to a number of in vitro techniques whose purpose is to promote the development of an immature or weak **embryo** into a viable plant. **Embryo rescue** has been widely used for producing plants from hybridizations in which failure of endosperm to properly develop causes **embryo** abortion. **Embryo rescue** is one of the earliest and successful forms of in-vitro culture techniques that is used to assist in the development of plant **embryos** that might not survive to become viable plants

# **20. Haploids**

## **Introduction**

A haploid is a cell or organism that has a single set of chromosomes that are not paired. The haploid gamete is normally produced during plant cell division. During fertilization, these cells normally merge with other similar haploid cells. A haploid cell only has half the number of chromosomes as are present in diploids.

## **Applications of Haploid Plants are:**

- In Vitro production of haploids can solve some problems in genetic studies
- The following points highlight the top applications of haploid plants

## **Development of Pure Homozygous Line**

In plant breeding, it is very much essential to get the pure homozygous line which is generally obtained through selfing for 6-7 generations. But by the use of anther/pollen culture it can be reduced to few months or a year. These genetically pure homozygous lines are used for breeding as well as genetic research purpose. This technique is also helpful for breeding of these plants which have more elongated juvenile phase.

### **Selection of Mutants Resistance to Diseases**

Selection of mutants with resistance to disease is of prime importance in crop improvement. Haploids provide a relatively easier system for the induction of mutations. Some examples of using anther culture technique in mutant successfully are tobacco mutants resistant to black shank disease and wheat lines resistant to scab.

### **Transfer of Desired Alien Gene**

Chromosomal instability in haploids makes them potential tools for introduction of alien chromosomes on genes during wider crossing programs.

### **Induction of Mutagenesis**

Haploid cell cultures are useful material for induction of mutations and to study the effect of mutation. This method can overcome the masking effect of presence of dominant gene. The screening method for detection of mutational effect Is also easier in this technique.

### **Induction of Genetic Variability**

The pollen/microspore is easy explant for production of genetically variable types by introducing the different foreign genes through different transformation procedure. These transformed or transgenic haploids can be used further in breeding program.

### **Development of Aneuploids**

Haploids have been used in the production of aneuploids like monosomies in wheat, trisomies in potato. In tobacco nullisomics were derived from haploids obtained from monosomies which could

not produce nullisomics on selfing. **Nullisomic** is a genetic condition involving the lack of both the normal chromosomal pairs for a species ( $2n-2$ ).

## 21. Somaclonal Variation

### Somaclonal variations

The genetic variations found in the in vitro cultured cells are collectively referred to as somaclonal variations. These are genetic variations in plants that have been produced by plant tissue culture and can be detected as genetic or phenotypic traits.

#### Basic features of somaclonal variations

Variations in number and structure of chromosomes are commonly observed. Regenerated plants with altered chromosomal changes often show changes in leaf shape and colour, growth rate and habit, and sexual fertility. It is generally heritable mutations and persists in plant population even after plantation into the field.

### Mechanism of Somaclonal Variations

- **Genetic (Heritable Variations)**
  - Pre-existing variations in the somatic cells of explant
  - Caused by mutations and other DNA changes
  - Occur at high frequency
- **Epigenetic (Non-heritable Variations)**
  - Variations generated during tissue culture
  - Caused by temporary phenotypic changes
  - Occur at low frequency

#### Causes of Somaclonal Variations are:

1. Physiological Cause

2. Genetic Cause
3. Biochemical Cause

### **1. Physiological Cause**

Physiological causes are: Exposure of culture to plant growth regulators and Culture conditions.

### **2. Genetic Cause**

- Genetic causes are: **Change in chromosome number:** Aneuploidy, gain or loss of 1 or more chromosomes, Polyploidy, gain or loss of an entire genome, Translocation, arms of chromosomes switched, inversion, piece of chromosome inverted.
- **Change in chromosome structure:** Deletion, Inversion, Duplication, Translocation,
- **Gene Mutation:** Transition, Traversions, Insertion and Deletion
- **Plasmagene Mutation :** It is a self-replicating extra nuclear determiner of hereditary characteristics.
- **Transposable element activation :** Transposable elements (TEs), also known as "jumping genes" or transposons, are sequences of DNA that move (or jump) from one location in the genome to **another**.
- **DNA sequence :** Change in DNA **and** Detection of altered fragment size by using Restriction enzyme
- **Change in Protein :** Loss or gain in protein band and Alteration in level of specific protein
- **Methylation of DNA:** Methylation inactivates transcription process.

### **Biochemical Cause**

It is the Lack of photosynthetic ability due to alteration in carbon metabolism e.g. biosynthesis of starch via carotenoid pathway and Nitrogen metabolism processes.

- **Advantages of Somaclonal Variations are:** help in crop improvement, creation of additional genetic variations, increased and improved production of secondary metabolites, selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity, suitable for breeding of tree species.

### **Applications of Somaclonal Variations**

**Production of agronomically useful plants:** As a result of somaclonal variations, several novel variants of existing crops have been developed, e.g., pure thorn-less blackberries, somaclonal variations are useful and improved morphological characters in different crops.

**Resistance to diseases:** Somaclonal variations have largely contributed towards the development of disease resistance in many crops e.g. rice, wheat, maize, sugarcane, tobacco, apple, tomato.

- **Resistance to abiotic stresses:** It has been possible to develop biochemical mutants with abiotic stress resistance.
  - i. Freezing tolerance e.g. wheat.
  - ii. Salt tolerance e.g., rice, maize, tobacco.
  - iii. Aluminium tolerance e.g., carrot, sorghum, tomato.

### **Resistance to herbicides**

Certain somaclonal variants with herbicide resistance have been developed. E.g.

- i. Tobacco resistant to glyphosate, sulfonylurea and picloram.
- ii. Carrot resistant to glyphosate.
- iii. Lotus resistant to 2, 4-dichlorophenoxy acetic acid

### **Improved seed quality**

A new variety of *Lathyrus sativa* (grass Pea) seeds with a low content of neurotoxin has been developed through somaclonal variations. **Neurotoxins** are an extensive class of exogenous

chemical neurological insults that can adversely affect function in both developing and mature nervous tissue.

## **22.Micropropagation**

Micropropagation is the practice of rapidly multiplying reserve plant material to produce a large number of progeny plants, using modern methods of plant tissue culture. Micropropagation is used to multiply noble plants, such as those that have been genetically modified or raised through conventional plant breeding methods. It is also used to provide a sufficient number of seedlings to plant from a common plant that does not produce seeds, or does not respond well to vegetative reproduction.

### **Micropropagation Technique**

Micropropagation is a complicated process and comprises mainly 4 stages (I, II, III and IV). But initial step 0 is also necessary.

**Step 0:** This is the initial step in micro-propagation, and involves the selection and growth of common plants for about 3 months under controlled conditions.

**Stage I – Establishment:** In this stage, the initiation and establishment of the culture is achieved in a suitable medium. The selection of appropriate explants is important. The most

commonly used explants are organs, shoot tips and axillary shoots. The explant selected is surface sterilized and washed prior to use.

**Stage II – Multiplication:** At this stage, the main activity of Micro propagation occurs in a defined culture medium. Phase II mainly involves the multiplication of shoots or the rapid formation of explant embryos.

**Stage III – Rooting:** This stage involves the transfer of shoots to a medium for rapid development in shoots. Sometimes sprouts are planted directly on the ground to develop roots. In vitro rooting of shoots is preferred while simultaneously handling a large number of species.

**Stage IV – Acclimatization:** This stage involves the establishment of seedlings in the soil. This is done by transferring seedlings from stage III of the laboratory to the environment of the greenhouse. For some plant species, stage III is omitted, and unrooted shoots of stage II are planted in pots or in a suitable compost mix.

**Factors Affecting Micro-propagation:** there is need of optimization of several factors is necessary for success in clonal propagation in vitro (micro-propagation).

**Genotype of the plant:** Selection of the correct genotype of plant species (by screening) is necessary to improve micropropagation. In general, plants with a vigorous germination and branching ability are more suitable for micro-propagation.

**Physiological status of explants:** The explants (plant materials) of the most recently produced parts of plants are more effective than those of the older regions. A good knowledge of the process of natural propagation of donor plants, with special reference to the stage of growth and seasonal influence, will be useful in the selection of explants.

**Culture media:** Conventional plant tissue culture media are suitable for micropropagation during stage I and stage II. However, for stage III, certain modifications are essential. The addition of growth regulators (auxins and cytokinins) and alterations in mineral composition is essential. This depends largely on the type of crop.

**Light:** The photosynthetic pigment in cultured tissues absorbs light and, therefore, influences micropropagation. Variations in daytime lighting also effect micropropagation. In general, a lighting of 16 hours of day and 8 hours of night is suitable for the proliferation of shoots.

**Temperature:** The majority of micropropagation culture necessitates optimum temperature around 25 °C. However, there are some exceptions.

### **Micropagation applications are:**

- Suitable alternative to traditional methods
- High prevalence of plants
- The production of disease-free plants
- Seed production in some crops
- Cost effective process
- Automated micro propagation
- Very small size explants can be used
- Only practical method of multiplying genetically modified cells or cells after protoplast fusion.
- Ease in keeping, packing and transport of material multiplied by micropagation.

## **23.Synthetic Seed**

**Synthetic seed:** Many fruit crops are difficult to multiply by conventional propagation methods and improve through traditional breeding programs. Among the innovative techniques of micro propagation, the concept of somatic embryogenesis with synthetic seed production or artificial seed technology is very promising. Synthetic seed is referred to as encapsulated somatic embryos, which functionally mimic seeds and can develop into seedling under suitable conditions. Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under in vitro or ex vitro conditions and that retain this potential also after storage. In simple words synthetic seed contains an embryo produced by somatic embryogenesis enclosed within an artificial medium that supplies nutrients and is encased in an artificial seed covering.

### **Why Synthetic Seeds:**

In some of the horticultural crops seeds propagation is not successful due to; heterozygosity of seeds particularly in cross pollinated crops, minute seed size e.g.; orchids, presence of reduced endosperm, some seeds require mycorrhizal fungi association for germination e.g.: orchids, no seeds are formed.

### **Characteristics of Synthetic Seeds:**

- i. High volume. Large scale propagation method
- ii. Maintains genetic uniformity of plants
- iii. Direct delivery of propagules to the field, thus eliminating transplants
- iv. Lower cost per plantlet
- v. Rapid multiplication of plants

### **Advantages of Synthetic Seeds are:**

- i. Ease of handling while in storage
- ii. Easy to transport
- iii. Has potential for long term storage without losing viability
- iv. Maintains the clonal nature of the resulting plants
- v. Serves as a channel for new plant lines produced through biotechnological advances to be delivered directly to the green house or field
- vi. Allows economical mass propagation of elite plant varieties

## **24.Germplasm conservation**

**Germplasm:** A germplasm is a collection of genetic resources for an organism. Germplasm is a living tissues from which new plants can be grown. It can be a seed or another plant part-a leaf,a piece of stem,pollen or even just a few cells that can be turned into the whole plant. For plants, the germplasm may be stored as a seed, stem, Callus, Whole plant in nurseries.

**Germplasm conservation:** Plant germplasm is genetic source material in the form of Seeds, Cultured cells Callus, Pollens. The in-situ /ex-situ preservation of this material is known as “Germplasm conservation”. Germplasm provide the raw material (genes) which the breeder uses to develop commercial crop varieties.

**What is the need of Preservation:** Preservation/Conservation of plant biodiversity is an important issue. Storage of Economically important, endangered, rare species and make them available when needed. The conventional methods of storage failed to prevent losses caused due to various reasons.

**Methods of Germplasm conservation are:**

1. In-situ Preservation
2. Ex-situ Preservation

**In-situ Preservation:** it is preservation of the germplasm in their natural habitat, conservation of domesticated and cultivated species in the farm or in the surroundings. However, there is a heavy loss or decline of species, populations and ecosystem composition, which can lead to a loss of biodiversity, due to habitat destruction and the transformations of these natural environments; therefore, in situ methods alone are insufficient for saving endangered species.

**Ex-situ preservation:** 1. It is used to maintain the biological material outside their natural habitats, for storage in seed banks, field gene collections, in vitro collections and botanical gardens. Ex situ conservation is a viable way for saving plants from extinction, and in some cases, it is the only possible strategy to conserve certain species. In vitro conservation is especially important for vegetatively propagated and for non-orthodox seed plant species. Non-Orthodox seeds are seeds which do not survive drying and/or freezing during ex-situ conservation.

**Approaches for the in vitro conservation of germplasm:**

- Cryopreservation (freeze-preservation)
- Cold storage
- Low-pressure and low-oxygen storage
- **Cryopreservation:** Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants. In this case the cells are preserved in the frozen state. The germplasm is stored at a very low temperature using
  - Solid carbon dioxide (at -790C)
  - Using low temperature deep freezers (at -800C)
  - Using vapour nitrogen (at- 1500C)
  - Liquid nitrogen (at-1960C).

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

**Low pressure and low oxygen storage:** In low- pressure storage, the atmospheric pressure surrounding the plant material is reduced. In the low oxygen storage, the oxygen concentration is reduced. The lowered partial pressure reduces the in vitro growth of plants. In the low-oxygen storage, the oxygen concentration is reduced and the partial pressure of oxygen below 50 mmHg reduces plant tissue growth. Due to the reduced availability of O<sub>2</sub>, and reduced production of CO<sub>2</sub>, the photosynthetic activity is reduced. It inhibits the plant tissue growth and dimension. This method has also helped in increasing the shelf life of many fruits, vegetables and flowers. The germplasm conservation through the conventional methods has several limitations such as short-

lived seeds, seed dormancy, seed-borne diseases, and high inputs of cost and labour. The techniques of cryo-preservation (freezing cells and tissues at -1960c) and using cold storages help us to overcome these problems.

**Applications or significance of germplasm conservation are:** The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock. It can be used at any time in future. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the existing and primitive plants will be lost. Main crops produce recalcitrant or short lived seeds. Similarly, in case of clonal crops seeds are not the best material to conserve due to their genetic heterogeneity and unknown worth. Their genes need to be conserved. The roots and tubers loose viability rapidly. Their storage requires large space, low temperature and is expensive. In addition, materials modified by genetic engineering may sometimes be unstable. Such materials are needed to be conserved intact for future use.

## 25. Genetic Markers in Plant Breeding

**Genetic marker:** A genetic marker is a gene or DNA sequence with a known location on a chromosome. It can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed. Any phenotypic difference controlled by genes, that can be used for studying recombination processes or selection of a more or less closely associated target gene. Anything in the genome that is variable and can be used to compare individuals. Detectable allelic variation on a chromosome it can be a phenotype, can also be a unique detectable sequence of DNA. They are points of variation that can be used to identify individuals or species, or may be used to associate an inherited disease with a gene through genetic linkage with nearby but possibly unidentified or uncharacterised genes. Examples include single nucleotide polymorphisms (SNPs) and minisatellites.

**Polymorphism:** In biology and zoology is the occurrence of two or more clearly different morphs or forms, also referred to as alternative phenotypes, in the population of a species.

**Single nucleotide polymorphisms (SNPs):** A single-nucleotide polymorphism, often abbreviated to SNP. A variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some appreciable degree within a population. For example, at a specific base position in the human genome, the C nucleotide may appear in most individuals, but in a minority of individuals, the position is occupied by an A. This means that there is an SNP at this specific position, and the two possible nucleotide variations – C or A – are said to be alleles for this position. As SNPs are thought to play a major role in the induction of phenotypic variation in plants, so it is very important to identify the functional SNPs regarding crop improvements. SNPs also can identify the genomic diversity of species to demonstrate the speciation and evolution, and associate genomic variations with phenotypic traits

**Types of genetic markers are:**

Molecular markers

Morphological markers

## **26.Molecular Markers**

**Molecular markers**

It is a sequence of DNA or protein that can be screened to reveal key attributes of its state or composition and thus used to reveal genetic variation, also known as “Genetic Marker”. Genetic markers are the sequences of DNA which have been traced to specific location on the chromosomes and associated with particular traits.

**Classification of molecular markers:**

Molecular Markers are classified as:

1. Protein Based Markers/ Biochemical Markers
2. DNA Based Markers

**Protein Based Markers/ Biochemical Markers:** Plant Breeding Markers related to the variations in protein and amino acid banding pattern.

Isozyme markers: Multiple forms of the same enzyme coded by the different genes

Allozyme : one enzyme, one locus; two or more alleles in a population

**Advantages: advantages of molecular markers are:**

They are simple, inexpensive, electrophoretically resolvable, and detectable, does not require DNA extraction or the availability of sequence information, primers or probes, quick and easy to use, codominant markers that have high reproducibility.

**Disadvantages of molecular markers are:** they are relatively low abundance and low level of polymorphism, can be affected by environmental conditions, they may change depending on the type of tissue used for the analysis.

**Applications of molecular markers are:**

They are used for detection of the gene introgression (gene movement) and recombination, for comparative mapping, for determination of the genetic diversity and phylogenetic relationships.

## 27. DNA based markers

### DNA Markers

A gene or other fragment of DNA whose location in the genome is known is called DNA marker. It refers to any unique DNA sequence which can be used in DNA hybridization, PCR or restriction mapping experiments to identify that sequence. It can be identified by a range of molecular techniques such as RFLPs, RAPDs, AFLP, SNPs, SCARs, microsatellites etc.

**Advantages of DNA markers:**

Advantages of DNA markers are presented below.

- They are highly polymorphic.
- They have simple inheritance (often co-dominant).
- They abundantly occur throughout the genome.
- They are easy and fast to detect.
- They exhibit minimum pleiotropic effect.
- Their detection is not dependent on the developmental stage of the organism.

**Properties of DNA Marker:** An ideal DNA marker should have some properties or characteristics

#### **Polymorphism:**

- Markers should exhibit high level of polymorphism.
- In other words, there should be variability in the markers.
- It should demonstrate measurable differences in expression between trait types and/or gene of interest.

#### **Co-Dominant:**

- Marker should be co-dominant.
- It means, there should be absence of intra-locus interaction.
- It helps in identification of heterozygotes from homozygotes.



### **Multi-Allelic:**

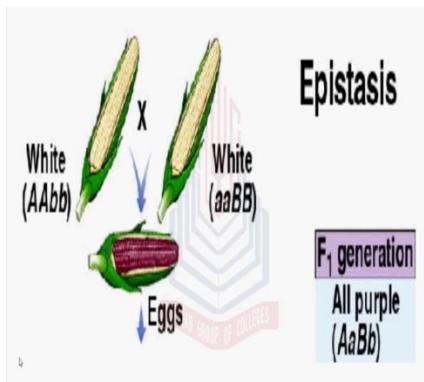
- The marker should be multi-allelic.
- It is useful in getting more variability/ polymorphism for a character.
- A multiallelic site is a specific locus in a genome that contains three or more observed alleles, again counting the reference as one, and therefore allowing for two or more variant alleles.



Different flower colors due to allelic variation in multiple genes

### **No Epistasis:**

- There should be absence of epistasis.
- It makes identification of all phenotypes (homo- and heterozygotes) easy.
- Epistasis: the interaction of genes that are not alleles, in particular the suppression of the effect of one such gene by another.



### **Neutral:**

- The marker should be neutral.
- The substitution of alleles at the marker locus should not alter the phenotype of an individual.
- This property is found in almost all the DNA markers

**No Effect of Environment:** Markers should be insensitive to environment. This property is also found in almost all the DNA markers.

#### **Applications of DNA Marker in Crop Improvement are:**

- i. DNA markers are useful in the assessment of genetic diversity in germplasm, cultivars and advanced breeding material.
- ii. DNA markers can be used for constructing genetic linkage maps.
- iii. DNA markers are useful in identification of new useful alleles in the germplasm and wild species of crop plants.
- iv. DNA markers are used in the marker assisted or marker aided selection. MAS has several advantages over straight selection.
- v. DNA markers are useful in the study of crop evolution.

## **28. Morphological markers**

### **Morphology:**

Morphology is a branch of biology dealing with the study of the form and structure of organisms and their specific structural features.

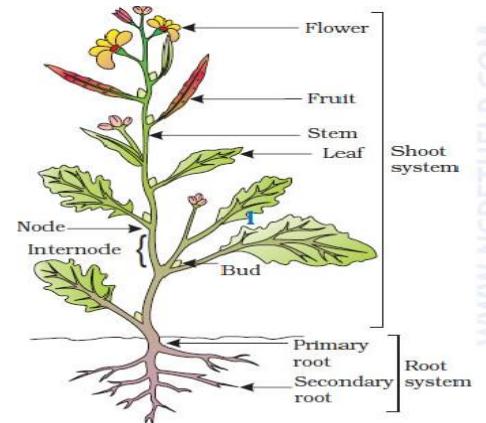
### **Plant Morphology:**

Plant morphology or phyto morphology is the study of the physical form and external structure of plants. This is usually considered distinct from plant anatomy, which is the study of the internal

structure of plants, especially at the microscopic level. Plant morphology is useful in the visual identification of plants.

### Morphological features of plants:

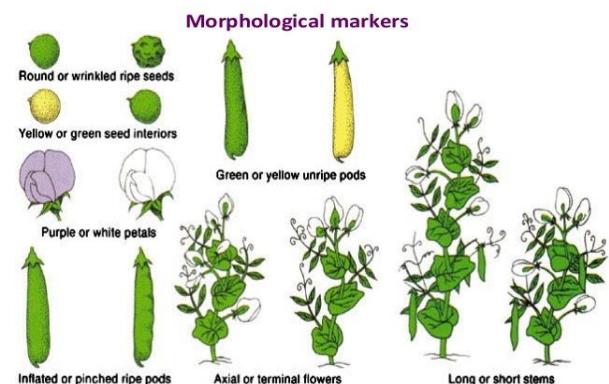
- Plants are characterized by following features:
- Roots
- Shoots
- Stem
- Leaves
- Flowers
- Fruits



### Morphological Markers

They are visually characterized phenotypic characters. e.g.

- Flower colour ,seed shape , growth habit, pigmentation
- It involves Germplasm characterization and indirect selection



### Advantages

#### Some advantages of morphological markers are:

- They are inexpensive to score and ready to experiments in natural populations

### Disadvantages

#### Some disadvantages of morphological markers are:

- visible polymorphisms relatively rare

- Most genetic variation not so easily observed (Variants are ambiguous)
- Genetic basis of variation can be complex, and is not necessarily easy to determine.

**Limitations: some limitations of morphological marks are enlisted here:**

- They do not represent the genome adequately, they give stable inheritance( Need repeated measures)
- They generally express late into the development of an organism. Hence their detection is dependent on the development stage of the organism.
- They usually exhibit dominance, sometimes they exhibit deleterious effects. They exhibit pleiotropy. They exhibit epistasis. They exhibit less polymorphism. They are highly influenced by the environmental factors.

