Module-3 Plant Tissue Culture and Edible vaccines

Scope- This module includes detailed information on the topic Plant Tissue Culture and an introduction to new approach of oral immunization that is Edible vaccines.

Learning outcomes-

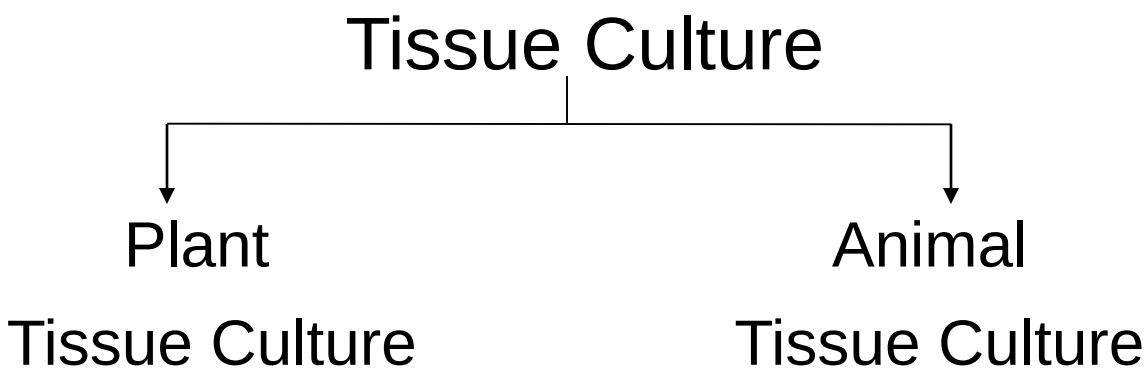
1. Student will able to learn about Plant tissue culture meaning, types, basic requirements and general procedure to culture a plant cell/ tissue/ organ.
2. Student will able to learn the basic technology to maintain a tissue culture lab and latest techniques of PTC.
3. Student will let to know about applications of PTC.
4. Student will learn about concept of edible vaccines, advantages, disadvantages, methods and applications.

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.

INTRODUCTION



- **Defination:**
- 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 explant 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 a multicellular tissue.
- When grown on agar medium, the tisse 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.

- The technique of *in-vitro* cultivation of plant cells or organs is primarily devoted to solve two basic problems:

1. To keep the plant cells or organs free from microbes

2. To ensure the desired development in cells and organs by providing suitable nutrient media and other environmental condition.

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 (Process through which the functional group of organic compound are modified by living cells) reactions are feasible using plant-cell cultures.

6. Disease free and desired propagule

Large scale production of plant with disease free and desired propagule could be stored and maintained without any damage during transportation for subsequent plantation.

7. Biosynthetic pathway

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

8. Immobilization of cells

Tissue culture can be used for plants preservation by immobilization (entrapment)of cell further facilitating transportation and biotransformation.

9 Continuous, uniform biomass is obtained.

10. Medicinally important compound can be synthesized, which can't be synthesized chemically.

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

12. Improvement of medicinal plant species.

13. 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.

1902	Haberlandt proposed concept of *in vitro* cell culture
1904	Hannig cultured embryos from several cruciferous species
1922	Kolte and Robbins successfully cultured root and stem tips respectively
1926	Went discovered first plant growth hormone –Indole acetic acid
1934	White introduced vitamin B as growth supplement in tissue culture media for tomota root tip
1939	Gautheret, White and Nobecourt established endless proliferation of callus cultures

1941 Overbeek was first to add coconut milk for cell division in Datura

1946 Ball raised whole plants of Lupinus by shoot tip culture

1954 Muir was first to break callus tissues into single cells

1957 Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation

1959 Reinert and Steward regenerated embryos from callus clumps and cell suspension of *Daucus carota*

1960	Cocking was first to isolate protoplast by enzymatic degradation of cell wall
1960	Bergmann filtered cell suspension and isolated single cells by plating
1962	Murashige and Skoog developed MS medium with higher salt concentration
1962	Kanta and Maheshwari developed test tube fertilization technique
1966	Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato

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

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 pippette 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 excies 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, strptomycin 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 B_1, B_6, B_3, B_5 etc. 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**

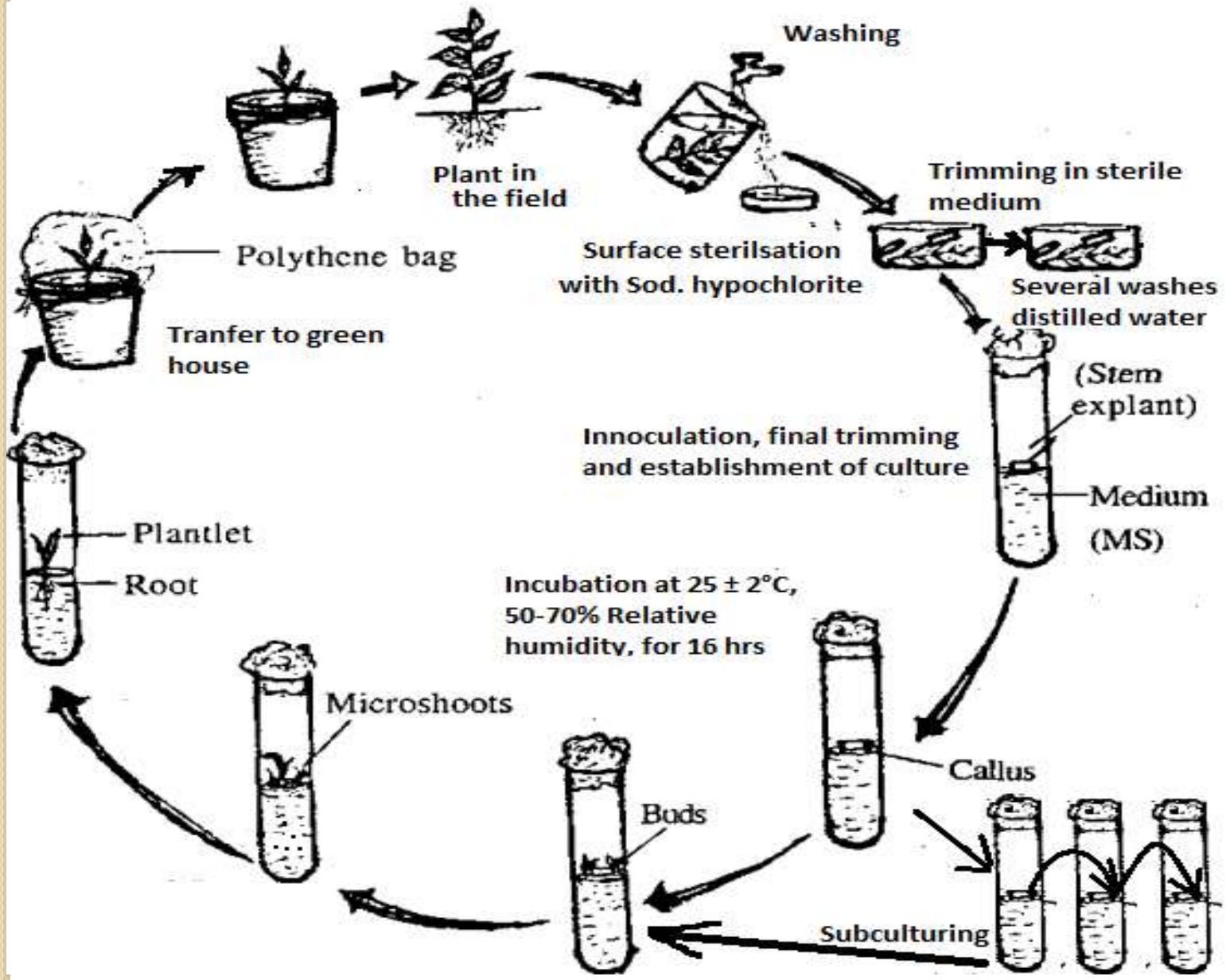
•At regular intervals growth and maintenance of the tissue culture in the incubators should be recorded. All observations should be done in aseptic environment.

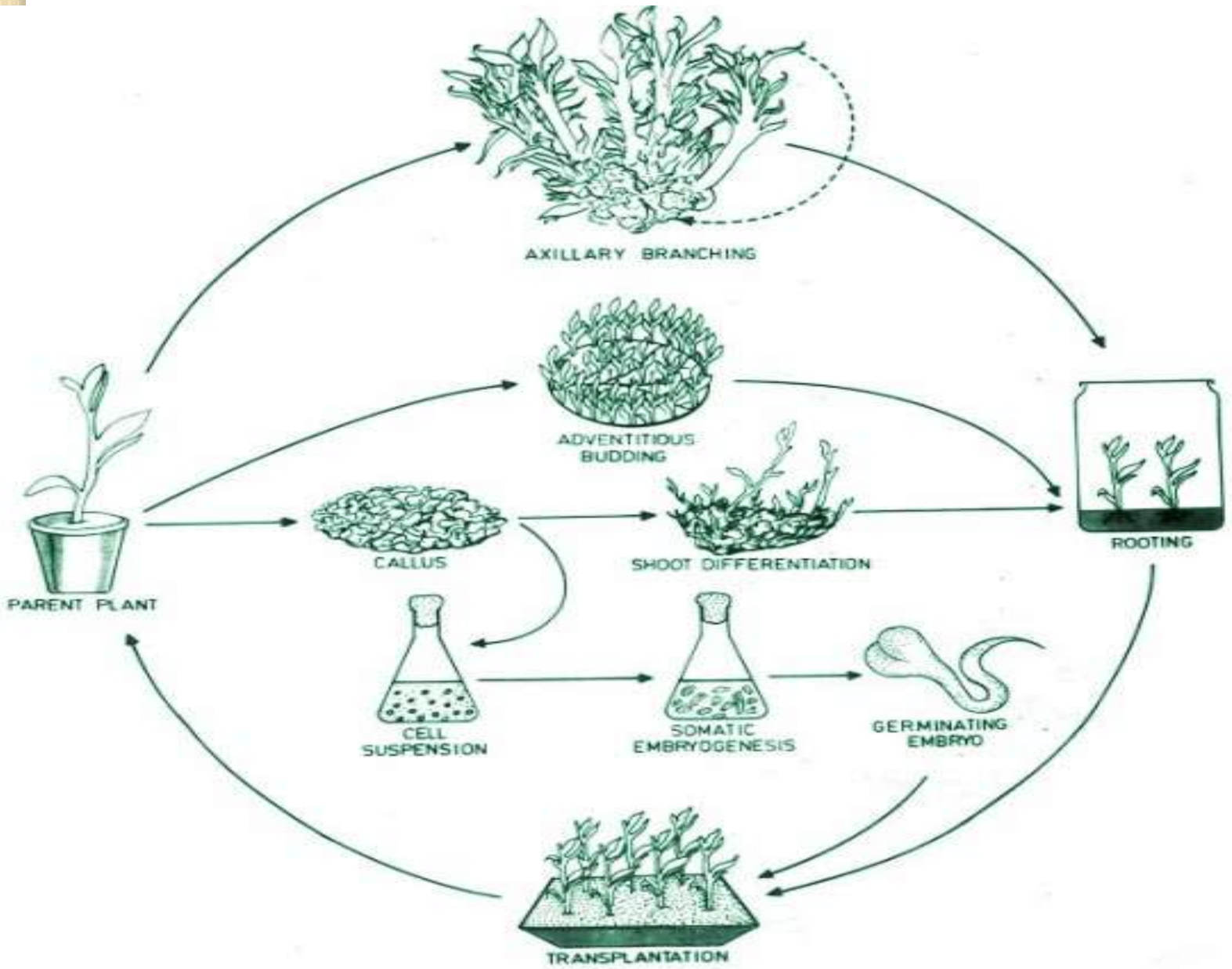
•For microscopic examination separate dust free space shold be marked for microscopic work.

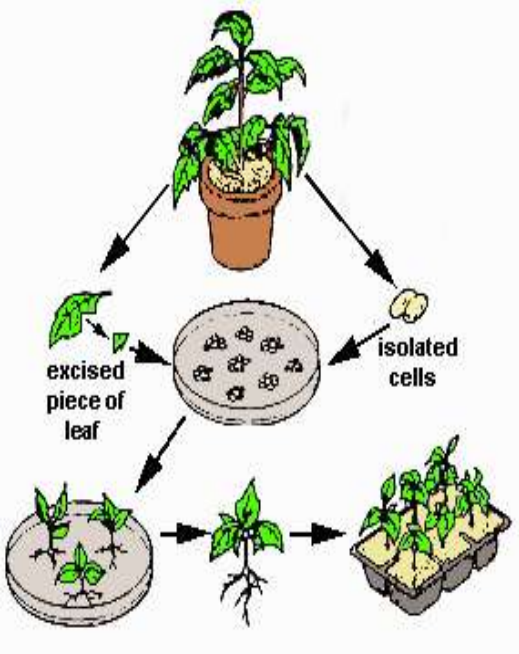
Basic Methodology/technique of Plant Tissue Culture

The general technique used in the isolation and growth of culture is described as follows:

1. Preparation of suitable nutrient medium: As per the selection of plant medium is autoclaved.
2. Selection of explant: Any excised part of health plant to be used e.g. Bud, leaf, root, seed etc.
3. Sterilisation of explants: by sodium hypochlorite, mercuric chloride etc. and washed asceptically for 6-10 times with sterilised water.
4. Inoculation (Transfer): The sterile explant is inoculated on solidified nutrient medium under asceptic condition.
5. Incubation: Cultures are incubated at of 25±2°C and at a relative humidity upto 50-70% fro 16 hrs of photo period.
6. Regeneration: Plantlets regenerated after transferring a portion of callus into another medium and induction of roots and shoots or directly from explants.
7. Hardening: Is the gradual exposure of plantlets for acclimatisation to environment condition.
8. Plantlet transfer: Plantlet are transferred to green house or field conditions.


