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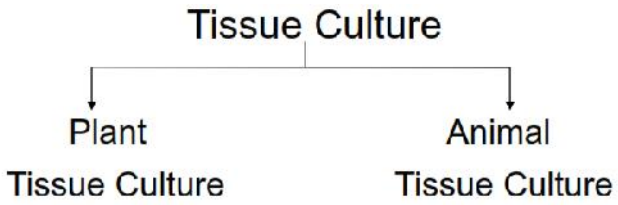
# UNIT III- PLANT TISSUE CULTURE

**Syllabus**

Historical development of plant tissue culture,types of cultures, Nutritional requirements, growth and their maintenance. Applications of plant tissue culture in pharmacognosy. Edible Vaccines




**DEFINITION:**

Plant-tissue culture is in-vitro cultivation of plant cell or tissue under aseptic and controlled environment conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant. In other words it is an experimental technique through which a mass of cells (callus) is produced from an explants tissue. The callus produced through this process can be utilized directly to regenerate palntlets or to extract or manipulate some primary and secondary metabolites.

The plant tissue culture refers to the cultivation of a plant cell which normally forms multicellular tissue. When grown on agar medium, the tissue forms a callus or a mass of undifferentiated cells. The technique of cell culture is convinient for starting and maintaining cell lines, as well as, for studies pertaining to organogensis and meristem culture.

**FIELDS OF TISSUE CULTURE:**

**AGRONOMICAL**

- Rapid multiplication of selected plants by the multiple production of plants identical to original plants can be done

- New plant obtained is different from original & more efficient according to certain defined criteria.
- Production of high-yielding, herbicide, drought, insect resistant and salt resistant crops.

**INDUSTRIAL :**

- Production of known molecule, using biosynthetic capacities of plant cells breed in a bioreactor
- An innovative aspect, employing the new source of variability accessible in vitro to obtain new molecules.

**ADVANTAGES OF TISSUE CULTURE**

**1. Availability of raw material**

Some plants are difficult to cultivate and are also not available in abundance and tissue culture technique is considered a better source for regular and uniform supply of raw material for medicinal plant industry for production of phytopharmaceuticals.

**2. Fluctuation in supplies and quality**

The method of production of crude drugs is variable in quality due to changes in climate, crop diseases and seasons. All these problems can be overcome by tissue culture.

**3. New methods for isolation**

It is possible to obtain new methods for isolation and newer compounds from plant by this technique and for which Patent rights can be obtained.

**4. Biotransformation (Biochemical Conversion)**

It is a process through which the functional group of organic compounds are modified by living cells.

Substrate ⟶ Chemically different product

**Chemical conversion by living cell culture**

This process can be done by using microorganism or plant cell suspension, hairy root culture and immobilized cell. Biotransformation by plant cell cultures yield a wide range of reactions, such as glycosylation, glucosyleserification, hydroxylation, oxido-reductions, hydrolysis, epoxidation, isomerisation, methylation, demethylation and dehydrogenation etc. It not only increases the

yield but also very economical for commercial production. Few examples of biotransformation are as follows:

| **Class** | **Substrate** | **Product** | **Plant** |
|---|---|---|---|
| phenolics | resorcinol | glucoside | *Datura innoxia* |
| Steroids | Digitoxin | digoxin | *Digitalius Lanata* |
| terpenoids | Citral | Citrol | *Lavandula angustifolia* |

**5. Genetic transformation (Transgenic plant)**

The plants obtained through genetic engineering contain a gene usually from an unrelated organism, such genes are called transgenes, and the plants containing transgenes are called as transgenic plants.

Genetic transformation can be defined as the transfer of foreign genes (DNA) or the recombinant DNA isolated from plants, viruses bacteria into a new genetic background.

The targeted cells for gene transformation are cultured cells or protoplast, meristem cells from embryos, pollens, zygote and cells from immature embryos, shoots and flowers.

**Application**

- Genes have been successfully transferred to many crops for resistance to various biotic stresses
- Genes resistant to abiotic stresses like herbicide resistance
- Resistance against viral infection
- Gene transfers to improve quality of food products
- Male sterility and fertility restoration in transgenic plants
- Transgenic plants have both basic and applied role in crop improvement

**E.g.** Tobacco, tomato, soybean, Satavari, papaya, liquorice, neem etc.

**6. Micropropagation (Clonal propagation)**

Micropropagation or Clonal propagation is a field dealing with the ability to regenerate plants directly from explants or from a single individual by asexual reproduction, constitute a clone.

It is defined as True-to-type propagation of selected genotypes using *in vitro* culture techniques.

Vegetative method of propagating plant is termed as micropropagation or cloning tissue culture or growing *in vitro*.

**Advantages** of this method is rapid multiplication of superior clones, maintenance of genetic uniformity, high yielding crops of the desirable characters in a short period of time, multiplication of sexually derived sterile hybirds and improvement of plant by developing virus-free, insect-resistant, disease–resistant, herbicide-resistant plant.

**Example:** Fennel is genetically heterozygous and produces wide variation in oil yield and composition. Also various plants like Garlic, Brahmi, Vinca, Eucalyptus, Gymnema, Liquorice etc. are propagation by this technique.

**7. Cryopreservation:**

The preservation of cell, tissue and organs in liquid nitrogen is called cryopreservation and the science pertaining to this activity is known as cryobiology. Cryopreservation is the non-lethal storage of biological material at ultra low temperature. At the temperature of liquid nitrogen (-196ºC) almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods. However, only few biological materials can be frozen to (-196ºC) without affecting the cell viability. Cryopreservation of few endangered medicinal plants e.g. Dioscorea, Chirata, Podophyllum etc. is done these days.

**8. Tracing the biosynthetic pathways of secondary metabolites**

Tissue culture can be used for tracing the biosynthetic pathways of secondary metabolites using labelled precursor in the culture medium.

9. **Generation novel compounds from plant**

By various methods of plant tissue culture isolation of novel compound and improvement in yield of the existing compound can be achieved.

10. Useful natural compounds can be produced, independent of soil condition & change in climatic conditions.

11. Improvement of medicinal plant species.

12. Propogation of plant without seeds in defined and controlled condition.

**Disadvantages of tissue culture**

1. High level of expertise is required.
2. A small error may lead to complete collapse of product/plant.
3. Lots of chemicals are required for plant tissue culture which must contain high purity.
4. There is no chance for evaluation of mutation.

5. Culture on artificial medium may lead to the depression of unusual metabolic pathways, which may not be beneficial to biotechnologist.

6. In majority cases amount of secondary metabolites produced is negligible.

7. The protocols for individual plants differ very widely and Change in the medium constitution & environmental parameters affect the rate of cell growth & accumulation of secondary metabolites.

8. To maximize on the cell mass produced the cell suspension culture eventually becomes very dense and these presents problems of even aeration.

9. Instability

10. Slow growth

11. Expensive process

12. Aseptic conditions are to be maintained through out the growth of plant.

**HISTORICAL DEVELOPMENT OF PLANT TISSUE CULTURE**

The principles of tissue culture were involved 1838-1839 in cell theory advanced by Schleiden and Schwann.

| Year | Worker | Advancement |
|---|---|---|
| 1902 | **Haberlandt** | First attempt of *in-vitro* culture of plant cell |
| 1904 | **Hannig** | Culture of embryogenic tissue of crucifers |
| 1922 | **Robbins** | *In-vitro* culturing of roots |
| 1925 | **Laibach** | Zygotic embryo culture in *Linum* |
| 1934 | **White** | Culture of roots of tomato plant |
| 1939 | **Gautheret, White and Nobecourt** | Successful establishment of indefinite callus culture |
| 1941 | **Braun** | Culture of Crown Gall Tissues |
| 1945 | **Loo** | Cultures from stem tip |
| 1955 | **Miller** | Hormone Kinetin discovered |
| 1957 | **Skoog, Miller** | Discovered that Auxin : Cytokinin ratio regulates the organ formation |
| 1960 | **Bergmann** | Development of Plating technique for isolation of single cell |
| 1970 | **Power** | Successful Protoplast fusion |
| 1970 | **Maheshwari and Guha** | Successful Anther Culture |
| 1971 | **Takabe** | Plants regenerated from protoplasts |
| 1974 | **Reinhard** | Biotransformation in plant tissue culture |
| 1978 | **Melchers** | Production of somatic hybrid *Pomato* |

**BASIC REQUIREMENTS OF PLANT TISSUE CULTURE:**

•Plant material

•Equipments and Glasswares

•Aseptic Condition

•Washing and storage facilities

•Media preparation room

•Sterilization room

•Nutrient medium

•Transfer room

•Culture room or incubators

•Proper and optimum aeration

•Well equipped observation or recording area

1. **Plant material**

The plant material should be disease free and should not be to old. Also the particular species/variety/genotype which are used should be the right one. Generally *in-vitro* germinated seedlings are frequently chosen as seed is often also much more readily sterilized than softer plant tissues. When plants are healthy and at the desired stage for use, it is often the case that only a specific part of these plants will give the best explants. E.g. A particular internode, the youngest fully expanded leaf etc.

