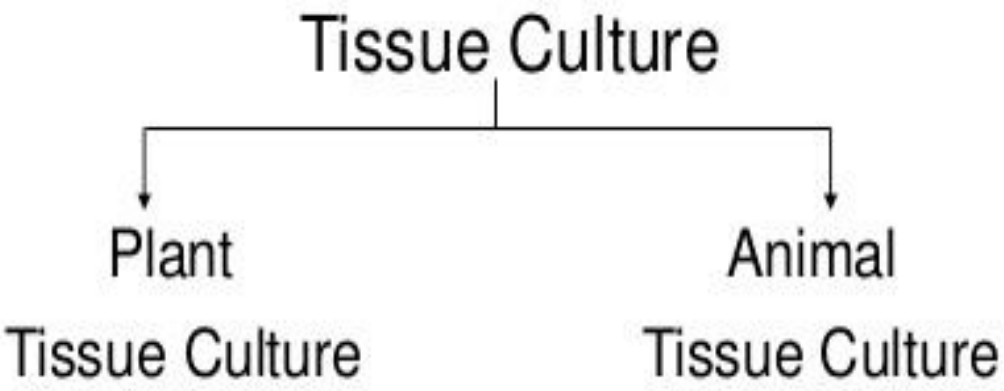
PLANT TISSUE CULTURE

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.

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 ± 0.5°C generally temperature recommended for most tissue culture studies is 36°C.

•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.

Basic Methodology/technique of Plant Tissue Culture

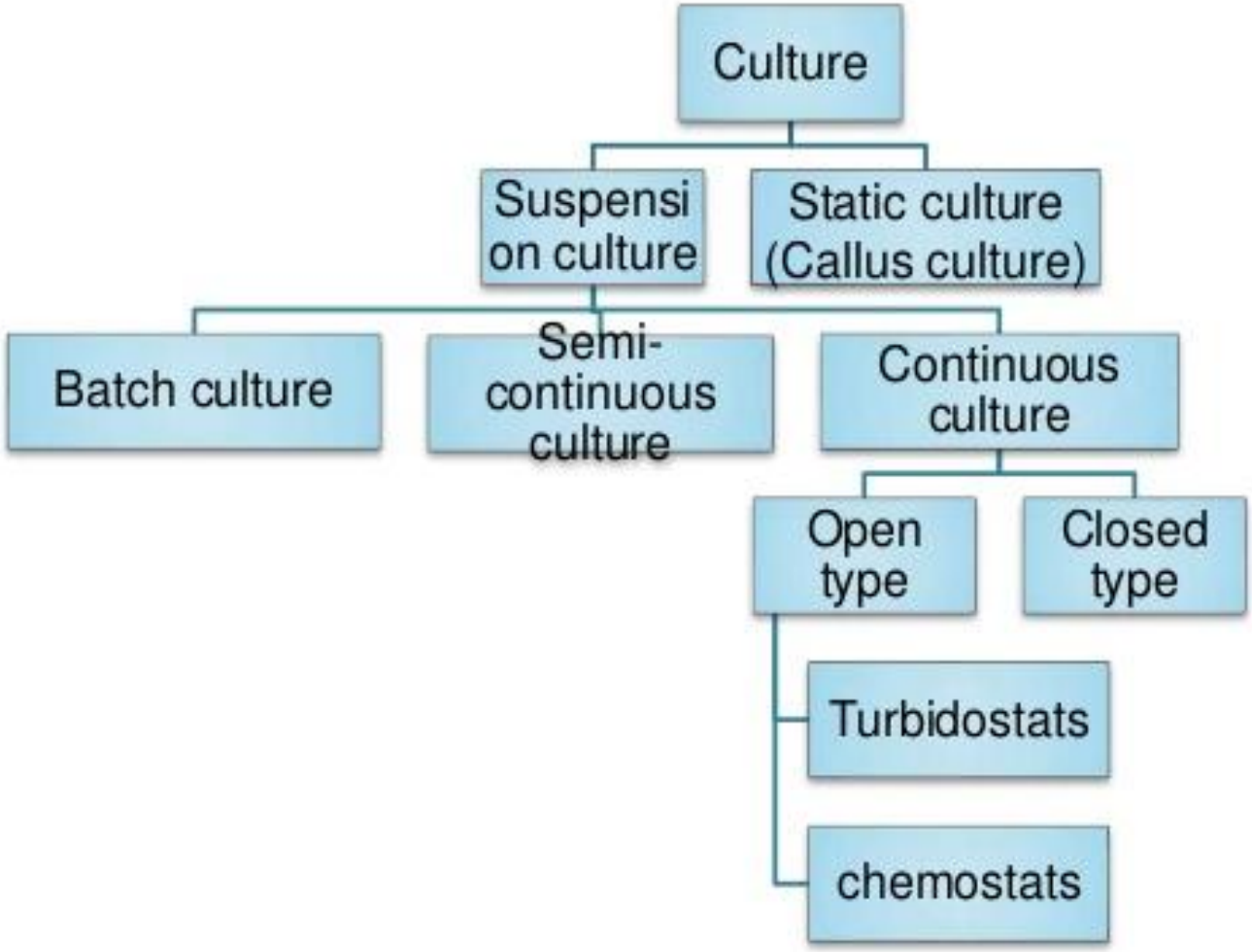
The general technique used in the isolation and growth of culture is described as follows:

