# UNIT I FUNDAMENTALS OF PLANT TISSUE CULTURE

## 1.1 INTRODUCTION TO PLANT TISSUE CULTURE AND TOTIPOTENCY OF PLANT CELL

**Introduction**

A fundamental and basic area of plant biotechnology, plant tissue culture aids in our understanding of the cellular processes involved in plant growth and development. Thorpe (2007) defines tissue culture as "culturing or cultivating desirable tissues, cells, or organs on a specially prepared sterile synthetic medium in well regulated light, temperature, and humidity." A group of methods called plant tissue culture are employed to sustain or cultivate, in sterile circumstances, plant cells, tissues, or organs on a nutrient a known-composition cultural medium. It is frequently employed to create clones of a plant via a process called "micro propagation." Plant tissue culture methods aided the business sector in producing a variety of plant-derived metabolites, tastes, oils, colors, and medicines.

The perfect media for plant tissue development should have all the necessary vitamins, minerals, and nutrients. The formulation of a plant tissue culture medium requires careful consideration of several key characteristics, including the explant's source, the kind of media appropriate for that specific explant, temperature, and several internal elements including vitamins, pH, and hardening agents. An essential step in the plant tissue culture process is the inclusion of necessary plant growth regulators for the appropriate development of the desired cell, tissue, or organ cultures.

Plant tissue culture techniques can provide various benefits over traditional propagation methods, such as:

1. The ability to grow perfect replicas of plants that produce exceptionally good flowers, fruits, or have other desirable features.
2. To rapidly generate fully grown plants.
3. When numerous plants are produced without seeds or essential pollination, seeds are produced.
4. The process by which genetically modified plant cells regenerate into whole plants.
5. Plants grown in sterile containers enable them to be moved about with a far lower risk of disease, insect, and pathogen transmission.

6. The process of developing plants from seeds that would otherwise have very little possibility of doing so, such as Nepenthes and orchids.
7. The aim is to eradicate viral and other illnesses from certain plants and swiftly propagate them as "cleaned stock" for horticultural and agricultural purposes.

**Historical events**

In 1902, Gottlieb Haberlandt proposed his views on plant tissue culture in the German Academy of Science by isolating photosynthetic leaf cells for the first time. He also stated that embryos can be developed from vegetative cells and defined the concept of "Totipotency", for which he is recognized as the "Father of plant tissue culture".

The first known plant tissue culture medium used for growing roots was developed by White in 1939, and the callus culture medium was developed by Gautheret. The idea for development of these media was taken from algae medium formulated by Uspenski and Uspenska medium and Knop's salt medium respectively.

In 1962. Murashige and Skoog developed another plant tissue culture medium that is most accepted today. Later, in 1968, Oluf L. Gamborg developed a plant tissue culture medium that helped to grow specific tissues like soybeans under controlled conditions. This medium is also called as B5 medium.

The concept of cell suspension cultures was first reported by Melchers and Engelmann, who highlighted culturing of individual cells and the production of economically important biomolecules.

The major development of plant tissue culture and its related biotechnological techniques started between 1940s and 1960s. Prominent researchers tried to understand the behavior of cells growing on the different nutrient natural media developed from coconut water, protein hydrolysates and modification of a growth medium by addition of many vitamins and minerals to check the quality of growth.

**Totipotency of Plant Cells**

The plant body is made up of different types of tissues and cells which are working in coordination functionally. The cells vary structurally in size, shape etc. and all of them have sane origin that is from a single cell called as 'Zygote'. The zygote divides mitotically and produce the entire plant. So, it is understandable that the information for the formation of entire plant lies in the single cell Zygote. Somatic cells also contain the same information which are produced later on. This inherent capacity or potential of single cell to form a whole new plant is termed as "Totipotency". The concept was first proposed by Schleiden and Schwann (1838) in their "Cell Theory". Later, Haberlandt (1902), defined the concept of totipotency.

Definition of totipotency is "the ability of a single cell to divide and produce a whole organism or plant". Plants exhibit a remarkable developmental plasticity. This is manifested, among others, in their high regeneration capacity. Plants, from time to time, need to cope with physical damages caused by their biotic or abiotic environment. To ensure survival, they have dedicated developmental pathways to close injuries and / or replace lost parts/ organs. These pathways have been exploited for vegetative plant propagation long since. Histological wound responses and callus formation had been observed and the term "dedifferentiation" was already used for this tendency quiet earlier.

**De-differentiation, Re-differentiation and Regeneration of whole plant:**

The three common phases of cellular growth in plants are cell division, cell enlargement and cell differentiation, which bring maturity to the cells. During in vitro growth of the living cells which are in the differentiated form i.e., they have lost the capacity to divide and then regain the power of division under suitable conditions, that is they become meristematic and the phenomenon is termed as "dedifferentiation". During dedifferentiation, functional forms of the cells revert to their early developmental stage. Later, these meristems or tissues are able to divide and produce cells that once again lose the capacity to divide but mature to perform specific functions, i.e., get re-differentiated. Thus "redifferentiation" can be defined as maturation or differentiation of dedifferentiated tissues.

Redifferentiation is the loss of the regained capacity to divide by differentiated cells. It allows differentiated cells to serve as functionally-specialized cells in the plant body. So, after preparing the plant body for physiological or structural change by dedifferentiation, the subjected differentiated cells in tissue culture revert to the redifferentiated form and thus forming a whole new plant.

So, the concept of totipotency was realized in plant cells with the ability to regenerate whole new plant starting from a single cell or few cells. The process involves dedifferentiation from partially differentiated cell/tissue type (e.g., parenchyma) to a meristem-like state followed by redifferentiation (regeneration) into well-organized structures. *De novo* organogenesis and somatic embryogenesis are the two major routes of regeneration of plants in tissue culture.

**Short Questions**

1. Totipotency of Plant cells
2. Advantages of Plant tissue culture over traditional methods of propagation

# 1.2 NUTRIENT REQUIREMENTS FOR PLANT TISSUE CULTURE

The plants growing in vivo or in field requires a medium (eg. soil) containing nutrients. The isolated plant tissues called 'explants' cannot synthesize their own food material and are grown on an artificial nutrient medium' which supplies nutrients exogenously to the growing cells. The nutrient medium basically is composed of two types of nutrients i.e., inorganic nutrients or salts like macronutrients and micronutrients and organic nutrients like carbon source, vitamins, amino acids, growth regulators ete. The nutrient media supply all the essential minerals required for *m vitro* growth and morphogenesis of the plant tissue.

## 1.2.1 Inorganic Nutrients

**Macronutrients**

The inorganic nutrients or salts needed in higher amounts (more than 30 ppm/L) are called as “macronutirents". They provide nutrients in both anion and cation forms. The elements namely, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) are important macronutrients. Macronutrients have structural and functional role in protein synthesis like cell wall synthesis, enzyme cofactors, membrane integrity etc. Most media contain N and K at 20-30 mM. While P, Mg, S, and Ca range from 1-3 mM.

i. **Nitrogen (N):** It is the major component of all plant tissue culture media. In inorganic form it is used as nitrates or ammonia. It helps to synthesis complex organic molecules.
ii. **Phosphorus (P):** It is supplied as sodium or potassium hydrogen phosphates. It is essential for energy metabolism.
iii. **Potassium (K):** It is essential for maintaining the ion balancing, osmatic pressure and osmatic regulation of cells. It helps in activation of many enzymes in the cells.
iv. **Calcium (Ca):** It is supplied in the form of calcium chloride and calcium nitrate. It provides strength to the cell wall and helps in regulation of cell membrane structure. It acts as a cofactor for many enzymes. It helps in cell division and multiplication.
v. **Magnesium (Mg):** It is supplied as magnesium sulphate. It is essential for enzymatic reactions and energy metabolism.
vi. **VE Sulphur (S):** It is supplied as magnesium sulphate and potassium sulphate. It involves in protein and chlorophyll synthesis.

**Micronutrients**

The nutrient elements namely, iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo) which are required in concentration less than 30 ppm are considered as 'micronutrients' or 'trace elements. Iron is the most critical of all the micronutrients. Iron and zine are commonly used in chelated form:

i. **Iron (Fe):** It is supplied as $Na_2$ FeEDTA. It is an important cofactor for many enzymes. It is essential for the growth and development of the plants.
ii. **Manganese (Mn):** It is supplied as manganese sulphate. It is essential for many enzymatic activities.
iii. **Zinc (Zn):** It is supplied as zine sulphate. It is essential for photosynthesis.
iv. **Boron (B):** It is supplied as boric acid. It involves in different enzymatic activities.
v. **Copper (Cu):** It is supplied as copper sulphate. It is also essential for photosynthesis.
vi. **Molybdenum (Mo):** It is supplied as sodium molybdate. It is essential for conversion of nitrates into ammonium.

**Table 1: Essential nutrients and their main functions**

| Elements | Function (s) |
|---|---|
| **Calcium** | Essential component of proteins, nucleic acids and come coenzyme. (Required in most abundant quantity). |
| **Magnesium** | Synthesis of cell wall, membrane function, cell signalling |
| **Potassium** | Component of chlorophyll, cofactor for some enzymes |
| **Phosphorus** | Major inorganic action, regulates osmotic potential. |
| **Sulfur** | Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer |
| **Manganese** | Cofactor for certain enzymes |
| **Iron** | Component of cytochromes, involved in electron transfer |
| **Chlorine** | Participated in photosynthesis |
| **Copper** | Involved in electron transfer reactions, Cofactor for some enzymes. |
| **Cobalt** | Component of vitamin $B_{12}$. |
| **Molybdenum** | Component of certain enzymes (e.g. nitrate reductase), cofactor for some enzymes. |
| **Zinc** | Required for chlorophyll biosynthesis, cofactor for certain enzymes. |

## 1.2.2 Organic Nutrients

- **Carbon Source:** Since most cultures cannot photosynthesize enough sugars for growth, a carbon source is supplied exogenously. This is usually provided in

the form of Sucrose (2-3%) in the medium. Glucose and fructose can also be used but occasionally. Other carbohydrates include m-inositol, maltose, lactose, galactose, raffinose and starch.

- **Vitamins:** Intact soil growing plants synthesize all vitamins required for their growth. But in vitro cultures require vitamins like thiamin (B1), nicotinic acid, pyridoxine (B6) and myo-inositol. All these vitamins are stable during autoclaving Other vitamins like biotin, pantothenic acid, folic acid, choline chloride, p-aminobenzoic acid, riboflavin, vitamin B12 and ascorbic acid are added at 1mg/L or less sometimes especially when cells or protoplasts are cultured.
- **Amino acids:** Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L-arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells. Glycine is the most commonly used amino acid.
- **Other organic supplements:** Addition of organic acids like citrate, malate, succinate or fumarate allow the growth of plant cells. Pyruvate also enhances the growth of cultured cells. Some plant tissues grow in the presence of natural products or organic extracts like protein (casein) hydrolysate, coconut milk, yeast and malt extract, ground banana, orange juice, tomato juice, watermelon juice, etc. Yeast extract is a good source of organic nitrogen and vitamins. Casein hydrolysate contains all the common amino acids. Fruit juices contribute a number of essential nutrients and vitamins. Mannitol, sorbitol or combination of these is used as an osmotic um. A concentration of 0.6M is generally used.

**Support System**

Apart from the inorganic and organic nutrients, nutrient media also contain other elements which do not supply any nutrients but are essential for preparation of nutrient media.

- **Gelling agents:** *In vitro culture* occurs either in liquid medium or on solid medium. In liquid medium (suspension culture), the tissues or cells are cultured in water containing only nutrients. The liquid medium has to be frequently agitated for aeration. The solid media are prepared by using gelling agents. Agar (0.5% - 1.0%) is the most widely used gelling agent as it is resistant to enzymes and does not react with media components. Agarose, a pure form of gel, gellant gums are also used.
- **Activated charcoal:** Supplementation of the medium with activated charcoal stimulates the growth and differentiation of certain plant cells (carrot, tomato, orchids). Some toxic inhibitory compounds (e.g. phenols) produced by cultured plants are removed (by adsorption) by activated charcoal, and this facilitates efficient cell growth in cultures. Addition of activated charcoal to certain

cultures (tobacco, soybean) is found to be inhibitory, probably due to adsorption of growth stimulants such as phytohormones.

- **Antibiotics:** It is sometimes necessary to add antibiotics to the medium to prevent the growth of microorganisms. For this purpose, low concentrations of streptomycin or kanamycin are used. As far as possible, addition of antibiotics to the medium is avoided as they have an inhibitory influence on the cell growth.
- **pH:** pH range of 5.0 to 6.0 is suitable for in vitro culture. Autoclaving nutrient media reduces pH by 0.3 to 0.5 units. pH above 6.0 leads to a hard medium while a pH less than 5.0 prevents gelling of agar. Hence, it must be adjusted by adding 0.1 N NaOH or HCl.

**Types of Media**

There are generally two types of media, a simple medium containing sucrose and inorganic salts and such medium is called as 'Minimal or Basal medium and the other medium also supplemented with vitamins, amino acids and growth regulators and such medium is referred as "Synthetic medium". Different types of media have been used by different workers during their experiments. But no single medium is capable of maintaining the optimum growth of all the plant tissues. So, the most suitable medium for particular tissue is determined by simple trial and error method.

The nutrient media are often named after the name of the person who formulated the media. Like MS medium (Murashige and Skoog), White's medium, Gamborg medium (B5 medium) or SH medium (Schenk and Hilde brant) etc.

The major types of nutrient media used in plant tissue culture are:

1. **White's Medium:** The medium was developed by P. R. White in 1963 for the establishment of the root culture of tomato. This was the earliest plant tissue culture media developed for root culture. It has a lower salt concentration and a higher concentration of $MgSO_4$. The concentration of nitrate is 19% lesser than the MS media. White's medium can be used for the purpose of shoot culture and callus culture. It is suitable for culture *Musa* and *Daucus* species
2. **Murashige and Skoog (MS) medium:** This medium was invented by two scientists named Toshio Murashige and Folke K. Skoog in 1962 while the two scientists were working on the discovery of plant growth regulators. It is the most commonly used medium in the tissue culture lab. Sometimes you may observe some numbers behind the MS, which indicate the concentration of sucrose in the medium. For example, MS0 indicates sucrose absence and MS10 indicated the presence of 10g/1 sucrose in the medium. The formulation is a blend of nutrients like inorganic salts, vitamins, and

amino acids. This medium is used to induce organogenesis, callus culture, micropropagation and cell suspension.

3. **Gamborg (B5) medium:** This medium was developed by O. L. Gamborg in 1968. He used the media for the callus and cell suspension culture of Glycine max belonging to the family of Fabaceae. This medium is a blend of nutrients like inorganic salts, vitamins and carbohydrates. The medium has a higher concentration of nitrate and potassium and a lower concentration of ammonia. Potassium nitrate is useful in inducing the soybean root callus formation and ammonium sulfate plays an essential role in cell growth. It is used for the purpose of protoplast culture.
4. **N6 medium:** Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.
5. **Nitsch and Nitsch (NN) medium:** The medium was developed by J. P. Nitsch in 1969 for the establishment of the *in vitro* anther culture of *Nicotiana*, from family *Solanaceae*. It contains a high concentration of thiamine, biotin, and folic acid that supports anther callus.

**Table 2: Nutrient composition of important media used in plant tissue culture**

| **Components** | **Amount ($mgl^{-1}$)** | | | | |
|---|---|---|---|---|---|
| | **White's** | **Murashige & Skoog (MS)** | **Gamborg (B5)** | **Chu (N6)** | **Nitsch's** |
| **Macronutrients** | | | | | |
| $MgSO_4 7H_2O$ | 750 | 370 | 250 | 185 | 185 |
| $KH_2PO_4$ | - | 170 | - | 400 | 68 |
| $NaH_2PO_4H_2O$ | 19 | - | 150 | - | - |
| $KNO_3$ | 80 | 1900 | 2500 | 2830 | 950 |
| $NH_4NO_3$ | - | 1650 | - | - | 720 |
| $CaCl_2 2H_2O$ | - | 440 | 150 | 166 | - |
| $(NH_4)_2SO_4$ | - | - | 134 | 463 | - |
| **Micronutrients** | | | | | |
| $H_3BO_3$ | 1.5 | 6.2 | 3 | 1.6 | - |
| $MnSO_4 4H_2O$ | 5 | 22.3 | - | 4.4 | 25 |
| $MnSO_4 H_2O$ | - | - | 10 | 3.3 | - |
| $ZnSO_4 7H_2O$ | 3 | 8.6 | 2 | 1.5 | 10 |
| $Na_2MoO_4 2H_2O$ | - | 0.25 | 0.25 | - | 0.25 |
| $CuSO_4 . 5H_2O$ | 0.01 | 0.025 | 0.025 | - | 0.025 |
| $CoCl_2 . 6H_2O$ | - | 0.025 | 0.025 | - | 0.025 |
| KI | 0.75 | 0.83 | 0.75 | 0.8 | - |
| $FeSO_4 7H_2O$ | - | 27.8 | - | 27.8 | 27.8 |
| $Na_2EDTA 2H_2O$ | - | 37.3 | - | 37.3 | 37.3 |
| Sucrose (g) | 20 | 30 | 20 | 50 | 20 |

| **Organic supplements** | | | | | |
|---|---|---|---|---|---|
| **Vitamins** | | | | | |
| Thiamine HCl | 0.01 | 0.5 | 10 | 1 | 0.5 |
| Pyridoxine (HCl) | 0.01 | 0.5 | 1 | 0.5 | 0.5 |
| Nicotinic acid | 0.05 | 0.5 | 1 | 0.5 | 5 |
| Myoinositol | - | 100 | 100 | - | 100 |
| **Others** | | | | | |
| Glycone | 3 | 2 | - | - | 2 |
| Folic acid | - | - | - | - | 0.5 |
| Biotin | - | - | - | - | 0.05 |
| **pH** | 5.8 | 5.8 | 5.5 | 5.8 | 5.8 |

**Short Questions:**

1. Write about Gamborg medium.
2. Organic nutrients added in Plant Nutrient medium

**Essay Questions**

1. Write in detail the various components that make up the plant tissue culture medium
2. Explain the different plant tissue culture mediums that are used in culture.

## 1.3 PLANT GROWTH REGULATORS (PGR)/ PHYTOHORMONES

These include both naturally occurring plant hormones or phytohormones and synthetic ones. Most of the synthetic ones are structural analogs of the natural hormones and bind to the same receptors. Plant growth regulators used in plant tissue culture are auxins, cytokinins, gibberellin and Abscisic acid. In most of the cultures only auxins and cytokinins are used.

**Auxins**

Auxins induce cell division, cell elongation, elongation of stem, internodes, tropism, apical dominance, abscission and rooting. The naturally occurring auxins are IAA (Indole 3-Acetic Acid), IBA (Indole 3 – Butyric Acid). The commonly used auxins are: IAA (indole 3 – Acetic Acid).

IBA (Indole 3-Butyric Acid) 2.4-1) (Dichloro Phenoxy Acetic Acid) NAA (Naphthylene Acetic Acid) NOA (Naphthoxy Acetic Acid) The 2,4-D is used for callus induction whereas, the other auxins are used for root induction.

In plant tissue culture, auxins are used either alone or with cytokinins or other PGR for callus induction, organogenesis or somatic embryogenesis. Auxins are used in the range of 1-5 μM, IAA is unstable in solution and is easily oxidized and

conjugated to inactive forms by plant cells. IBA is more stable in solution. IAA and IBA are not heat stable. Hence, they cannot be autoclaved with the medium. They are filter sterilized and incorporated in the medium in sterile form. 2,4 D and NAA can be autoclaved Auxins should be dissolved in minimum amount of 0.1N NaOH or ethyl alcohol and final volume is made up to one ml.

**Cytokinins**

Cytokinins are used to induce shoot formation, axillary shoot proliferation and to inhibit root formation. They promote cell division. They have been shown to activate RNA synthesis and to stimulate protein and the enzymatic activity. The commonly used cytokinins are BAP (6-Benzyl Amino Purine) BA (Benzy adenine) 2iPA (Isopentyl adenine) Kinetin (6 - furfural laminopurine) and Zeatin (purine derivative).

Zeatin was the first naturally occurring cytokinin to be identified. Cytokının like activity is exhibited by adenine and adenosine, particularly at higher concentrations. Most of the cytokinins except zeatin and its derivatives can be autoclaved with the media and are used in the range of 1 - 50 μM. Cytokinins should be dissolved in minimum amount of 0.1N NaOH or ethyl alcohol and final volume made upto one ml.

Diphenyl Urea, a growth factor present in coconut milk exhibits cytokinin like response. So as a source of cytokinin 10-15% v/v coconut milk is added to the medium.

**Gibberillins**

Gibberellins were first obtained from the ‘foolish seedling disease’ of rice caused by *Gibberella fujikuori*. Gibberellins cause stem elongation, flowering, breaking of dormancy of seeds, parthenocarpy development of fruits etc in plants. There are more than 50 different compounds classified as gibberellins. However, only $GA_3$, Gibberellic acid and $GA_{417}$ are mostly used plant tissue culture. In plant tissue culture, it inhibits induction of organogenesis, especially adventitious root formation. It enhances callus growth and simulates the elongation of dwarf or stunted plantlets.

**Abscisic acid (ABA)**

Abscisic acid regulates closure of stomata and cause bud and seed dormancy. In plant issue culture it promotes maturation and germination of somatic embryos.

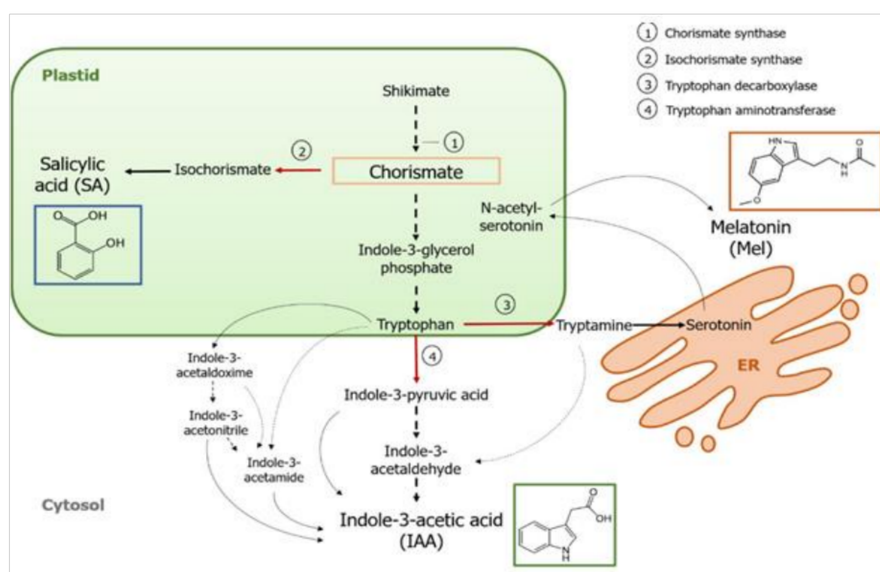
**Effect of growth regulators:**

- More cytokinin/low auxin ratio regenerated to shoot part.
- Low cytokinin/more auxin regenerated to only root part.
- Medium cytokinin/medium auxin regenerated to both shoot and root.
- Medium cytokinin, low auxin only regenerated to callus.

# 1.3.1 BIOSYNTHETIC PATHWAY

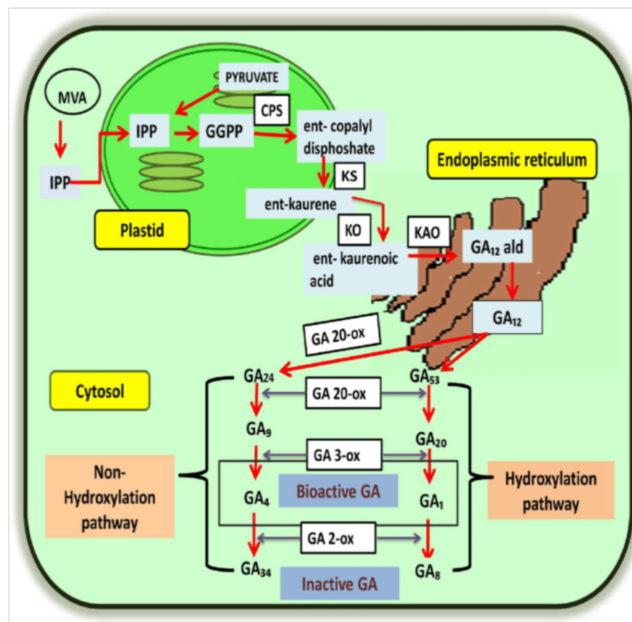
**AUXIN BIOSYNTHETIC PATHWAY**

Chorismate, the last product of the shikimate pathway, plays a crucial role in phytohormone biosynthesis| Chorismate produces tryptophan and other aromatic amino acids through a number of reactions. One of these reactions is the use of tryptophan aminotransferase (TA) to convert tryptophan to indole-3-pyruvic acid; It is capable of producing indole-3-acetic acid (IAA), an auxin| TA has been suggested as a universal key enzyme for IAA biosynthesis in both vegetative organs and reproductive organs development, including fruit growth and ripening. On the other hand, chorismate can be changed into isochorismate, which can then be changed into SA, if isochorismate synthase (ICS) is active. This route is presently well established in plants even though it was initially discovered in bacteria. Lastly, the enzyme tryptophan decarboxylase (TDC) has the ability to convert tryptophan into tryptamine. Despite the fact that tryptamine converts to indole-3-acetaldehyde and then to IAA, this is just one of the several pathways for IAA production.



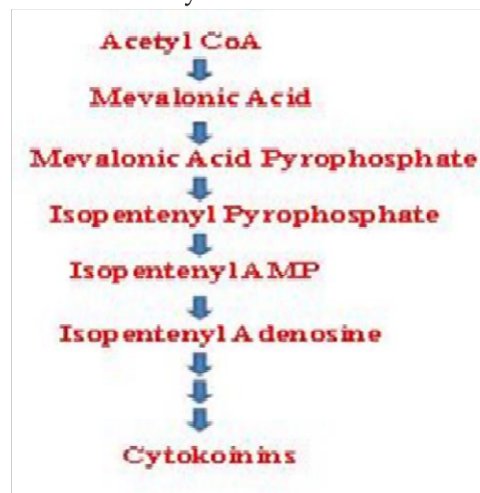

**GIBBERELLIC ACID BIOSYNTHETIC PATHWAY**

Gibberellic acid is a hormone present in fungi and plants, commonly known as gibberellin A3 or GA3. $C_{19}H_{22}O_6$ is its chemical formula. It becomes a white to pale-yellow solid after purification. In their regular state, plants generate a lot of GA3. Microorganisms can be used to industrially generate the hormone. Simple gibberellin, a pentacyclic diterpene acid that stimulates cell growth and elongation, is known as gibberellic acid. When applied sparingly, it influences plant breakdown and promotes growth, but with time, plants become tolerant to it.[GA causes the cells in seeds to create more mRNA molecules, which code for hydrolytic enzymes, when the seeds germinate. Gibberellic acid is a powerful hormone that naturally occurs in plants and regulates their growth.




## CYTOKININ BIOSYNTHETIC PATHWAY

The first reaction in the biosynthesis of isoprene cytokinins is catalyzed by adenosine phosphate-isopentenyltransferase (IPT). It can use AMP, ADP, or ATP as substrates and dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyldiphosphate (HMBDP) as prenyl donors. DMAPP and HMBDP, which are used in the production of cytokines, are generated via the methylerythritol phosphate route (MEP).Plants and microorganisms can also manufacture cytokinins through the recycling of tRNA. tRNA-isopentenyltransferase is responsible for prenylating these adenines. Auxin is known to control the production of cytokinin.




## Physiological roles of Cytokinins

- Accelerates cell division, cell elongation and morphogenesis.
- Initiation in protein and nucleic acid metabolism.
- Counteract the influence of apical dominance.
- Helps to delay senescence.

- Provide resistance to plant injured by high temperature and low temperature.
- Can break seed dormancy and promotes germination.

**Short Questions**

1. Plant Growth regulators
2. Role of Plant growth regulators in Plant tissue culture

**Essay Questions:**

1. Write about the various plant growth regulators and highlight on their significance in Plant tissue culture.

## 1.4 PREPARATION OF MEDIA

The nutrients required for optimal growth of plant organ tissue and protoplast in vitro generally vary from species to species. No single media can be suggested as for all types of in vitro culture. In order to formulate a suitable medium for a new system a well-known basal medium such as MS medium (Murashigel and Skoog), B5 (Gamborg *et al*), White media etc. can be used By making minor quantitative and qualitative changes a new media can develop to accommodate the specific requirements of the desired plant material.

**Methods of media preparation**

**Principle:**

*In vivo* plant cells, tissues and organs get their appropriate nutrient and growth requirements from the intact plant body for their organized growth and development. Isolated cell, tissues and organs also need nutrients for their *in vitro* growth and development. So, nutrients are supplied artificially according to the medium formulated by several workers. The main objective of medium preparation is to culture the cell, tissue and organ *in vitro*.

**Procedure 1**

The most suitable method for preparing media now is to use commercially available dry powdered media. The powder is dissolved in distilled water generally 10% less than final volume of medium and after adding sugar, agar and other desired supplements. The final volume is made up with distilled water. The pH is adjusted and media is autoclaved.

**Procedure 2**

Another method of preparing media is to prepare concentrated stock solutions. The use of stock solutions reduces the number of repetitive operations involved in media preparation and, the chance of human or experimental error. Also, direct weighing of media components (eg., micronutrients and hormones) required only

in milligram or microgram quantities in the final formulation cannot be performed. For these components, preparation of concentrated stock solutions by dissolving required quantities of chemicals in distilled water and subsequent dilution into the final media is standard procedure. All the stock solutions are stored in proper containers at low temperature in refrigerators at 2° - 4°C.

Stock solutions of macronutrients can be prepared at 10 times the concentration of the final medium. A separate stock solution for calcium salts may be required to prevent precipitation.

Micronutrient stock solutions are generally made up at 100 times their final strength and can be stored in a refrigerator for up to 1 year. Vitamins are prepared as 100X or 1000X suck solutions and stored in a freezer at -20°C for 2-3 months. Auxin stock solutions are generally prepared at 100-1000 times the final desired concentrations. The auxins NAA and 2.4-D are considered to be stable and can be stored at 4°C for several months, Stock solution of Iron is stored in amber coloured bottles. Substances which are unstable in frozen state must be freshly added to the final mixture of stock solution at the time of medium preparation, Contaminated (or) precipitated stock solution should not be used

pH range of 5.0 to 6.0 is suitable for *in vitro* culture. Autoclaving of nutrient media reduces pH by 0.3 to 0.5 units. plf above 6.0 leads to a hard medium while a pH less than 5.0 prevents gelling of agar Hence, it must be adjusted by adding 0.IN NaOH or HCl.

**Example: Preparation of MS medium**

**To make 1 litre of MS medium:**

i. Dissolve 30gms cane sugar in 200 ml distilled water. Mix 1-2 gm activated charcoal and filter through filter paper, set inside the Buchner funnel fitted on a special conical flask with small side arm attachment. Filtering is done by using a suction pump
ii. Take distilled water in another flask and add in sequence the appropriate amount of stock solution as follows:
    a. Desired concentrations of auxin and/or cytokinin are added from stock solution according to the formula:
    b. Desired concentration/Stock concentration = amount (mi) of stock solution to be taken for one litre medium.
    c. If the quantity of the medium is less than one litre, then hormones are added using another formula:
    d. Required concentration × Volume of medium / Stock concentration × 1,000 amount (ml) of stock solution to be added.

iii. In Pour filtered sucrose solution and salt, vitamins, amino acid, hormone solution mixture into a one litre measuring cylinder, Make the final volume to one litre with distilled water Shake well to mix up uniformly

iv. Adjust the pH of the liquid medium 5.6-5.8 with the aid of 0.1(N) HCl or 0.1(N) NaOH. This operation is done using the pH metre.

v. Add 5% to 8% agar to the liquid medium to make solid medium, Heat to 60°C to dissolve the agar completely. Otherwise, without adding agar, liquid medium can be used for culture.

vi. Dispense the culture medium into culture tube (20 ml/tube) or wide mouth conical flask (25-40 ml/flask). Insert non-absorbent cotton plug wrapped with gauge cloth. Cover the plug with the help of brown paper and rubber band.

vii. Medium is finally sterilized by autoclaving.

## Sterilization

**Principle**

The culture medium, especially when it contains sugar, will also support the growth of micro-organisms like bacteria, fungi etc. So, if they come in contact with medium either in cellular form or in spore form, the micro-organisms grow faster than the higher plant tissue due to their brief life cycle and will kill the issue. The micro-organisms may come from glass vials, instruments, nutrient medium used for culture and even from plant material itself. Therefore, the surface of plant tissue and all non-living articles including nutrient medium should also be sterilized.

**Methods**

Sterilization is the process of killing all microorganisms (bacterial, viral, and fungal) with the use of either physical or chemical agents. A disinfectant is a chemical substance that kills microorganisms on inanimate objects, such as exam tables and surgical instruments. Sterilization in the microbiological laboratory denotes sterilization process implemented in preparation of culture media, reagents and equipment where the work warrants maintaining sterile condition.

**A. Sterilization of non-living Articles**

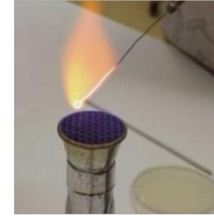
Sterilization in the laboratory is done by following methods:

1) Physical method i.e., use of heat, filters, radiation
2) Chemical method i.e., by use of chemicals

**1) Physical Methods:**

a) **Dry heat sterilization:** Inoculation loops or needles are sterilized by heating to 'red' in Bunsen burner or spirit lamp flame. Sterilization in hot air oven is performed at a temperature of 160 °C and maintained or holding for one hour. Spores are killed at this temperature and this is the most common method of sterilization of glassware, swab sticks, pestle

and mortar, mineral oil etc. Dry heat sterilization causes protein denaturation, Oxidative damage, toxic effect of elevated electrolyte in absence of water.



- **Red heat:** Sterilized by holding them in Bunsen flame till they become red hot Bacteriological loops, straight wires, tips of forceps and searing spatulas etc., are heated by this method. It is limited to those articles that can be heated to redness in flame.

- **Flaming:** This method is done by passing the article over a flame, but not heating it to redness. e.g., scalpels, mouth of test tubes, flasks, glass slides etc.
- **Hot air Oven:** Standard operating procedure for the setting up of hot air oven Pack all the glassware such as pipette with pipette can, glass petridishes, sample dish, test tubes, pestle and mortar, mineral oil to be sterilized by hot air oven sterilization with suitable wrapping Switch on the hot air oven until to reach 160°C. Hold on in that temperature for 1 hour. Switch off the heating of hot air oven and open the door once come below 65°C Standard operating procedure for the setting up of filtration. Once the bio safety cabinet is ready for filtration. Switch on the blower.

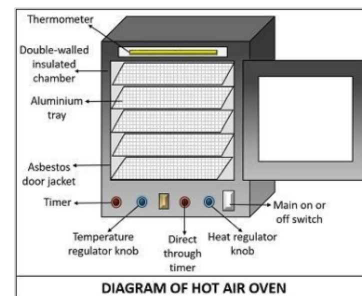


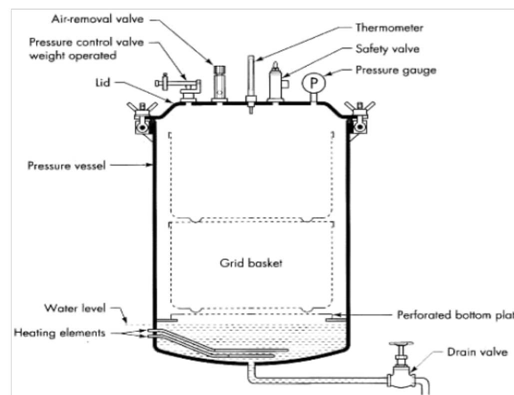
DIAGRAM OF HOT AIR OVEN

b) **Wet heat/Moist heat sterilization:** Moist heat sterilization is accomplished by following

- **Boiling:** Boiling at 100°C for 30 minutes is done in a water bath. Syringes, rubber goods and surgical instruments may be sterilized by this method. Almost all bacteria and certain spores are killed in this method.
- **Steaming:** Steaming at 100°C for 20 to 30 minutes under normal atmospheric pressure are more effective than dry heat at the same temperature because bacteria are more susceptible to moist heat, Steam has more penetrating power and sterilizing power as more heat is given up during condensation. Suitable for sterilizing media which may be damaged at a temperature higher than 100°C).
- **Tyndallization (Fractional Sterilization):** It is the steaming process performed at 100°C is done in steam sterilizer for 20 min followed by incubation at 37°C overnight and this cycle is repeated for

successive 2 days. Spores, if any, germinate to vegetative bacteria during incubation and are destroyed during steaming on second and third day Heat labile media containing sugar, milk, gelatin can be sterilized using this method.

- **Autoclaving:** It is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. This is achieved by employing a higher pressure. The autoclave is closed and made air-tight for pressure development and at 15 lbs per sq. inch pressure. 121°C temperatures will be reached and this temperature is given as sterilizing holding time for further 15 minutes. This process kills spores and this works like a pressure cooker and one of the most common methods of sterilization.



**Autoclaving Diagrams**

- **Pasteurization:** It is another one method of moist heat sterilization which works below 100°C heat. This process is used in heating of milk and other liquid food. The product is held at temperature and for a period of time to kill pathogenic bacteria that may be present in the product. This process does not destroy complete organism including spores. All these moist heat sterilization causes denaturation and coagulation of protein, breakage of DNA strands, and loss of functional integrity of cell membrane.

**c) Filtration:** This method of sterilization is used for media particularly heat labile in nature (e.g. sera an media containing proteins or labile metabolites). If the study warrants bacteria free filtrates it can be obtained through 0.45 micron sized filter membranes and if the study requires viral particle free solution, then 0.22 micron sized filter membranes are use. In earlier days absorptive liters of asbestos or diatomaceous earth were replaced by unglazed porcelain or sintered glasses are used. Nowadays these are replaced by nitrocellulose membrane filters of graded porosity, PVDF etc.

d) **Ultraviolet Radiation:** UV radiations at wavelength between 330nm and 400nm causes sterilizing effect. This method is used in surface sterilization of laminar airflow, biosafety cabinet and in certain cases in laboratory.

2) **Chemical method**

**Eg:** Phenolic compounds, halogens, alcohols, aldehydes, gases, surface active agents, oxidizing agents, dyes, heavy metals, acids and alkalis etc.

**Sterilization of Plant Material (Explant)**

**Method 1:**

Plant material which is to be cultured, should be surface sterilized to remove the surface borne microorganisms. This procedure is done in front of a laminar air flow or inside the inoculation chamber before the plant material is inoculated onto the culture medium.

The steps for surface sterilization of plant material are follows:

- **i.** Thoroughly washed plant material or explant in tap water is immersed in 5% v/v solution of liquid detergent such as "Teepol" for 10-15 minutes.
- **ii.** Then wash the material thoroughly in tap water and finally in distilled water. This step can be done in the general laboratory. Subsequent steps are done in front of a laminar air flow or the pre-sterilized inoculation chamber.
- **iii.** Dip the explants in 70% ethyl alcohol for 60 seconds.
- **iv.** Immediately transfer the material into an autoclaved jaw bottle and pour 5-10% Sodium hypochlorite (v/v) solution.
- **v.** Keep them for 10-15 minutes. During that period, the bottle is frequency swirled for shaking so that all surfaces of plant material come equally in contact with sterilant.
- **vi.** After 10-15 minutes, decant the sterilant and wash the explants thoroughly with several changes of autoclaved distilled water to remove all traces of sterilant.
- **vii.** Then the explants are ready for culture

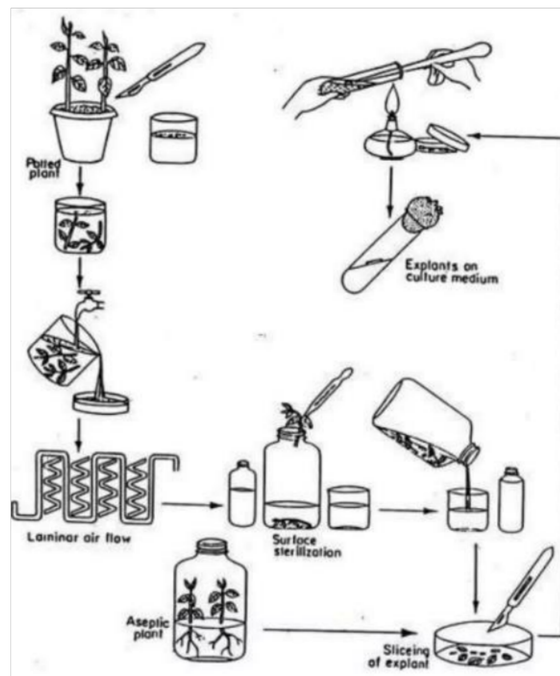



**Fig: Demonstration of surfaces sterilization of explant (leaf)**

**Method 2:**

Surface sterilization of plant tissue may cause some deleterious effect because most of the sterilants are toxic chemicals. Seeds can more or less resist such deleterious effect due to the presence of its seed coat. So to avoid the surface sterilization of plant tissue, seeds are surface sterilized and are cultured on simple basal nutrient medium. Seeds in culture germinate and give rise to an aseptic seedling. Explants from such seedlings grown under aseptic and controlled conditions are the most suitable material for culture and need no further surface sterilization.

The steps for surface sterilization of plant material are follows:

i. Wash the dry seeds thoroughly with tap water.
ii. Dip the seeds in 5% Teepol solution (v/v) for 10-15 minutes. Decant the Teepol solution and wash the seeds again with tap water and finally with distilled water.
iii. Rinse the seeds with 70% ethyl alcohol for 1 minute.
iv. In front of laminar air flow, transfer the seeds into an autoclaved bottle and pour 0.1% $HgCl_2$ solution (w/v) so that seeds are immersed. Leave for 10-15 minutes. Stir the bottle frequently.
v. Decant the sterilant and wash 3-4 times with autoclaved distilled water.
vi. Transfer the seeds from bottle to autoclaved petridish with the aid of sterile forceps
vii. Open the closure of the culture vial containing the basal nutrient medium. Flame the neek of the culture vial and in quick succession transfers a few seeds on to the medium, Replace the closure.

viii. Incubate the seeds in continuous dark either at room temperature or at 25-28°C .

ix. After a week or two, seedlings will emerge, explants taken from these seedlings will be ready made sterilized explants which can be used for inoculation.

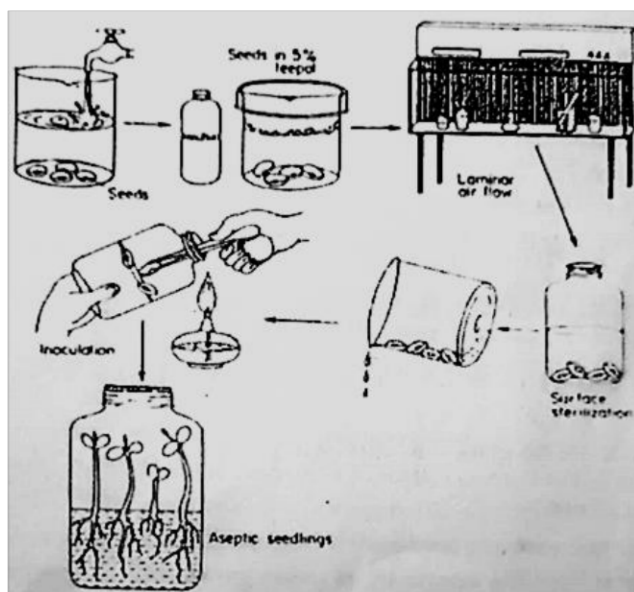



**Fig: Demonstration of surface sterilization of explant (seeds)**

**Inoculation Technique**

**Principle:**

Transferring of the surface sterilized explants on the nutrient medium is called as inoculation. Precautions must be taken to prevent the entry of any microorganism at the time of inoculation using the sterilized instruments and under aseptic conditions.