Washing
Plant in the field
Trimming in sterile medium
Polythene bag
Surface sterilsation with Sod. hypochlorite
Several washes distilled water
Tranfer to green house
(Stem explant)
Innoculation, final trimming and establishment of culture
Medium (MS)
Plantlet
Root
Incubation at 25 ± 2°C, 50-70% Relative humidity, for 16 hrs
Microshoots
Callus
Buds
Subculturing


AXILLARY BRANCHING
ADVENTITIOUS BUDDING
PARENT PLANT
CALLUS
SHOOT DIFFERENTIATION
ROOTING
CELL SUSPENSION
SOMATIC EMBRYOGENESIS
GERMINATING EMBRYO
TRANSPLANTATION



Selection of plant

Preparation of an explant

Inoculation

After incubation

Plant ready to be transferred into green house or hardening stage

Various stages of plant growth

Regeneration of a plant from an explant

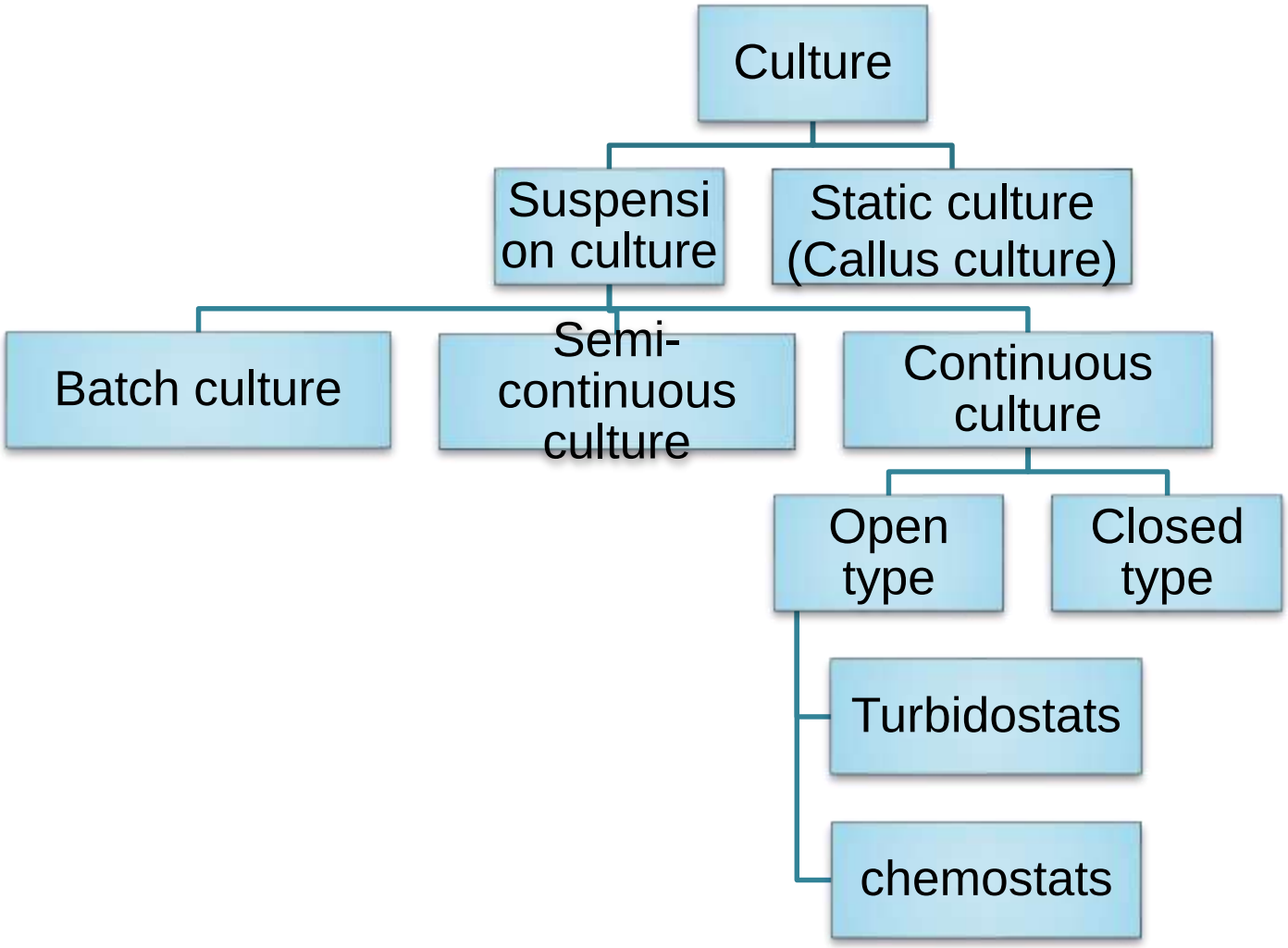
Laminar air flow

Tissue culture rack

Types of Cultures

1. Depending upon the type of medium
2. Depending on the part used for culture

1. Depending upon the type of medium



2. Depending on the part used for culture

a) Organ Culture

i. Root tip culture (Meristem - root tip culture)
ii. Shoot tip culture (Meristem - shoot tip culture)
iii. Leaves or leaf primordia culture
iv. Flower culture (Meristem - floral culture)
v. Anther and pollens culture
vi. Ovule and embryo culture
vii. Ovaries culture
viii. Nucellus culture
ix. Seed culture
x. Cotyledon culture
xi. Endosperm culture
xii. Fruit culture
xiii. Plant cell culture

b) Hairy Root Culture

c) Protoplast Culture and Somatic Hybridization

STATIC CULTURE (CALLUS CULTURE)

- **Callus** – a mass of undifferentiated plant cells grown on solid media from plant part/explant.
- It may initiate from explants of any multi-cellular plant.
- The organs such as root, stem tips, leaves, flowers and fruit are grown on solid media.
- The cell groups are initiated from:
  - Explant/Segments of root, stem or leaf either from the mature or embryogenic plant
  - Explant/Excised fragments of parenchyma or mixed tissue containing cambium or endosperm
- The longer the tissue explant the more complex the range of cell types & greater the possibilities of initiating a culture of mixed cells.
- Callus can be induced to undergo organogenesis and/or embryogenesis and eventually whole plant by providing suitable nutrient medium.
- To study the biosynthetic pathway of various metabolic processes by using tracer elements in callus culture.
- It is useful for the production of secondary metabolites.

SUSPENSION CULTURE

- It involves active proliferation of callus as undivided unit suspended or submerged in a liquid medium.
- The nutrient medium in this case is in constant agitation so as to prevent the cells from settling or aggregating in to clumps.
- Suspension cultures are normally initiated by transferring pieces of undifferentiated callus to a liquid medium which is agitated during incubation.

Batch Suspension Culture

- It means 'Culture in a fixed volume of culture medium'.
- In general, a nutrient medium and cellular inoculum are mixed, aerated and allowed to grow
- In Batch cultures, as the cells grow, the medium is depleted of nutrients and metabolic byproducts from the cells accumulate.
- Batch cultures are characterized by

  - continuous changes in the medium
  - continuous internal changes in cellular composition
  - accumulation of metabolic products.
- The system is "closed" with respect to additions or removal of culture, except for circulation of air.

Semicontinuous Suspension Culture

- Here, the system is ‘open’.
- There is periodic removal of culture and the addition of fresh medium, due to which growth of the culture is continuously maintained.

Continuous Suspension Culture

- The system is ‘open’ here also.
- In this systems, volume of culture remains constant and fresh medium is added continuously to a fixed volume of growing culture and withdrawn.

- Cell proliferation takes place under constant condition.
- This system allows
  - Establishment of steady states of growth and metabolism.
  - Study of the changes which occur in transitions from one steady state to another.
  - Identification of the controlling factors.

Two types of Continuous Suspension Culture

1. **Open type**

   Regulated new medium and balancing harvest of equal volume of culture

- **Chemostat**
- Continuous new medium input is set at a predetermined rate and determine the nature of the resulting equilibrium.
- Useful to study the steady states.
- Desired rate of growth is maintained by adjusting the level of concentration of nutrient by constant inflow of

- **Turbidostat**
- Cell density is set at a predetermined level (as monitored by the optical properties of the culture) and new medium is added to maintain cell density within limits.
- Particularly valuable for work at low cell densities.
- Offers a simplified system for study of effects of physical factors and growth regulating substance in growth rate and associated metabolism.

2. Closed type

- The used medium is replaced with fresh medium, hence, the cells from used medium are mechanically separated and added back to the culture and thus, the cell biomass keeps increasing.

CULTIVATION OF PLANT CELLS

Organogenesis

- Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds or root or even whole plantlets.

- In short, the formation of organs is called organogenesis. The *de-navo* genesis of plant organ is broadly defined as organogenesis.

- In plants, development is the process that results in a functional mature organism which includes all the events during the life of the plant that produce the body of the organism and provides the capacity to obtain food, to reproduce and deal with the hazards of its environment. Therefore, organogenesis is a process that is in some way unique to plants.

- The shoot buds are monopolar structures which in turn give rise to leaf primordial and the apical meristem.

- The stimulation of shoot bud differentiation in plants depends on many factors which differ for different plant species.

- Skoog showed that auxin could stimulate rooting and inhibit shoot formation.

- Other factors affecting organogenesis are size and source of the explant.

- Light intensity plays an important role in organogenesis.

- High light intensity has been shown to be inhibitory for shoot bud formation in tobacco.

❖Even the quality of light has effect as blue light has been shown to induce shoot formation while red light stimulates rooting in tobacco.

❖The optimum temperature required may vary with plant species.

❖A medium solidified with agar favours bud formation although there are some reports about the development of leafy shoot buds on cultures grown as suspension.

Embryogenesis

- Embryo is defined as the earliest recognisable multicellular stage of an individual that occurs before the development of characteristic organs of the given species.
- Production of embryo like structure from callus is known as embryogenesis. In higher plants such embroys usually arise from zygote formation and are termed as zygotic embryos.
- Various types of cells and tissues can be used as source of embryogenic cells. It may be microspores (1n), zygote (2n), somatic cells (2n) or somatic hybrids (4n).
- Embryogenesis can be induces in such cells by nonproliferative or proliferative direct embryogenesis or by growing embryogenic callus which in turn germinates and develops into whole plant.
- Embryogenesis can be initiated in an explant only from the more juvenile or meristematic tissues.
- Immature zygotic embryos, cotyledons and hypocotyl dissected from ungerminated seeds are common explants.
- Isolated somatic cells can develop into embroys.

- Embryo development occurs through an organised sequence of cell division, enlargement and differentiation.
- The final stages of development towards maturation are distinguished by overall enlargement and matured embryo morphology.
- Somatic embryogenesis involves three distinct steps which are absent in organogenesis

1. Induction

- It is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called embryogenic clumps (ECs).
- These ECs develop into initial stages of somatic embryo i.e. globular stage.

2. Maturation

- In this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped and the mature embryo here undergoes biochemical changes to acquire hardness.

3. Conversion

- Embryos germinate to produce seedlings.

Advantages of Organogenesis & Embryogenesis

1. Efficiency of process (reduction in labour cost and time, the formation of plantlets is fewer steps)

2. The potential for the production of much higher number of plantlets and morphological and cytological uniformity of the plantlets.

3. Production of several plants on commercial scale includes food crops, vegetables, spices, and fruits, medicinal and aromatic plants.

2. Depending on the part used for culture

a) Organ Culture

i. Root tip culture (Meristem - root tip culture)
ii. Shoot tip culture (Meristem - shoot tip culture)
iii. Leaves or leaf primordia culture
iv. Flower culture (Meristem - floral culture)
v. Anther and pollens culture
vi. Ovule and embryo culture
vii. Ovaries culture
viii. Nucellus culture
ix. Seed culture
x. Cotyledon culture
xi. Endosperm culture
xii. Fruit culture
xiii. Plant cell culture

b) Hairy Root Culture

c) Protoplast Culture and Somatic Hybridization

2. Depending on the part used for culture

a) Organ Culture

i. Root tip culture (Meristem – root tip culture)

- Root apical meristem is mainly responsible for the growth of roots by cell division, cell enlargement and cellular differentiation.
- Tips of the lateral roots are sterilised, excised and transferred to fresh medium.
- The lateral roots continue to grow and provide several roots, which after seven days, are used to experimental cultures.
- By this culture method it is 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.

ii. Shoot-tip culture (Meristem – shoot tip culture)

- The shoot apex or shoot-tip (100-1000 µm long) consists of the apical meristem and one to three adjacent leaf primordia.
- Actively growing shoot-tip is surface sterilized and is placed on a defined culture medium under sterile conditions.

- Shoot Node culture is simplified form of shoot culture, which is yet another method for production from pre-existing meristem. It is the simplest method but is associated with the least genetic variation.