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.

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

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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

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.

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

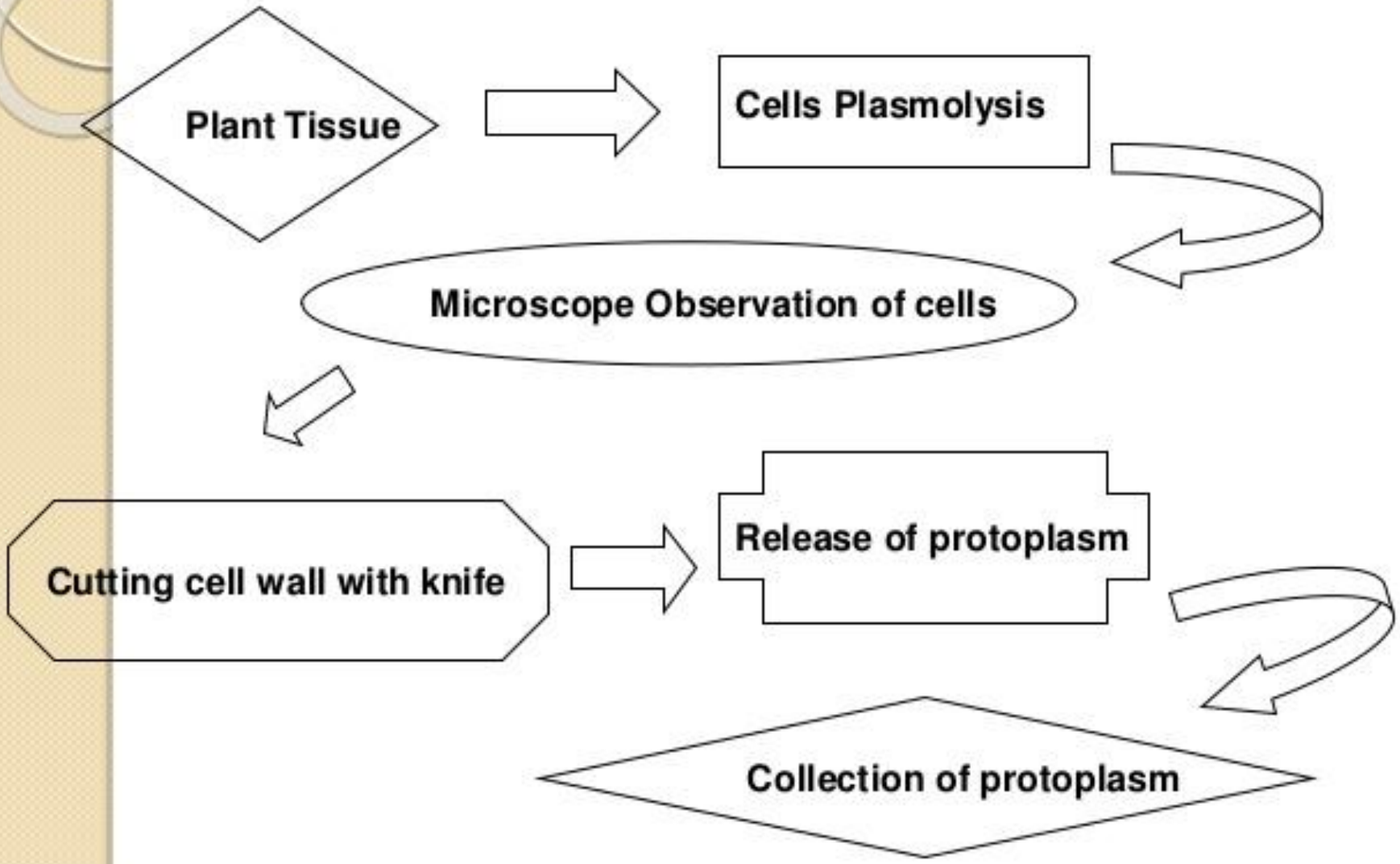
❖The cells were kept in a suitable plasmolyticum (lysis of plasma membrane) and cut with a fine knife.