## **29. Transformation**

### **Transformation**

In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surroundings through the cell membrane. Transformation occurs most commonly in bacteria and in some species occurs naturally. Transformation can also be effected by artificial means. Bacteria that are capable of being transformed, whether naturally or artificially, are called competent

### **Competent cells**

Competent Cells are more likely to incorporate foreign DNA if their cell walls are altered so that DNA can pass through more easily. Such cells are said to be competent

### **Types of competence (there is two types of competence):**

- i. Natural competence
- ii. Artificial competence

- i. **Natural competence:** Bacteria are able to take up DNA from their environment by three ways; conjugation, transformation, and transduction. In transformation the DNA is directly entered to the cell. Uptake of transforming DNA requires the recipient cells to be in a specialized physiological state called competent state.

### **Artificial competence:**

- It is a laboratory procedure by which cells are made permeable to DNA, with conditions that do not normally occur in nature. This procedure is comparatively easy and simple, and can be used in the genetic engineering of bacteria but in general transformation efficiency is low. There are two main methods for the preparation of competent cells They are **Calcium chloride method and Electroporation.** Transformation may also be used to describe the insertion of new genetic material into nonbacterial cells including animal and plant cells

### **History:**

Transformation was first demonstrated in 1928 by British bacteriologist Frederick Griffith. Griffith discovered that a harmless strain of *Streptococcus pneumoniae* could be made virulent after being exposed to heat-killed virulent strains. Griffith hypothesized that some "transforming principle" from the heat-killed strain was responsible for making the harmless strain virulent.

In 1944 this "transforming principle" was identified as being genetic by Oswald Avery, Colin MacLeod, and Maclyn McCarty. They isolated DNA from a virulent strain of *S. pneumoniae* and using just this DNA was able to make a harmless strain virulent. They called this uptake and incorporation of DNA by bacteria "transformation." Transformation using electroporation was developed in the late 1980s, increasing the efficiency of in-vitro transformation and increasing the number of bacterial strains that could be transformed. Transformation of animal and plant cells was also investigated with the first transgenic mouse being created by injecting a gene for a rat growth hormone into a mouse embryo in 1982. In 1907 a bacterium that caused plant tumors, *Agrobacterium tumefaciens*, was discovered and in the early 1970s the tumor inducing agent was found to be a DNA plasmid called the Ti plasmid. By removing the genes in the plasmid that caused the cancer and adding in novel genes researchers were able to infect plants with *A. tumefaciens* and let the bacteria insert their chosen DNA into the genomes of the plants. Not all

plant cells are susceptible to infection by *A. tumefaciens* so other methods were developed including electroporation and micro-injection. Particle bombardment was made possible with the invention of the Biolistic Particle Delivery System (gene gun) by John Sanford in 1990.

## 30. Mechanisms of Transformation

### Bacterial Transformation:

Bacterial transformation may be referred to as a stable genetic change brought about by the uptake of naked DNA (DNA without associated cells or proteins) and competence refers to the state of being able to take up exogenous DNA from the environment.

Two forms of competence exist:

1. Natural and
2. Artificial.
  1. **Natural competence:** About 1% of bacterial species are capable of naturally taking up DNA under laboratory conditions; many more are able to take it up in their natural environments. Such bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane(s). DNA material can be transferred between different strains of bacteria, in a process called horizontal gene transfer.
  2. **Artificial competence:** Artificial competence is induced by laboratory procedures and involves making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature. Calcium chloride transformation is a method of promoting competence. Chilling cells in the presence of divalent cations such as  $\text{Ca}^{2+}$  (in  $\text{CaCl}_2$ ) prepares the cell membrane to become permeable to plasmid DNA. The cells are incubated on ice with the DNA and then briefly heat shocked (e.g., 42°C for 30–120 seconds) thus allowing the DNA to enter the cells. This method works very well for circular plasmid DNA. An excellent preparation of competent cells will give  $\sim 10^8$  colonies per microgram of plasmid. A poor preparation will be about  $10^4/\mu\text{g}$  or less. Good, non-commercial preparations should give  $10^5$  to  $10^6$  transformants per microgram of plasmid. The method, however, usually does not work well for linear DNA, such as fragments of chromosomal DNA, probably because the cell's native exonuclease enzymes rapidly degrade linear DNA.

Interestingly, cells that are naturally competent are usually transformed more efficiently with linear DNA than with plasmid DNA.

**Electroporation:** Electroporation is another method of promoting competence. In the method the cells are briefly shocked with an electric field of 10-20 kV/cm that creates holes in the cell membrane through which the plasmid DNA enters. This method is ready to the uptake of large plasmid DNA. After the electric shock the holes are rapidly closed by the cell's membrane-repair mechanisms. The efficiency with which a competent culture can take up exogenous DNA and express its genes is known as transformation efficiency.

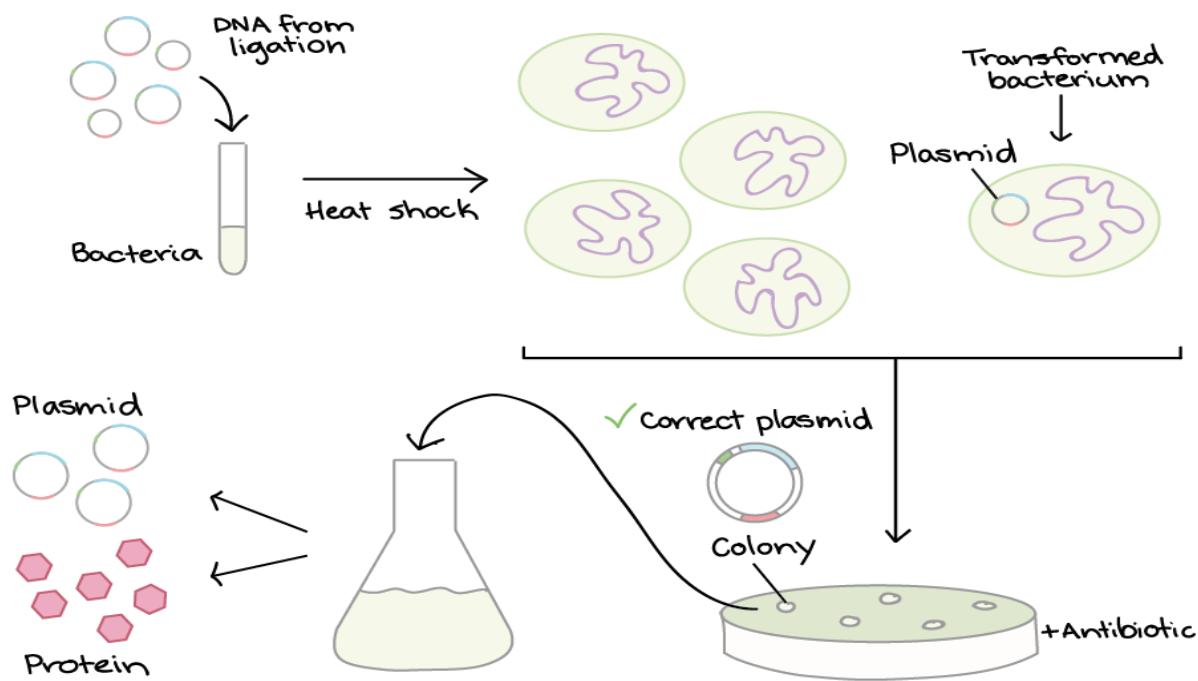
## 31. Bacterial transformation & selection

Bacteria can take up foreign DNA in a process called transformation. Transformation is a key step in DNA cloning. It occurs after restriction digest and ligation and transfers newly made plasmids to bacteria. After transformation, bacteria are selected on antibiotic plates. Bacteria with a plasmid are antibiotic-resistant, and each one will form a colony. Colonies with the right plasmid can be grown to make large cultures of identical bacteria, which are used to produce plasmid or make protein

### DNA cloning

Transformation and selection of bacteria are key steps in DNA cloning. DNA cloning is the process of making many copies of a specific piece of DNA, such as a gene. The copies are often made in bacteria. In a typical cloning experiment, researchers first insert a piece of DNA, such as a gene, into a circular piece of DNA called a plasmid. This step uses restriction enzymes and DNA ligase and is called a ligation. After a ligation, the next step is to transfer the DNA into bacteria in a process called transformation. Then, we can use antibiotic selection and DNA analysis methods to identify bacteria that contain the plasmid we're looking for.

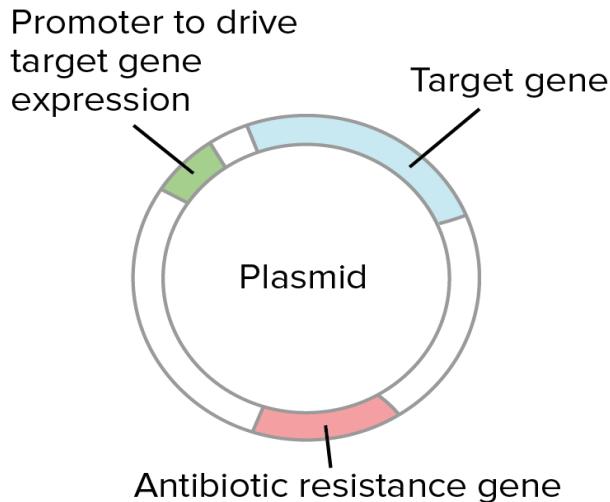
### Steps of bacterial transformation and selection



## Steps

Specially prepared bacteria are mixed with DNA (e.g., from a ligation). The bacteria are given a heat shock, which causes some of them to take up a plasmid. Plasmids used in cloning contain an antibiotic resistance gene. Thus, all of the bacteria are placed on an antibiotic plate to select for ones that took up a plasmid. Bacteria without a plasmid die. Each bacterium with a plasmid gives rise to a cluster of identical, plasmid-containing bacteria called a colony. Several colonies are checked to identify one with the right plasmid (e.g., by PCR or restriction digest).

A colony containing the right plasmid is grown in bulk and used for plasmid or protein production.

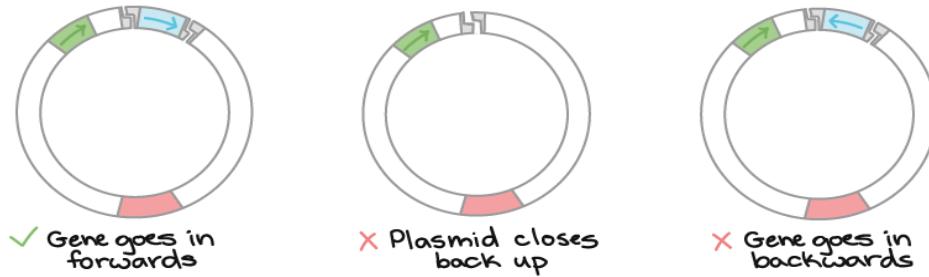


### Why do we need to check colonies?

The bacteria that make colonies should all contain a plasmid (which provides antibiotic resistance). However, it's not necessarily the case that all of the plasmid-containing colonies will have the same plasmid.

### How does that work?

When we cut and paste DNA, it's often possible for side products to form, in addition to the plasmid we intend to build. For instance, when we try to insert a gene into a plasmid using a particular restriction enzyme, we may get some cases where the plasmid closes back up (without taking in the gene), and other cases where the gene goes in backwards.



### Why does it matter if a gene goes into a plasmid backwards?

In some cases, it doesn't. However, if we want to express the gene in bacteria to make a protein, the gene must point in the right direction relative to the **promoter**, or control sequence that drives gene expression. If the gene were backwards, the wrong strand of DNA would be transcribed and no protein would be made. Because of these possibilities, it's important to collect plasmid DNA from each colony and check to see if it matches the plasmid we were

trying to build. Restriction digests, PCR, and DNA sequencing are commonly used to analyze plasmid DNA from bacterial colonies.

## 32. Transformation in Plants

Gene Transfer is introduction of foreign genetic material, either DNA or RNA artificially or naturally into a cell. It is often also referred to as transformation and is one of the foundations of molecular biology. It is now possible to introduce and express DNA stably in nearly 150 different plant species. 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, Terminator, Selectable marker and other genes that deliver the DNA into the host plant (Ex. vir genes of *Agrobacterium*)

### Plant transformation

Plant transformation is a scientific approach whereby DNA from any organism is inserted into the genome of a species of interest. The inserted DNA is called a “transgene”, and the resulting plant is said to be “transgenic”. Transgenic plants are plants derived from cells in which genes (often of nonplant origin) have been stably introduced by transformation to give the plant a new and useful trait. Transgenic plants can be obtained after transformation of single cells and the subsequent regeneration into complete, fertile plants by tissue culture protocols. Transformed plant cells can be identified by their ability to grow on selective media containing an antibiotic or a herbicide as transformation vectors contain selection genes conferring such properties. Novel functions are expressed in transformed plant cells if the coding regions are surrounded by promoter and terminator regions that are recognized by the plant transcription machinery. The most preferred methods for plant transformation use either the particle gun or the natural transformation system of *Agrobacterium tumefaciens*, as they can cope with cells present in whole plants or tissues. *Agrobacterium tumefaciens* can be disarmed by deletion of the *onc*-genes that are naturally present between the 25-bp repeats of the T-DNA. Any gene introduced between these repeats is translocated into plant cells by *Agrobacterium tumefaciens*. Transformed cells with a single copy of the transgene usually show higher and more stable expression than multicopy lines, in which expression may suffer from posttranscriptional gene silencing. (T)-DNA integration in plant cells occurs at random sites in the genome by nonhomologous end-joining and related backup pathways.

Targeted integration of transgenes can be accomplished in plant cells by using either a site-specific recombination system (e.g. Cre-*lox*) or by homology-directed integration in combination with a site-specific nuclease (e.g. a zinc-finger nuclease). *Agrobacterium tumefaciens*-mediated

transformation can be applied not only in dicotyledonous plants, but also in monocots such as cereals and in yeasts and fungi. The T-DNA can be used as a mutagen causing insertion mutations. Libraries of *Arabidopsis thaliana* T-DNA transformants are in use now in mutant screens to identify insertion mutations in genes of interest (reverse genetics).

**TABLE 49.1 Gene transfer (DNA delivery) methods in plants**

<b>Method</b>	<b>Salient features</b>
<b>I. Vector-mediated gene transfer</b>	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
<b>II. Direct or vectorless DNA transfer</b>	
<b>(A) Physical methods</b>	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
<b>(B) Chemical methods</b>	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

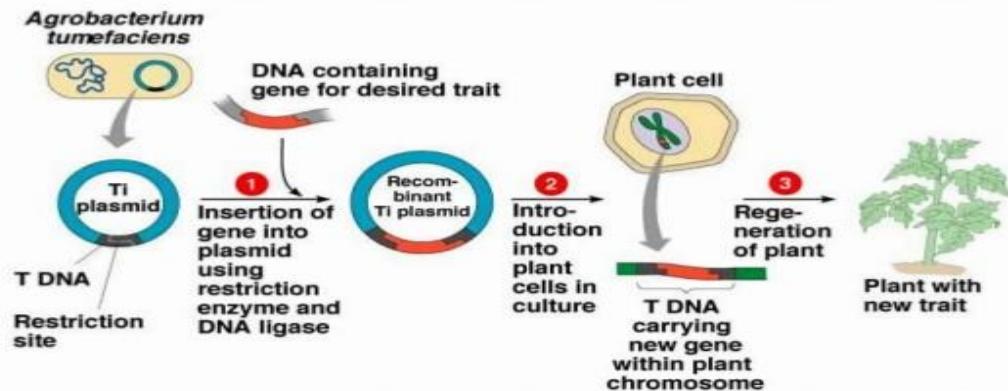
### Vector-Mediated Gene Transfer:

#### Agrobacterium-Mediated Gene Transfer

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium. It is rod shaped and motile, and belongs to the bacterial family of Rhizobiaceae. A. tumefaciens is a phytopathogen, and is treated as the nature's most effective plant genetic engineer.

Some workers consider this bacterium as the natural expert of inter-kingdom gene transfer. In fact, the major credit for the development of plant transformation techniques goes to the natural unique capability of A. tumefaciens. Thus, this bacterium is the most beloved by plant biotechnologists.

# Agrobacterium mediated Transformation.



## species of Agrobacterium:

- A. tumefaciens that induces crown gall disease.
- A. rhizogenes that induces hairy root disease.

## Crown Gall Disease and Ti Plasmid

Almost 100 years ago (1907), Smith and Townsend postulated that a bacterium was the causative agent of crown gall tumors, although its importance was recognized much later. As *A. tumefaciens* infects wounded or damaged plant tissues, it induces the formation of a plant tumor called crown gall. The entry of the bacterium into the plant tissues is facilitated by the release of certain phenolic compounds (acetosyringone, hydroxyacetosyringone) by the wounded sites. Crown gall symptoms include round, wart-like growths 2 inches or larger in diameter that appear at or just above the soil line, or on lower branches and stems. Plants with several galls may be unable to move water and nutrients up the trunk and become weakened, stunted and unproductive. Young plants can be killed by developing gall tissue. Crown gall formation occurs when the bacterium releases its Ti plasmid (tumor-inducing plasmid) into the plant cell cytoplasm. A fragment (segment) of Ti plasmid, referred to as T-DNA, is actually transferred from the bacterium into the host where it gets integrated into the plant cell chromosome (i.e. host genome). Thus, crown gall disease is a naturally evolved genetic engineering process.

The T-DNA carries genes that code for proteins involved in the biosynthesis of growth hormones (auxin and cytokinin) and novel plant metabolites namely opines, amino acid derivatives and agropines, sugar derivatives. The growth hormones cause plant cells to

proliferate and form the gall while opines and agropines are utilized by *A. tumefaciens* as sources of carbon and energy. As such, opines and agropines are not normally part of the plant metabolism (neither produced nor metabolised). Thus, *A. tumefaciens* genetically transforms plant cells and creates a biosynthetic machinery to produce nutrients for its own use.

As the bacteria multiply and continue infection, grown gall develops which is a visible mass of the accumulated bacteria and plant material. Crown gall formation is the consequence of the transfer, integration and expression of genes of T-DNA (or Ti plasmid) of *A. tumefaciens* in the infected plant. The genetic transformation leads to the formation of crown gall tumors, which interfere with the normal growth of the plant. Several dicotyledonous plants (dicots) are affected by crown gall disease e.g. grapes, roses, stone-fruit trees.

## 33. Virus Mediated Gene Transfer

### Viral transformation

Viral transformation is the change in growth, phenotype, or indefinite reproduction of cells caused by the introduction of inheritable material. Through this process, a virus causes harmful transformations of an in vivo cell or cell culture. Viral transformation can occur both naturally and medically. Natural transformations can include viral cancers, such as human papillomavirus (HPV) and T-cell Leukemia virus type I. Hepatitis B and C are also the result of natural viral transformation of the host cells. Viral transformation can also be induced for use in medical treatments. Cells that have been virally transformed can be differentiated from untransformed cells through a variety of growth, surface, and intracellular observations.

### Transduction

Transduction is the process by which foreign DNA is introduced into a cell by a virus or viral vector.

### Viral transformation (transduction)

Package the desired genetic material into a suitable plant virus and allow this modified virus to infect the plant. If the genetic material is DNA, it can recombine with the chromosomes to produce transformant cells. However genomes of most plant viruses consist of single stranded RNA which replicates in the cytoplasm of infected cell. For such genomes this method is a form of transfection and not a real transformation, since the inserted genes never reach the

nucleus of the cell and do not integrate into the host genome. The progeny of the infected plants is virus free and also free of the inserted gene.

### **Plant Viruses as Vectors**

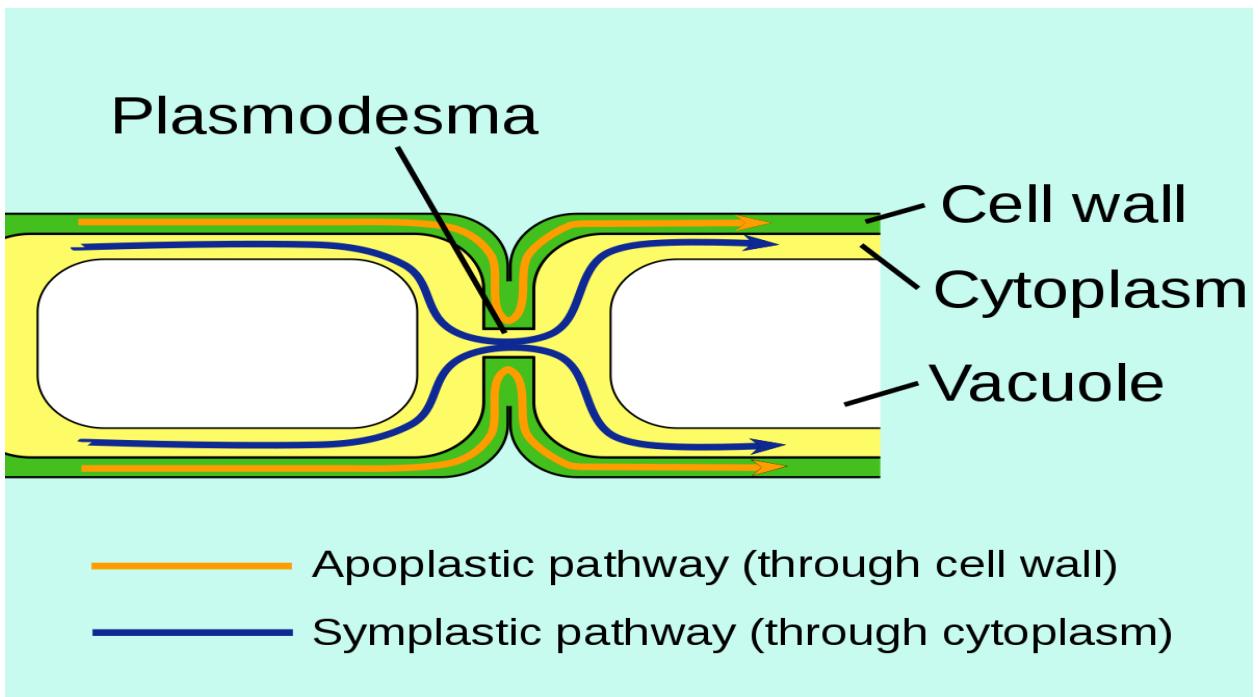
Plant viruses are considered as efficient gene transfer agents as they can infect the intact plants and amplify the transferred genes through viral genome replication. Viruses are natural vectors for genetic engineering. They can introduce the desirable gene(s) into almost all the plant cells since the viral infections are mostly systemic.

#### **Plant viruses are non-integrative vectors:**

The plant viruses do not integrate into the host genome in contrast to the vectors based on T-DNA of *A. tumefaciens* which are integrative. The viral genomes are suitably modified by introducing desired foreign genes. These recombinant viruses are transferred, multiplied and expressed in plant cells. They spread systemically within the host plant where the new genetic material is expressed.