**Procedure:**

A typical procedure of transfer of explant to media or inoculation is given below:

i. Put all the sterilized articles (media, instruments, glass goods etc.) for inoculation on the glass racks of the inoculation chamber. Alternatively, if laminar air flow is available, keep all articles on the table of air flow cabinet. Laminar air flow blows bacteria- free air over the working surface.

ii. Put on the switch of UV lamps of inoculation chamber for one hour before work. In case of laminar air flow, the power switch is put on and allows the air flow to blow air for at least 15 minutes before work.

iii. Put off the UV lamp before entering inside the inoculation chamber. Do not put off laminar air flow

iv. The working glass table top of the inoculation chamber or the table of laminar air flow is swabbed with alcohol before starting work.

v. Wear a clean apron and use a mask. Clean the hands with alcohol and dry it.
vi. Pour alcohol in a clean coupling jar and dip all instruments into it. Light the spirit lamp. Take the surface sterilized or aseptic plant material in a, sterile petri dish.
vii. Flame the neck of culture tube or flask and in quick succession remove the plug of glass vials. Transfer the tissue onto the medium and replace the closure. Each time, the instruments are passed through the flame of the spirit lamp.

**Precautions to be taken**

- Always keep away the hands moistened with alcohol from the spirit lamp. So dry the alcohol first.
- Exposure to UV light builds up a high concentration of Ozone gas (toxic) inside the closed chamber. It is, therefore, healthy to enter the chamber only 15-30 minutes after switching off the UV lamp.
- Do not dip hot instruments in alcohol and don't use hot instrument for cutting or holding the plant material.
- Work carefully and try to ensure that media and plant tissues are exposed for the plant material.
- Don't heat the neck of the glass vials excessively.

**Incubation of the culture**

**Principle:**

High temperatures are likely to lead to dissociation of the culture medium and tissue damage while at very low temperatures tissue growth is well in dark while others need both light arid dark conditions. Desiccation of culture medium and high humidity is favourable medium. Therefore, cultures are incubated in a culture room humidity are controlled.

**Procedure**

i. After inoculating the tissue onto the culture medium, cultures are incubated on culture rack at 25-28°C constant temperature.
ii. Culture tubes are placed at 30-45° inclined position. For this purpose a long wooden stick is placed on the middle of culture rack and lay the plugged end of the culture tube on the support.
iii. Illumination is provided by cool-white fluorescent light placed about 18 inches above the culture to give a light intensity of 4–10x $10^3$ lux for 16 hours.
iv. The relative humidity of the culture room is maintained above 50%.
v. If light is not necessary, then put off the light and cover the whole rack with a block cloth.

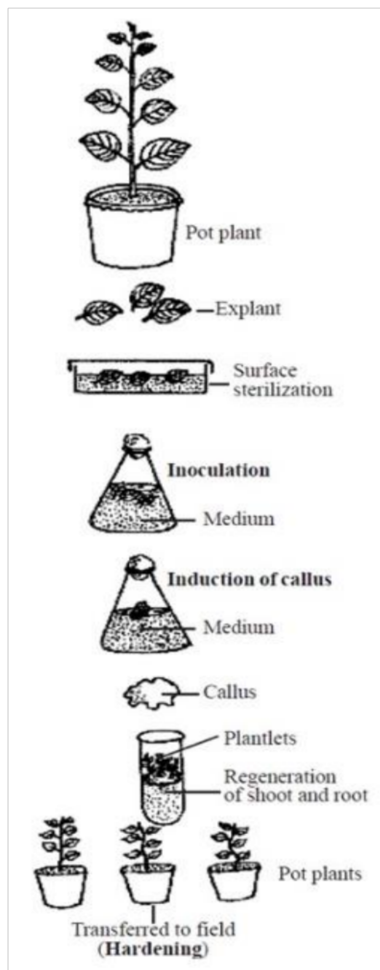



**Fig: Basic techniques used in plant tissue**

**Short Questions:**

1. Explain Autoclaving mode of Sterilization
2. Filtration method of sterilization

**Essay Questions**

1. Explain the various physical methods of sterilization of Plant tissue culture medium.
2. Schematically explain in detail the complete procedure for sterilization of plant explant.

## 1.5 INDUCTION OF CALLUS AND CELL SUSPENSION CULTURES

**Induction of callus or callus culture**

Callus refers to unorganized mass of undifferentiated cells. Callus is formed by the proliferation of the parent tissue. The cells of a callus are parenchymatous,

amorphous and unorganized. Generally callus is formed as a result of injury at the cut ends of a stem or a root.

When tissues on culture medium produce unorganized mass of cells with no regular form then it is called 'callus culture'. In some cases, it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis. Genotype, composition of nutrient medium and physical growth factors influence the formation of callus. The size and shape of the explants is also important.

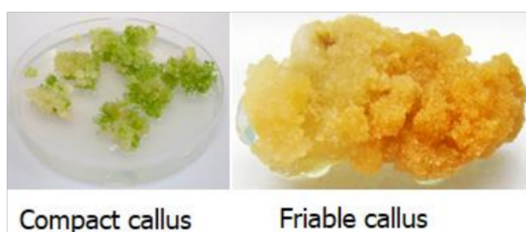
**History**

Callus formation from isolated stem segments of *Populous* was first observed by Rechinger in 1893. Working with cambial tissues of carrot and tobacco first prolonged callus culture were simultaneously reported by Gautheret in Paris. Nobecourt in England and White in Princeton, USA in 1930.

Gautheret cultured explants of carrot root on a medium containing inorganic salts, sugar (glucose), thiamine, cystine and IAA. On culture the explants grew forming undifferentiated tissue or callus. By repeated sub-culturing this callus was maintained for a prolonged period. White (1939) cultured the stem pro-cambium of hybrid *Nicotiana glauca × N- langsdorfii* on agar medium where callus was formed.

**Characteristics of Callus in Tissue culture**

- **Types:** Callus differs in compactness or looseness, i.e. cells may be tightly joined and the tissue mass is one solid piece and called as "compact callus" or cells are loosely joined and individual cells readily separate and are called as 'friable callus'. This can be due to the genotype or the medium composition. A friable callus is often used to initiate a liquid cell suspension culture and also a source of protoplasts. Both the types are sometimes interchangeable.
- **Colour:** It varies from culture to culture and usually depends on the colour of the tissue from which it is taken. The callus may be colourless or green containing chlorophyll or yellow having carotenoids or flavonoids or it may be purple due to the presence of anthocyanin. Callus may be uniformly or partially coloured.
- **Anatomy:** Callus may be homogenous, consisting of uniform parenchyma cells of it may be heterogenous, having differentiation into tracheid's, sieve tubes, trichomas, secretory cells and suberized cells etc. Callus which grows actively may be composed of large vacuolated parenchyma cells and small closely arranged dividing cells. Such variations among the cells of the callus tissue are due to the origin and age of the callus and the composition of the culture medium.


Compact callus Friable callus

- **Cytology:** Callus on prolonged culture shows changes in the nuclear cytology of the cells. Changes both in chromosome structure and chromosome number have been noted. Chromosomal aberrations, nuclear fragmentation or endopolyploidy etc. are observed.

**Requirements for callus culture**

**Selection of explant**

Callus may initiate from explants of any multicellular plant. Explants from stem, root, leaf, flower, fruit or seed etc. may be taken for culture. Callus formation has been recorded from storage parenchyma, pericyclic cells of roots, cambial cells of vascular bundles, provascular cells, secondary phloem, pith cells, mesophyll cells and cotyledons. Size of the explants depend on the average size of the tissues to be cultured. Usually large pieces of tissue are selected for culture.

**Nutrient medium**

Some standard media, such as, MS medium can be successfully used for callus culture. For initiation and maintaining callus kinetin is widely used in the medium. For callus initiation usually an exogenous supply of hormone is required. But explants having cambial cells of not require a supply of hormone. According to hormone requirements callus culture may be of five types. Auxin requiring cultures. Cytokinin requiring cultures,. Cultures requiring both auxin and Cytokinin, Gibberellin requiring cultures and cultures requiring other natural extracts, such as, yeast extract, coconut milk, casein hydrolysate or tomato juice etc.

**Procedure**

- Explant is first washed with liquid detergent like 5% Teepol and fun surface sterilized with 1.6% sodium hypochlorite solution of 1% aqueous solution of bromine for 10 min and rinsed sterile distilled water.
- Then the inner uncontaminated issue is excised. If the tissue (such as, root hypocotyl, cotyledon etc.) is taken from a seedling then the seed before germination is surface sterilized and allowed to germinate under aseptic conditions.
- The sterile explants are inoculated aseptically on a nutrient medium.
- Incubation of culture material done under controlled physical conditions. Generally 50-60% relative humidity and 25±2 °C temperatures are ideal for callus induction
- Dark or light condition is provided depending on the nature of plant

**Stages of callus formation**

Callas formation from explants occurs in three stages

i. **Induction stage:** Metabolism is stimulated and the cells prepare to divide. Cell size remains unchanged.
ii. **Cell division stage:** Cells divide actively and the cell size decreases. Cell division is mainly periclinal and occurs towards the periphery giving rise to wound cambial cells.
iii. **Differentiation stage:** Cells differentiate by expansion and maturation. Rapidly growing calluses are more or less alike but as the growth rate decreases the calluses show their characteristic structures and forms.

**Sub-culturing of callus**

Callus is sub-cultured by moving a fragment of callus from initial inoculum to fresh medium in another vessel. Sub-culturing is required to produce more callus or to maintain the callus Generally after 4-6 weeks, sub-culturing can be done.

For sub-culturing the inoculum should not be very small, as very small inoculum fails to grow or shows little growth. The inoculum should be about 0.5-1 cm and weighing about 20 100 mg. Active growth can be maintained even after several subcultures. Repeated sub-culturing can be avoided by freeze preservation of the culture.

**The callus is sub-cultured because:**

- The nutrient may be exhausted.
- Agar may be desiccated, or
- Cell metabolites may accumulate and cause toxicity.

**Protocol for callus culture from carrot roots**

Generally, different plant tissues of many plant species can be used to induce callus formation but carrot rots are highly specific and it is taken as typical example for callus culture.

The following are steps taken in callus culture of carrot root:

**i.** Fresh and healthy carrot root washed thoroughly in running tap water.
**ii.** External 1-2 mm is scraped. Upper 1 cm of carrot root is discarded and then it is cut into 0.5 cm thick slices.
**iii.** These slices are placed immediately in a beaker containing water.
**iv.** These are then transferred to a beaker containing sodium hypochlorite solution and kept there for 10 minutes.
**v.** Slices are taken out with a sterile force from the hypochlorite solution and washed successively in 3 beakers containing double distilled water keeping the slices for 20-30 seconds in each the slices are kept in the third beaker.
**vi.** A carrot slice is taken and is placed on a petri dish. Tissue cylinders are cut out from the cambial region by a sterilized cork borer, after cutting

maximum number of tissue cylinders from the cambial region remaining portion of the slice is discarded

**vii.** Tissue cylinders are placed in a petridish containing double distilled water.

**viii.** A tissue cylinder is transferred to a petridish and its two sides are trimmed with a sterile scalpel and discarded.

**ix.** Remaining cylinder is cut into explants measuring 5 mm diameter and 2 mm thickness.

**x.** These explants are placed in a petri dish containing double distilled water.

**xi.** Explants are then transferred with a sterile forceps on the surface of a sterile filter paper on a petri dish. The upper and lower surfaces of each explants are blotted.

**xii.** One such explant is transferred to each culture tube containing the nutrient medium.

**xiii.** Culture tubes are kept in a glass storage jar, wrapped in aluminium foil and placed in an incubator at 25°C.

**xiv.** The surface of the explants after few days becomes somewhat rough, indicating initiation of the callus. Callus can be maintained from few weeks to three months depending on the rate of growth.

**xv.** Generally after 6-8 weeks the callus is sub-cultured. The callus is divided into small parts of 100 mg approximately.

**xvi.** Each piece is transferred to a new flask containing 30 c.c. of culture medium and sub- cultured at a temperature of 25°C or above.

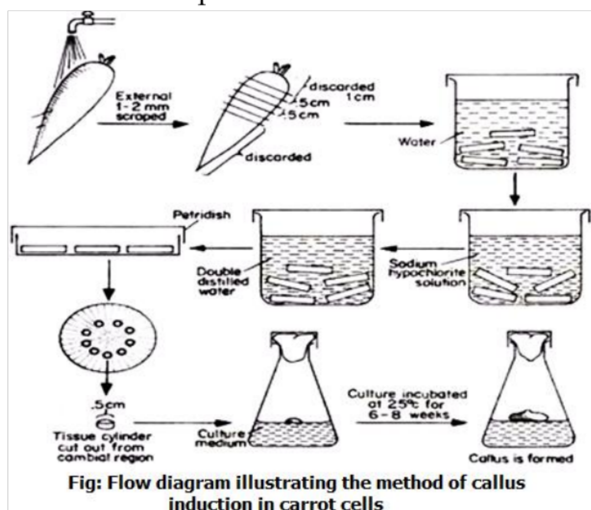



**Fig: Flow diagram illustrating the method of callus induction in carrot cells**

**Application of callus culture**

**i.** The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium which is called as organogenesis or morphogenesis. Similarly, callus can be induced to form somatic embryo which can gives rise to whole plant.

ii. Callus tissue is good source of generic or karyotypic variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

iii. Cell suspension culture in moving liquid medium can be initiated from callus culture.

iv. Callus culture is very useful to obtain commercially important secondary metabolites. If a bit tissue from a medicinally important plant is grown in vitro and produced callus culture, then secondary metabolites or drugs can be directly extracted from the callus tissues without sacrificing the whole plant.

v. Several biochemical assays can be performed from callus culture.

**Induction of Cell Suspension Cultures**

A plant cell suspension culture is a liquid culture where cells from a soft or friable callus divide and multiply. The culture is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters.

In a plant cell suspension, the cells divide, multiply and grow. Plant cell suspension cultures are used to obtain secondary metabolites in pharmaceuticals, cosmetics, or used in the food industry. Researchers use plant cell suspension cultures to analyze metabolic pathways for basic science and even knockdown experiments.

A cell suspension culture refers to culturing of cell aggregates which are dispersed and growing in a moving liquid media. It is normally initiated by transferring pieces of undifferentiated and friable callus to a liquid medium, which is continuously agitated by a suitable device.

The cells are suspended in the liquid culture by constant agitation by keeping in a gyratory shaker at 100-250 rpm which facilitates aeration and dissociation of cell clumps into smaller pieces. A good suspension culture is one which consists of a high percentage of single cells along with small cluster of cells. Orbital shakers are widely used for the initiation and serial propagation of plant cell suspension culture. They should have a variable speed control (30-150 rpm) and the stroke range should be of 4-8 cm orbital motion. Suspension cultures grow much faster than callus cultures and it should be sub-cultured about every week.

**Procedure**

There are five steps to establishing a cell suspension:

i. **Prepare the explant:** Plant material is critical when it comes to establishing a cell suspension. Cell suspensions can be raised from leaf discs, seed fragments, meristems, stem pieces, mature or immature embryos, anthers, pollen, microspores and ovules, flowers, or root sections.

ii. **Sterilize and isolate explants:** Once the explant, has been selected the tissue needs superficial disinfection to remove contaminants that can be introduced into the plant tissue culture. The contaminant removal is essential because microbes grow faster than the plant tissues in a culture medium, exhausting the nutrients of the media. The disinfection can be done with disinfectants like sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride ($HgCl_2$). Once the explant is disinfected, it is generally cut to induce wounding and transferred into a solid medium to induce callus production.

iii. **Initial stage of cell suspension:** The calli formed over plant tissues are generally compact. After successive sub culturing of the calli from old solid media to fresh media, the calli become friable. The friable calli are ideal for establishing a cell suspension because their texture favors nutrient uptake. Also, cells can be easily distributed over the liquid medium. After calli become friable, they are transferred from a solid medium to a liquid.

iv. **Grow the cell suspension:** In this step, the cells from the calli in liquid media follow a growth curve, where there are lags, exponential and stationary phases. The friable calli is adapted to the new liquid environment in the lag phase. In the exponential phase, cells grow and multiply until they reach the stationary phase, when the cell growth stops.

v. **Metabolite's production:** This step is adjusted according to the research goals. Cell suspensions can be used for basic science, such as studying the behavior of a given metabolite or protein in a liquid medium or for massive production of metabolites.

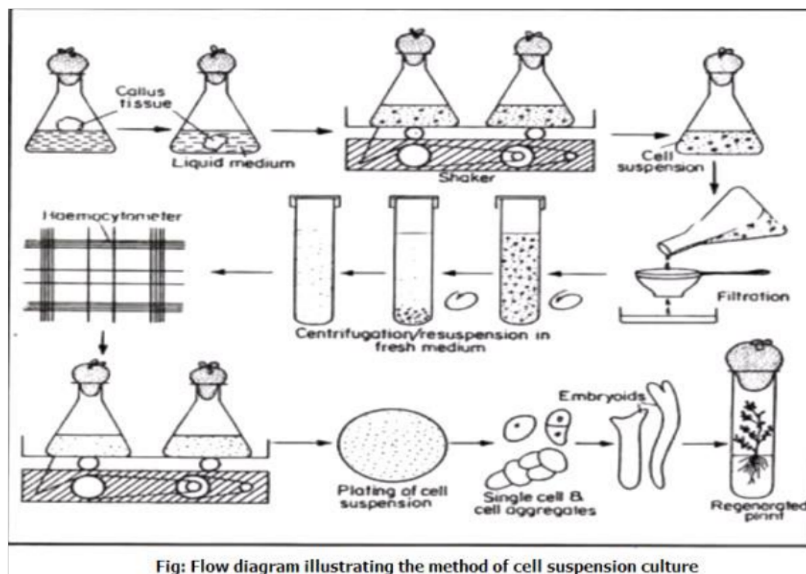



**Fig: Flow diagram illustrating the method of cell suspension culture**

The metabolite's production in cell suspensions is commonly a two-step process. In the first step, the cell suspension needs to grow enough to provide mature cells that can produce metabolites.

Then, the metabolite production is induced in a second step. Modifying the liquid media with specific reagents (e.g. coconut water, jasmonic acid) or changing environmental conditions (such as light and temperature is typically used to stimulate metabolite production.

**Measurement of growth in cell suspensions**

A plant cell suspension's growth can be measured by following methods:

i. **Fresh weight:** Fresh weight is a measurement of growth for a cell suspension and requires you harvest cells from a cell suspension every so often to determine the fresh weight of cells per volume of cell suspension

First, mix the cell suspension culture well and collect cells of cells with the help of a pipette daily or every two days, according to the experimental setup and volume of cell suspension culture. Place the volume on a pre-weighted container and place the container on a bench scale and take the value. Then, calculate the difference between the data for the pre-weighted container and the container plus the fresh sample. The resulting data will be the measure of fresh weight in the cell suspension.

ii. **Dry weight:** Dry weight is a measurement of growth on a cell suspension where dried cells are weighted every so often to determine the dry weight of cells per volume of cell suspension.

First, mix the cell suspension culture well and collect cells of cells with the help of a pipette daily or every two days, according to the experimental setup and volume of cell suspension culture Proceed to dry the cells in an oven to low temperature (~45°C) or use a freeze drier. Place the dried cells on a pre-weighed container, and put the container on a bench scale, and take the value. Then, calculate the difference between the data for the pre weighed container and the container plus the dry sample. The resulting data will be the dry weight in the cell suspension.

iii. **Packed cell volume (PCV):** The Packed cell volume (PCV) is a measurement of the growth non-invasive in cell culture. It consists in decanting the cells within a flask, marking the top of the decanted cells and proceeding with a rule to measure how high is the packed cell volume

First, allow the cells to decant within the flask. Slightly tilt the flask and make a mark with a marker pen. Then use a ruler to measure the cell's height and write down the data in your notebook. Repeat the process every sampling day. At the end of the sampling, you will notice how much your cell suspension grew.

iv. **Medium conductivity/osmolality:** The medium conductivity is a measurement of the growth in plant cell suspensions. Conductivity is a measure of the ability of water to pass an electrical current. Cells absorb

nutrients (present in the form of salts) from the medium, and then as the cells take nutrients from the medium, the electrical conductivity decreases.

The medium conductivity is calculated using a conductivity meter. Introduce the conductivity meter in the cell suspension, take note of the value and record the data every sampling day.

In the end, you can plot the data and expect a decreasing conductivity curve which indicates the cells are absorbing the nutrients of the medium and growing.

**Short Questions**

1. Applications of callus culture.
2. Write about the various characteristics of callus culture.

**Essay Questions**

1. Describe in detail the callus induction.
2. Explain in detail steps involved in suspension cell culture highlighting on the applications.

## 1.6 ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

*In vitro* plant regeneration is the process of growing plants using tissue culture techniques. Plant regeneration can be achieved by culturing explants to form organs (organogenesis), or by developing plant embryos (somatic embryogenesis). Both techniques can lead to regenerating entire plants.

**Organogenesis**

It refers to the formation of plant organs such as roots and shoots directly on the explant which lacks a preformed meristem or de novo origin from callus and cell suspension culture induced from the explant

**Phases of organogenesis**

Organogenesis can be split into three phases

- In the first phase, cells in the explants acquire 'competence' which is defined as the ability to respond to hormonal signals of organ induction, During this phase, the process leading to organ genic competence is called dedifferentiation where differentiated cells become undifferentiated.
- During the second phase, competent cells in cultured explants are destined and determined for specific organ formation under the influence of the phytohormones balance.

- Lastly, the third phase is where morphogenesis proceeds independently of the exogenously supplied phytohormones. It means, plant organs take the shape of roots or shoots when phytohormones are removed from the culture medium.

**Types of organogenesis**

Organogenesis is of two types namely

i. **Direct organogenesis:** Direct organogenesis or Adventitious regeneration refers to the development of organs such as roots, buds, shoots, flowers etc., or embryo like structures on an explant directly, bypassing the callus stage. The shoots or roots are induced on tissues that normally do not produce these organs. This pathway is less common.

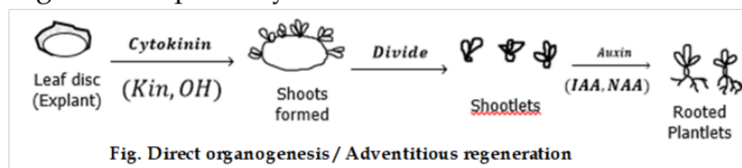



Fig. Direct organogenesis / Adventitious regeneration

ii. **Indirect organogenesis:** In this pathway, explant gives rise to initiation of callus, which is an unorganized mass of undifferentiated cells, from which shoots and roots are formed. Hence, it is called as indirect organogenesis. The callus consists of an aggregation of meristem-like cells that are developmentally plastic. This process involves formation of callus from a matured explant (by dedifferentiation) and formation of various organs from the callus or adventitious meristems (by re-differentiation).

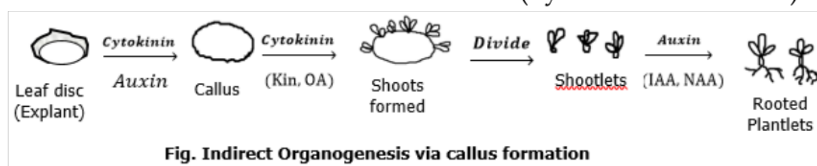



Fig. Indirect Organogenesis via callus formation

It is made possible by altering the concentration of plant growth hormones in the nutrient medium. Skoog and Muller demonstrated that high ratio of cytokinin to auxin stimulated the formation of shoots in tobacco callus while high auxin to cytokinin ratio induced root regeneration. Thus the organ formation depends upon the ratios rather than the absolute concentration of auxin and cytokinin. The negative side of this method is that it introduces mutations *in vitro* (somaclonal variations). The callus phase also makes it more technically challenging than shoot tip micropropagation.

**Factors influencing the in vitro organogenesis**

There are three primary factors influencing in vitro organogenesis. Among these factors, organogenesis depends mainly on the balance of auxins and cytokinins and the tissue's ability to respond to phytohormones during specific culture conditions.

i. **Explant:** A broad range of explants has been used to induce organogenesis. They include leaf disks, petioles, stems, peduncles, stipules, roots, embryos, sepals, and protoplasts. The optimal explant in organogenesis is usually adjusted for each plant species.

ii. **Plant growth regulators:** The kind of plant growth regulators (PGRs) and the amount used for plant regeneration varies considerably. In general, a combination of auxin and cytokinin is necessary for successful regeneration. In recent years, the potent cytokinin TDZ has been commonly used as a unique PGR in the medium favoring the shoot regeneration.

iii. **Culture conditions:** Light is essential in influencing organogenesis, mainly for shoot regeneration, In exceptional cases, darkness favors in vitro organogenesis, as is the case of strawberry shoot

**Applications of Organogenesis**

Organogenesis has been used mainly for applications like:

- **Plant multiplication:** This application is ideal especially for clonal propagation. As there is more chance to induce direct organogenesis, desirable traits can be preserved from the mother plant. Transgenic plants produced from organs induced in vitro also retain a great potential to be faithfully cloned if direct organogenesis is promoted.
- **Germplasm preservation:** Bud or shoots can be stored using gelling agents, to preserve and delay the developmental process for the organs. The organs can be later released from the gelling agents to continue the regeneration process.

**Somatic Embryogenesis**

Normally a zygote is formed after an egg has been fertilized by a sperm. The zygote then develops into an embryo (zygotic embryo). The process by which the embryos are regenerated from somatic cells, issues or organs is called as somatic embryogenesis. Such embryos are also called as non-zygotic embryos.

Somatic embryogenesis is the opposite of zygotic or sexual embryogenesis. Somtic embryos are formed from a single cell and it requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant. The bipolar structure of the somatic embryo contains both shoot and root meristems. The embryos develop by forming structural steps of the globular, heart, torpedo, cotyledonary and mature stages. While in organogenesis, it requires two different hormonal signals to induce first a shoot organ and then a root organ. No endosperm or seed coat is formed around a somatic embryo.

**Types of somatic embryogenesis**

Somatic embryos could be induced either directly from the explant tissue by passing the callus formation stage or via the formation of callus from the explant. It is of two types:

i. **Direct somatic embryogenesis:** It can be initiated directly from the explants through "pre-embryogenic determined cells." Such cells are found in embryonic tissues of scutellum, hypocotyls and nucellus.
ii. **Indirect somatic embryogenesis:** It is done through the establishment of callus from which embryos are developed. Here the embryo arises from "induced embryogenic determined cells."

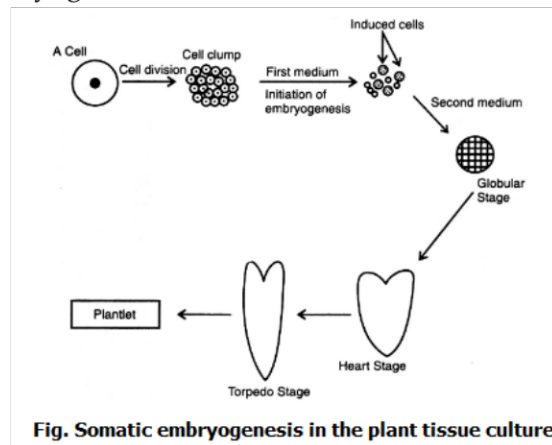



**Fig. Somatic embryogenesis in the plant tissue culture**

**Phases of somatic embryogenesis**

Somatic embryogenesis can be split into four phases:

i. The first phase in where embryogenic masses (callus retaining cells with embryogenic competence) initiate from vegetative cells or tissues.
ii. In the second phase, embryogenic cell lines (a group of cells with an embryogenic fate) are maintained and developed.
iii. The third phase involves somatic embryo formation (embryo undergoes globular, heart shaped, torpedo and cotyledonary stages) and maturation (accumulation of reserve substances).
iv. Somatic embryos are converted (germinated) into viable plantlets.

**Factors influencing somatic embryogenesis**

There are five factors influencing somatic embryogenesis explant, plant growth regulators, minor components, culture conditions, and embryo maturation.

i. **Explant:** The type of explant and the age of the explant can have an impact on the success of somatic embryogenesis. Young explants especially yield more somatic embryos than older explants. Different explant tissue (root, flower, shoots, etc.) from the same mother plant produce embryogenic calli at different frequencies.

ii. **Plant growth regulators:** If media is supplemented with auxins, it can help promote callus proliferation and inhibit differentiation. And if auxins are decreased, this will help the development of somatic embryos. Auxins are also responsible for establishing cell polarity in the embryo (apical and basal axis). Although cytokinins are also suitable candidates for induction of somatic embryogenesis, fewer cytokinins are used to stimulate somatic embryogenesis.

iii. **Minor components:** Amino acids like glutamine, proline, tryptophan, polyamines (putrescine, spermidine), and brassinosteroids (e.g., 24-epibrassinolid) have been reported to enhance somatic embryogenesis in some species.

iv. **Culture conditions:** Light condition is a critical aspect for somatic embryogenesis. For instance, white light enhances growth but at the same time increases the production of phenolic compounds and Abscisic acid levels. These substances induce oxidative reactions generating browning of the tissues.

v. **Embryo maturation:** Maturation is regarded as a crucial stage of embryogenesis Maturation is a preliminary stage for embryo development, which is essentially required for effective germination. One critical factor in embryo maturation is water loss. Most of the tissue culture treatments promoting the maturation of the embryo use osmoticums (osmotic stress inducers) like sugars.

**Applications of Somatic embryogenesis**

Somatic embryogenesis has been used in applications such as:

- **Cell selection:** As the origin of the embryos can be unicellular, a cell line can be derived from a single cell.
- **Plant multiplication**: SE is the preferred choice technique to propagate plants, especially genetically transformed plants (by *Agrobacterium* and other transformation protocols)
- **Somatic hybrid regeneration:** Through protoplast fusion, two genetic materials can be joined to produce a plant with better traits. Here, SE is also med to regenerate plants from these hybrid protoplasts.
- **Regeneration of homozygous plants:** plants can he regenerated from pollen or ovules as explants. It produces homozygous plants (plants derived from germinal lines)
- **Germplasm preservation:** Somatic embryos can be stored as synthetic seed (using gelling agents), to preserve the embryo and resembling the seed structure (embryo plus nutritional substances).
- **Virus elimination:** As an embryo can be derived from non-vascular tissues usually virus transport around there) and the origin can be unicellular, plants produced in these conditions can be free from virus.

**Short Questions**

1. Factors influencing somatic embryogenesis.
2. Applications of somatic embryogenesis
3. Applications of Organogenesis.
4. What are the four phases of somatic embryogenesis.

**Essay Questions**

1. Describe in detail the process of somatic embryogenesis and mention about the various applications of Somatic embryogenesis.
2. Explain in detail steps involved in suspension cell culture highlighting on the applications.
3. Write in detail about the process of Organogenesis and state the various factors that affect the process of organogenesis.

# UNIT II APPLICATIONS OF PLANT TISSUE CULTURE

## 2.1 MERISTEM CULTURE, MICROPROPAGATION AND THEIR APPLCIATIONS

**Meristem Culture**

Meristem culture refers to regeneration of whole plant from tissues of an actively dividing plant part such as stem tip, root tip or axillary bud. The apical meristem refers to dome like extreme shoot tip of 0.25 to 0.30 mm in length and 0.1 mm in diameter. To grow virus free plants meristem tips of 0.2-0.3 mm is used. For shoot tip culture large explants measuring up to 2 cm in length is used. This technique is widely used in vegetatively propagated plants such as sugarcane, potato, banana and several timber species.

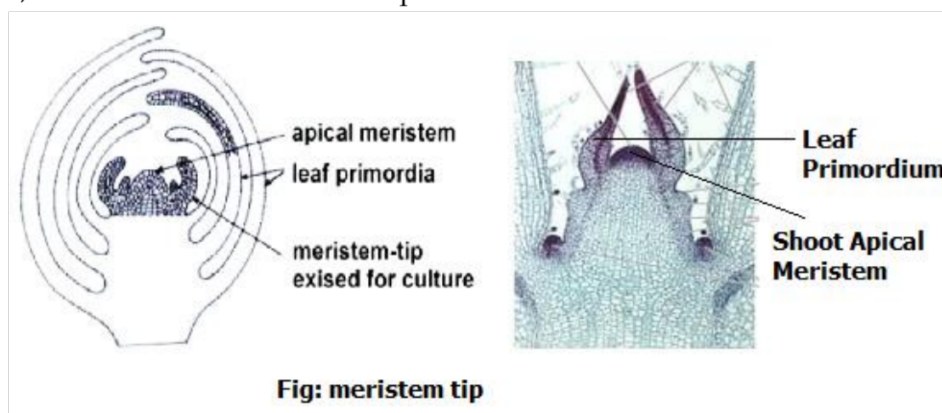



Fig: meristem tip

Morel and Martin (1952) isolated 100μm long meristem from virus – infected plants, and cultured them to obtain virus – free shoots in dahlia. Plants free from viruses, viroid's, mycoplasma and even fungi and bacteria in a range of crops can be accomplished by this technique. Virus free clones of potato, sugarcane have been produced from valuable virus infected stocks through meristem culture.

**Procedure**

1) Dissect out the shoot apical meristem (100-500 μm in length) with one or two leaf primordia.
2) The larger the meristem explant, the greater the chances of its survival and shoot development. But the risk of infection by the virus also increases with explant size. Therefore, a compromise has to be reached between these two opposing forces in deciding the explant size

3) Viruses are eliminated by thermotherapy of whole plants, in which plants are exposed to temperatures between 35-40°C for a few minutes to several weeks depending on the host virus combination.
4) In general, it is preferable to excise larger shoot-tips from heat-treated plants. Also, cultured meristems may also be given thermotherapy
5) A prolonged exposure to a low temperature (5°C), followed by shoot-tip culture, has also proved quite successful in virus elimination. This technique is called cryotherapy.
6) Some chemicals, e.g., virazole (ribavirin), cyclohexamide, actinomycin D, etc., which interfere with virus multiplication, may be added into the culture medium for making the shoot-tips free from Viruses, this is known as chemotherapy.

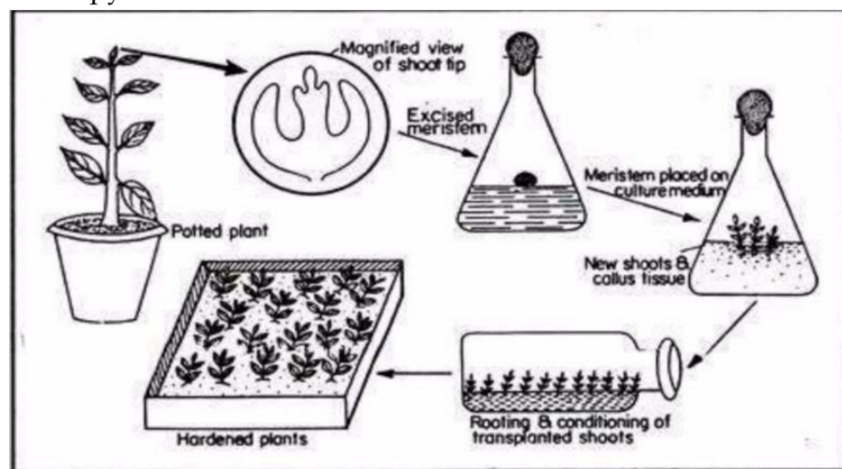



**Fig: Flow diagram illustrating the technique of meristem culture (shoot tip)**

**Applications of meristem culture**

**1)** Virus free plants can be obtained through this technique as meristematic cells remain free from virus even in the virus infected plants.
**2)** Useful in germplasm exchange of asexually propagated plant species as the plantlets obtained by meristem culture are free from pathogens.
**3)** Meristems are suitable for cryopreservation by storing the germplasm at - 196° C in the liquid nitrogen for long period of time.
**4)** Virus-free plants serve as excellent experimental materials for evaluating the detrimental effects of infections by various viruses.
**5)** The virus free bulbs grew more rapidly, plants were more vigorous, and they produced a greater number of larger flowers that had richer colour than the virus infected stock.
**6)** The virus-free plants are deliberately infected by known viruses to study the effects of the infection on performance of the host.
**7)** Meristem culture can also help eliminate other pathogens like mycoplasma, bacteria and fungi. Bacteria and fungi present in explants show up when they

are cultured *in vitro* since tissue culture media provide excellent nutrition for the microbes.

**Micropropagation**

Propagation of plants vegetatively by cutting, budding, grafting etc., involves only mitotic cell division. The progeny obtained by vegetative propagation of a single plant is called as a clone. Tissue culture also enables rapid clonal propagation of plants. *In vitro* clonal propagation of plants by tissue culture is called as "micropropagation"

The process involves selection of plant tissues (explant) from a healthy and vigorous mother plant. Any part of the plant such as leaf, apical meristem, bud and root can be used as explant.

**Pathways for Micropropagation**

The main objective of micropropagation is to produce progeny plants which are identical to the parent plants in genotype. This is achieved by three pathways: (i) Proliferation from pre-existing meristems (Axillary bud proliferation) (ii) Organogenesis and (m) Somatic embryogenesis

**1) Proliferation of Pre-Existing Meristem/Axillary Bud Proliferation**

- This method makes use of already existing meristem to initiate *in vitro* culture (e.g. shoot-tip nodal explant). The merit of using axillary bud proliferation from a node or bud is that, the shoot has already differentiated and only its elongation and root differentiation are required. The size of the shoot tip ranges between 1 and 10 mm in length.
- Shoot tips are easy to excise from the plant. They are genetically stable and have high survival and growth rates. They contain preformed incipient shoot and are phenotypically homogeneous. Axillary and terminal buds also have the advantages of shoot tips but they are more difficult to disinfect.
- Cytokinin in the media stimulates the pre-existing meristem in the explant (apical meristem in shoot tips and axillary buds in nodal explants) to develop into shoots.
- Each leaf on such shoot has an axillary bud which are sub-cultured after 4-6 weeks onto a fresh medium.
- In most plant species, each explant produces 5-6 shoots in 4-5 weeks which would result in 510 to 612 plants in one year from a single explant, assuming 100% survival.
- In some species, (Eg. Blueberry) when the axillary buds do not produce new shoots then the shoot bud developed from the explant is excised and cut into small pieces to obtain nodal explants which are then subcultured to

initiate a new cycle of micropropagation. This is termed as 'single node culture

**Protocol**

The following stages are involved in micro propagation:

- **Stage 0:** This is the initial step in micro propagation and involves the selection and growth of stock plants for about 3 months under controlled conditions.
- **Stage 1:** In this stage, the initiation and establishment of a culture in a suitable medium are achieved. Selection of appropriate explants is important. The most commonly used explants are organs, shoot tips and axillary buds. The chosen explant is surface sterilized and washed before use.
- **Stage 11:** It is in this stage, the major activity of micropropagation occurs in a defined culture medium. Stage II mainly involves multiplication of shoots or rapid embryo formation from the explant. A growth chamber set at 20-24 °C is used, with a 2000 to 4000-lux light intensity, and a lighting period of 16 hours or so.
- **Stage III:** This stage involves the transfer of shoots to a medium for rapid development into shoots. Sometimes, the shoots are directly planted in soil to develop roots. *In vitro* tooting of shoots is preferred while simultaneously handling a large number of species
- **Stage IV:** This stage involves the establishment of plantlets in soil and is called "hardening”. This is done by transferring the plantlets of stage III from the laboratory to the environment of the greenhouse for 'acclimatization". For some plant species, stage III is skipped, and un- rooted stage II shoots are planted in pots or in the suitable compost mix.

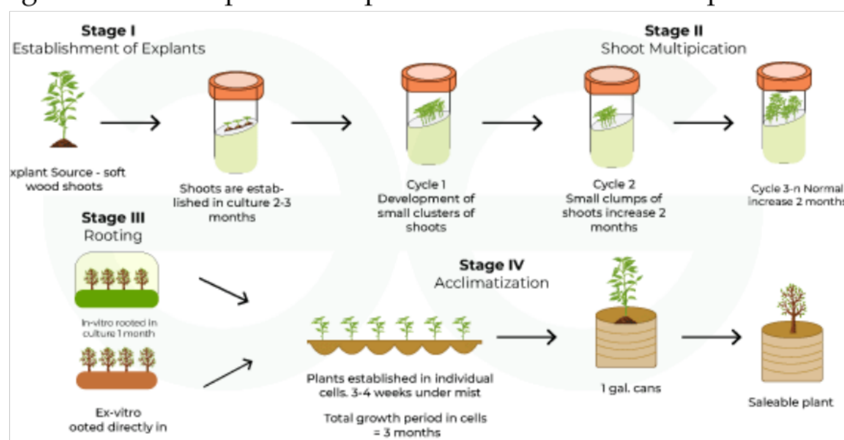



**Fig: Stages involved plant tissue culture during micropropagation**

## Types of Micropropagation techniques

- Micropropagation techniques are of three types based on the way of propagation, first, the propagation from shoots with cytokinin like

benzyladenine or kinetin; second, multiple shoot differentiation from dedifferentiating tissue, callus, with an auxin-like indole acetic acid; and finally, the embryo differentiation from callus.

- The former two methods need the rooting process with an auxin-like indole acetic acid and with naphthaleneacetic acid thereafter.
- Nowadays, the method of propagation from shoots is the most preferred one, because the latter two methods present the possibility of genetic variation owing to the dedifferentiated phase, callus.

**Application of Micro Propagation**

- Production of large number of genetically uniform plants.
- A small explant is enough to produce millions of true to type plants.
- Rapid multiplication of rare and elite genotypes in a small area.
- This technique is possible alternative in plants species which do not respond to conventional bulk propagation technique
- In plants with long seed dormancy, micro propagation is faster than seed propagation.
- Useful to obtain virus free stocks
- In dioecious species plants where one sex is more desirable than the other sex. Eg:- Male asparagus and Female papaya etc. In such cases plants of desired sex can be selectively multiplied by micropropagation.
- This technique is carried out throughout the year independent of seasons.
- Plant tissue in small amounts is sufficient for the production of millions of clones in a year using micropropagation. It would take a great deal of time to produce an equal number of plants using conventional methods.
- The technique of micropropagation provides a good alternative for those plant species that show resistance to practices of conventional bulk propagation.
- An alternative method of vegetative propagation for mass propagation is offered through micropropagation. Plants in large numbers can be produced in a short period. Any particular variety may be produced in large quantities and the time to develop new varieties is reduced by 50%.
- Large amounts of plants can be maintained in small spaces. This helps to save endangered species and the storage of germplasm
- The micropropagation method produces plants free of diseases. Hence, disease-free varieties are obtained through this technique by using meristem tip culture.
- The proliferation of *in vitro* stocks can be done at any time of the year. Also, a nursery can produce fruit, ornamental, and tree species throughout the year.

- Increased yield of plants and increased vigor in floriculture species are achieved.
- Fast international exchange of plant material without the risk of disease introduction is provided. The time required for quarantine is lessened by this method.
- The micropropagation technique is also useful for seed production in certain crops as the requirement of genetic conservation to a high degree is important for seed production.
- With micropropagation having various advantages over conventional methods of propagation, this method holds better scope and future for the production of important plant-based phytopharmaceuticals.
- Independent of availability of plants, micropropagation offers a lucrative alternative approach to conventional methods in producing controlled amounts of biochemical.

Therefore, intense and continuous efforts in this field will direct controlled and successful production of valuable, specific, and yet undiscovered plant chemicals.

**Short Questions**

1. Types of Micropropagation.
2. Applications of Micropropagation.
3. Applications of Meristem culture.

**Essay Questions**

1. Describe in detail the procedure for meristem culture and mention about the various applications of Meristem culture.
2. Explain the procedure involved in micro propagation to generate tissue cultured Plants and list the various application of Micropropagation

## 2.2. ENCAPSULATION AND PRODUCTION OF SYNTHETIC SEEDS

**Introduction to Synthetic Seeds / Artificial Seeds**

Somatic embryos are not enclosed by seed coats and due to microbial contamination and desiccation they are not able to survive if these are sown directly into field soil. The aim of somatic embryos encapsulation is to produce an analog to true seeds,

Somatic embryo (embryoids), shoot buds or any other plant material obtained as a result of *in vitro* culture are covered (encapsulated) with a chemical membrane. Such encapsulated materials behave as seeds. These are called artificial seeds or synthetic seeds. The artificial covering acts as an artificial seed coat. Such seeds are bead like and can "germinate" and plantlets are also formed. Several substances are

used as artificial seed coats. Some of them are agar, agarose, carrageenin, polyacrylamide, nitrocellulose, ethyl cellulose and sodium alginate. Sodium alginate is most commonly used.