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

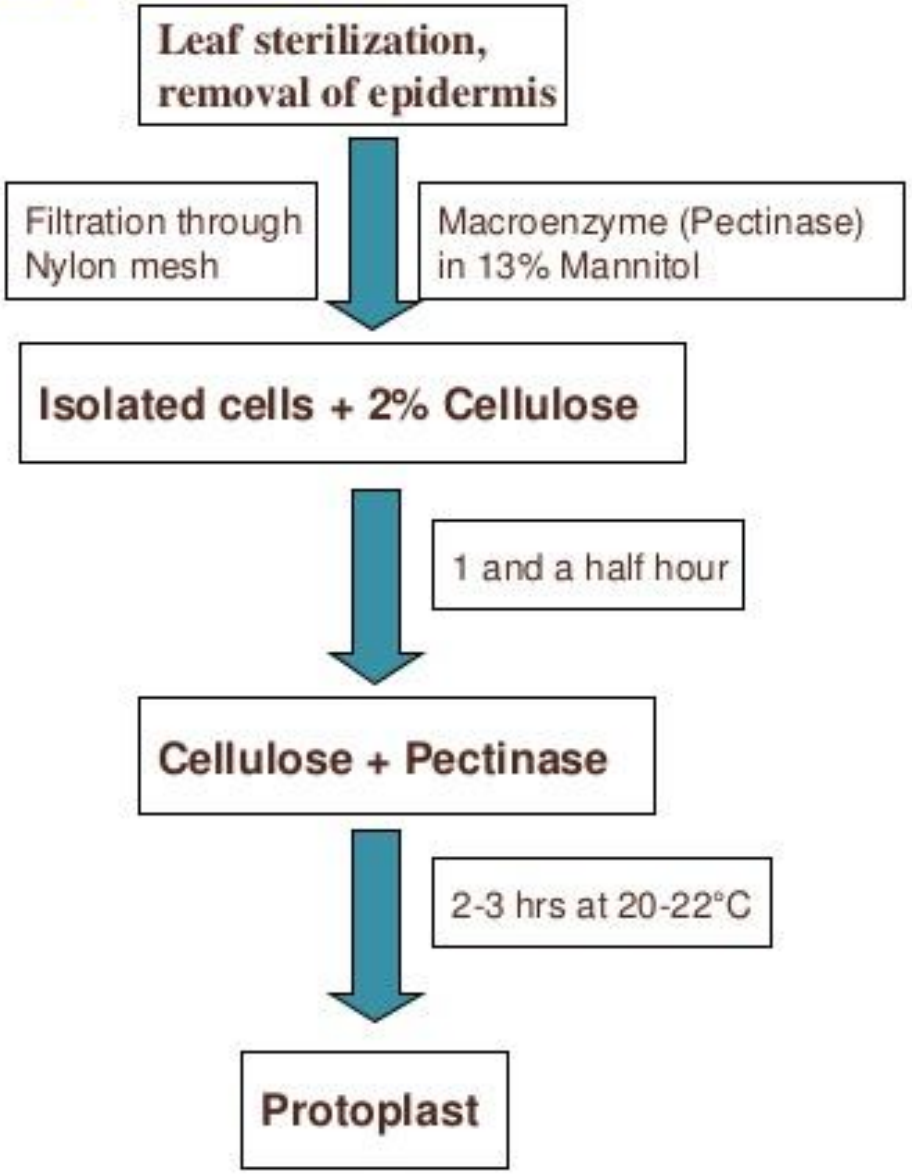
❖This mechanical procedure gave low yield of protoplasts and could be utilized for only highly vacuolated and non meristematic cells.