- Some of the crop species that have been freed of viruses by this technique, they include soyabean, sweet potato, sugar cane and rhubarb.
- This method is used with both monocot and dicot species.

iii. Leaves or leaf primordia culture

- The growth of leaves on the culture medium depends upon the stage of the leaves during excision.
- It is observed that explants from immature young leaves grow better than explants from older leaves.
- Leaves (800 µm long) may be detached from shoots, surface sterilized and placed on a solidified medium where they will remain in a healthy conditions for a long period.
- It is believed that leaf culture depends upon the physiological state and the age of the leaf.
- The shoot forming potentials differ in the leaf cultures as per the derivation of the explant and the hormonal factors involved.

iv. Flower culture (Meristem - floral culture)

- Flowers two days after pollination are excised, sterilized by immersion in 5% calcium hypochlorite, repeatedly washed with sterilized water and transferred to culture tubes containing an agar medium.
- When cultured, such flowers produce fruits. Larger fruits are obtained on medium supplemented with growth hormones. Flowers excised before pollination do not produce fruits.
- This culture system is useful in studying microclimates or nutritional effects on the vegetative and reproductive processes of the plant.

v. Anther and pollens culture

- Young flower buds are removed from the plant and surface sterilized.
- Immature stage of anther or late stage of anther containing starch filled pollen usually grow abnormally or the development is generally ineffective and hence for better response always select mature anther or pollen.
- The anthers are then carefully excised and transferred to an appropriate nutrient medium.
- The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period.
- Anther or pollen grains of different species have been successfully culture to obtain large number of haploid plants.
- Pollen grains removed from the anther either mechanically or by naturally dehiscence. Anthers placed in 5 ml of liquid medium in a petri dish containing pollen grains in the culture media are sealed with parafilm and incubated. After incubation haploid plantlets are developed.

vi. Ovule and embryo culture

- Mature embryos are excised from ripened ovule/seeds and cultured mainly to avoid inhibition in the seed for germination. Very small globular embryos require a delicate balance of the hormones.
- Embryo is dissected from the ovule/seed and put into culture media.
- This type of culture is relatively easy as the embryos require a simple nutrient medium containing mineral salts, sugar and agar for growth and development.
- Also, multicellular immature embryos are dissected out and cultured aseptically to obtain viable hybrids. Once the embryo is rescued, two genomes are needed to be combined together to produce a fertile plant.
- By this method dormancy period of seeds can be shortened, as well as haploids can be produced.
- By ovule culture, it is possible to grow, study various nutritional requirements and stages young embryos or zygote.

vii. Ovaries 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 such a simple medium but can develop into seedless fruits on a medium supplemented with hormones.
- By this culturing 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.

viii. Nucellus culture

- Nucellar tissues excised from unfertilised ovules have no adventitive embryoids. If cultured on a medium containing malt extract and adenine, embryoids are formed but they are unable to germinate.

- When such embryoids are excised and cultured on a medium containing gibberellin, plantlets may be formed which can then be transplanted to the field.

- Disease-free clones can be obtained by nucellus culture.

ix. 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 pre-sterilized 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.

x. Cotyledon culture

- Immature cotyledon develops into somatic embryos, shoot buds and complete plants if cultured on a suitable nutrient medium.

xi. Endosperm culture

- If endosperm is cultured on a proper nutrient medium continued proliferation of callus mass takes place and subsequently plant is regenerated.
- It is useful for plant breeding and horticultures.
- It also useful in the production of plantlets as an alternative to the conventional methods of crossing i.e. tetraploids with diploids or triploids induction which is applicable to fruit trees.

xii. Fruit culture

- The culture of fruit tissues as whole organ or isolated tissue section such as ovary has been successfully cultured to give rise to mature fruits e.g. strawberry.
- Usually when an isolated portion of the fruit tissue is introduced into a sterile environment.
- It immediately loses structural integrity and degenerates into a rapidly dividing callus mass.
- Loss of structural integrity is associated correspondingly with an alteration of physiology that is subsequently reflected in the production of an altered metabolism.
- So it is not possible to make a meaningful study of fruits developing using callus derived culture.
- The use of fruit culture is to serve as a bioassay system to study fruits maturation events within a controlled environment.

xiii. Plant cell culture

- Culture of isolated cells or very small aggregates remaining disperswed in liquid medium.

b) HAIRY / TRANSFORMED ROOTS (TRANSINFECTION)

- Certain soil bacteria of the genus *Agrobacterium* (Gram negative bacteria) infects a wide range of plant species and causes the infection in plant termed as “Hairy root” disease.
- The disease is transformed bye their genome t-DNA from a bacterial plasmid to plant hairy root cells.
- A large number of small fine hairy roots covered with root hairs originated directly from the explant in response to *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*.
- The hairy roots are produced by inoculating the host plant when grown in a hormone-free medium give rise to copious roots referred to as 'transformed roots' or 'hairy roots'.
- These are fast growing, highly branched adventitious roots at the site of infection.
- They are genetically stable and affect a wide range of dicotyledonous plants and have same metabolic features.

Establishment/Methodology of Hairy Root Culture

- The explant material is inoculated with a suspension of *Agrobacterium rhizogenes* generated by growing bacteria in YMB medium for two days at 25°C with gyratory (round circle) shaking, pelleting by centrifugation (5×10 rpm; 20min) and suspending the bacteria in YMB medium to form a thick suspension.
- Transformation may be induced as asceptic plants grown from seeds, of on detached leaves, leaf discs, petioles of stem segments from green house plants following sterilization of excised tissue with 10% (v/v) domestos for 20 minutes.
- Scratching the midrib of a leaf or the stem of a plantlet, with the needle of a hypodermic syringe containing the thick bacterial suspension allows inoculation with small (about 5-10µl) droplets containing *Agrobacterium rhizogenes*.
- In some species a profusion of roots may appear directly at the site of inoculation, but in other a callus will form initially and roots emerge subsequently from it.
- In either case hair rot normally appear with in 1-4 weeks although the susceptibility of species to infection is variable.

Advantages of Hairy Root Culture

- Genetic and growth kinetic stability over prolonged period of growth *in vitro*.
- Ease of culture *in vitro* using simple media lacking phytohormones.
- Many plant cell culture systems, which did not produce adequate amount of desired compounds is being reinvestigated using hairy root culture methods.
- A diversified range of plant species has been transformed using various bacterial strains.
- Plant regeneration, plant improvement and genetic manipulation in plant can be done.
- Reproducible & predictable levels of product synthesis.
- Capability to synthesize (novel) secondary metabolites specific to that plant species from which they have developed in equal or even higher amount compared to field grown plants.
- More accumulation of secondary metabolites.
- Eg. Levels of steroidal alkaloid solasodine is significantly higher in hairy root cultures than callus or suspension cultures.

Problems associated with Hairy Root Culture

Excess bacterial growth.

- Slow regeneration.
- Decreased cell division and transformation due to stress.
- No transformation.
- Loss of gene effects.

Applications of Hairy Root Culture

Sr. No.	Plant	Product obtained
1	*Artemisia annua*	Artimisinin
2	*Hyoscyamus muticus*	Hyoscyamine
3	*Nicotiana tabacum*	Nicotine, Anatabine
4	*Podophyllum spp.*	Lignans
5	*Panax ginseng*	Polyacetylene analogues
6	*Solanum aviculare*	Solasodine
7	*Withania somnifera*	Withanolides
8	*Valeriana wallichi*	Valpotriates

c) Protoplast culture

❖Protoplast are plant cells with a plasma membrane but without cell wall, because of this the protoplast provide the starting point for many of the technique of genetic manipulator of plants, in particular the induction of somaclonal variation, somatic hybridization and genetic transfer.

❖They are cultivated in liquid as well as on solid media.

Isolation of Protoplasts is by two methods.

Protoplasts can be isolated from almost all plant parts i.e., roots, leaves, fruits, tubers, root nodules, endosperm, pollen cells, and cells of callus tissue.

1. **Mechanical method**
2. **Enzymatic method**

1. Mechanical method

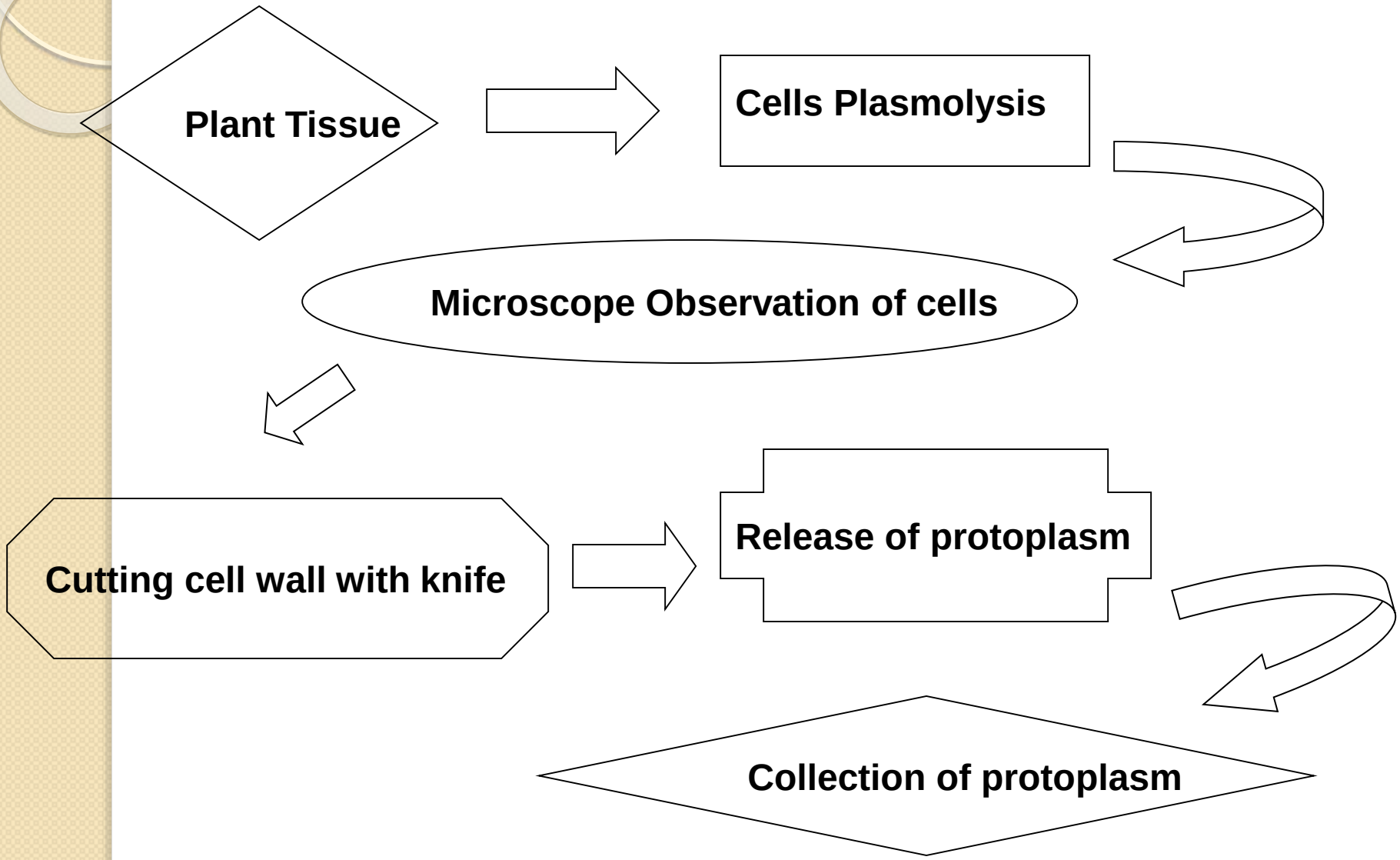
❖The cells were kept in a suitable plasmolyticum (lysis of plasma membrane) and cut with a fine knife.

❖Cells were cut only through the cell wall, releasing intact protoplast.

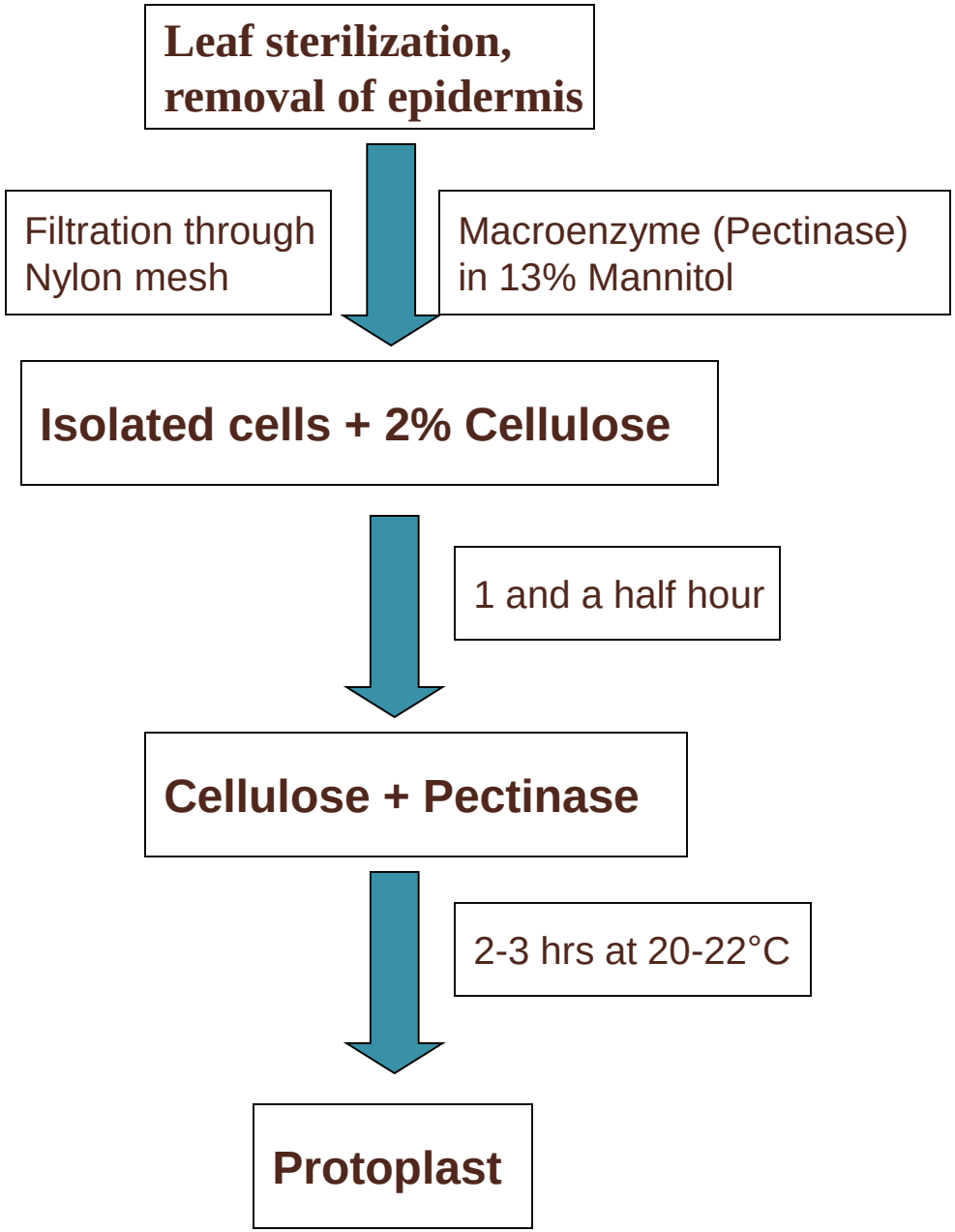
❖This mechanical procedure gave low yield of protoplasts and could be utilized for only highly vacuolated and non meristematic cells.