The procedure is to mix the somatic embryos in a substance to make it well protected in that matrix and it is rigid enough to allow for rough handling. So, the artificial or synthetic seeds are the seed like structures where the somatic embryos derived from tissue culture are encapsulated by hydrogels and can be used directly to be sown in soil as a substitute of natural seeds.

These will germinate normally into plants. This covering helps to give physical protection, avoid desiccation and encapsulation should carry nutrients, growth regulators to help in germination. Antibiotics may be added to avoid contamination and also it should be durable and non-toxic.

**Table: Difference between natural seeds and artificial seeds**

| Natural seeds | Synthetic seeds |
|---|---|
| Hard seed coat present. | No seed coat, only encapsulated. |
| Embryos are much protected within cotyledons or endosperm. | Embryo are not protected within any kind of maternal tissue. |
| Embryos undergo controlled desiccation by the maternal tissue and have a natural dormancy period. | Embryos do not pass through any kind of desiccation and they the do not have any dormancy period. |
| The natural seeds have their own storage reserves like endosperm or cotyledons to provide food during germination | The artificial seeds do not have their own storage tissue, the nutrients or growth regulators can be supplied within the encapsulating material |

The technology designed in combine the advantages of clonal propagation with those of seed propagation and storage. The first synthetic seeds were produced by Kitto and Janick in 1982 using cartel somatic embryos

**Need of 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 eg; orchids
- Presence of reduced endosperm
- Some seeds require mycorrhizal fungi association for germination eg: orchids
- No seeds are formed

**Characteristics of synthetic seeds**

1) Large scale propagation method
2) Maintains genetic uniformity of plants

3) Direct delivery of propagules to the field, thus eliminating transplants
4) Lower cost per plantlet
5) Rapid multiplication of plants

**Types of synthetic seeds**

i. **Desiccated synthetic seeds:** The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri-dishes and leaving them on the bench overnight to dry. Such types of synseeds are produced only in plant species whose somatic embryos are desiccation tolerant.
ii. **Hydrated synthetic seeds:** The hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules.

**Procedure for Synthetic Seed Production:**

The somatic embryos for synthetic seeds are produced in the lab through culturing of somatic cells and treating with different hormones to produce somatic embryos directly or indirectly through callus formation. The following are the different steps involved in artificial seeds production:

**1)** Establishment of callus culture
**2)** Establish somatic embryogenesis
**3)** Mature somatic embryos
**4)** Synchronize and Stimulate somatic embryos
**5)** Mass production of embryos
**6)** Encapsulation of matured somatic embryos
**7)** Desiccation
**8)** Field planting

**Establishment of callus culture and somatic embryogenesis: (Refer unit 1)**
**Encapsulation of somatic embryos**

**Gel agents**

Somatic embryos produced naked embryos without storage materials and protective layer (seed coat). This is very difficult for handling so this demand the encapsulation and coating

The somatic embryos produced ar encapsulated using gel agents like agar, alginate, polyco, carboxy methyl cellulose, guar gum, sodium pectate etc. Among these, alginate encapsulation found to be more suitable and practicable.

Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its moderate viscosity and low spinnability of solution, low toxicity for somatic embryos and quick gellation, low cost and bio-compatibility characteristics. The use of agar as gel matrix was deliberately avoided as it is considered inferior to alginate with respect to long term storage.

Alginate was chosen because it enhances capsule formation and also the rigidity of alginate beads provides better protection to the encased somatic embryos against mechanical injury.

Since somatic embryos lack seed coat and endosperm, the matrix of encapsulation can be added with nutrients and growth regulator, which will serves as an artificial endosperm. This will increase the efficiency of germination and viability of seeds.

Addition to these nutrients other useful materials fungicides, pesticides, antibiotics and microorganism can also be incorporated.

**Table: List of important gelling agents for synthetic seeds**

| Gel | Conc. % W/V | Complexing agents | Conc. mM |
|---|---|---|---|
| Sodium Alginate | 0.5-5.0 | Calcium salts | 30-100 |
| Sodium Alginate | 2.0 | Calcium Chloride | 30-100 |
| with Gelatin | 5.0 | Potassium or Ammonium Chloride | 500 |
| Carragenan | 0.2-0.8 | | |
| with Locust Beam Gum | 0.4-1.0 | Temperature lowered | |
| Gel-rite™ | 0.25 | | |

**Methodology**

Two standardized methods have been used to coat somatic embryos:

i. **Gel complexation via a dropping procedure:** In this method, isolated somatic embryos are mixed with 0.5 to 5% (W/V) Sodium alginate and dropped into 30-100 μM Calcium nitrate solution. Surface complexation begins immediately and the drops are gelled completely within 30 min. The somatic embryos are mixed with sodium alginate (2%) and the suspension is dropped into the calcium salts solution (200mM). The principle involved is when sodium alginate dropped into the calcium salt solutions it from round firm beads due to the son exchange between $Na^+$ in sodium alginate and $Ca^{2+}$ in calcium salt solutions and sodium alginate form calcium alginate in 20-30 minutes.

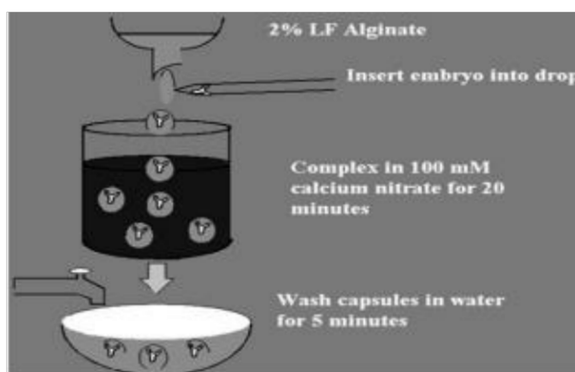



**Fig: Dropping method for synthetic seed formation**

ii. **Moulding:** In this method, isolated somatic embryos are mixed in a temperature-dependent gel such as Gel-rite and placed in the well of a micro-titer plate and it forms gel when the temperature is cooled down.

**Fig: Moulding method for synthetic seed formation**

**Assessment of germination of artificial seeds**

After encapsulation, initially, the effect of coating on somatic embryos is very difficult assess because the germination and continued development of the encapsulated embryos are sometimes very inconsistent after planting into soil

So, to overcome this problem, embryo response in terms of embryo to plant development or conversion is tested under aseptic conditions. Embryo conversion is the percent of the frequency is somatic embryos that produce green-plants having a normal phenotype

After encapsulation, the artificial seeds are tested by two steps

1. Test for embryo to plant conversion
2. Green-house and field planting

Maturation of somatic embryos means the completion of embryo development through some stages. Initially, embryo develops as globular-shaped stage, then heart-shaped stage and finally torpedo-shaped stage. In the final stage, embryo attains maturity and develops the opposite poles for shoot and root development at the two extremities.

This embryo then starts to germinate and produces plantlet. However, in some plant species, such sequential development may not be followed. Again, in some species requiring cold treatment for embryo germination, it may be necessary to

chill young or mature embryos for their normal maturation and development into plantlets.

**Germination of synthetic seeds**

Embryo to plant conversion includes the following steps:

i. Encapsulated embryos are placed aseptically on simply agar medium with minimal nutrients.
ii. Uniform germination of somatic embryos and growth and development of root and shoot systems.
iii. In Production of true leaves.
iv. A green-plant with a normal phenotype.

This assay should be very critical before sowing the artificial seed in green-house or in the field. Otherwise, some modifications are to be required. The final assessment will be the green- house or field performance of artificial seed and their yield in comparison to plants derived from true seeds.

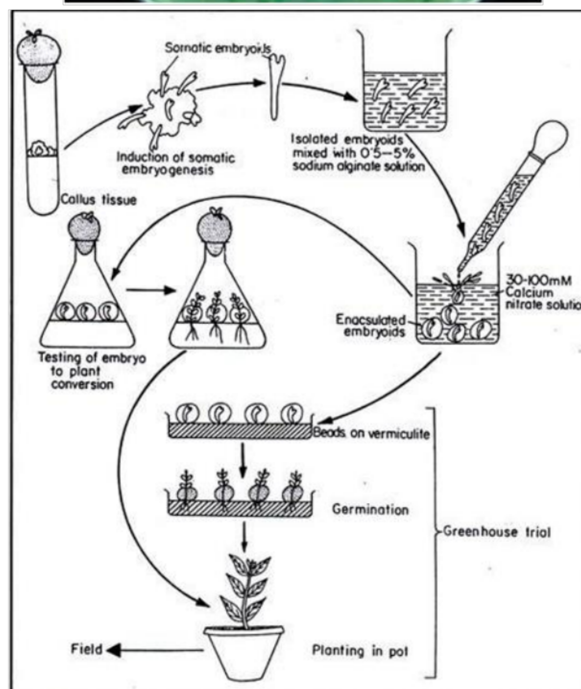



**Fig: Steps for Synthetic seed production**

**Storage of artificial seeds**

Storage of artificial seeds is a great limitation. When the artificial seeds are stored at low temperature, the embryos show a characteristic drop in conversion. The limited storage time of artificial seeds is probably due to an anaerobic environment in the capsule.

This is a problem for somatic embryos because they are not developmentally arrested and continue very active respiration. To overcome this limitation, two possible solutions are, to have a smaller ratio of capsule volume to embryo volume so that gas diffusion can readily occur or to induce an arrested state in the embryo using growth control agent in the encapsulation medium.

Rai et al. reported that a high concentration of sucrose or ABA could be useful for short-term conservation of guava because of their temporary inhibition in encapsulated somatic embryos germination. Ray and Bhattacharya indicated that 4 °C, where storage achieved up to 14 weeks with high regrowth percentage in Rauvolfia. However, while short artificial seed storage can be obtained by applying several procedures-such as using suitable temperature (usually 4°C), suitable capsulation materials, and optimal storage conditions (reduced heat, light, oxygen, etc.) long storage can be achieved using dehydration and/or cryopreservation techniques.

**Potential Uses of Synthetic Seeds**

i. In some plants it requires a long time to reach at reproductive phase and seed production. In these cases the synthetic seeds can be helpful to get the propagules in a short period
ii. Generally the reproduction phase in a plant is season dependent, in these cases the somatic embryos and the synthetic seeds can be produced at any time as required.
iii. In cpe of natural seeds there is a particular dormancy period, but in artificial seeds there is no dormancy period, thus it is more helpful for propagation. It can reduce the life cycle period of a plant.
iv. Artificial seeds can also be helpful where there is no successful seed production after sexual hybridization. The somatic embryos can be obtained from somatic hybrids obtained through protoplast fusion.
v. Artificial seeds are helpful in case of meiotically unstable genotypes, where the normal seed set is of low frequency.
vi. In cases where the embryo germination is difficult, the artificial seed can provide the beneficial adjuvants i.e., growth promoting seed substances, plant nutrients, etc. through the artificial coats.
vii. In genetic manipulation of crop plants the production of artificial seed may be useful.

viii. For somatic hybrid plant production through protoplast culture and fusion the artificial seed technology is helpful.
ix. In the production of genetically modified (GM) crops through transgenics, the synthetic seed production may be applied.
x. In general, synthetic seed technology is useful for rapid easiest way of non-conventional method of propagation in crop plants.

**Limitations**

i. Limited production of viable micro-propagules that are useful in synthetic seed producer
ii. Asynchrous development of somatic embryos
iii. Improper maturation of somatic embryos that makes them inefficient for germination
iv. and conversion in to normal plants AV
v. Lack of dormancy and stress tolerance in somatic embryos that limit the storage of
vi. synthetic seeds
vii. Somaclonal variations which may alter the genetic constituent of the embryos
viii. The initial cost for the production of artificial seed is more than that for the natural seeds.
ix. Production and germination of artificial seeds require aseptic conditions. Any deviation will affect the quality of the seeds and their subsequent development.

**Short Questions**

1) List the potential uses of synthetic seeds
2) Write about the various limitations of synthetic seeds.
3) Steps involved in the germination of synthetic seeds

**Essay Questions**

1) Describe in detail the procedure for production of somatic embryos highlighting on the various applications of somatic embryos.
2) Write in detail about encapsulation of somatic embryos.

## 2.3 CELL SUSPENSION CULTURES AND APPLICATIONS

**Types of suspension cultures:** The suspension cultures are broadly grouped as follows: (a) Batch cultures, (b) Continuous cultures, and (c) Immobilized cell cultures.

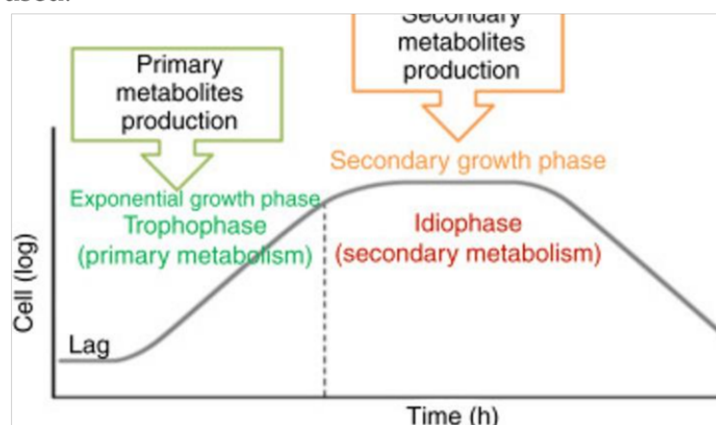
a. **Batch Culture Batch culture:** It is a type of cell suspension culture that is grown in a fixed volume of nutrient culture medium. Here, the cell suspension

increases in biomass by cell division and cell growth until a factor in the culture medium becomes limiting and then the growth ceases. The cells in batch culture exhibits a typical sigmoidal curve with the following five phases of a growth cycle:

- **i.** Lag phase, where the cells prepare to divide.
- **ii.** Exponential or log phase, where the rate of cell division is the highest.
- **iii.** Linear phase, where cell division slows down but the rate of cell expansion increases.
- **iv.** Deceleration phase, where the rates of cell division and elongation decreases.
- **v.** Stationary phase, where the number and size of cells remain constant.

The lag phase duration depends mainly on inoculum size and growth phase of the culture from which the inoculum is taken. The log phase lasts about 3-4 cell generations (a cell generation is the time taken for doubling of cell number), and the duration of a cell generation may vary from 22-48 hr, depending mainly on the plant species. The stationary phase is forced on the culture by depletion of the nutrients and possibly due to an accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period, the cells may die.

Batch cultures are maintained by sub-culturing at weekly intervals. The exact time and dilution required must be determined for each cell line. Dilutions of 1:4 after one week or 1:10 after two weeks are commonly used. It is recommended that a small sample should be withdrawn to determine the cell density before sub-culturing. Batch cultures are unsuitable for studies on cell growth and metabolism as there is a constant change in cell density and nutritional status of the medium. But batch cultures are much more convenient than continuous cultures and, hence are routinely used.




**b. Continuous Culture:** In a continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the used or spent medium by fresh medium Continuous cultures are of two types:

i. **Closed type:** In a closed continuous culture, the cells which are separated while the used medium is taken out for replacement are added back to the culture so that cell biomass keeps on increasing.

ii. **Open type:** In open continuous cultures, both cells and the used medium are taken out and replaced by equal volume of fresh medium. The replacement volume is so adjusted that the cultures remain at submaximal growth indefinitely. The open cultures are of either turbidostat or chemostat types. In a turbidostat, cells are allowed to grow upto a preselected turbidity (usually, measured as OD) when a predetermined volume of the culture is replaced by fresh normal culture medium. But in a chemostat, a chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while other nutrients are still in concentrations higher than required.

c. **Immobilized Cell Culture:** Plant cells and cell groups may be encapsulated in a suitable material, e.g., agarose and calcium alginate gels, or entrapped in membranes or stainless steel screens. The gel beads containing cells may be packed in a suitable column or, alternatively, cells may be packed in a column of a membrane or wire cloth. Liquid medium is continuously run through the column to provide nutrients and aeration to cells.

Immobilization of cells changes their cellular physiology in comparison to suspension culture cells. The advantages of immobilized cell reactors are many like no risk of cell washing out, protection of cells from liquid shear by protective covering, low contamination, better control on cell aggregate size and regular removal of cellular wastes.

After a period of time, it becomes necessary to transfer organs and tissues to fresh media chiefly due to nutrient depletion and medium drying. This is particularly true of tissue and cell cultures where a portion of tissue is used to inoculate new culture tubes or flasks; this is known as sub-culturing. In general, suspension cultures need to be sub-cultured every 3-14 days.

Plant cell and tissue cultures may be maintained indefinitely by serial sub-culturing. In case of suspension cultures, sub-culturing should be done about or somewhat prior to the time of their maximum growth. The inoculums volume should be 20-25% of the fresh medium volume; in any case, the initial cell density of the fresh culture (just after inoculation) should be around $5 \times 10^4$ cells $ml^{-1}$ or higher otherwise the cells may fail to divide.

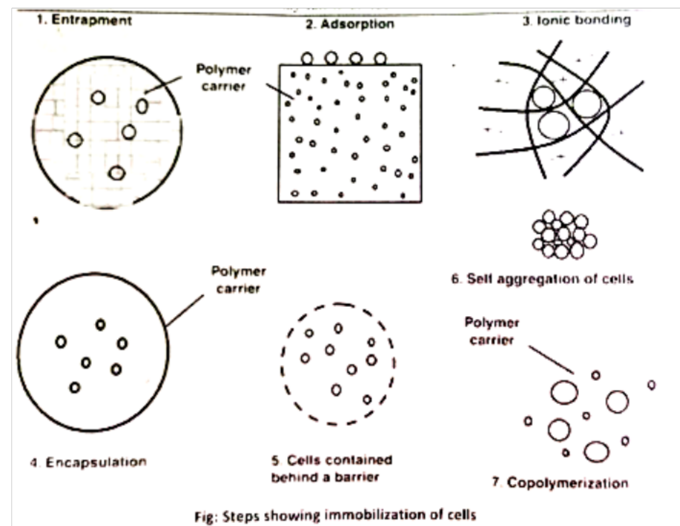


Fig: Steps showing immobilization of cells

**Applications of cell suspension culture**

i. The culture of single cells and small aggregates in moving liquid medium is an important experimental technique for a lot of studies that are not correctly possible to do from the callus culture. Such a system is capable of contributing many significant information's about cell physiology, biochemistry, metabolic events at the level of individual cells and small cell aggregates.

ii. It is also important to build up an understanding of an organ formation or embryoid formation starting from single cell or small cell aggregates. The technique of plating out cell suspension on agar plates is of particular value where attempts are being made to obtain single cell clones.

iii. Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids and a considerable amount of industrial effort is being placed on the exploitation and expansion of this area.

iv. Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised. Cell population in a suspension can be subjected to a range of mutagenic chemicals e.g. ethyl methane-sulphonate (EMS), N. nitroso N-methyl urea etc.

v. Plants could be raised from the mutant cell clones and the mutant plants are selected from the population either by morphological differences or by metabolic/biochemical differences. The selected plants can then be grown on and propagated further to produce a mutant population for evaluation studies.

**Short Questions**

1. Applications of cell suspension cultures
2. Immobilized cell cultures.

**Essay Questions**

1. Write in detail about different methods of cell suspension cultures.

# 2.4 PROTOPLAST ISOLATION, CULTURE AND FUSION

A plant cell without its cell wall is known as a protoplast. It is called as a naked plant cell because the cell wall has been removed either by a mechanical or an enzymatic method Protoplast can be isolated from almost all plant parts *viz*. root, leave fruits, tuber, endosperm, pollen etc. Protoplast culture refers to the aseptic isolation and in vitro culture of protoplast to obtain viable plants.

**Historical events**

The term protoplast was introduced in 1880 by Hanstein. The first isolation of protoplasts was achieved by Klereker (1892) employing a mechanical method. A real beginning in protoplast research was made in 1960 by Cocking who used an enzymatic method for the removal of cell wall.

Rakabe and his associates (1971) were successful to achieve the regeneration of whole tobacco plant from protoplasts. Rapid progress occurred after 1980 in protoplast fusion to improve plant genetic material, and the development of transgenic plants.

**Methods of protoplast isolation**

**1) Mechanical method:**

In this method large and highly vacuolated cells (eg. onion bulbs, scales, radish root & beet root tissue) are plasmolysed in an osmotic solution, causing the protoplast to shrink away from the cell wall. The tissue is dissected to release the protoplasts. Mechanical method for protoplast isolation is no more in use because of the following limitations:

i. Yield of protoplasts and their viability is low..
ii. It is restricted to certain tissues with vacuolated cells.
iii. The method is laborious and tedious.

However, some workers prefer mechanical methods if the cell wall degrading enzymes (of enzymatic method) cause deleterious effects to protoplasts.

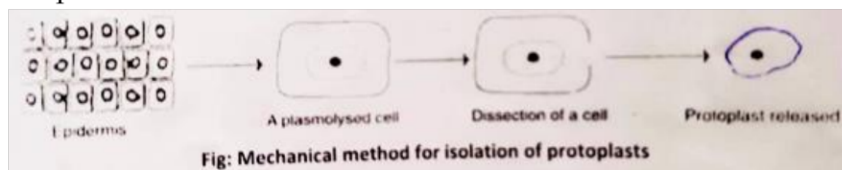


Fig: Mechanical method for isolation of protoplasts

**2) Enzymatic method**

Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells and minimal or no damage to the protoplasts. Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices, fruits,

embryos and microspores. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots are most frequently used. In addition, callus and suspension cultures also serve as good sources for protoplast isolation.

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose and pectin which can be respectively degraded by the enzymes cellulases, hemicellulase and pectinase. The enzymes are usually used at a pH 4.5 to 6.0, temperature 25-30°C with a wide variation in incubation period that may range from half an hour to 20 hours.

After the digestion of cell wall the isolated protoplast is subject to osmotic stress. If an osmotic stabilizing agent is not included in the medium the isolated protoplast would take in water by the process of osmosis and would eventually burst as there is no cell wall to constrain the cell.

The isolated cells are macerated with macro enzyme (Pectinase) in 13% mannitol. Pectinase mainly degrades the middle lamella while cellulase are required to digest the cell wall. The cells are purified by filtration through nylon mesh. Then the cells are incubated in 2% cellulose for about 90 min.

The enzymatic isolation of protoplasts can be carried out by two approaches:

i. **Two step or sequential method:** The tissue is first treated with pectinase (macerozyme) to separate cells by degrading middle lamella. These free cells are then exposed to cellulose in release protoplasts. Pectinase breaks up the cell aggregates into individual cells while cellulose removes the cell wall proper.
ii. **One step or simultaneous method:** This is the preferred method for protoplast isolation. It involves the simultaneous use of the enzymes, macroenzyme und cellulose.

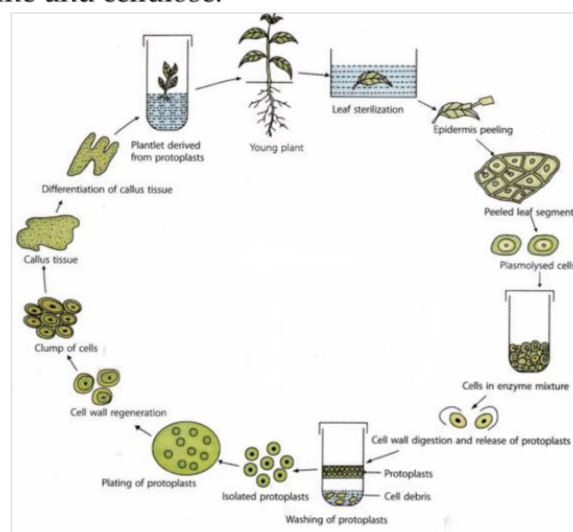



**Fig: Isolation of protoplasts by enzymatic method**

**Purification of protoplasts**

The enzyme digested plant cells, besides protoplasts contain undigested cells, broken protoplasts and undigested tissues. The cell clumps and undigested tissues can be removed by filtration. This is followed by centrifugation and washings of the protoplasts. After centrifugation, the protoplasts are recovered.

**Viability test of protoplasts**

It is essential to ensure that the isolated protoplasts are healthy and viable before culturing them and are capable of undergoing sustained cell divisions and regeneration

There are several methods to assess the protoplast viability.

i. Fluorescern diacetate (FDA) staining method - The dye accumulates inside viable protoplasts which can be detected by fluorescence microscopy
ii. Phenosafranine stain is selectively taken up by dead protoplasts (turn red) while the viable cells remain unstained.
iii. Exclusion of Evans blue dye by intact membranes.
iv. Measurement of cell wall formation-Calcofluor white (CFW) stain binds to the newly formed cell walls which emit fluorescence.
v. Oxygen uptake by protoplasts can be measured by oxygen electrode.
vi. Photosynthetic activity of protoplasts.
vii. The ability of protoplasts to undergo continuous mitotic divisions (this is a direct measure)

**Protoplast Culture**

**Methods:** The very first step in protoplast culture is the development of a cell wall around the membrane of the protoplast. This is followed by the cell divisions that give rise to a small colony. With suitable manipulations of nutritional and physiological conditions, the cell colonies may be grown continuously as cultures or regenerated to whole plants. Protoplasts are cultured either in agar medium or liquid medium. Sometimes, protoplasts are first allowed to develop cell wall in liquid medium, and then transferred to agar medium.

**Agar culture Method**

Agarose is the most frequently used agar to solidify the culture media. The concentration of the agar should be such that it forms a soft agar gel when mixed with the protoplast suspension. The plating of protoplasts is carried out by Bergmann's cell plating technique. In agar cultures, the protoplasts remain in a fixed position, divide and form cell clones. The advantage with agar culture is that clumping of protoplasts is avoided.

**Liquid culture Method**

Liquid culture is the preferred method for protoplast cultivation for the following reasons:

i. It is easy to dilute and transfer.
ii. Density of the cells can be manipulated as desired.
iii. For some plant species, the cells cannot divide in agar medium, therefore liquid medium is the only choice.
iv. Osmotic pressure of liquid medium can be altered as desired.

**Culture Media**

**Nutritional components:** In general, the nutritional requirements of protoplasts are similar to those of cultured plant cells (callus and suspension cultures). Mostly, MS and B5 media with suitable modifications are used.

**Some of the special features of protoplast culture media are listed below:**

**i.** The medium should be devoid of ammonium, and the quantities of iron and zinc should be less.
**ii.** The concentration of calcium should be 2-4-times higher than used for cell cultures. This is needed for membrane stability.
**iii.** High auxin/kinetin ratio is suitable to induce cell divisions while high kinetin/auxin rate is required for regeneration.
**iv.** Glucose is the preferred carbon source by protoplasts although a combination of sugars (glucose and sucrose) can be used.
**v.** The vitamins used for protoplast cultures are the same as used in standard tissue culture media.

**Osmoticum and osmotic pressure**

Osmoticum broadly refers to the reagents/ chemicals that are added to increase the osmotic pressure of a liquid. The isolation and culture of protoplasts require osmotic protection until they develop a strong cell wall. In fact, if the freshly isolated protoplasts are directly added to the normal culture medium, they will burst. Thus, addition of an osmoticum is essential for both isolation and culture media of protoplast to prevent their rupture. The osmotica are of two types non-tonic and ionic.

- **Non-ionic osmotica:** The non-ionic substances most commonly used are soluble carbohydrates such as mannitol, sorbitol, glucose, fructose, galactose and sucrose. Mannitol being metabolically inert, is most frequently used.
- **Ionic osmotica:** Potassium chloride, calcium chloride and magnesium phosphate are the ionic substances in use to maintain osmotic pressure. When the protoplasts are transferred to a culture medium, the use of metabolically active osmotic stabilizers (e.g., glucose, sucrose) along with metabolically inert osmotic stabilizers (mannitol) is advantageous. As the growth of protoplasts and cell wall regeneration occurs, the metabolically active compounds are utilized, and this results in the reduced osmotic pressure so that proper osmolarity is maintained.

**Culture Techniques**

The culture techniques of protoplasts are almost the same that are used for cell culture with suitable modifications. Some important aspects are briefly given:

- **Multiple Drop Array (MDA) Screening:** This technique was developed for systematic screening of multiple combinations of media constituents for protoplast culture. MDA screening method uses hanging drop technique in which each droplet measuring 40µl represents one combination of factors to be tested as one experimental unit. The droplets are arranged in a regular array of 7x7 drops on the lid of a Petridish (9 cm). Each droplet represents one combination of factors to be tested. To test seven different auxins in combination with four different Cytokinins in the medium, each Auxin or Cytokinin is used in at least seven different concentrations. Whole experiment includes 4x7 petri dishes and each petri dish has 4 x 7 x 49 = 1372 two-factor combinations.
- **Feeder layer technique:** For culture of protoplasts at low density feeder layer technique is preferred. This method is also important for selection of specific mutant or hybrid cells on plates. The technique consists of exposing protoplast cell suspensions to X-rays (to inhibit cell division with good metabolic activity) and then plating them on agar plates.
- **Co-culture of protoplasts:** Protoplasts of two different plant species (one slow growing and another fast growing) can be co-cultured. This type of culture is advantageous since the growing species provide the growth factors and other chemicals which help in the generation of cell wall and cell division. The co-culture method is generally used if the two types of protoplasts are morphologically distinct.
- **Micro-drop culture:** Specially designed dishes namely 'cuprak dishes with outer and inner chambers are used for micro drop culture. The inner chamber carries several wells wherein the individual protoplasts in droplets of nutrient medium can be added. The outer chamber is filled with water to maintain humidity. This method allows the culture of fewer protoplasts for droplet of the medium.
- **Other Techniques:** Electroporation treatment of protoplasts is reported to stimulate division and regeneration in them. Protoplasts suspended in buffer solution (4 times its plating density) if exposed to high voltage (250-2000 volt) DC pulses for 10-50 µs after intervals of every 10 seconds could enhance higher DNA synthesis and promote early gene expression for differentiation and regeneration. Heat-shock treatment (45°C for 5 min, followed by 10 sec on ice) gives similar stimulatory effect as that of electroporation.

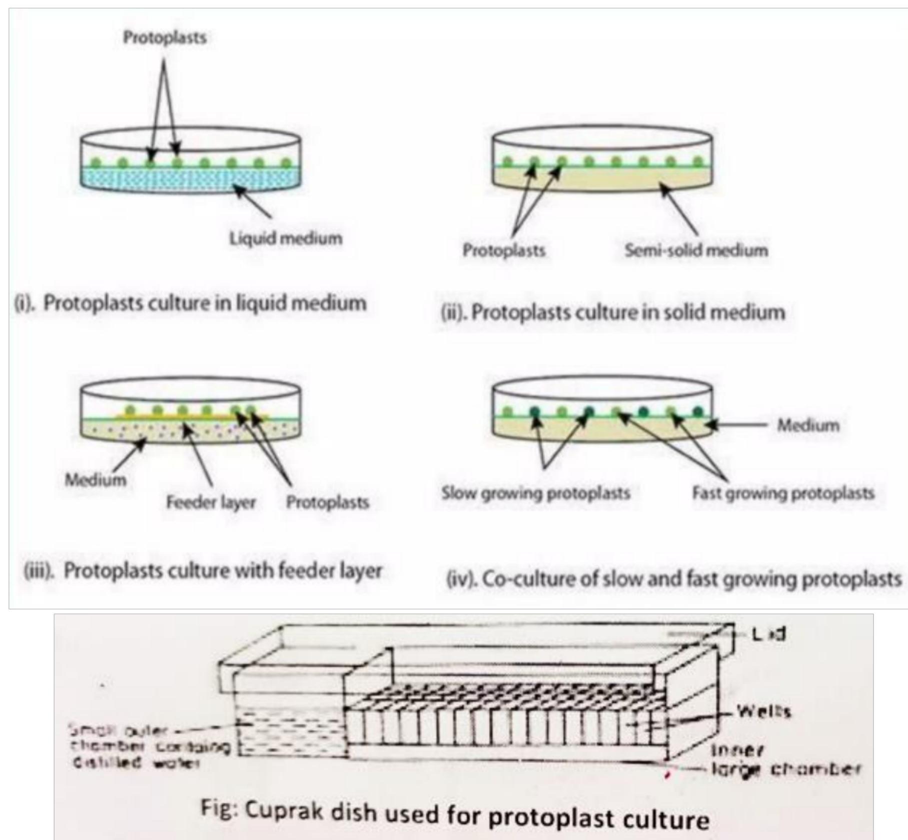



Fig: Cuprak dish used for protoplast culture

**Regeneration from protoplasts**

Protoplast regeneration which may also be regarded as protoplast development occurs in two stages:

i. **Formation of cell wall:** The process of cell wall formation in cultured protoplasts starts within a few hours after isolation that may take two to several days under suitable conditions. As the cell wall development occurs, the protoplasts lose their characteristic spherical shape The newly developed cell wall by protoplasts can be identified by using calcofluor white fluorescent stain. The freshly formed cell wall is composed of loosely bound micro fibrils which get organized to form a typical cell wall. This process of cell wall development requires continuous supply of nutrients, particularly a readily metabolised carbon source (e.g sucrose), Cell wall development is found to be improper in the presence of ionic osmotic stabilizers in the medium. The protoplasts with proper cell wall development undergo normal cell division. On the other hand, protoplasts with poorly regenerated cell wall show budding and fail to undergo normal mitosis.

ii. **Development of callus/whole plant:** As the cell wall formation around protoplasts is complete, the cells increase in size and the first division generally occurs within 2-7 days. Subsequent divisions result in small colonies, and by the end of third week, visible colonies (macroscopic

colonies) are formed. These colonies are then transferred to an osmotic-free (mannitol or sorbitol-free) medium for further development to form callus. With induction and appropriate manipulations, the callus can undergo organogenic or embryo genic differentiation to finally form the whole plant. A general view of the protoplast isolation, culture and regeneration

Plant regeneration can be done from the callus obtained either from protoplasts or from the culture of plant organs. There are however, certain differences in these two calluses. The callus derived from plant organs carries preformed buds or organized structures, while the callus from protoplast culture does not have such structures. The first success of regeneration of plants from protoplast cultures of *Nicotiana tabacum* was achieved by Takebe *et al* (in 1971). Since then, several species of plants have been regenerated by using protoplasts.

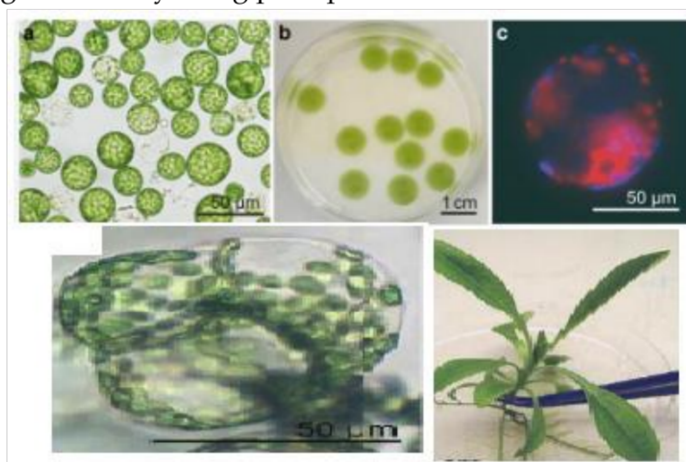



**Fig. Regeneration of plants from protoplast culture**

**Table: Few examples of plant species regenerated from protoplasts**

| Category | Plant Species |
|---|---|
| **Cereals** | Oryza sativa<br>Zea mays<br>Hordeum vulgare |
| **Vegetables** | Cucumis sativus<br>Brassica oleracea<br>Capsicum annuum |
| **Woody trees** | Larix eurolepsis<br>Coffea canephora<br>Prunus avium |
| **Ornamentals** | Rosa sp<br>Chrysanthemum sp<br>Pelagronium sp |

| Tubes and roots | Beta vulgaris<br>Ipomoca batatas |
|---|---|
| Oil crops | Helianthus annuces<br>Brassica napus |
| Legumes | Glycine max |

**Sub-Protoplasts**

The fragments derived from protoplasts that do not contain all the contents of plant cells are referred to as sub-protoplasts. It is possible to experimentally induce fragmentation of protoplasts to form sub-protoplasts. This can be done by application of different centrifugal forces created by discontinuous gradients during centrifugation. Exposure of protoplasts to cytochalasin B in association with centrifugation is a better approach for fragmentation of protoplasts.

There are three types of sub-protoplasts:

i. **Mini-protoplasts:** These are also called as karyoplasts and contain the nucleus. Mini-protoplasts can divide and are capable of regeneration into plants.
ii. **Cytoplasts:** These are sub-protoplasts containing the original cytoplasmic material (in part or full) but lack nucleus. Thus, cytoplasts are nuclear-free sub-protoplasts which cannot divide, but they can be used for cybridization.
iii. **Micro-protoplasts:** This term was suggested for sub-protoplasts that contain not all but few chromosomes.

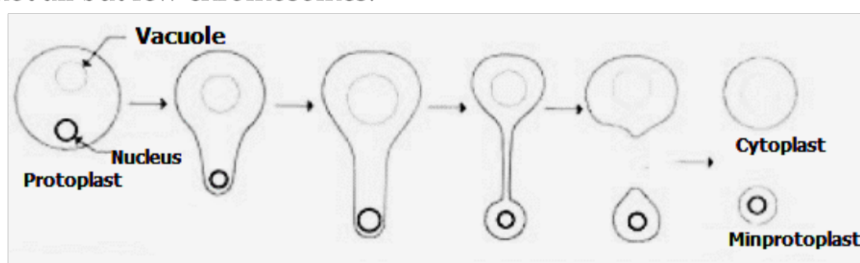



**Fig: Fragmentation of protoplast to form sub-protoplasts**

**Applications of protoplast culture**

i. Two or more protoplasts can be induced to fuse & then fusion product carefully nurtured to produce a hybrid plant. In some cases, hybrids that cannot be produced by conventional plant genetics because of sexual or physiological incompatibility can be produced by protoplast fusion.
ii. After removal of cell wall the isolated protoplast is capable of ingesting foreign material into the cytoplasm by a process similar to endocytosis as described for certain animal cells & protozoans.
iii. The cultured protoplast rapidly regenerates a new cell wall & this developmental process offers a novel system for the study of wall biosynthesis & deposition.

**iv.** Population of protoplasts can be studied as a single cellular system that is their manipulation is similar to that of microorganisms.

**Protoplast fusion**

Protoplast fusion technique is the best means of producing unique hybrid plants (somatic hybrids), which are otherwise difficult to be produced by conventional sexual hybridisation method. Protoplasts being devoid of walls make them useful of removing the naturally existing incompatibility barriers during fusion of different cells at interspecific, inter generic, or even inter kingdom levels. This technique was envisaged to offer exciting possibilities for studies in the field of somatic cell genetics and crop improvement.

**Methods of protoplast fusion**

Fusion of protoplasts of two plants can be achieved by physical or chemical methods.

Important methods for protoplast fusion are as follows:

1) **Mechanical Fusion:** Protoplasts can be fused mechanically by pushing them towards each other in the medium without the use of fusion inducing agents. Freshly isolated protoplasts being without cell wall readily fuse by gentle tapping in a depression slide but through this procedure there is maximum damage/injury to protoplasts.
2) **Induced fusion:** Irrespective of their origin, freshly isolated protoplasts are induced to undergo fusion with the help of a range of fusogens. Treatment with following fusion inducing agents have yielded successes in producing somatic hybrid plants:
   - **$NANO_3$ treatment:** This method was first demonstrated by Powar et al (1970) Isolated protoplasts were floated in sucrose osmoticum for cleaning and then transferred to 0.25M $NANO_3$ solution and subsequent centrifugation promoted fusion process. This method was used to produce first somatic hybrid plant by fusing protoplasts of *Nicotiana glauca and Nicotiana langsdorffii* by Carlson et al (1972). This procedure results in low frequency of heterokaryon formation.
   - **High pH/Ca++ treatment:** Keller and Melchers (1973) developed this method for fusing protoplasts of two different lines of tobacco and is now used commonly. Isolated protoplasts are incubated in a solution of 0.4 M mannitol containing 0.05 M $CaCl_2$, with pH at 10.5 and temperature 37°C. Aggregation of protoplasts takes place immediately and fusion occurs within 10 minutes. Many intraspecific and interspecific somatic hybrids have been produced using this procedure.

- **PEG (Polyethylene glycol) treatment:** PEG has been used as a fusogen for producing somatic hybrids in large number of plant species because of high frequency of heterokaryon formation. Approximately 0.6 ml of PEG solution (dissolve 1 g of PEG, mol. wt. 1500, in 2ml of 0.1 M glucose, 10 mM $CaCl_2$ and 0.7 mM $KH_2PO_4$) is added in drops to a pellet of isolated protoplasts in the tube and after having capped the tube protoplasts in PEG are incubated at room temperature for 40 minutes. Gentle rocking of tubes helps to bring the protoplasts in contact. Incubation is followed by elution of PEG by addition of 0.5 $ml^{-1}$ of protoplast culture medium in the tube after every ten minutes. Eluted fusogen-treated protoplasts are washed by centrifugation to remove fusogen and resuspended in the culture medium. Both the molecular weight and the concentration of PEG are important in inducing successful fusions. Low molecular weight PEG (less than 100 mol. wt.) is unable to produce tight adhesions while PEG ranging up to 6000 mol. wt. per mol. can be more effective in inducing fusions. Elution of PEG in presence of high pH/Ca increases the fusion frequency and survivability of protoplast.

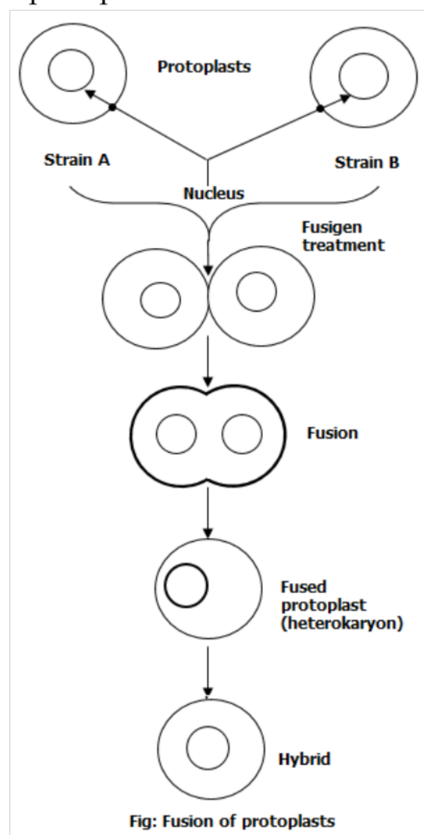


Fig: Fusion of protoplasts

- **Electrofusion:** Fusion of protoplasts by application of electric field has been found to be simpler, quicker and more efficient method than chemically induced fusion Cells after electrofusion do not show

cytotoxic responses which are generally found in protoplasts or heterokaryons subjected to PEG treatment. Using electric pulses to introduce foreign DNA into plant cells (electroporation) has further developed interest in the application of electrofusion in somatic cell genetic studies. Senda et al (1979) first attempted electrofusion by positioning two micro electrodes with the help of a micro manipulator to adjoining Rauwolfia protoplasts. Zimmermann and Schewrich (1981) fused batches of protoplasts by electric fields by devising a protocol which is now widely used. This protocol involves a two-step process: i. Protoplasts are introduced into a small fusion chamber containing parallel wires or plates which serve as electrodes ii. A low-voltage and rapidly oscillating AC field is applied which aligns protoplasts into chain of cells (pearl chains) between the two electrodes. Once alignment is complete, the fusion is induced by application of a brief spell of high-voltage DC pulses 0.125-1 KV $CM^{-1}$. A high voltage DC pulse induces a reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and reorganization of membrane. Entire process takes less than 5 minutes. Shoots or complete somatic hybrid plants regenerated from heterokaryons formed by electrofusion of protoplasts have been reported in combinations: *Nicotiana tabacum* (+) *N. tabacum. Nicotiana plumbaginifolia (+) N. tabacum. Nicotiana glauca (+) N langsdorffii. Solonum tuberosum (+) S. phureja*

**Somatic Hybridization**

The improvement of domesticated plants has long been accomplished by sexual hybridization between closely related species. Unfortunately, the majority of the time sexual hybridization is restricted to cultivars within a species or, at best, to a small number of closely related wild species. Thus, the value of sexual hybridization for agricultural enhancement is constrained by species barriers.