**Equipments and Glasswares**

**Incubating chamber or laminar airflow cabinet** with UV light fitting for aseptic transfer. Incubator with temperature control ± 0.5ºC generally temperature recommended for most tissue culture studies is 36ºC.

**Autoclave**-for sterilization of glassware, media etc.

**Refrigerators and freezers**-For storage of reagents, tissue culture stock solutions, chemicals etc.

**Hot air oven**-for dry sterilization of glassware, media etc.

**Microscope**-Simple and special microscope with a provision to take camera are required. The stage of this microscope should be large enough to accommodate large roller bottles in specific cases.

**pH meter**- for adjusting the pH of the medium

A spirit burner or gas micro burner for flame sterilization of instruments

**Washing up equipments**- Washing facilities for glassware, pipette etc. in deep soaking baths or washing sinks of stainless steel or polypropylene are suitable for manual washing and rinsing of almost all types of glassware except pipettes. Standard siphon type pipette washers are suitable for washing the pipettes soaked in detergent for overnight. The washed pipettes should be rinsed with deionised water and dried in a stainless steel pipette dryer.

**Water purifier**- Pure water is required at most of the plant tissue culture study.

**Centrifuge-** To increase the concentration of cell suspension culture

**Shakers**- To maintain cell suspension culture

**Balance**- To weigh various nutrients of the preparation of the medium

**Shelves**- Build from rigid wire mesh to allow maximum air movement and minimum shading should be used in the culture room.

**Scissors, scalpels and forceps**- For explant preparation from excised plant parts are for their transfer

**Culture vessels**- Usually borosilicate glass vessels are preferred, it includes test tubes, conical flasks, bottles, special flat tubes etc. Now, the common vessels are 100 ml conical flasks or large test tubes of 25× 150 mm size.

**Glasswares**- Like measuring cylinders, beakers, funnels, petri dishes, graduated pipette, conical flask etc. Are required for preparation of nutrient media.

**Miscellaneous**-Non absorbent cotton plug, screw cap or polyurethane foam is required to close the mouth of the culture vessel. Aluminium foil is required to cover the exposed part of plug from becoming wet when autoclaved. Labels, marking pencils, hand lens, plastic disposables like syringes, plastic bottles, hot plate, stirrer etc.

**Microwave-** not essential but it melts the solidified media for pouring in culture vessels like petri dishes etc.

**Aseptic Condition**

•The plant materials (tissues), equipments, culture media and the room should be free from microorganisms. Usually dry heat, wet heat, ultrafiltration and chemicals are used for the sterilisation process. Surface sterilisation of plant materials such as seed, fruit, stem, leaf etc. by agents like

9-10% calcium hypochlorite for 5-30 minutes

2% sodium hypochlorite solution for 5-30 minutes. The materials need to be washed thoroughly in double-distilled water, after sterilising in these solutions.

10-12% of hydrogen peroxide solution for 5-15 minutes.

1-2% bromine water, for 2-10 minutes

1% solution of chlorine water, mercuric chloride, silver nitrate or antibiotics etc. can also be used.

Absolute alcohol is used for hard tissues

- Dry heat method is used for sterilisation of equipments in hot air oven.
- Sterilisation of equipment with chromic acid-sulphuric acid mixture, hydrochloric acid, nitric acid strong detergent solution, alcohol, incubator or autoclaves etc. are use for this purpose.
- Wet heat method is used for sterilisation of glassware, culture media in autoclave at 121°C and 15 lb pressure for 15 minutes.
- Ultrafiltration is used for sterilisation of liquid media which are unstable at high temperature.
- Antibiotics are added to medium to prevent the growth of the microorganisms e.g. Potassium benzyl penicillin, streptomycin sulphate, gentamycin etc.
- Chemicals like alcohol are used for sterilisation of working area and the instruments.
- Sterilisation of the environment is done by fumigation method, the inoculation chamber is generally laminar airflow cabinet is widely used these days.

**Washing and storage facilities**

Fresh water supply and disposal of waste water facility should be available. Space for distillation unit for the supply of distilled and double distilled water and de-ionized water should be available. Working table, sink or wash basin for apparatus/equipment washing should be acid and alkali resistant. Sufficient space is required for lacing hot air oven, washing machine, pipette washers etc. For storage of dried glassware separate dust proof cupboards or cabined should be provided.

**Media preparation room**

It should be spacious to accommodate lab ware, culture vessels, equipments, chemicals etc. The preparation room should also be well equipped with refrigerator, freezer etc. for storage of media and stock solutions.

**Sterilization room**

In the tissue culture lab it is desirable to have separate sterilization room for sterilization of culture media, glassware, metallic equipments like scissors, scalp etc. Generally sterilisation is done in autoclave or hot air oven.

**Nutrient medium**

Media is composed of

**Inorganic nutrients** which includes macronutrients like nitrogen, phosphorous, potassium, calcium etc. and micronutrients like boron, copper, iron, manganese, zinc etc.

**Organic nutrients** includes Vitamins like Vitamin B1, B3, B5, B6, Amino acids like L-arginine, L-asparagine, L-cysteine HCL, L-glutamine etc, Carbon source like glucose or maltose, Growth hormones/regulators like auxin, cytokinins and gibberellins, ethylene, abscisic acid.

**Others media substances** like protein hydrolysates, yeast extaracts,fruit (e.g. banana) extracts, coconut milk, solidifying agents like agar, alginate, gelatin etc., Iron source e.g.EDTA, Antibiotics.

**pH** of the medium should be in a range of 5.6-6.0 before autoclaving the culture medium

**Transfer room**

It is provided with the laminar flow hood where most of the work of culture initiation and subsequent sub culturing is performed. Culture re-plantation, transfer or re-initiation in a clean media, harvesting of 'ripe' cultures is also performed in this area.

**Culture room or incubators**

Cultures are incubated on shelves or in incubators under specific condition of temperature, humidity, air circulation and light. Incubation chamber or area should have both light and temperature controlled devices managed for 24 hours period. Generally high output, cool, white fluorescent light is preferred for a photo-period duration (specified period for total darkness as well as for higher intenesity light) with a temperature range of 25±2°C (range 18-25°C).

The rooms are required to be maintined at a relative humidity upto 70-75% (range of 20-90% controllable to ±3%) and uniform forced air circulation.

**Proper and optimum aeration**

Adequate aeration is required for cell to grow. Tissues which are cultured on semisolid media do not require any special method for aeration, but tissues which are grown in suspension cultures, require special devices for aeration.

Aeration for submerged cultures can be provided by following methods:

Placing the culture vessel with the liquid medium on an automatic shaker. The two ends of the filter paper are dipped in a medium and the middle horizontal portion on which the tissue is placed

remains above the level of the medium this method is called as filter paper bridge method.

Passing sterilised air through the medium and by stirring the medium. The culture vessels are closed with non-adsorbent cotton covered in cheese cloth. This process allows proper aeration but prevents the entry of microorganisms.

**Well equipped observation or recording area**

At regular intervals growth and maintenance of the tissue culture in the incubators should be recorded. All observations should be done in aseptic environment. For microscopic examination separate dust free space should be marked for microscopic work.