❖The method is laborious and tedious.

1. Mechanical Method



2. Enzymatic Method



2. Enzymatic method

❖Commercial preparations of purified cell wall degrading enzymes such as macroezyme, cellulase and hemicellulose became available that gave further progress to enzymatic isolation of protoplasts.

❖By this method very large number of protoplast are obtained compared to mechanical method.

❖Cells are not damaged or broken

❖Osmotic shrinkage of protoplast is much less.

❖Enzymatic method of protoplast isolation can be classified into two groups.

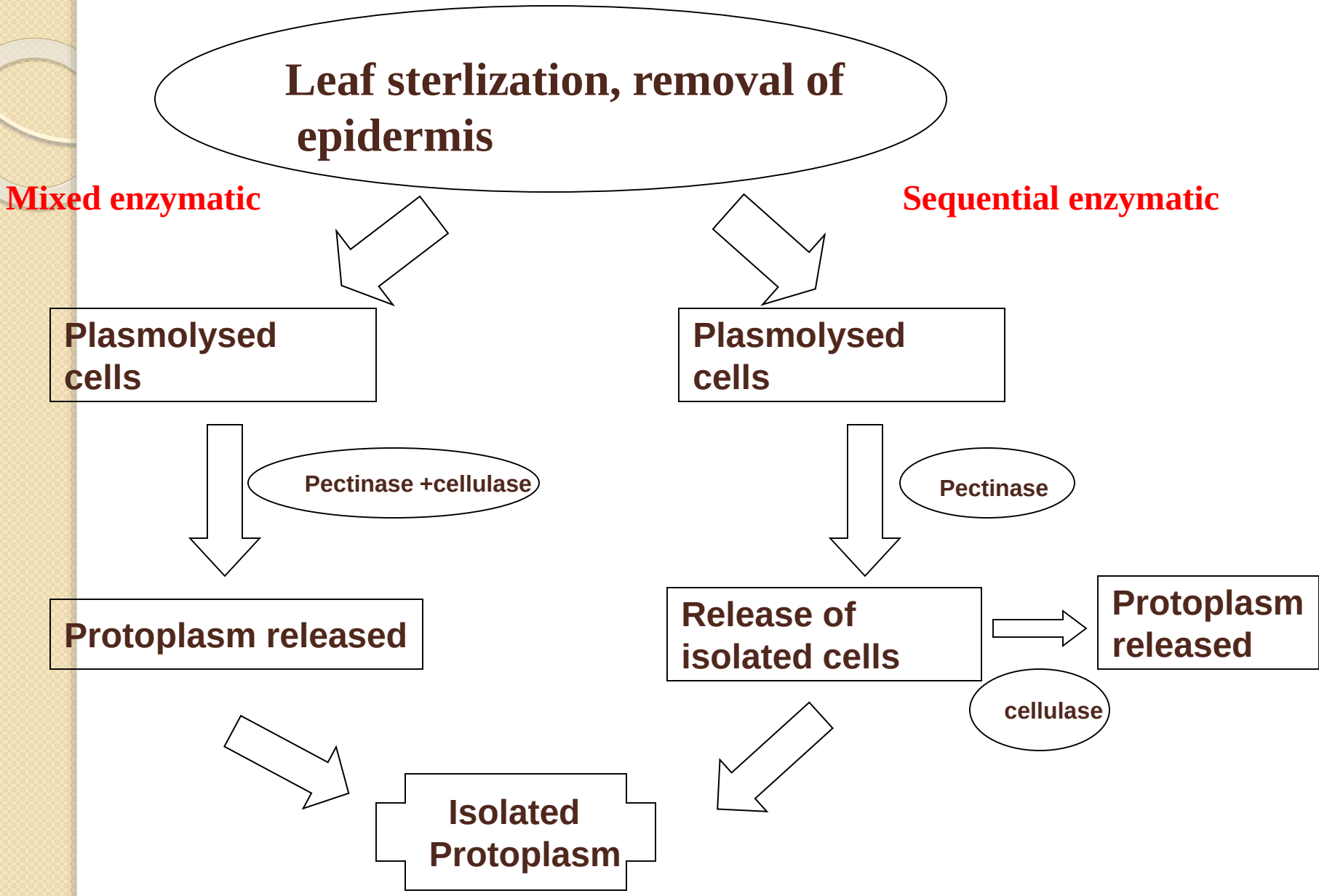
2.1 Sequential enzymatic

This involves two steps where first macerated plant tissues are incubated with pectinase to get single cells followed by cellulase treatment to get protoplast.

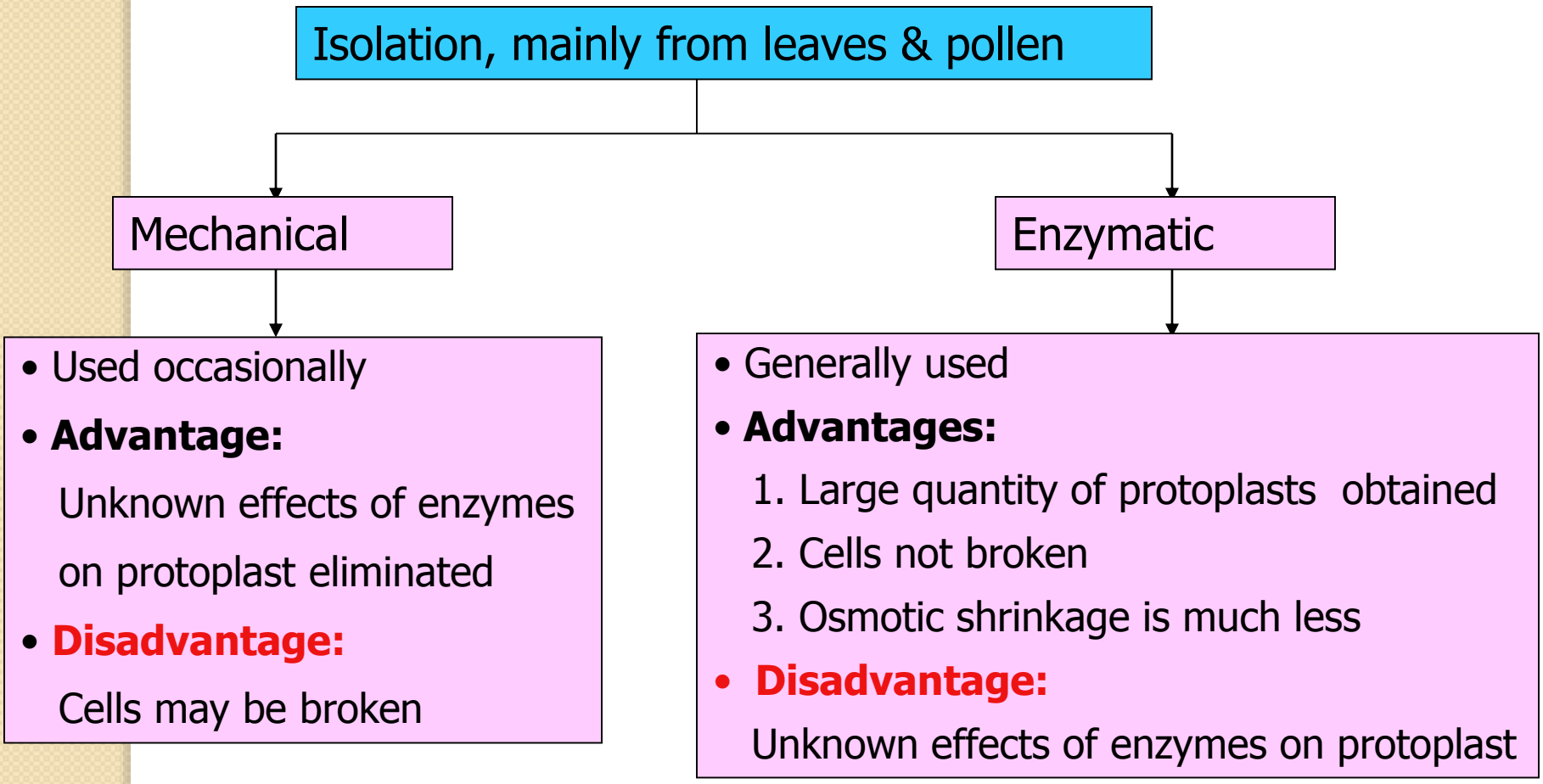
2.2Mixed enzymatic

This involves simultaneous separation of cells and degradation of their walls to convert protoplast by immersing plant tissues in mixture of pectinases and cellulases.

2. Enzymatic method



ISOLATION OF PROTOPLASTS in brief



PROTOPLAST CULTURE

Isolated protoplast are usually cultured in either liquid or semisolid agar media plates. They require somatic protection in culture medium until they generate a strong cell wall. Methodology of Protoplast culture is described below:

Isolated Protoplast is cleaned by centrifugation and decantation method

↓

The protoplast solution (1× 105 protoplast/ml) is poured on sterile and cooled molten nutrient medium

↓

Mix the two gently but quickly by rotating each petridish

↓

Allow the medium to set, seal petridishes with paraffin film and incubate

↓

Protoplasts capable of dividing undergo cell divisions from callus within 2-3 weeks

↓

The callus is then subculture on fresh medium

↓

Embryogenesis begin from callus when it is transferred to a proper nutrient medium and subsequently whole plant develops

Applications of protoplast culture

1. To develop Novel hybrid plant through protoplast fusion.
2. In single cell derived colony, isolation of mutants through mutagens is easier.
3. Single cell cloning can be easily performed with protoplasts.
4. Regeneration of entire plant or plant improvement through protoplast culture.
5. Genetic transformation through DNA uptake can be achieved.
6. Reproducible protoplast to plant systems are now available for many plants of agronomic value.

Protoplast fusion

•Protoplast fusion

• It is the technique in which two or more protoplast are fused into a single cell protoplast.

•The protoplast fusion allows us to bring an desirable plant traits in combination that are not possible by sexual means.

•May occur between same or different plant

•Mutinucleate protoplasm obtained after fusion

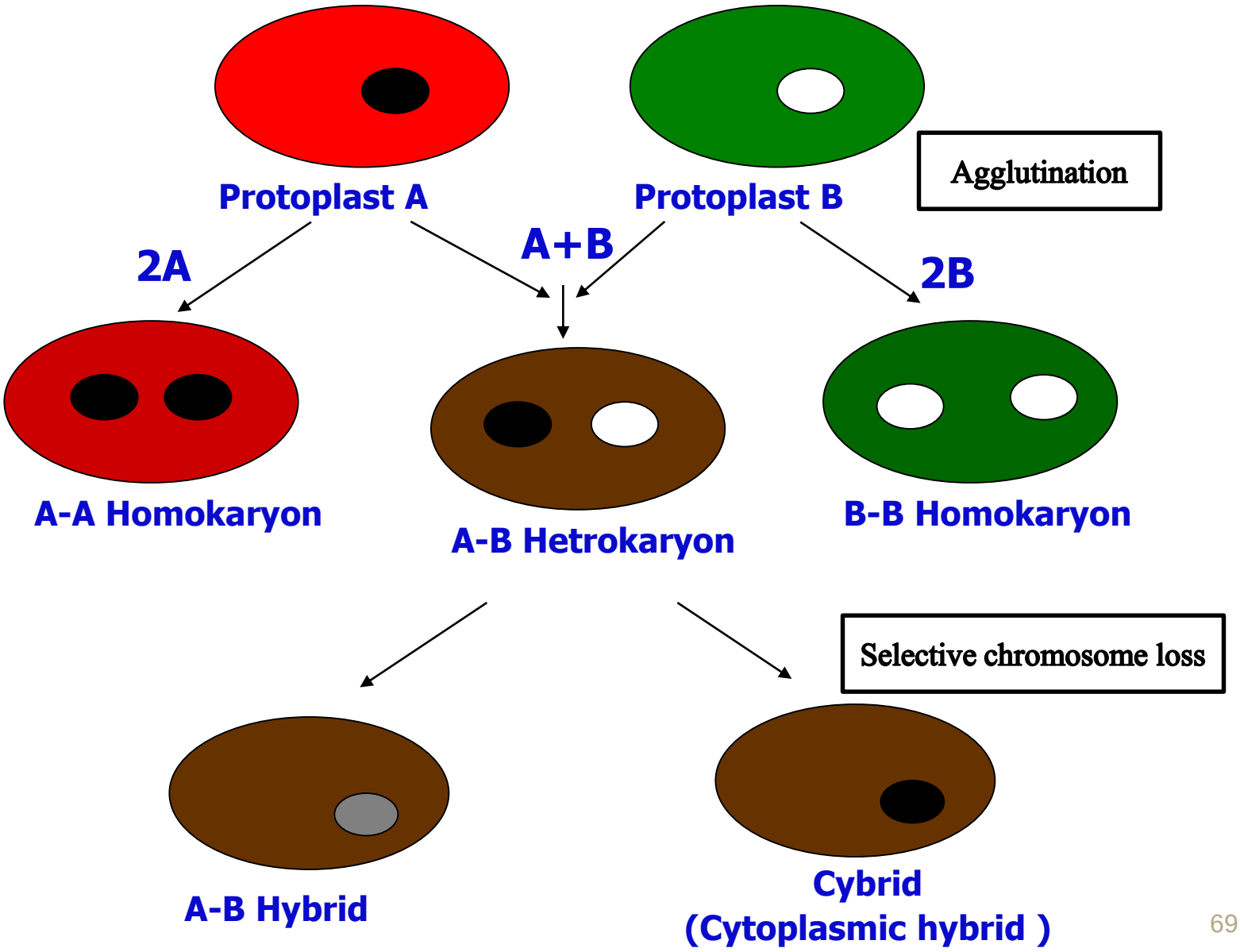
Three main phases are there for fusion:

1. **Agglutination**: Plasma membrane of two or more protoplast are brought into close proximity. (A and B)

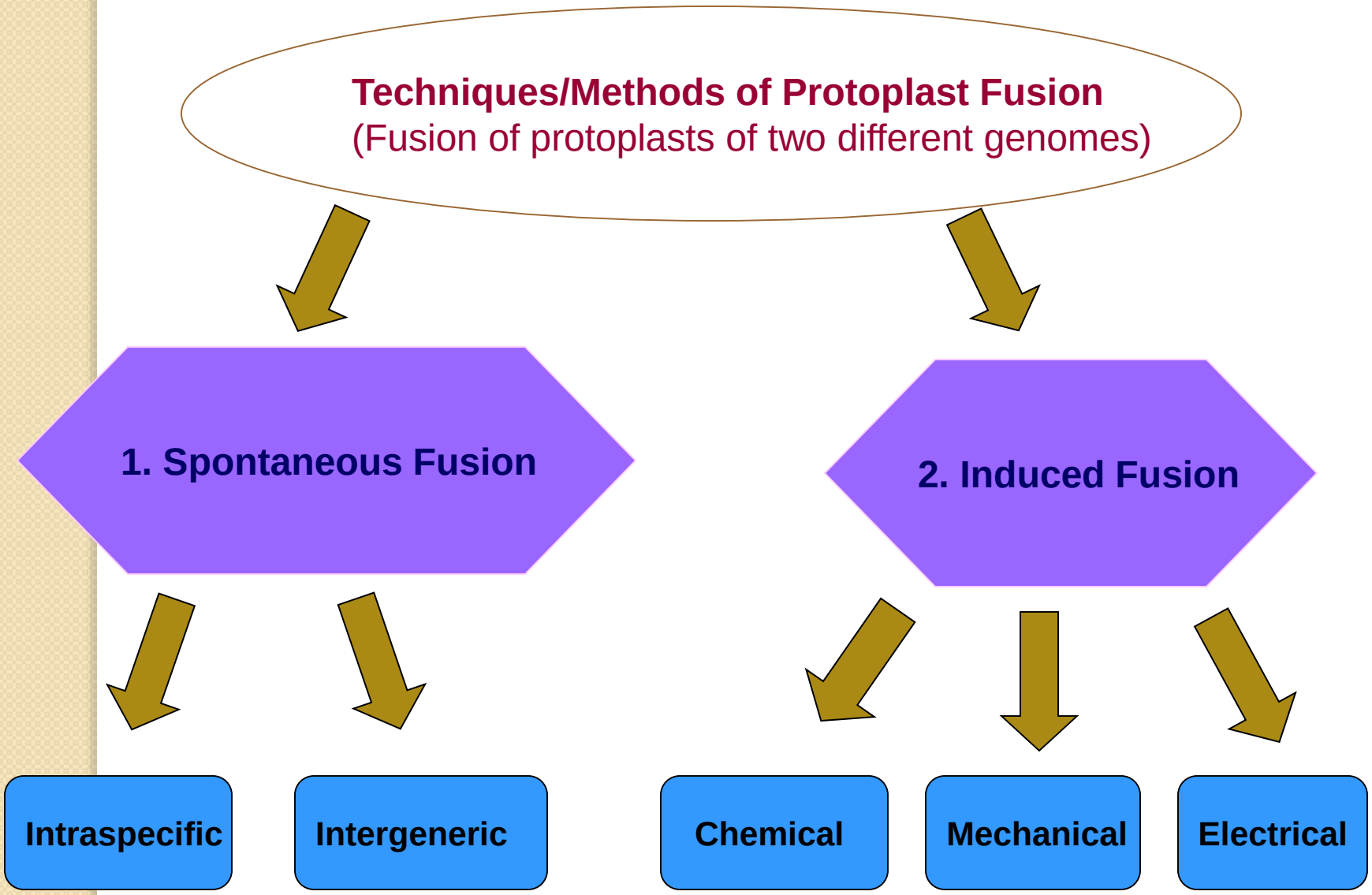
2. Membranes of protoplasts agglutinated by fusogen get fused at the point of adhesion. This results in the **formation of cytoplasmic bridge** between the protoplast.

3. **Rounding off** of the fused protoplast due to the expansion of cytoplasmic bridge froming a spherical hetrokaryon (A-B) or homo karyons (A-A or B-B). Binulceate hetero karyons. The fusion of the nuclei results in a tetraploid hybrid cell. Also cybrid cell is formed with a selective chromosome loss.

Stages of Protoplast fusion and Hybridization



Methods of Protoplast fusion



Techniques/Methods of Protoplast Fusion

Spontaneous Fusion

- Protoplast fuse spontaneously during isolation process mainly due to physical contact among similar parental protoplast.
- Protoplast from their adjoining cells fuse through their plasmodesmata to form a multinucleate protoplast.
- Two types of spontaneous fusion
  - **Intraspecific** produce homokaryones
  - **Intergeneric** have no importance

Induced Fusion

- Freely isolated protoplast from different sources are fused with the help of fusion inducing agents.
- Fusion needs an inducing agent that actually reduces electro negativity and allows the isolated protoplast to fuse.
- The various fusogens used for induced fusion are classified under various class as below:
  - a) Chemical fusion
  - b) Mechanical fusion
  - c) Electrical fusion

a) Chemical Fusion

Types of fusogens are used like PEG, $NaNo_3$, Ca^{2+} ions, Polyvinyl alcohol (PVA) etc. The cell membrane posses negative charge and after treatment with such chemical agents when cell membrane are brought into close physical contact they fuse.

b) Mechanical Fusion

It is not dependent upon the presence of fusion inducing agent. Physical fusion of protoplasts is done under microscope by using micromanipulator and perfusion micropipette.

c) Electrical Fusion

Protoplast are placed into a small culture cell containing electrodes and a potential difference (10kv m^{-1}) is applied then the protoplast will line up (form chain) between the electrodes.

Fusion of protoplasts of chain is induced by the application of high strength electric field (100kv m^{-1}) for

Somatic hybridization

Defination:

The technique of hybrid production through the fusion of isolated **somatic** protoplast under *in-vitro* conditions and subsequent development of their product to a hybrid plant is called somatic hybridization.

Various stages of somatic hybridization are as follows

1. Isolation of protoplast

↓

2. Fusion of the protoplasts of desired species/varieties

↓

3. Identification and Selection of somatic hybrid cells

↓

4. Culture of the hybrid cells

↓

5. Regeneration of hybrid plants

1. **Isolation of Protoplast**

   Same as discussed before
2. **Fusion of isolated Protoplast**

   Same as discussed before
3. **Identification and Selection of somatic hybrid cells**

The protoplasts of two parents may be labelled by different fluorescent compounds, which will then enable for the identification and selection of hybrids. **Identification** is based on difference between the parental cells and hybrid cell with respect to Pigmentation, Cytoplasmic markers, Presence of chloroplast, Nuclear staining etc.

In the mixture of both fused and unfused protoplast **Selection of hybrid** is done on the basis of several procedures like Genetic complementation, antibiotec-resistant cell lines, isoenzyme analysis, Phytotoxins, Specific amino acid, Auxin autotrophy, Auxotrophic and metabolic mutants, Chromosomal analysis by hybird cell, Herbicides etc.

4. **Culture of the hybrid cells**

Hybrid cells are cultured on suitable medium provided with the appropriate culture conditions.

5. **Regeneration of hybrid plants**

Plants are induced to regenerate from hybrid cells. These hybrid plants must be at least partially fertile, in addition to having some useful property, to be of any use in breeding schemes.