Somatic hybridization is the process of creating hybrid plants by fusing isolated somatic (plant body/vegetal cells other than the reproductive cells) protoplasts in a lab setting. The resulting heterokaryon is then developed into a hybrid plant. A new hybrid cell with traits from both parent plants can be created by fusing the protoplasts of two separate plant cells using the somatic hybridization process.

Somatic cell fusion is the joining of two separate cells' protoplasts to create functional cell hybrids called as 'somatic hybrids'. In the hybrid cell, the cytoplasm and nuclei of both parents are combined. Sometimes a fused hybrid, also known as a 'cybrid' or 'cytoplasmic hybrid contains the nuclear genome of just one parent but the cytoplasmic genes (plastome) of both parents. These hybrids produced by the

fusion of somatic cells are an excellent approach to getting around any species' barriers to sexual hybridization.

**Somatic Hybridization Technique**

Somatic Hybridization involves the following three steps:

**A.** Fusion of protoplasts
**B.** Selection of hybrid cells
**C.** Identification of hybrid plants

**A. Fusion of protoplasts (Mechanism)**

The fusion of protoplasts involves three phases agglutination, plasma membrane fusion and formation of heterokaryons.

1) **Agglutination (adhesion):** When two protoplasts are in close contact with each other, adhesion occurs. Agglutination can be induced by fusogens e.g. PEG, high pH and high $Ca^{2+}$
2) **Plasma membrane fusion:** Protoplast membranes get fused at localized sites at the points of adhesion. This leads to the formation of cytoplasmic bridges between protoplasts. The plasma membrane fusion can be increased by high pH and high $Ca^{2+}$ high temperature and PEG, as explained below:
    a) High pH and high $Ca^{2+}$ ions neutralize the surface charges on the protoplasts. This allows closer contact and membrane fusion between agglutinated protoplasts.
    b) High temperature helps in the intermingling of lipid molecules of agglutinated protoplast membranes so that membrane fusion occurs.
    c) PEG causes rapid agglutination and formation of clumps of protoplasts. This results in the formation of tight adhesions of membranes and consequently their fusion.
3) **Formation of heterokaryons:** The fused protoplasts get rounded as a result of cytoplasmic bridges leading to the formation of spherical homokaryon or heterokaryon.

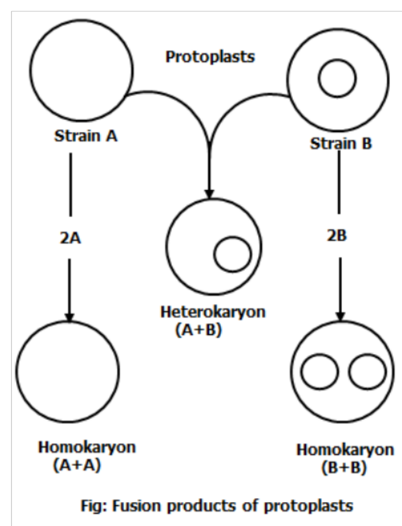


Fig: Fusion products of protoplasts

B. **Selection of Hybrid Cells**

About 20-25% of the protoplasts are actually involved in the fusion. After the fusion process, the protoplast population consists of a heterogenous mixture of un-fused chloroplasts, homokaryons and heterokaryons. It is therefore necessary to select the hybrid cells (heterokaryons). The commonly used methods employed for the selection of hybrid cells are biochemical, visual and cytometric methods.

1. **Biochemical methods:** The biochemical methods for selection of hybrid cells are based on the use of biochemical compounds in the medium (selection medium). These compounds help to sort out the hybrid and parental cells based on their differences in the expression of characters.
   - **Drug sensitivity:** This method is useful for the selection hybrids of two plant species, if one of them is sensitive to a drug. Protoplasts of *Petunia hybride* (species A) can form macroscopic callus on MS medium, but are sensitive to (inhibited by) actinomycin D. *Petunia parodii* protoplasts (species B) form small colonies, but are resistant to actinomycin D. When these two species are fused, the fused protoplasts derive both the characters-formation of macroscopic colonies and resistance to actinomycin D on MS medium. This helps in the selection of hybrids. The parental protoplasts of both the species fail to grow. Protoplasts of P. *parodii* form very small colonies while that of P. *hybrida* are inhibited by actinomycin D. Drug sensitivity technique was originally developed by Power et al (1976) for the selection of hybrids of *Petunia sp*.

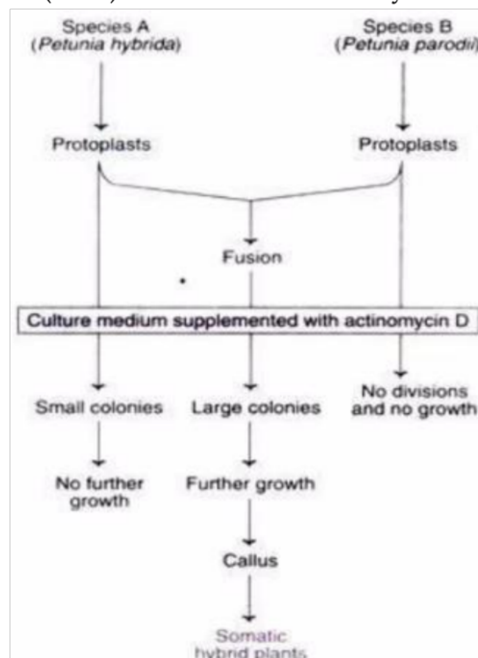



**Fig: Drug sensitivity for the isolation of hybrid cells**

- **Auxotrophic mutants:** Auxotroph's are mutants that cannot grow on a minimal medium and therefore require specific compounds to be added to the medium. Nitrate reductase deficient mutants of tobacco (*N. tabacum*) are known. The parental protoplasts of such species cannot grow with nitrate as the sole source of nitrogen while the hybrids can grow. Two species of nitrate reductase deficiency-one due to lack of apoenzyme (nia-type mutant) and the other due to lack of molybdenum cofactor (enx- type mutant) are known. The parental protoplasts cannot grow on nitrate medium while the hybrid protoplasts can grow. The selection of auxotrophic mutants is possible only if the hybrid cells can grow on a minimal medium. Another limitation of the technique is the paucity of higher plant auxotrophs.

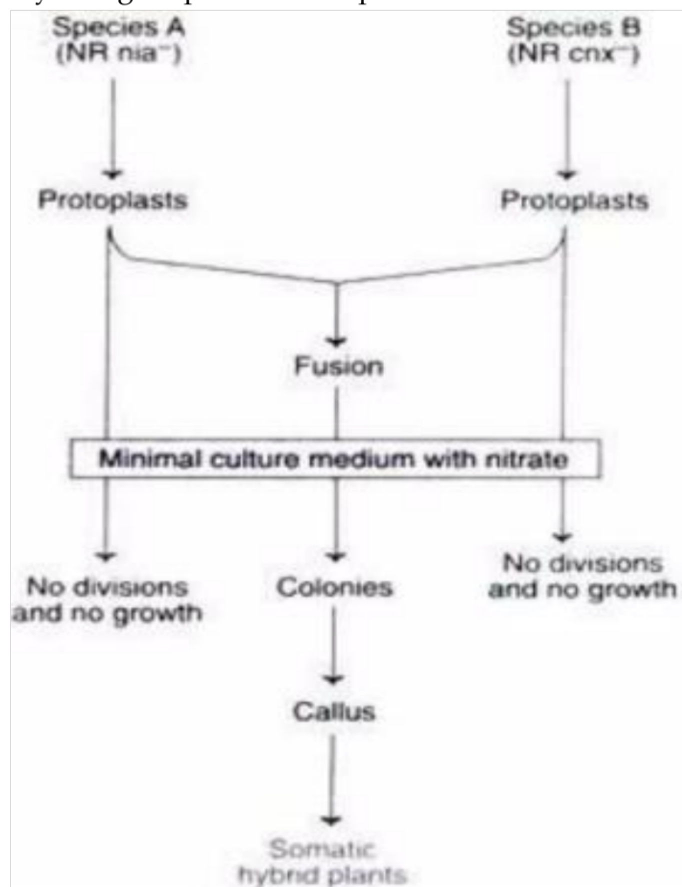



**Fig: Selection of hybrid cells based on auxotrophic mutant**

2. **Visual method:** Visual selection of hybrid cells, although tedious is very efficient. In some of the somatic hybridization experiments, chloroplast deficient (albino or non-green) protoplasts of one parent are fused with green protoplasts of another parent. This facilitates the visual identification of haterokaryons under light microscope. The heterokaryons are bigger and green in colour while the parental protoplasts are either small or colourless. Further identification of these heterokaryons has to be carried out to

develop the specific hybrid plant. There are two approaches in this direction - growth on selection medium and mechanical isolation:

- **Visual selection coupled with differential media growth:** There exist certain natural differences in the sensitivity of protoplasts to the nutrients of a given medium. Thus, some media can selectively support the development of hybrids but not the parental protoplasts. A diagrammatic representation of visual selection coupled with the growth of heterokaryons on a selection medium.
- **Mechanical isolation:** The visually identified heterokaryons under the microscope can be isolated by mechanical means. This involves the use of a special pipette namely Drummond pipette. The so isolated heterokaryons can be cloned to finally produce somatic hybrid plants. The major limitation of this method is that each type of hybrid cell requires a special culture medium for its growth. This can be overcome by employing micro drop culture of single cells using feeder layers.

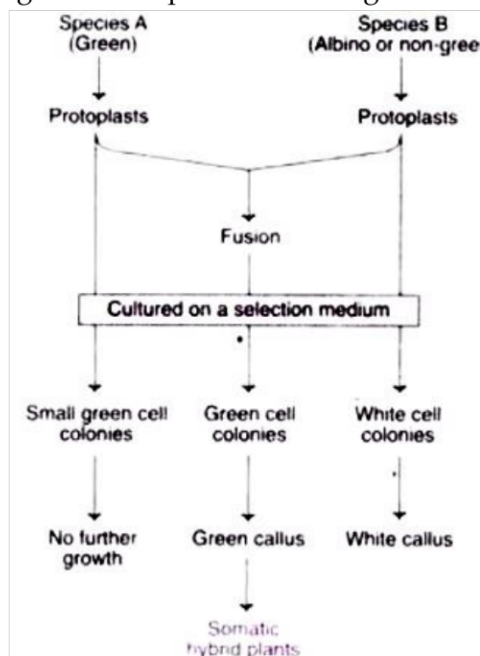



**Fig: Visual selection of hybrid cells**

3. **Cytometric methods:** Some workers use flow cytometry and fluorescent-activated cell sorting techniques for the analysis of plant protoplasts while their viability is maintained. The same techniques can also be applied for sorting and selection of heterokaryons. The hybrid cells derived from such selections have proved useful for the development of certain somatic hybrid plants.

## C. Identification of Hybrid Plants

The development of hybrid cells followed by the generation of hybrid plants requires a clear proof of genetic contribution from both the parental protoplasts.

The hybridity must be established only from euploid and not from aneuploid hybrids. Some of the commonly used approaches for the identification of hybrid plants are briefly described below:

- **Morphology of hybrid plants:** Morphological features of hybrid plants which usually are intermediate between two parents can be identified. For this purpose, the vegetative and floral characters are considered. These include leaf shape, leaf area, root morphology, flower shape, its structure, size and colour, and seed capsule morphology. The somatic hybrids such as pomatoes and topatoes which are the fused products of potato and tomato show abnormal morphology, and thus can be identified. Although the genetic basis of the morphological characters has not been clearly known, intermediate morphological features suggest that the traits are under the control of multiple genes. It is preferable to support hybrid morphological characters with evidence of genetic data.
- **Isoenzyme analysis of hybrid plants:** The multiple forms of an enzyme catalysing the same reaction are referred to as isoenzymes. Electrophoretic patterns of isoenzymes have been widely used to verify hybridity. Somatic hybrids possess specific isoenzymes (of certain enzymes) of one or the other parent or both the parents simultaneously. There are many enzymes possessing unique isoenzymes that can be used for the identification of somatic hybrids e.g. amylase, esterase, aspartate aminotransferase, phosphodiesterase, isoperoxidase, and hydrogenases (of alcohol, lactate, malate). If the enzyme is dimeric (having two subunits), somatic hybrids usually contain an isoenzyme with an intermediate mobility properties. The isoenzymes are often variable within the same plant. Therefore, it is necessary to use the same enzyme from each plant (parents and somatic hybrids), from a specific tissue with the same age.
- **Chromosomal constitution:** The number of chromosomes present in the hybrid cells can be directly counted. This provides information on the ploidy state of the cells. The somatic hybrids are expected to possess chromosomes that are equal to the total number of chromosomes originally present in the parental protoplasts. Sometimes, the hybrids are found to contain more chromosomes than the total of both the parents. The presence of chromosomal markers is greatly useful for the genetic analysis of hybrid cells.
- **Molecular techniques:** Specific restriction pattern of nuclear, mitochondrial and chloroplast DNA can be used to characterize the hybrid plants (Hybrid will show unique bands from both the parents). For restriction based studies marker techniques like RFLP, RAPD, ISSR can be used to characterize variations and similarity in banding pattern of hybrid/cybrid plants. Southern blot analysis using specific repetitive DNA (r DNA) radioactive

and non- radioactive probes can be used to analyze nuclear genomes in somatic hybrids or cybrids-ECO RI restriction nuclease fragmentation of cp DNA followed by separation of fragments on gel electrophoresis is used for characterization of chloroplast genome in plants obtained by protoplast fusion.

**Applications of somatic hybridization**

1) Novel interspecific and intergeneric crosses which are difficult to produce by conventional methods can be easily obtained.
2) Important characters, such as resistance to diseases, ability to undergo abiotic stress and other quality characters can be obtained in hybrid plant by the fusion of protoplasts of plant bearing particular character to the other plant which may be susceptible to diseases.
3) Protoplasts of sexually sterile haploid, triploid, aneuploid plants can be fused to obtain fertile diploids and polyploids.
4) Most of the agronomically important traits, such as cytoplasmic male sterility, antibiotic resistance and herbicide resistance, are cytoplasmically encoded, hence can he easily transferred to other plant.
5) Plants in juvenile stage can also be hybridized by means of somatic hybridization.
6) Somatic hybridization can be used as a method for the production of autotetraplonds.
7) Creation of brand new interspecific and intergeneric fusions between plants that are difficult or impossible to hybridize by traditional methods. It removes obstacles caused by sexual incompatibility.
8) **Disease Resistance:** Somatic hybridization has allowed the spread of disease resistance genes from one plant to numerous others. Tomatoes now have the ability to resist a number of illnesses, including TMV, the spotted wilt virus, insect pests, and cold tolerance.
9) **Resistance to Abiotic Stress:** Research on somatic hybridization for resistance to abiotic stress has been focused on the families Fabaceae, Brassicaceae, Poaceae, and Solanaceae and relates to cold and frost resistance.
10) Cytoplasmically encoded features such as some forms of male sterility and specific antibiotic and herbicide resistance traits are among the traits that are useful for agriculture. Resistance to antibiotics, herbicides as well as CMS has been introduced in so many cultivated species

**Limitations of somatic hybridization**

1) Application of protoplast methodology requires efficient plant regeneration system from isolated protoplasts. Protoplasts from two species can be fused, however, production of somatic hybrids is not easy.

2) The end product of somatic hybridization are often unbalanced (sterile, misformed and unstable)
3) Somatic hybridization of two diploids leads to formation of amphidiploids which is unfavorable.
4) It is not sure for a character to completely express after somatic hybridization.

**Cybrids**

Somatic hybrids can be obtained where nucleus is derived from one parent and cytoplasm is derived from both the parents, thus resulting 'cytoplasmic hybrids' also called as 'cybrids' Whereas, sexual hybridization is an exact mixture of parental nuclear genes but the cytoplasm is derived from the maternal parent only.

In cytoplasmic hybridization, nucleus from one protoplast is inactivated or segregated out in carly stage such that one protoplast contributes the cytoplasm while the other contributes the nucleus alone or both nucleus and cytoplasm. In cybrids, there is fusion between protoplasts containing the full component of nucleus, mitochondria and chloroplasts with functional cytoplasmic component of second protoplast.

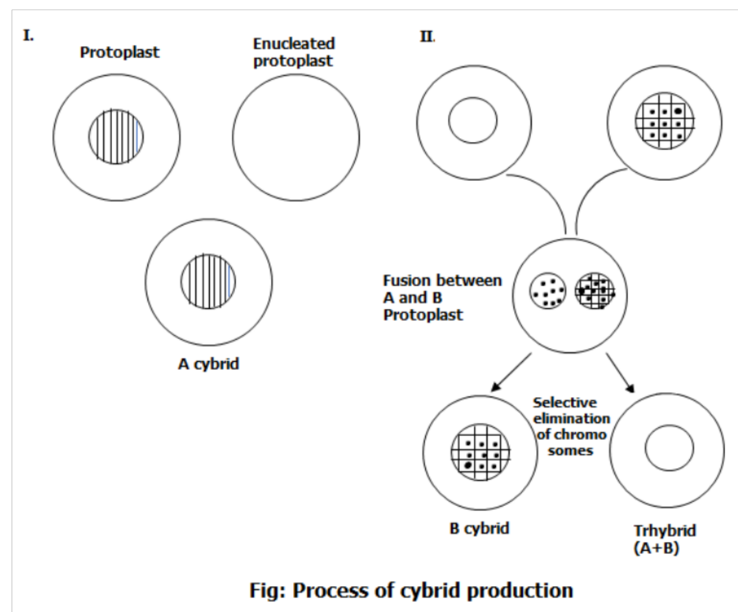



Fig: Process of cybrid production

**Methods to obtain cytoplasmic hybrids (inactivation of protoplasts)**

i. **By application of lethal dosages of X-rays or gamma rays to one parental protoplast population:** Ionizing radiation treatment damages the nucleus, thus the protoplasts become inactivated and non-dividing but they function as an efficient donor of cytoplasmic genophores when fused with recipient protoplasts. *Nicotiana* protoplasts can be inactivated by 5-kr dose of X-rays. Other protoplasts may require different doses.

ii. **By treatment with iodoacetate to metabolically inactivate the protoplasts:** Pre-treatment with iodoacetate will cause the degeneration of non-fused and auto-fused protoplasts while fusion of iodoacetate pre-treated protoplasts with non-treated protoplasts will cause metabolic complementation and result in viable hybrids. In an experiment, iodoacetate treated *Nicotiana plumbaginifolia* cell suspension was fused with X-rays irradiated N. morphology but most of them contained N. *tabacum* chloroplasts. The iodoacetate treatment does not impair the nucleus of the treated protoplasts. Thus, the latter can complement an X- rays irradiated protoplast.

iii. **Fusion of normal protoplasts with enucleated protoplasts:** The high-speed centrifugation (20,000-40,000x g) for 45-90 minutes in an iso-osmotic density gradient with 5-50% percoll will yield enucleated protoplasts. Additional exposure of isolated protoplasts to cytochalsin B in combination with centrifugation has also been found beneficial for enucleation.

iv. **Fusion of cytoplasts with protoplasts:** Isolated protoplasts can be experimentally induced to fragment into types of sub-protoplasts called mini-protoplasts or cytoplasts. The term mini-protoplast was coined by Wallin et al. (1978) for sub-protoplasts having nuclear material which can divide and may be able to regenerate into plants.

**Application of cytoplasmic hybridization**

1) **Production of hybrid organisms:**
   - Production of novel interspecific and intergeneric crosses between plants that are difficult or impossible to hybridize conventionally.
   - Both interspecific and intergeneric hybrids can be acquired by somatic hybridization.
2) **Overcomes sexual incompatibility barriers:**
   - Somatic hybridization overcome the sexual incompatibility barriers during breeding or cross fertilization.
   - For example, fusion between protoplasts of Lycopersicon esculentum (tomato) and Solanum tuberosum (potato) created the pomato first achieved by Melchers et al. (1978).
3) **Somatic hybridization for gene transfer:**
   i. for production of Disease resistance variety:
      - Many disease resistance genes viz. potato leaf roll virus, leaf blight, Verticillium, Phytophthora, etc. have been transferred to Solanum tuberosum from other species where normal crossings would not be possible due to taxonomic or other barriers.
      - Resistance has been established in tomato against various diseases like TMV, spotted wilt virus, insect pests and also cold tolerance.
   ii. **for production of Abiotic stress resistance:**

- Work related to somatic hybridization for abiotic stress has been mainly done on families Fabaceaa, Brassicaceae, Poaceae, Solaneae and relates to cold and frost resistance.

iii. **for production of Quality characters:**

- Somatic hybrids produced between Brassica napus and Eruca sativa were fertile and had low concentration of erucic acid content (Fahleson et al., 1993).

4) **Transfer of Cytoplasmic male sterility:**
   - Various agriculturally functional traits are cytoplasmically encoded, including some types of male sterility and certain antibiotic and herbicide resistance factors,
5) **Production of resistant variety:**
   - Resistance to antibiotics, herbicide as well as CMS has been introduced in so many cultivated species.
6) **Production of auto-tetraploids:**
   - Somatic hybridization can be used a as an alternative to obtain tetraploids and, if this is unsuccessful, colchicine treatment can be used.
   - Protoplasts of sexually sterile (haploid, triploid, aneuploid, etc.) plants can be fused to produce fertile diploids and polyploids.
7) **Hybridization becomes possible between plants that are still in the juvenile phase.**
8) **Production of heterozygous lines within a single species that normally could only be propagated by vegetative means, e.g. potato and other tuber and root crops.**
9) **To study cytoplasmic genes:**
   - Somatic cell fusion is useful in the study of cytoplasmic genes and their activities. This information can be employed in plant breeding experiments.
10) **Production of unique nuclear-cytoplasmic combinations:**
    - Mitochondrial and chloroplast recombination has also been reported to result in unique nuclear- cytoplasmic combinations.
    - These unique combinations using protoplasts will aid the development of novel germplasm not obtainable by conventional methods.

**Limitation of cytoplasmic hybridization**

There are certain limitations to the use of these types of somatic hybridization:

i. Plants regenerated from some of the combinations in somatic hybridization are often sterile, deformed, and unstable and are thus not viable, particularly if the fusion partners are taxonomically far apart.

ii. Application of protoplast methodology requires efficient plant regeneration from protoplasts. Protoplasts from any two species can be fused. However, production of somatic hybrid plants has been limited to a few species.

iii. Sometimes, the major problem is the lack of an efficient selection method for fused product.

iv. The development of chimaeric calluses in place of hybrids. This is usually due to the nuclei not fusing after cell fusion and dividing separately. Plants that are regenerated from chimaeras usually lose their chimeric characteristics, since adventitious shoots or embryos usually develop from a single cell.

v. Somatic hybridization of two diploids leads to the formation of an amphidiploid which is generally unfavourable (except when tetraploids are formed intentionally). For this reason in most cases, the hybridization of two haploid protoplasts is normally recommended.

vi. Regeneration products after somatic hybridization are usually variable because of the somacional variation, chromosome elimination, translocation, organelle segregation etc.

vii. It is never certain that a particular characteristic will be expressed after somatic hybridization.

viii. The genetic stability during protoplast culture is poor.

ix. To achieve successful integration into a breeding programme, somatic hybrids must be capable of sexual reproduction.

x. In all cases reported, somatic hybrids containing a mixture of genes from two species must be backerossed to the cultivated crop to develop new varieties.

**Short Questions**

1. Write about the viability test of protoplasts.
2. Limitations of cytoplasmic hybridization.
3. Applications of cytoplasmic hybridization.
4. Application of somatic

**Essay Questions**

1. Explain in detail the steps involved in somatic hybridization.
2. Describe in detail the methods to obtain cytoplasmic hybridization and list the various application of cytoplasmic hybridization.

## 2.5 SOMACLONAL VARIATION

The term somaclonal variation by Larkin and Scoweroft (1981) was given for the variability generated by the use of a tissue culture cycle. Somaclonal variation is defined as genetic variation observed among progeny plants obtained after somatic tissue culture *in vitro*.

Theoretically all progeny plants regenerated from somatic cells should be identical clones However, variations might occur in number of progeny which are

known as somaclones and they are genetically variable from their explant. The initiating explant for a tissue culture cycle may come virtually from any plant organ or cell type including embryos, microspores, roots, leaves and protoplasts. So, all somatic tissue culture can result in somaclonal variation. Somaclonal variation is a phenotypic changes as a result of chromosomal rearrangement during tissue culture.

**Basis of somaclonal variation:**

Following are some of the basis of chromosomal rearrangement which results in somaclonal Variation

1) **Karyotype changes:**
   - Variant plants with altered chromosome number have been reported by several workers.
   - Polyploidy is the most frequently observed chromosome abnormality e.g. aneuploidy in oats, potato, barley.
   - The change in chromosome number in a variant plant is commonly associated with reduced fertility and with altered genetic ratios in the progeny of self-fertilized plants.
2) **Changes in chromosome structure:**
   - In contrast to gross changes in chromosome number, more cryptic chromosome rearrangements may be responsible for genetic variation.
   - Translocations have been reported in potato, ryegrass, oats, etc. Published work also suggests that chromosome deletions, duplications, inversions and other minor reciprocal and non-reciprocal rearrangements occur among regenerated plants e.g. ryegrass, barley.
   - Chromosome irregularities such as breaks, acentric and centric fragments, ring chromosomes and micronuclei were observed in garlic somaclones.
   - Cytological abnormalities such as multilobed nuclei, multinucleate cells, abnormal anaphase and mixoploidy were observed in self-fertilized, SC3 generation of barley.
3) **Single gene mutations:**
   - Single gene mutations have been detected by several workers.
   - Recessive single gene mutations are suspected if the variant does not appear in Ro or SCI generation but the self-fertilized R1 or SC2 progeny segregate in 3:1 Mendelian ratio for the trait of interest. This type of analysis has been reported for maize, *Nicotiana sylvestris*, rice, wheat, etc.
4) **Cytoplasmic genetic changes:**
   - The most detailed work on this has been done on maize.
   - Many studies have implicated mitochondria as the determinants of male sterility and *Drechslera maydis* T toxin sensitivity in maize,
   - The original lines used had all the Texas male sterile cytoplasm, which results in plants being normally highly susceptible to this disease.

- However, when selection pressure was applied by subculturing callus cultures in the presence of toxin, some regenerants were found to be not only resistant to the toxin but also fertile.
- When restriction analysis of the mitochondrial DNA (mtDNA) was evaluated it was evident that significant changes had occurred in the mtDNA of plants derived from cell cultures.
- Molecular analyses based on restriction endonuclease digest patterns of mitochondrial and chloroplast genomes of regenerated potato plants derived from protoplasts have revealed significant change in mitochondrial genomes but not in chloroplast genomes.
- It was suggested that the variation results from substantial DNA sequence rearrangements (e.g. deletion, addition and intramolecular recombination) and cannot be explained by simple point mutations.
- However, point mutations can also occur in the genomes of organelles of regenerated plants.
- Variations in chloroplast DNA have been detected in tomato and wheat somaclones.

**5) Mitotic crossing over:**

- Mitotic crossing over (MCO) could also account for some of the variation detected in regenerated plants.
- This could include both symmetric and asymmetric recombination. MCO may account for the recovery of homozygous recessive single gene mutations in some regenerated plants.
- Somatic cell sister chromatid exchange, if it is asymmetric can also lead to deletion and duplication of genetic material and hence variation.

**6) Gene amplification and nuclear changes:**

- Studies have shown that nuclear genes are affected by tissue culture stress.
- It has been seen that heritable quantitative and qualitative changes can be observed in the nuclear DNA content of doubled haploid *Nicotiana sylvestris* obtained from pollen cultured plants.
- These plants contain generally increased amounts of total DNA and an increasing proportion of highly repeated sequences.
- Both AT and GC rich fractions are amplified in tissue culture derived plants. Durante et al. (1983) showed similar amplification of AT and GC rich fractions in DNA from *Nicotiana glauca* pith explants within hours of culture.
- These studies suggest the presence of a differential replication process during the early stages of dedifferentiation.

- It has been seen that plant cells like those of other eukaryotes can increase of decrease the quantity of a specific gene product by differential gene amplification and diminution.
- For instance, ribosomal RNA gene amplification and diminution are now known to be widespread in wheat, rye, and tobacco, and in lax, ribosomal DNA is known to alter directly in response to environmental and cultural pressures.
- Reduction in ribosomal RNA genes (rDNA) has been found in potato plants regenerated from protoplasts.
- Additionally, both structural rearrangement within DNA and methylation of nucleotide sequences of DNA have been observed.

**7) Transposable elements:**

- Several authors have speculated that transposable elements may also be responsible for somaclonal variation,
- Heterozygous light green (Su/su) somaclones with a high frequency of coloured spots on the leaf surface have been detected for a clone of N. tabacum and for a N. *tabacum* + *N. sylvestris* somatic hybrid. The somatic hybrid has an unstable pattern of inheritance that would be consistent with an unstable gene.
- A causal relationship between genetic instability possibly related to tissue culture induced transposition and somaclonal variation was speculated.
- Recently, genomic changes in a maize line and mutations in tobacco line due to activation of transposable elements under in vitro tissue culture conditions have been demonstrated.
- It has also been shown that several types and copies of non-active transposable elements, including retro-transposable elements, are present in the genome of potato.
- Thus, it is likely that a few active transposable elements may also be present in potato and could be responsible for generating some degree of somaclonal variation in regenerated potato plants.

**8) DNA methylation:**

- DNA methylation plays an important role in the regulation of gene expression and its implication in somaclonal variation.
- Many genes show a pattern in which the state of methylation is constant at most sites but varies at others.
- A majority of sites are methylated in tissues in which the gene is not expressed but non-methylated in tissues where the gene is active. Thus, an active gene may be described as unmethylated.
- A reduction in the level of methylation is part of some structural change needed to permit transcription to proceed.

- Important changes in the methylation level of genomic DNA in course of dedifferentiation and somatic embryogenesis have been reported for higher plants.
- Wherever the gene expression and its regulation is playing a role, DNA methylation becomes an important factor for getting the somaclonal variants.
- Unstability of gene may arise through the methylation pattern of that particular gene and thus a causal relationship between genetic instability possibly related to tissue culture-induced transposition, and somaclonal variation.
- In higher plants many controlling factors act together to achieve the desired gene regulation.
- One of these levels of control is provided by adding a small "tag" called a methyl group onto "C" one of the bases that make up the DNA code.
- The methyl group tagged C's can be written as mC.
- Simpler organisms, such as many types of bacteria and the single celled yeast, usually do not use methyl group tagged C's in regulating their genes, some bacteria but not all use methyl group tagged A's "mA" for this purpose.
- However, most bacteria have specific patterns of mC and mA for this purpose i.e. for signalling in their DNA called methylated DNA.
- Thus the process in which the methyl group is added to certain bases as in cytosine (C) and adenine (A) by the help of enzyme DNA methylase at the C5 atom of cytosine and N6 position of adenine is called methylation.
- When only one strand of DNA is methylated it is called hemi-methylated DNA if it is in both strands of DNA this is called fully methylated DNA.
- In hemi-methylated condition gene is able to express while in fully methylated state gene cannot be expressed.
- In general rule, methylation prevents the gene expression but the de-methylation respond to the expression of a gene.
- Hence in terms of somaclonal variation (i.e. variation occurs in the in vitro culture of plants) the DNA methylation by regulating the gene expression and sometimes by inducing the mutations may cause variation in the culture.
- In *Arabidopsis thaliana*, a new variant has been developed from the normal one this is a tiny dwarf plant, shriveled, a mere shadow of its genetically identical neighbour.
- This dwarf plant is named as "bal", because of its shape.
- It constantly perceives a pathogen attack even though it has the exact same DNA sequence of parental plant.

- It was found that the difference between the two plants is not due to changes in the DNA sequence but actually the 'bal dwarf' is caused by increased activation of a single gene.
- This is something looks like a mutation and behaves like a mutation but it is actually caused by the packaging of DNA (caused basically by DNA methylation) rather than change in the DNA sequence.
- The gene affected in '*bal variant*' is involved in the disease resistance and is called an R- gene.
- The R-gene is more active in 'bal plant' and as a result the plant defense system becomes hyperactivated, constantly fighting off disease even when no pathogens are present to pose a threat.
- The yielded dwarf plant is more resistant to bacterial infection.
- The precise molecular change leading to the increased R-gene activation is most probably the changes in DNA methylation, chemical modification of cytosine

**Applications of Somaclonal Variation:**

i. **Production of Novel variants:**
   - An implication of somaclonal variation in breeding is that novel variants can arise and these can be agronomically used.
   - A number of breeding lines have been developed by somaclonal variation.
   - **Example:** An enhanced scented Geranium variety named 'Velvet Rose' has been generated. An example of heritable somaclonal variation is the development of pure thornless blackberries Lincoln Logan (Rubus), Hasuyume, a protoplast derived rice cultivar, and somaclone T-42 has been generated.
   - In India two varieties namely Pusa Jai Kisan in mustard Brassica and CIMAP Bio13 in Citronella have been released for cultivation.
   - An improved variety of rice 'DAMA' has been released through pollen haploid somaclone method which combined microspore culture with somatic tissue culture (see Heszky and Simon-Kiss, 1992).
   - Somacionally derived mutants in tomato with altered color, taste, texture and shelf life are being marketed in USA by Fresh World farms

ii. **Production of disease resistance variety:**
   - The greatest contribution of somaclonal variation towards plant improvement is in the development of disease resistant genotypes in various crop species.
   - Resistance was first reported in sugarcane for eye spot disease (*Helminthosporium sacchari*), downy mildew (*Sclerospora sacchari*) and Fiji virus disease by regenerating plants from the callus of susceptible clones and screening the somaclones.

**Table: List of few successful examples of somaclonal variants obtained**

| Food crops | Pathogen |
|---|---|
| Barley | Rhynchosporium secalis |
| Maize | Helminthosporium maydis |
| Rice | Helminthosporium oryzae |
| Rape | Phoma lingam, Alternaria brassicicola |
| Sugar-cane | Fijivirus, Sclerospora saccharli, Helminthosporium sacchari |

iii. **Production of abiotic stress resistance variety:**
- Somaclonal variation has resulted in several interesting biochemical mutants, which are being successfully used in plant metabolic pathway studies, i.e. amino acid and secondary metabolic pathways.
- Investigations have shown that the level of free amino acids, especially proline, increases during cold hardening.
- In vitro selection has also been used to obtain plants with increased acid soil, salt, aluminium and herbicide resistance.

iv. **Production of Cold tolerance:**
- Lazar et al. (1988) developed somaclonal variants for freezing tolerance in Norstar winter wheat.
- A significant positive correlation between proline level and frost tolerance has been found in a broad spectrum of genotypes.
- In vitro selection and regeneration of hydroxyproline resistant lines of winter wheat with increased frost tolerance and increased proline content has been reported (Dorffling et al., 1997).
- The results showed strong correlation of increased frost tolerance with increased proline content.

v. **Production of Salt tolerance:**
- Plant tissue culture techniques have been successfully used to obtain salt tolerant cell lines or variants in several plant species, viz tobacco, alfalfa, rice, maize, *Brassica juncea, Solanum nigrum*, sorghum, etc.
- In most cases, the development of cellular salt tolerance has been a barrier for successful plant regeneration, or if plants have been obtained they did not inherit the salt tolerance.
- Only in a few cases it was possible to regenerate salt tolerant plants. Mandal et al. (1999) developed a salt tolerant somaclone BTS24 from indigenous rice cultivar Pokkali.
- This somaclone yielded 36.3 q/ha under salt stress conditions as compared to 44 q/ha under normal soil.
- Some of the reports for successful production of healthy, fertile and genetically stable salt tolerant regenerants from various explants.

vi. **Production of Aluminium tolerance:**

- Plant species or cultivars greatly differ in their resistance to aluminium stress.
- In recent years, considerable research has been focused on the understanding of physiological, genetic and molecular processes that lead to aluminium tolerance.
- Despite the problems encountered in adapting culture media for in vitro selection for aluminium resistance, cell lines have been isolated in several species, e.g. alfalafa, carrot, sorghum, tomato, tobacco.
- Aluminium tolerant somaclonal variants from cell cultures of carrot were selected by exposing the cells to excess ionic aluminium in the form of aluminium chloride (Arihara et al., 1991).
- Jan et al. (1997) elicited aluminium toxicity during in vitro selection in rice by making several modifications in the media viz. love pH, low phosphate and calcium concentrations, and unchelated iron and aluminium along with aluminium sulphate
- After selection on aluminium toxic media, callus lines were maintained on aluminium toxic free media and 9 tolerant plants were obtained. Transmission of aluminium resistance character was identified till the fourth generation.

vii. **Production of Drought tolerance:**

- Wang et al. (1997) reported a sorghum somaclonal variant linc (R111) resistant to drought stress.
- A novel hybrid was developed by crossing R111 with several sterile lines, suggesting that selection of somacional variants is an effective method for creating new sources of genetic variation.
- Drought tolerant rice lines were obtained by in vitro selection of seed induced callus on a media containing polyethylene glycol as a selective agent which simulated the effect of drought in tissue culture conditions.

viii. **Production of Herbicide resistance:**

- Through in vitro selection several cell lines resistant to herbicides have been isolated and a few have been regenerated into complete plants.
- Among the important achievements are tobacco, soybean, wheat, maize, etc., resistant to various herbicides such as glyphosate, sulfonylurea, imidazolinones, etc.

ix. **Production of Insect resistance:**

- Zemetra et al. (1993) used in vitro selection technique for generation of somacional variants for Russian wheat aphid (Diuraphis noxia) in wheat. Calli from susceptible wheat cultivar "Stephens" were exposed to an extract from aphid. Resistance to aphid was observed in both R2 and R3 generations.

**x. Seed quality improvement:**

- Recently, a variety Bio L 212 of lathyrus (Lathyrus sativa) has been identified for cultivation in central India which has been developed through somaclonal variation and has low ODAP (β-N-oxalyl-2-α, β diamino propionic acid), a neurotoxin (Mehta and Santha, 1996), indicating the potential of somaclonal variations for the development of varieties with improved seed quality.

**xi. Introgression of Alien gene:**

- The increase in genome rearrangement during tissue culture provides a new opportunity for alien gene introgression which can help widen the crop germplasm base, particularly by culturing immature embryos of wide crosses where crop and alien chromosomes cannot replicate through meiosis.
- Introgression of genes may be better achieved by imposing one or more cell culture cycles on interspecific hybrid material.
- The enhanced somatic genome exchange is likely to produce regenerants where part of the alien genome has been somatically recombined into the chromosomes of crop species.

**Limitations of somaclonal variation:**

- Uncontrollable and unpredictable nature of variation and most of the variations are of no apparent use.
- The variation is cultivar dependent.
- The variation obtained is not always stable and heritable. The changes occur at variable frequencies.
- Not all the changes obtained are novel. In majority of cases, improved variants have not been selected for breeding purposes.
- must be backcrossed to the cultivated crop to develop new varieties.

**Short Questions**

1. Gene amplification and nuclear changes resulting in somaclonal variation
2. Limitations of somaclonal variations.

**Essay Questions**

1. Describe in detail the basis for somaclonal variations.
2. List the various applications of somaclonal variations.

## 2.6. ANTHER AND POLLEN CULTURES

Regeneration of whole plant from anther (or) pollen in the culture medium is called 'anther culture. Haploid plants can be developed by anther culture. The optimum stage differs from species to species. Production of haploid plants through anther culture is known as "androgenesis".

Haploids can be identified by cytological studies at callus stage or at plant level by biochemical studies or marker genes linked with haploidy. In general, the haploids are much weaker highly sterile and difficult to maintain when compare to the normal plants of concerned species. Therefore, chromosome number of all haploids are doubled usually by treating with colchicine to produce doubled haploids which have the normal somatic chromosome complement (2n) of the species and are fully fertile. The double haploid plants are completely homozygous and fully vigorous and can be used for the evaluation of performance and selection for desirable traits.

**Principle of Anther and Pollen Culture**

The basic principle of anther and pollen culture is the production of haploid plants exploiting the totipotency of microspore and the occurrence of single set of chromosome (n) in microspore In this process, the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcibly to a new metabolic pathway for vegetative cell division.

In culture, pollen may divide mitotically or can follow the normal pathway of forming vegetative and generative nuclei. The generative nucleus remains quiescent and abort. The vegetative nucleus divides repeatedly, forming a multinucleate pollen. The multinucleate pollen undergoes segmentation which may lead to form either organized embryoid structure or callus tissue. Both types of development are utilized to form haploid plantlets.

**Modes of androgenesis**

There are two modes of androgenesis:

1. **Direct androgenesis:** In this type, microspore behaves like a zygote and undergoes change to form embryoid which ultimately gives rise to a plantlet.
2. **Indirect androgenesis:** In contrast to the direct androgenesis, the microspore, instead of undergoing embryogenesis, divide, repeatedly to form a callus tissue which differentiates into haploid plantlets.

**Factors affecting Androgenesis**

1. **Genotype of donor plant:** Genotypic differences among the donor plants greatly affect the ability of pollen grains to form haploid plants. For example, In tobacco, *N. langsdorffii* only few pollen embryos could be induced than in other species. Also, in rice, japonica types respond better than indica types.
2. **Physiological status of donor plants:** Physiology of the donor plant is affected by the following:
   i. **Age:** The buds from the first flush of flowers show better respond than those born subsequently.
   ii. **Environment:** The response in culture is predominantly influenced by different external conditions like light intensity, photo period temperature,

nutritional status and concentration of $CO_2$. Androgenic response was greater when the anthers were taken from plants are grown in short day (8hrs) with high light intensity as compared to those grown in long days (16hrs) with low light intensity.

iii. **Stage of pollen development:** The optimum stage of pollen varies with the species. Usually the anthers containing pollen at early to mid uninucleate stage is used Generally the bud size is used as an index of the pollen stage.

iv. **Size of anthers:** Spikelets and texture of spikelets are correlated with the optimum development stage of the pollen which influences the culture.

v. **Anther wall factors:** The anther wall, whole anthers (or) extract of anthers were found to play an important role in androgenic response by acting as a conditioning factor.

vi. **Culture medium:** Sucrose is essential for androgenesis, the usual level of sucrose is 2-4%. However higher concentration of 6-12% favours androgenesis in cereals. The media requirements vary with the genotype, age of anther and the conditions under which the donor plants are grown. Basal medium of MS, Nitsch and Nitsch, white, N6 for solonaceous crops, B5 and its modifications for Brassica and B5, N6, for potato are commonly used. Rich medium may encourage the proliferation of the diploid tissue of anther wall and should be avoided, Incorporation of activated charcoal into the medium has stimulated the induction of androgenesis. The iron in the medium also plays a very important role for the induction of haploids. Potato extract, coconut milk and growth regulators like auxin and cytokinin are also used for anther and pollen culture due to their stimulatory effect on androgenesis.

**Procedure for anther culture**

1) **Pretreatment:** Induction of haploids can be enhanced by keeping the anther or flower bud at low temperature. Cold treatment may also act to help the embryogenesis by repressing the gametophytic differentiation or by lowering the abscisic acid content of the anther which is considered to be inhibitory for the production of haploids.
2) After pre-treatment, the anthers are dissected under sterile conditions. In plants with minute Dowers Eg:- *Brassica and Trifolium* it may be necessary to use a stereo microscope for dissecting the anthers. In case of cereals whole panicles may be inoculated in the medium. Anthers should be placed horizontally and not in upright position usually about 50-60 anthers should be placed in 10ml of liquid medium. Anthers can also be plated on solid agar media at the rate normally 10-20 anthers in a 6 cm petridish.
3) The young flower buds are collected and washed under running tap water to remove the dirt.