#### **Criteria for a plant virus vector**

An ideal plant virus for its effective use in gene transfer is expected to possess the following characteristics. The virus must be capable of spreading from cell to cell through plasmodesmata. The viral genome should be able to replicate in the absence of viral coat protein and spread from cell to cell. This is desirable since the insertion of foreign DNA will make the viral genome too big to be packed. The recombinant viral genome must elicit little or no disease symptoms in the infected plants. The virus should have a broad host range. The virus with DNA genome is preferred since the genetic manipulations involve plant DNA.

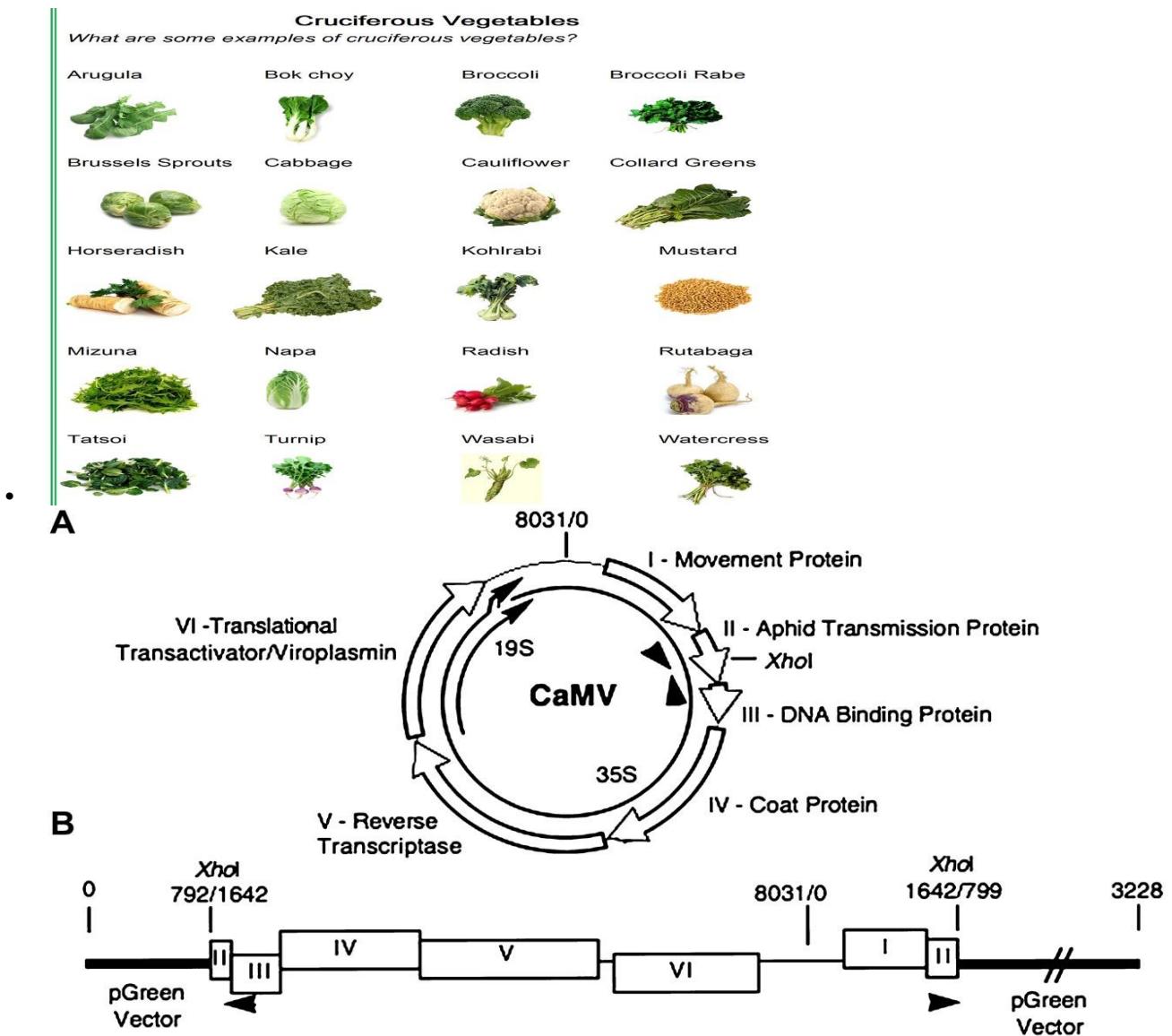


The three groups of viruses — caulimoviruses, Gemini viruses and RNA viruses that are used as vectors for gene transfer in plants are briefly described.

#### **Caulimoviruses as Vectors:**

The Caulimoviruses contain circular double-stranded DNA, and are spherical in shape. Caulimoviruses are widely distributed and are responsible for a number of economically important diseases in various crops. The cauliflower mosaic virus (CaMV) is the most important for gene transfer. The other caulimoviruses include carnation etched virus, dahlia mosaic virus, mirabilis mosaic virus and strawberry vein banding virus.

**Cauliflower mosaic virus (CaMV):** CaMV infects many plants (e.g. members of Cruciferae, Datura) and can be easily transmitted, even mechanically. Another attractive feature of CaMV is that the infection is systemic, and large quantities of viruses are found in infected cells. A diagrammatic view of the CaMV genetic map is depicted in Figure. The genome of CaMV consists of a 8 kb (8024 bp) relaxed but tightly packed circular DNA with six major and two minor coding regions. The genes II and VII are not essential for viral infection.



### Use of CaMV in gene transfer:

For appropriate transmission of CaMV, the foreign DNA must be encapsulated in viral protein. Further, the newly inserted foreign DNA must not interfere with the native assembly of the virus. CaMV genome does not contain any non-coding regions wherein foreign DNA can be inserted. It is fortunate that two genes namely gene II and gene VII have no essential functions for the virus. It is therefore possible to replace one of them and insert the desired foreign gene. Gene II of CaMV has been successfully replaced with a bacterial gene encoding dihydrofolate reductase that provides resistance to methotrexate. When the chimeric CaMV was transmitted to turnip plants, they were systemically infected and the plants developed resistance to methotrexate.

### Limitations of CaMV as a vector

CaMV vector has a limited capacity for insertion of foreign genes. Infective capacity of CaMV is lost if more than a few hundred nucleotides are introduced. Helper viruses cannot be used since the foreign DNA gets expelled and wild-type viruses are produced.

## **34. Virus Mediated Gene Transfer 2**

### **Gemini Viruses as Vectors**

The Gemini viruses are so named because they have geminate (Gemini literally means heavenly twins) morphological particles i.e. twin and paired capsid structures. These viruses are characterized by possessing one or two single-stranded circular DNAs (ss DNA). On replications, ss DNA forms an intermediate double-stranded DNA. The Gemini viruses can infect a wide range of crop plants (monocotyledons and dicotyledons) which attract plant biotechnologists to employ these viruses for gene transfer. Curly top virus (CTV) and maize streak virus (MSV) and bean golden mosaic virus (BGMV) are among the important Gemini viruses. It has been observed that a large number of replicative forms of a Gemini virus genome accumulate inside the nuclei of infected cells. The single-stranded genomic DNA replicates in the nucleus to form a double-stranded intermediate. Gemini virus vectors can be used to deliver, amplify and express foreign genes in several plants/ explants (protoplasts, cultured cells). However, the serious drawback in employing Gemini viruses as vectors is that it is very difficult to introduce purified viral DNA into the plants. An alternate arrangement is to take the help of Agrobacterium and carry out gene transfer.

### **RNA Plant Viruses as Vectors**

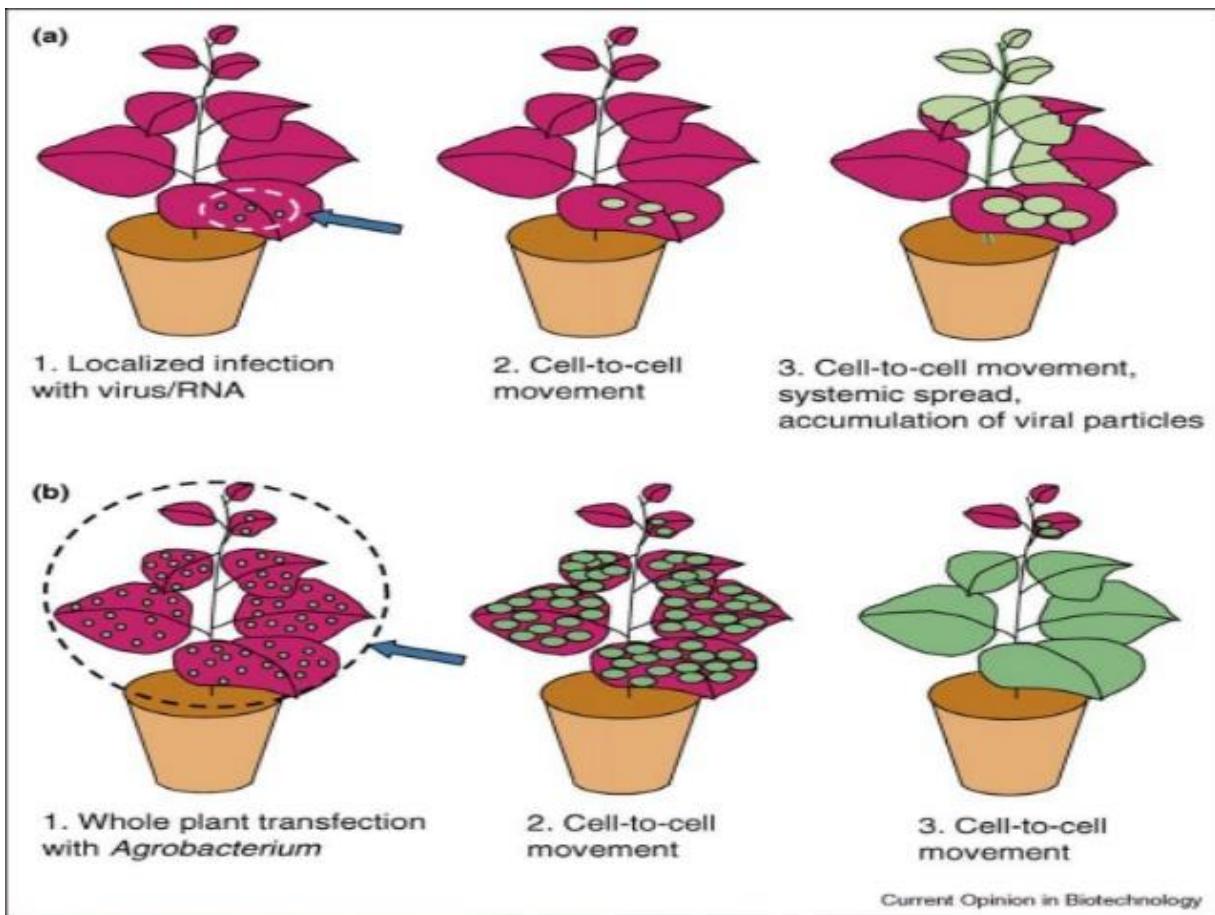
**There are mainly two type's single-stranded RNA viruses:**

#### **Mono-partite viruses:**

These viruses are usually large and contain undivided genomes for all the genetic information e.g. tobacco mosaic virus (TMV).

#### **Multipartite viruses:**

The genome in these viruses is divided into small RNAs which may be in the same particle or different particles, e.g. brome mosaic virus (BMV). HMV contains four RNAs divided between three particles. Plant RNA viruses, in general, are characterized by high level of gene expression, good efficiency to infect cells and spread to different tissues. But the major limitation to use them as vectors is the difficulty of joining RNA molecules in vitro.



### Use of cDNA for gene transfer:

Complementary DNA (cDNA) copies of RNA viruses are prepared in vitro. The cDNA so generated can be used as a vector for gene transfer in plants. This approach is tedious and slow or complicated and therefore inefficient. However, some success has been reported. A gene sequence encoding chloramphenicol resistance (enzyme- chloramphenicol acetyltransferase) has been inserted into brom mosaic virus genome. This gene expression, however, has been confined to protoplasts.

### Limitations of Viral Vectors in Gene Transfer

The ultimate objective of gene transfer is to transmit the desired genes to subsequent generations. With virus vectors, this is not possible unless the virus is seed-transmitted. However, in case of vegetatively propagated plants, transmission of desired traits can be done e.g. potatoes. Even in these plants, there is always a risk for the transferred gene to be lost anytime. For the reasons referred above, plant biotechnologists prefer to insert the desired genes of interest into a plant chromosome.

## 35. 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 methods used for direct gene transfer in plants are

1. Chemical method
2. Physical method

➤ **Chemical mediated gene transfer**

1. Chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts.
2. Calcium phosphate is also used to transfer DNA into cultured cells.

**Liposome mediated gene transfer or Lipofection**

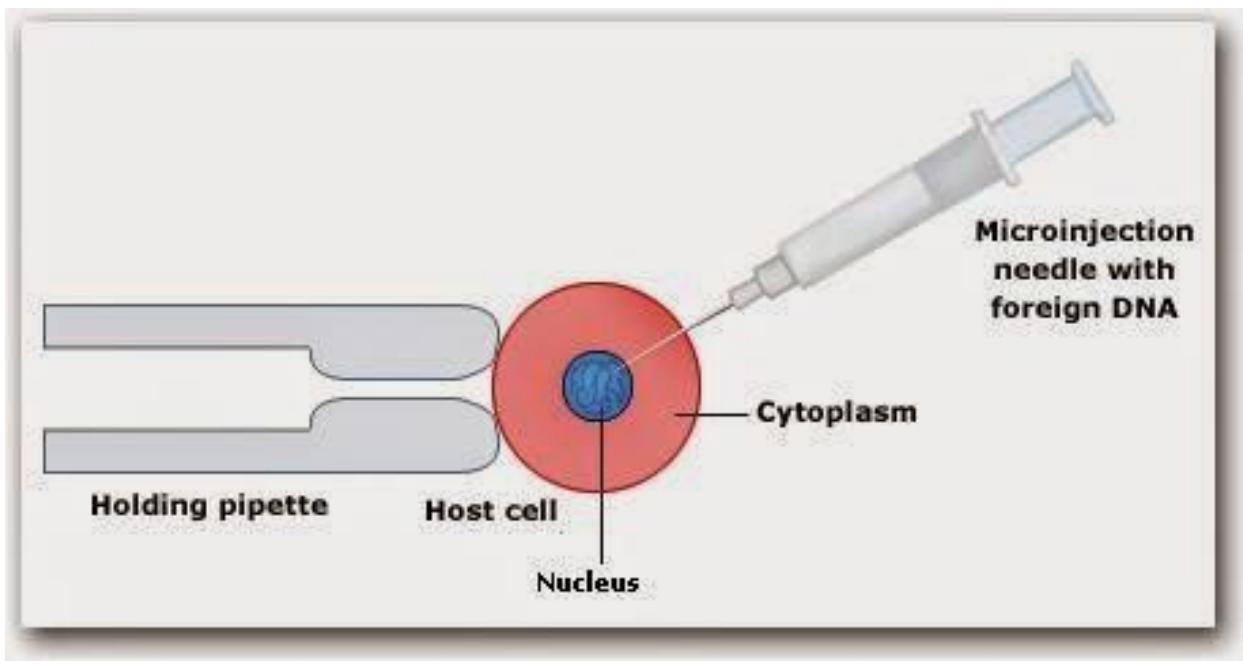
Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adhere to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient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

**Silicon carbide method**

In this method, fibres of organic material like silicon carbide are used for gene transfer. These fibres, when mixed with plasmid DNA and plant tissue or cells, help in penetration of the foreign DNA into the plant tissue.

**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 micrometerdiameter) 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<sub>2</sub> (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<sub>2</sub> breaks the cell wall at certain regions and binds the DNA to the cell surface.

## **Conjugation**

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

## **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).

## **36. Electroporation**

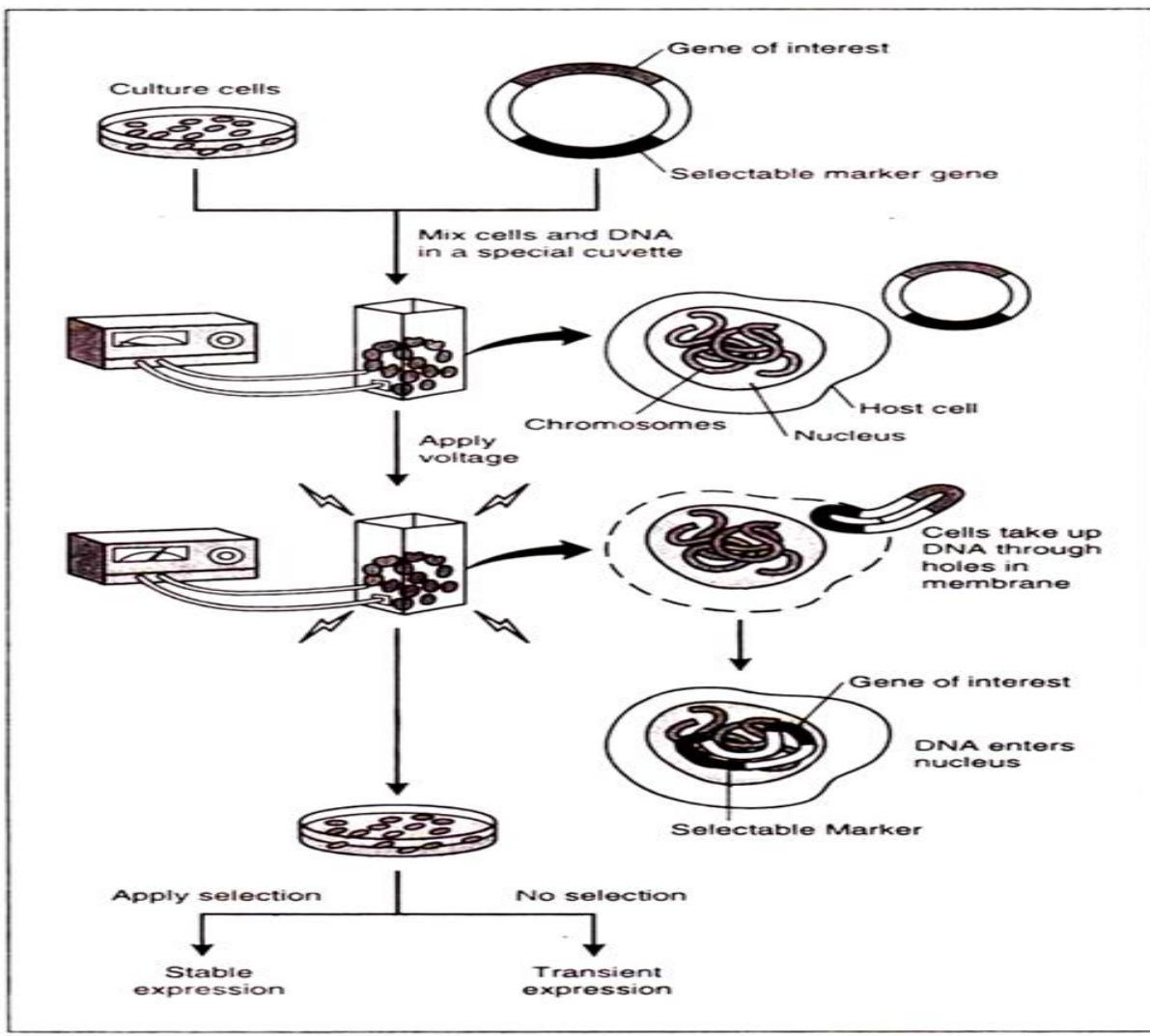
Electroporation or electro-permeabilization is the process of applying electrical field to a living cell for a brief duration of time in order to create microscopic pores in the plasma membrane called electro-pores. This technique is used for transferring the recombinant DNA molecule into wide range of hosts starting from bacteria to plant (plant protoplasts) and animal cells

### **Principle:**

The phospholipid molecules of the plasma membrane are not static. When we apply electric field to them their kinetic energy increases resulting in the increase in the membrane permeability at certain points. This is exactly where we see the formation of electro-pores. The recombinant DNA can pass through these transient pores before they close.

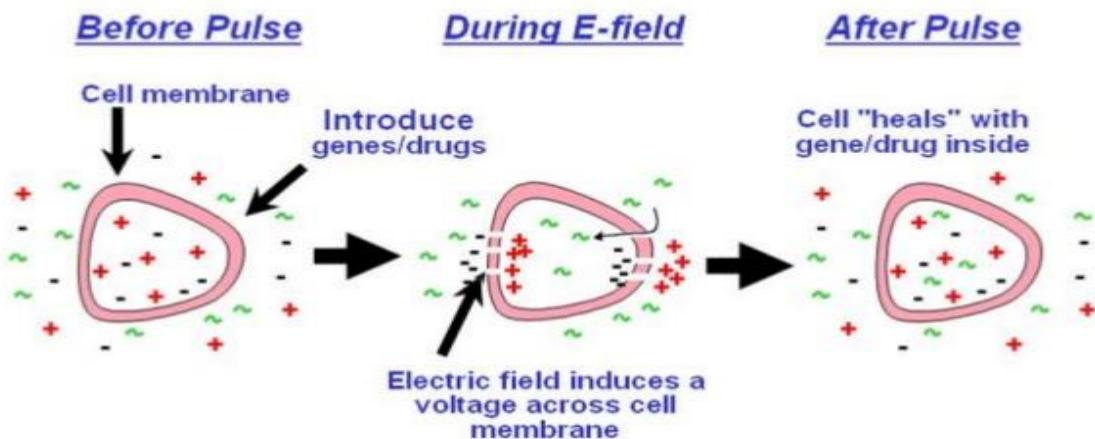
### **Procedure**

In this process cells are mixed with the recombinant DNA and the mixture is placed in a small chamber with electrodes connected to a specialized power supply. Then a brief electric impulse is discharged across the electrodes, which makes pores (holes) in the plasma membrane. These pores remain for some time and are again resealed themselves. Recombinant DNA enters the cell which are removed and plated in fresh selective medium. The process of selection is then applied to isolate cells carrying recombinant DNA.



**Fig. 5.6:** Gene transfer by electroporation

# Electroporation Cell Process



## Advantages of electroporation:

1. This technique is simple, convenient and rapid, besides being cost-effective.
2. The transformed cells are at the same physiological state after electroporation.
3. Efficiency of transformation can be improved by optimising the electrical field strength, and addition of spermidine.

## Limitations of electroporation:

1. Under normal conditions, the amount of DNA delivered into plant cells is very low.
2. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.
3. Regeneration of plants is not very easy, particularly when protoplasts are used.