## BASIC METHODOLOGY OF TISSUE CULTURE

The general technique used in the isolation and growth of culture:

**Preparation of suitable nutrient medium**: depends on plant

**Selection of explant**: excised part of healthy plant e.g. Bud, leaf, root, seed

**Sterilization of explants**: by sodium hypochlorite, mercuric chloride etc and washed aseptically for 6-10 times with sterilised water.

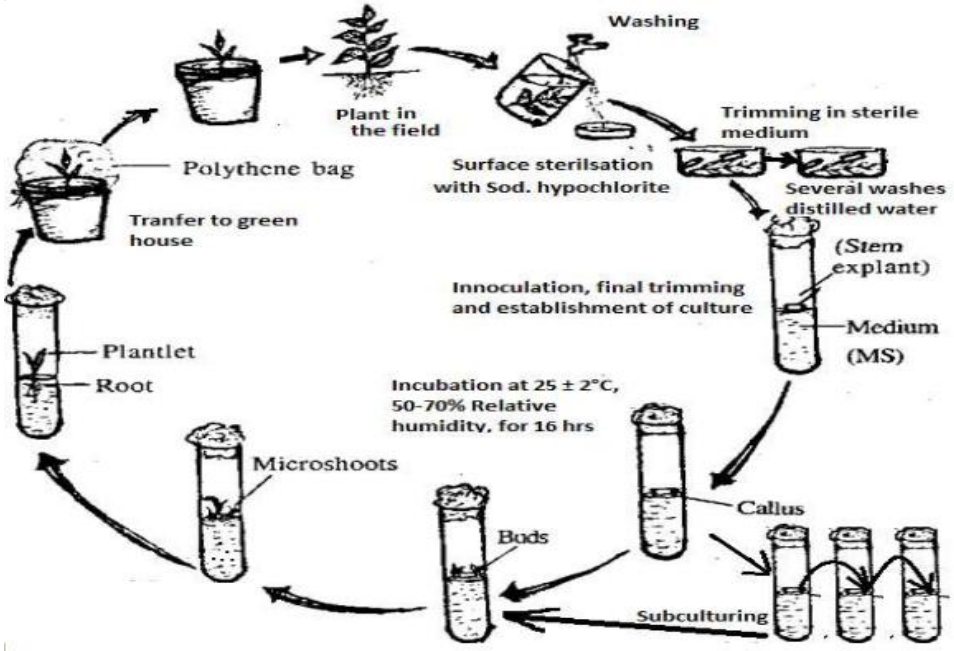
**Inoculation** (Transfer): The sterile explant inoculated on solidified nutrient medium under aseptic condition.

**Incubation**: Cultures are incubated at of 25±2°C and at RH 50-70%. 16 hrs of photo period.

**Regeneration**: Plantlets regenerated after transferring a portion of callus into another medium and induction of roots and shoots

**Hardening**: Is the gradual exposure of plantlets for acclimatization to environment condition

**Plantlet transfer**: Plantlet are transferred to green house or field conditions



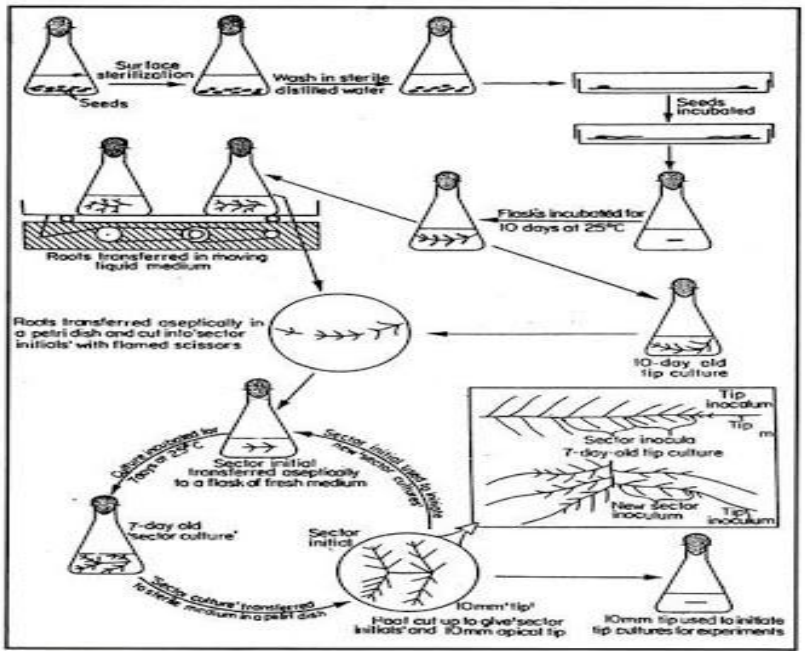

**TYPES OF PLANT TISSUE CULTURES based on part used**

**Root Tip Culture (Meristem root tip culture)**

**Function of Root apical meristem:** Cell division/differentiation/ Enlargement

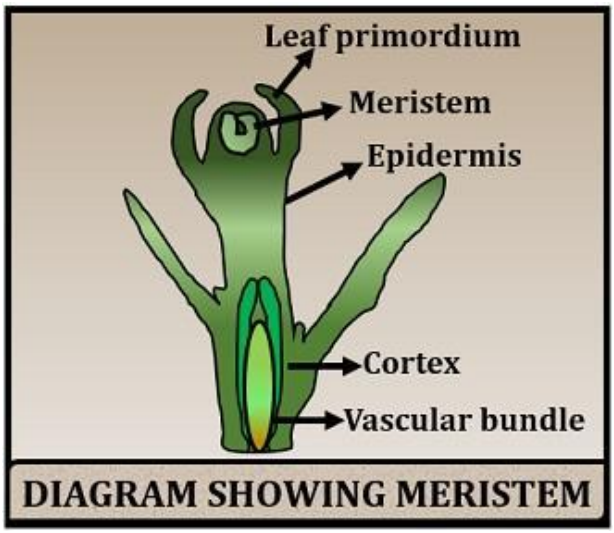
Tips of the lateral roots are sterilized, excised and transferred to fresh medium. The lateral roots continue to grow and provide several roots, which after seven days, are used to initiate stock or experimental cultures. Thus, the root material derived from a single radical could be multiplied and maintained in continuous culture; such genetically uniform root cultures are referred to as a clone of isolated roots.

Possible to study the nutritional requirements of roots, shoot and root growth, conditions required for the development of secondary vascular tissues, lateral root and bud formation, nodulation etc.




**LEAVES OR LEAF PRIMORDIA CULTURE**

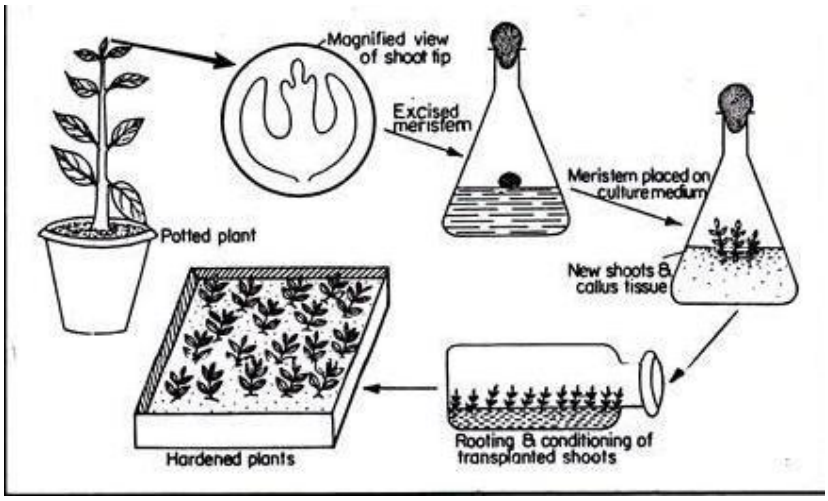
Leaves (800 µm) detached from shoots, surface sterilized **and placed on a solidified medium where they** remains in a healthy conditions for a long periods. Growth rate in culture depends on its stage of maturity at excision. Young leaves have more growth potential than the nearly mature ones.




**SHOOT TIP CULTURE**

The excised shoot tips (100–1000 µm long) of many plants species can be cultured on simple nutrient media, with growth hormones and form roots and develop into plants.