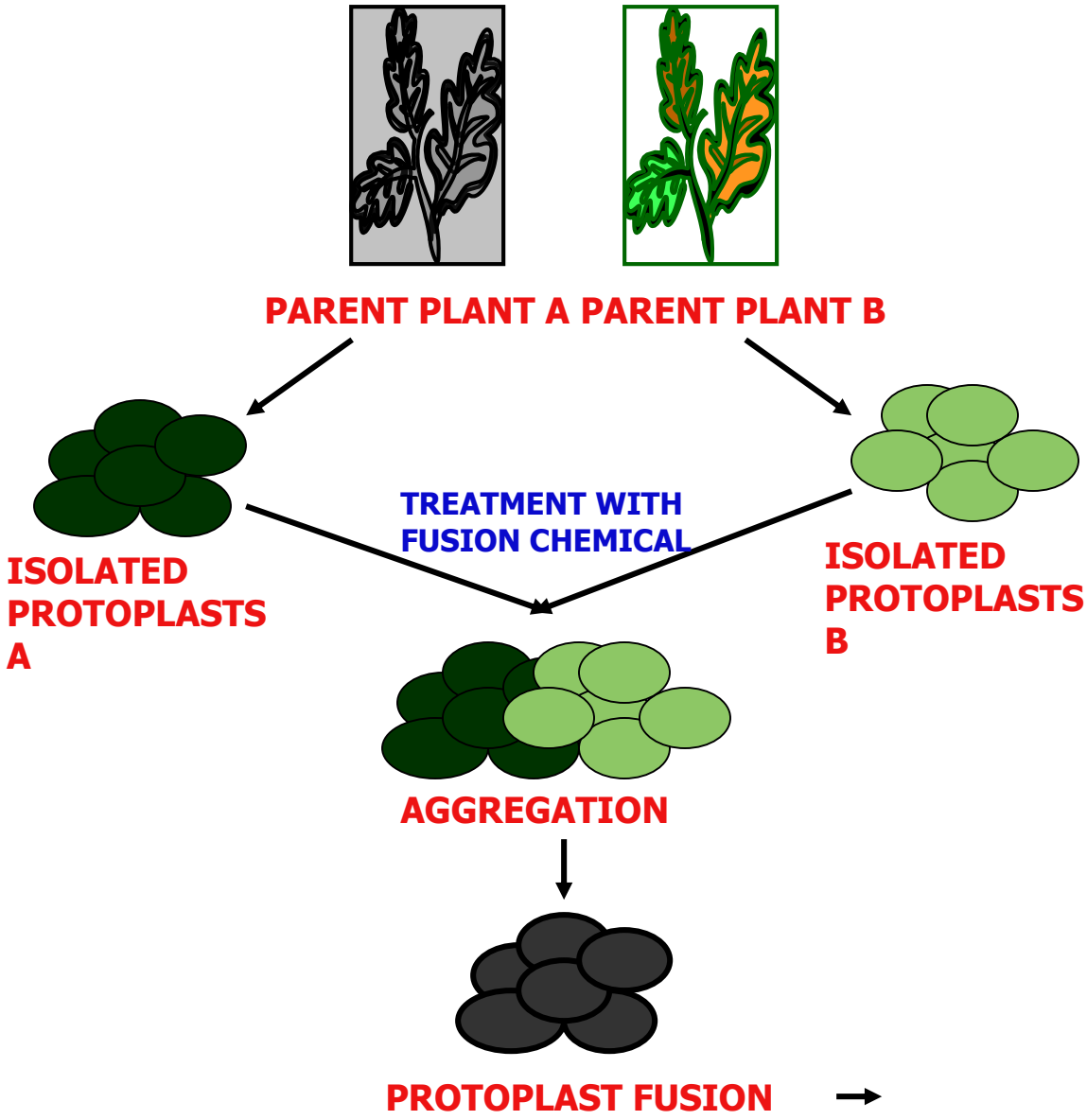
Advantages of somatic hybridization

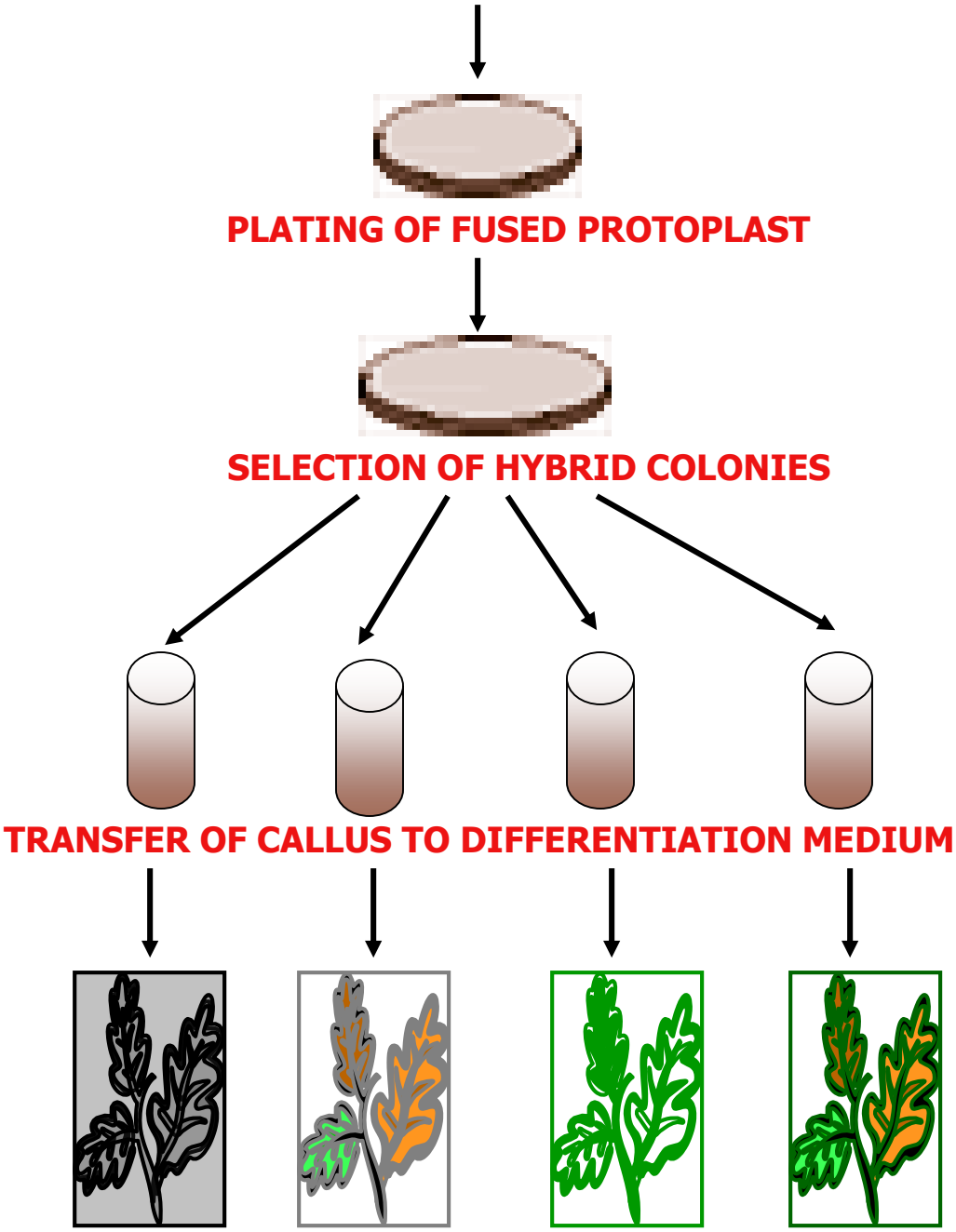
- Novel approach for introducing or increasing genetic variability at nuclear or extra nuclear organelle genome level, in higher plants
- Production of novel interspecific and intergenic hybrid
  - e.g. Pomato (Hybrid of potato and tomato)
- Somatic hybridization is significant in improvement of plants such as banana, potato, sugarcane, and yam.
- Production of fertile diploids and polypoids from sexually sterile haploids, triploids and aneuploids
- Transfer gene for disease resistance, abiotic stress resistance, herbicide resistance and many other quality characters.
- Production of heterozygous lines in the single species which cannot be propagated by vegetative means
- Studies on the fate of plasma genes
- Production of unique hybrids of nucleus and cytoplasm

Limitations of Somatic hybridization

- Poor regeneration of hybrid plants
- Non-viability of fused products
- Not successful in all plants.
- Production of unfavorable hybrids
- Lack of an efficient method for selection of hybrids
- No confirmation of expression of particular trait in somatic hybrids.
- Elimination of chromosomes from the hybrid cell

PROTOPLAST FUSION FROM 2 DIFFERENT PLANT SPECIES & LATER PLATING & SELECTION OF HYBRID COLONIES & REGENERATION OF "SOMATIC HYBRID"

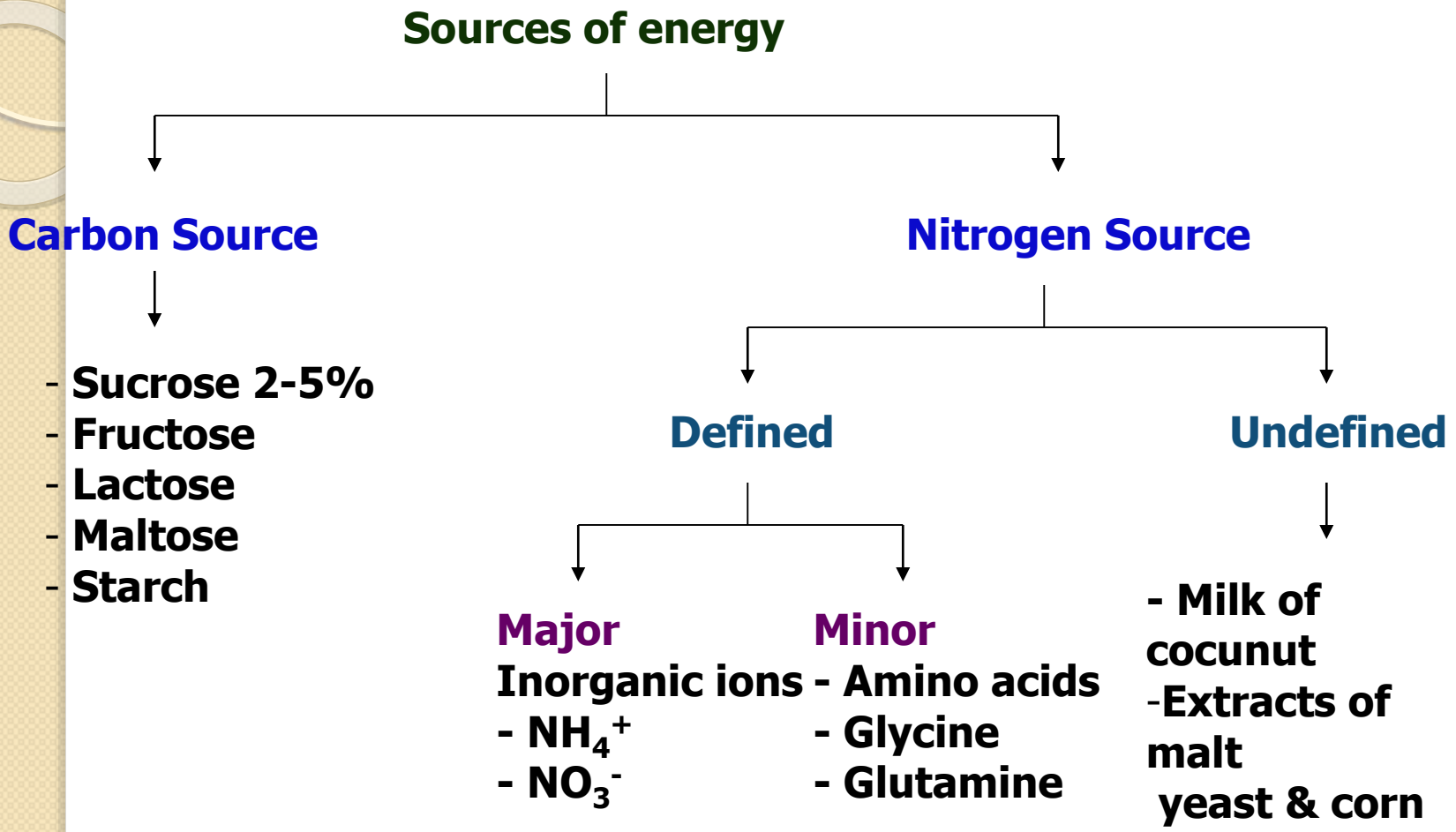



PLATING OF FUSED PROTOPLAST
SELECTION OF HYBRID COLONIES
TRANSFER OF CALLUS TO DIFFERENTIATION MEDIUM
SELECTION OF SOMATIC HYBRID PLANTS

Nutrient Media for Plant Tissue Cultures

Functions of medium

- Provide water
- Provide mineral nutritional needs
- Provide vitamins
- Provide growth regulators
- Access to atmosphere for gas exchange
- Removal of plant metabolite waste


Sources of energy
Carbon Source
Nitrogen Source
- Sucrose 2-5%
- Fructose
- Lactose
- Maltose
- Starch
Defined
Undefined
Major
Inorganic ions
- NH_4^+
- NO_3^-
Minor
- Amino acids
- Glycine
- Glutamine
- Milk of cocunut
-Extracts of malt
yeast & corn

Composition of Culture media

- Cultre 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 B_1, B_6, B_3, B_5 etc. 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

Inorganic Macro nutrients for plant tissue cultures

Constituents	Heller	Nitsch & Nitsch	White	Hildebrandt Ricker & Dugger	Musashige & Skoog	Gautheret
KCl	750	1500	65	65		
$NaNO_3$	600					
$MgSO_4$ $7H_20$	250	250	720	180	370	125
NaH_2PO_4 H_20	125	250	16.5	33		
$CaCl_2$ $2H_20$	75				440	
KNO_3		2000	80	80	1900	125
$CaCl_2$		25				
Na_2SO_4			200	800		
$Ca(NO_3)_2$						
NH_4NO_3					1650	
KH_2PO_4					170	125
$MgSO_4$						
$Ca(No_3)_2$ $4H_20$			300	400		500

Inorganic Micro nutrients for plant tissue cultures

Constituents	Heller	Nitsch & Nitsch	White	Hildebrandt Ricker & Dugger	Musashige & Skoog	Gautheret
$NiSO_4$						0.05
$FeSO_4\ 7H_20$					27.8	0.05
$MnSO_4\ 4H_20$	0.01	3	7	4.5	22.3	3
KI	0.01		0.75	3.0	0.83	0.5
$NiCl_2\ 6H_20$	0.03					
$CoCl_2$					0.025	
$Ti(SO_4)_3$						0.2
$ZnSO_4\ 7H_20$	0.01	0.5	3	6	8.6	0.18
$CuSO_4\ 5H_20$	0.03	0.025			0.025	0.05
$BeSO_4$						0.1
H_3BO_3	1.0	0.5	1.5	0.38	6.2	0.05
H_2SO_4						1.0
$FeCl_3\ 6H_20$	1.0					
Mg_2MO_4		0.025			0.25	
$AlCl_3$	0.03					
$Fe_2\ (SO_4)_3$			2.5			
Ferrictartarate				40		

Inorganic nutrients:

- Mineral elements play very important role in the growth of plant Function of nutrients in plant growth. Essentially about 15 elements found important for whole plant growth have also been proved necessary for the growth of tissue(s) in culture.
- Macronutrient: Elements required in the life of a plant greater than 0.5 mmol/lit are referred as macronutrients.
- The macronutirents include six major elements as follows:

  Nitrogen (N), Potassium (K), Phosphorous (P), Calcium (Ca), Magnesium (Mg), Sulfur (S).

  Nitrogen 2-20mmol/lit – Influences plant growth rate, essential in plant nucleic acids (DNA), proteins, chlorophyll, amino acids, and hormones.

- **Phosphorus** 1-3 mmol/lit– Abundant in meristimatic and fast growing tissue, essential in photosynthesis, respiration.

- **Potassium** 20 -30 mmol/lit– Necessary for cell division, meristematic tissue, helps in the pathways for carbohydrate, protein and chlorophyll synthesis.

- **Calcium** 1-3 mmol/lit - Involved in formation of cell walls and root and leaf development. Participates in translocation of sugars, amino acids, and ties up oxalic acid (toxin)

- **Magnesium** 1-3 mmol/lit - Involved in photosynthetic and respiration system. Active in uptake of phosphate and translocation of phosphate and starches.

- **Sulfur** 1-3 mmol/lit - Involved in formation of nodules and chlorophyll synthesis, structural component of

Inorganic nutrients: contd…

- Micronutrient: Elements required in the life of a plant less than 0.5 mmol/lit are referred as micronutrients.
- Overall the plant thrives on seventeen elements out of which four like carbon, hydrogen, oxygen and nitrogen are derived from the atmosphere and the rest thirteen microelements like Boron, copper, iron, manganese, zinc, cobalt, molybdenum, nickel, aluminium, iodine, ferrous, sodium, chlorine.
- A media lacking in these micronutrient does not support healthy and wholesome growth and the plant exhibits deficiency symptoms such as pigmentation, absence of vessels, presence of narrow cambial zone, cellular hypertrophy and symptoms of chlorosis due to absence of ferrous and sulphur.