❖The method is laborious and tedious.

1. Mechanical Method



2. Enzymatic Method



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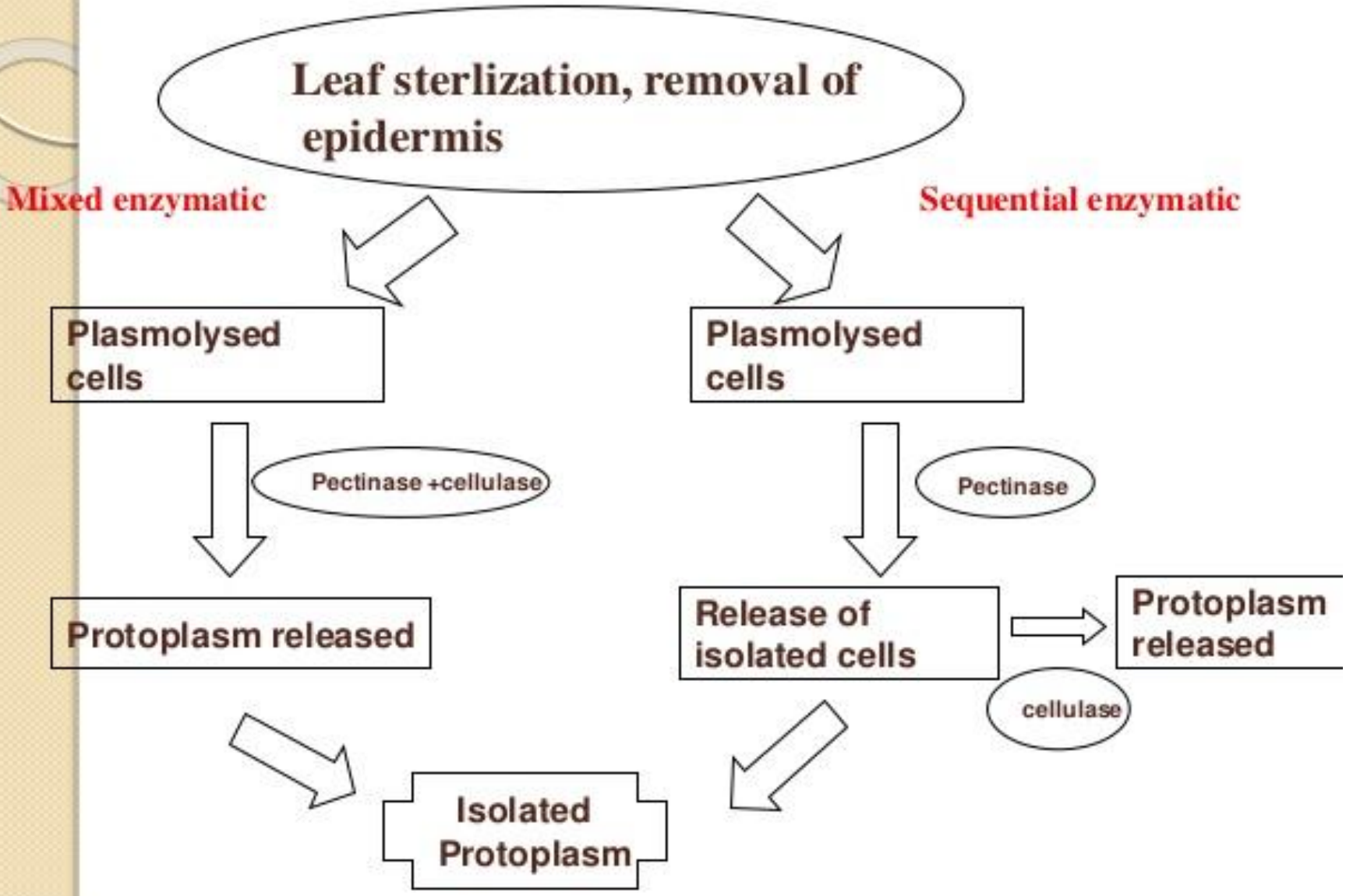