**Virus free** species: potato, sugarcane, rhubarb. Used for both monocot and dicot plants




**COMPLETE FLOWER CULTURE**

**By** Nitsch**in 1951 Culture of the** flowers of dicotyledonous species;

The flowers remain healthy and develop normally to produce mature fruits. Used to study microclimates or nutritional effects on the vegetative and reproductive processes of the plant.

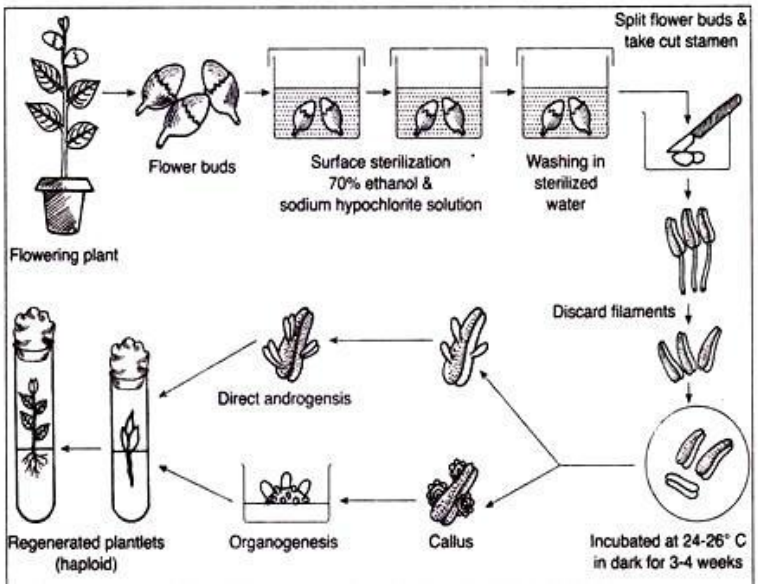
Flowers (2 days after **pollination) are excised, sterilized** by immersion in5% calcium hypochlorite, washed with sterilized water and transferred to culture tubes containing an agar medium.

The fruits that develop are smaller than natural ones, size can be increased by supplementing the medium with growth hormones.

**Anther and Pollens Culture**

- Young flower buds removed from the plant and surface sterilized.
- The anthers carefully excised and transferred to nutrient medium.• Immature stage usually grows abnormally and there is no development of pollen grains from pollen mother cells.• Anther at a very young stage (containing microspore mother cells or tetrads) and late stage (containing binucleate starch-filled pollen) of development are generally ineffective, therefore select mature anther or pollen.
- Mature anther or pollen grains of gymnosperms can be induced to form callus by spreading them out on the surface of a suitable agar media.
- Mature pollen grains of angiosperms do not usually form callus, with few exceptions.

- Pollen grains removed from the anther either mechanically or by natural dehiscence. Anthers placed in 5 ml of liq. medium in a petri dish containing pollen grains in the culture media, sealed with parafilm and incubated.
- After incubation haploid plantlets are developed.



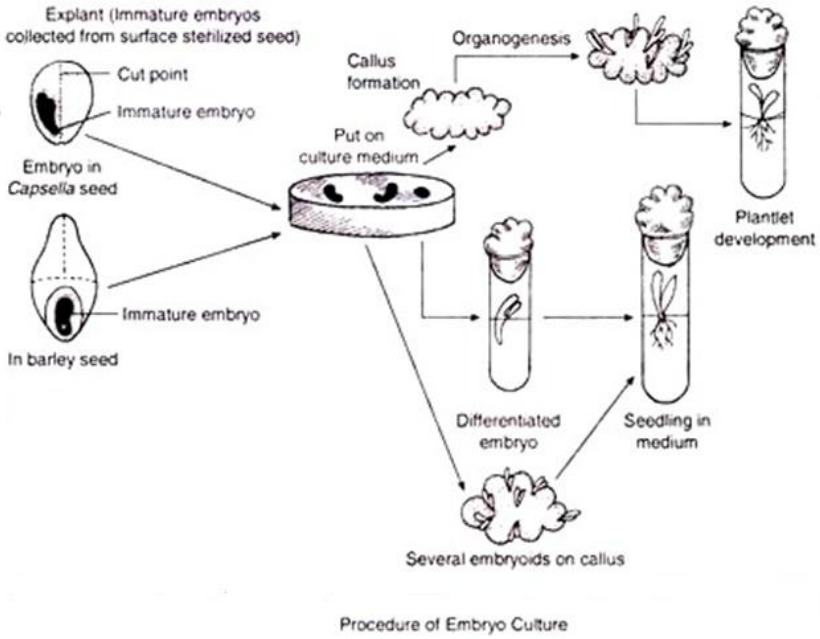

**OVULE AND EMBRYO CULTURE**

Embryo is dissected from the ovule and put into culture media. Very small globular embryos require balanced hormones. Hence, mature embryos are excised from ripened seeds and cultured to avoid inhibition in the seed for germination.

Is relatively easy, requires simple nutrient medium containing mineral salts, sugar and agar for growth and development.

The seeds treated with 70% alcohol for about 2 min, washed with sterile distilled water, treated with surface sterilizing agent for specific period, *once again rinsed with* sterilized distilled water and kept for germination by placing them on double layers of presterilized filter paper placed in petridish moistened with sterilized distilled water or placed on moistened cotton swab in petridish.

The seeds are germinated in dark at 25–28°C and small part of the seedling isutilized for the initiation of callus. Dormancy period of seeds can be shortened & production of haploids. By ovule culture, possible to grow, study various nutritional requirements and stages young embryos or zygote.




Procedure of Embryo Culture

**OVARY CULTURE**

Ovaries excised after pollination can produce fruits on a simple medium containing mineral salts, sugar and vitamins.

Ovaries taken from un-pollinated flowers fail to produce fruits on a simple medium but can develop into seedless fruits on a medium supplemented with hormones.

- By this method, physiology of fruit development can be studied.
- Haploids can be produced.
- Rare hybrids can also be produced by ovary culture.
- Dormancy period of seeds can be reduced

**SEED CULTURE**

• The seeds are treated with 70% alcohol for about two minutes, washed with sterile distilled water, treated with surface sterilizing agent for specific period.

• Once again rinsed with sterilized distilled water and kept for germination by placing them on double layers of presterilized filter paper, placed in petri-dish moistened with sterilized distilled water or placed on moistened cotton swab in petri-dish.

• The seeds are germinated in dark at 25-28°C and small part of the seedling is utilized for the initiation of callus.

**HAIRY ROOT CULTURE**

- By Steward et al. (1900).• A large number of small fine hairy roots covered with root, hairs originate directly from the explants in response to *Agrobacterium rhizogenes infection* are termed hairy roots.
- These are fast-growing, highly branched adventitious roots at the site of infection and can grow even on a hormone-free culture medium.
- Many plant cell culture systems, which do not produce adequate amount of desired compounds, are being reinvestigated using hairy root culture methods
- A diversified range of plant species has been transformed using various bacterial strains. One of the most important characteristics of the transformed roots is their capability to synthesize secondary metabolites specific to that plant species from which they have been developed.
- Growth kinetics and secondary metabolite production by hairy roots is highly stable and are of equal level and even they are higher to those of field grown plants

Hairy Root in solid and in liquid media

**ESTABLISHMENT AND MAINTENANCEOF VARIOUS CULTURES**

3 main culture systems, **selected on the basis of the objective.**

1.Growth of callus masses on solidified media (**callus culture** also known as **static culture**).

2. Growth in liquid media (**suspension culture**) consists of mixture of single cells or cell aggregates.

3. Protoplast culture: Callus culture (static tissue culture) or Suspension culture

## CALLUS CULTURE

- Callus is an amorphous aggregate of loosely arranged parenchyma cells, which proliferate from mother cells.
- Cultivation of callus on a solidified nutrient medium under aseptic conditions is known as callus culture

**A. Initiation of callus culture (SP,CM,T,I,M)**

1. **Selection and preparation of explant**: organ or culture is selected such as segments of root or stem, leaf primordia, flower structure or fruit, etc.

Excised part washed with tap water sterilized → 0.1% $HgCl_2$ or 2% NaOCl,15 min. → detergent to sterilization solution to reduce water repulsion → wash with sterile glass D water → cut to small segments (2-5mm) aseptically.