- **Micronutrients concentrations and there role**
- Iron (Fe)-1 μM/l - Involved in Cell division, respiration, chlorophyll synthesis and photosynthesis. Eg. FeNaEDTA = sodium salt of EDTA.
- Manganese (Mn) 20-90 μM/l - Involved in Cell elongation, regulation of enzymes and growth hormones. Assists in photosynthesis and respiration.
- Boron (B) 2-5100 μM/l responsible for cell division and cell elongation
- Copper (Cu) 0.1 μM/l
- Molybdenum (Mo) 5μM/l
- Cobalt (Co) 0.1 μM/l
- Zinc (Zn) 1.5-30 μM/l
- Iodine (I) Nickel (Ni), Aluminum (Al), Ferrous, chlorine (Cl) and sodium (Na) are also required

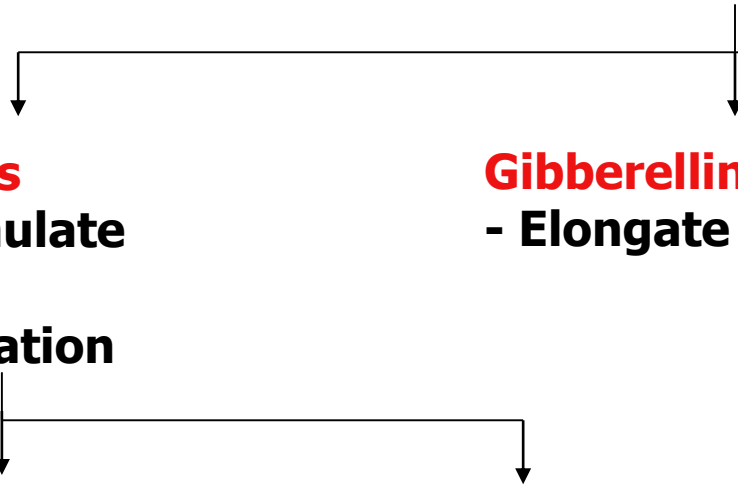
- **Organic nutrients**

- It includes Nitrogen substances, Vitamins, Amino acids, Carbon source, Growth hormones/regulators
- **Nitrogen source**
- Most cultured plant cells are capable of synthesising essential vitamins but not in sufficient amount.
- To achieve best growth it is essential to supplement the tissue culture medium with one or more vitamins and amino acid.
- **Vitamins**
- Thiamine (Vitamin B_1) - essential as a coenzyme in the citric acid cycle. It is required mostly in tissue culture and is considered to be essential. Thiamine hydrocholoride in 0.1-1mg/lit concentration is required.
- Nicotinic acid (niacin-Vitamin B_3) 0.5 mg/lit, Pyridoxine (Vitamin B_6) 0.5 mg/lit, Calcium pentothenate (Vitamin B_5) 0.1 mg/lit, are known to improve growth of the tissue culture material.
- Myo-inositol - part of the B complex, in phosphate form is part of cell membranes, organelles and is not essential to growth but beneficial and have important role in many biosynthetic pathways.
- Cynocobalamin (Vitamin B_{12}), Riboflavin (Vitamin B_1), Folic acid (Vitamin M) 0.5 mg/lit, Biotin (Vitamin H), p-amino benzoic acid (PABA), Ascorbic acid (Vitamin C), α-tocopherol (vitamin E) are added in special cases but their exact role is not yet well established.

- **Amino Acids**
- Some cultured plant-cells can synthesize all amino acids, none are considered essential.
- The most common sources of organic nitrogen used in culture media are amino acid mixtures, (e.g., casein hydrolysate), L-glutamine, L-asparagine, orginine, methionine and adenine.
- When amino acids are added alone, they can be inhibitory to cell growth.
- Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. L-tyrosine - stimulates shoot formation.
- Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.
- **Carbon source**
- Carbohydrates are used in tissue culture media as an energy source of carbon. Most plant tissue culture are nonautotropic and are therefore entirely dependent on an external source of carbon.
- The most commonly used carbon source is **Sucrose (2-5% or 20-30 g/lit)**
- **Glucose** and **Fructose** are used for good growth.

- **Maltose** and **raffinose** are used in some cases.
- In general excised dicotyledonous roots grow better with sucrose where as monocots do best with dextrose (glucose).
- Other carbohydrates like mannose, sorbitol, pentoses, sugar alcohol, glycols, hexoses, uronic acid, lactose, galactose, potato starch, grain starch and even glycosides can be used depending on the experimental conditions.
- **Growth hormones/regulators/ Modulators**
  - The success of plant tissue, cell and organ culture will depends on the amount of plant hormones and growth substance added into nutrient medium.
  - Auxins, ethylene, abscisic acid, cytokinins and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones.
  - The requirement of these hormones varies considerable with their endogenous levels.
  - Other plant hormones like polyamines, jasmonates, salicylates are also used depending on the experimental conditions and plants to be cultured.

Plant Growth Regulators (Hormones)



Auxins
- Stimulate cell elongation

Natural
IAA

Synthetic
NAA
2,4-D

Gibberellins
- Elongate internodes

Cytokinins
- Promote cell division

Natural
Adenine
Zeatin

Synthetic
Kinetin
Benzyladenine

Auxins

❖Auxins show a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division and organization of meristems giving rise to either callus or defined organs.

❖In organized tissue, auxins cause root formation, delaying leaf senescence, fruit ripening and used in embryogenesis.

❖Commonly used natural auxin is indole-3-acetic acid (IAA-1-50 mg/lit), but depending on the species, other natural auxins are 4-chloroindole-3-acetic acid, indole-3-butyric acid (IBA).

❖Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA-0.1-10 mg/lit) and 2, 4 dichlorophenoxyacetic acid (2, 4-D-0.05-0.5 mg/lit).

Cytokinins

- Cytokinins are useful in culture for stimulation of cell division (cytokinesis), release of lateral bud dormancy and induce adventitious bud formation.
- Cell division is regulated by the joint actions of auxins and cytokinins.
- Auxins affect DNA replication where as cytokinins seems to exert some control over the events leading to mitosis.
- In intact plants, cytokinins promote lateral bud growth and leaf expansion, promote chlorophyll synthesis and enhance chloroplast development.
- The most commonly used cytokinins are the substituted purines such as synthetic derived kinetin (0.1-10 mg/lit), BA (6-benzyladenine).
- Zeatin and 2-iP (6-γ-γ-dimethylamino purine) are naturally occurring cytokinins.
- Other cytokinins are adenosine and adenylic acid.
- Kinetin is 30,000times more potent than adenine.

Gibberellins

- Gibberellins will promote flowering, seed germination and stem or shoot elongation.
- There are over 20 known Gibberellins. Gibberellin (GA_3) is usually used to increase the shoot elongation.
- Gibberellins are used rarely compared to auxin and cytokinin.
- Cultured callus cells synthesize their own Gibberellins.

Abscisic acid (ABA)

- Abscisic acid (ABA) is naturally produced in plant tissues. ABA and other structurally related natural compounds are most likely produced by the cleavage of xanthophyll.
- ABA is often regarded as being an inhibitor, as it maintains bud and seed dormancy, inhibits auxin-promoted cell wall acidification loosening and slows cell elongation.
- ABA plays a key role in closing of stomatal apertures (reducing transpiration) and abscission of leaves.
- ABA also control of water and ion uptake by roots.

Ethylene

❖It is a gaseous hormone synthesied in cultured cell, fungi and bacteria.
❖Ethylene gas promotes fruit ripening, senescence, and leaf abscission.
❖At higher concentrations the gas decreased cell elongation but increased cell expansion.
❖The role of ethylene can be difficult to understand because its effects vary with development stage and because low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect.
❖Ethylene is synthesized from methionine. Auxin stimulate the production of ethylene but the physiological significance of ethylene in tissue culture is quite obscure.

Growth regulators/Hormones and their functions

- -auxin – promote roots growth and Cell division.
- -cytokinin – promote shoots growth and Cell division
- -gibberellin – promote cell enlargement and shoot elongation
- -abscisic acid – plant stress hormone and inhibits auxin
- -ethylene – low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect

- **Others media substances** which promotes growth of the tissue culture like protein hydrolysates (e.g., soy-protein hydrolyzates), yeast extracts, fruit (e.g. banana) extracts, coconut milk fresh/pasteurized.
- **Phenolic compounds** like Phloroglucinol - Stimulates rooting of shoot sections.
- Activated **charcoal** is used as a detoxifying agent. Detoxifies wastes from plant tissues and impurities.
- Adsorption quality vary, concentration normally used is 0.3 % or lower. It adsorbs the secondary products secrted by the culture tissue.
- Charcoal for tissue culture acid washed and neutralized never reused.
- Controls the supply of endogenous growth hormones.
- De-mineralises water.

Solidifying agent

❖Solid media are often preferred because its improved oxygen supply and support culture growth.

❖Therefore, substance with strong gelling capacity is added into the liquid media.

❖The most commonly used substance is agar and others are alginate, carrageenan, gelatin, starch, silica gel, hydroxy ethyl cellulose and polyacrylamide.

❖Agar is extraordinary resistant to enzymatic hydrolysis at incubation temperature and only few bacteria exist which are capable of producing degrading enzyme.

- **Iron source** EDTA can be used as a iron source.

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

- **Antibiotics** are used for prevention of excessive contamination in the culture medium. Generally fungicides and bactericides are used in culture medium but are not been very useful because they can be toxic to the explant and the contaminant sometimes reappears as soon as they are removed. Commonly used antibiotics are Carbonicillin (500mg/lit) and Augmentin (250 mg/lit)

FIELDS OF APPLICATION

Agronomical

- Rapid multiplication of selected plants by the multiple production of plants identical to original plants
- 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.

Application of plant tissue culture

- Plant tissue culture technology has been used in almost all the field of biosciences. Its applications include

1. Production of phytopharmaceuticals and secondary metabolites.
   a) Biotransformation (Biochemical Conversion)
   b) Plant cell immobilization
   c) Genetic transformation (Transgenic plant)
   d) Elicitors
2. Micropropagation (Clonal Propagation)
3. Synthetic seed
4. Protoplast culture and somatic hybridization
5. Hairy root culture
6. Cryopreservation
7. Tracing the biosynthetic pathways of secondary metabolites
8. Generation novel compound from plant
9. Respiration, organ function and metabolism in plant tissue culture can be studied.
10. Plant improvement by studying diseases of plant and their elimination with the help of plant tissue culture.
11. Mutant cell selection is done by addition of toxic substance to cells followed by isolation of resistant cells.
12. Production of economical valuable chemicals by plant tissue culture which are not possible by other chemical methods.

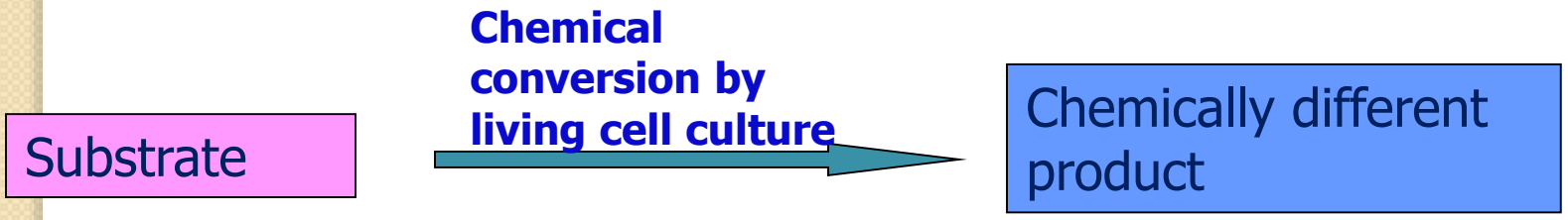
1. **Production of phytopharmaceuticals and secondary metabolites**

Secondary plant metabolites like alkaloids, terpenoids, flavonoids, lipids, oils, tannins, anthraquinones, flavones, napthaquinones, vitamins, proteins, anticancer agents, antiviral agents etc. are isolated from plant tissue culutre. Following table list few phytopharmaceuticals or secondary metabolities derived from plant tissue culture.

Compound	Plant species	Culture type
Anthraquinones	*Cassia angustifolia*	Callus
Caffeine	*Coffee arabica*	Callus
Cardenolides	*Digitalis purpurea*	Suspension & Callus
Codeine	*Papaver somniferum*	Suspension
Diosgenin	*Dioscorea composita*	Callus
Glycyrrhizin	*Glycyrrhiza glabra*	Suspension
Papain	*Carica papaya*	Callus
Reserpine	*Rauwolfia serpentina*	Suspension
Rosmarinic acid	*Coleus blumei*	Callus & Suspension
Trigonelline	*Trigonella foenum-graecum*	Suspension
Vinblastine	*Catharanthus roseus*	Callus
Visnagin	*Ammi visnaga*	Suspension
Xanthotoxin	*Ruta graveolens*	Suspension

a) Biotransformation (Biochemical Conversion)

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



❖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.

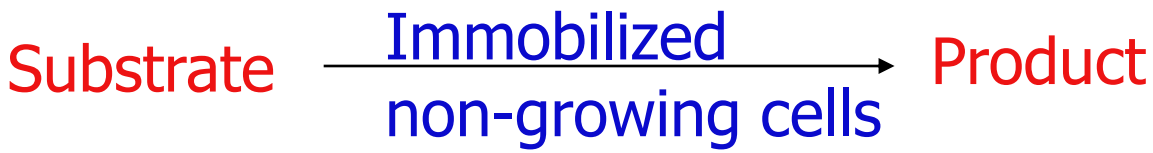
❖Few examples of biotransformation are as follows:

Class	Substrate	Product	Plant
Alkaloids	Codeinone	codeine	*Papaver somniferum*
phenolics	resorcinol	glucoside	*Datura innoxia*
Steroids	Digitoxin	digoxin	*Digitalius Lanata*
Phenolic flavonoids	liquiritigenin	Glucoside	*Datura innoxia*, *Catharanthus roseus*
Phenolic anthraquinones	Rhein	Glucoside	*Perilla frutescens*
Phenolic courmarins	Umbelliferone	Umbelliferone-7-glucoside	*Datura innoxia*, *Catharanthus roseus*
terpenoids	Citral	Citrol	*Lavandula angustifolia*

b) Plant cell Immobilization

❖It is defined as a technique, which confines to a catalytically active enzyme (or to a cell) with in a reactors system and prevents its entry into the mobile phase, which carries the substrate and product.

❖It involves the entrapment of cells within a gel or passive adsorption on solid support materials, thus creating a situation for cell to imitate membership on a tissue of a whole plant.



❖It is a controlled process of agglutination, formation and adhesion on a matrix under controlled condition.

❖It is a process in which highly reactive and sensitive enzyme is embedded in a matrix so that only substance and product can pass through matrix.

❖Cells cease to grow & accumulate metabolites.

❖In immobilization technique the plant cells are entrapped in different polymerize matrices. E.g. alginate, agar, agarose etc. and converted into hetrogeneous catalyst.