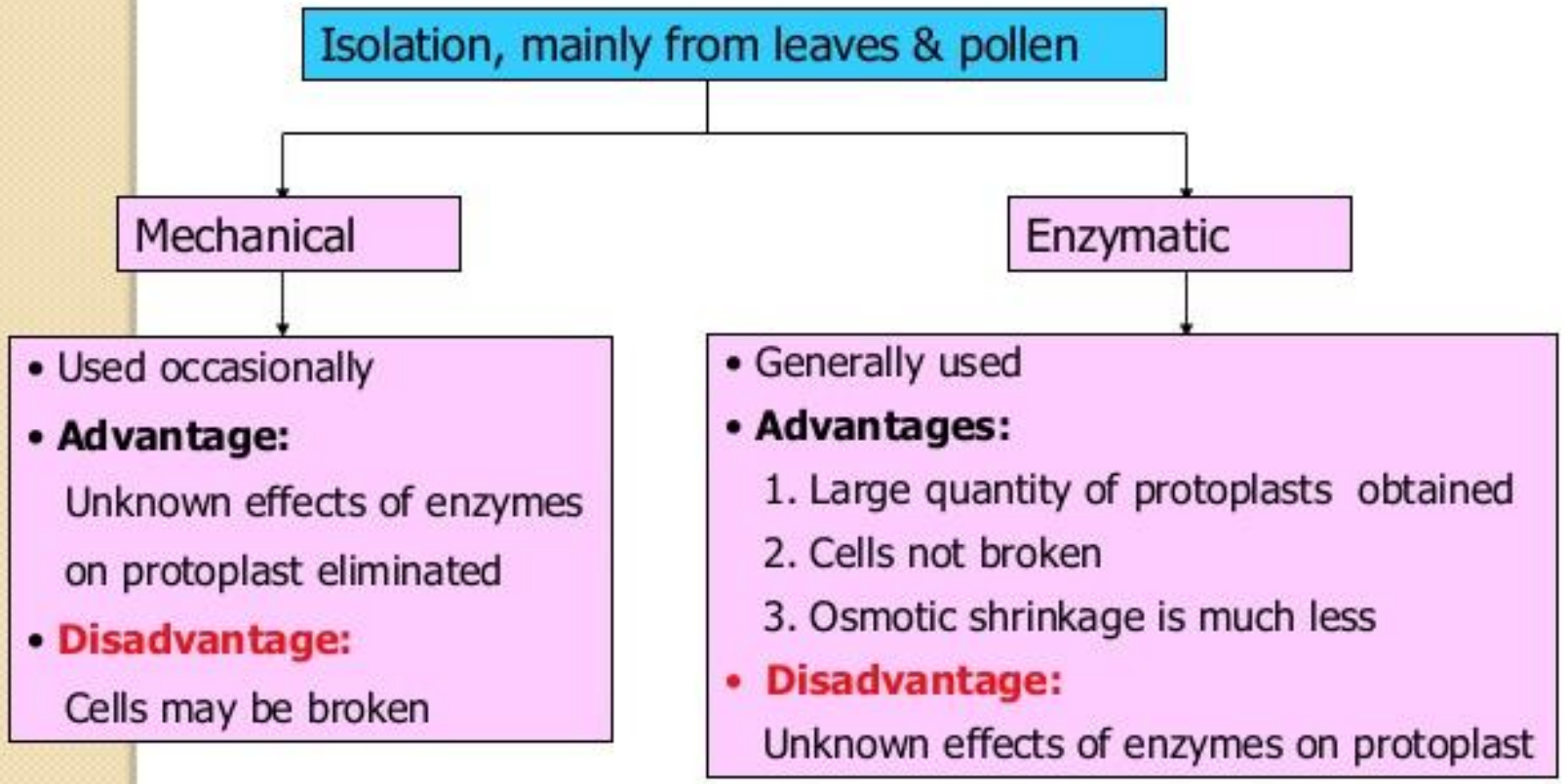


ISOLATION OF PROTOPLASTS in brief



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.