***2.*** **Selection of culture medium: Depends on species of plant & Objective**

**Well-defined nutrient medium**- inorganic and organic nutrients and vitamins.

MS Medium : has ↑ conc. Of $NO_3$, K, $NH_4$ ions → useful for dicot tissues

Growth **hormones (auxin, cytokinin) :** Auxins, IBA and NAA **f**or rooting + cytokinin for shoot proliferation.

2, 4-D and 2, 4, 5-T for good growth of the callus culture. Favourable for monocot tissues or explant. Selected semisolid nutrient is prepared. pH of the medium (5.0–6.0)

Pour into culture Vessels (15 ml for 25 x 150 mm culture tubes or 50 for 150 Ml flasks) plugand sterilize by autoclaving.

**3. Transfer of explant**

Surface sterilized organs (explant) → into vessel (semisolid culture medium)

**4. Incubation of culture**

Inoculated vessels → BOD incubator, Incubate at 25–28°C, light and dark cycles for 12-h duration.

Nutrient medium is supplemented with auxin to induce cell division.

After 3-4 weeks → callus (size of callus gets five times the size of the explant)

Commercially important secondary metabolites can be obtained from static culture by manipulating the composition of media and growth regulators (physiological and biochemical conditions), but on the whole it is a good source for the establishment of suspension culture.

**Callus is formed through three stages of development**

- Induction , Cell division and Cell differentiation ***(ICC)***

***1. Induction***

metabolic activities of the cell increase; cell accumulates organic contents and finally divides into a number of cells.

The length of this phase depends- functional potential of the explant and the environmental conditions of the cell division stage.

***2. Cell division***

This is the phase of active cell division as the explant cells revert to meristematic state.

***3. Cell differentiation***

This is the phase of cellular differentiation, i.e. morphological and physiological differentiation occur leading to the formation of secondary metabolites.

5. **Maintenance (Sub culturing)**

- After sufficient time of callus growth on the same medium → Depletion of nutrients, loss of water, *a*ccumulation ***of metabolic toxins.***
- To maintain of growth in callus culture → sub-culture of callus in fresh medium.
- Healthy callus tissue of sufficient size (5–10 mm in diameter) and weight 20–100 mg) is transferred under aseptic conditions to fresh medium, sub-culturing repeated after 4-5 weeks.
- Many callus cultures remain healthy & grow at slow rate for longer period without sub-culturing also if incubation is done at low temperature (5–10°C)
- Normally, total depletion takes about 28 days.

**CALLUS CULTURE- COLOR OF CALLUS**

- White: If grown in dark due to the absence of chlorophyll
- Green: If grown in light
- Yellow: Due to development of carotenoid pigments in greater amounts
- Purple: Due to the accumulation of anthocyanins in vacuole
- Brown: Due to excretion of phenolic substance and formation of quinones

## SUSPENSION CULTURE

- Contains a uniform suspension of separate cells in liquid medium.
- To prepare suspension culture, callus fragments → to liquid medium (without agar) → agitated in rotary shaker (50-150 rpm) to keep the cells separate → sufficient number of cells → subculturing in fresh liquid medium.
- Single cells can also be obtained from fresh plant organ (leaf).
- ***Initiation of suspension culture***

**(a) Isolation of single cell from callus culture:**

**Healthy** callus tissue → petridish on a sterile filter paper , cut to pieces with sterile scalpel → Selected piece of callus 300–500 mg → into flask with 60 ml of liquid nutrient media no gelling agent → agitation at 50–150 rpm to separate cells → Decant medium, resuspend residue by slowly rotating the flask → transfer 1/4th of the entire residue to fresh medium, followed by sieving the medium to get uniformity of cells.

**(b) Isolation of single cell from plant organ:**

**From the** plant organ (leaf tissue) single cell isolation:

- Mechanical method
- Enzymatic method

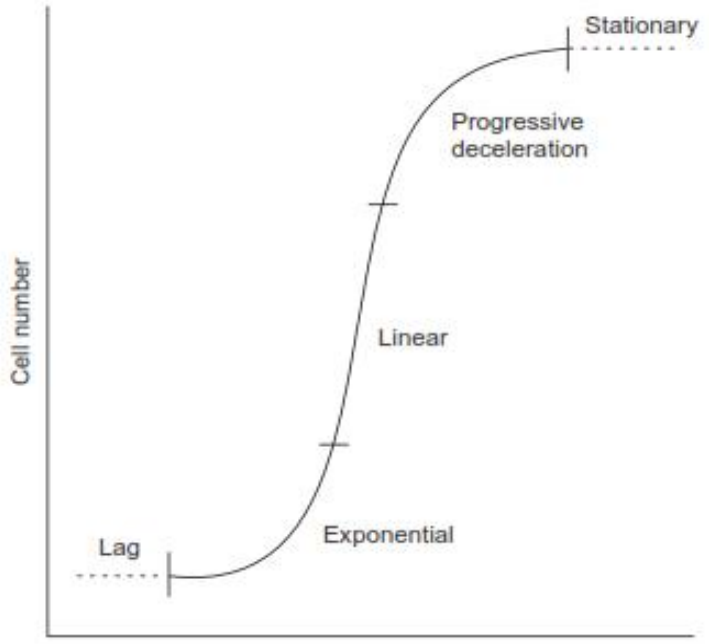
**Mechanical method:**

**surface sterilized fresh leaves →** grinded in (1:4) (20 µmol sucrose; 10 µmol $MgCl_2$ , 20 µmol tris-HCl buffer, pH 7.8) in glass pestle mortar → homogenate → passed muslins cloth → washed with sterile D $H_2O$ → centrifuged with culture medium → sieved → placed on culture dish for inoculation

**Enzymatic method:**

Leaves from 60- to 80-day-old plant → sterilized in 70% ethanol → in hypochlorite solution → washed sterile DD water → on sterile tile, peel off lower surface with sterile forceps → cut into small pieces (4 cm) → Transfer (2 g leaves) to flask (100ml) containing 20 ml filtered sterilized enzyme solution (macerozyme 0.5% solution, 0.8% mannitol and 1% potassium dextran sulphate)→ Incubate at 25°C for 2 h (change the enzyme solution with the fresh one at every 30 min) → wash the cell twice with culture medium → place them in culture dish.

**CURVE SHOWING THE GROWTH PATTERN IN THE SUSPENSION CULTURE**




1. **Lag phase:** Period where the cells adjust themselves to the nutrient medium and undertake all the necessary synthesis prior to cell division.
2. **Logarithmic phase or exponential phase:** Very rapid cell division, logarithmic increase in cell number
3. **Linear phase :** Rapid cell division results in a linear increase in number
4. **Stationary phase** : As nutrients are depleted and some of the cells of the culture being to show senescent characteristics, the rate of cell division within the culture declines and it passes through the stationary phase.

**PARAMETERS FOR MEASURING GROWTH OF CULTURED CELLS**

*1.* ***Cell Fresh weight****:* can be determined by collecting cells on a pre-weighed (in wet condition) circular filter of nylon fabric supported in a funnel, washing the cells with water to remove the medium, draining under vacuum, and reweighing.

2. **Cell *Dry weight****:* pre-weighed dry nylon filter and after collecting the cells on the filter dry them for 12 h at 60°C and reweigh. Cell weight is expressed as per culture or per $10^6$ cells.

*3.* ***Packed cell volume (PCV).*** *T*ransfer a known volume of uniformly dispersed suspension to a 15-ml graduated centrifuge tube and spin at 200 rpm for 5 min. PCV is expressed as ml pellet/ m1 culture.

4. ***Cell counting****:* cell colonies are of various sizes. Specific procedure is followed. 1 volume of culture + 2 volumes of 8% chromic trioxide, heat to 70°C for 2-15 min. Cool, and shake vigorously for 10 min before counting the cells in a haemocytometer.

**ASSESSMENT OF VIABILITY OF CULTURED CELLS**

1. ***Phase contrast microscopy****:* based on cytoplasmic streaming and the presence of a healthy nucleus

*2.* ***Reduction of tetrazolium*** *salts.* respiratory efficiency of cells is measured by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazan. Formazan can be extracted and measured spectrophotometrically.