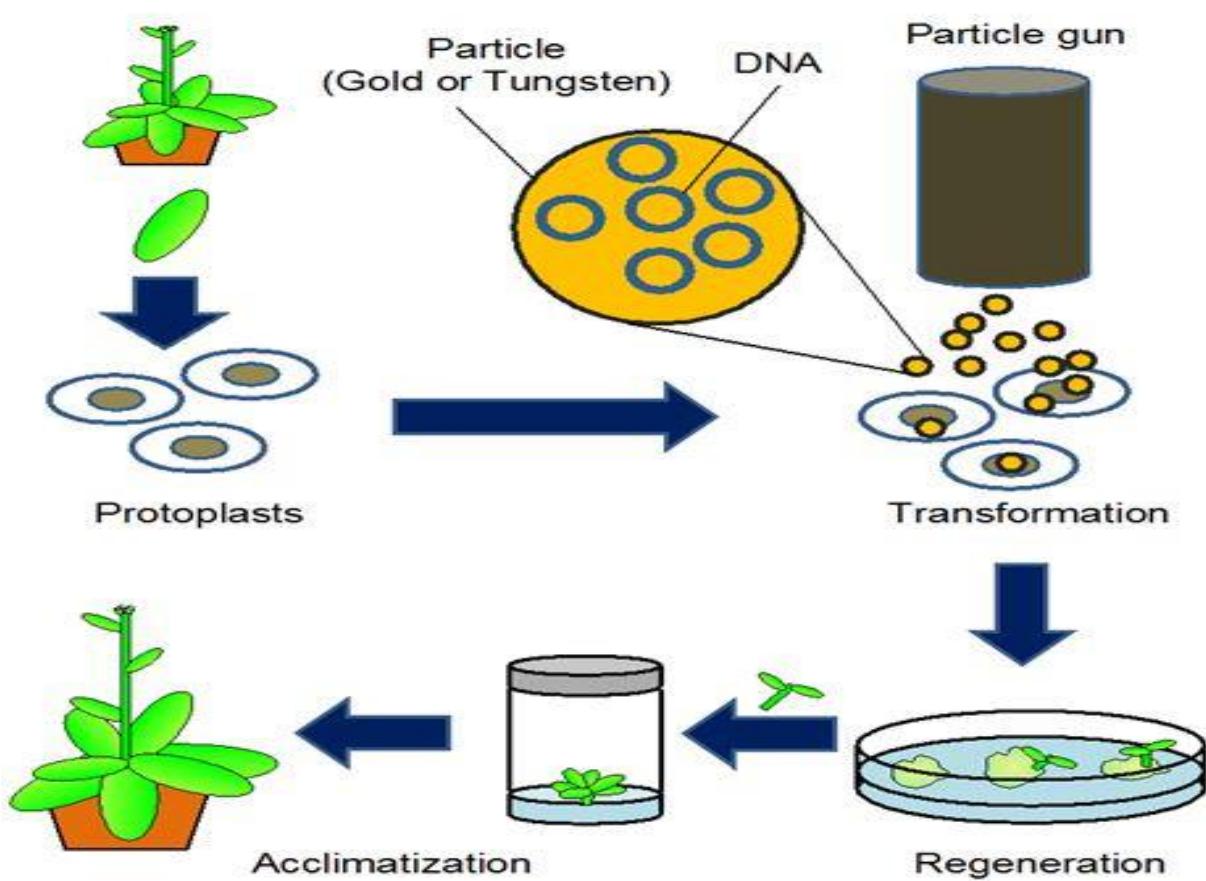
## 37. Particle Bombardment (Biolistics)

Biolistics is a method where cells are physically impregnated with nucleic acids or other biological molecules. A ballistic particle delivery system is a device for plant transformation where cells are bombarded with heavy metal particles coated with DNA/RNA. This technique was invented by John Stanford in 1984 for introduction of DNA into cells by physical means to avoid the host-range restrictions of *Agrobacterium*. *Agrobacterium*-mediated geneti

transformation system works well for dicotyledonous plants but has low efficiency for monocots. Biolistic particle delivery system provides an effective and versatile way to transform almost all type of cells. It has been proven to be a successful alternative for creating transgenic organisms in prokaryotes, mammalian and plant species.



In this process, construct having gene of interest is coated on the surface of tiny particles of gold or tungsten (0.6 – 1 mm in size). Prior to coating, DNA is precipitated with calcium chloride, spermidine and polyethylene glycol. These coated microparticles are loaded onto the macrocarrier and accelerated to high speed by using pressurized helium gas. Plant cell suspensions, callus cultures, or tissues could be used as the target of these microprojectiles. As the microprojectiles penetrate the plant cell walls and membranes to enter the cells, coated DNA is released from its surface and incorporated into the plant's genome. In biolistics, use of binary vectors with T-DNA border sequences is not required. This method is especially important for monocots, for which efficiency of other transformation methods is not satisfactory. A wide range of tissues such as apical and floral meristems, embryos, seedlings, leaves, cultured cells and floral tissues could be used as target in this method.



A number of parameters should be carefully considered before using particle bombardment.

These can be classified under three categories

### **Physical parameters**

Nature, chemical and physical properties of the metal particles utilized to carry the foreign DNA. The nature and preparation of DNA, binding of DNA on the particles and target tissues.

### **Environmental parameters**

Variables such as temperature, photoperiod and humidity of donor plants, explants, and bombarded tissues affect physiology of tissues and influence receptiveness of the target tissue.

### **Biological parameters**

Choice and nature of explants, pre- and post bombardment culture conditions, osmotic pre- and post-treatment of explants.

### **Factors affecting bombardment**

Several attempts are made to study the various factors, and optimize the system of particle bombardment for its most efficient use.

Some of the important parameters are described.

#### **Nature of micro particles:**

Inert metals such as tungsten, gold and platinum are used as micro particles to carry DNA.

These particles with relatively higher mass will have a better chance to move fast when bombarded and penetrate the tissues.

#### **Nature of tissues/cells:**

The target cells that are capable of undergoing division are suitable for transformation.

Some more details on the choice of plant material used in bombardment are already given.

#### **Amount of DNA**

The transformation may be low when too little DNA is used. On the other hand, too much DNA may result in high copy number and rearrangement of transgenes. Therefore, the quantity of DNA used should be balanced. Recently, some workers have started using the chemical aminosiloxane to coat the micro particles with low quantities of DNA adequate enough to achieve high efficiency of transformation.

#### **Advantages of particle bombardment over Agrobacterium-mediated DNA transfer**

This system is species independent and can be used successfully for a wide range of organisms. Many species which are recalcitrant to other direct transfer methods or are not ready to Agrobacterium-mediated transformation have been transformed by this technique. Introduced DNA does not need sequences necessary for T-DNA replication and transfer as complex interaction between bacterium and plant tissue does not take place. Transformation of organelle DNA (mitochondria and chloroplasts) has also been achieved by this method. Multiple genes can be introduced in a single plant. Particles can be coated with DNA/RNA/siRNA/large fragments of nucleic acids.

#### **Limitations of particle bombardment method:**

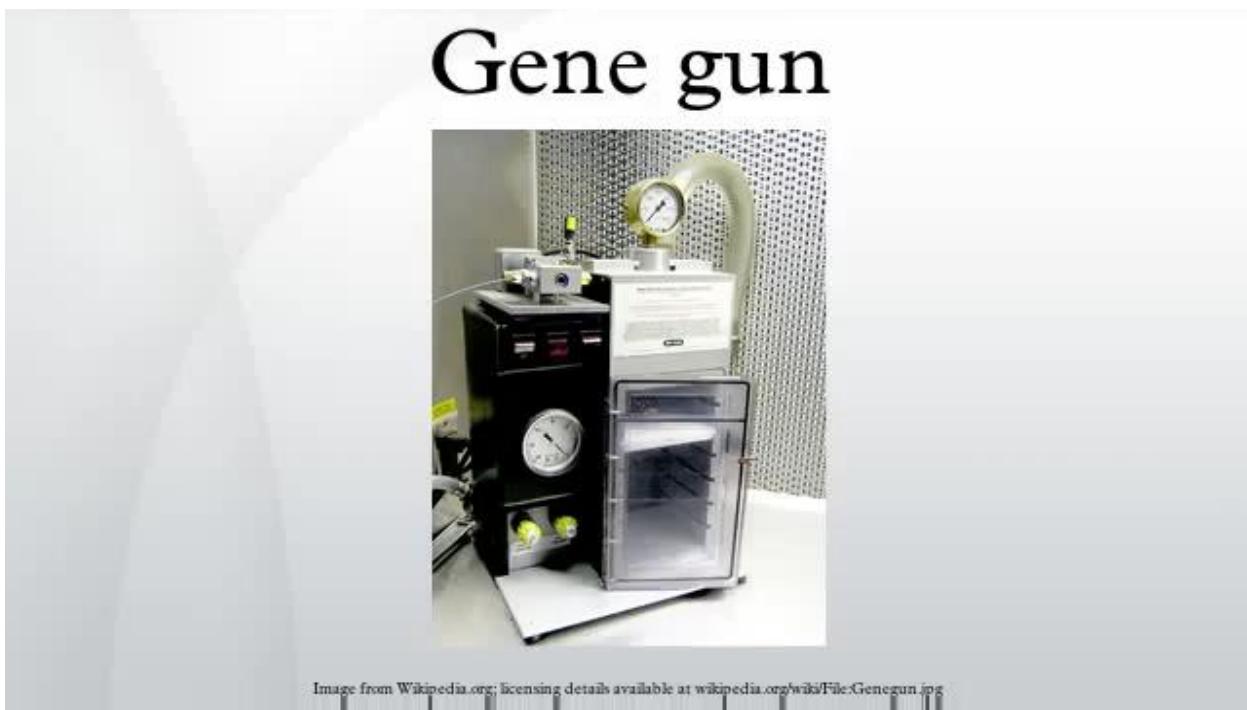
- Limited regeneration capacity of tissue being bombarded
- Efficiency of stable integration of DNA.
- Insertion of multiple copies of the gene
- Integration of rearranged and/or truncated DNA sequences
- Damage to the cellular tissue.
- Specialized and expensive equipment's are required

## 38. Particle Bombardment (Biolistics) 2

### Gene Delivery System:

This system has many names and also Known as Particle Bombardment, Biolistics, Microprojectile bombardment, Particle acceleration, Particle inflow gun, Gene gun. A gene gun or a ballistic particle delivery system, originally designed for plant transformation, is a device for delivering exogenous DNA (transgenes) to cells. Using a gene gun directly shoots a piece of DNA into the recipient plant tissue.

Tungsten or gold beads are coated in the gene of interest and fired through a stopping screen, accelerated by Helium, into the plant tissue. The particles pass through the plant cells, leaving the DNA inside.



### Advantage:

This method can be use to transform all plant species.

No binary vector is required.

Transformation protocol is relatively simple.

### Disadvantage:

Difficulty in obtaining single copy transgenic events.

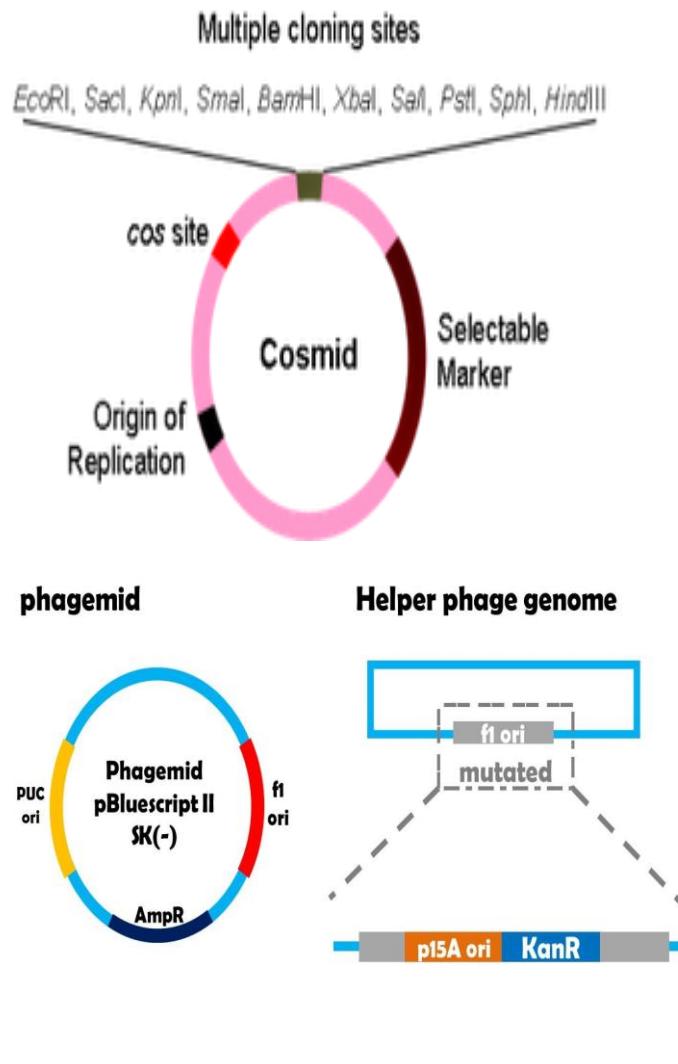
High cost of the equipment and microcarriers.

Intracellular target is random (cytoplasm, nucleus, vacuole, plastid, etc.).

Transfer DNA is not protected.

## 39. Microinjection

The process of using a fine glass micropipette to manually inject transgene at microscopic or borderline macroscopic level is known as microinjection. The transgene, in the form of plasmids, cosmids, phage, YACs, or PCR products, can be circular or linear and need not be physically linked for injection



Microinjection involves direct mechanical introduction of DNA into the nucleus or cytoplasm using a glass micro capillary injection pipette. The protoplasts are immobilized in low melting

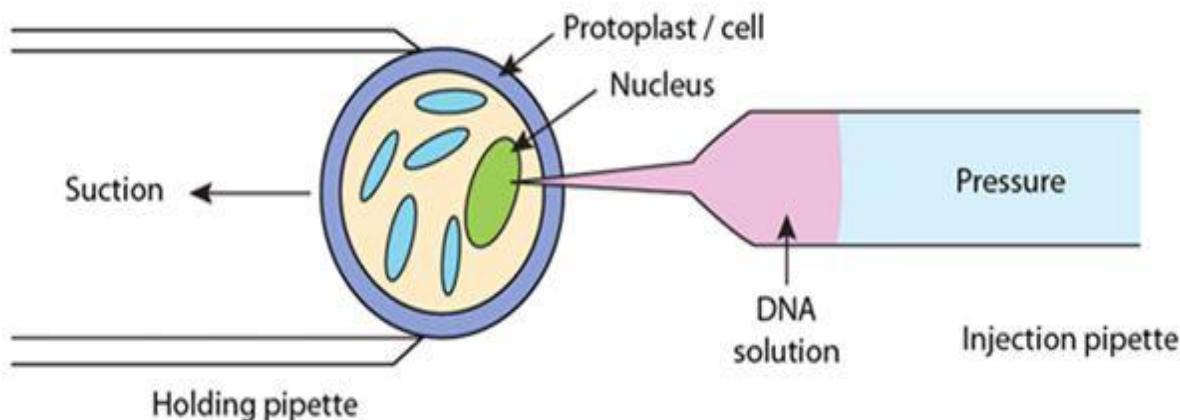
agar, while working under a microscope, using a holding pipette and suction force. DNA is then directly injected into the cytoplasm or the nucleus.

The injected cells are then cultured *invitro* and regenerated into plants. Successful examples of this process has been shown in rapeseed, tobacco and various other plants. Stable transformants can be achieved through this method but it requires technical expertise and is a time consuming process.

Also, microinjection has achieved only limited success in plant transformation due to the thick cell walls of plants and a lack of availability of a single-cell-to-plant regeneration system in most plant species.

In this technique a traditional compound microscope (around 200X magnification) or an inverted microscope (around 200x magnification) or a dissecting stereomicroscope (around 40-50x) is used.

Under the microscope target cell is positioned and cell membrane and nuclear envelope are penetrated with the help of two micromanipulators. One micromanipulator holds the pipette and another holds the micro capillary needle.



There are two types of microinjection systems; constant flow system and pulsed flow system

In the **constant flow** system the amount of sample injected is determined by the duration for which needle remains in the cell. The constant flow system is relatively simple and inexpensive but outdated.

The **pulsed flow** system has greater control over the volume of substance delivered, needle placement and movement and has better precision. This technique results in less damage to the receiving cell, however, the components of this system are quite expensive.

## 40. Chemical Gene Transfer Methods

There are two types of chemical methods used for plant transformation

1. Polyethylene glycol (PEG)-mediated transfer
2. DEAE Dextran-Mediated transfer

### **Polyethylene glycol (PEG)-mediated transfer**

Polyethylene glycol (PEG), in the presence of divalent cations (using  $\text{Ca}^{2+}$ ), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA.

In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome.

The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

#### **Advantages of PEG-mediated transformation:**

- A large number of protoplasts can be simultaneously transformed.
- This technique can be successfully used for a wide range of plant species.

#### **Limitations of PEG-mediated transformation:**

- The DNA is susceptible for degradation and rearrangement.
- Random integration of foreign DNA into genome may result in undesirable traits.
- Regeneration of plants from transformed protoplasts is a difficult task.

### **(DEAE) Dextran Mediated transfer**

This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer. Diethyl aminoethyl dextran (DEAE-dextran) is a soluble poly cationic carbohydrate that promotes interactions between DNA and endo cytotic machinery of the cell.

In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.

Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line

### **Advantages**

This method is Simple and inexpensive, more sensitive and it can be applied to a wide range of cell types and Can be used for transient transfection.

### **Disadvantages**

DEAE is a toxic to cells at high concentrations. Transfection efficiency varies with cell type. It can only be used for transient transfection but not for stable transfection. Typically produces less than 10% delivery in primary cells.

## **41. Plastid transformation**

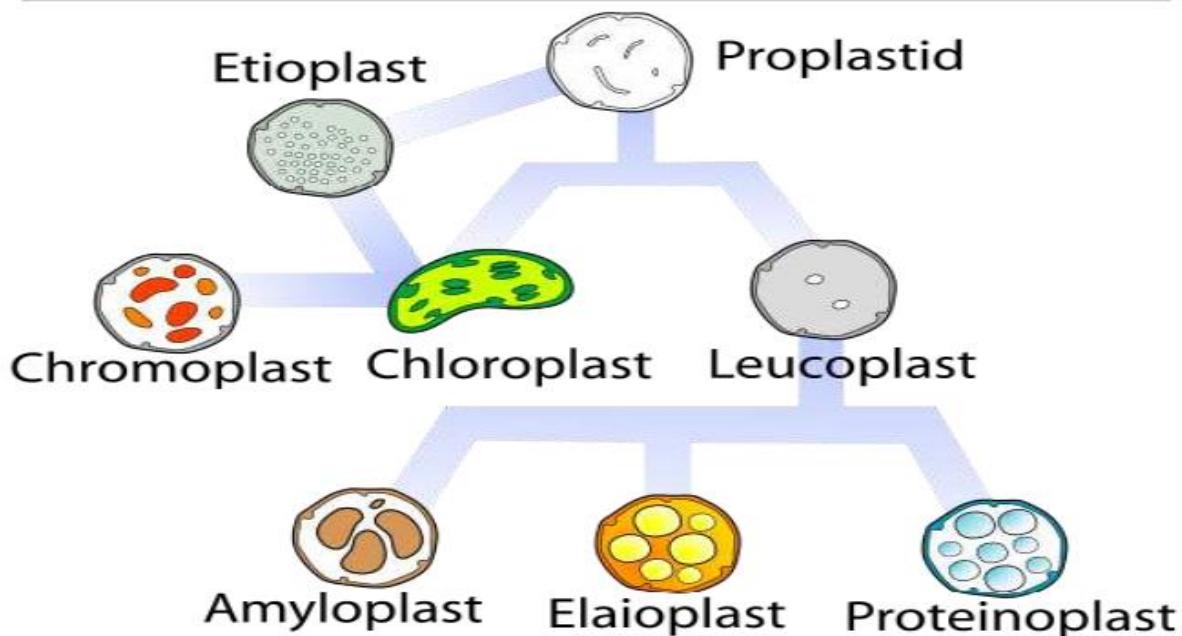
### **Plastid**

Plastids are the major organelle of plant and algal cells. These are the site of manufacture and storage of important chemical compounds. It has circular, dsDNA copies and it replicates autonomously of the cell. Plastids are thought to have been originated from endosymbiotic bacteria and plastid genes show maternal inheritance.

### **Derived from proplastids in meristem**

## **Plastids**

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### **Plastids Have diverse functions**

- Chloroplasts – green plastids – for photosynthesis
- Chromoplasts – coloured plastids – for pigment synthesis and storage
- Gerontoplasts – control dismantling of photosynthetic apparatus during senescence
- Leucoplasts – colourless plastids – monoterpene synthesis
- Leucoplasts include amyloplasts (starch), elaioplasts (fats), proteinoplasts (proteins) and tannosomes (tannins)

### **Comparison of the nuclear and plastid genomes of angiosperm**

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	<b>Nuclear genome</b>	<b>Plastid genome</b>
Chromosomes	Two copies of each of many chromosomes; the number of chromosomes per diploid cell is species-specific	~60 copies of a single circular chromosome per plastid ~50–60 chloroplasts per cell
Genes per chromosome	Could be thousands	~120–150
Arrangement and operons transcription of genes	Each gene is separate	Many genes are in (individually transcribed) (transcribed together)

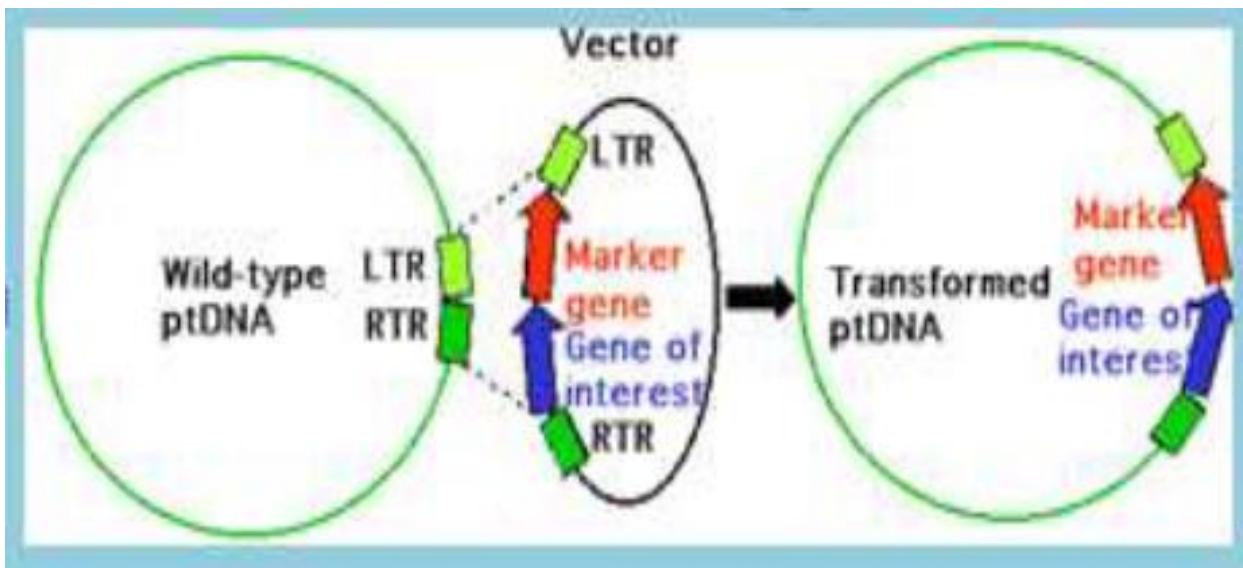
### **Why plastid is used for transformation?**

Due to the High protein expression levels and absence of epigenetic effects. Plastids have uniparental inheritance is commercially favored and easy transgene stacking in operons. Since plastids are maternally inherited, they aren't transmitted by pollen these are biologically safe.