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

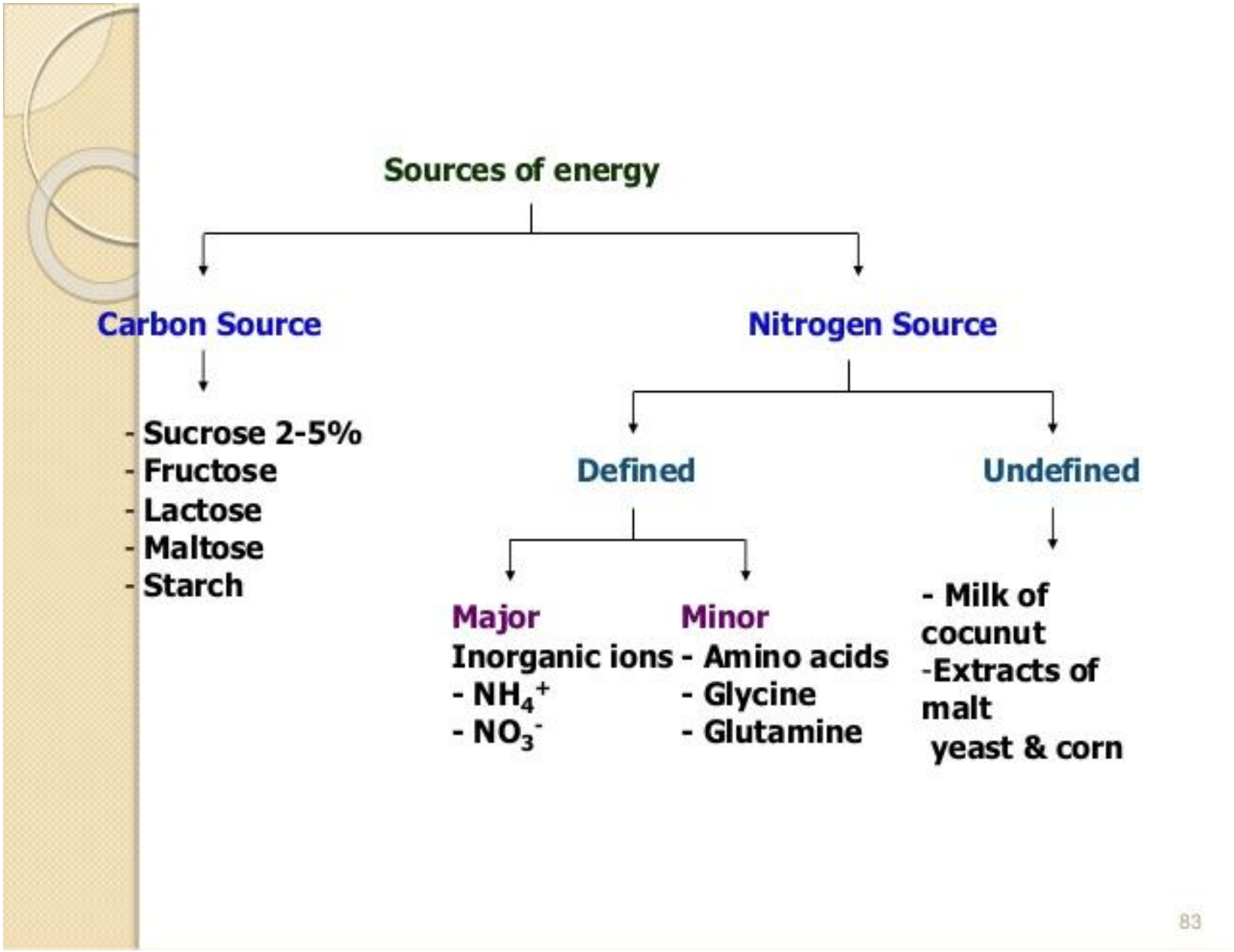
Protoplast are placed into a small culture cell containing electrodes and a potential difference (10kv m^{-1}) is applied then the protoplast will line up (form chain) between the electrodes.