Different types of immobilization are

1. Adsorption-Direct intracellular binding due to natural affinity (adhesion or agglutination)
2. Covalent linkage-Covalent coupling on otherwise inert matrices.
3. Cross linking-Intracellular connection via bi or poly functional reagent
4. Embedding-Mixing with suitable materials by changing their consistency with temperature.
5. Entrapment-Physical retention within the frame work of diverse pore size and permeability (Micro encapsulation)

Mechanism/methods of immobilization system

1. **Entrapment**
   a. Gel entrapment by polymerization with polymers like polyacrylamide e.g. vinca
   b. Gel entrapment by ionic network formation: Entrapment of cell in calcium alginate

c. Gel entrapment by formation by precipitation of some natural and synthetic polymer by changing one or more parameters such as pH, temperature, salinity etc.

d. Entrapment in performed structures like hollow fibre reactors e.g. Polyurethane foam.

2. **Surface immobilization** with help of nylon, cellulose etc.
3. **Immobilization by embedding** in agar, agarose etc.

Applications of plant cell immobilization:

- ❖ Biotransformation
- ❖ Rapid biosynthesis of secondary metabolites
- ❖ Synthesis from precursor
- ❖ The biosynthesis of secondary metabolites with increased yield over extended period of time.

Examples: Immobilization of various plant like Capsicum, Coffee, Vinca, Mentha, Tobacco etc.

c) 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.

d) Elicitors

❖Elicitors are organic/inorganic agents used in tissue culture to trigger rapid and increase production of secondary metabolites or rapid growth of plant cell culture/organs.

❖Induction of stress in plant cultures in terms of specific environmental, physiological & biological conditions, to enhance the production of secondary metabolites, is known as Elicitation.

❖The secondary compounds synthesized & accumulated in response to such conditions are called '**Phytoalexins**', which act as defense agent to invading pathogens. The signals triggering the formation of phytoalexins are called **elicitors**.

Classification of Elicitors

A. Based on Origin

1. **Exogenous elicitors:** Enzymes, metal ions, U.V. Light, chitosan etc.
2. **Endogenous elicitors:** Hepta-β-glucoside, Dodeca β-1,4 D-galacturonide etc.

B. Based on nature

1. **Biotic elicitors**
2. **Abiotic elicitors**

1. Biotic elicitors

❖They are derived from microorganisms or produced within the plant cells by plant defensive process against microbial infection.

❖They include mainly β-Linked glucans, chitosan, enzymes, cell wall derived polysaccharides like pectin, pectic acid, cellulose, etc.

❖These elicitors when added to medium in low concentration (50-250ng/l) enhance metabolite production.

2. Abiotic elicitors

❖Product accumulation also occurs under stress caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, freezing and thawing cycles, non-essential components of media (agarose, tin, and agaropectin), certain chemicals (methyl jasmonate, copper sulphate, silver nigtrate etc.), and high salt concentration grouped under abiotic elicitors.

Elicitors stimulated the accumulation of secondary metabolites in different plant culture like Opium, Dioscorea, Datura, Vinca, Capsicum, Carrot etc.

2. 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*.

❖Methods of Micropropagation are as follows:

a) Micropropagation by proliferation of axillary buds
b) Adventitious shoot proliferation
c) Artificial seed.
d) Plant regeneration by organogenesis from callus and cell suspension cultures.
e) Plant regeneration from callus and cell suspension culture by somatic embryogenesis.
f) Direct (adventitious) somatic embryogenesis (Non-zygotic embryogenesis)

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.

3. Synthetic seed (Artificial seed or Somatic seed or Synerts or Synseed)

❖Synthetic seeds are defined as **artificially encapsulated** somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing a seed and that possess the ability to convert into a plant under *in vitro* or ex vitro conditions and that retain this potential also after storage.

❖Various plant species which are reported for artificial seed production are Carrot, Alfa alfa (somatic embryos), Banana, Cardamom (Shoot buds or shoot tips), Ecalyptus (Axillary buds) etc.

❖They are classified as Desiccated and Hydrated. **These two are again classified into encapsulated and uncoated**.

❖The **desiccated synthetic seeds** are produced from somatic embryos either naked or encapsulated in polyoxy ethylene glycol followed by their desiccation. E.g. Wheat, Soyabean (Uncoated), Carrot (Encapsulated)

❖**Hydrated synthetic seeds** are produced in those plant species where somatic embryos are recalcitrant and sensitive to desiccation. Hydrated seeds are produced by encapsulating the somatic embryos or somatic propagules in hydrogel capsules. E.g. Carrot, tomato (Uncoated), Mango, Alfa alfa (Encapsulated)

Applications of synthetic seeds

1. Micropropagation through artificial seeds.
2. For development of plants for breeding purpose
3. Propagation of variety of crop plants especially crops for which true seeds are not used or not readily available for multiplication or the true seeds are expensive. Hybrid plants may vegetatively propagated plants which are prone to infections e.g. Garlic, potato, tomato, hybrid rice etc.
4. Transplanting improved or selected material in forestry to reduce the cost of breeding e.g. European larch, white spruce etc.
5. Germplasm conservation of endangered species through cryopreservation of synseeds in near future.

4. Protoplast culture and somatic hybridization

Already discussed before

5. Hairy root culture

Already discussed before

6. 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.

7. **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.

8. **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.

9. Respiration, organ function and metabolism in plant tissue culture can be studied.
10. Plant improvement by studying diseases of plant and their elimination with the help of plant tissue culture.
11. Mutation and selection of the mutant cell is done for significant contribution to new genetic variability.
12. Production of economical valuable chemicals by plant tissue culture which are not possible by other chemical methods.

THANK YOU

EDIBLE VCCINES:

A New Approach to oral immunization

CONTENTS :-

- *Vaccines*
- *Edible Vaccines*
    1. *Developing*
    2. *Candidates required*
    3. *Recent developments*
    4. *Applications*
- *Advantages*
- *Limitations*
- *Conclusion*

What are vaccines ?

- Vaccines are designed to elicit an immune response without causing diseases.
- Typically vaccines are composed of killed or attenuated disease causing organisms.
- Successful vaccination programs lead to far fewer individuals ever showing symptoms of diseases, thus reducing the need for costly treatment procedures.

Edible Vaccines

- Vaccines that one can eat are called edible vaccines, which are among the most unusual approaches for administering new vaccines.

- They are engineered to contain antigen, but bear no genes that would enable whole pathogen to form.

- Again these are mucosal-targeted vaccines, which cause stimulation of both systematic and mucosal immune response.

- When they are taken orally, undergoes the mastication process and the majority of plant cell degradation occur in the intestine as a result of action of digestive or bacterial enzyme on these vaccines.

- The first report of edible vaccines in tobacco, at 0.02% of total leaf protein level appeared in 1990.

- These vaccines are currently being developed for a no. of human and animal diseases including measles,cholera & hepatitis B.

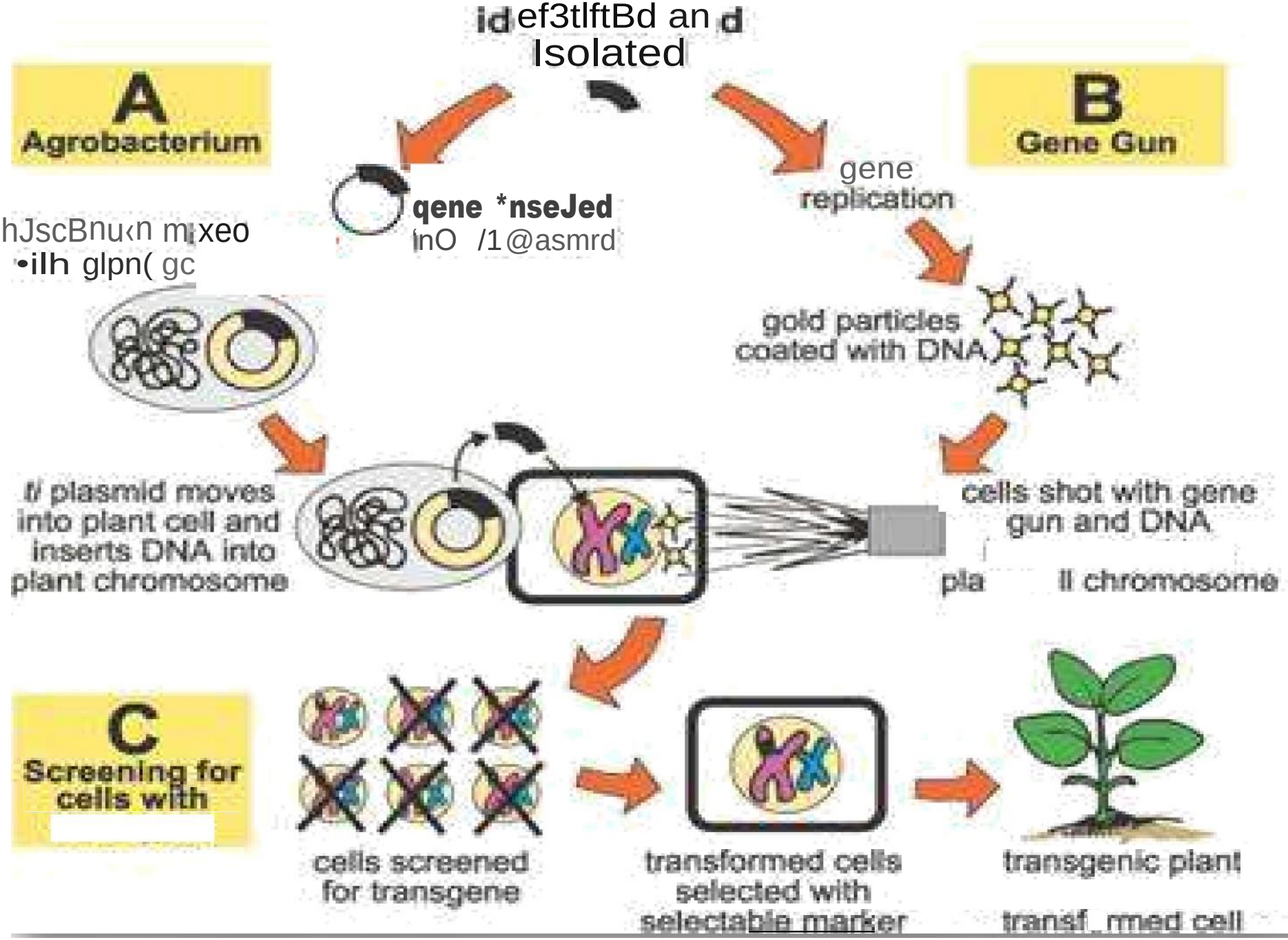
Developing an edible vaccine

Methods for transformation of DNA/Gene into plants :-

- There are several methods but two methods are sited here.

1. *Agrobacterium tumifaciens* method.

2. Microprojectile bombardment method ‘or’ Gene gun method.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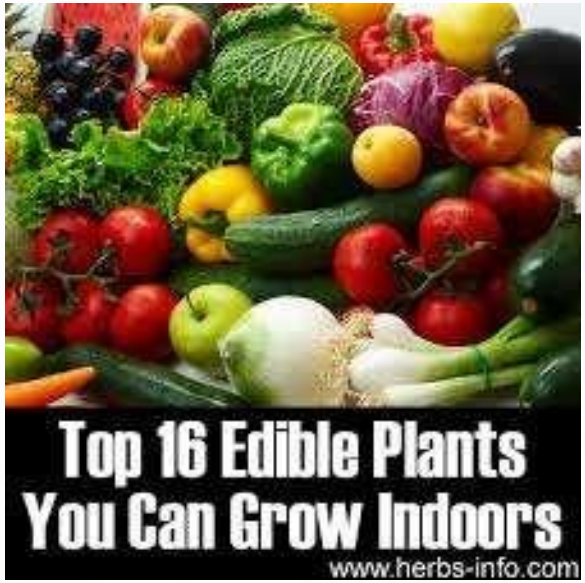
Candidates for Edible Vaccines

- Edible vaccines include bananas, potatoes , lettuce and tomatoes as well as rice, wheat, soybean etc.

- Choosing a plant to be used as a vaccine is important i.e it must be hardy, Palatable with high nutritive and protein content.



What are the recent developments

- Edible vaccines have been made to study the expression of ORF2 gene of hepatitis ‘E’ virus in tomatoes.
- Genetically engineered tobacco plant made to produce Interleukin ten which is used to test intestinal disorder i.e crohn’s disease.

Application of Ediblevaccines.

Major successes have been achieved on these edible vaccines are,

- **Malaria:-**

Three antigens are currently being investigated for the development of a plant based malaria vaccine, Merozoite Surface Protein (MSP) 4 & (MSP) 5 from *P.falciparum*, & MSP 4/5 from *P.yoelli*.

- Measles :-

MV-H antigen was selected for the development of edible vaccine, which can be transformed in tobacco plants.

- Diabetes :-

Transgenic potato and tobacco plants with gene GAD67, fed to diabetic mice , developed insulin dependent diabetes.

Advantages

- *Reduced need for medical personnel and sterile injection conditions.*
- *Therapeutic proteins are free of pathogens & toxins.*
- *Economical in mass Production and transportation.*
- *Delivery of multiple antigens.*

Limitations:-

- Stability of vaccine in fruit is unknown.
- Selection of best plant is difficulty.
- Consistency of dosage from fruit to fruit, plant to plant & generation to generation is not similar.
- Certain foods like potato are not eaten raw & cooking the food might weaken the medicine present in it.

Conclusion:-

Edible vaccines might be a solution that will enable the positive effects of vaccines. They offer a way to deliver a vaccine orally thus decreasing the cost of production and shipping .

Hence these novel inventions of edible vaccines did a man's service to humanity or mankind.