Difficulty in delivering foreign DNA through double membrane of the plastid. The enormous copy number (polyploidy) of the plastid genome. The desired genetic modification must be in each copy of plastid genome in each cell. Failure to achieve homoplasmy results in rapid somatic segregation and genetic instability. Repeated rounds of selection and regeneration are required.

## **Chloroplast transformation requirements**

For chloroplast transformation chloroplast specific expression vector is needed and a method for DNA delivery through a double membrane of the chloroplast. An efficient selection for the transplastome is required.



### DNA delivery into plastids

Successful methods include biolistic and polyethylene glycol-mediated transfer. Biolistic is preferred as it is less time-consuming and demanding. Integration of foreign DNA into plastid genome occurs via homologous recombination. Homologous recombination operates in plastids at a high efficiency.



Transformation of the chloroplast genome by bombarding tobacco leaves with micro projectiles coated with DNA. Following bombardment, leaf discs are placed onto antibiotic containing medium (panel A). Transgenic plants are regenerated from the transformed tissue that is able to develop green chloroplasts (panel B).

## 42. Methods of chloroplast transformation

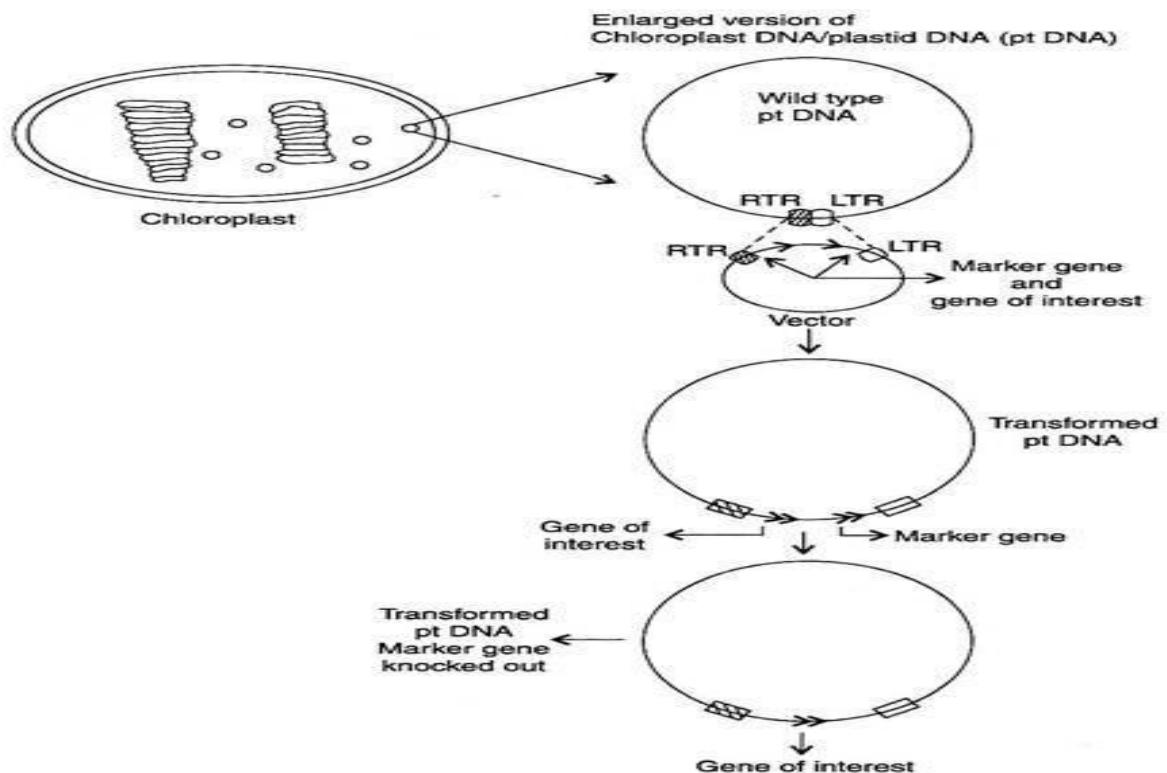
The following points highlight the top five methods of chloroplast transformation in higher plants.

The methods are:

1. Vectors for Plastid Transformation
2. Engineering for High-Level Protein Production
3. Biolistic Transfer
4. PEG Mediated Transformation
5. Galistan Expansion Femtosyringe Method.

### Vectors for Plastid Transformation:

Vectors used for plastid transformation utilize left (LTR) and right (RTR) targeting regions to direct inserting of transgene into the plastid region. Some of the commonly used plasmid transformation vectors are plasmid repeat vector (pRV) and vector pRB94 and pRB95. Expression vector for chloroplast transformation contains two-open reading frame under the control of chloroplast-specific promoter and termination signal.



**Fig. 19.1 Transformation of plastid DNA by recombination events**

Presence of homologous sequence facilitate two recombination events which consequently responsible for the insertion of marker gene and genome of interest into the LRT and RTR regions of the plastid. Some of the well-known plastid transformation vectors are plasmid repeat vector, and other is pRB95. Some of the expression system facilitated the expression of ribosome binding site region inserted at intergenic regions allowing production of target protein from polycistronic mRNA transcript

### **Engineering for High-Level Protein Production:**

Strategies for production of high amount of recombinant proteins have been adopted like utilization of strong promoter, and stable mRNA transcript determined by 5' untranslated (UTR) and 3' untranslated (UTR) of the transgene. Considering these facts several plastid expression vectors are designed to contain 5' regulatory region PL cassette and 3' regulatory region (T cassette), strong sigma 70-type PEP promoter of the rRNA operon promoter (prnn).

The 3' UTR regulatory sequence of mRNA include RNA stem loop structure, which acts as a inefficient transcription terminator. Most 3' UTR T cassettes are derived from PbSA, rbcL and rps 16 genes. The 5' UTR (PL cassette) region play important role in translation efficiency. In addition, careful optimization of transgene codons, despite its prokaryotic nature of expression system, resulted in high protein production.

**Biostatic Transfer:** Biostatic is one of the efficient approaches for plastid transformation.

Effective penetration and high transfer frequency are some of the plus points of biostatic method. There are number of bacteria and viruses are known to infect chloroplast. It's envelop is made up of double membrane and actually considered that chloroplast transformation was considered to be virtually impossible.

However, invention of biostatic gene gun technology paved the pathway of direct delivery of target gene into the living cell. It is fortunate that DNA is deposited in a chloroplast and successfully integrated into the chloroplast genome.

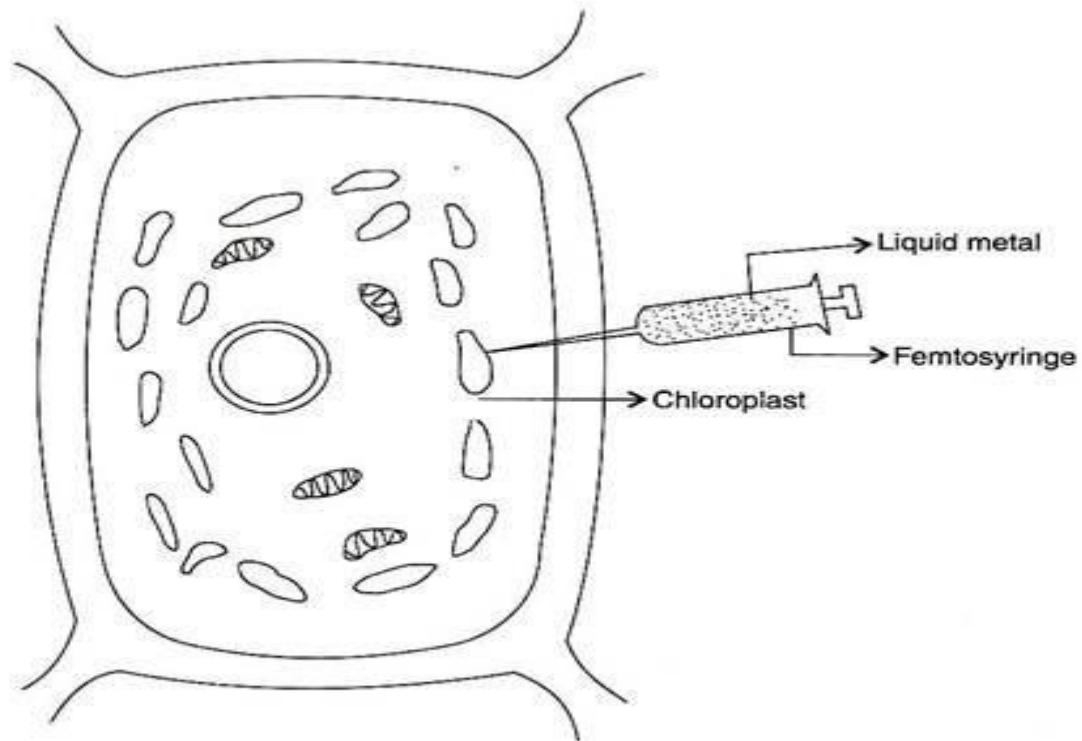
### **PEG Mediated Transformation:**

Polyethylene glycol is widely used in transformation work. Despite its efficiency, PEG mediated transformation is far behind than the biostatic approach. Foreign DNA is taken by protoplast in presence of PEG and transported by unknown process from cytoplasm into the chloroplast and finally integrated into the genome.

### **Galinstan Expansion Femtosyringe Method:**

The existing microinjection method in which recipient cells damaged by the release of cellular contents into the needle after injection have raised fresh look into the designing of novel approach for chloroplast transformation of wide range of species. A novel galinstan

femtosyringe method designed for chloroplast transformation involves microinjection of foreign DNA into chloroplast



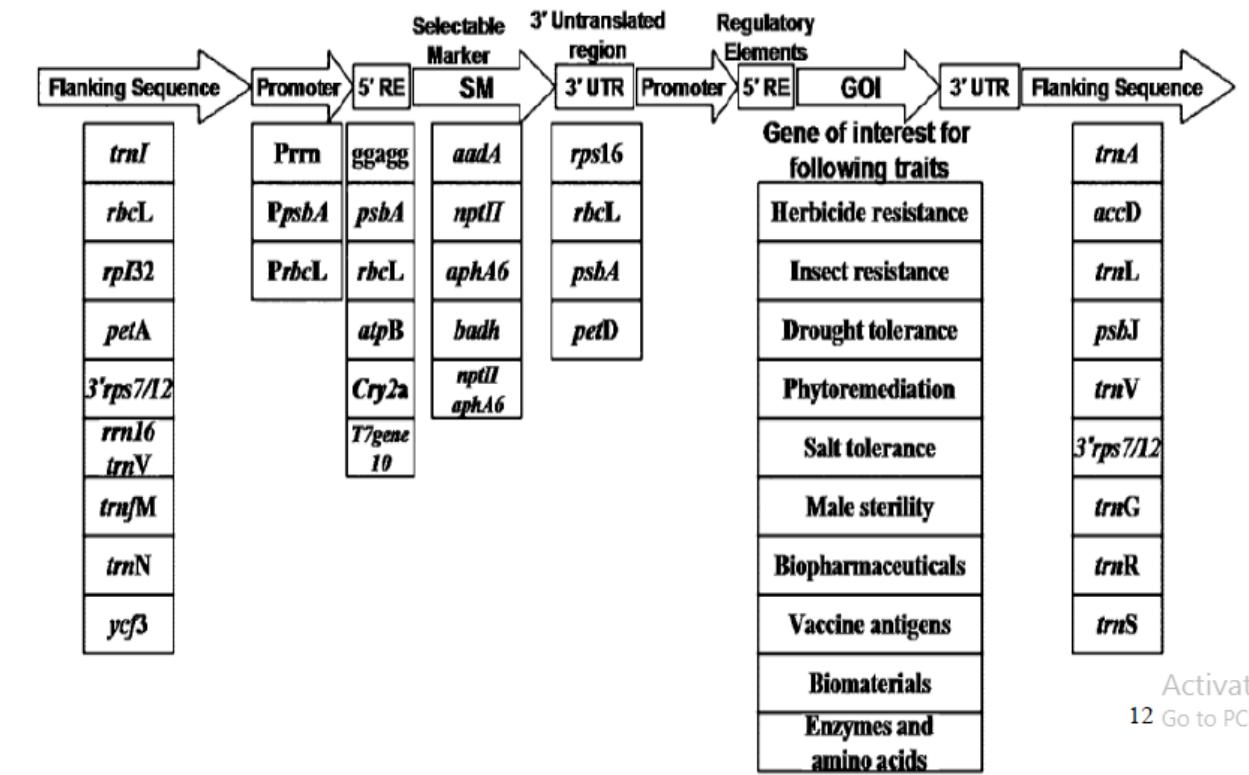
**Fig. 19.3** Galistan femtosyringe method of plastid transformation  
(After Daniel et al 1999)

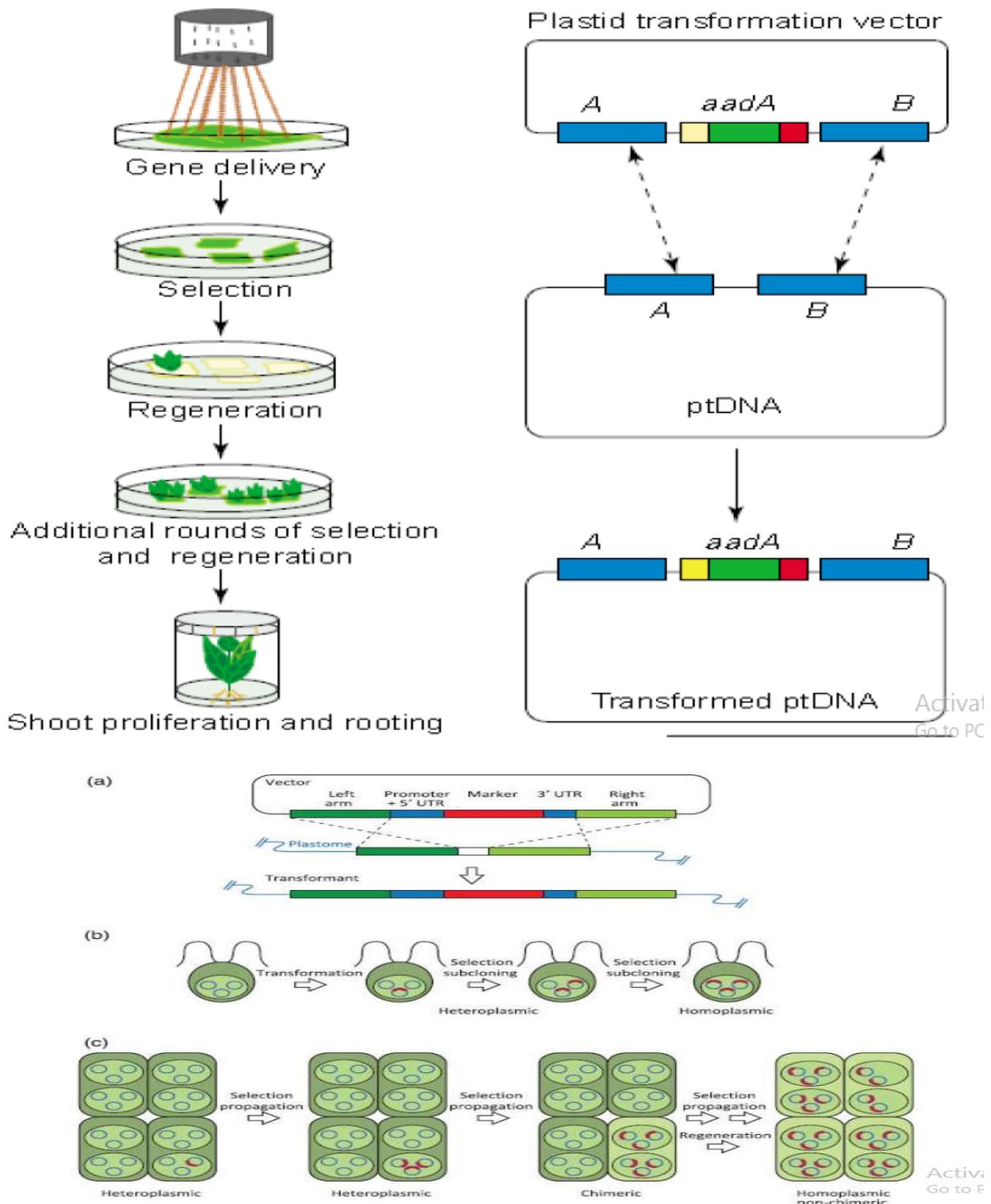
The heat induced expansion of a liquid metal within a glass syringe forces the foreign DNA through a minute capillary top with a diameter of approximately  $0.1 \mu\text{m}$ . The liquid metals employed in the specialized femtosyringe are generally galinstan, an alloy of gallium, indium and tin.

### 43. Molecular biology of Chloroplast Transformation

Stable transformation system depends on integration of the transforming DNA into the plastid genome by homologous recombination. Sequence to be introduced into the plastid genome must flanked on both side by region of homology with the chloroplast genome. Primary transplastomic event results hetroplasmic cells. Hetroplasmy is unstable so it will resolve into homoplasmy.

**For optimal levels of gene expression in leaves**  
***trnI-Prrn-ggagg-aadA-Trps16-PpsbA-GOI-TpsbA-trnA***





### Marker removal

- Recombination between directly repeated sequences excises the intervening DNA sequence and one copy of the direct repeat.

- The breakage and joining of DNA strands involved in recombination can be mediated by the native homologous recombination machinery present in plastids.

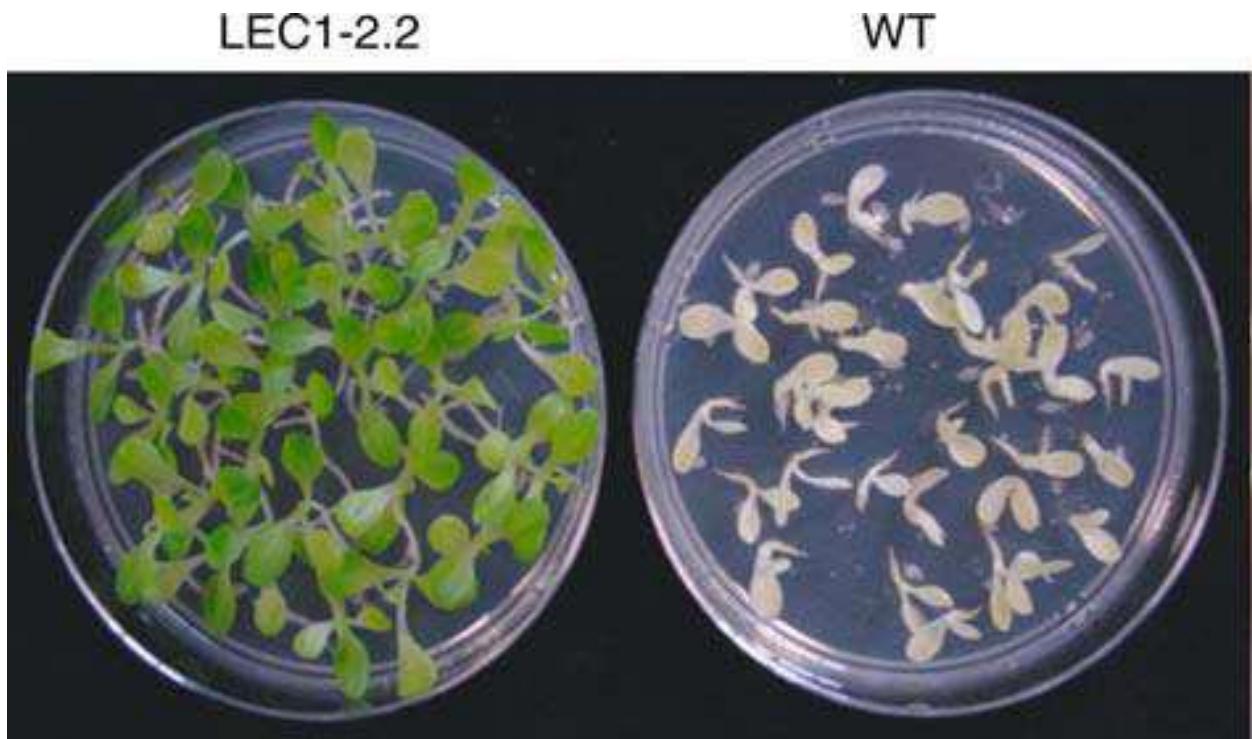
### **Case Study – *Lactuca sativa***

#### 1. Protoplast isolation

- Lettuce seeds were sterilized and sown on MS medium with 2% sucrose
- Shoot tips from leaves obtained were transferred to MS medium with 3% sucrose
- The leaves were cut into pieces and incubated in PG solution, followed by enzyme solution consisting of 1% cellulase and 0.25% macerozyme
- Protoplast suspension was filtered through nylon mesh
- Protoplasts were collected at surface after centrifugation at 70g for 8min

#### **2. Transformation and culture**

- 10µl transforming DNA and 0.6ml PEG solution was added to protoplast suspension and incubated at 25°C for 10min
- Protoplasts were mixed with 1:1 solution of B5 and 2% agarose to a density of 3.6 X 10<sup>4</sup> protoplasts per ml
- The suspension was plated onto Petri dishes and cultured at 25°C in the dark
- Selection was initiated on the 7th day by fresh medium containing spectinomycin dihydrochloride



- 100% of spectinomycin-resistant lettuce cell lines were true plastid transformants
- A limitation was the high frequency of polyploid cell lines

### **Analyses**

- PCR – specific primers were used to assess the presence of *aadA* gene in resistant cell lines
- Immunoblot analysis – using HRP-conjugated secondary antibodies
- Southern and Northern blots were performed to look for target genes and their transcripts

### **Production of human therapeutic proteins**

#### **Why lettuce is favoured over tobacco?**

- Most of the plant is leaf tissue and this tissue contains the greatest number of plastids per cell
- Unlike tobacco, lettuce has no toxic alkaloids that need to be removed - low purification and downstream processing costs
- Lettuce is a relevant human foodstuff that can be consumed without cooking

Year	Milestone	DNA delivery	Approach	Selection	Reference
1988	<i>Chlamydomonas reinhardtii</i> 1 <sup>st</sup> stable plastid transformation	Biolistic	Homologous targeting	Photosynthetic competence	Boynton & Gillham (Science, 240)
1990	<i>Nicotiana tabacum</i> 1 <sup>st</sup> stable plastid transformation	Biolistic	Homologous targeting	Spectinomycin (rrn16)	Svab et al (PNAS, 87)
1993	<i>Nicotiana tabacum</i> 1 <sup>st</sup> high level foreign protein (2.5% GUS)	PEG	Homologous targeting	Spectinomycin Kanamycin	Golds et al (Biotech. 11) O'Neill et al (Plant J. 3)
1995	<i>Nicotiana tabacum</i> New agronomic trait: <i>B. thruegiensis</i> Marker gene elimination: co-transformation	Biolistic	Homologous targeting	Spectinomycin	McBride et al (Biotech. 13) Carrer and Maliga (Biotech. 13)
1998	<i>Arabidopsis thaliana</i> 1 <sup>st</sup> stable plastid transformation	Biolistic	Homologous targeting	Spectinomycin	Sikdar et al (Plant Cell Rep. 18)
1999	<i>Solanum tuberosum</i> (potato) 1 <sup>st</sup> stable plastid transformation <i>Oryza sativa</i> (rice) 1 <sup>st</sup> stable plastid transformation	Biolistic	Homologous targeting	Spectinomycin	Sidorov et al (Plant J. 19) Khan and Maliga (Nat. Biotech. 17)

Activat  
Go to PC

Year	Milestone	DNA delivery	Approach	Selection	Reference
2000	<i>Nicotiana tabacum</i> 1 <sup>st</sup> human protein expression	Biolistic	Homologous targeting	Spectinomycin	Staub et al (Nat. Biotech. 18)
2001	<i>Lycopersicon esculentum</i> (tomato) 1 <sup>st</sup> foreign protein in fruit Marker gene elimination: CRE-lox New agronomic traits: glyphosate tolerance and PPT resistance	Biolistic	Homologous targeting	Spectinomycin	Ruf et al (Nat. Biotech. 19) Corneille et al (Plant J. 19) Ye et al (Plant J. 25) Lutz et al (Plant Physiol. 125)
2002	<i>Porphyridium</i> sp. 1 <sup>st</sup> stable plastid transformation	Biolistic	Homologous targeting	Spectinomycin	Lapidot et al (Plant Physiol. 129)
2003	<i>Chlamydomonas reinhardtii</i> : Foot-and-mouth disease virus VP1 protein expression <i>Brassicaceae</i> (oil seeds) 1 <sup>st</sup> stable plastid transformation Phytoremediation: Mercury	Biolistic	Homologous targeting	Spectinomycin	Sun et al (Biotechnol Lett. 25) Skarjinskaia et al (Transgenic Res. 12) Ruiz et al (Plant Physiol. 132)
2004	<i>Gossypium hirsutum</i> (cotton) 1 <sup>st</sup> stable plastid transformation <i>Glycin max</i> (soybean) 1 <sup>st</sup> stable plastid transformation <i>Linum usitatissimum</i> L. (flax): PHB polymer expression	Biolistic	Homologous targeting	aph A-6 npt II Spectinomycin	Kumar et al (PMB. 56) Dufourmantel et al (PMB. 55) Wrobel et al (J. Biotech. 107)

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## 44. Pesticide

Pesticides are substances that are meant to control pests, including weeds. Any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals, causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs. Substances that may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant, or agent for thinning fruit or preventing the premature fall of fruit. Also used as substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport

### Classification

Pesticides can be classified by target organism.