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

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
- Sucrose 2-5%
- Fructose
- Lactose
- Maltose
- Starch
Nitrogen Source
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 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…

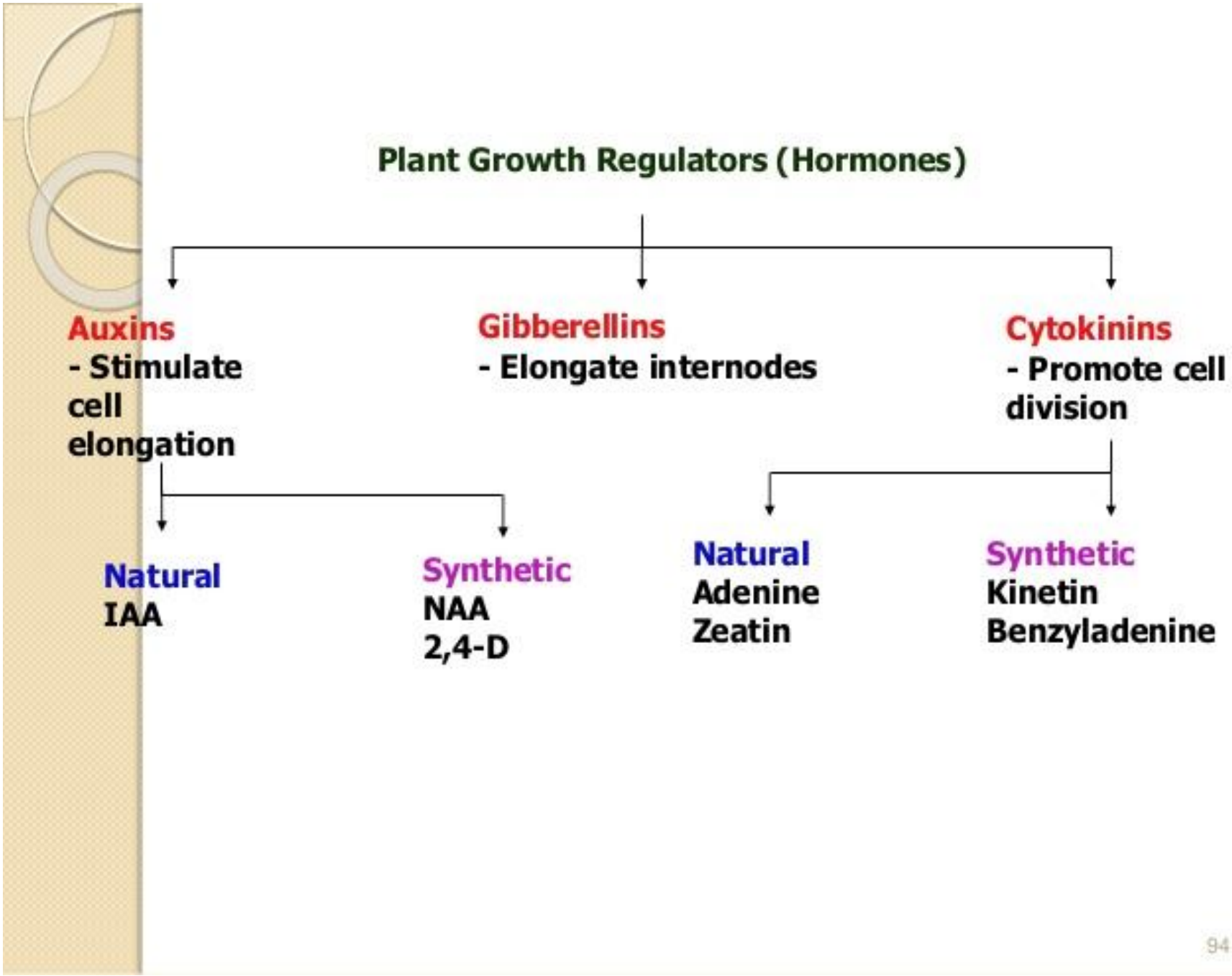
- Micronutrient: Elements required in the life of a plant less than 0.5 mmol/lit are referred as micronutrients.
- Overall the plant thrives on seventeen elements out of which four like carbon, hydrogen, oxygen and nitrogen are derived from the atmosphere and the rest thirteen microelements like Boron, 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
Gibberellins
- Elongate internodes
Cytokinins
- Promote cell division
Natural
IAA
Synthetic
NAA
2,4-D
Natural
Adenine
Zeatin
Synthetic
Kinetin
Benzyladenine

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.

- **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)

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.