3. ***Fluorescein diacetate (FDA) method****:* Stock solution of FDA at a concentration of 0.5% prepared in acetone, stored at 0°C.

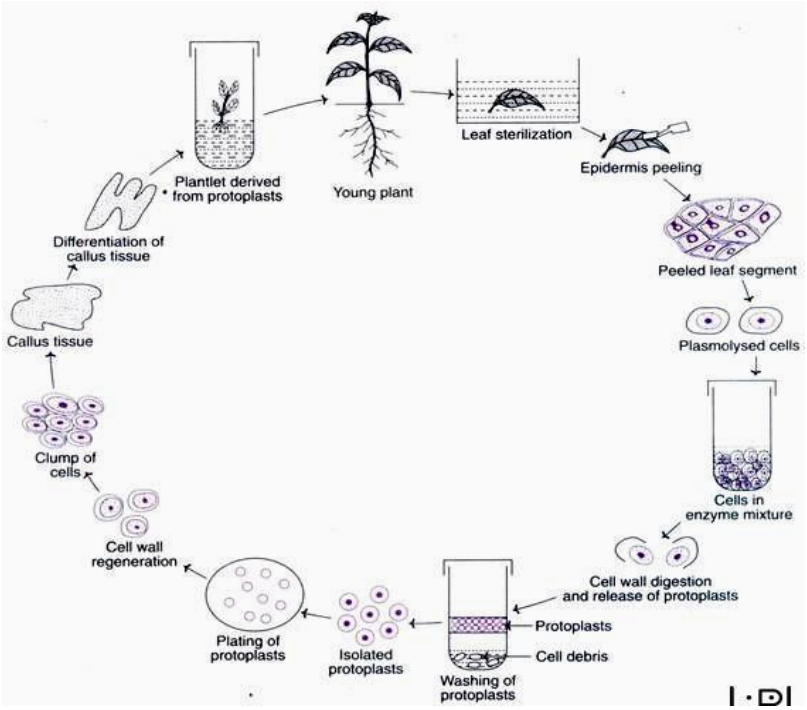
To test viability, add to the cell or protoplast suspension at a final concentration of 0.01%. Incubate for 5 min, examine the cells under mercury vapour lamp.

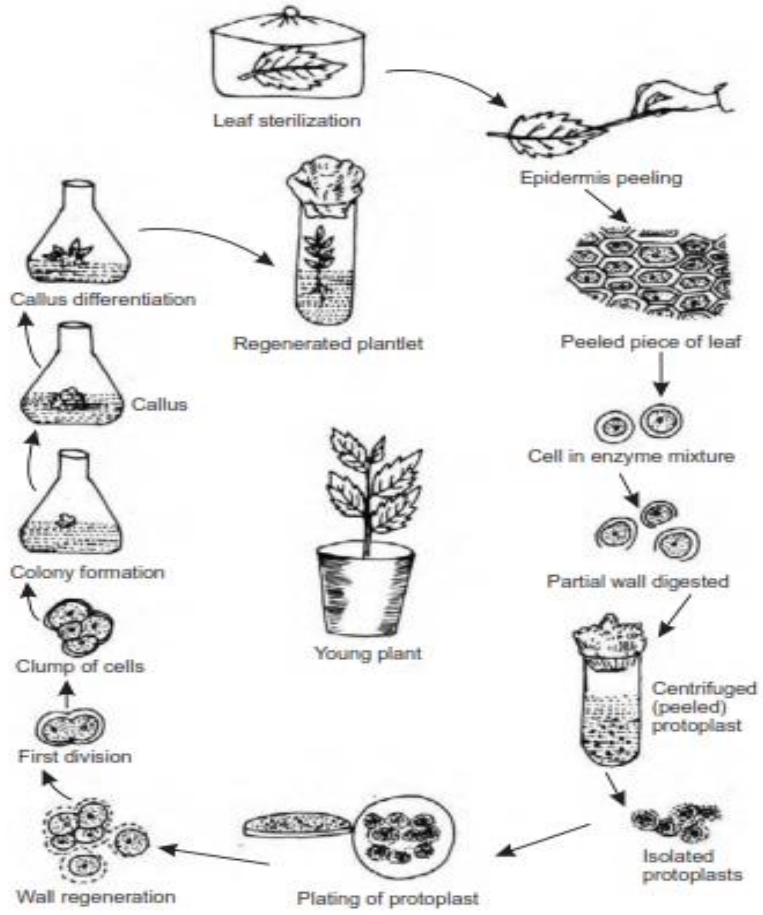
FDA is non-fluorescing and non- polar, and freely permeates across the plasma membrane. Inside the living cell it is cleaved by esterase activity, releasing the fluorescent polar portion fluorescein. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, but in dead and broken cells it is lost. When illuminated with UV light it gives green fluorescence.

**PROTOPLAST CULTURE**

- Protoplasts are the naked cells of varied plant origin without cell walls, which are cultivated in liquid as well as on solid media.
- Protoplasts can be isolated by mechanical or enzymatic method from almost all parts of the plant: roots, tubers, root nodules, leaves, fruits, endosperms, crown gall tissues, pollen mother cells and the cells of the callus tissue but the most appropriate is the leaves of the plant.
- Fully expanded young leaves from the healthy plant are collected, washed with running tap water and sterilized by dipping in 70% ethanol for about a minute and then treated with 2% solution of sodium hypochlorite for 20–30 min, and washed with sterile distilled water to make it free from the trace of sodium hypochlorite.

- The lower surface of the sterilized leaf is peeled off and stripped leaves are cut into pieces (midrib).
- The peeled leaf segments are treated with enzymes (macerozyme and then treated with cellulase) to isolate the protoplasts.
- The isolated protoplasts cleaned by centrifugation and decantation method.
- Then the protoplast solution of known density (1 × 105 protoplasts/ml) is poured on sterile and cooled down molten nutrient medium in petridishes.
- Mix the two gently by quickly rotating each petridish. Allow the medium to set and seal petridishes with paraffin film.
- Incubate the petridishes in inverted position in BOD incubator.
- The protoplasts, which are capable of dividing undergo cell divisions and form callus within 2–3 weeks. The callus is then sub-cultured on fresh medium.
- Embryogenesis begins from callus after transferring to a medium with auxin and cytokinin, where the embryos develop into plantlets which may be transferred to pots






**NUTRITIONAL REQUIREMENTS :**

- ✓ vary with the species, Trial and error basis
- ✓ Gautheret (1942), White (1943), Haberblandt etal. (1946), Haller (1953), Nitsch and Nitsch (1956), Murashige and Skoog (1962), Eriksson (1965) and B5 (Gamberg et al., 1968)
- ✓ To maintain the vital functions of a culture, the basic medium consisting
- inorganic nutrients (macronutrients and micronutrients)
- organic components (amino acids, vitamins),
- growth regulators (phytohormones)
- utilizable carbon (sugar) source
- gelling agent (agar/phytogel)
- ✓ inorganic nutrients (macronutrients and micronutrients)

- ***Macronutrients:*** *The macronutrients include six major* elements: N, P, K, Ca, Mg and S as salts. Concentration of Ca, P, S  Mg 1–3 mmol/l, N 2–20 mmol/l.
- *Micronutrients: required in trace qty.* but essential, B, Cu, Fe, Mn, Zn and Mo. In addition, Co, $I_2$ and Na.

✓ ***Organic nutrients***

- *Nitrogenous substances:* thiamine (vitamin B) pyridoxine (vitamin B6), nicotinic acid (vitamin B3) and calcium pentothenate (vitamin B5) and ionositol
- Complex nutritive mixtures of undefined composition- casein hydrolysate, coconut milk, corn milk, malt extract, tomato juice and yeast extract promotes growth
- *Carbon Source:* utilizable source of carbon: sucrose at a concentration of 2–5%. Glucose and fructose, maltose, galactose, mannose, lactose, sorbitol, starch etc. Dicotyledonous roots grow better with sucrose where as monocots do best with dextrose (glucose).