Type of pesticide	Target pest group
<a href="#">Algicides or algaecides</a>	<a href="#">Algae</a>
<a href="#">Avicides</a>	<a href="#">Birds</a>
<a href="#">Bactericides</a>	<a href="#">Bacteria</a>
<a href="#">Fungicides</a>	<a href="#">Fungi</a> and <a href="#">oomycetes</a>
<a href="#">Herbicides</a>	<a href="#">Plant</a>
<a href="#">Insecticides</a>	<a href="#">Insects</a>
<a href="#">Miticides or acaricides</a>	<a href="#">Mites</a>
<a href="#">Molluscicides</a>	<a href="#">Snails</a>
<a href="#">Nematicides</a>	<a href="#">Nematodes</a>
<a href="#">Rodenticides</a>	<a href="#">Rodents</a>
<a href="#">Virucides</a>	<a href="#">Viruses</a>

- Algaecides are used for killing and/or slowing the growth of algae.
  - Antimicrobials control germs and microbes such as bacteria and viruses.
  - Biopesticides are made of living things, come from living things, or they are found in nature.
  - Desiccants are used to dry up living plant tissues.
  - Defoliants cause plants to drop their leaves.
- 
- Disinfectants control germs and microbes such as bacteria and viruses.
  - Fungicides are used to control fungal problems like molds, mildew, and rust.
  - Herbicides kill or inhibit the growth of unwanted plants, aka weeds.
  - Illegal and Counterfeit Pesticides are imported or sold illegally.

- Insecticides are used to control insects.
- Insect Growth Regulators disrupt the growth and reproduction of insects.
- Miticides control mites that feed on plants and animals. Mites are not insects, exactly.
- Molluscicides are designed to control slugs, snails and other molluscs.
- Mothballs are insecticides used to kill fabric pests by fumigation in sealed containers.
- Natural and Biological Pesticides control pests using things found in nature, or man-made versions of things found in nature.
- Ovicides are used to control eggs of insects and mites.
- Pheromones are biologically active chemicals used to attract insects or disrupt their mating behavior. The ratio of chemicals in the mixture is often species-specific.
- Repellents are designed to repel unwanted pests, often by taste or smell.
- Rodenticides are used to kill rodents like mice, rats, and gophers.

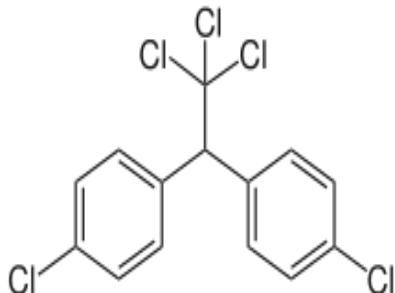
## **45. Uses of pesticides**

Pesticides are used to control organisms that are considered to be harmful, for example, they are used to kill mosquitoes that can transmit potentially deadly diseases like West Nile virus, yellow fever, and malaria. They can also kill bees, wasps or ants that can cause allergic reactions. Insecticides can protect animals from illnesses that can be caused by parasites such as fleas. Pesticides can prevent sickness in humans that could be caused by moldy food or diseased produce. Herbicides can be used to clear roadside weeds, trees, and brush. They can also kill invasive weeds that may cause environmental damage. Herbicides are commonly applied in ponds and lakes to control algae and plants such as water grasses that can interfere with activities like swimming and fishing and cause the water to look or smell unpleasant. Uncontrolled pests such as termites and mold can damage structures such as houses. Pesticides are used in grocery stores and food storage facilities to manage rodents and insects that infest food such as grain. Each use of a pesticide carries some associated risk. Proper pesticide use decreases these associated risks to a level deemed acceptable by pesticide regulatory agencies. DDT, sprayed on the walls of houses, is an organochlorine that has been used to fight malaria since the 1950s. Recent policy statements by the World Health Organization have given stronger support to this approach.

However, DDT and other organochlorine pesticides have been banned in most countries worldwide because of their persistence in the environment and human toxicity. DDT use is not always effective, as resistance to DDT was identified in Africa as early as 1955, and by 1972 nineteen species of mosquito worldwide were resistant to DDT.

Dichlorodiphenyltrichloroethane, commonly known as DDT, is a colorless, tasteless, and almost odorless crystalline chemical compound, an organochlorine, originally developed as an insecticide, and ultimately becoming infamous for its environmental impacts. First synthesized in 1874, DDT's insecticidal action was discovered by the Swiss chemist Paul Hermann Müller in

1939. DDT was used in the second half of World War II to control malaria and typhus among civilians and troops.



### Benefits

Pesticides can save farmers' money by preventing crop losses to insects and other pests; in the U.S., farmers get an estimated fourfold return on money they spend on pesticides. One study found that not using pesticides reduced crop yields by about 10%.

Another study, conducted in 1999, found that a ban on pesticides in the United States may result in a rise of food prices, loss of jobs, and an increase in world hunger. There are two levels of benefits for pesticide use, primary and secondary. Primary benefits are direct gains from the use of pesticides and secondary benefits are effects that are more long-term.

### Primary benefits

- Controlling pests and plant disease vectors
- Improved crop/livestock yields
- Improved crop/livestock quality
- Invasive species controlled

### Controlling human/livestock disease vectors and nuisance organisms

- Human lives saved and disease reduced. Diseases controlled include malaria, with millions of lives having been saved or enhanced with the use of DDT alone.
- Animal lives saved and disease reduced
- Controlling organisms that harm other human activities and structures
- Drivers view unobstructed
- Tree/brush/leaf hazards prevented
- Wooden structures protected

## 46. Effects of pesticides

### Health effects

Pesticides may cause acute and delayed health effects in people who are exposed. Pesticide exposure can cause a variety of adverse health effects, ranging from simple irritation of the skin and eyes to more severe effects such as affecting the nervous system, mimicking hormones causing reproductive problems, and also causing cancer.

A 2007 systematic review found that "most studies on non-Hodgkin lymphoma and leukemia showed positive associations with pesticide exposure" and thus concluded that cosmetic use of pesticides should be decreased. Non-Hodgkin lymphoma is a cancer that starts in white blood cells called lymphocytes, which are part of the body's immune system. Leukemia involves the production of abnormal white blood cells -- the cells responsible for fighting infection. There is substantial evidence of associations between organophosphate insecticide exposures and neurobehavioral alterations. Limited evidence also exists for other negative outcomes from pesticide exposure including neurological, birth defects, and fetal death.

### **Environmental effects**

Widespread use of pesticides in agriculture has experts worried due to their long-term environmental damage. Some pesticides can stick around for years, posing a very real threat to the ecological system and hence human health. Excessive and careless use of pesticides can contaminate water sources and soil, make fruits and vegetables less nutritious, and reduce biodiversity. Some pesticides have also been linked to the dramatic reduction in the number of bees across the world, posing a huge threat to agriculture and food security, given that bees pollinate more than 70% of all crops.

### **Economics**

In one study, the human health and environmental costs due to pesticides in the United States was estimated to be \$9.6 billion: offset by about \$40 billion in increased agricultural production.

Additional costs include the registration process and the cost of purchasing pesticides: which are typically borne by agrichemical companies and farmers respectively. The registration process can take several years to complete (there are 70 different types of field test) and can cost \$50–70 million for a single pesticide.

At the beginning of the 21st century, the United States spent approximately \$10 billion on pesticides annually.

## **47. Effects of pesticides on human health**

- Experts broadly classify the effects of pesticides as topical or systemic.
- Topical reactions are usually limited to areas of the body that have come in direct contact with a pesticide.
- Inflammation of the skin (dermatitis) such as a rash or blisters is usually the most common topical symptom.
- Other topical reactions may include sneezing, wheezing, and coughing, usually triggered by petroleum distillates that many pesticides contain.
- Severe pesticide poisoning can cause seizures, change in heart rate, and sometimes even coma and death

### **Short-Term Effects of Pesticides**

- Short-term pesticide poisoning or acute toxicity from pesticides is usually the result of a single and brief exposure to a pesticide.
- This kind of poisoning can happen due to exposure via the skin, inhalation, through the eyes, or orally.
- Symptoms of acute toxicity can become apparent instantly or take as long as 48 hours
- Short-term effects of pesticides can manifest as:
- Nausea and vomiting
- Diarrhea
- Loss of consciousness
- Seizures
- Coughing and sore throat
- Extreme weakness

### **Long-Term Effects Of Pesticides**

- While continual, low-dose exposure to pesticides don't usually show immediate effects, they cause serious harm to human health in the long term.
- Repeated exposure to pesticides, even in small doses, has been linked to a number of diseases such as cancer, Parkinson's, Alzheimer's, sterility, and developmental disorders.
- Chronic exposure to pesticides can also lead to genetic changes and serious nerve disorders.
- Some studies have even linked pesticides to asthma, ADHD, depression, and anxiety

- Some pesticides contain chemicals that may be endocrine disruptors. These types of pesticides can be especially damaging because they interfere with our hormones and hormonal balance.
- Over a period of time, even low concentrations of these chemicals can cause obesity, diabetes, thyroid tumors, decreased fertility, uterus abnormalities, and early puberty.
- Lastly, pesticides are also known to cause neurological issues such as loss of memory and coordination, visual impairment, mood instability, and reduced motor skills

### **Effects Of Pesticides On Pregnant Women**

- Exposure to pesticides and pesticide residue can lower fertility in women.
- A Harvard study found that women who ate more than two servings of fruits or vegetables with high pesticide residue each day were 18% less likely to become pregnant and 26% less likely to have a live birth compared to women with lower exposure

### **Effects Of Pesticides On Kids**

Children are especially susceptible to harmful effects of pesticides. They can easily become exposed to pesticides (via inhalation or skin contact) in schools, daycare, playgrounds, hospitals, and any other public areas, no matter how careful you are. Kids' bodies are smaller and still growing, they take more breaths per minute, and they also eat and drink more relative to their weight – all factors that make them more likely to absorb pesticides and residue. Their little kidneys and liver also cannot eliminate pesticides from their bloodstream as effectively as an adult's.

## 48. Effects of pesticides on environment

**What the mean of herbicide & pesticide.....**

- Simply can say chemical substances use to control weeds & pests



**What is environment.....**

- Environment** is living things and what is around you. It includes physical, chemical and other natural forces.



Natural Environment

Built Environment

Although each pesticide is meant to kill a certain pest, a very large percentage of pesticides reach a destination other than their target. Pesticides easily contaminate the air, ground and water when they run off from fields, escape storage tanks, are not discarded properly, and especially when they are sprayed aerially.

## **Water**

Pesticides can be found in rain, ground water, streams, rivers, lakes and oceans. There are four major ways that pesticides can reach the water:

- it can drift outside of the area of where it was sprayed,
- it can leach through the soil,
- it can be carried as runoff,
- or it may be spilled accidentally.

## **Soil**

The use of pesticides decreases the general biodiversity in the soil. Soil quality is higher without chemicals and this allows for higher water retention, necessary for plants to grow.

## **Plants**

Nitrogen fixation, which is necessary for the growth of many large plants, is hindered by pesticides that can be found in soil. This can lead to a large decline in crop yields. Application of pesticides to crops that are in bloom can kill honeybees, which act as pollinators. This also decreases crop pollination and reproduction.

## **Animals**

Animals may be poisoned by pesticide residues that remain on food after spraying.

An application of pesticides in an area can eliminate food sources that certain types of animals need, causing the animals to relocate, change their diet, or starve.

Poisoning from pesticides can even make its way up the food chain; for example, birds can be harmed when they eat insects and worms that have consumed pesticides.

## **Birds**

Birds are being harmed by pesticide use. Rachel Carson's book Silent Spring discusses the loss of several bird species due to accumulation of pesticides in their tissues. Types of

fungicides used in farming are only slightly toxic to birds and mammals, but may kill off earthworms, which can in turn reduce populations of the birds and mammals that feed on them

### Aquatic Life

Fish and other aquatic biota may be harmed by pesticide-contaminated water. Application of herbicides to bodies of water can cause plants to die, diminishing the water's oxygen and suffocating the fish. Repeated exposure of some pesticides can cause physiological and behavioural changes in fish that reduce populations, such as abandonment of nests, decreased immunity to disease, and increased failure to avoid predators. Additionally, as some pesticides come in granular form, birds and other wildlife may eat the granules, mistaking them for grains of food. A few granules of a pesticide are enough to kill a small bird. Herbicides may also endanger bird populations by reducing their habitat.



### Negative effects of pesticides

- Are carried on the wind
- Leaves residue on produce
- Remains inside produce and animals through bio accumulation
- Runs off into open water which contaminates public water supply as well as fish and other seafood.
- Pesticides can enter the body through the skin, eyes, mouth, and nose.
- Fetuses can suffer from exposure and develop behavioral problems as well as growth issues
- Babies can develop lower cognitive scores and fewer nerve cells and can have a lower birth rate as well as being born prematurely.

- Toxins from pesticides can stay in the body and build up in the liver
- There are many possible reactions that include fatigue, skin irritations, nausea, vomiting, breathing problems, brain disorders, blood disorders, liver and kidney damage, reproductive damage, cancer, and in some cases, death.

## **49. Signs and symptoms by pesticide poisoning**

### **How do pesticides enter our bodies?**

Pesticides can enter your body during mixing, applying, or clean-up operations. There are generally three ways a chemical or material can enter the body through the skin (dermal) through the lungs (inhalation) by mouth (ingestion).

#### **Dermal (absorption through skin or eyes)**

In most work situations, absorption through the skin is the most common route of pesticide exposure. People can be exposed to a splash or mist when mixing, loading or applying the pesticide. Skin contact can also occur when you touch a piece of equipment, protective clothing, or surface that has pesticide residue on it. Pesticides can also be absorbed through your eyes. In addition, pesticides, can cause injuries to the eye itself.

#### **Inhalation (through the lungs)**

Inhalation may occur when working near powders, airborne droplets (mists) or vapors. The hazard from low-pressure applications is fairly low because most of the droplets are too large to remain in the air. Applying a pesticide with high pressure, ultra-low volume, or fogging equipment can increase the hazard because the droplets are smaller and they can be carried in the air for considerable distances. Pesticides with a high inhalation hazard will be labelled with directions to use a respirator.

#### **Ingestion (by mouth)**

While ingestion (by mouth) is a less common way to be exposed, it can result in the most severe poisonings. There are numerous reports of people accidentally drinking a pesticide that has been put into an unlabeled bottle or beverage cup/container (including soft drink cans or bottles). Workers who handle pesticides may also unintentionally ingest the substance when eating or smoking if they have not washed their hands first.

### **Types of pesticide poisoning:**

There are two types of pesticide poisoning:

#### **Acute poisoning**

This happens when someone has been exposed to a high dose of pesticide. This could occur when the pesticide is being mixed, for example, or if a hose breaks drenching the person or bystanders with liquid pesticide solution. Another example might be accidental ingestion of a pesticide, such as a child swallowing the chemical.

## **Chronic poisoning**

This results from a person being exposed to a small amount of pesticide on many occasions over a long period of time. Chronic poisoning may happen when the operator repeatedly uses pesticide improperly, especially if they do not wear protective clothing and equipment or wears protective clothing which is not clean or is worn out, like wearing cracked or torn gloves.

### **Symptoms of pesticide poisoning**

Symptoms of mild poisoning includes headache, sweating, diarrhea irritation of nose and throat eye irritation, nausea, fatigue, changes of mood, skin irritation, insomnia, loss of appetite, thirst, weakness, restlessness, dizziness, sore joints and nervousness.

### **Symptoms of severe poisoning**

Symptoms of severe poisoning includes vomiting, convulsions, loss of reflexes, unconsciousness, inability to breathe, fever muscle twitching, thirst, constriction of eye pupils (eye pupils become small), increased rate of breathing

## **50. Treatment for Pesticide Poisoning**

When pesticides are released into the air, we breathe them in through our nose and mouth. Once in the lungs, the pesticides quickly enter the blood and spread poison through the whole body. Because some pesticides have no smell, it is often hard to know if they are in the air. The most common forms of air-borne pesticides are fumigants, aerosols, foggers, smoke bombs, pest strips, sprays, and residues from spraying. You can also inhale pesticide dust in a storage area, when it is being used in an enclosed area, such as a greenhouse, or when it is being transported to the fields. Pesticide dust in the air can travel miles to pollute an area far from where it was used. It is easy for pesticide dust to get into houses.

If you think you have breathed in pesticides, get away from the pesticides right away! Do not wait until you feel worse.

### **Treatment**

If you or someone else breathes in pesticides: Get the person away from the area where she breathed in the poison, especially if it is an enclosed area. Get fresh air. Loosen clothing to make breathing easier. Sit with head and shoulders raised.

If the person is unconscious, lay her on her side and watch her to make sure there is nothing blocking her breathing. If the person is not breathing, quickly do mouth-to-mouth breathing. Seek medical help. Take the pesticide label or name of the pesticide with you.



## 51. Treatment for Pesticide Poisoning 2

Like other toxic chemicals, pesticides can poison people in different ways:

They can poison through the skin and eyes, through the mouth (by swallowing) or through the air (by breathing). Each kind of poisoning needs a different kind of treatment.

### **When pesticides get on the skin**

Most pesticide poisonings are from pesticides being absorbed through the skin. This can happen when they spill while being moved, when they splash during mixing, during spraying, or when you touch crops that have just been sprayed. Pesticides can also get on your skin through your clothes, or when you wash clothes with pesticides on them.

Rashes and irritation are the first signs of poisoning through the skin. Because skin problems may be caused by other things, such as a reaction to plants, insect bites, infections, or allergies, it can be hard to know if the problem is caused by pesticides.

Talk to other workers to find out if the crop you are working with causes this kind of reaction. If you work with pesticides and get any unexpected skin rashes, it is safest to treat them as if they are caused by pesticides.

### **Treatment**

If you or someone else gets pesticides on the body:

Quickly remove any clothing the pesticides spilled onto. Wash the pesticides off the skin as soon as possible with soap and cool water. If it got into the eye, rinse the eye with clean water for 15 minutes.

### **If the skin is burned from pesticides:**

Rinse well with cool water. Do not remove anything stuck to the burn. Do not apply lotions, fats, or butter. Do not break blisters. Do not remove loose skin. Cover the area with a sterile dressing, if available.

### **When pesticides are swallowed**

People can swallow pesticides by eating, drinking, or smoking cigarettes in the fields while working with pesticides, or by drinking water polluted with pesticides.

Children can drink or eat pesticides, especially if pesticides are stored in containers also used to hold food, or left in the open or low to the ground.

### **Treatment**

When someone swallow's pesticides:

If the person is unconscious, lay her on her side and make sure she is breathing. If the person is not breathing, quickly do mouth-to-mouth breathing. Mouth-to-mouth breathing can also expose you to the pesticide, so cover your mouth with a pocket mask, a piece of cloth, or thick plastic wrap with a hole cut in the middle, before you start mouth-to-mouth breathing. Find the pesticide package and read the label right away. The label will tell you if you should make the person vomit up the poison or not. If the person can drink, give her lots of clean water.

Seek medical help. If it is available, always take the pesticide label or name with you.

Do not vomit if the label says not to. Never vomit after swallowing a pesticide that contains gasoline, kerosene, xylene, or other petroleum-based liquids. This will make the problem worse. Never make the person vomit or drink if she is unconscious, confused, or shaking badly. If you are sure vomiting is OK, give the person: a glass of very salty water or 2 tablespoons of pounded strong-tasting edible plant (such as celery, basil, or another local herb) followed by 1

or 2 glasses of warm water. Keep the person moving around. This can help her vomit sooner. After vomiting, activated or powdered charcoal can help absorb any poison still in the stomach

## **52. Approaches to pesticide poisoning**

When effectively applied, pesticides can kill or control pests, including weeds, insects, fungi, bacteria, and rodents. Chemical pest control has contributed to dramatic increases in yields for most major fruit and vegetable crops

### **Pesticide poisoning**

A pesticide poisoning occurs when chemicals intended to control a pest affect non-target organisms such as humans, wildlife, or bees

#### **Types of pesticide poisoning.**

Humans may be harmed by pesticides in two ways: they may be poisoned or injured.