4) These are then surface sterilized by immersing in 70% ethanol or sodium hypochlorite solution.
5) Then washed in sterile water and transferred into a sterile petridish.
6) With the help of sharp scalpel and using forceps the buds are split open and anther lobes are taken out.
7) One of the anther lobes of each bud is checked by crushing into acetocarmine stain under microscope for the proper stage of microspore development.
8) The filament portions are removed from the selected anther lobes.
9) The damaged anther lobes should be discarded and intact anther lobes are placed into proper media.
10) Explants used for anther culture is very critical as the antherlobes bearing the PMC (pollen mother cell) of correct divisional stage signify for being divided to form the callus mass or the direct haploid embryo.
11) Incubated at 24°-28°C in dark for 3-8 weeks.
12) The haploid embryos or plantlets develop, come out by bursting the anther lobes.
13) The haploid plantlets are self-sterile due to presence of single set of chromosome which are not able to participate in meiotic segregation. By colchicine treatment, haploids are made homozygous diploid, or isogeneic diploid which are fertile.
14) Haploids or homozygous diploid grown in vitro are transferred to pot and grown to maturity in the glasshouse.

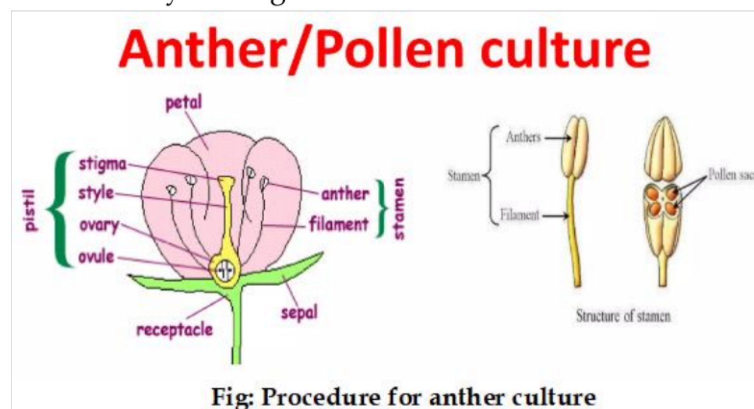



**Fig: Procedure for anther culture**

**Applications of anther culture**

Haploid production Major application of pollen / anther culture is production of haploid plants. It takes much time to produce haploid plants by conventional breeding methods (many generations of inbreeding or backcrossing). As pollens are haploid, plants developed from these are homozygous (haploids).

- **Protoplast isolation** - Used for protoplast isolation as single pollens (unicellular) are available.

- **Transformation** - Used in transgenic plant formation, it can be done with less time consumption.
- **Crop improvement** - In-vitro anther culture is used for improvement in vegetable and cereal crops e.g. asparagus, sweet pepper, watermelon, cabbage, broccoli, wheat etc.
  1. To obtain haploids plants, main and foremost advantage of the in vitro production of haploid over the conventional plant breeding method is the saving of time.
  2. By anther and pollen culture, homozygous diploid or isogenic diploid plant can be produced within a year as compared to the long inbreeding method which might take four to six years.
  3. Isogenic lines are also beneficial where plants are self-incompatible, e.g. rye.
  4. Anther culture is applied in mapping population.
  5. The success of any crop improvement de-pends on the extent of genetic variability in base population. In this regard callus cul-tures are a rich source of genetic variability. By anther culture, not only haploids but plants of various ploidy level and mutants can be regenerated. The anther culture de-rived callus of *Arachis hypogea*, *A. villosa, Cajanus cajan and Cicer arietinum* show a wide range of genetic variability and thus can be incorporated into the breeding programmes.
  6. Haploids derived from anther and pollen culture are useful in cytogenetic studies.
  7. By comparing the heterozygous diploid with haploid or homozygous diploid population, recessive phenotypic characters can be identified very easily.
  8. Achievemems in anther culture In Japan, a commercial tobacco variety N. tabaccum F-211 has been produced by anther culture which is resistant to bacterial wilt and has mild smoking quality. In china, 81 varieties and strains of rice have been developed through anther culture Eg. Hua yu-1. Xin-Xiu, Xhonghua -8 and xhonghua -9 with high yield and blast resistance have been produced.

**Short Questions**

1. Define the term androgenesis and write about the two different modes of androgenesis.
2. Write about the various factors effecting androgenesis.
3. List the various applications of anther culture.

**Essay Questions**

1. scribe in detail the procedure of anther culture

# 2.7 CRYOPRESERVATION

Many of the important crop species produce recalcitrant seeds with early embryo degeneration. Also many of the plants are vulnerable to insects, pathogens and various climatic hazards. Maintenance of these plants are very difficult. Mainly the plant species which are endangered, rare and threatened with extinction are needed to be conserved by ex-situ method of germplasm conservation.

Plant tissue culture may be applied for this purpose. In vitro germplasm storage collection provides a cost effective alternative to growing plants under field conditions, nurseries or greenhouses.

Furthermore, the cryopreservation of cells and tissue, revival of these tissue and regeneration of plants from tissue through tissue culture technique really effective in conservation biotechnology. Cryopreservation involves storage of cells, tissues, etc. at a very low temperature using liquid nitrogen.

The word cryo comes from the Greek word "kayos" meaning "frost". It means preservation in a "frozen state". It is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. Cryopreservation is a technique in which low temperature is used to preserve the living cells and tissue. In this technique, tissues can be preserved for a very long time. The science that deals with cryopreservation is known as "cryobiology".

It can be done over the following temperature:

- Solid carbon dioxide (at-79°C)
- Low-temperature deep freezer (at -80°C)
- In vapor phase nitrogen (at-150°C)
- In liquid nitrogen (at-196°C)

**The temperature which is normally used is:** Using solid carbon dioxide -80°C or Using liquid nitrogen -196°C. The main aim of the Cryopreservation technique is to achieve low temperatures without incurring further harm due to ice crystal formation during freezing. In the Past, Cryopreservation was based on coating the material to be frozen with cryoprotectants. Due to the intrinsic toxicity of many cryoprotectants, new techniques are being studied and worked upon.

**Steps of Cryopreservation**

The technique followed by the regeneration of plants involves the following steps.

- **Selection of Material:** For cryopreservation, the selection of proper plant material important. Two important factors depend on it such as nature and density. Any tissue can he selected for this purpose, for example embryo, meristem, ovules seeds, etc. The density should be high.

- **Addition of Cryoprotectant:** The chemical material is important as it prevents cryo destruction. Some examples of cryoprotectants are Glycerol, Proline, Mannitol, Sucrose, Glucose, Polyethylene glycol alcohol. Mainly two cryoprotectants should be used together instead of a single one as they are considered to be more effective.
- **Freezing:** Different species of plants show different types of sensitivity to low temperatures. They are different types of methods:
    a. **Slow Freezing Method -** In this process, the tissue or plant material is slowly frozen at a slow cooling rate. The major advantage is that the plant cells are partially hydrated and serve in a better manner.
    b. **Rapid Freezing Method -** The vials are plunged in liquid nitrogen. In this process, the temperature decreases from -300 to 1000 degrees rapidly.
    c. **Dry Freezing Method** - In this method hydrated cells and seeds are stored.
- **Storage in Liquid Nitrogen:** It is also important for the maintenance of the sale or material at a specific temperature. In general, the temperature is kept - 70 to - 196°C. Prolonged storage is done at the temperature of -196°C in liquid nitrogen. A continuous supply of nitrogen is needed to prevent damage.
- **Vitrification:** Vitrification is a process of conversion of liquid into solid in the absence of crystallization. When the cells have properly undergone the process of slow freezing, it will result in vitrification where ice formation does not take place because here the aqueous solution is much concentrated which results in permitting the formation of ice cubes. Instead, the water gets solidified into a glassy clear state.
- **Thawing:** The thawing process is usually carried out by plunging the vials into a warm water bath with vigorous swirling. It also causes the vials to get transferred or move to another bath at 0°C.

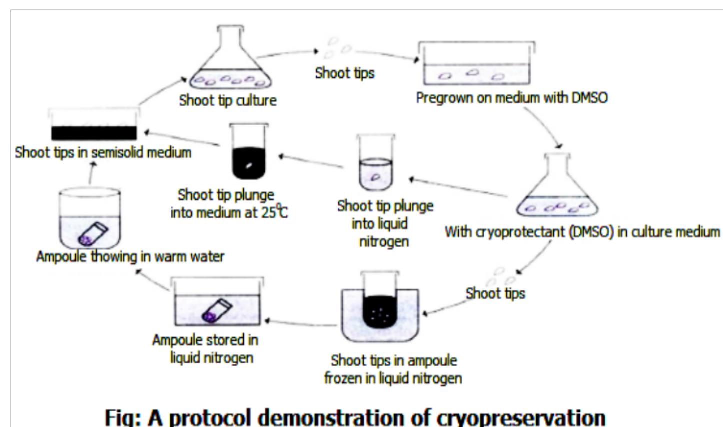



**Fig: A protocol demonstration of cryopreservation**

- **Washing & Reculturing:** The preserved material is washed to remove the cryoprotectant. Furthermore, the material is recultured in a fresh medium.

- **Measurement of Viability:** Due to storage stress, there is a possibility of cell death. The presence of viability can be seen in most cases. It is calculated by the formula (no of cells growing no of cells thawed) x 100.
- **Regeneration of Plants:** After that, the viable seeds are cultured on a non-specific growth medium. Suitable environmental conditions are maintained.

**Applications of cryopreservation**

1. Cryopreservation is one of the most reliable strategies for preserving plant genetic resources for the long term.
2. In agriculture, germplasm cryopreservation is used to improve domestic varieties, genetics and adaptability to environmental changes.
3. These strategies can now be used for plant genotypes also. New cryogenic methods utilizing cryopiates (V and D) have recently been developed. These technologies have advantages such as ease of application and excellent regeneration rates after cryopreservation.
4. Cryopreservation is a powerful tool for preserving endangered species' germplasm. It can also help to maintain plant fertility.
5. Disease-free plants can be conserved and propagated and recalcitrant seed can be maintained for a long time.
6. Pollen can be maintained to increase longevity.
7. No change or contamination of fungus or bacteria takes place after the storage process is completed and material is preserved.
8. Minimal space is required for the purpose of cryopreservation.
9. Minimal labor is required for the purpose of cryopreservation.
10. Due to the gradual disappearance of economic and rare species the necessity for storage of genetic resources increases. The convent journal method of the storage fails to prevent losses caused by:
    - Attack of pathogen and pest
    - Climatic disorders
    - Natural disorder
    - Political and economic causes

**Short Questions**

1. Define the term androgenesis and write about the two different erodes of androgenesis.
2. Write about the various factors effecting androgenesis.
3. List the various applications of Cryopreservation.

**Essay Questions**

1. Describe in detail the procedure of Cryopreservation and mention the various steps involved.

# UNIT III PRODUCTION OF TRANSGENIC PLANTS

## 3.1 DIRECT GENE TRANSFER TECHNIQUES - PHYSICAL METHODS

**Gene transfer technologies in plants:** The process of transfer, integration and expression of transgene in the host cells. i. known is genetic transformation. A foreign gene (transgenes encoding the trait be incorporated into plant cells, along with a "cassette" of extra gene material to add a desirable trait to a crop. The cassette inch des a sequence of DNA called a "promoter", which determines where and when the foreign gene is expressed in the host, and "maker gene" which allow, breeders to determine by screening or selection which plants contain the sorted gene. To example marker genes may take plants resistant to antibiotics not need routinely (e.g., Agrimycin, kasetyanter tolerant of some herbicides.

Various genetic transfer techniques are grouped into two main categories:

1) Vector mediated gene transfer (Indirect method)
2) Vector less gene transfer (Direct method)

**1) Direct methods of gene transfer**

When the foreign DNA is directly inserted into the plant genome, the word direct or vector less DNA transmission is used. Direct DNA transfer methods rely on naked DNA being delivered into the plant cells. This contrasts with the transfer of agrobacterium or vector mediated DNA that can be considered indirect methods. The majority of the methods for direct transfer of DNA are simple and effective. And in addition to this process has been used to develop other transgenic plants. The introduction of DNA into plant cells without biological agents such as *Agrobacterium* being involved and leading to stable transformation is called "direct gene transfer",

**Types or direct IVNA transfer**

The direct DNA transfer can be broadly divided into three categories:

1. **Physical gene transfer methods:** Electroporation, Particle bombardment, Micro injection. Macro injection Liposome-Mediated Transformation, Silicon Carbide Fibre-Mediated Transformation.
2. **Chemical gene transfer methods:** Poly-ethylene glycol (PEG) - mediated. 2.2 Diethyl amino ethyl (DEAL), dextran-mediated, Calcium phosphate precipitation

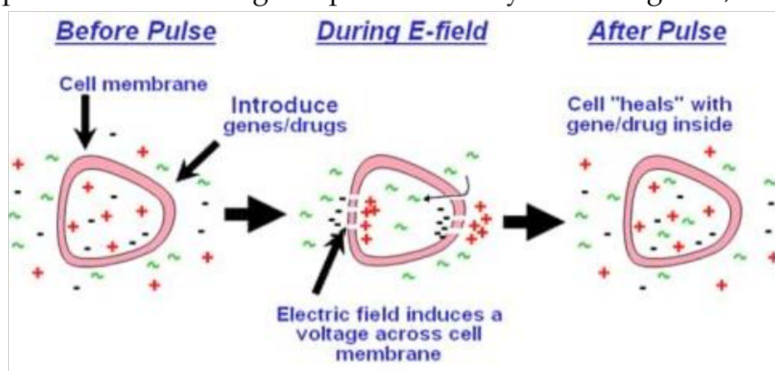
3. DNA imbibition by cells/tissues/organs
4. Pollen transformation
5. Delivery via growing pollen tubes
6. Laser induced transformation etc.

**Physical gene transfer methods**

1. **Electroporation**

Electroporation is the incorporation of DNA into the cell by exposing them to high voltage electrical pulses for a very short period of time to cause temporary pores in the plasma lemma. Plant cell electroporation generally uses protoplast, while thick plant cell walls restrict the movement of macromolecule.

Thus method can be used in those crop species in which regeneration from protoplast is possible. In the early years, only protoplasts were used for gene transfer by electroporation. Now a day, intact cells, callus cultures and immature embryos can be used with suitable pre-and post-electroporation treatments, Electroporation has been successfully used for the production of transgenic plants of many cereals e.g. rice, wheat, maize




**Steps:**

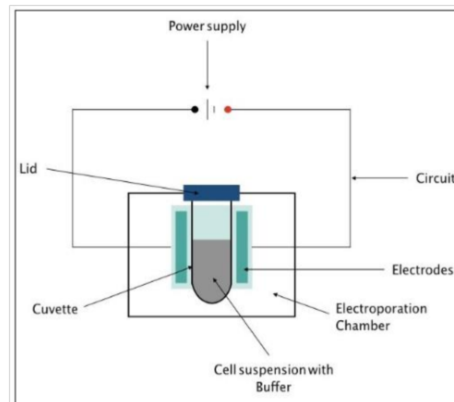
- ✓ The plant material is incubated in a buffer solution containing the desired foreign target DNA, and subjected to high voltage electrical impulses
- ✓ The electric current leads to the formation of small temporary holes in the membrane of the protoplasts through which the DNA can pass.
- ✓ After entry into the cell, the foreign DNA gets incorporated with the host genome, resulting the genetic transformation the protoplasts are then cultured to regenerate in to whole plants.

**Advantages of electroporation**

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

**Limitations of electroporation**

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




**a) Particle bombardment/microprojectile/biolistic/gene gun/particle acceleration**

Particle bombardment is a technique used to introduce foreign DNA into plant cells. Particle (or micro projectile) bombardment is the most effective method for gene transfer and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms. The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics.

**Plant material used in bombardment:** Two types of plant tissue are commonly used for particle bombardment:

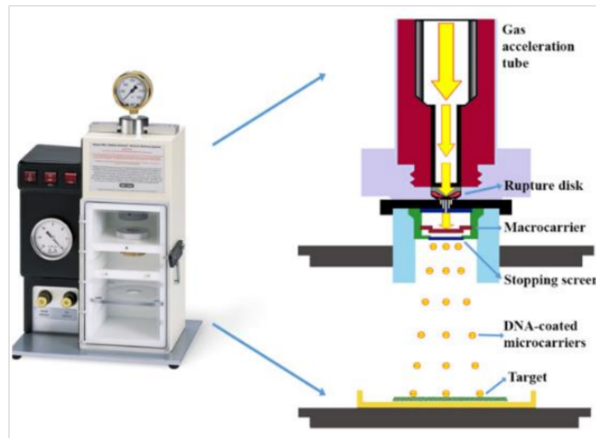
1) Primary explants which can be subjected to bombardment that are subsequently induced to become embryo genic and regenerate
2) Proliferating embryonic tissues that can be bombarded in cultures and then allowed proliferate and regenerate.

   In order to protect plant tissues front being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

**Steps:**

1. The process of transformation employs foreign DNA coated with minute 0.2-0.7 µm gold (or) are tungsten particles to deliver into target plant cells.
2. The coated particles are loaded into a particle gun and accelerated to high speed (By using pressurized helium gas or by electro static energy released by a droplet of water exposed to a high voltage).
3. The target could be plant cell suspensions, callus cultures, or tissues.
4. The projectiles penetrate the plant cell walls and membranes.

5. As the micro projectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA.




b) **Transgene integration in bombardment:** It is believed (based on the gene transfer in rice by biolistics) that the gene transfer in particle bombardment is a two stage process.
   1. In the pre-integration phase, the vector DNA molecules are spliced together. This results in fragments carrying multiple gene copies
   2. Integrative phase is characterized by the insertion of gene copies into the host plant genome. The integrative phase facilitates further transgene integration which may occur at the same point or a point close to it. The net result is that particle bombardment is frequently associated with high copy number at a single locus. This type of single locus may be beneficial for regeneration of plants.

**Advantages of particle bombardment**

i. There has been tremendous success in transforming plant species. It is due to its rapid assessment of the transient expression of genetic constructs introduced into cells of intact tissues. After the plant cell genome gets impregnated by the DNA-coated gold particles, the DNA is used as a template strand for transcription by the cell. This is called transient expression.

ii. The capability of biolistics to optimize the delivery of DNA into the cells is done on strict parameters. These include the target tissue, behavior in tissue culture, and available marker genes. If the delivered DNA construct contains a selectable marker, then stably transformed cells can be selected and cultured accordingly using tissue culture methods.

iii. For example, transformed cells in the culture with a DNA construct containing a gene that confers resistance to any particular antibiotic or herbicide, are selected including that antibiotic or herbicide in the tissue culture media.

iv. The selected transformed cells are allowed to divide and differentiate into the organized specialized, tissue cells of an entire plant. It happens when the transformed cells are treated with a series of plant growth hormones, such as auxins and gibberellins. This capability in plants for total regeneration is called totipotency.

v. Thus, the desired gene can be introduced into callus cultures or even tissues of whole seedlings.

**Limitations of particle bombardment**

- The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing
- The target tissue may often get damaged due to lack of control of bombardment velocity.
- Sometimes, undesirable chimeric plants may be regenerated.

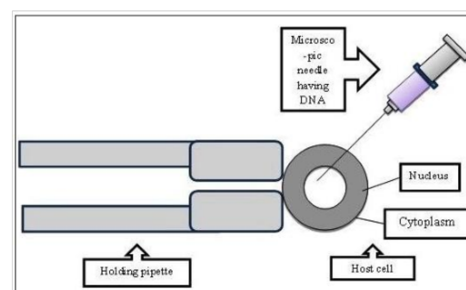
**c) Microinjection**

Microinjection is a direct method to introduce DNA into either cytoplasm or nucleus. It is a microsurgical procedure conducted on a single cell, using a glass needle (i.e., a fine, glass microcapillary pipette), a precision positioning device (a micromanipulator) to control the movement of the micropipette, and a micro injector. Extrusion of fluid containing the genetic material through the micropipette uses hydrostatic pressure. Injections are typically carried out under direct visual control, using a microscope. The small tip diameters of these micropipettes, combined with the high precision of the micromanipulator, allow accurate and precise DNA delivery. This technique is based on the experiments of Barber and forms the basis of developments observed today.

It is the use of a glass micropipette to inject a liquid substance at a microscopic or borderline macroscopic level. The target is often a living cell but may also include intercellular space. Microinjection is a simple mechanical process usually involving an inverted microscope with a magnification power of around 200x.

**Steps:**

- ✓ For processes, such as cellular or pronuclear injection the target cell is positioned under the microscope




- ✓ Using very small bore glass needles (outer diameter of usually less than 0.2 μm), DNA or other material is injected into the cell for subsequent integration and/or expression.
- ✓ Two micromanipulators-one holding the pipette and one holding a microcapillary needle are also used. In this way the process can be used to introduce a vector into a single cell.

**Advantages:**

i. Unlike many other approaches, the direct nature of the approach ensures delivery in almost every cell treated. Further, the exact copy number or number of DNA molecules delivered into each cell can be precisely controlled.

ii. This technique is now established as one of the most flexible technique for introducing DNA and even RNA into living cells.

iii. These developments have also helped researchers to study single cells for complicated cellular processes, structure, and functions *in vitro*.

**II. Chemical gene transfer methods**

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

Polyethylene glycol (PEG), in the presence of divalent cations (using $Ca^{2+}$), 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**

i. A large number of protoplasts can be simultaneously transformed.

ii. This technique can be successfully used for a wide range of plant species

**Limitations of PEG-mediated transformation**

i. The DNA is susceptible for degradation and rearrangement.

ii. Random integration of foreign DNA into genome may result in undesirable traits.

iii. Regeneration of plants from transformed protoplasts is a difficult task.

**III. DEAE dextran-mediated transfer**

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The efficiency increased to 80% when DMSO shock is given. The major limitation of this approach is that it does not yield stable trans-formants.

**IV. Calcium phosphate co-precipitation-mediated transfer**

The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the cells get transformed. The success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate Addition of dimethyl sulfoxide (DMSO) increases the efficiency of transformation.

**Short Questions**

1) Write about the various chemical methods of gene transfer.
2) Write about microinjection method of gene transfer.

**Essay Questions**

1) Write in detail about Particle bombardment/microprojectile/biolistic/gene gun/particle acceleration highlighting on its applications, advantages and limitations.
2) Write in detail about electroporation method of gene transfer highlighting on its applications, advantages and limitations.

## 3.2 GENE TRANSFROMATION *AGROBACERIUM* MEDIATED GENE TRANSFER

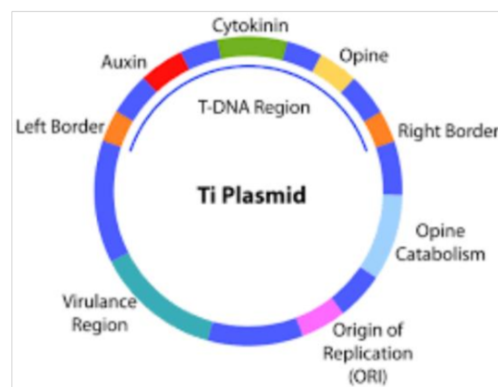
**Vector mediated gene transfer (indirect method)**

Vector-mediated gene transfer is carried out either by *Agrobacterium* mediated transformation or by use of plant viruses as vectors. In this approach the transgene is combined with a vector which takes it to the target cells for integration. The term plant gene vector applies to potential vectors both for transfer of genetic information between plants and the transfer of genetic information from other organisms (bacteria fungi and animals) to plants.

**Agrobacterium mediated transformation**

The Agrobacterium system was historically the first successful plant transformation system, marking the breakthrough in plant genetic engineering in 1983. The Agrobacterium is naturally occurring gram negative soil bacterium with two common species. A *tumifacience* and *Arhizogenes* there are known as natural gene engineers for their ability to transform plants.

A *tumifacience* induces tubers called crown galls, whereas *A. rhizogenes* causes hairy root diseases. Large plasmids in these bacteria are called tumour inducing (Ti plasmid) and root inducing (Ri plasmid) respectively. The major credit for the development of plant transformation techniques goes to the natural unique capability of *A. tumefaciens.*




The Ti plasmid has two major segments of interest in transformation that is T DNA and virus region. The T DNA region of the Ti plasmid is the part which is transferred to plant cell and incorporated into nuclear genome of cells. The transfer of T DNA is mediated by genes in the another region of Ti plasmid called vir genes (virulence genes).

Modified Ti plasmid are constructed that lack of undesirable Ti genes but contain a foreign gene (resistant to a disease) and a closely linked selectable marker gene (E.g.: for antibodies resistance).

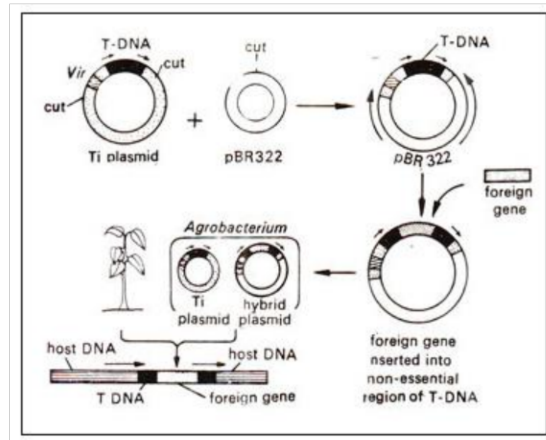



**Fig: Transfer of genes using Agrobacterium**

**Process**

The process of Agrobacterium-mediated gene transfer:

- ✓ It begins by inserting the desired gene(s) into a plasmid vector, which is a small circular piece of DNA that can replicate independently of the host genome. The plasmid vector also contains the necessary regulatory sequences to control the expression of the inserted gene(s).
- ✓ The plasmid vector is introduced into the *Agrobacterium tumefaciens* through a process called transformation. Once inside the bacterium, the plasmid vector is recognized by the bacterium's natural transfer machinery, which packages the T-DNA and delivers it into the host plant's genome.
- ✓ The T-DNA is integrated into the host plant's DNA, resulting in the transfer of the desired gene(s) into the plant's genome.
- ✓ The transformed plant cells can then be selected and regenerated into whole plants using tissue culture techniques.

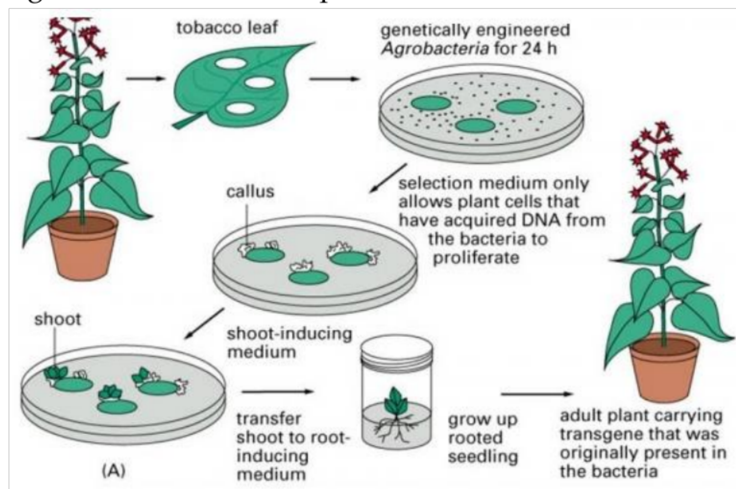



**Fig: Steps involved in Agrobacterium mediated gene transfer in tobacco**

**Factors affecting the gene transfer**

Agrobacterium-mediated gene transfer is a widely used technique for introducing foreign DNA into plants. However, successful gene transfer depends on several factors, including:

1. **Agrobacterium strain:** Different strains of Agrobacterium have varying abilities to transfer genes into plants. Therefore, the choice of the strain used is crucial.
2. **Vector design:** The vector used to transfer the gene must be designed in can integrate into the plant genome and express the desired gene. The vector also needs to have appropriate regulatory elements that control the expression of the gene.
3. **Plant species and tissue type:** Different plant species and tissue types vary in their susceptibility to Agrobacterium-mediated gene transfer. The choice of the plant species and tissue type should be carefully considered.
4. **Wounding:** Wounding of the plant tissue can enhance gene transfer efficiency. Therefore, some protocols include wounding the plant tissue before Agrobacterium infection.
5. **Co-cultivation conditions:** The co-cultivation conditions, such as temperature, humidity, and light, can also affect gene transfer efficiency. These conditions should be optimized for each specific plant species and tissue type.
6. **Antibiotics:** Antibiotics are often used to select for transformed plant cells. However, the concentration of antibiotics and the duration of selection can also affect gene transfer efficiency.
7. **Contamination:** Contamination with other microorganisms can also reduce gene transfer efficiency. Therefore, it is crucial to maintain sterile conditions throughout the entire process.
8. **Explants:** The type and size of the plant tissue used as an explant can influence gene transfer efficiency. Generally, young and actively growing tissues are more amenable to transformation than mature or differentiated tissues.
9. **Plant Growth Regulators (PGR):** The use of PGRs such as auxins and cytokinins can affect the efficiency of gene transfer. For instance, the addition of cytokinins to the culture medium can enhance shoot regeneration, which can increase the number of transformed plants.
10. **Light:** Light is an important factor that affects plant growth and development. The quality and quantity of light can influence the efficiency of gene transfer. For example, the use of blue light can enhance Agrobacterium-mediated gene transfer in some plant species.
11. **Temperature:** The temperature of the culture environment can also affect gene transfer efficiency. In general, the optimal temperature range for

Agrobacterium-mediated gene transfer is between 20-25°C. Higher or lower temperatures can decrease the efficiency of gene transfer.

**Advantages**

Agrobacterium-mediated gene transfer is a preferred method for transforming plants for several reasons:

1. **High transformation efficiency:** Agrobacterium-mediated gene transfer can result in high transformation efficiencies, meaning that a large number of plants can be efficiently transformed with the desired gene(s).
2. **Precise and targeted gene insertion:** Agrobacterium–mediated gene transfer allows for the precise and targeted insertion of new genes into specific sites in the plant genome, resulting in a more stable and predictable gene expression.
3. **Versatility:** Agrobacterium-mediated gene transfer can be used to introduce a wide range of genes into plant genomes, including genes from other species, synthetic genes, and regulatory elements.
4. **Safe and natural process:** Agrobacterium-mediated gene transfer is a natural process that occurs in nature, and does not require the use of harsh chemicals or physical methods that can damage plant cells.
5. **Compatibility with tissue culture techniques:** Agrobacterium–mediated gene transfer is compatible with tissue culture techniques, which allows for the regeneration of whole plants from transformed cells.

Overall, Agrobacterium-mediated gene transfer is a reliable and effective method for introducing new genes into plant genomes, which has led to the development of genetically modified crops with improved traits and increased yields.

**Applications**

Some of the most common applications include:

1. **Crop improvement:** Agrobacterium-mediated gene transfer can be used to introduce desirable traits into crops, such as disease resistance, herbicide tolerance, and improved yield
2. **Functional genomics:** The technique can be used to study the function of genes by introducing gene constructs that alter their expression or function.
3. **Molecular farming:** Agrobacterium-mediated gene transfer can be used to produce recombinant proteins in plants. This approach can be more cost effective and environmentally friendly than traditional methods of protein production.
4. **Conservation of rare and endangered species:** The technique can be used to preserve the genetic diversity of rare and endangered plant species by

storing their DNA in a seed bank or by regenerating plants from tissue culture.

5. **Biosynthesis of novel compounds:** Agrobacterium-mediated gene transfer can be used to introduce biosynthetic pathways for the production of novel compounds, such as pharmaceuticals or industrial chemicals, into plants.
6. **Plant-microbe interactions:** The technique can be used to study plant-microbe interactions, such as the role of plant defense genes in response to pathogenic bacteria.

**Limitations**

- ✓ **Limited host range:** Agrobacterium-mediated gene transfer is not effective in all plant species. Some plant species are inherently resistant to Agrobacterium infection, and it can be challenging to develop transformation protocols for them.
- ✓ **Insertion site variability:** The site where the transferred DNA integrates into the plant genome is variable and unpredictable. This can lead to positional effects on gene expression, gene silencing, and other unintended effects.
- ✓ **Tissue culture requirements:** The process of Agrobacterium-mediated gene transfer often requires tissue culture, which can be technically challenging, time-consuming, and expensive.
- ✓ **Epigenetic effects:** The transferred DNA can sometimes induce epigenetic changes, such as DNA methylation, that can affect the expression of endogenous genes and the stability of the transgene.
- ✓ **Unintended effects:** The introduction of foreign DNA into plants can sometimes have unintended effects, such as affecting plant development or altering metabolic pathways.
- ✓ **Regulatory hurdles:** The use of Agrobacterium-mediated gene transfer in agriculture is subject to regulatory approval in many countries. This can make it difficult and expensive to commercialize genetically modified plants.

**Short Questions**

1) Applications of Agrobacterium mediated gene transfer.
2) Write about the various factors effecting gene transfer by agrobacterium.
3) List the limitations of Agrobacterium mediated gene transfer.

**Essay Questions**

1) Describe in detail Agrobacterium mediated gene transfer in plants to raise transgenic plants

# 3.3 BINARY VECTORS AND CO-INTEGRATED VECTORS

We know that the Ti plasmid of *Agrobacterium tumefaciensis* a natural vector for genetic engineering of the plant cells, because it can transfer its T-DNA from the bacterium to the plant genome. However the methods to clone gene of interest into the TDNA was cumbersome due to various reasons. The Ti plasmid is present in low copy number in *Agrobacterium* and it does not have unique restriction sites that are very essential for cloning gene of interest. In addition, the oncogenes present in T-DNA region cause disorganized growth and affect regeneration potential of the recipient plant cells, thus making it difficult to have the transformation event inherited over generations in a stable manner.

On the other hand it is much easier to clone genes in *E.coli* and several vectors had been developed for gene cloning in this bacterium, like pBR322 for example. Taking a cue from these vectors, attempts were made to develop T-DNA based vectors for genetic transformation of plants.

**Features of a gene cloning vector**

A typical plasmid gene cloning vector of *E.coli* possesses the following features:

- **a.** An origin of replication permitting multiple copies of the plasmid to exist in the bacterial cell.
- **b.** Plasmid mobility function to allow transfer of the plasmid by conjugal transfer between bacterial cells.
- **c.** A multiple cloning site that has unique restriction enzyme sites, not present anywhere else on the plasmid.
- **d.** Selectable markers that allow only the bacterial cells having the plasmid to proliferate
- **e.** Screenable markers that allow selection of those bacterial cells that have the gene of interest inserted within the multiple cloning site.

The wild type Ti plasmids are not suitable as gene cloning vectors because they do not possess these features. The size of the Ti plasmid is very large and lacks unique restriction sites. It is a low copy number plasmid in Agrobacterium and hence not suitable for gene cloning. Besides this, the presence of oncogenes within T–DNA borders lead to abnormal phenotypes and are not desirable. These problems have been overcome by the development of T–DNA based vectors, suitable for genetic transformation of plants.

There are two basic types of vectors, namely the co–integrate vectors and binary vectors. Both vectors make use of disarmed Ti plasmids, ie. Ti plasmids lacking the oncogenes (hormone biosynthesis genes present within the T-DNA region), for transforming plants.

### Binary vectors

The binary vector system consists of an *Agrobacterium* strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in *E. coli* and *Agrobacterium*.

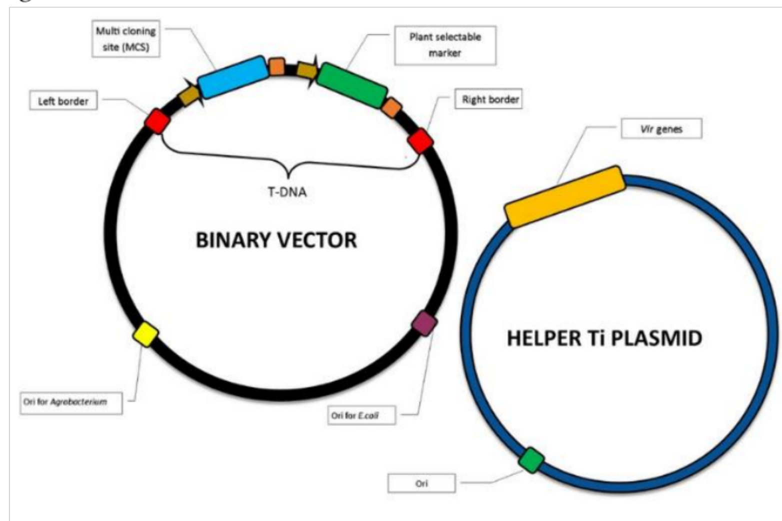



**Fig: A diagrammatic representation of a typical binary vector system**

### Components of binary vectors

T-DNA binary vectors generally contain a number of features important for their use in genetic engineering experiments:

1) T-DNA left and right border repeat sequences to define the T-DNA.
2) A plant-active selectable marker gene to select the plant cells after successful transformation.
3) Restriction endonuclease sites (multiple cloning sit) within T-DNA into which the gene of interest can be inserted.
4) Origin(s) of replication to allow maintenance in *E. coli* and *Agrobacterium*.
5) Selectable marker gene for the selection of the binary vector in *E. coli* and *Agrobacterium*

### Utilization of binary vectors

There are many binary vectors developed for genetic transformation of plants eg. pBH21, PCAMBIA series of vector etc. While all of them have T-DNA borders within which lies a multiple cloning site (MCS), they differ in the promoters and terminators flanking the MCS and in the plant selectable and screenable markers. The recombinant binary vectors are mobilised into *Agrobacterium* either by tri-parental mating or transformation. The Ti plasmid in *Agrobacterium* lacks the T region and provides the vir genes in trans for excising the T-DNA containing the gene of interest and bringing about its transfer and integration into the plant genome.

Further, improvements in the binary vectors include super binary vectors, which carry additional virulence genes on the vector backbone. These are especially useful in genetic transformation of recalcitrant plants like cereals. Another improvisation was the introduction of multiple left borders in the binary vector. This ensured correct cleavage at the left border of T- DNA and prevented transformation of the vector backbone into host plant. A reduction in the size of the binary vectors has been achieved in the $_{p}$GREEN $_{p}$SOUP vector system, where the binary vector has a smaller sized origin of replication.

**Advantages of binary vectors**

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.
ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

**Co-integrate vectors**

Co-integrate vectors are produced by integrating a suitably modified *E. coli* plasmid containing the gene of interest into a disarmed Ti plasmid. The co-integration of the two plasmids is achieved within the *Agrobacterium* by homologous recombination. For homologous recombination to occur, the *E. coli* plasmid and the disarmed Ti plasmid must have some sequences common to both for the recombination to occur.

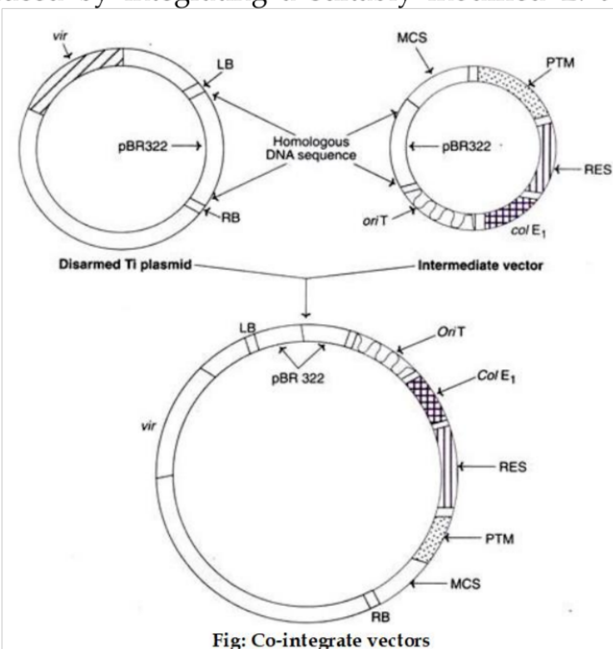


Fig: Co-integrate vectors

These vectors were developed during the early eighties to transform gene into plants. Zambryskiet al.(1983), substituted almost all of the T-DNA of the Ti plasmid pTiC58 with pBR 322 sequences, retaining the left and right border regions and nos genes. This was called as pGV3850. The gene of interest was cloned in the commonly used E.coli vector (pBR322), into which were also inserted selectable marker genes for both the *Agrobacterium* and plant cells. The recombinant pBR322 was then transferred to *Agrobacterium* and the gene of interest inserted through

homologous recombination between the pBR322 vector sequences present in the T-DNA region of Ti plasmid and the transferred recombinant pBR322.

The steps involved in the development of Co-integrate vectors are as follows:

1) The transfer of recombinant IV containing the gene of interest into the disarmed pTi by a "tri parental mating'. The three bacterial strains,
   a) E.coli strain having a helper plasmid.
   b) The E. coli strain carrying the DNA insert as recombinant IV
   c) *Agrobacterium tumefasciens* carrying the disarmed pTi, were mixed together.
2) The conjugation between the two E. coli strains results in the transfer of the helper plasmid into the E. coli strain carrying the recombinant IV. Then the helper plasmid induces the transfer of recombinant IV into the Agrobacterium. Now the Agrobacterium contains both the recombinant IV and the disarmed pTi.
3) Homologous recombination between the T-DNA sequences of the pTi and IV results in the formation of a large co-integrate vector in which the recombinant IV gets inserted into the T DNA region of the disarmed pTi. The recombinant T-DNA was then transferred to the plant genome.

Agrobacterium cells containing the co-integrate vectors are selected due to the presence of selectable marker gene contributed by the recombinant IV. Binary vectors Intermediate vectors used for the development of co-integrate vectors has resulted in the development of a large co- integrate plasmids. But this large size is not necessary for the transformation as this large size involves tedious and cumbersome handling procedures during cloning.

**Advantages of co-integrate vector:**

1) Target genes can be easily cloned
2) The plasmid is relatively small with a number of restriction sites.
3) Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

**Short Questions**

1) List the various components of a co-integrate vector.
2) Write about the various components of a binary vector.
3) List the typical feature a plasmid gene cloning vector of E.coli must possesses to be used a vector for transformation.

**Essay Questions**

1) Describe in detail co-integrate vector mediated gene transfer in plants to raise transgenic plants.
2) Write in detail about binary vectors and its use in gene transfer.

# 3.4 VIRAL VECTORS FOR GENE TRANSFER

Plant viruses have been used as heterologous gene expression vectors since the beginning of genetic engineering. The advent of molecular biology and high-throughput sequencing technologies have enabled the manipulation of the viral genome to express heterologous proteins and RNAs in plants. Several recent studies have highlighted the potential use of plant virus vectors as transient delivery vehicles for CRISPR-Cas reagents in plants. To date, the best alternative approach to Agrobacterium transfer method to deliver the CRISPR-Cas reagents into the plant cells is the plant viruses. Recent development in GE technologies have urged scientists to incorporate viral vectors and utilize them for the efficient delivery of GE reagents in plant cell.

| Virus Type | gRNA | Nucleases Type | Plant Species | Mutation Heritability |
|---|---|---|---|---|
| **DNA viruses** | | | | |
| CaLCuV | AtU6-gRNA | - | SpCas9-overproducing tobacco (*N.benthamiana*) | No |
| WDV | TaU6-gRNA | SpCas9 | Wheat (T.aestivum) | No |
| | OsU6-gRNA | SpCas9 | SpCas9 – overproducing rice *(O.sativa)* | No |
| BeYDV | AtU6-gRNA | ZFN,TALEN, SpCas9 | Tobacco *(N.tabacum)* | No |
| | AtU6-gRNA | TALEN.SpCas9 | Tomato *cv.MicroTom* | No |
| | AtU6-gRNA | TALEN.SpCas9 | Potato | No |
| | AtU6-gRNA | SpCas9 | Potato | No |
| | AtU6-gRNA | SpCas9 | Tomato *cv.MicroTom* | No |

**Genome Editing by Geminiviruses**

Geminiviridae, the largest virus family consists of circular, single-stranded (ss) DNA viruses infecting a wide variety of hosts ranging from staple to fiber crops worldwide, such as cotton, maize, wheat cucurbits, tomato, and several ornamental and weed plants. Geminiviral genomes are highly reduced in size, ranging from ~2.7 to 5.5 kb encoding four to eight functional proteins present in both sense and complementary sense strand. The insect pests which are involved in transmitting geminiviruses are whitefly (Bemisia tabaci) and leafhoppers. Geminiviruses-derived vectors have been extensively used in the production of proteins, vaccines, and in inducing gene silencing in functional genomic methods. In the perspective of GE, geminivirus-based replicons have attracted much attention and proved successful for genome-editing technologies.