***Plant growth regulators***

- ***Auxins:*** cell division and cell growth: chemical analogues of IAA, 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin
- ***Cytokinins:*** promote cell division: zeatin and 2iP (2-isopentyl adenine) natural, synthetic analogues, kinetin and BAP (benzylaminopurine)
- ***Gibberellins:*** cell elongation, agronomically important in plant height and fruit set. GA3 being the most common.
- ***Abscisic acid:*** inhibits cell division, used to promote distinct developmental pathways such as somatic embryogenesis

✓ ***Solidifying agents for solidification of the media***

- improved oxygen supply and support to the culture growth
- agar–agar 0.8–1.0%, (Ca, Mg, K, Na and trace elements as impurities)
- Agar (Agarose) resistant to enzymatic hydrolysis

✓ pH of the medium adjusted between 5.0 and 6.0 before sterilization. pH higher than 6.0 gives hard medium and pH below 5.0 does not allow satisfactory gelling of the Agar.

**METHODS TO PREPARE CULTURE MEDIA**

✓ 2 methods of preparation of media:

(i) weigh the required qty of nutrient, dissolve separately & mix at the time of medium preparation.

(ii) Prepare the stock solution separately for macro-nutrients, micro-nutrients, iron solution and organic components, store in the refrigerator till not used

e.g. Murashige and Skoog's media stock solution →

Group I: 20x concentrated solution

Group II: 200x

Group III Iron salts at 200x

Group IV organic ing. except sucrose 200x

| Stock solution ingredients | Amount (mg/L) |
|---|---|
| **Group I** | |
| $NH_4NO_3$ | 1,650 |
| $KNO_3$ | 1,900 |
| $CaCl_2 \cdot 2H_2O$ | 440 |
| $MgSO_4 \cdot 7H_2O$ | 370 |
| $KH_2PO_4$ | 170 |
| **Group II** | |
| KI | 0.83 |
| $H_3BO_3$ | 6.2 |
| $MnSO_4 \cdot 4H_2O$ | 22.3 |
| $ZnSO_4 \cdot 7H_2O$ | 8.6 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 |
| $CuSO_4 \cdot 5H_2O$ | 0.025 |
| $CoCl_2 \cdot 6H_2O$ | 0.025 |
| **Group III** | |
| $FeSO_4 \cdot 7H_2O$ | 27.8 |
| $Na_2EDTA \cdot 2H_2O$ | 37.3 |
| **Group IV** | |
| Inositol | 100 |
| Nicotinic acid | 0.5 |
| Pyridoxine HCl | 0.5 |
| Thiamine HCl | 0.1 |
| Glycine | 2 |

✓ **Stock Solution preparation** (1 or 10 mmol l-1)

- Each component - weigh, dissolve separately DD $H_2O$, stored in refrigerator till used.
- For Fe solution, dissolve $FeSO_4$ $7H_2O$ and $Na_2EDTA2H_2O$ separately in 450 ml dis. $H_2O$ by heating and stirring. Mix the 2 solutions, adjust pH to 5.5 and adjust vol. to 1 L with dis. $H_2O$

✓ **Semisolid media preparation**

- Agar and sucrose weighed, dissolved in $H_2O$ by heating on water bath.
- Req. quantities of stock solution (for 1L: 50 ml of stock solution of Group 1, 5 ml of stock solution II, III and IV group) and other special supplements are added and final volume is made up with DD $H_2O$
- After mixing well, pH of the medium is adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl.

- ✓ **Sterilization of Culture Media**

- Pack culture media, seal with cotton, cover with Al foil, autoclave at 2-2.2 atm press. At 121°C, 15-40 min (time to be fixed **from the time when temperature** reaches the required temperature). The exposure time depends on the volume of the liquid to be sterilized.

**EDIBLE VACCINES**

VACCINE

• A biological preparation that improves immunity to a particular disease. It contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe. It stimulate the body's immune system to recognize the agent, destroy it, and keep a record of it for later encounters. It reduces mortality rate caused by various organisms. It is one of the safe and effective measure to control various infectious diseases.

• A protein which acts as the vaccine, present in food and consumed as the internal composition of food is known as **EDIBLE VACCINE**

**EDIBLE VACCINE- WHY?**

• Immunization through DNA vaccines is an alternative but is an expensive approach

• Edible vaccine gives cost-effective, easy-to-administer, easy-to-store and socio-culturally readily acceptable vaccines for their delivery systems.

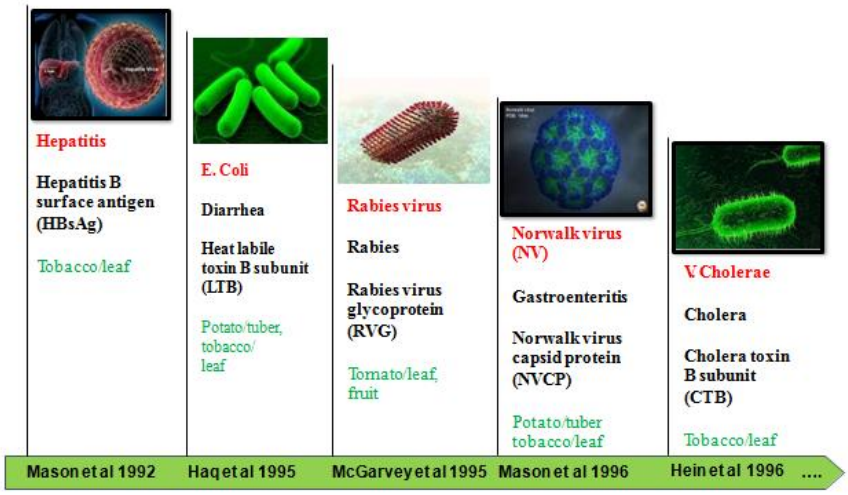
• Oral vaccines provide “mucosal immunity” at various sites by secreting antibodies.

• Don’t need to worry about re-use, misuse and lack of sterilization. Thus, low risk of infection.

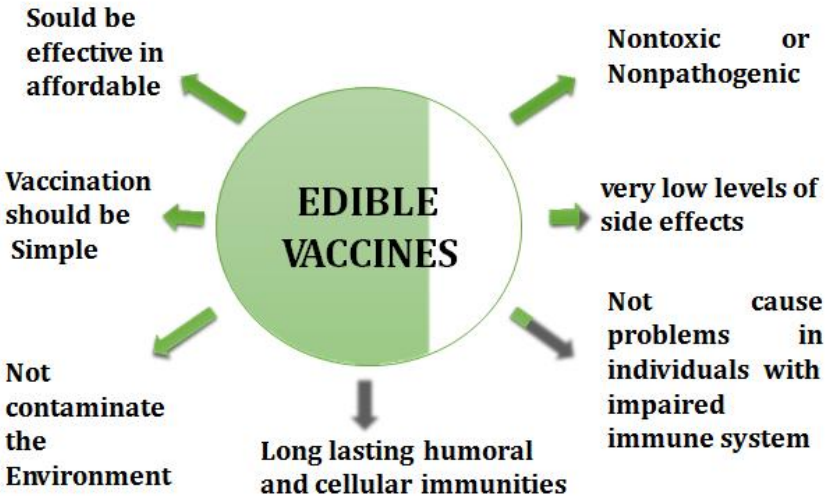
**PLANTS USED FOR EDIBLE VACCINES**

Tobacco, Potato, Banana, Tomato, Rice, Lettuce, Soybean, Alfalfa, Carrot, Peanuts, Wheat, corn ETC.