Pesticide poisoning is caused by pesticides that harm internal organs or other systems inside the body. Pesticide-related injuries usually are caused by pesticides that are external irritants.

#### **Hazard**

Hazard is the risk of harmful effects from pesticides. Hazard depends on both the toxicity of the pesticide and your exposure.

Hazard = Toxicity x Exposure

#### **Exposure**

When a pesticide contacts a surface or organism, that contact is called a pesticide exposure.

For humans, a pesticide exposure means getting pesticides in or on the body. The toxic effect of a pesticide exposure depends on how much pesticide is involved and how long it remains there.

#### **Types of Exposures**

- Pesticides contact your body in four main ways:
- Oral exposure (when you swallow a pesticide),

- Inhalation exposure (when you breathe in a pesticide),
- Ocular – (through the eyes), or
- Dermal (through the skin)

### **Strategies to exposure/poison**

- Diagnosis
- Prevention
- Treatment

## **53. Herbicides**

A herbicide is a chemical substance used to control or manipulate undesirable vegetation, especially weeds. Herbicides are extensively used in gardening, farming, and landscape turf management. Herbicides tend to have wide-ranging effects on non-target species (other than those the pesticide is meant to control or kill). Herbicides, also commonly known as weedkillers, are chemical substances used to control unwanted plants. Modern herbicides are often synthetic mimics of natural plant hormones which interfere with growth of the target plants. The term organic herbicide has come to mean herbicides intended for organic farming. Some plants also produce their own natural herbicides, such as the genus *Juglans* (walnuts), or the tree of heaven; such action of natural herbicides, and other related chemical interactions, is called allelopathy.

### **Herbicides are classified into two categories**

selective and non-selective.

Selective herbicides kill specific unwanted plants while leaving desirable vegetation relatively unharmed.

Non-selective herbicides (total weed killers) kill all or most plant species.

### **Methods of application**

A herbicide can be applied directly to the plant, applied to the soil, or sprayed onto the foliage. Herbicides are applied before, during, or after crop planting in row-crop farming to maximize crop production by diminishing the development of unwanted plants. Herbicides are also applied in ponds and lakes to control aquatic plants, in forests to prepare logged areas for replanting, and to golf courses, lawns, parks, and other areas to clear out unwanted vegetation.

### **Herbicide Application time**

Herbicides generally are applied at different times, depending upon the emergence time of the weeds and upon the type of fruit plants. Herbicides that are applied at specific times include the following:

Preplant herbicides are used before the crop is planted to control germinating weed seeds, and are usually mixed into the top 2 to 3 inches of soil. No preplant herbicides are labeled for fruit plants.

Preemergence herbicides are used after the crop has been planted, but before the weeds or crop emerges. Restrictions on the age of plants to be treated must be followed.

Postemergence herbicides are used after the crop and/or weeds have emerged from the soil surface and are growing. The most common of these is Round-Up®, which can be purchased without a pesticide license.

Herbicides usually are more effective when temperatures before application have favored uniform germination and rapid weed growth. Rapidly growing weeds are easiest to kill. High temperatures at the time of application also tend to increase the activity of the herbicide but also increase the possibility of crop injury. Moderate temperatures between 70 and 85°F are the most favorable for spraying. Wind can also be a factor in herbicide application. It can cause improper distribution over the weeds, reducing herbicide effectiveness while increasing the danger of drift onto desirable plants. Fewer problems occur if sprays are used when the wind velocity is low and the wind is blowing away from desirable plants.

### **Maximum Yield explains Herbicide**

Besides selective and non-selective classifications, a herbicide can also be categorized according to three other characteristics:

Persistence - How long the herbicide remains potent

Mechanism of action that how does it works. Means of uptake - How the plants will absorb it (e.g., through the roots, aboveground foliage, etc.)

### **Mode of action**

A herbicide's effectiveness is strongly influenced by its toxic mode of action and the application method. Herbicides can act by inhibiting a plant's amino acid production, growth, photosynthesis, cell division, or by mimicking natural auxin hormones to cause deformities.

Most modern herbicides are synthetic mimics of a natural plant's hormones that obstruct the target plant's growth. Some plants such as the tree of heaven and juglans (walnuts) produce their own natural herbicide. Organic herbicides are useful and are commonly used in organic gardens, but they are less effective and more costly than synthetic herbicides because they based on natural materials. For difficult cases, a combination of several herbicides is recommended when dealing with herbicide resistance.

## **First herbicides**

Although research into chemical herbicides began in the early 20th century, the first major breakthrough was the result of research conducted in both the UK and the US during the Second World War into the potential use of herbicides in war. The first modern herbicide, 2,4-D, was first discovered and synthesized by W. G. Templeman at Imperial Chemical Industries. In 1940, he showed that "Growth substances applied appropriately would kill certain broad-leaved weeds in cereals without harming the crops." By 1941, his team succeeded in synthesizing the chemical. In the same year, Pokorny in the US achieved this as well.

## **54. Types of Herbicides**

The different types of herbicides are all designed to kill plant tissue. However, they accomplish it by two basic methods. They are known as Contact Herbicides and Systemic Herbicides.

Contact herbicides: Contact is a word that means the chemical in that specific type of herbicide will kill the parts of the plant it contacts. For broadleaf weeds this means it will kill the above ground leafy part of the plants. It will not directly kill the below ground plants parts, such as roots, bulbs, tubers, or rhizomes. Contact herbicides are popular because they work quickly by killing the tissue in as fast as one day. Some herbicides will combine contact with systemic chemicals for a faster effect. New Round Up Weed and Grass Killer has combined both for faster control. For some plants, killing the above ground portions will not be enough to wipe out the plant completely. Most plants will regrow plant tissue and the herbicide will need to be reapplied. However, each time the plant has to use energy to start growth again will weaken the plant and eventually kill it.

### **Systemic Herbicide**

For systemic types of herbicides, the word "Systemic" means the plant absorbs through the leaves or stems and transports it internally throughout the plant. The chemical travels with the sap so it usually doesn't have the quick "knockdown" effect. The greatest benefit of a systemic type of herbicide is that it will kill the entire plant, roots and all. The speed of chemical movement in the plant is largely dependent on soil and air temperature.

A chemical sprayed in early spring may take a couple weeks longer to work than the same chemical sprayed in mid-summer. The speed of kill is also dependent on the "mode of action" of the chemical (how the chemical works inside the plant).

### **Five types of herbicides:**

**Broad spectrum** - these work on a wide variety of plants.

**Selective** - these work on a narrow range of plants.

**Contact** - these kill plant tissue at or near the point of contact with it (they do not spread around the plant). Therefore, they require even coverage in their application.

**Systemic** - these move through the plant tissues via the plant's circulation system, and these can be injected into the plant.

**Residual** - these can be applied to the soil in order to kill weeds by root uptake. They remain active in the ground for a certain length of time, and can control germinating seedlings.

## 55. Effect of Herbicides on People

Herbicides are poisonous chemicals that are used to kill unwanted plants, and are considered to be a type of pesticide. They are frequently used around the home and farm and represent a serious health hazard to adults, but they are especially hazardous to children and pets.

### Types

Herbicides are commonly found as liquids or powders, and are sometimes premixed into fertilizer products.

Herbicides are classified according to the types of plants that they affect.

Broad-spectrum herbicides will kill any plant on which they are applied, while selective herbicides are designed to target only certain types of plants.

Contact herbicides affect only the part of a plant that the chemical touches, while systemic herbicides are designed to be drawn up into the plant through its roots or absorbed through its leaves and stems.

Systemic herbicides kill the entire plant. Although many modern herbicides are less toxic than their predecessors, they are still poisons and should always be handled with caution.

### Skin Irritations and Allergic Reactions

According to the University of Missouri, herbicides are designed to be toxic to plants but in general are not highly toxic to mammals. Skin irritations are some of the most common effects when a person comes into contact with herbicides, and are most likely to happen on exposed areas, such as the hands and forearms. Some chemicals may burn the skin and should be washed off immediately with cold water. The Department of Veterans Affairs confirms that chloracne, a form of acne, is associated with exposure to Agent Orange. It can be mild or severe, and last up to several years. In severe cases, the skin may thicken and flake off. During the Vietnam War, the U.S. military sprayed millions of gallons of the herbicide Agent Orange between 1961 and 1971, in an attempt to defoliate trees in the jungle and deprive the enemy of food and cover.

### Cancers

The Department of Veterans Affairs acknowledges that the herbicide Agent Orange is responsible for a wide range of health problems in Vietnam veterans, including several types of cancer. The National Academy of Sciences concluded that there was a positive correlation between the incidence of Hodgkin's disease, a cancer of the lymph system, and exposure to

the herbicide Agent Orange. The VA also notes that non-Hodgkin's lymphoma is also associated with exposure to the defoliant. Herbicides are also suspected as causes of prostate cancer, cancers of the lungs and bronchial tubes, and cancers of the larynx and trachea.

### **Nervous System Disorders**

Some herbicides can cause nervous system disorders, such as peripheral neuropathy.

The early symptoms of this disease include numbness and tingling in the toes and fingers, gradually spreading to include the hands and feet. Pain may be present, as well as muscle weakness and sensitivity to touch. Acute peripheral neuropathy occurs within a few weeks of being exposed. The VA cites peripheral neuropathy as another symptom of exposure to Agent Orange.

### **Effects on Children**

Children and infants are at a higher risk for illnesses from herbicides than adults. According to the EPA, because children are still developing, their immune systems are less able to protect them from damage from herbicides. Children are also more likely to play in areas that expose them to chemicals, such as rolling on the floor or lawn. Mild exposure can result in complaints of dizziness and nausea, but herbicides may also cause neurological and developmental damage to children.

### **Pets**

Pets can be poisoned by herbicides by coming into contact with the chemicals when they are outside, but herbicides kept in the home may also be a problem if they are stored where pets can get to them. Pets can ingest herbicides by chewing on plants or toys that have been contaminated, or when they lick themselves after coming into contact with the chemical. Animals that bring herbicides into the house may spread the chemicals around the home and leave residue on furniture and carpets.

## **56. Herbicide Resistance**

Weeds are unwanted & useless plants that grow along with the crop plants. Weeds compete with the crops for light & nutrients, besides harboring various pathogens. So it is estimated that the world's crop yield is reduced by 10 – 15 % due to the presence of weeds. To tackle the problem of weeds, modern agriculture has developed a wide range of weed killers (herbicides). Herbicides are broad spectrum as they can kill a wide range of weeds.

**An ideal herbicide is to possess the following characters:**

- Capable of killing weeds without affecting crop plants
- Not toxic to animals & microorganisms
- Rapidly translocated within the target plant
- Rapidly degraded in the soil

- Commercially available herbicides is that they can not discriminate weeds from the crop plants .
- For this reason , crops are also affected by herbicides hence the need to develop herbicide resistance plants

### **Herbicide resistance**

Herbicide resistance is the inherited ability of an individual plant to survive a herbicide application that would kill a normal population of the same species. Herbicide resistance does not equate to poor performance of a herbicide. Resistant weeds can often survive application of herbicide at rates that are much greater than the recommended rate. Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In contrast, tolerance can be defined as the inherent ability of a plant to survive a herbicide treatment at a normal use rate. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering. Resistance may occur in plants by random and infrequent mutations; no evidence has been presented to demonstrate herbicide-induced mutation. Through selection, where the herbicide is the selection pressure, susceptible plants are killed while herbicide resistant plants survive to reproduce without competition from susceptible plants. Thus, the appearance of herbicide resistance in a field is an example of rapid weed evolution."

### **Factors Leading to the Development of Herbicide Resistance**

Because weeds contain a tremendous amount of genetic variation that allows them to survive under a variety of environmental conditions the development of a resistant species is brought about through selection pressure imposed by the continuous use of an herbicide. Factors that can lead to or accelerate the development of herbicide resistance include weed characteristics, Herbicide characteristics and cultural practices.

#### **Weed characteristics**

- Annual growth habit.
- High seed production.
- Relatively rapid turnover of the seed bank due to high percentage of seed germination each year (i.e., little seed dormancy).
- Several reproductive generations per growing season.
- Extreme susceptibility to a particular herbicide.
- High frequency of resistant gene(s), (e.g. *Lolium rigidum*).

#### **Herbicide characteristics**

- Herbicide characteristics which lead to rapid development of herbicide resistance in weed biotypes include:

- A single site of action
- Broad spectrum control.
- Long residual activity in the soil.

### **Cultural practices**

Cultural practices can also increase the selective pressure for the development of herbicide resistant biotypes. In general, complete reliance on herbicides for weed control can greatly enhance the occurrence of herbicide resistant weeds.

Other factors include:

- Shift away from multi crop rotations towards mono cropping.
- Little or no cultivation or tillage for weed control or no elimination of weeds that escape herbicide control.
- Continuous or repeated use of a single herbicide or several herbicides that have the same mode of action.
- High herbicide use rate relative to the amount needed for weed control.
- Orchard and vineyard systems.
- Roadsides.

## **57. Mechanisms of Herbicide Resistance**

For a herbicide to reach its active site in the plant, it must be taken into the plant and moved in lethal concentrations to the site where it has activity. Once it reaches the target site, it must be able to bind to the active site and stop that particular pathway. For a plant to be resistant, there must be a change that will allow it to avoid one or more of these steps. Theoretically, there could be a change in any one of these necessary steps beginning with uptake of the herbicide into the plant.

### **Potential mechanisms that could be responsible for resistance**

Target-site mutation – there is a change in the binding site that prevents the herbicide from binding or interacting.

Metabolism – the herbicide is modified into a nontoxic molecule before it reaches the target site.

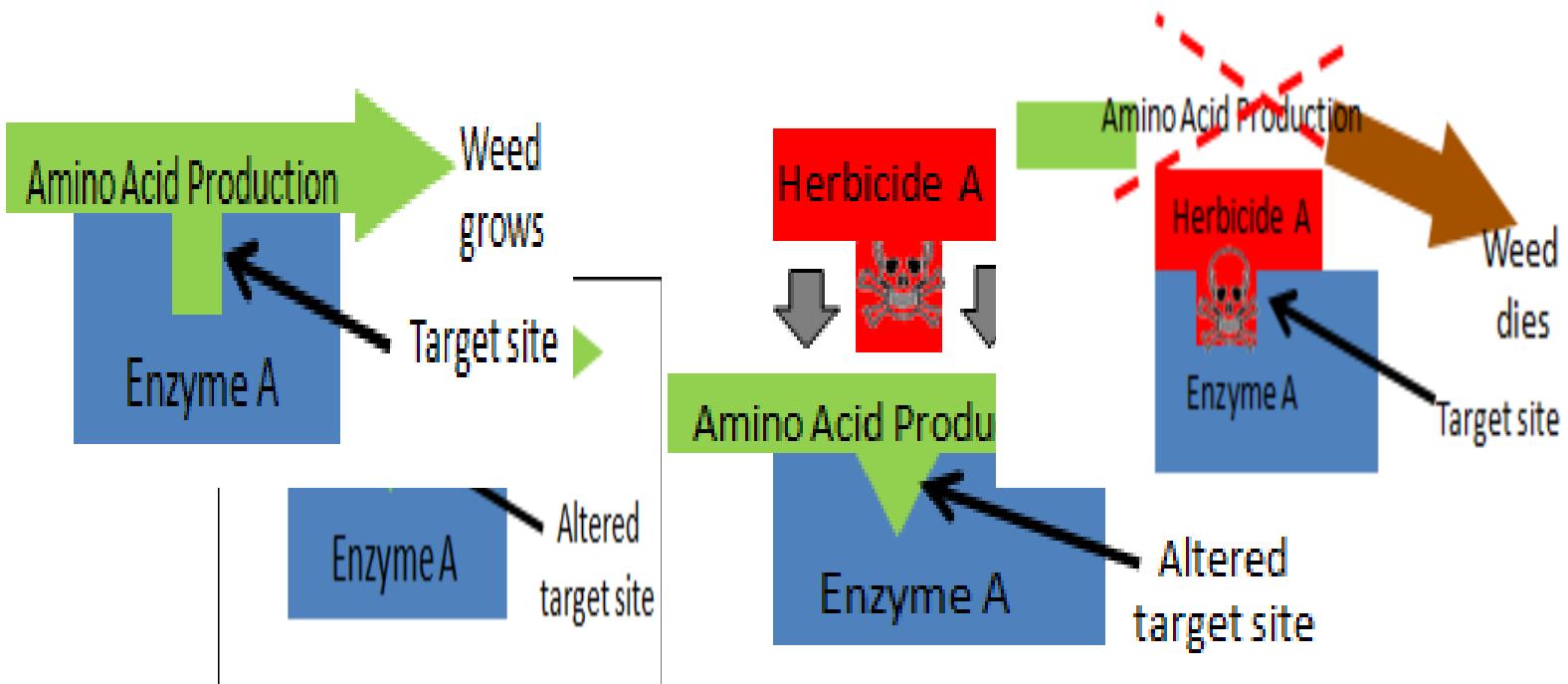
Sequestration – the herbicide is physically removed from the target site.

Reduced uptake – the herbicide is not taken up in lethal quantities.

Reduced translocation – the herbicide is not transported to the site in the plant where it has activity.

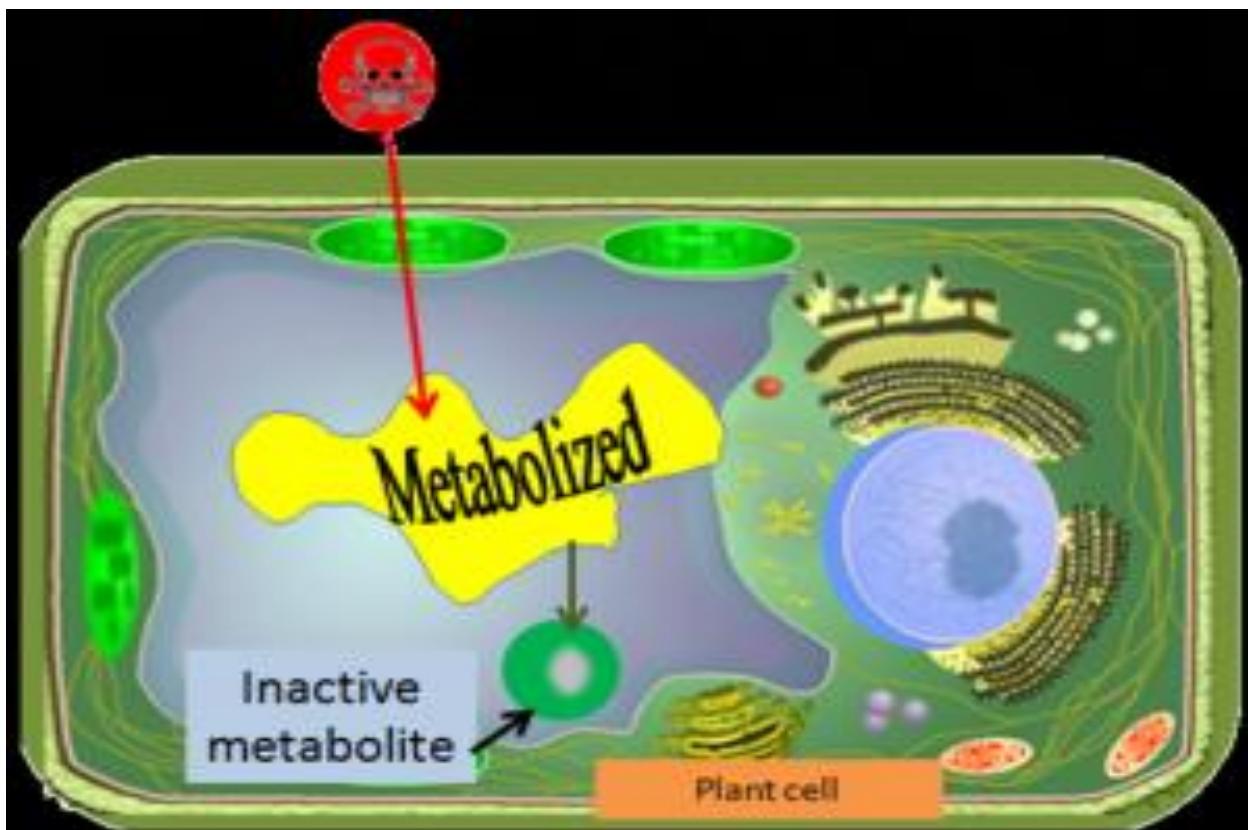
### **Altered target site**

- An herbicide has a specific site (target site of action) where it acts to disrupt a particular plant process or function (mode of action).
- If this target site is somewhat altered, the herbicide no longer binds to the site of action and is unable to exert its phytotoxic effect.
- This is the most common mechanism of herbicide resistance.



### **Enhanced metabolism:**

Metabolism within the plant is one mechanism a plant uses to detoxify a foreign compound such as an herbicide. A weed with the ability to quickly degrade an herbicide can potentially inactivate it before it can reach its site of action within the plant.



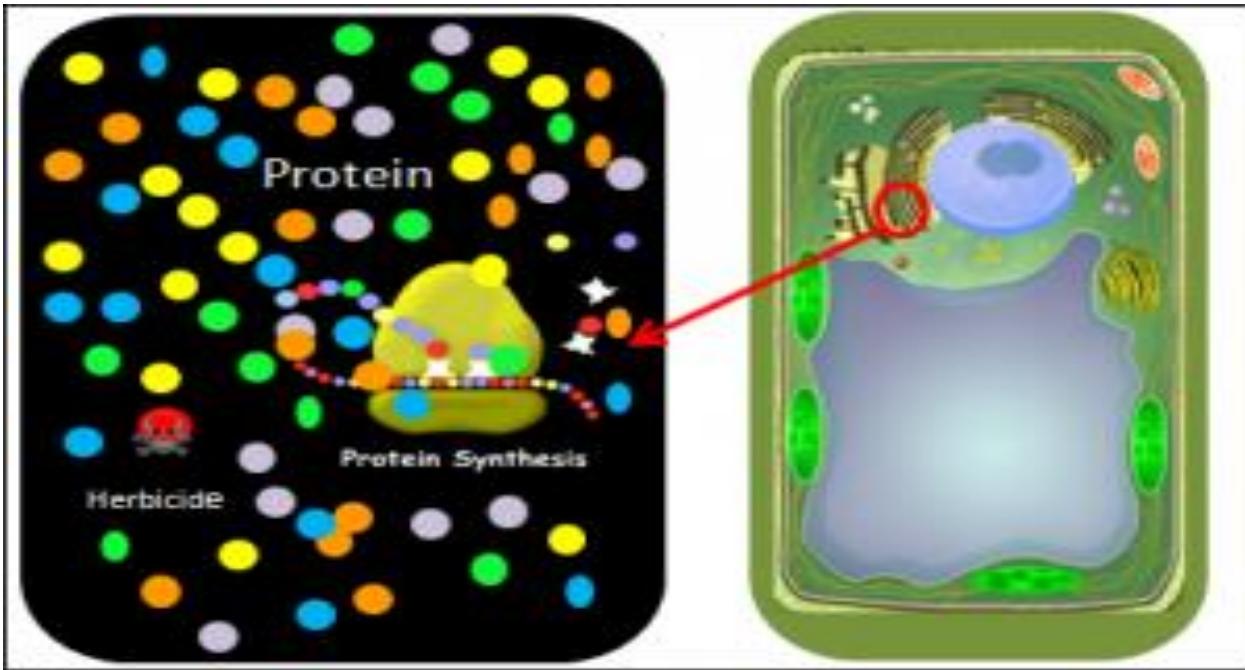
### **Compartmentalization or sequestration**

Some plants are capable of restricting the movement of foreign compounds (herbicides) within their cells or tissues to prevent the compounds from causing harmful effects.