The following remarkable properties of geminiviruses style them as suitable vectors for plant genome engineering:

1. having the ability to make infection in a wide range of plants belonging to various species at once
2. requiring a very smaller number of proteins for initiation of replication inside hosts (in case of mastreviruses, replication associated protein: Rep)
3. its expression is regulated by its own natural promoter present in the intergenic region and any user-specific inducible/constitutive promoters
4. independently replicate inside the host by homologous recombination (HR)-dependent replication, it reverts the host cell into S phase fit for HR if associated with SSN and complementary target sequences and
5. it produce a large amount of amplicons via replication inside the host cell, in turn developing a high number of SSNs and target sequences when used as a vector for genome engineering thereby increasing targeting efficiency.

The engineering of geminiviruses as vector has been used for the heterologous protein expression in plants. Cermak et al. (2015) employed geminivirus-based replicons to generate heritable modifications at a 10- fold higher frequency compared to the traditional delivery methods (i.e., Agrobacterium) to the tomato genome by inserting promoter to the upstream of a gene that regulates the anthocyanin biosynthesis. The resulting gene editing is the overexpression and ectopic accumulation of the anthocyanin biosynthesis pigments in tomato tissues and the targeted modifications was transmitted to the next progeny in a Mendelian manner. In addition, they conclude that by employing geminivirus based replicons, high-frequency, precise modification in the tomato genome was achieved, and it can also overcome the efficiency barrier that made gene targeting in plants more challenging

**RNA Viruses a Potential Delivery System In-Planta**

The use of DNA viruses creates a possibility for inserting foreign DNA into the plant genome while RNA viruses provide an advantage over DNA viruses because the infectious cycles take place in the host cytoplasm, thus resulting the plants free from foreign DNA, which also avoids raising ethical issues and regulatory concerns.

Numerous plant RNA viruses including tobacco rattle virus (TRV), tobacco mosaic virus (TMV), pea early browning virus (PEBV), barley strip mosaic virus (BSMV), foxtail mosaic virus (FoMV) and beet necrotic yellow vein virus (BNYVV) have been used exclusively as vector for the delivery of sgRNAs into the plant cells and editing efficiencies up to 80% have achieved.

Tobacco rattle virus (genus Tobravirus, family Virgaviridae) is a bipartite, positive single stranded RNA (+ssRNA) virus that infects more than 400 different

plant species belonging to 50 families. It is transmitted by nematodes (family; Trichodoridae), mechanically and through seed transmission. It has two genome components, TRVI (or RNAI) which is essential for viral movement and replication proteins whereas TRV2 (or RNA2) which encodes the coat protein (CP) and many other nonstructural proteins. TRV also contains important genes encoding 134 and 194 kDa movement protein (MP), and 16 kDa cysteine-rich protein, whose function has not been identified yet. TRV2 encodes the CP and non-structural proteins involved in nematode transmission, though they are non-functional for infection cycle. Therefore, by using TRV2 as a vector, two non-structural protein encoding genes can be changed with multiple cloning sites for integrating segments of interest, and for heterologous protein expression and host genes for VIGS.

**TRV has many advantages including:**

1. Vast host range (more than 400 host species) and migrating ability to the growing tissues.
2. Their smaller genome size provides cloning, library construction, and multiplexing and agroinfections.
3. Their RNA genome does not integrate in the plant genome.

TRV-based vectors were the pioneers to deliver ZFNs and TALENs into petunia and tobacco plants which leads to the permanent and heritable genome modifications in the infected plants. TRV-based vector can be used to target native genes for the generation of crop plants with novel traits. For the delivery of CRISPR-Cas reagents, Ali et al. (2015) demonstrated a TRV- based vector to successfully edit the N. benthamiana and A. thaliana genomes and the detection of targeted changes in the progeny of transgenic plants provides evidence of the TRV efficiency to infect the germline cells. Furthermore, another RNA virus has also been used to deliver different nucleic acids into various plant species, such as pea early browning virus (PEBV).

**Advantages**

The plant virus-derived vectors provide numerous advantages:

1. Easy to manipulate
2. Higher transient expression owing to high gene copy number leading to the swift development of the desired product
3. Genome can be used as repair template
4. Accumulate at high levels (repair template and sgRNAs)
5. It can spread systemically in plants resulting high expression and gene editing efficiency include expression of multiple sgRNAs from a single viral genome that allows multiple targeted gene editing (VIGE)
6. Capability to screen various construct variants among diverse host plant genotypes, therefore evading poor construct and interruptions in stable plant

regeneration/transformation of host plants, even in the plants those are difficult to be transformed

7. Capability to achieve spatio-temporal gene expression at different growth stages of plants by making changes in the timings of inoculation; and
8. Efficient gene expression in all vulnerable plant hosts, without certain position effects among various transgenic lines.

**Limitations**

Limitations of plant viral based vectors include:

i. The small genome size of geminiviruses causes hindrance and impotent to transfer long DNA chunks e.g., Cas nucleases (~4.2 kb).
ii. Only transient expression usually without any transfer of desirable characters to subsequent generations through breeding or through seed
iii. Due to mutation or deletion, the introduced genes may be lost over time (more problematic with larger inserts)
iv. Adverse effects could be possible on the host or interactions with other viruses, and
v. Transmission to the other susceptible crops or wild hosts may also be possible.

## 3.5 SELECTION OF TRANSGENIC PLANTS USING SELECTABLE MARKER GENES AND REPORTER GENES

**Marker genes**

Marker systems are tools for studying the transfer of genes into an experimental organism. In gene transfer studies, a foreign gene, called a transgene, is introduced into an organism, in a process called transformation. A common problem for researchers is to determine quickly and easily if the target cells of the organism have actually taken up the transgene. A marker allows the researcher to determine whether the transgene has been transferred, where it is located, and when it is expressed.

Marker genes exist in two broad categories: 1. Selectable marker genes and II. Reporter

**Selectable Marker Genes**

The selectable marker genes are usually an integral part of plant transformation system. They are present in the vector along with the target gene. In a majority of cases, the selection is based on the survival of the transformed cells when grown on a medium containing a toxic substance (antibiotic, herbicide, antimetabolite). This is

due to the fact that the selectable marker gene confers resistance to toxicity in the transformed cells, while the non-transformed cells get killed. A large number of selectable marker genes are available and they are grouped into three categories antibiotic resistance genes, antimetabolite marker genes and herbicide resistance genes.

a) **Antibiotic Resistance Genes:** In many plant transformation systems, antibiotic resistance genes (particularly of *E. coli*) are used as selectable markers. Despite the plants being eukaryotic in nature, antibiotics can effectively inhibit the protein biosynthesis in the cellular organelles, particularly in chloroplasts. Eg: Neomycin phosphotransferase II (npt II gene): The most widely used selectable marker is npt II gene encoding the enzyme neomycin phospho-transferase II (NPT II). This marker gene confers resistance to the antibiotic kanamycin. The trans-formants and the plants derived from them can be checked by applying kanamycin solution and the resistant progeny can be selected.
b) **Antimetabolite Marker Genes:** Eg: Dihydrofolate reductase (dhfr gene) The enzyme dihydrofolate reductase, produced by dhfr gene is inhibited by the antimetabolite methotrexate. A mutant dhfr gene in mouse that codes for this enzyme which has a low affinity to methotrexate has been identified. This dhfr gene fused with CaMV promoter results in a methotrexate resistant marker which can be used for the selection of transformed plants.
c) **Herbicide Resistance Markers:** Eg: Enolpyruvylshikimate phosphate synthase (epsps/aroA genes): The herbicide glyphosate inhibits photosynthesis. It blocks the activity of enolpyruvylshikimate phosphate (EPSP) synthase, a key enzyme involved in the biosynthesis of phenylalanine, tyrosine and tryptophan. Mutant strains of *Agrobacterium* and *Petunia hybrida* that are resistant to glyphosate have been identified. The genes epsps/aroA confer resistance to transgenic plants which can be selected.

**Reporter Genes**

A reporter gene may be regarded as the test gene whose expression can be quantified. The plant transformation can be assessed by the expression of reporter genes. In general, an assay for the reporter gene is carried out by estimating the quantity of the protein it produces or the final products formed.

Some of the important reporter genes are as follows:

- **β-Glucuronidase (GUS gene):** β-Glucuronidase producing gene (gus/uidA) is the most commonly used reporter gene in assessing plant transformation for the following reasons: i. β-Glucuronidase assays are very sensitive. ii. Quantitative estimation of the enzyme can be done by fluorometric method (using substrate 4-methylumbelliferryl P-D-glucuronide which is hydrolysed to 4-methylumbelliferone), iii. Qualitative data on the enzyme

can be obtained by histochemical means (enzyme localization can be detected by chromogenic substance such as substrate X-gluc). iv. No need to extract and identify DNA.

- **Green fluorescent protein (GFP gene):** Green fluorescent protein (GFP), coded by gfp gene, is being widely used in recent years. In fact, in many instances. GFP has replaced GUS since assays of GFP are easier and non-destructive. Thus, screening of even the primary transplants can be done by GFP which is not possible with other reporter genes. Gene for GFP has been isolated from jelly fish *Aequorea victoria* which is a luminescent organism. The original gip gene has been significantly modified to make it more useful as a reporter gene. GFP emits fluorescence which can be detected under a fluorescent microscope.
- **Bacterial luciferase (luxA/luxB genes):** The bacterial luciferase genes (luxA and luxB) have originated from *Vibrio harveyi*. They can be detected in some plant transformation vectors. The detection assay of the enzyme is based on the principle of bioluminescence. Bacterial luciferase catalyses the oxidation of long-chain fatty aldehydes that results in the emission of light which can be measured.
- **Firefly luciferase (lue gene):** The enzyme firefly luciferase, encoded by the gene lue, catalyses the oxidation of D-luciferin (ATP dependent) which results in the emission of light that can be detected by sensitive luminometers. The firefly luciferase gene, however, is not widely used as a marker gene since the assay of the enzyme is rather cumbersome.

## 3.6 Genome Editing - CRISPR/Cas Technology

The terms genome engineering, genome editing, and gene editing, refer to modifications Insertions, deletions, substitutions) in the genome of a living organism.

Genome editing through programmable endonucleases is the most recent approach to genetic engineering. Endonucleases are used to specifically induce double strand breaks in target genes of interest. The cellular DNA repair pathway then acts on the double strand break to restore the damage through non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the process, insertions, deletions, substitutions, and DNA recombination may occur.

Three kinds of programmable endonucleases are currently being used for plant genome editing. Zine finger nucleases, transcription activator-like effector nucleases (TALEN$_S$) and CRISPR-Cas9. The most widely used approach to genome editing nowadays is based on Clustered Regularly Interspaced Short Palindromic Repeats and associated protein 9 (CRISPR- Cas9).

In prokaryotes, CRISPR-Cas9 is an adaptive immune system that naturally protects cells from DNA virus infections. CRISPR-Cas9 has been modified to create a versatile genome editing technology that has a wide diversity of applications in medicine, agriculture and basic studies of gene functions.

CRISPR-Cas9 has been used in a growing number of monocot and dicot plant species to enhance yield, quality, and nutritional value, to introduce or enhance tolerance to biotic and abiotic stresses, among other applications. Although biosafety concerns remain, genome editing is a promising technology with potential to contribute to food production for the benefit of the growing human population.

**Mechanism**

CRISPR Cas-edited plants are obtained by mutagenesis techniques using site-directed nucleases (SDNs) which can introduce targeted changes into specific DNA sequences of the genome to improve desired traits.

A distinction can be made between site-directed nuclease type I (SDN-1), site-directed nuclease type II (SDN-2), and site-directed nuclease type III (SDN-3) techniques because they result in different editing outcomes.

**Step 1: Targeting:**

Cas9-guide RNA complex is introduced into a cell, where it randomly associates and dissociates with the DNA. Cas9 recognizes and binds to a three-nucleotide sequence motif called PAM (proto-spacer adjacent motif. It is usually a three-nucleotide sequence consisting of 5 prime NGG-three prime in which the N represents any nucleotide (A, C, G, or T) followed by two guanine (G) nucleotide bases) that is abundant throughout the genome. One way to think of the Cas9-guide RNA complex is as a molecular scissor (Cas9) with a programmable GPS (guide RNA). Cas9 is a nuclease, a type of enzyme that cleaves DNA. It recognizes and binds to a three-nucleotide sequence motif called PAM that is ubiquitous throughout a cell's genome.

Scientists synthesize the guide RNA to contain a 20-nucleotide sequence that matches a particular sequence in a cell's DNA that they want to target. When the guide RNA is added to Cas9, it will guide Cas9 to this target sequence. The target sequence can be nearly any sequence as long as it occurs near a PAM motif, it can be part of a gene's coding region or a regulatory sequence that scientists want to change in some way.

**Step 2: Binding:**

Once it binds to a PAM motif, Cas unwinds the DNA double helix. If the DNA at that location perfectly matches a 20-nucleotide sequence within the guide RNA,

the DNA and matching RNA will bind through complementary base pairing. Cas recognizes and binds to PAM motifs in the cell's DNA. The motif consists of any nucleotide (designated "N") followed by two guanines, when looking at a DNA sequence in a 5 prime to 3 prime direction (N- G-G). Cas9 unwinds and pulls apart the DNA double helix upstream of PAM–in other words, closer to the 5 prime end of the DNA strand relative to PAM. If the sequence of the unpaired DNA strand is not an exact match to the 20–nucleotide sequence within the guide RNA, Cas9 disengages from the DNA, which zips back up into a double helix. If the sequences are a perfect match, the guide RNA base pairs with the complementary DNA sequence, forming a DNA-RNA helix.

**Step 3: Cleaving:**

The DNA-RNA pairing triggers Cas to change its three-dimensional structure and activates its nuclease activity. Cas cleaves both DNA strands at a site upstream of PAM. When the guide RNA perfectly aligns with the target DNA, the RNA and DNA will form a DNA–RNA helix. This binding event activates Cas9's nuclease, or DNA–cutting, activity. It makes specific cuts in the DNA at a position three nucleotides upstream from the PAM site. Two active sites (regions where molecules bind to undergo chemical reactions) on the nuclease domain of Cas9 generate the cuts and cleave both strands of the DNA double helix, resulting in a double-stranded DNA break.

**Step 4: DNA Repair:**

Cells contain enzymes that repair double-stranded DNA breaks. The repair process is naturally error-prone and will lead to mutations that may inactivate a gene NON-HOMOLOGOUS END-JOINING CRISPR-induced double-stranded DNA breaks can be repaired by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) NHEJ is the more frequently used, faster repair mechanism, because the cell does not use a template to join broken DNA ends together. It is, however, an error-prone process that can introduce mutations in the target sequence. Errors are rare, but when the break is repaired correctly, Cas9 will once again recognize the target sequence and cleave it. Repeated cycles of cleavage and repair eventually result in a mutation. The type of mutation is random, but it will occur precisely within the desired target sequence. If the target sequence is within a gene's coding region, the mutation will likely inactivate that gene.

By designing different repair templates, scientists can change the target DNA sequence into a new sequence. These templates could also correct an existing mutation by replacing it with a non-mutated sequence of DNA.

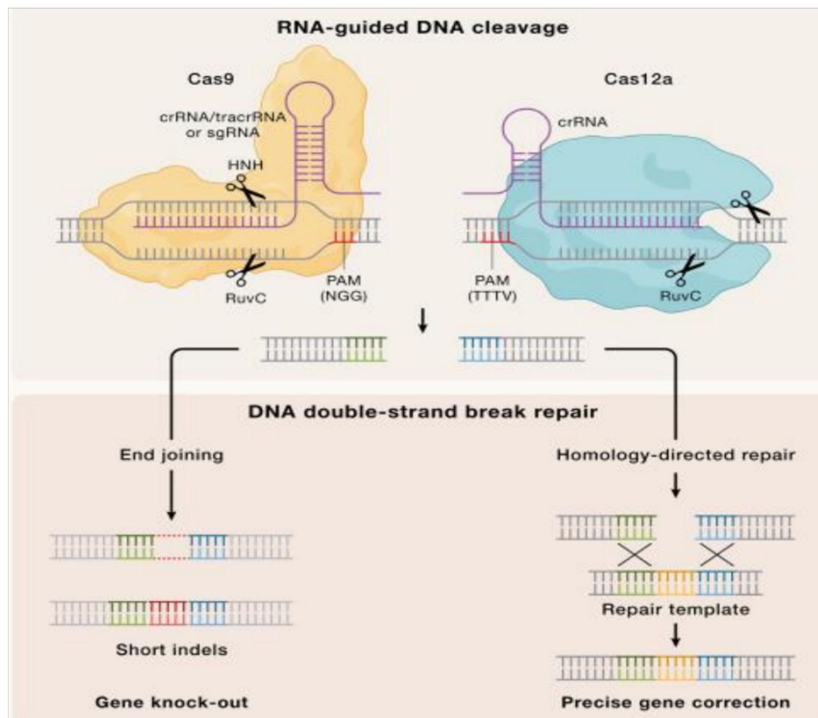



**Fig: targeted genome editing using CRISPR – Cas9. (A) The CRISPR-Cas9 system consist of a Cas9 protein and one or several guide RNA. Guide RNAs determine target DNA specificity by sequence complementarity. (B) Guide RNA and Cas9 protein form a binary complex that specifically cleaves target DNA creating a double – strand DNA break. (C) Cellular DNA repair mechanisms, non-homologous end joining (NHEJ) and homology – directed repair (HDR), repairs the double – strand DNA break. In the process, short insertions, deletions, nucleotide substitutions, or gene insertion may occur.**

## The Genome Editing Process using *Agrobacterium tumefaciens*

- A fundamental part of the genome editing process is the identification of target genes that determine phenotypes of interest, such as susceptibility to viruses, other pathogens, resistance to herbicides or adverse environmental factors.
- Guide RNAs are artificially designed to specifically direct Cas9 to the target gene to be edited. Bioinformatic programs that generate candidate guide RNAs while accounting for the possibility of off-targets are available (http://crispr.mit.edu/). Dynamic expression vectors have also been designed to clone and co-express guide RNAs and Cas9.
- Although variations have been developed recently, transformation of plant cells to express guide RNAs and Cas9 follows a process similar to these established for the generation of transgenic plants. The expression cassettes contain constitutive or inducible promoters, transcription terminators and antibiotic and/or herbicide resistance markers used for selection purposes.
- The vector carrying the Cas9 protein and the guide RNA is then introduced into *Agrobacterium tumefaciens* or *Rhizobium rhizogenes*. Colonies containing the CRISPR-Cas9 construct are further used to transform plants by *Agrobacterium*–mediated transformation and first generation transgenic plants are identified by antibiotic or herbicide selection.

- Green fluorescent protein (GFP) has also been used to distinguish cells or calluses containing the CRISPR-Cas9 cassette. In all cases, sequencing the target gene is required in order to identify the mutations introduced by genome editing.
- The presence of the CRISPR-Cas9 cassette renders the plants transgenic and thus subject to the corresponding biosafety regulations. However, in sexually propagated plants, after identification of the genome edited plants, the CRISPR-Cas9 transgene can be eliminated by Mendelian segregation. This key part of the process removes the transgene in the third or subsequent generations resulting in the formation of genome-edited plants without a transgene Because of the absence of the transgene in these plants, they resemble those with mutations generated by natural means or chemical mutagenesis.
- Not all plant species are susceptible to *A. tumefaciens*. In species recalcitrant to Agrobacterium-mediated transformation, alternatives include *Rhizobium rhizogenes*- mediated or protoplast transformation.

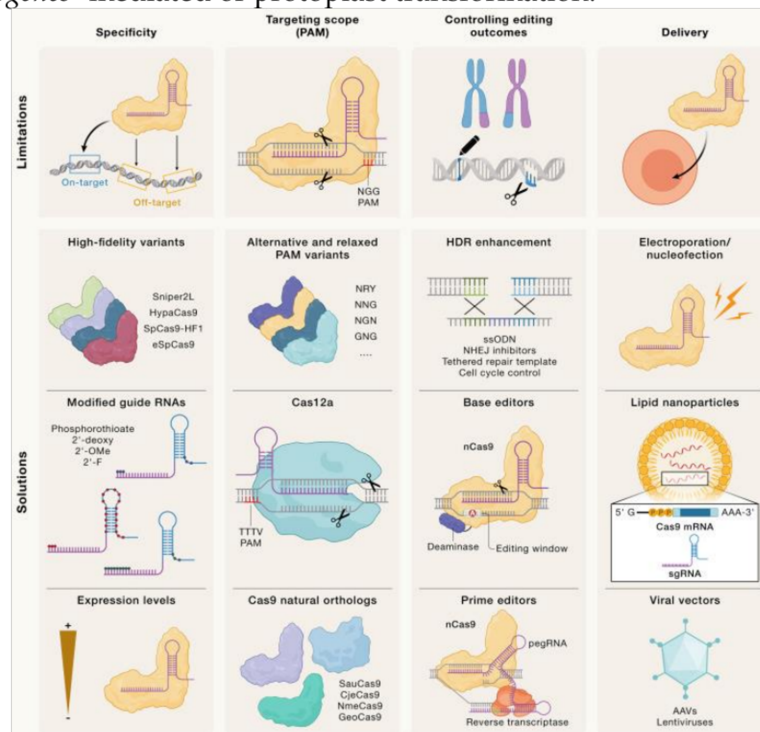



**Fig: Genome editing process using CRISPR – Cas9 and Agrobacterium tumefaciens. (A) Cas9 protein and guide RNAs are cloned into the same plasmid vector containing transfer DNA (T-DNA) signals. Expression is driven by strong constitutive (U6, 35S, or other), inducible or tissue specific promoters. Transcription termination is programmed by addition of terminator such as the U6 or Nopaline synthase (NOS). For plant genome editing purposes, Cas9 has been codon – optimized and might contain an epitope tag to determine expression. (B) *A. tumefaciens* or *R. rhizogens* is transformed with the plasmid vector carrying the cassette for Cas9 protein and guide RNAs expression. (C) Bacteria is used to transform embryos, ovules in flowers, protoplasts, roots, or cells in leaves. Integration site of the T-DNA is random. (D) Expression of Cas9 protein and guide RNAs lead to editing of the target DNA. The T-DNA Insertion site and the DNA target are likely not linked. (E) The T-DNA Insertion and edited part of the genome can be separated by Mendelian segregation.**

**Applications of Genome Editing in plants**

- Recent advances in genome editing such as gene engineering using CRISPR/Cas have opened many opportunities to accelerate plant breeding and to bridge the gap between conventional breeding and the knowledge acquired through plant molecular biology to study and improve (complex) traits.
- CRISPR/Cas-mediated genome editing enables very precise and efficient targeted modification in most crops, and thus largely increases the speed of crop improvement compared to conventional breeding.
- Since the first description of CRISPR/Cas as a plant genome-editing technique, the technology has been successfully applied in close to 120 crops and model plants, with reports of wide applications.
- Genome editing with CRISPR–Cas9 is amendable to edit any gene in any plant species. Because of its simplicity, efficiency, low cost, and the possibility to target multiple genes, it allows faster genetic modification than other techniques. It also can be used to genetically modify plants that were previously neglected. The potential that this represents for crop breeding and the development of sustainable agriculture is incommensurable.
- Impressive genetic modifications have been achieved with CRISPR-Cas9 to enhance metabolic pathways, tolerance to biotic (fungal, bacterial or viral pathogens), or abiotic stresses (cold, drought, salt), improve nutritional content, increase yield and grain quality, obtain haploid seeds, herbicide resistance, and others.
- Notable cases include thermosensitive genic male sterility in maize and wheat, improved nutritional properties in sorghum and wheat, tolerance or resistance to pathogens and resistance to herbicides.
- In potato CRISPR-Cas9 was used to knockout the gene encoding granule bound starch synthase (GBSS) in one round of transfection resulting in the development of potato plants that produce amylopectin starch, a highly desirable commercial trait.
- In cucumber CRISPR-Cas9 system was used to inactivate the eukaryotic translation initiation factor gene elF4E. The resulting non-transgenic homozygotic mutant plants were immune to Cucumber vein yellowing virus (Genus Ipomovirus) and resistant to the potyviruses Zucchini yellow mosaic virus and Papaya ring spot mosaic virus.
- Engineering genetic resistance to viruses and other pathogens has immense potential to manage diseases for which no natural resistance has been detected, such as maize lethal necrosis disease and tomato brown rugose fruit virus

**Short Questions**

1. List the various applications of genome editing in plant.

**Essay Questions**

1. Describe in detail genome editing in plants using CRISPR-Cas9.
2. Write in detail about genome editing in plants using *Agrobacterium tumefaciens* or *Rhizobium rhizogenes*.

| UNIT IV | APPLICATIONS OF TRANSGENIC PLANTS |
|---|---|

**Introduction**

Recently, several transgenic plants have been produced to meet specific needs of agriculture and human life. Some of these are given below:

a) **Transgenic crop plants having resistance to pathogens and pests:**
   1. Transgenic papaya is resistant to papaya ring spot virus
   2. Bt. Cotton is resistant to insects.
   3. Transgenic tomato plants are resistant to the bacterial pathogen Pseudomonas.
   4. Transgenic potato plants are resistant to the fungus Phythophthora.

b) **Transgenic plants suitable for food processing technology:** Transgenic tomato "Flavr Savr is bruise resistant i.e., suitable for storage and transport due to delayed ripening and offers longer shelf-life.

c) **Transgenic plants with improved nutritional value:** Transgenic golden rice obtained from 'Taipei' is rich in vitamin A and prevents blindness.

d) **Transgenic plants useful for hybrid seed production:** Male sterile plants of Brassica napus are produced. This will eliminate the problem of manual emasculation and reduce the cost of hybrid seed production.

e) Transgenic plants tolerant to abiotic stresses caused by chemicals, cold, drought, sale, heat etc. 1. Basmati variety of rice was made resistant against biotic and abiotic stresses. 2 Round-up ready soybean is herbicide tolerant

f) Besides these, genetically modified crops have evolved as alternative resources to industries, in the form of starches, fuels and pharmaceuticals. Transgenic plants have been shown to express the genes of insulin, interferon, human growth hormones, antibiotics, antibodies etc. These biochemical produced by plants are as food as or sometimes better than those produced in bacteria.

g) Utilization of plants as biofactories or bioreactors for obtaining commercially useful products, specialized medicines, chemicals and antibodies on a large scale is described as molecular farming. In the near future this field is expected to revolutionise both the farming as well as biochemical industry.

**Short Questions**

1. Write in detail about the various transgenic plants produced and highlight on their qualities.

# 4.1 HERBICIDE RESISTANT TRANSGENIC CROPS

Genetically modified herbicide tolerant crops are one of the biotechnological inventions commercially accepted and exploited world-wide. Weeds grow with crop plants and compete for water and nutrients. They significantly decrease the crop yields and productivity and are one of the major problems in crop management. Herbicides are chemicals that kill weeds.

Herbicide development and production is a tough task as herbicides cannot differentiate between weeds and crop plants. Therefore selective herbicides that can kill only a specific weed were used in crop management programs. These selective herbicides do not harm the crop plant, but are ineffective in killing all types of weeds. Non-selective herbicides often known as "broad- spectrum" herbicides are effective at removing wide range of weeds but they can also kill valuable crop plants.

Several crops have been genetically modified to make them resistant to non-selective herbicides. These genetically modified herbicide resistant crops harbor genes that enable them to degrade the active component in the herbicide, making the modified plant harmless to herbicide Farmers can therefore easily control weeds during the entire growing season and have more flexibility in choosing times for spraying

There are four mechanisms of resistance to herbicide action:

a) **Altered target site:** An herbicide has a specific target site of action where it binds and disrupts a particular plant process. If this target site is altered, the herbicide can no longer bind to its specific site and is unable to exert its toxic effect. This is one of the most common mechanisms of herbicide resistance.
b) **Enhanced metabolism:** A weed can degrade an herbicide and detoxifies it before it can reach its site of action within the plant.
c) **Compartmentalization:** Some plants have the ability of sequestering the active compounds of herbicides within their cells or tissues to render the compounds ineffective. Herbicides can be inactivated by binding to a sugar molecule or sequestered to metabolically active regions of the cell like cell wall.
d) **Over-expression of the target protein:** If the target protein can be produced in significantly large quantities by the plant, then the effect of the herbicide becomes insignificant.

Two herbicide resistant cropping systems are routinely used for soybean, maize, rapeseed and cotton: Monsanto's Roundup Ready with active agent as glyphosate and Bayer's Liberty Link with active agent as glufosinate. These are actually broad spectrum herbicides which are effective in killing all green plants

except those which are protected as a result of the genetic modification against these components. Use of these herbicide resistant cropping systems enables effective weed control by herbicide application.

**Roundup ready Soyabean**

Roundup ready resistant Soyabean was developed in 1974 and commercialized in 1996. Scientists from Monsanto genetically modified soyabean to contain in-plant resistance genes to Roundup Weather MAX herbicide. With roundup crops, farmers can spray broad spectrum herbicides without killing valuable crops.

Glyphosate, the active agent of roundup ready, interferes with the synthesis of essential amino acids like phenylalanine, tyrosine and tryptophan. Plants and microorganisms can make these essential amino acids with the help of an enzyme called 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). The enzyme catalyzes the penultimate step of the shikimate pathway for the biosynthesis of above mentioned essential aromatic amino acids. This enzyme is present only in plants and lower organisms but is absent in animals. Animals obtain these aromatic amino acids from their diet.

Roundup Ready Soybeans have been genetically modified to express a version of EPSPS. Scientists inserted the genetically modified plasmid containing necessary elements for EPSPS expression into soyabean germplasm. The plasmid harbors EPSPS from the CP4 strain of the bacteria, *Agrobacterium tumefaciens* known as CP4 EPSP synthase, 355 promoter (E355) from cauliflower mosaic virus (CaMV), (CTP4) coding sequence from *Petunia hybrida* encoding a Chloroplast transit peptide, and a transcriptional termination element called nopaline synthase (nos 3') from *Agrobacterium tumefaciens.*

Glyphosate is an inexpensive herbicide, and is environmentally friendly. Using the combination of glyphosate resistant crops (Roundup Ready crops) and glyphosate (Roundup Ready herbicide) has become widely popular.

**Advantages of using herbicide tolerant crops**

1. There is excellent weed control and hence higher crop yields are obtained.
2. It is possible to control weeds later in the plant's growth and hence there is flexibility in timing of herbicide application.
3. With herbicide resistant crops, there are reduced numbers of sprays in a season. Additionally because of less spraying there is reduced fuel use.

**Short Questions**

1. Write about round up ready Soybean.

**Essay Questions**

1. Write in detail about various mechanisms of herbicide resistance seen in various plants and list the advantages of using herbicide tolerant plants.

# 4.2 INSECT PEST RESISTANT TRANSGENIC PLANTS

Pests are a major threat to agriculture leading to reduced plant productivity. Insects can cause major yield losses, both in the fields and during storage. Breeders have also developed cultivars that can survive and produce higher yields in presence of insects. With the advent of new techniques crop plants have been genetically modified so that they can survive in presence of pests by being toxic to them.

Cotton being an important cash crop plays an important role in the Indian economy. Millions of people are engaged in cultivation, processing and trade of cotton. India ranks third in cotton production after China and USA. Insect pests (cotton aphids, bags, whitefly, caterpillars, spider mites, thirps) are responsible for low yields of cotton. These insects harm cotton plants in various stages of growth as cap-suckers, defoliators and tissue borers. Despite huge spray of chemical insecticides, bollworm infection was not being controlled leading to huge economic losses

**Bt Cotton**

The soil bacterium *Bacillus thuringiensis*, It is a gram–negative aerobic spore forming bacterium found world-wide. It produces proteins toxic to various herbivorous insects especially to the larvae of insect order Lepidoptera, which includes cotton bollworms, moths, butterflies, beetles and flies. It is harmless to mammals including humans, birds, fishes, or other beneficial insects.

The bacterium produces a toxic protein which will be in inactive crystalline form and is converted to active toxic form called as "delta endotoxin” only when it is consumed by insects is known as "cry proteins” as it is produced as crystalline proteins inclusions during sporulation.

The protein binds to certain receptors (the aminopeptidase N (APN) receptors and the cadherin-like receptor) in insect's intestine and causes wounding of epithelial midgut cells. This toxin destroys the gut of insect, ultimately leading to death of insect.

In USA, *Bacillus thuringiensis* was registered as a biopesticide but the performance of Bt msecticide on cotton points was limited. The insecticide can be degraded by light, heat, UV, high pH and desiccation. Even the areas where these pests of cotton (bollworms) feed are difficult to treat. The insect must eat sufficient treated plant to accumulate lethal dose of toxin.

Genetic engineering techniques permits the scientists to isolate the bacterial genes required for production of Bt toxins and introduce them to plants. Recombinant plants overcome the limitations of insecticidal spray as Bt proteins are protected from environmental degradation.

The proteins are produced in all the tissues of plant ensuring that larva will eat Bt protein wherever it feeds. These transgenic cotton plants are resistant to insect pests.

Almost 100 diverse forms of Bt toxin have been isolated from variable strains of *Bacilius thuringiensts*. The cry proteins and genes encoding them have been classified on the basis of their structure, activity spectrum and antigenic properties into four major groups. Cryl genes are lepidoptera specific. Cryll genes are lepidoptera- and diptera-specific. Crylll genes are colcoptera-specific and CryIV genes are diptera-specific. The toxicity of the protein changes with the molecular modification of the cry proteins.

First Bt recombinant transgenic plants (Bt cotton) were registered in 1995, by the United States Environmental Protection Agency (EPA). Bt technology has been used for other crop as well. Apart from lepidopteran resistant cotton, other Bt crops include: European corn borer resistant corn, corn rootworm resistant corn, Bt eggplant, Colorado potato, beetle resistant Bt potato, potato tuber moth resistant, Bt potato, Bt soybean, and lepidopteran resistant tomato.

Use of Bt plants has replaced the use of insecticides and led to global economic benefits from Bt cotton. Important agronomic traits like fiber quality, yield, harvestability, were maintained in recombinant cotton plants harboring Bt gene. There was tremendous increase in the yield and significant decrease in production cost

**Proteinase Inhibitors**

Proteinase inhibitors are the proteins that inhibit the activity of proteinase enzymes. Certain plants naturally produce proteinase inhibitors to provide defense against herbivorous insects. This is possible since the inhibitors when ingested by insects interfere with the digestive enzymes of the insect. This results in the nutrient deprivation causing death of the insects. It is possible to control insects by introducing 'proteinase inhibitor genes' into crop plants that normally do not produce these proteins.

**Cowpea trypsin inhibitor gene:**

It was observed that the wild species of cowpea plants growing in Africa were resistant to attack by a wide range of insects. Research findings revealed that insecticidal protein was a trypsin inhibitor that was capable of destroying insects

belonging to the orders Lepidoptera (eg. Heliothis virescans), Orthaptera (e.g. Locusta migratoria) and Coleoptera (eg. *Anthonous grandis*). Cowpea trypsin inhibitor (CpTi) has no affect on mammalian trypsins; hence it is non-toxic to mammals. CpTi gene was introduced into tobacco, potato and oilseed rape for developing transgenic plants. Survival of insects and damage to plants were much lower in plants possessing CpTi gene.

**Advantages of proteinase inhibitors:**

i. Many insects, not controlled by Bt, can be effectively controlled.
ii. Use of proteinase gene along with Bt gene will help to overcome Bt resistance development in plants.

**Limitations of proteinase inhibitors:**

i. Unlike Bt toxin, high levels of proteinase inhibitors are required to kill insects.
ii. It is necessary that the expression of proteinase inhibitors should be very low in the plant parts consumed by humans, while the expression should be high in the parts of plants utilized by insects.

**Lectins**

Lectins are plant glycoproteins and they provide resistance to insects by acting as toxins. The lectin gene (CNA) from snowdrop (*Calanthus nivalis*) has been transferred and expressed in potato and tomato. The major limitations of lectin are that it acts only against piercing and sucking insect, and high doses are required.

**Mechanism of toxicity and insecticidal properties of lectins**

The insecticidal activity of plant lectins, against an array of insects belonging to homoptera, coleoptera, diptera and lepidoptera are reported. Although the precise mode of action of plant lectins is not fully understood, it appears that resistance to proteolytic degradation by the insect digestive enzymes and binding to gut structures are two pre-requisites for lectins to evert their deleterious effects on insects.

After binding to the surface of the intestinal epithelial cells, lectins interfere with the digestive, protective or secretory functions of the intestine. Most probably, binding of a lectin to the receptor decreases the absorption of nutrients and/or disrupt the midgut epithelial cells of insects

A wide range of lectins, viz., GNA, Con A. PSA and ASA, exhibiting mannose of mannose glucose sugar binding affinity, revealed palpable anti metabolic effects towards members of the homopteran insects both under *in vitro and in planta* conditions. Mannose- binding lectin GNA encoding gene has been introduced and expressed in diverse crop plants, viz.. rice, wheat, tobacco and potato to protect against different pests.

**GNA transgenics and their insecticidal activity**

*Galanthus nivalis* agglutinin (GNA), commonly known as snowdrop lectin, is a mannose- specific tetrameric protein. GNA proved to be toxic to important insect pests belonging to the orders such as homoptera, coleoptera and lepidoptera. GNA protein was the most toxic among various lectins tested against BPH. Immuno-histochemical localisation of GNA in intoxicated adults and nymphs disclosed that GNA could cross the insect-gut barrier. The snowdrop lectin has exerted systemic effects via transport from the gut contents to the haemolymph across the gut epithelium.

In view of the marked entomotoxic effects exhibited by the GNA, it has been introduced into a number of dicot and monocot plants. The gna was initially expressed in tobacco and mustard, and the transgenic plants showed increased resistance against aphids. Transgenic potato plants expressing GNA disclosed resistance against potato aphid, potato peach aphid and a lepidopteran tomato moth. It was reported that transgenic rice containing gna conferred substantial resistance against BPH insects. Similarly, expression of GNA in wheat, conveyed resistance against the wheat grain aphid.

**Short Questions**

1. Write about Protease inhibitors and their role as insecticides emphasizing on their advantages and limitations.
2. Write about lectins, their mechanism of toxicity and insecticidal properties.

**Essay Questions**

1. Write in detail about toxins that act as biopesticides like Bt toxin, Lecitins and Protease inhibitors.

## 4.3 VIRUS, BACTERIAL AND FUNGAL RESISTANT TRANSGENIC PLANTS

Continuing attention is being devoted to the development of substitute strategies in plant – disease management and reducing dependency on synthetic chemicals. Viral, fungal and bacterial diseases are unquestionably the most versatile for environmental adaption and in the destruction of plant growth. Among the strategies, resistance breeding has generated proven data and been exploited in depth. However, conventional methods alone are not sufficient to control the novel races of viral, fungal and bacterial pathogens in crops due to a scarcity in required crop variations.

The current situation encourages the search for variation against biotic stress through identification of genes across species

Over the last two decades, significant efforts have been initiated in plant–disease management via genetic engineering. In addition, several molecular techniques have emerged to disentangle multifaceted plant pathogen systems and associated disease-resistance candidate genes. Besides describing many promising candidate genes from viruses, fungi and bacteria. Numerous plant disease–resistance genes have been identified and evaluated in crop improvement programs by transformation. Advancement in plant transformation techniques enables transferring useful genes for the rational creation of disease–resistant plants. Success has been achieved in transgenic crops against various diseases of important crop plants.

**Transgenic plants against viral diseases**

Engineering plants for virus resistance has grown considerably since evidence emerged in the 1980s showing that viral genes can be used to engineer plants for antiviral resistance, as elucidated for tobacco mosaic virus (TMV). This was followed by the development of virus resistant tobacco expressing the coat protein (CP) gene of several plant viruses. The finding in the late 1990s that this mode of resistance was due to the newly discovered RNA silencing further expanded the use of genetic engineering to control plant viruses.

The first commercially produced virus resistant GM crop was squash, which exhibited resistance to watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV). This was followed by transgenic papaya with resistance to papaya ringspot virus (PRSV).

Today, about seven GE crops with enhanced virus resistance have been approved for commercial production in the United States of America (USA) and China, and greenhouse and field trials of transgenic plants with resistance to viruses are underway in many other countries, especially in South America and Africa.

Since its elucidation in 1990, many RNA and DNA viruses have been successfully controlled using RNA silencing approaches, which suppress gene expression in a sequence–specific manner. Many RNA silencing precursors, including: sense/antisense, small interfering RNA (siRNA), microRNA (miRNA), and hairpin RNA (hpRNA) have been harnessed to generate virus resistant transgenic crops.

Some of the viral genes used to produce hpRNA constructs for processing into the silencing complex include genes coding for the CP, replication associated

protein (Rep), RNA–dependent RNA polymerase (RdRP), movement protein (MP), and proteases. The RNA transcripts produced from these gene constructs are processed into small interfering RNAs (siRNAs) that are key antiviral molecules. More recently, the shortcomings of the hpRNA strategy have at least partially been addressed using artificial microRNAs (amiRNAs) and transacting siRNAs (tasiRNA) based strategies that are high throughput approaches, which are amenable to multiplexing and have been used to simultaneously control diverse RNA and DNA viruses of different families.

Non-viral antiviral genes, including especially R genes and ribosome-interacting proteins, as well as protease inhibitors have also been incorporated into crop genomes to generate resistance against viruses. Mechanically, most of these non-viral genes silence host genes that are involved in virus replication. Other gene silencing approaches have developed virus resistance in insect vectors in order to limit virus transmission.

**Squash (Resistance to ZYMV, WMV and CMV):**

Squash was one of the first crops that was genetically engineered in the early 1990s in an effort to contain epidemics of zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and cucumber mosaic virus (CMV) Like other early efforts in the use of genetic engineering to generate resistance to viruses, the CP genes of ZYMV and WMV were used to transform squash. A transgenic line with a strong resistance to ZYMV and WMV, designated "ZW20", was approved for cultivation in the USA in 1994 Subsequently, the CP genes of all three viruses were used in the transformation and line "CZW-3", which exhibited resistance to all three viruses, was approved for cultivation in 1996. These transgenic lines represented about 12% of all USA squash production and it was estimated that growers benefitted about $24 million from the technology in 2006.

**Papaya (Resistance to PRSV Resistant):**

Papaya ringspot virus (PRSV)-resistant papaya was one of the first virus-resistant transgenic plants to be approved in response to the PRSV epidemic in Hawaii (USA) in 1990s. This is because of the inability to develop resistance through conventional breeding to contain the epidemic. Transgenic papaya lines developed under the trade names "Rainbow" and "SunUp" and expressing the coat protein gene of PRSV were first introduced in Hawaii in 1998. Since then, these transgenic lines have exhibited broad-spectrum resistance to PRSV strains in Hawaii.

**Transgenic plants against bacterial and fungal pathogens**

In contrast to herbicide- or insect-resistant transgenic plants, which have been grown extensively worldwide for more than 10 years, the development of

transgenic plants with enhanced resistance to fungal and bacterial pathogens has received only limited success. Much of the limitation towards successful implementation in transgenic strategies for increasing plant tolerance towards pathogens stems from generally achieving low levels of resistance that are below the threshold desired by producers, or high levels of resistance against only a specific pathogen or even a single strain. This generally observed low levels of resistance coupled with the negative perception of GM plants has resulted in a relatively small number of transgenic lines being brought to late stage field testing and even fewer that have been successfully brought to market.

There are different strategies for increasing disease resistance caused by bacteria or fungal pathogens in transgenic plants that include: expressing R–genes, pathogenesis–related (PR) antimicrobial genes, detoxification of pathogen virulence factors, increasing structural barriers. RNAi and the modification of defense–signaling pathways.