## HISTORY OF EDIBLE VACCINES



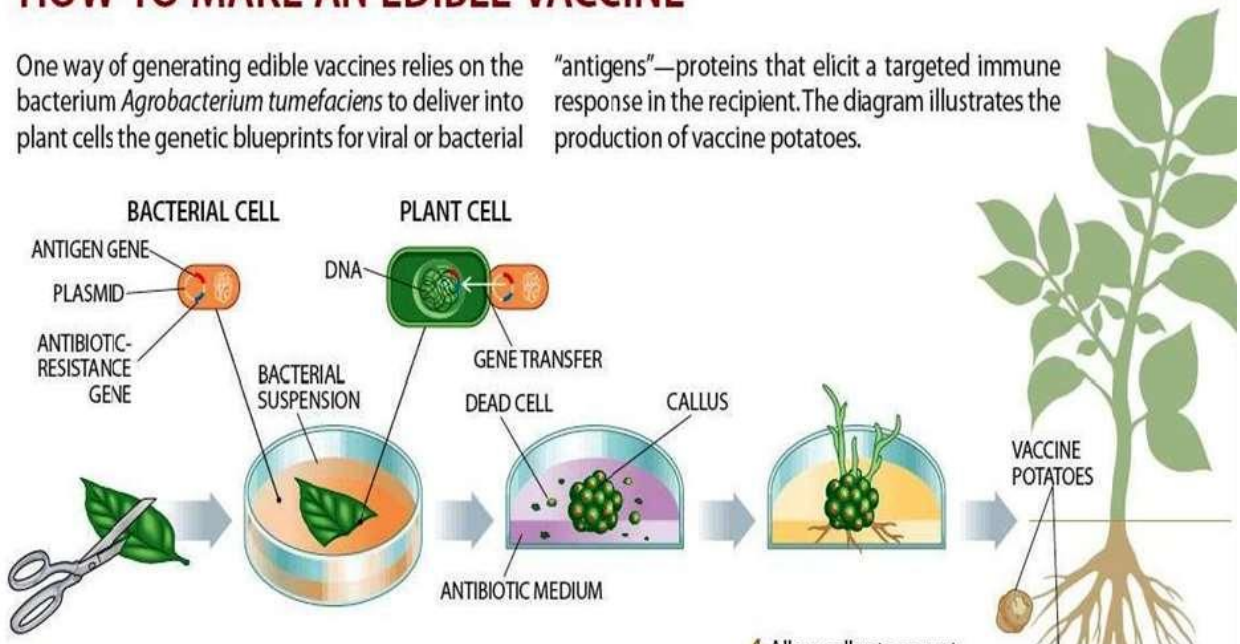

## IDEAL PROPERTIES OF EDIBLE VACCINES



| TARGET PATHOGENS | EXPRESSED IN | MODE OF ADMINESTRATION |
|---|---|---|
| Enterotoxigenic Ecoli (humans) | Potato, tobacco | Immunogenic and protective when administered orally. |
| Vibrio cholera(humans) | Potato | Immunogenic and protective when administered orally. |
| Hepatitis B virus (humans) | Tobacco | Extracted proteins is Immunogenic when administered by injection |
| Hepatitis B virus (humans) | Potato | Immunogenic and protective when administered orally. |
| Norwalk virus(humans) | Potato | Virus like particles form and Immunogenic when administered orally. |
| Rabies virus (humans) | Tomato | Intact glycoproteins |
| Foot and mouth disease (agricultural domestic animals) | Arabidopsis | Immunogenic and protective when administered orally |
| Foot and mouth disease (agricultural domestic animals) | Alfalfa | Immunogenic and protective when administered by injection or orally |
| Transmissible gastroenteritis corona virus | Maize | Protective when administered oral |

# HOW TO MAKE AN EDIBLE VACCINE

One way of generating edible vaccines relies on the bacterium *Agrobacterium tumefaciens* to deliver into plant cells the genetic blueprints for viral or bacterial "antigens"—proteins that elicit a targeted immune response in the recipient. The diagram illustrates the production of vaccine potatoes.




1 Cut leaf.

2 Expose leaf to bacteria carrying an antigen gene and an antibiotic-resistance gene. Allow bacteria to deliver the genes into leaf cells.

3 Expose leaf to an antibiotic to kill cells that lack the new genes. Wait for surviving (gene-altered) cells to multiply and form a clump (callus).

4 Allow callus to sprout shoots and roots.

5 Put in soil. Within three months, the plantlets will grow into plants bearing antigen-laden vaccine potatoes.

**Developed by Arntzen in the 1990s.**

Introduce genes of interest into plants (Transformation)

↓

Genes expressed in the plant tissues edible parts (Transgenic plants)

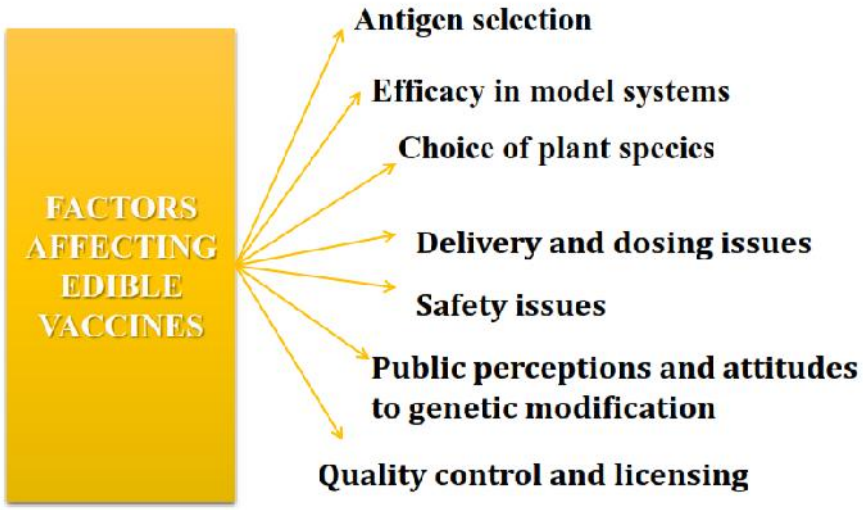
↓

Genes encode putatively protective vaccine antigens from viral, bacterial, and parasitic pathogens that cause disease in humans and animals

↓

Ingestion of the edible part of the transgenic plant (Oral delivery of vaccine)

## FACTORS AFFECTING EFFICACY OF EDIBLE VACCINES




**1. CHOLERA**

plants were transformed with the gene encoding B subunit of the *E. coli* heat liable enterotoxin (LT-B). Transgenic potatoes expressing LT-B were found to induce both serum and secretory antibodies when fed to mice; these antibodies were protective in bacterial toxin assay *in vitro*. This is the first "proof of concept" for the edible vaccine.

**2.MALARIA**

Three antigens are currently being investigated for the development of a plant-based malaria vaccine, merozoite surface protein (MSP) 4 and MSP 5 from *Plasmodium falciparum*, and MSP 4/5 from *P. yoelli*. Wang *et al* have demonstrated that oral immunization of mice with recombinant MSP 4, MSP 4/5 and MSP1, co-administered with CTB as a mucosal adjuvant, induced antibody responses effective against blood stage parasite.

**3. HEPATITIS B**

Significantly exceeded the protective level of 10 mIU/mL in humans**.** potato-based vaccine against hepatitis B have reported The amount of HBsAg needed for one dose could be achieved in a single potato.

**4. MEASLES**

Mice fed with tobacco expressing MV-H (measles virus haemagglutinin from Edmonston strain) could attain antibody titers five times the level considered protective for humans and they also demonstrated secretory IgA in their faeces. Ex: Carrot, banana and rice are the potential candidates

**5. STOPPING AUTOIMMUNITY**

The transgenic potato and tobacco plants when fed to no obese diabetic mice showed increased levels of IgG, an antibody associated with cytokines that suppress harmful immune response.

**ADVANTAGES OF EDIBLE VACCINES**

DO not require administration by injection.

Possible production of vaccines with low costs.

Do not require separation and purification of vaccines from plant materials.

Necessary syringe & needles not required.

Economical in mass production and transportation.

Heat stable, eliminating the need for refrigeration.

**DISADVANTAGE OF EDIBLE VACCINE**

Development of immunotolerance to vaccine peptide or protein.

Consistency of dosage form fruit to fruit, plant to plant, and generation to generation is not similar.

Stability of vaccine in fruit is not known.

Dosage of vaccines would be variable.

Selection of best plant is difficult.

Certain foods like potato are not eaten raw, and cooking the food might weakens the medicine present in it.

Not convenient for infants.

**SAFETY ASPECTS**

• Contamination through cross pollination.

• Vaccine antigen may affect browsing animals.

• Vaccine contamination via plant debris spreading on surfaces and ground waters.

• Affect on humans living in the area drinking vaccine polluted water or breathing vaccine polluted dust.

• Cultivation and production of pharmaceutical crops should be limited to control the production facilities like greenhouse, or in plant tissue culture, that prevent the environmental release of biopharmaceuticals.