In this case, an herbicide may be inactivated either through binding (such as to a plant sugar molecule) or removed from metabolically active regions of the cell to inactive regions, the cell wall, for example, where it exerts no effect.

### **Over-expression of the target protein:**

If the target protein, on which the herbicide acts, can be produced in large quantities by the plant, then the effect of the herbicide becomes insignificant.



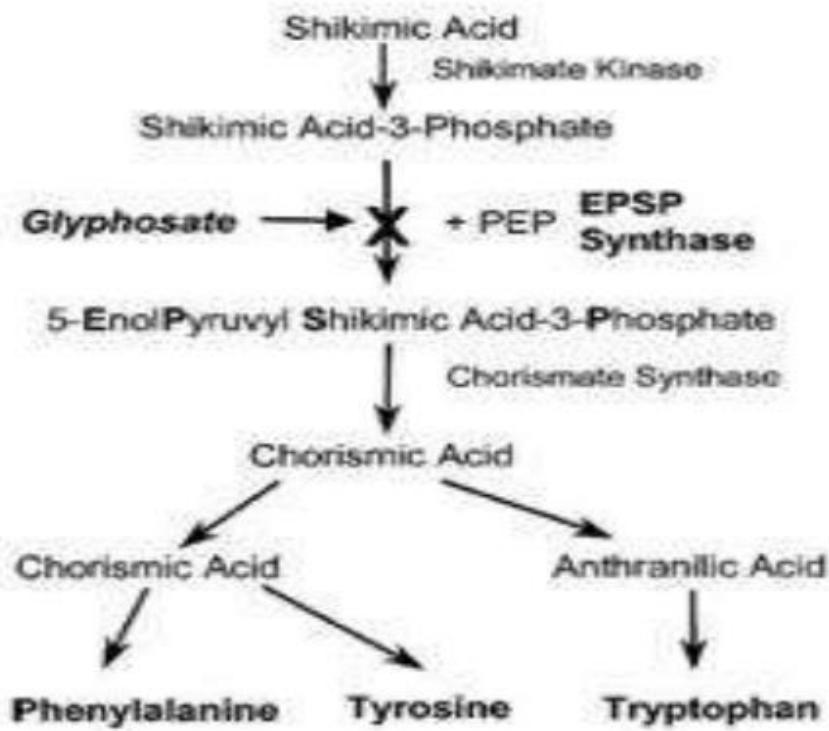
## 58. Mechanisms of Herbicide Resistance 2

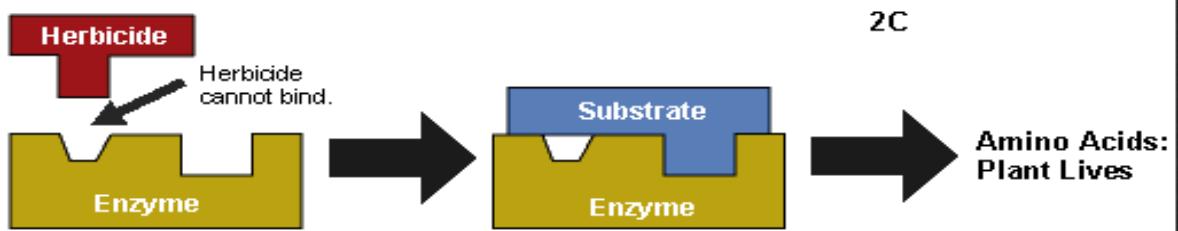
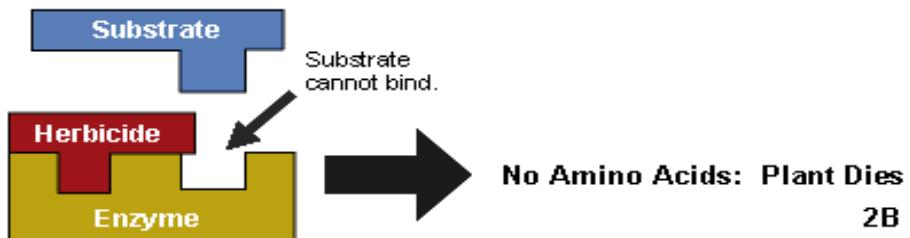
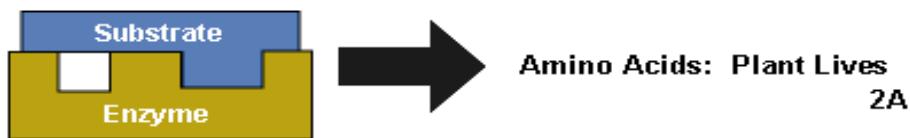
### Glyphosate

It is a broad spectrum herbicide , effective against 76 of worlds worst 78 weeds. Less toxic to animals , is rapidly degraded & short life span. The American company (Monsanto) market it as round up .

### Mechanism of Glyphosphate action

Capable of killing the plants in low concentration. Rapidly transported to growing tissues. It is competitive inhibitor of EPSPS (a key enzyme shikimic acid path way





Shikimic acid pathway results in the formation of amino acids, phenols, metabolites.

Glyphosate binds with EPSPS & blocks metabolism. Thus biosynthesis of aa & other products is inhibited. So cell division & plant growth is blocked. Shikimic acid pathway doesn't occur in animals.

So it is not toxic to animals

### Advantages of using herbicides

- Broad spectrum of weeds controlled
- Reduced crop injury
- Reduced herbicide carryover
- New mode of action for resistance management
- Crop management flexibility and simplicity
- Use of herbicides that are more environmentally friendly

### Disadvantages of herbicides

- Mammalian toxicity
- Eco toxicity

- Weeds become super weeds
- Reduced crop yield
- Creates soil and air pollution
- Herbicides also damage the Crop plants along with weed

### **Herbicide resistant Crops/Plants**

- A number of biological manipulations involved in genetic engineering are in use to develop herbicide resistance plant
- Over expression of EPSPS gene
- Use of mutant EPSPS gene
- Detoxification of herbicide by a foreign gene

## **59. Herbicide Resistance Crops/Plants**

### **How to produce herbicide resistant crops?**

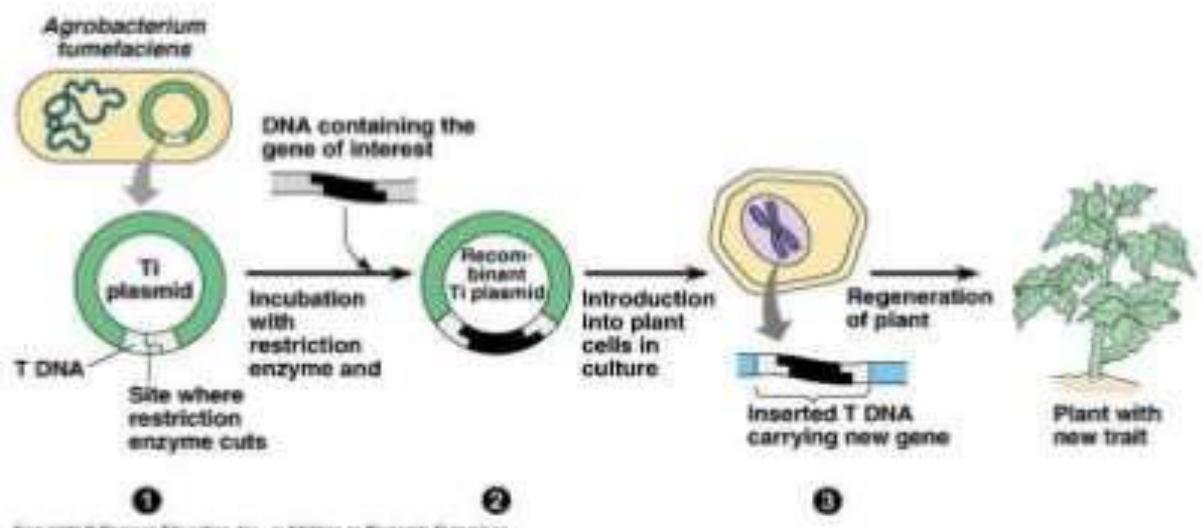
A number of biological manipulations involved in genetic engineering are in use to develop herbicide resistance plant by Over expression of EPSPS gene, Use of mutant EPSPS gene, Detoxification of herbicide by a foreign gene.

### **Glyphosphate resistance in crop/plants**

#### **1.Over expression of EPSPS gene**

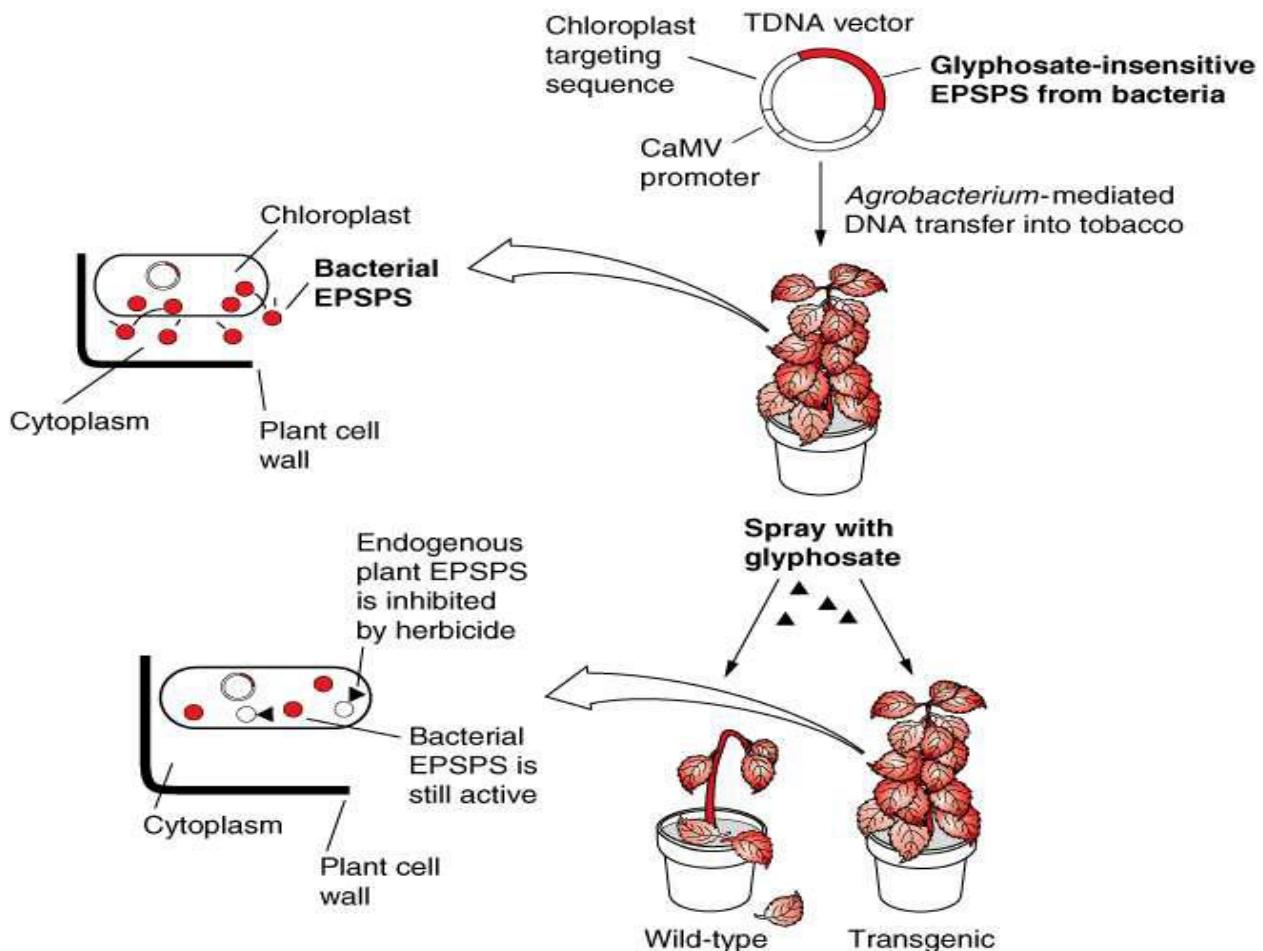
An over expression gene of EPSPS was detected in petunia. Gene from petunia was isolated & introduced in to other plants. The transgenic plants can tolerate glyphosphate 2 -4 times higher than that required to kill wild type weed plants

#### **Transfer of petunia gene into bacterium and then into plant**



## 2. Use of mutant EPSPS

EPSPS mutant gene resistant to glyphosphate was found in *s. typhimurium* it was found that single base substitution ( C to T ) change in amino acid from proline to serine. This enzyme cannot bind to glyphosphate using agrobacterium as vector mutant EPSPS was introduced in to tobacco plants but this is failed. It was later known that shikimic acid Pathway occurs in chloroplast, mutant EPSPS was produced in cytoplasm. This gene is not capable of transported to chloroplast. Later years mutant EPSPS gene was tagged with chloroplast specific transit EPSPS enzyme that freely enter chloroplast & confer resistance against herbicide



### 3. Detoxification of glyphosphate

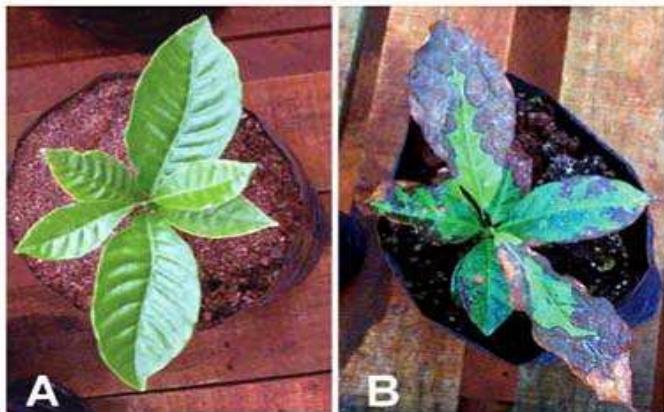
The soil microorganisms possess enzymes **glyphosphate oxidase** that converts **glyphosate** to **glyoxylate + amp**.

That gene was isolated from *ochrobactrum anthropi* & was introduced in to crop plants e.g: oil seed rape



### Use of combine strategy

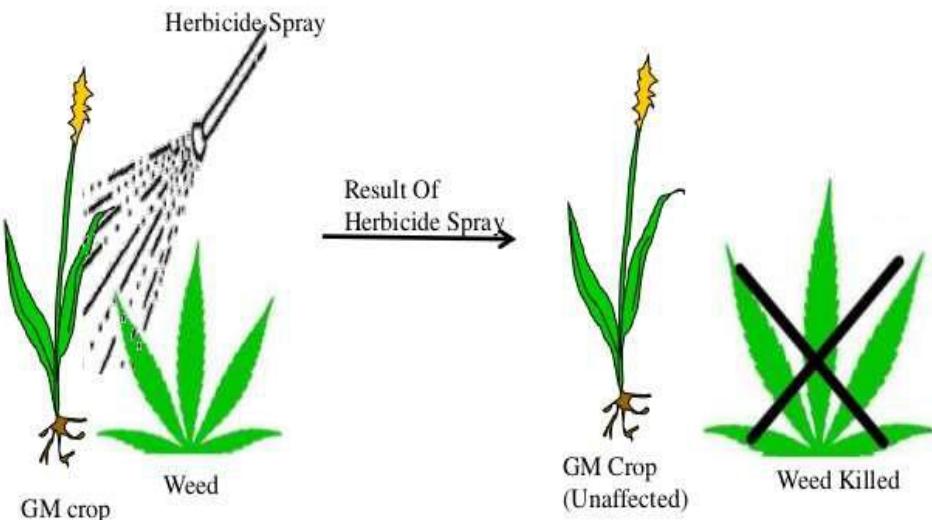
High resistance is acquired when the above 3 strategies combine together by this approach mutant, detoxification, over expression genes were employed in the same organism thus provides resistance.



**Figure 1.** Herbicide tolerant coffee plant (A) and non-transformed plant (B), one week after spraying with ammonium glufosinate at 200 mg.L<sup>-1</sup> (Ribas et al., 2006).

## Herbicide Tolerance

- Over 63% of Gm crops grown globally have herbicide tolerance traits.
- Herbicide tolerance is achieved through the introduction of a gene from a bacterium conveying resistance to some herbicides. In situations where weed pressure is high, the use of such crops has resulted in a reduction in the quantity of the herbicides used.



## 60. Level of Herbicide Resistance

The level of herbicide resistance in weeds varies by weed biology and resistance mechanism. In some cases, resistance occurs when the species survives application of a labeled rate, while in other cases, the species can survive up to 1000 times the labeled rate. (1X equals the labeled rate.) This is important in terms of being able to identify herbicide resistance in the field.

## **Herbicide Resistance Characteristics**

- Low-Level Resistance
- High-Level Resistance

### **Low-Level Resistance**

- A continuum of plant responses from slightly injured to nearly dead
- The majority of plants display an intermediate response
- Susceptible plants will be present in the population, especially when herbicide resistance is determined early

### **High-Level Resistance**

- Plants are slightly injured to uninjured
- Few plants have an intermediate response
- Susceptible plants can be present in the population

### **Low-Level Resistance**

- A continuum of plant responses from slightly injured to nearly dead
- The majority of plants display an intermediate response
- Susceptible plants will be present in the population, especially when herbicide resistance is determined early

### **High-Level Resistance**

Plants are slightly injured to uninjured

Few plants have an intermediate response

Susceptible plants can be present in the population

## **Herbicide Resistance Types**

- Single Herbicide Resistance
- Cross Herbicide Resistance
- Multiple Herbicide Resistance
- Cross Resistance

### **Single Herbicide Resistance**

Resistant to only one herbicide

**Herbicide A**  
**MOA #1**



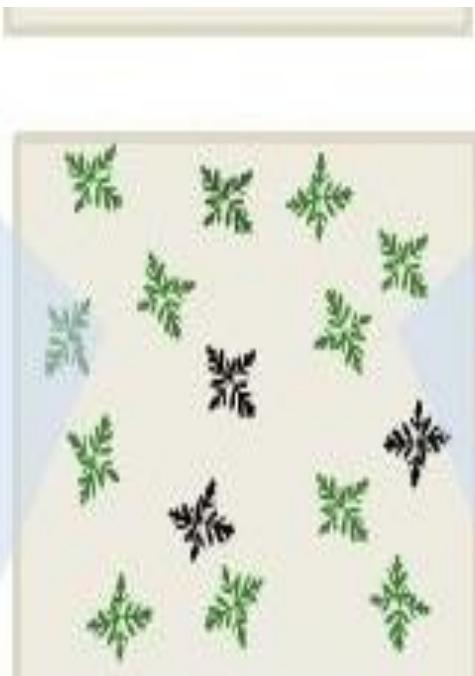
### Cross Herbicide Resistance

Resistant to two or more herbicide families with same mechanism of action

Single resistance mechanism

Herbicide A  
MOA #1

Herbicide B  
MOA #1

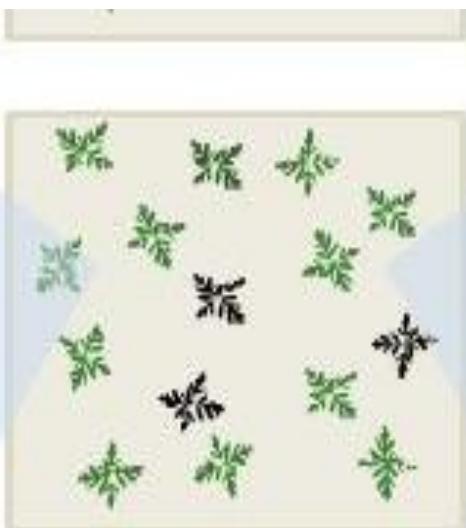


### Multiple Herbicide Resistance

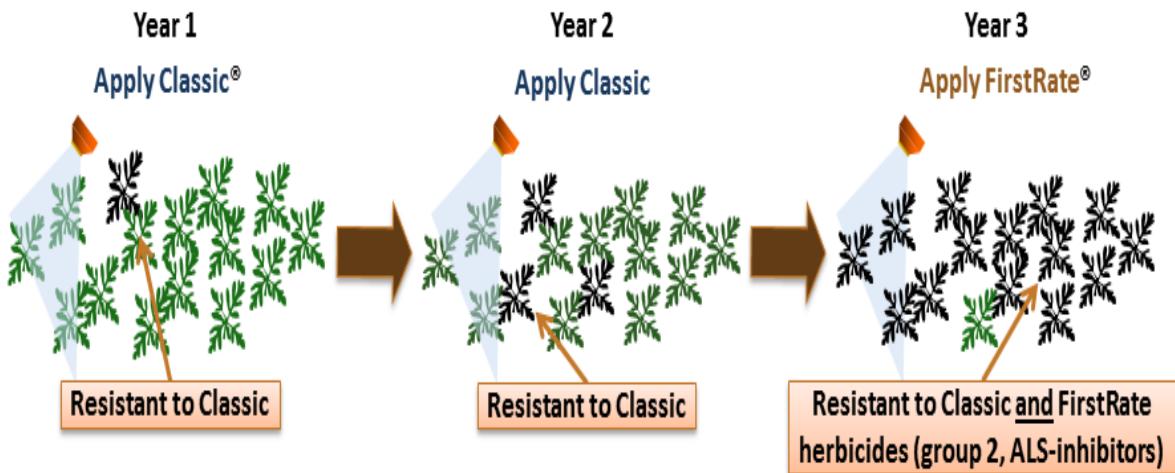
Resistant to two or more herbicides with different mechanisms of action. May be the result of two or more different resistance mechanisms

Herbicide A  
MOA #1

Herbicide C  
MOA #2



## Cross Resistance: same mechanism of action



## Multiple Resistance

Multiple resistance can occur following repeated applications of a single herbicide and selection for herbicide-resistant biotypes followed by repeated applications of another herbicide and selection for herbicide-resistant biotypes.