**R genes**

Genetic engineering allows for introduction of R–genes from unrelated plant species, which often remain functional in the new host plant. The R-gene Rxol from maize was successfully introduced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae pv. oryzicola*. Additional examples of this strategy involve the R-gene RCTI from *Medicago truncatula* that was expressed in alfalfa and conferred resistance to *Colletotrichum trifolii,* and RPI-BLB2 from wild potato *Solanum bulbocastanum* conferring resistance to *Phytophtokora infestans* in cultivated potato.

**Detoxification of Virulence Factors**

In contrast to biotrophic pathogens, necrotrophs produce copious amounts of pathogenicity factors, including toxins and cell–wall–degrading enzymes, as a means of successfully establishing infections. Mutants lacking these pathogenicity factors often have reduced virulence or in some instances are completely avirulent. Polygalacturonase–inhibitory proteins (PGIPs) serve to inhibit the activity of the fungal cell wall–degrading polygalacturonases. Overexpression of PGIPs in transgenic plants has successfully reduced disease symptoms due to *B. cinerea and Bipolaris sorokininia*.

The main non–host selective mechanism for *Sclerotinia sclerotiorum* infection involves the secretion of oxalic acid. Oxalic acid functions through a variety of mechanisms, including: lowering the pH of the plant to near optimal for cell–wall degrading enzyme (CWDE) activity, repression of the oxidative burst, decreasing the activity of plant defense enzymes, weakening of plant cell walls through chelating $Ca^{++}$ ions, as well as being directly toxic to the plant cells and being a

potent mediator of plant–programmed cell death. Oxalic acid-deficient *S. sclerotiorum* mutants are unable to infect many plant species, indicating its significance in pathogenicity. Proteins that can degrade oxalic acid include wheat oxalate oxidase and oxalate decarboxylase, converting oxalic acid to $CO_2$ and hydrogen peroxide or $CO_2$ and formate, respectively.

Overexpression of these enzymes in lettuce, sunflower, soybean, rape seed, tomato and tobacco all demonstrated at least partial resistance to *S.sclerotiorum*.

**Expression of Antimicrobial Peptides/Metabolites**

The most commonly used approach for engineering fungal and bacterial resistance in plants is through the expression of antimicrobial peptides, PR–proteins and proteins involved in the production of antimicrobial metabolites. Naturally occurring PR proteins are constitutively expressed at low levels and are induced to high levels during pathogen challenge or application of either salicylic acid or jasmonic acid. They have a variety of functions, including degrading the fungal cell walls, membranes, RNA or are involved in generating secondary metabolites or increasing cell physical barriers.

Chitinases and β1-3 glucanases have been investigated extensively since they are hydrolytic enzymes that serve to break down the main structural components of fungal cell walls, chitin and laminarin. Overexpression of both chitinases and glucanases from a wide range of donor organisms has been examined in a variety of plant species. Chitinase overexpression has been moderately successful in increasing tolerance to diseases caused by both biotrophic and necrotrophic fungal pathogens. However, this chitinase–derived resistance was rarely at a level high enough to pursue commercial development. There has been limited success reported from overexpression of β-1, 3 glucanases, with little to no increased disease resistance reported in nearly all cases. However, combined expression of a chitinase and β-1, 3 glucanase often resulted in a synergistic effect, further enhancing the resistance in several plant species. Additionally, chitinases originating from mycoparasitic biocontrol agents, most notably *Trichoderma harzianum*, that can exhibit higher anti–fungal activity than plant chitinases have been proven to be more effective source for enhancing fungal disease resistance in transgenic plants.

**Modification of defense signaling pathway**

Potentially the most promising candidate for increasing pathogen resistance through signaling modifications is NPRI. The loss of function *Arabidopsis* mutant nprl (non-expressor of pr genes) was originally identified from mutant screens where nprl plants were unable to either mount a SAR response or accumulate PR transcripts and were hypersensitive to biotrophic pathogens. To date, NPRI-like

orthologs have since been characterized from over 15 different plant species. NPR1 has been identified as the key master control switch of SAR, with implications in other JA/ ethylene–controlled signaling pathways NPRI is constitutively expressed at low levels and transcript accumulation increases up to two–fold during pathogen challenge or when treated with SA. The gene product is located as an inactive multimeric protein in the cytosol. During pathogen infection, or application of an elicitor, the NPRI complex is reduced and the structure is changed to the monomeric form that is transported to the nucleus and induces expression of several PR genes. Overexpression of Arabidopsis NPRI (AtNPRI) or an endogenous NPRI ortholog has resulted in increased resistance to biotrophic and necrotrophic pathogens in a variety of different plant species.

**Short Questions**

1. Write about Transgenic plants raised against viral diseases.
2. Write about detoxification of virulence factors.

**Essay Questions**

1. Write in detail about various strategies used for increasing resistance in plants for bacterial and fungal diseases.

## 4.4 TRANSGENIC CROPS FOR RESISTANT AGAINST DIFFERENT ABIOTIC STRESSES

Abiotic stresses such as heat, drought and salinity are the major environmental constraints affecting production and productivity of almost all the crops. Conventional plant breeding has not been proved that much successful in addressing abiotic stress mitigation so far. The reason might be that the traits are controlled by a number of genes present at a quantitative trait locus (QTL). To combat the negative effects of various abiotic stresses, it is pre–requisite to identify potential candidate genes or QTLs (gene networks) associated with broad–spectrum multiple abiotic stress tolerance.

Various abiotic stresses including drought, high temperature, salinity, frost and flood, etc. adversely affect overall crop growth and productivity by affecting the vegetative and reproductive stages of growth and development. These stresses generally trigger a series of physiological, biochemical and molecular changes in the plants which often result in damage to the cellular machinery. These changes include the disruption of cellular osmotic balance leading to dysfunctional homeostasis, ion distribution and oxidative stresses which cause denaturation of integral proteins of plants.

Plants respond to such stresses in a variety of mechanisms which trigger the cell signalling process, transcriptional controls and production of a number of stress conditions related tolerant proteins, antioxidants and osmotic solutes to maintain homeostasis and to protect and repair the damaged integral proteins.

Generally, plants which are stress sensitive are unable to synthesize such compounds under stress conditions and, thus, are rendered liable to various stresses which hamper their overall growth.

A number of genes have been identified in a number of plants/organisms, closely or distantly related, which code for the synthesis of these stress protecting compounds and thus can be targeted for genetic transformation into sensitive genotypes.

**Such genes have been classified into three categories as:**

**a)** Genes which code for the synthesis of various osmolytes such as mannitol, glycine betain, proline, and heat shock proteins

**b)** Genes responsible for ion and water uptake and transport like aquaporins and ton transporter, etc. and

**c)** Genes regulating transcriptional controls and signal transduction mechanism, examples *MAPK, DREBI*, etc.

**Drought tolerance**

Various genes controlling signaling and gene regulatory pathways offer certain key targets for genetic engineering for abiotic stress tolerance. Transcription factors (*TFs*) that regulate or switch on the expression of a number of genes involved in imparting abiotic stress tolerance in plants have been proposed as the most efficient targets for genetic transformation. These transcription factors include *DREBl* gene family, Myb gene family, etc.

The following are the examples of successful crops with drought tolerance:

**a)** Transformed tomato plants with a DNA cassette containing an Arabidopsis C repeat/dehydration-responsive element binding factor 1 (*CBF*l) cDNA and a nos terminator, driven by a cauliflower mosaic virus 35S promoter. These transgenic tomato plants were more resistant to water deficit stress than the wild-type plants.

**b)** Improved tolerance to cold and drought stress in transgenic apple by the over–expression of a cold–inducible *Osmyb* 4 gene from rice, which codes for a *TF* belonging to *Myb* family is reported.

**c)** The over-expression of *DREBlb TF* gene had also been reported to induce cold tolerance and drought tolerance in transgenic grapevine.

**Heat tolerance**

Under heat stress, many reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and superoxide are produced inside the plant cells, leading to various kinds of physiological disorders in plants which affect crop growth and productivity. These ROS denature enzymes and damage various cellular components inside the plant cells. Tolerance to heat stress is straightway correlated with the increased capacity of plants to scavenge ROS. Thus, it is very important to scavenge ROS to maintain normal growth and metabolism of plants.

Plants have developed a variety of mechanisms to combat ROS by the production of various enzymatic systems like superoxide dismutase (SOD) to remove superoxide ions, glutathione reductase (GR) and peroxidase to scavenge peroxide ions ($H_2O_2$), etc. Thus, over-expression of ROS scavenging enzymes in plants via genetic transformation offers a much potential strategy to overcome heat stress.

**The following are important examples of transgenic plants tolerant to heat:**

- ☑ Over-expression of cytosolic ascorbate peroxidase (*cAPX*) gene improved tolerance to heat stress in transgenic apple.
- ☑ Transgenic tomato plants which over-expressed *cAPX* gene has shown enhanced tolerance to heat (40°C), In field tests, detached fruits from field grown transgenic tomato plants showed enhanced resistance with the exposure to direct sunlight as compared to the fruits from wild- type (non - transgenic) plants.
- ☑ Over-expression of *Cu/Zn superoxide dismutase (Cu/Zn SOD)* gene (derived from *Manihot esculenta*) under an oxidative stress inducible promoter *SWPA2* in potato led to enhanced heat stress tolerance. *Cu/Zn SOD* is an ROS scavenging enzyme and, thus, helps in quenching of free radicals released under heat stress in plants.
- ☑ The non-enzymatic methods involve the production of a variety of chemical compounds including polyamines, carotenoids, ascorbic acid, tocopherol, etc., which directly react with ROS, scavenge them and thus provide protection to the plants against heat stress.
- ☑ Polyamines play an important role in imparting thermal stress tolerance in plants. *S adenosyl-1-methionine decarboxylase* (*SAMDC*) is one of the key regulatory target enzymes in polyammes biosynthesis. Over-expressed *SAMDC* cDNA, isolated from Saccharomyces *cerevisae*, in tomato plants for enhanced polyamines production.

**Salinity tolerance**

Salinity or salt stress is one of the most prevalent abiotic stresses that severely affect the quality and quantity of crops. Around 20% of the world irrigated

agricultural land is affected with salinity problem. Salinity tolerance is a complex mechanism governed by many genes. Plants which are exposed to abiotic stress conditions produce several pathogenesis–related proteins to compensate the adverse effect of stress conditions.

The following are the important examples of transgenic plants tolerant to salinity:

- ☑ *Osmotin* is one of the important pathogenesis–related proteins, which is produced by the plants to combat various biotic and abiotic stresses. Over–expression of tobacco *osmotin* gene in strawberry (*Fragaria × ananasa* Duch.) found that the transgenic strawberry plants exhibited tolerance to salt stress.
- ☑ Tolerance of chilli pepper (*Capsicum annum L.*) plants was obtained by the ectopic expression of tobacco *osmotin* gene via *Agrobacterium tumefaciens*-mediated gene transfer technique. $T_2$ generation of transgenic pepper plants revealed enhanced levels of chlorophyll, proline, glycine betaine, ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and relative water content (RWC) in biochemical analysis and survived in salinity level up to 300 mM NaCl concentration.
- ☑ Carrizo citrange, an excellent rootstock of citrus with a yeast-derived halotolerance gene, *HAL* 2, found to possess salt tolerance mechanism. *HAL2* gene is involved in the methionine biosynthetic pathway and confers tolerance to lithium and sodium ions. It encodes for a salt-sensitive *biphosphate nucleotidase*, which is required for sulfate accumulation. The transgenic lines expressing *HAL2* protein showed improved tolerance to salinity than the wild–type plants.
- ☑ Transgenic tomato plants expressing tolerance to chilling and salt stress is developed by incorporation of *cytosolic ascorbate peroxidase* (CAPX) gene, derived from pea (*Pisum sativum L.*). *Ascorbate peroxidase* plays a key role in quenching hydrogen peroxide ($H_2O_2$) in plant cells, thus providing protection against oxidative injury induced by chilling and salt stress.
- ☑ The transgenic plants showed better seed germination rate (26–37%) than the wild type (3%) when the seeds were placed at 9°C for 5 weeks. *APX* activity was found 10–25 folds higher in transgenic plants under salinity stress (200-250 mM) conditions, thus ensuring minimum damage to the leaves comparatively.

**Short Questions**

1. A short note on how plants in general respond to various abiotic stresses:
2. Write about various drought tolerant plants.
3. List the various heat tolerant plants

**Essay Questions**

1. Write in detail about various salinity tolerant plants.

# 4.5 TRANSGENIC CROPS WITH HIGH NUTRITIONAL VALUES

Plants are the chief food of human beings. They not only provide calories but also fulfill the need for nutrients, essential amino acids, vitamins etc. required by our body. Around 870 million people suffer from hunger worldwide. In developing countries undernourishment and micronutrient deficiency are amongst common problems affecting people causing serious illness and death with increasing worldwide population. Staple crops rich in starch do not provide micronutrients, so physical and mental health of human population gets affected. The solution for this problem is production of staple crops with improved nutritional quality called "biofortification' through genetic engineering and biotechnology.

Transgenic crops are generated with improved nutritional traits beneficial for human beings. The examples are rare so far, but their number is increasing at a tremendous rate. Our diet is rich in phytochemicals which include proteins, carbohydrates and fats which are the major constituents present in grams per 100g of food and minor constituents namely vitamins, minerals and secondary metabolites found in mg per 100g of food.

Qualitative changes have been more common in major constituents like proteins, carbohydrates and fats as quantitative changes are difficult to achieve. Knowledge of biosynthetic pathways involved in engineering of any metabolic trait is essential which can be elucidated through functional genomics and gene delivery methods.

**Vitamins**

Vitamin deficiency is a common health problem worldwide. Most of the cereal crops are deficient in essential vitamins. Therefore, transgenic manipulations have been done for improvement of vitamin content of many crops of commercial value the focused vitamins being vitamin A, B, C, and E.

**Golden Rice**

Vitamin A deficiency (VAD) causes blindness in 5 million children annually resulting in death of most of these children. Globally, nearly 124 million children suffer from VAD. It is a matter of concern in Southeast Asia, Africa, Caribbean and Latin America. One to two million deaths may be avoided by providing Vitamin A enriched nutrition among children 1-4 years and 0.5 million during higher age group of children. One approach is providing vitamin A capsules to children and new mothers and alternatively provitamin A can be provided in the form of b

carotene in rice which is one of the best known examples of nutritional improvement of a food crop.

A variety of rice was produced through genetic engineering capable of synthesizing betacarotene, a precursor of Vitamin A in the endosperm of transgenic rice. This variety, known as "Golden rice', differs from the parental variety by having two additional beta-carotene synthesis genes. Golden rice was produced by transforming rice with two beta-carotene synthesis genes: (i) psy (phytoene synthase) from Daffodil (*Narcissus pseudomarcissus*) (ii) crt I (carotene desaturase) from a soil bacterium (*Erwinia uredovora*).

Both these genes were inserted into rice nuclear genome under the control of an endosperm specific promoter to express them only in endosperm. The end product of this pathway is lycopene, but plants do not accumulate it otherwise the rice would have been red. An endogenous enzyme inside the plant converts lycopene to beta-carotene giving golden yellow color after which it is named. The details of golden rice were first published in Science in 2000.

This was product of an eight year project by Ingo Potrykus of Swiss Federal Institute of Technology and Peter Beyer of University of Freiburg. In the year 2005, a new variety of rice, called Golden rice 2 producing 23 times more beta–carotene in comparison to original Golden rice was announced.

**Amino acids**

Out of twenty amino acids, humans can synthesize only ten amino acids. Remaining have to be obtained exogenously through diet and are called essential amino acids. Proteins obtained from cereals and legumes are poor proteins as they lack or have low levels of these essential amino acids particularly lysine and methionine. Cereals are poor source of lysine and legumes are poor in methionine. Therefore, improving the content of methionine is a target in case of cereal crops.

The inability of humans and many farm animals to synthesize certain amino acids has long triggered tremendous interest in increasing the levels of these so-called essential amino acids in crop plants. Knowledge obtained from basic genetic and genetic engineering research has also been successfully used to enrich the content of some of these essential amino acids in crop plants. Among the Enriching crop plants in essential amino acids has both economical and humanitarian interest. In developed countries, the interest is mostly for the livestock feeding industry because farm animals generally provide sufficient amount of essential amino acids for human diets. In developing countries, where plants directly account for the majority of the food, the interest is both humanitarian and economical.

So far, the success of genetic approaches has been mostly restricted to maize *(Zea mays)* by generating quality protein maize (QPM) cultivars, which are enriched in Lys and to some extent Trp in their seeds. However, breeding approaches have resulted in relatively limited success in other crop species. This is mostly due to limited availability of genetic resources for plant breeding, and the fact that genetic traits for high contents of Lys. Trp, or Met are generally associated with abnormal plant growth because these traits do not operate in a seed-specific manner.

In contrast, results from genetic engineering research appear to be more promising. Particularly because this approach allows seed-specific expression of specific traits of interest, using seed-specific promoters. In fact, one high Lys maize cultivar, LY038, developed by genetic engineering, represents the first genetically modified (GM) crop with high nutritional value to be approved for commercial use in a number of countries.

The potential to increase the contents of Trp and Met in a seed-specific manner have already been proven successful in basic research studies. Another advantage of genetically engineered traits is that they can be transformed into multiple plant species and genotypes and function synergistically with many other agronomically important traits. These genetic engineering approaches were generally aimed at tailor-made improvements of essential amino acid metabolie pathways and expressing native and genetically engineered proteins enriched in essential amino acid contents.

However, improvements of metabolic pathways by genetic engineering also requires a detailed understanding of how these pathways interact with regulatory networks that fine tune plant development. These are now beginning to be elucidated by modern systems biology approaches, including transcriptomics, proteomics, and metabolomics.

**Oils**

Fatty acid modification is another area of interest of biotechnologists. To increase the content of unsaturated fatty acids in plants, one of the strategies is to alter the expression of desaturase genes, responsible for introducing double bonds in the fatty acids at specific locations. In several crops like maize, soyabean and oilseed rape, nutritionally improved oils are produced through transgenic methodology. Progress has been made to engineer fatty acid synthase pathway and accumulation of polyunsaturated fatty acids in oil crops whereas both quantitative and qualitative alterations in minor constituents have been widely reported.

Two discrete paths were adopted to ultimately produce EPA and DIHA in the seeds of transgenic plants, though both started from the same position of having a

"toolkit" of suitable biosynthetic genes isolated from microalgae and other omega-3 accumulators, validated by heterologous expression in yeast.

The first approach, exemplified by Petrie and colleagues at CSIRO (Canberra, Australia), took advantage of Agrobacterium-mediated transient expression systems which allow the rapid evaluation of multiple gene combinations (via co-infection) in the leaves of *Nicotiana benthamiana.* This allowed the identification of an optimal set of non-native genes which directed the efficient synthesis of EPA and DHA in plants, albeit non-seed tissues. However, by the further co-expression in leaves of a "master regulator" transcription factor LEC2, the authors were able to "reprogramme" lipid metabolism in this tissue so that it now more closely resembled that of seeds, with the specific capacity to synthesize seed oil in the form of triacylglycerol.

Thus, the host leaf cells under evaluation came to more closely resemble the metabolic context observed in seeds. In addition, an added benefit of this transcription factor–mediated reprogramming was that instead of having to use constitutive promoters to drive the expression of the non-native genes, it was now possible to use seed-specific promoters (since they were now active as a result of the LEC2 expression), meaning that both regulatory elements and biosynthetic enzymes were validated in a plant host.

To date, two oilseed crop species have been identified as potential hosts for the omega–3 LC–PUFA biosynthetic trait-canola, a cultivar of rapeseed (*Brassica napus L.*) and (*Camelina sativa*).

**Short Questions**

1. A short note on Golden rice.
2. Write about Quality protein maize.

**Essay Questions**

1. Write in detail about fatty acid modification to increase the content of fatty acids in plants.

## 4.6. TRANSGENIC AS BIOREACTORS

Advances in molecular biology, immunology and plant biotechnology have changed the paradigm of plant as a food source to so-called 'plant bioreactor' to produce valuable recombinant proteins. These include therapeutic or diagnostic monoclonal antibodies, vaccines, and other biopharmaceutical proteins.

The plant as a bioreactor for the production of therapeutic proteins has several advantages, which include the lack of animal pathogenic contaminants, low cost of

production, and ease of agricultural scale-up compared to other currently available systems. Thus, plants are considered to be a potential alternative to compete with other systems such as bacteria, yeast, or insect and mammalian cell culture.

**Edible vaccines**

Edible vaccines are also called as food vaccines, oral vaccines, subunit vaccines and green vaccines. They seem to be a viable alternative especially for the poor and developing countries. They have come up as great boon in medicinal science for which biotechnologists should be given all credit. The concept of edible vaccines lies in converting the edible food into potential vaccines to prevent infectious diseases. It involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. It has also found application in prevention of autoimmune diseases, birth control, cancer therapy, etc. Edible vaccines are currently being developed for a number of human and animal diseases.

In 1998 a new era was opened in vaccine delivery when researchers supported by the National Institute of allergy and infectious diseases (NIAID) have shown for the first time that an edible vaccine can safely generate significant immune responses in people. The report by collaborators from the University of Maryland in Baltimore, the Boyce Thompson Institute for Plant Research in Ithaca, N.Y., and Tulane University in New Orleans appeared in the May issue of Nature Medicine. According to the then Director of NIAID "Edible vaccines offer exciting possibilities for significantly reducing the burden of diseases like hepatitis and diarrhea, particularly in the developing world where storing and administering vaccines are often major problems."

**Plants utilized**

To date, many plant species have been used for vaccine production. The choice of the plant species is important. An edible, palatable plant is necessary if the vaccine is planned for raw consumption. In case of vaccine for animal use, the plant should preferentially be selected among those consumed as normal component of the animal's diet. Some food vehicles are tobacco, potato, tomato, rice, banana etc.

The first edible vaccine was produced in tobacco in 1990 in which 0.02% recombinant protein (a surface protein from Streptococcus) of the total soluble leaf proteins was found. Transgenic tobacco is successfully engineered for the production of edible vaccines against hepatitis B antigen using 's' gene of hepatitis B virus (HBV).

**Production methods**

Two main strategies are used for the production of candidate vaccine antigen in plant tissues:

1. **Stable genomic integration:** Under this method the genetic line is produced that can be propagated either by vegetative (stem cuttings) or sexual (seeds) reproduction methods. The stable expression strategy provides an opportunity to introduce more than one gene for possible multicomponent vaccine production. Stable transformation causes the desired gene to be incorporated either in nucleus or chloroplast. Agrobacterium mediated gene transfer is used for transforming the plants in which the gene is integrated in nucleus. Besides, direct delivery of DNA into the tissue or biolistic method are also used.
2. **Transient expression using viral vectors:** In this method viral vectors are used as a tool to deliver genetic material into cells. A recombinant plant virus is selected that can carry the vaccine gene and can cause the plant to express the antigen by systemic infection. As compared to stable expression, transient expression is difficult to initiate, because the viral vectors must be inoculated into individual host plants, but gives higher level of expression as it allows the virus to replicate and amplify the gene copy number.

**Advantages**

- ☑ Edible vaccines activate both mucosal and systemic immunity, as they come in contact with the digestive tract lining which is not possible with subunit vaccines which provide poor mucosal response. This dual effect of edible vaccines provides first-line defense against pathogens invading through mucosa, such as *Mycobacterium tuberculosis* and agents causing diarrhea, pneumonia, STDs, HIV, etc.
- ☑ No need of medical personnel and syringes. Sterile injection conditions are no more required.
- ☑ Economical in mass production by breeding compared to an animal system.
- ☑ Easy for administration and transportation.
- ☑ Effective maintenance of vaccine activity by controlling the temperature in plant cultivation.
- ☑ Heat stable, thus eliminating the need of refrigeration.
- ☑ Enhanced compliance (especially in children).
- ☑ Delivery of multiple antigens. Integration with other vaccine approaches.
- ☑ Plant-derived antigens assemble spontaneously into oligomers and into virus like particles.
- ☑ Reduced risk of anaphylactic side effects from edible vaccine over injection system is one benefit reported
- ☑ Edible vaccines would also be suitable against neglected/less common diseases like dengue, hookworm, rabies, etc. They may be integrated with other vaccine approaches and multiple antigens may also be delivered.

**Limitations and Challenges**

One of the key goals of the edible-vaccine pioneers was to reduce immunization costs but later many limitations were reported as given below:

- ☑ Consistency of dosage from fruit to fruit, plant to plant, lot to lot, and generation to generation is not similar.
- ☑ Stability of vaccine in fruit is not known.
- ☑ Evaluation of dosage requirement is tedious.
- ☑ Selection of best plant is difficult.
- ☑ Certain foods like potatoes are generally not eaten raw and cooking the food might weaken the medicine present in it.
- ☑ Not convenient for infants as they might spit it, eat a part or eat it all, and throw it up later. Concentrating the vaccine into a teaspoon of baby food may be more practical than administering it in a whole fruit.
- ☑ There is always possibility of side effects due to the interaction between the vaccine and the vehicle.
- ☑ People could ingest too much of the vaccine, which could be toxic, or too little, which could lead to disease outbreaks among populations believed to be immune.
- ☑ A concern with oral vaccines is the degradation of protein components in the stomach due to low pH and gastric enzymes.
- ☑ Potential risk of spreading the disease by edible vaccine delivery is a concern of many. Potential contamination of the oral delivery system is a possible danger.

**Antibodies production**

Antibodies (also known as an immunoglobulins) are complex proteins produced by vertebrates that recognize antigens (or molecular patterns) on pathogens and some dangerous compounds in order to alert the Adaptive immune system that there are pathogens within the body.

A plantibody' is an antibody that is produced by plants that have been genetically engineered with animal DNA encoding a specific human antibody known to neutralize a particular pathogen or toxin. The transgenic plants produce antibodies that are similar to their human counterparts, and following purification, plantibodies can be administered therapeutically to acutely ill patients or prophylactically to at–risk individuals (such as healthcare workers). The term plantibody and the concept are trademarked by the company Biolex.

**Production**

A plantibody is produced by insertion of genes encoding antibodies into a transgenic plant. The plantbodies are then modified by intrinsic plant mechanisms (N-glycosylation). Plantbodies are purified from plant tissues by mechanical

disruption and denaturation/removal of intrinsic plant proteins by treatment at high temperature and low pH, as antibodies tend to be stable under these conditions, Antibodies can further be purified away from other acid- and temperature–stable proteins by capture on commercially produced protein A resins. Production of antibodies in whole transgenic plants, such as species in the genus *Nicotiana*, is cheaper and safer than in cultured animal cells.

Commercial use is not yet legalized, but clinical trials are underway to implement the use of plantibodies for humans as injections. So far, companies have started conducting human tests of pharmaceutical products, creating plantibodies that include:

- ☑ Hepatitis B vaccine
- ☑ Antibody to fight cavity causing bacteria
- ☑ Antibodies to prevent sexually transmitted diseases
- ☑ Antibodies for non-Hodgkin's -cell lymphoma
- ☑ Vaccine against HIV virus
- ☑ Anthrax vaccine (from tobacco)
- ☑ Antibodies against Ebola virus

**Applications**

- ☑ Transgenic plants offer an attractive method for large-scale production of antibodies for immunotherapy. Antibodies produced in plants have many advantages that are beneficial to humans, plants, and the economy as well. They can be purified cheaply and in large numbers.
- ☑ Production of many seeds by the plants allows for ample storage and have no risk of transmitting diseases to humans because the antibodies are produced without the need of the antigen or infectious microorganisms.
- ☑ Plants could be engineered to produce antibodies which fight off their own plant diseases and pests, for example, nematodes, and eliminate the need for toxic pesticides.
- ☑ Antibodies generated by plants are cheaper, easier to manage and safer to use than those obtained from animals. The applications are increasing because recombinant DNA is very useful in creating proteins that are identical when exposed into a plant.
- ☑ Plants are likely to make the plant systems a useful alternative for small, medium and large scale production throughout the development of new antibody-based pharmaceuticals.
- ☑ The main reason plants are being used to produce antibodies is for treatment of illnesses such as immune disorders, cancer and inflammatory diseases, given the fact that the plantibodies also have no risk of spreading diseases to humans.

- ☑ Plants are more economical than most forms of creating antibodies and the technology for harvesting and maintaining them is already present.
- ☑ Plantibodies can be made at an affordable cost and easier manufacturing due to the availability and relatively easy manipulation of genetic information in crops such as potatoes, soybean, alfalfa, rice, wheat and tobacco.

**Biodegradable plastics**

Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers naturally synthesized and accumulated as intracellular energy and carbon reserves by a wide range of bacteria. Production of PHAs in microbial cells is intensified under specific conditions such as the lack of nitrogen, phosphorus, sulfur, potassium, zinc, iron, magnesium or oxygen.

PHAs are round shape granules with a diameter of 0.1–0.2 μm which are accumulated in the cytoplasm. PHAs polymers are made of identical monomers whose number ranges from 600 to 35,000. PHAs have properties similar to those of conventional petrochemical plastics. In addition, products made of these materials are natural, nontoxic, renewable and biocompatible, which make them more attractive than non-biodegradable petrochemical plastics.

For the above-mentioned reasons, from among other alternatives to petroleum-based plastics, PHAs have enjoyed great interest in the academia and industry. The differences in traits of a wide range of PHAs depend mainly on their monomers' compositions. The first observed and most studied PHA is polyhydroxybutyrate (PHB).

PHB is stiff and brittle, however, in the form of fibers behaves such as elastic material. One of the greatest advantages of PHB is air impermeability and water insolubility. It has a helical crystalline structure which seems to be a crucial feature that determines the possibility of the chemical and mechanical processing of the polymer. Furthermore, a non-toxic and thermoplastie nature of PHB makes it a very attractive material as a replacement for conventional plastics.

Interestingly, some properties of PHAs are similar to those of conventional plastics such as polypropylene. However, the whole process of bioplastic production is expensive than obtaining petrochemically based plastics products. For the economic reasons, i.e., high costs of nutrient substrates for bacteria, PHAs mass scale production using microorganisms is not popular, which stimulates efforts aimed at reducing the cost of production of these compounds.

One of the possibilities is to try to obtain transgenic plants that would be used as PHAs natural bioreactors. However, the greatest difficulty of biosynthesis of

complex PHAs structures in plant cells is to control the appropriate ratio and composition of the monomers that are formed in them. These attempts were successful in the 1990s when the *Arabidopsis thaliana* was transformed with genes encoding the enzymes needed for PHB biosynthesis, isolated from *Alcaligenes eutrophus*.

The first genetically modified plant producing PHAs was *A. thaliana*. The transformation was made in the beginning of 1990s and it has successfully brought about PHB accumulation in plant tissues. The PHB accumulation was at a level of 0.01% fresh mass and took place in cytoplasm, nucleus and vacuoles. Although the yield was poor, the impact of the studies was enormous and the study has been a basis for further work on transgenic plants capable of biosynthesis of PHAs. 2 years later a breakthrough studies brought transgenic *A thaliana* plants capable of PHB accumulation at a level of 14% of dry mass (DW). This success was possible because of the localization of the enzymes catalyzing PHB biosynthesis in plastids The transformation involved the use of three separate constructs comprising genes coding the enzymes needed for PHB biosynthesis. Each construct was a modified binary plasmid Ti originating from *Agrobacterium tumefaciens,* pB1 121. Each of the three vectors contained a sequence of chloroplast transit peptide from a Rubisco small subunit isolated from pea, a gene coding one of the PHAs enzymes and a synthetic linking sequence, under the control of cauliflower mosaic virus promoter. Three independent constructs were obtained: pBI-TP-Thio. PBI-TP-Red, pBI-TP-Syn, containing the genes of 3-ketothiolase, acetoaceryl-CoA reductase and PHB synthase. The most effective PHB biosynthesis was observed in the plants containing all three genes in their genome, which were obtained by cross-breeding.

**Short Questions**

1. A short note on Edible vaccines.
2. Write about Biodegradable plastics Polyhydroxyalkanoates (PHAs)

**Essay Questions**

1. Write in detail about Edible vaccines, their production methods. Advantages and limitations.
2. Write in detail about production of Plantibodies production, list the Plantibodies produced and highlight on their applications.

# PRACTICALS FOR PLANT BIOTECHNOLOGY

## CONTENT

1. Preparation of media for plant tissue culture
2. Sterilization of explants and inoculation (Inoculation Technique)
3. Establishment of callus culture
4. Preparation of synthetic seeds
5. Meristem culture
6. Cell suspension culture
7. Protoplast isolation and culture
8. Agrobacterium mediated gene transfer

## SPOTTERS

1. Callus cultures
2. Sterilization techniques
3. Somatic embryos
4. Synthetic seeds
5. Meristem culture
6. Cell suspension cultures
7. Isolation of protoplasts
8. Particle bombardment gene transfer method
9. Binary or co-integrate vectors
10. Gus gene expression in transgenic plant tissues
11. Golden rice

| EXPERIMENT 1 | PREPERATION OF MEDIA FOR PLAN TISSUE CULTURE |
|---|---|

**Aim:** To prepare MS medium for plant tissue culture.

**Principle:** In vivo plant cells, tissues and organs get their appropriate nutrient and growth requirements from the intact plant body for their organized growth and development. Isolated cell, tissues and organs also need nutrients for their in vitro growth and development. So, nutrients are supplied artificially according to the medium formulated by several workers. The main objective of medium preparation is to culture the cell, tissue and organ in vitro.

**Procedure:** To make 1 litre of MS medium:

i. Dissolve 30 rams cane sugar in 200 ml distilled water. Mix 1-2 gm activated charcoal and filter through filter paper, set inside the Buchner funnel fitted on a special conical flask with small side arm attachment. Filtering done by using a suction pump.
ii. Take distilled water in another flask and in sequence the appropriate amount of stock solution as follows.
iii. Desired concentrations of auxin and /or cytokinin are added from stock solution according to the formula:
   **Desired concentration/ Stock concentration = amount (ml) of stock solution to be taken for one litre medium.**
   If the quantity of the medium is less than one litre, then hormones are added using another formula:
   **Required concentration X Volume of medium Stock concentration × 1,000 = amount (ml) of stock solution to be added.**
iv. Pour filtered sucrose solution and salt, vitamins, amino acid, hormone solution mixture into a one litre measuring cylinder. Make the final volume to one litre with distilled water. Shake well to mix up uniformly.
v. Adjust the pill of the liquid medium 5.6-5.8 with the aid of 0.1(N) HCI or 0.1(N) NaOH. This operation is done using the pH metre.
vi. Add 5% to 8% agar to the liquid medium make slid medium. Heat to 60c to dissolve the agar completely. Otherwise, without adding sugar. Liquid medium can be used for culture.
vii. Dispense the culture medium into culture tube (20 ml/tube) or wide mouth conical flask (25-45 ml/flask). Insert non-absorbent cotton plug wrapped with gauge cloth. Cover the plug with the help of brown paper and rubber band.
viii. Medium is finally sterilized by autoclaving.
   **Result:** 1 litre of MS medium is prepared in aseptic conditions.

| EXPERIMENT 2 | STERILIZATION OF EXPLANTS AND INCULATION |
|---|---|

**Aim:** To sterilize the explants under aseptic conditions.

**Principle:** Plant material which is to be cultured, should be surface sterilized to remove the surface borne microorganisms. This procedure is done in front of a laminar air flow or inside the inoculation chamber before the plant material is inoculated onto the culture medium.

**Procedure:** The steps for surface sterilization of plant material are follows:

i. Thoroughly washed plant material or explant in tap water is immersed in 5% v/v solution of liquid detergent such as "Teepol" for 10-15 minutes.
ii. Then wash the material thoroughly in tap water and finally in distilled water. This step can be done in the general laboratory. Subsequent steps are done in front of a laminar air flow or the pre-sterilized inoculation chamber.
iii. Dip the explants in 70% ethyl alcohol for 60 seconds.
iv. Immediately transfer the material into an autoclaved jaw bottle and pour 5-10% Sodium hypochlorite (v/v) solution.
v. Keep them for 10-15 minutes. During the period, the bottle is frequently swirled for shaking so that all surfaces of plant material come equally in contact with sterilant.
vi. After 10-15 minutes, decant the sterilant and wash the explants thoroughly with several changes of autoclaved distilled water to remove all traces of sterilant.
vii. Then the explants are ready for culture.
**Result:** The explant is sterilized by surface sterilization method.

## Inoculation Technique

**Aim:** To inoculate the explant onto the surface of the agar medium under aseptic conditions.

**Principle:** Transferring of the surface sterilized explants on the nutrient medium is called as inoculation. Precautions must be taken to prevent the entry of any microorganism at the time of inoculation using the sterilized instruments and under aseptic conditions.

**Procedure:** A typical procedure of transfer of explant to media or inoculation is given below:

i. Put all the sterilized articles (media, instruments, glass, goods etc) for inoculation on the glass racks of the inoculation chamber. Alternately, if

laminar air flow is available, keep all articles on the table of air flow cabinet. Laminar air flow blows bacteria-free air over the working surface.

ii. Put on the switch of UV lamps of inoculant chamber for one hour before work. In case of laminar air flow, the power switch is put on and allows the air flow to blow air for at least 15 minutes before work.

iii. Put off the UV lamp before entering inside the inoculation chamber. Do not put off laminar air flow.

iv. The working glass table top of the inoculant camber or the table of laminar air flow.

v. Wear a clean apron and use a mask. Clean the hands with alcohol and dry it.

vi. Pour alcohol in a clean coupling jar and dip all instruments into it. Light the spirit lamp. Take the surface sterilized or aseptic plant material in a, sterile petri dish.

vii. Flame the neck of culture tube of flask and in quick succession remove the plug of glass vials. Transfer the tissue onto the medium and replace the closure. Each time, the instruments are passed through the flame of the spirit lamp.

viii. **Result:** The explant is inoculated on the surface of the agar medium under aseptic conditions.

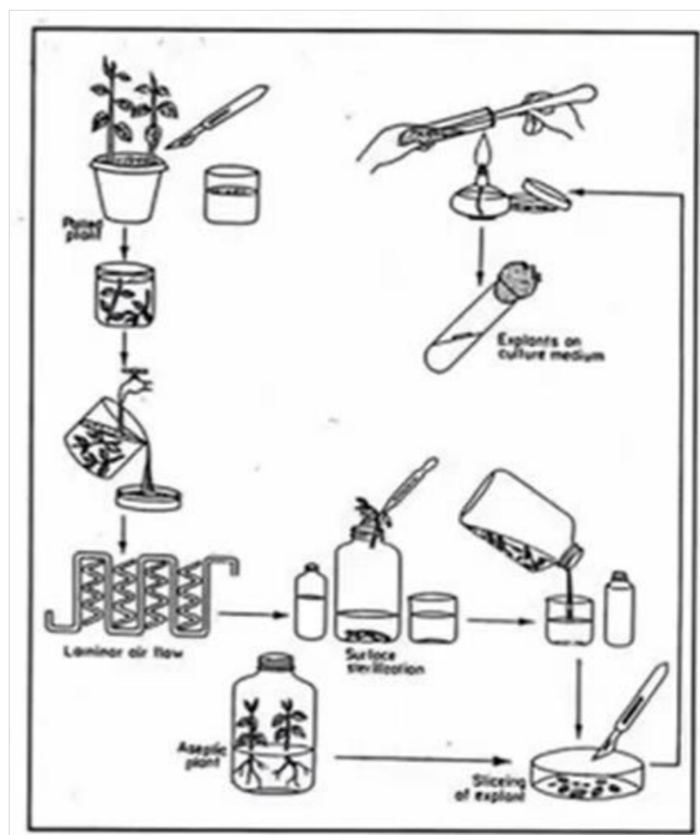



**Fig: Sterilization and inoculation of explant on the agar medium under aseptic conditions**

| EXPERIMENT 3 | ESTABLISHMENT OF CALLUS CULTURE |
|---|---|

**Aim**: To induce callus culture from carrot cells under aseptic conditions

**Principle:** Generally, different plant tissues of many plant species can be used to induce callus formation but carrot rots are highly specific and it is taken as typical example for callus culture

**Procedure:** The following are steps taken in callus culture of carrot root

i. Fresh and healthy carrot root washed thoroughly in running tap water
ii. External 1-2 mm is scraped. Upper 1 cm of carrot root is discarded and then it is cut into 0.5 cm thick slices.
iii. These slices are placed immediately in a beaker containing water.
iv. These are then transferred to a beaker containing sodium hypochlorite solution and kept there for 10 minutes
v. Slices are taken out with a sterile force from the hypochlorite solution and washed successively in 3 beakers containing double distilled water keeping the slices for 20 30 seconds in each. The slices are kept in the third beaker.
vi. A carrot slice is taken and is placed on a petridish. Tissue cylinders are cut out from the cambial region by a sterilised cork borer, after cutting maximum number of tissue cylinders from the cambial region remaining portion of the slice is discarded
vii. Tissue cylinders are placed in a petridish containing double distilled water.
viii. A tissue cylinder is transferred to a petridish and its two sides are trimmed with a sterile scalpel and discarded.
ix. Remaining cylinder is Y cut into explants measuring 5 mm diameter and 2 mm thickness.
x. These explants are placed in a petridish containing double distilled water.
xi. Explants are then transferred with a sterile forceps on the surface of a sterile filter paper on a petridish. The upper and lower surfaces of each explants are blotted.
xii. One such explant is transferred to each culture tube containing the nutrient medium.
xiii. Culture tubes are kept in a glass storage jar, wrapped in aluminium foil and placed in an incubator at 25°C.
xiv. The surface of the explants after few days becomes somewhat rough, indicating initiation of the callus. Callus can be maintained from few weeks to three months depending on the rate of growth.
xv. Generally after 6-8 weeks the callus is sub-cultured. The callus is divided into small parts of 100 mg approximately.

**xvi.** Each piece is transferred to a new flask containing 30 c.c. of culture medium and sub- cultured at a temperature of 25°C or above

**Result:** Callus is induced from the carrot cells after incubation under aseptic conditions.

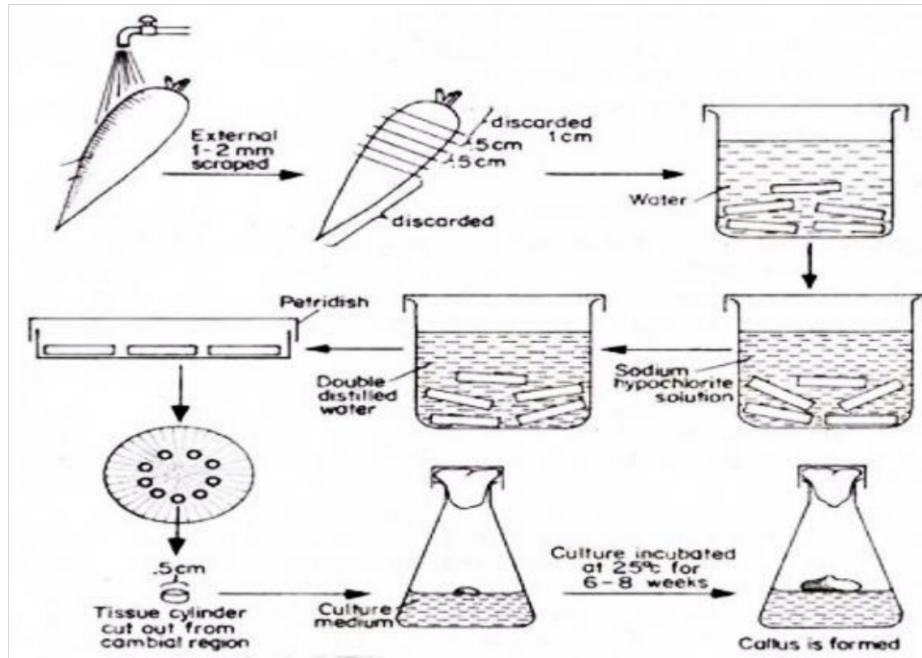



**Fig: Flow diagram illustrating the method of callus induction in carrot cells**

| EXPERIMENT 4 | PREPARATIO OF SYNTHETIC SEEDS |
|---|---|

**Aim:** To prepare synthetic seeds by dropping method.

**Principle:** The somatic embryos for synthetic seeds are produced in the lab through culturing of somatic cells and treating with different hormones to produce somatic embryos directly or indirectly through callus formation. Somatic embryos produced naked embryos without storage materials and protective layer (seed coat). This is very difficult for handling so this demand the encapsulation and coating. They are encapsulated using gel agents like agar, alginate, polyco, carboxy methyl cellulose, guar gum, sodium pectate etc.

**Procedure:** The following steps are involved in the formation of synthetic seeds using gel complexion through dropping procedure:

i. The isolated somatic embryos are mixed with 0.5 to 5% (W/V) Sodium alginate solution and dropped into 30-100 µM Calcium nitrate solution.
ii. Surface complexation begins immediately and the drops are gelled completely within 30 min.
iii. Or the somatic embryos are mixed with sodium alginate (2%) and the suspension is dropped into the calcium salts solution (200mM).
iv. When sodium alginate dropped into the calcium salt solutions it from round firm beads due to the ion exchange between $Na^+$ in sodium alginate and $Ca^{2+}$ in calcium slat solutions and sodium alginate form calcium alginate in 20-30 minutes.

**Result:** Somatic embryos are encapsulated in calcium alginate hydrogel beads to form synthetic seeds.

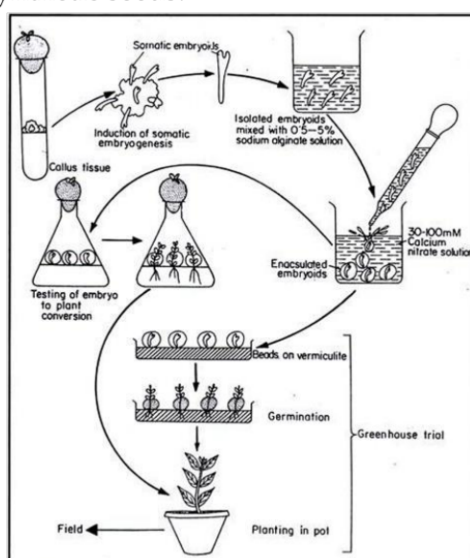



**Fig: Steps for synthetic seed production by dropping method**

| EXPERIMENT 5 | MERISTEM CULTURE |
|---|---|

**Aim:** To culture shoot apical meristem on agar medium under aseptic conditions.

**Principle:** Meristem culture refers to regeneration of whole plant from tissues of an actively dividing plant part such as stem tip, root tip or axillary bud. The apical meristem refers to dome like extreme shoot tip of 0.25 to 0.30 mm in length and 0.1 mm in diameter. To grow virus free plants meristem tips of 0.2-0.3 mm is used. This technique is widely used in vegetatively propagated plants such as sugarcane, potato, banana and several timber species.

**Procedure:** The following are the steps involved in shoot apical meristem culture:

i. Dissect out the shoot apical meristem (100-500 μm in length) with one or two leaf primordia. 2. The larger the meristem explant, the greater the chances of its survival and shoot development. But the risk of infection by the virus also increases with explant size. Therefore, a compromise has to be reached between these two opposing forces in deciding the explant size.
ii. Viruses are eliminated by thermotherapy of whole plants, in which plants are exposed to temperatures between 35-40°C for a few minutes to several weeks depending on the host- virus combination.
iii. In general, it is preferable to excise larger shoot-tips from heat-treated plants. Also, cultured meristems may also be given thermotherapy.
iv. A prolonged exposure to a low temperature (5°C), followed by shoot-tip culture, has also proved quite successful in virus elimination. This technique is called cryotherapy.
v. Some chemicals, e.g., virazole (ribavirin), cyclohexamide, actinomycin D, etc., which interfere with virus multiplication, may be added into the culture medium for making the shoot-tips free from Viruses; this is known as chemotherapy.

**Result:** Shoot tip apical meristem is cultured on agar medium under aseptic conditions.

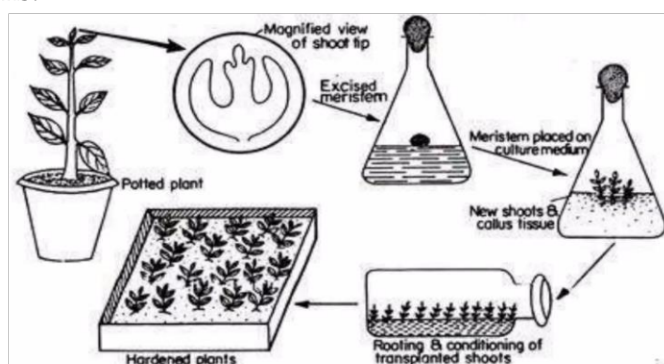



**Fig: Flow diagram illustrating the technique of meristem culture (shoot tip)**

| EXPERIMENT 6 | CELL SUSPENSION CULTURE |
|---|---|

**Aim:** To culture suspension culture from the callus under aseptic conditions.

**Principle:** A cell suspension culture refers to culturing of cell aggregates which are dispersed and growing in a moving liquid media. It is normally initiated by transferring pieces of undifferentiated and friable callus to a liquid medium, which is continuously agitated by a suitable device.

**Procedure:** The following are steps to establishing a cell suspension:

i. The calli formed over plant tissues are generally compact. After successive subculturing of the calli from old solid media to fresh media, the calli become friable. The friable calli are ideal for establishing a cell suspension because their texture favors nutrient uptake. Also, cells can be easily distributed over the liquid medium. After calli become friable, they are transferred from a solid medium to a liquid.

ii. In this step, the cells from the calli in liquid media follow a growth curve, where there are lags, exponential and stationary phases. The friable calli is adapted to the new liquid environment in the lag phase. In the exponential phase, cells grow and multiply until they reach the stationary phase, when the cell growth stops.

iii. This step is adjusted according to the research goals. Cell suspensions can be used for basic science, such as studying the behavior of a given metabolite or protein in a liquid medium or for massive production of metabolites.

iv. The metabolite's production in cell suspensions is commonly a two-step process. In the first step, the cell suspension needs to grow enough to provide mature cells that can produce metabolites.

v. Then, the metabolite production is induced in a second step. Modifying the liquid media with specific reagents (e.g., coconut water, jasmonic acid) or changing environmental conditions (such as light and temperature) is typically used to stimulate metabolite production.

**Result:** Cell suspension culture is induced in liquid medium by using friable callus under aseptic conditions.

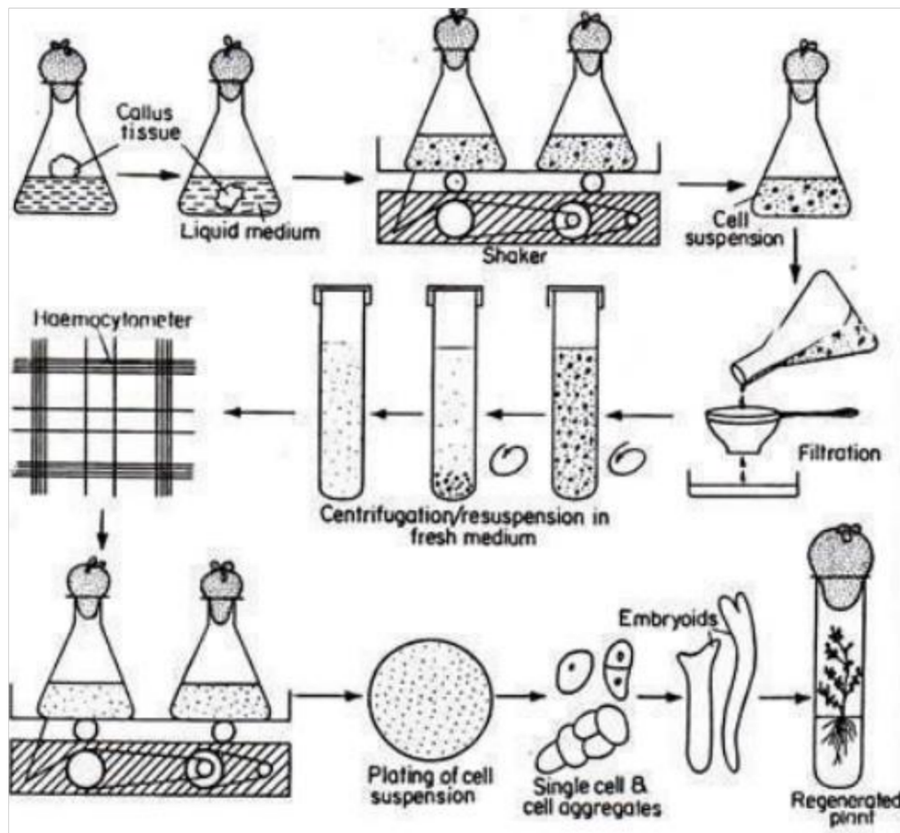



**Fig: Flow diagram illustrating the method of cell suspension culture**

| EXPERIMENT 7 | PROTOPLAST ISOLATION AND CULTURE |
|---|---|

**Aim:** To isolate the protoplast using enzymatic digestion method.

**Principal:** A plant cell without its cell wall is known as a protoplast. It is called as a naked plant cell because the cell wall has been removed either by a mechanical or an enzymatic method. Protoplast can be isolated tom almost all plant parts *viz.* root, leave fruits, tuber, endosperm, pollen etc. Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells and minimal or no damage to the protoplasts.

**Procedure:**
**Methods of protoplast isolation**

- **i.** Surfaces sterilize the leaves in 70% ethyl alcohol.
- **ii.** Wash the leaves with sterilized distilled water
- **iii.** Peel the leaf pieces from the lower epidermis of the leaves
- **iv.** Place the leaf pieces in a chemical mixture containing 13% mannitol for plasmolysis and shaken in a water bath
- **v.** Place the treated leaf pieces are macerated with cell wall degrading enzyme mixture (pectinase cellulose) and incubate for 24hrs at 25°C.
- **vi.** Filter the above mixture and centrifuge for few minutes (100 rpm)
- **vii.** Collect the sediment containing protoplast pellets after the removal of supernatant enzyme medium.
- **viii.** Resuspend and wash the protoplast pellet in sorbitol and subject it for spinning
- **ix.** Place the protoplast in 20% sucrose solution
- **x.** The above solution is again centrifuged and protoplasts are removed with a pipette and placed finally in a suitable culture medium
  **Result:** Protoplasts are isolated from the leaf cells by enzymatic method.

**Methods of protoplast culture**

**Aim:** To culture the protoplast under aseptic conditions.

**Principle:** The very first step in protoplast culture is the development of a cell wall around the membrane of the protoplast. This is followed by the cell divisions that give rise to a small colony with suitable manipulations of nutritional and physiological conditions, the cell colonies may be grown continuously as cultures or regenerated to whole plants. Protoplasts are cultured ether in agar medium or liquid medium. Sometimes, protoplasts are first allowed to develop cell wall in liquid medium, and then transferred to agar medium.

**Procedure:** Isolated protoplast can be cultured in several ways or methods. Of which Agar embedding technique is a common method and widely practiced.

i. Protoplast suspension is mixed with equal volume of melted agarified medium.
ii. The protoplast agar mixture is poured into petridishes. This is called plating.
iii. This mixture is allowed to spread uniformly, after replacing the lid on the petridish.
iv. After solidification of the medium, the petridish is inverted and incubated at 25°C with illumination.
v. The cultures are subcultured periodically in the same agar medium.
vi. Protoplasts gradually develop cell walls and become regenerated cells.
vii. Later these cells divide and redivide and form colonies.
viii. The colonies are subcultured on agar medium to produce new cultures.
ix. Shoots and roots are inducted on the callus formed.
x. **Result:** Isolated protoplasts are cultured by using agar embedding technique

| EXPERIMENT 8 | AGROBACTERIUM MEDIATED GENE TRANSFER |
|---|---|

**Aim:** To transfer the genes by Agrobacterium mediated gene transfer method.

**Principle:** Vector-mediated gene transfer is carried out either by Agrobacterium mediated transformation or by use of plant viruses as vectors. In this approach the transgene is combined with a vector which takes it to the target cells for integration. The term plant gene vector applies to potential vectors both for transfer of genetic information between plants and the transfer of genetic information from other organisms (bacteria fungi and animals) to plants.

The Ti plasmid has two major segments of interest in transformation that is T DNA and virus region. The T DNA region of the Ti plasmid is the part which is transferred to plant cell and incorporated into nuclear genome of cells. The transfer of T DNA is mediated by genes in the another region of Ti plasmid called vir genes (virulence genes).

**Procedure:** The process of Agrobacterium-mediated gene transfer:

i. It begins by inserting the desired gene(s) into a plasmid vector, which is a small circular piece of DNA that can replicate independently of the host genome. The plasmid vector also contains the necessary regulatory, sequences to control the expression of the inserted genets).
ii. The plasmid vector is introduced into the *Agrobacterium tumefaciens* through a process called transformation. Once inside the bacterium, the plasmid vector is recognized by the bacterium's natural transfer machinery, which packages the T-DNA and delivers it into the host plant's genome.
iii. The T-DNA is integrated into the host plant's DNA, resulting in the transfer of the desired gene(s) into the plant's genome,
iv. The transformed plant cells can then be selected and regenerated into whole plants using tissue culture techniques.

**Result:** Gene is transferred by using indirect method called Agrobacterium mediated gene transfer.

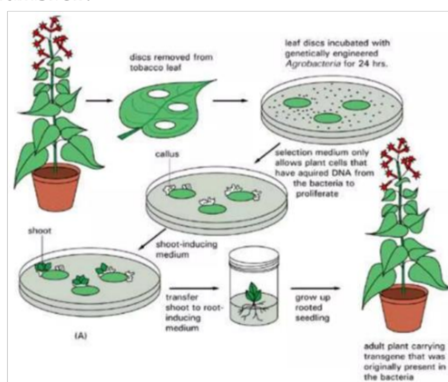



**Fig: Steps involved in Agrobacterium mediated gene transfer in tobacco**

**1) Callus cultures**

i. When tissues on culture medium produce unorganized mass of cells with no regular form then it is called 'callus culture'.

ii. In some cases, it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis.

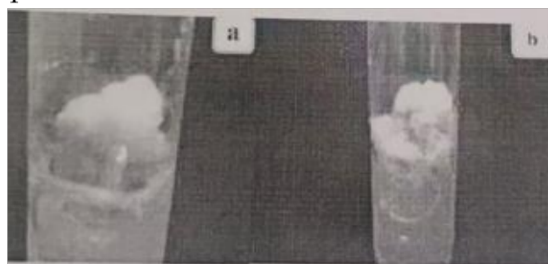
iii. Genotype, composition of nutrient medium and physical growth factors influence the formation of callus. The size and shape of the explants is also important.

iv. Callus differs in compactness or looseness, i.e. cells may be tightly joined and the tissue mass is one solid piece and called as 'compact callus' or cells are loosely joined and individual cells readily separate and are called as 'friable callus'.

v. This can be due to the genotype or the medium composition. A friable callus is often used to initiate a liquid cell suspension culture and also a source of protoplasts. Both the types are sometimes interchangeable.

vi. The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium which is called as organogenesis or morphogenesis.

vii. Similarly, callus can be induced to form somatic embryo which can gives rise to whole plant.




**2) Sterilization techniques - Autoclave**

i. Autoclave is an instrument used for sterilization in biology experiments. It was first developed by Chamberland in 1884.

ii. Autoclave is usually of pressure cooker type made up of double walled cylindrical metal vessel made up of stainless steel or copper.

iii. The lid is provided with the pressure gauze for monitoring the pressure, exhaust valve to remove the air and safety valve to avoid explosion during operation.

iv. The articles are kept loosely in the autoclave chamber in a basket.

v. Water is boiled with the help of heater and steam is released into the autoclave's chamber.

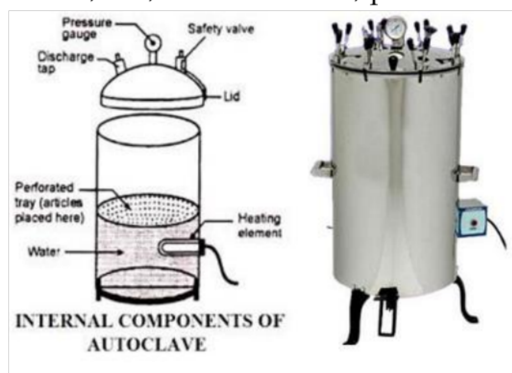
vi. The exhaust valve is kept open till the air in the chamber is driven out. The exhaust valve is closed and steam pressure in the chamber is allowed to reach the desired value.

vii. Autoclaving or Moist or steam sterilization:

viii. The temperature of the steam inside the chamber depends on pressure in the autoclave. Generally, a pressure of 15 pound with temperature at 121°C is employed for 15-20 minutes which is enough to kill all the cells and spores of organisms.

ix. Steam condenses on the cooler surface of the object and transfers heat energy to the object and sterilizes it.

x. Autoclave is used for sterilizing culture media, glass wares, other heat resistant instruments, etc., but not for oils, powders and plastics.




**Hot air oven**

i. Hot an oven is a sterilization instrument used in science laboratory

ii. It contains a double walled insulated cabinet with outer wall made up of mild steel sheets and an inner wall made up of anodized aluminium or stainless steel.

iii. The space between the walls is filled with glass wool to provide thermal insulation.

iv. Hot an oven is fitted with an electric heating mechanism and thermostat control, using which the required constant temperature can be obtained by trial and error.

v. A circulating fan is fitted to circulate air within the cabinet. For proper circulation of hot air, the shelves are perforated. It has a single insulated metallic door and control panel containing all the switches and indicators.

vi. There is air ventilator on both the sides of cabinet through which moisture laden air escapes out from the oven Hot air oven sterilizes the object by hot dry air.

vii. It kills the microorganisms by dehydrating and oxidizing the cellular constituents. Dry heat is less effective in killing microorganism than moist heat.

viii. Higher temperature for longer time period is used with dry air because it has less penetration power.

ix. It removes moisture from microorganisms and thus interferes with coagulation of microbial proteins. Hot air oven can be used at different temperatures.

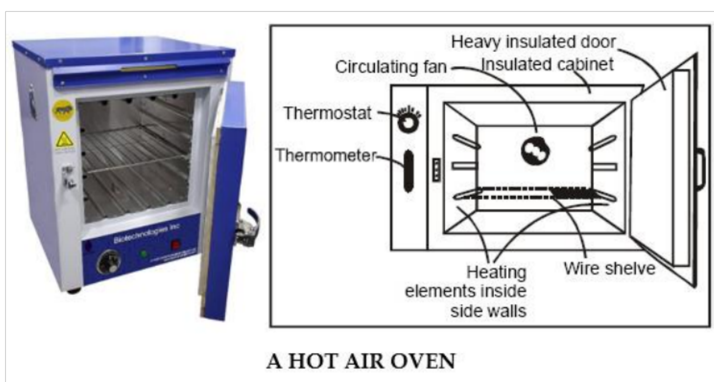
x. A hot air oven is used to sterilize glass wares such as test tubes, petriplates, corrodible metal instruments, powders, oils etc., which can tolerate prolonged heat exposure but get spoiled by moist heat.

xi. However, it is not suitable for sensitive materials like plastic and rubber items. Liquid substances, such as prepared media and saline solutions cannot be sterilized in oven, as they lose water due to evaporation.

xii. Pack all the glassware such as pipette with pipette can, glass petridishes, sample dish, test tubes, pestle and mortar, mineral oil to be sterilized by hot air oven sterilization with suitable wrapping Switch on the hot air oven until to reach 160 °C.

xiii. Hold on in that temperature for 1 hour

xiv. Switch off the heating of hot air oven and open the door once come below 65°C.




A HOT AIR OVEN

**3) Somatic embryos**

i. Somatic embryogenesis is an artificial process in which a plant or embryo is derived from a single somatic cell.

ii. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. ordinary plant tissue. No endosperm or seed coat is formed around a somatic embryo.

iii. Cells derived from competent source tissue are cultured to form an undifferentiated mass of cells called a callus. Plant growth regulators in the tissue culture medium can be manipulated to induce callus formation and subsequently changed to induce embryos.

iv. The ratio of different plant growth regulators required to induce callus or embryo formation varies with the type of plant.

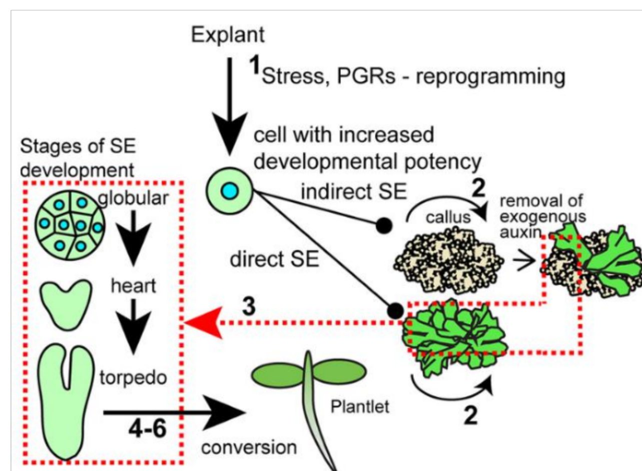
v. Somatic embryos are mainly produced in vitro and for laboratory purposes, using either solid or liquid nutrient media which contain plant growth regulators (PGR's). The main PGRs used are auxins but can contain cytokinin in a smaller amount.

vi. Shoots and roots are monopolar while somatic embryos are bipolar, allowing them to form a whole plant without culturing on multiple media types.

vii. Somatic embryogenesis has been described to occur in two ways ie., direct embryogenesis or indirect embryogenesis.

viii. Direct embryogenesis occurs when embryos are started directly from explant tissue creating an identical clone. In other words without callus formation of embryo from explant, that is called direct embryogenesis.

ix. Indirect embryogenesis occurs when explants produced undifferentiated, or partially differentiated, cells (often referred to as callus) which then is maintained or differentiated into plant tissues such as leaf, stem, or roots. 2,4-Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP) and Gibberellic acid (GA) has been used for development of indirect somatic embryos.




**4) Synthetic seeds**

i. Somanc embryo (embryoids), shoot buds or any other plant material obtained as a result of *in vitro* culture are covered (encapsulated) with a chemical membrane. Such encapsulated materials behave as seeds. These are called artificial seeds or synthetic seeds.

ii. The artificial covering acts as an artificial seed coat. Such seeds are bead like and can "germinate" and plantlets are also formed.

iii. Several substances are used as artificial seed coats. Some of them are agar, agarose carrageenin, polyacrylamide, introcellulose, ethyl cellulose and sodium alginate Sodium alginate is most commonly used.

iv. These will germinate normally into plants. This covering helps to give physical protection, avoid desiccation and encapsulation should carry nutrients, growth regulators to help in germination.

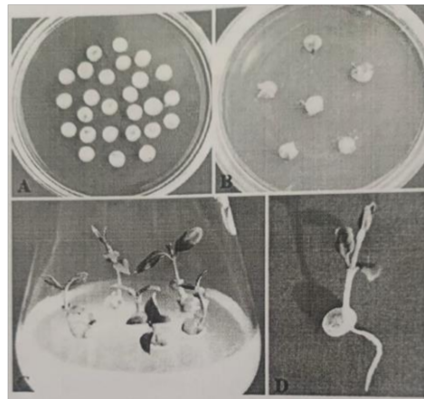
v. Antibiotics may be added to avoid contamination and also it should be durable and non- toxic.

vi. Artificial seeds can also be helpful where there is no successful seed production after sexual hybridization. The somatic embryos can be obtained from somatic hybrids obtained through protoplast fusion.

vii. Artificial seeds are helpful in case of meiotically unstable genotypes, where the normal seed set is of low frequency.

viii. In cases where the embryo germination is difficult, the artificial seed can provide the beneficial adjuvants i.e., growth promoting seed substances, plant nutrients, etc. through the artificial coats.

ix. In genetic manipulation of crop plants the production of artificial seed may be useful.



**5) Meristem culture**

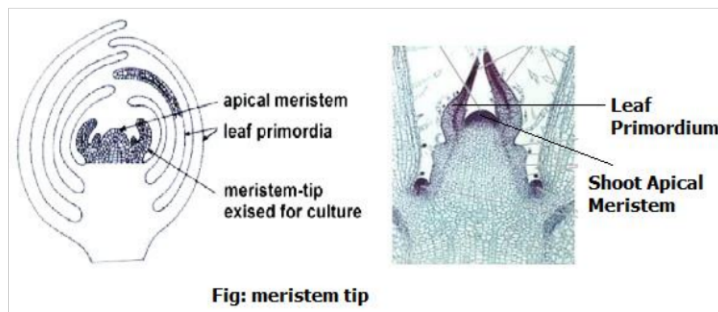



Fig: meristem tip

**i.** Meristem culture refers to regeneration of whole plant from tissues of an actively dividing plant part such as stem tip, root tip or axillary bud.

**ii.** The apical meristem refers to dome like extreme shoot tip of 0.25 to 0.30 mm in length and 0.1 mm in diameter. To grow virus free plants meristem tips of 0.2-0.3 mm is used.

**iii.** For shoot tip culture large explants measuring up to 2 cm in length is used.

**iv.** This technique is widely used in vegetatively propagated plants such as sugarcane, potato, banana and several timber species.

**v.** Plants free from viruses, viroids, mycoplasma and even fungi and bacteria in a range of crops can be accomplished by this technique.

**vi.** Virus free clones of potato, sugarcane have been produced from valuable virus infected stocks through meristem culture.

vii. Useful in germplasm exchange of asexually propagated plant species as the plantlets obtained by meristem culture are free from pathogens.

viii. Meristems are suitable for cryopreservation by storing the germplasm at –196° C in the liquid nitrogen for long period of time

ix. Virus-free plants serve as excellent experimental materials for evaluating the detrimental effects of infections by various viruses.

**6) Cell suspension cultures**

i. A plant cell suspension culture is a liquid culture where cells from a soft or friable callus divide and multiply. The culture is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters.

ii. In a plant cell suspension, the cells divide, multiply and grow. Plant cell suspension cultures are used to obtain secondary metabolites in pharmaceuticals, cosmetics, or used in the food industry. Researchers use plant cell suspension cultures to analyze metabolic pathways for basic science and even knockdown experiments.

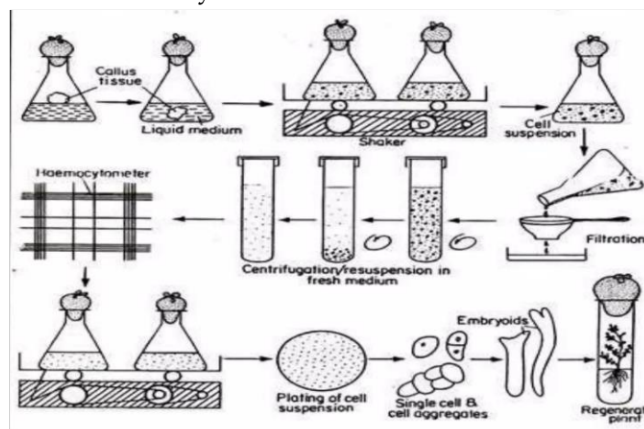
iii. A cell suspension culture refers to culturing of cell aggregates which are dispersed and growing in a moving liquid media. It is normally initiated by transferring pieces of undifferentiated and friable callus to a liquid medium, which is continuously agitated by a suitable device.

iv. The cells are suspended in the liquid culture by constant agitation by keeping in a gyratory shaker at 100-250 rpm which facilitates aeration and dissociation of cell clumps into smaller pieces.

v. A good suspension culture is one which consists of a high percentage of single cells along with small cluster of cells.

vi. Orbital shakers are widely used for the initiation and serial propagation of plant cell suspension culture. They should have a variable speed control (30-150 rpm) and the stroke range should be of 4-8 cm orbital motion.

vii. Suspension cultures grow much faster than callus cultures and it should be sub-cultured about every week.



7) **Isolation of protoplasts**

A plant cell without its cell wall is known as a protoplast. It is called as a naked plant cell because the cell wall has been removed either by a mechanical or an enzymatic method. Protoplast can be isolated from almost all plant parts viz. root, leave fruits, tuber, endosperm, pollen etc. Protoplast culture refers to the aseptic isolation and in vitro culture of protoplast to obtain viable plants.

1. **Mechanical method:** In this method large and highly vacuolated cells (eg. onion bulbs. scales, radish root & beet root tissue) are plasmolysed in an osmotic solution, causing the protoplast to shrink away from the cell wall. The tissue is dissected to release the protoplasts.

   Mechanical method for protoplast isolation is no more in use because of the following limitations:

   i. Yield of protoplasts and their viability is low.
   ii. It is restricted to certain tissues with vacuolated cells.
   iii. The method is laborious and tedious.

However, some workers prefer mechanical methods if the cell wall degrading enzymes of enzymatic method) cause deleterious effects to protoplasts.

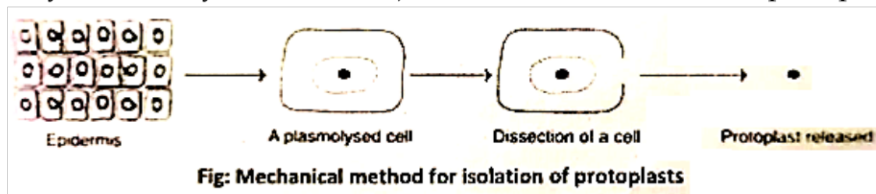



**Fig: Mechanical method for isolation of protoplasts**

2) **Enzymatic method:**

Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells and minimal or no damage to the protoplasts. Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices, fruits, embryos and microspores. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots are most frequently used. In addition, callus and suspension cultures also serve as good sources for protoplast isolation.

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose and pectin which can be respectively degraded by the enzymes cellulose, hemicellulose and pectinase. The enzymes are usually used at a pH 4.5 to 6.0, temperature 25-30°C with a wide variation in incubation period that may range from half an hour to 20 hours.

After the digestion of cell wall the isolated protoplast is subject to osmotic stress. If an osmotic stabilizing agent is not included in the medium

the isolated protoplast would take in water by the process of osmosis and would eventually burst as there is no cell wall to constrain the cell.

The isolated cells are macerated with macroenzyme (Pectinase) in 13% mannitol. Pectinase mainly degrades the middle lamella while cellulase are required to digest the cell wall. The cells are purified by filtration through nylon mesh. Then the cells are incubated in 2% cellulose for about 90 min

**8) Particle bombardment gene transfer method**

Particle bombardment is a technique used to introduce foreign DNA into plant cells. Particle for micro projectile) bombardment is the most effective method for gene transfer and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms. The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics.

Plant material used in bombardment: Two types of plant tissue are commonly used for particle bombardment:

i. Primary explants which can be subjected to bombardment that are subsequently induced to become embryo genic and regenerate
ii. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

i. There has been tremendous success in transforming plant species. It is due to its rapid assessment of the transient expression of genetic constructs introduced into cells of intact tissues. After the plant cell genome gets impregnated by the DNA-coated gold particles, the DNA is used as a template strand for transcription by the cell. This is called transient expression.
ii. The capability of biolistics to optimize the delivery of DNA into the cells is done on strict parameters. These include the target tissue, behavior in tissue culture, and available marker genes. If the delivered DNA construct contains a selectable marker, then stably transformed cells can be selected and cultured accordingly using tissue culture methods.
iii. For example, transformed cells in the culture with a DNA construct containing a gene that confers resistance to any particular antibiotic or herbicide, are selected including that antibiotic or herbicide in the tissue culture media.
iv. The selected transformed cells are allowed to divide and differentiate into the organized, specialized, tissue cells of an entire plant. It happens

when the transformed cells are treated with a series of plant growth hormones, such as auxins and gibberellins. This capability in plants for total regeneration is called totipotency.

v. Thus, the desired gene can be introduced into callus cultures or even tissues of whole seedlings.

**Steps:**

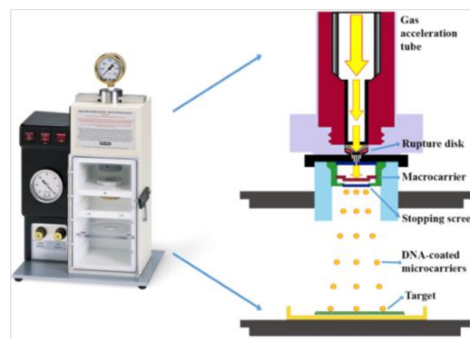
i. The process of transformation employs foreign DNA coated with minute 0.2-0.7 µm gold (or) are tungsten particles to deliver into target plant cells.

ii. The coated particles are loaded into a particle gun and accelerated to high speed (By using pressurized helium gas or by electro static energy released by a droplet of water exposed to a high voltage).

iii. The target could be plant cell suspensions, callus cultures, or tissues.

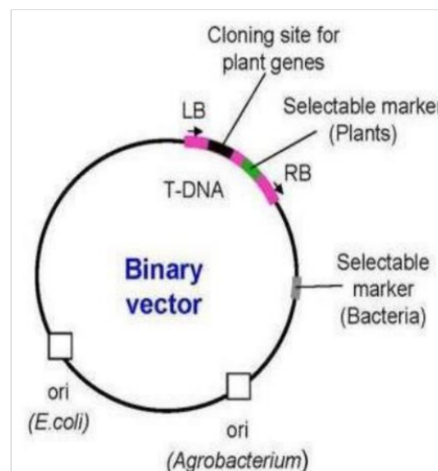
iv. The projectiles penetrate the plant cell walls and membranes.

v. As the micro projectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA.




**9) Binary or co-integrate vectors**

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in *E. coli* and *Agrobacterium*.



The binary vector has the following components:

i. Left and right borders that delimit the T-DNA region.
ii. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells
iii. A multiple cloning site (MCS) for introducing target/foreign genes.
iv. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.
v. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.
vi. A broad host-range origin of replication such as RK2 that allows the replication of binary vector in Agrobacterium.

**Production and use of binary vector:**

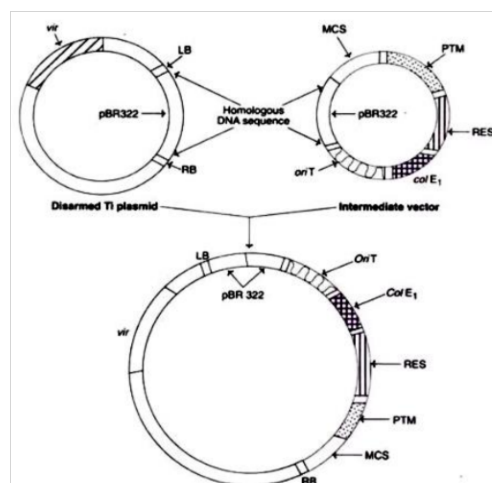
The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in *E. coli*. By a mating process, the binary vector is mobilised from *E. coli* to *Agrobacterium*. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

**Advantages of binary vectors:**

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.
ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

**Co-integrate vector**

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid.



**Production of disarmed Ti plasmid:**

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

**Construction of intermediate vector:**

The intermediate vector is constructed with the following components:

1) A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.
2) A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant cells and thus permits their isolation.
3) A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.
4) A multiple cloning site (MCS) where foreign genes can be inserted.
5) A Co/El origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.
6) An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

**Production and use of co-integrate vectors:**

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (eg. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

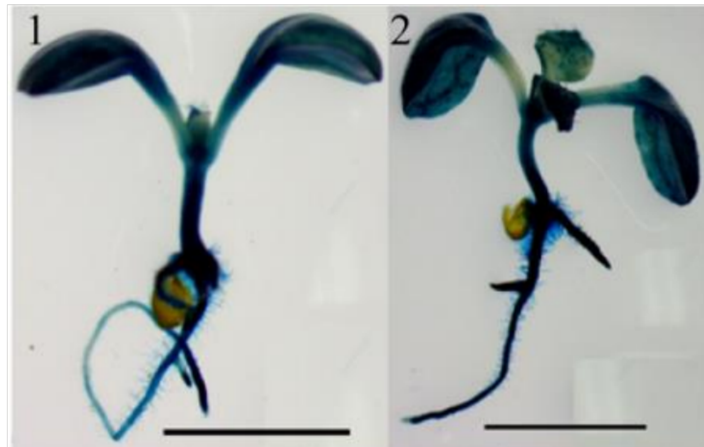
**Advantages of co-integrate vector:**

i. Target genes can be easily cloned

ii. The plasmid is relatively small with a number of restriction sites.
iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

**10) Gus gene expression in transgenic plant tissues**

**i.** For examination of the expression location and expression pattern of specific genes, GUS histochemical assay is done.
**ii.** Substrate used in the assay is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), which gives an insoluble indigo-blue precipitate at the site of GUS activity.
**iii.** GUS staining is a very powerful tool in plant molecular biology, delivers great versatility for various applications.
**iv.** As a sensitive and reliable method that is also easy to perform, GUS staining has become the most popular way to evaluate gene expression in plants.
**v.** GUS is short for *β-Glucuronidase*, a gene from *Escherichia coli (E.coli)* that encodes a sugar consuming enzyme-Glucuronidase.
**vi.** As there is no detectable GUS gene expression in plants, and the bacterial GUS gene does not interfere with plant intrinsic gene expressions, GUS provides an ideal reporter, being orthogonal to native genetic machinery, for gene expression in the plant system.
**vii.** By fusing the promoter of genes of interest to the GUS coding region, the expression patterns of plant genes can be reflected by the activation of GUS.




**11) Golden rice**

**i.** A variety of rice was produced through genetic engineering capable of synthesizing beta-carotene, a precursor of Vitamin A in the endosperm of transgenic rice.
**ii.** This variety, known as 'Golden rice', differs from the parental variety by having two additional beta-carotene synthesis genes.

iii. Golden rice was produced by transforming rice with two beta-carotene synthesis genes: (i) psy (phytoene synthase) from Daffodil (*Narcissus pseudomarcissus*) (ii) crt I (carotene desaturase) from a soil bacterium (*Erwinia uredovora*).

iv. Both these genes were inserted into rice nuclear genome under the control of an endosperm specific promoter to express them only in endosperm.

v. The end product of this pathway is lycopene, but plants do not accumulate it otherwise the rice would have been red.

vi. An endogenous enzyme inside the plant converts lycopene to beta-carotene giving golden yellow color after which it is named.

vii. This was product of an eight year project by Ingo Potrykus of Swiss Federal Institute of Technology and Peter Beyer of University of Freiburg.

viii. In the year 2005, a new variety of rice, called Golden rice 2 producing 23 times more beta-carotene in comparison to original Golden rice was announced.

Code No: F- 15448

**FACULTY OF SCIENCE**

**B.Sc (CBSC) V-Semester Examination, December 2023/January 2024**

**Subject: Biotechnology**

**Paper- V (A): Plant Biotechnology**

**Time: 3 Hours** **Max. Marks: 80**

**Part – A**

**Note: Answer any eight questions.** **(8 × 4 = 32 Marks)**

1. What is de-differentiation? Explain.
2. Write short note on organogenesis.
3. List out the application of tissue culture.
4. Explain meristem culture and give is applications.
5. Write a short note on production of synthetic seeds.
6. What is somaclonal variation? Explain.
7. Explain electroporation gene transfer method.
8. Differentiate binary and co-integrated vectors.
9. What are the methods for selection of transgenic plants?
10. How are transgenic plants used as bioreactor? Explain
11. What are edible vaccines?
12. Explain about Bt cotton.

**PART - B**

**Note: Answer all the questions** **(4 × 12 = 48 Marks)**

13. (a) Discus on preparation and sterilization of tissue culture media.

**(OR)**

(b) Elaborate on callus induction and cell suspension culture.

14. (a) What is micropropagation? Describe the methods and applications of micropropagation.

**(OR)**

(b) What is cryopreservation? Explain the steps involved in cryopreservation and give its applications.

15. (a) What is gene editing? Describe CRISPR CAS technology and give its advantages.

**(OR)**

(b) Elaborate on the molecular mechanism of agrobacterium infection.

16. (a) Discuss on the production of transgenic plants for herbicide resistance and applications.

**(OR)**

(b) What is abiotic stress? Explain the production of transgenic plants for drought stress toleranc.

Code No. 6548

**FACULTY OF SCIENCE**

**B.Sc (CBCS) V Semester Examination, March 2022**

**Subject: Biotechnology**

**Paper –V(A) : Plant Biotechnology**

**PART - A**

**Note: Answer any eight questions.** **(8 × 4= 32 Marks)**

1. Differentiate organogenesis and somatic embryogenesis.
2. Discuss the role of gibberellins in plant tissue culture.
3. Explain the terms regeneration and differentiation.
4. What is micropropagation and differentiation?
5. What is plant germplasm? Why it should be conserved?
6. Discuss the origin of somaclonal variation.
7. Describe microinjection method.
8. What are selection markers? Give examples of selection marker genes.
9. Discuss the mechanism of genome editing.
10. Define edible vaccines. Explain the production of edible vaccine with an example.
11. How are transgenic plants used as bioreactors?
12. What are biodegradable plastics? How are they produced?

**PART – B**

**Note: Answer any four questions** **(4 × 12= 48 Marks)**

13. Discuss the induction of callus and cell suspension cultures.
14. Give a general account on nutritional requirements of plant tissue culture.
15. Write an essay on encapsulation methods, production of synthetic seeds and applications.
16. Describe the production of haploids using pollen cultures. Add note on application of haploids.
17. With a detailed description of TI plasmid explain the molecular mechanism of agrobacterium mediated transformation.
18. Discuss the use of viral vectors in gene transfer to plants.
19. Give a detailed account on bacterial and fungal resistant transgenic plants.
20. Discuss the generation of salinity tolerance using transgenic approach in plants.

**FACULTY OF SCIENCE**

**B.Sc V semester (CBSC) examination, July 2021**

**Subject: Biotechnology**

**(Plant Biotechnology)**

**Paper: VI A – (DSE E-1)**

**Time: 2 Hours** **Max. Marks: 80**

**PART – A**

**Note: Answer any four questions.** **(4 × 5 = 20 Marks)**

1. Write a short note on redifferentiaton and Dedifferentiation.
2. Explain role of gitterellins in plant growth and development
3. Write a short note on somatic embryogenesis.
4. Explain the method of preservation of plant germplasm.
5. Write about transgenic production of insect resistant plants.
6. Explain the strategies for production of light stress resistant plants.
7. Give strategies for production of drought resistant plants.
8. How can transgenic plants be useful for antibody production?

**PART – B**

**Note: Answer any two questions.** **(2 × 20 = 40 Marks)**

9. Describe preparation of media and sterilization techniques used in plant tissue culture.
10. Discuss the nutritional requirements for plant tissue culture.
11. Explain the development of stomatic hybrids and cybrids and application
12. Detail the technique of cell suspension cultures and their use for the production of secondary metabolites.
13. Explain the transgenic technique for the production of virus resistant plants.
14. Detail transgenic production of herbicide resistant plants.

Code No. 8216

# FACULTY OF SCIENCE

## B.Sc. V- Semester (CBSC) examination. November/ December 2019

## Subject: Bio-Technology

## Plan Biotechnology

## Paper – VI – A (DSE E – 1)

**Time: 3 Hours** **Max.Marks: 60**

**Part – A** **(5 × 3 = 15 Marks)**

**[Short Answer Type]**

**Note: Answer any five of the questions. Each question carries equal marks.**

1. Differentiate dedifferentiation and dedifferentiation
2. What are the micro nutrients used in plant tissue culture media?
3. What is meristem culture? How can it be useful in production of virus free plants?
4. Give a brief note on batch and continuous cell suspension cultures
5. How are glyphosate tolerant plant produced?
6. Explain the use of transgenic plants for production of biodegradable plastics
7. Define encapsulation. What are the encapsulating agents used in production of synthetic seeds?
8. How are explants sterilized?

**PART – B** **(3 × 15 = 45 Marks)**

**[Essay Answer Type]**

**Note: Answer all questions from the following.**

9. a) Describe the processes organogenesis and somatic embryogenesis.

**OR**

b) Write an account on induction of callus cultures.

10. a) What is micropropagation? Explain the stages of micropropagation and its application.

**OR**

b) How can protoplasts be useful for production of somatic hybrids and cybrids? Discuss.

11. (a) Describe the production of transgenic plants for viral resistance.

**OR**

(b) How can transgenic plants be enhanced for vitamin A and E nutritive values? Discuss.