MBO34A PLANT TISSUE CULTURE

NOTES PREPARED BY

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PLANT TISSUE CULTURE

DEFINATION:

Tissue culture is in vitro cultivation of plant cell or tissue under aseptic condition or on defined nutrients medium for the production of metabolites or regenerate plants.

The process requires a-1.Well-equipped culture laboratory 2.Nutrient medium

These process involves various steps: 1.Prepration of nutrient medium with inorganic and organic salts 2.Supplemented with vitamins

3.Plants growth hormones

4. Amino acids

HISTORICAL DEVELOPMENTS IN PLANTS TISSUE CULTURE TECHNOLOGY Year DEVELOPMENTS

1954	**Muir etal first suspension culture of single cell -nicotiana**
1966	Kohenbach first cell division and culture of mesophyll cell - mocleaga
1971	Nagata and Takebe regeneration of plant from culture used protoplast-Nicotiana
1981	Used of hallow fiber reactor for sec. metabolite production

Historical Development

- KLercker first attempt to isolt protoplast mechanical.
- Haberlandt 1902 first cultivation exp with isolated plant cell.
- Kotte and robbens intrvitro cultivation of root tips.
- White 1934 first permanent root culture .
- Burashies and skup 1962 development of M.S media.
- Shuler 1981 production of secondary metabolites by biotransformation.
- Phytone 1995 taxon production from plant tissue culture.

BASIC REQURIMENTS FOR A TISSUE CUITURE LABORATORY

General basic facilities

1. Equipment's and apparatus
2. Washing and storage facilities
3. Media preparation room
4. Sterilization room
5. Aseptic chamber for culture transferring
6. Incubation room
7. Observed area or data recorder room ,equipped area computer for data processing
8. Cold room

1.EQUIPEMENT AND APPARATUS CULTURE VESSELS AND GLASSWARE

1. Difference types of culture vessels are used for growing culture
2. culture can be grow in large test tubes and wide mouth conical flask [erlen merge flask]
3. For making preparation graduates pipettes, measuring cylinders, beakers, filters, funnels, and petri dishes are also required

EQUIPEMENT

1. Scissors, scalpes,and forceps for explants preparation form excised plants parts
2. A separates burners or gas micron burners for flame sterilization of instruments
3. An autoclave to sterilization the media of glassware
4. A P^H meter adjusting the P^H of the medium.
5. Shaker to mentation suspension culture or liquid culture
6. A balance
7. Incubating chamber or laminar air flow
8. A BOD incubator

2. Washing and storage facilities

1.Fresh water supply
2.Disposal of the waste
water

3. Distillation unite of supply of de-ionized water and double distilled water

4. Storage of dried glassware dust proof cupboards should be provided

3.Media preparation room

Room should have sufficient space to accommodate chemical ,tab ware ,vessels, and equipment for mixing ,hot plate ,ph. meter, water bath, oven ,autoclave , refrigerator, for storage of prepared media and stock sol^{n}.

4. Sterilization Room

For sterilization of culture media a good quality autoclave is require for sterilization of glassware and metallic equipment hot air oven with adjustable tray is required .

5 . Aseptic chamber

For transfer of culture into sterilized media, contaminate free environmental is mandatory. The simple type of area require small wooden hood, having glass or plastic door fitted with UV tube.

The advantages of working in the laminar air flow cabinets is that the flow of air does not hamper the us of burner and moreover the cabinets occupies relatively small space within the laboratory.

6. Incubation room or incubator

Environmental factor have great effect on the growth of cultured tissue. Therefore, It is very essential to incubate all type of culture in well controlled environmental condition like temp. Humidity and air circulation.

Air condition are required to maintain the temp. At 25$^{+}_{-}$ 2°. Light is adjusted in the terms of specific period for total darkness as well as higher intensity light. The humidity range of 20-90% controllable to ±03% and forced air circulation can be achieved. Ex – BOD incubator.

7. data collection and recording of the observation

The growth of the tissue culture in the incubator should be observed end recorded at regular intervals. For microscopic examination separate dust free space should be marked for microscopic work. All the recorded data should be feeded in computer.

Ingredient for nutritional requirement for tissue culture :-

1.Preparation of explant

Explant means detach portion of plant. Like root, stem, leaf, meristematic tissue,stamans,etc.

1. Somatic embryo genesis

Types of somatic embryo genesis

i) Direct

Means →tissue → embryo

ii) Indirect

Means →tissue→ calluses→ embryo

Nutritional ingredient for tissue culture

(a)Nutrients (b)Vitamin (c)Growth hormones (d)Solidified media (e)Complex extract (f)Sugar source (g)Distilled water (h)PH

a. Nutrients:

The two nutrients are used to preparation of culture media as

1.Marceonutrients:

- Phosphorous-maintain osmotic pressure
- Sulphur-synthesis of amino acids
- Magnesium – synthesis of co-enzyme

- Nitrogen-contain cell growth nucleus
- Calcium-prepared cell membrane gt -0.5 $mmol^{-1}$

b.MICRONUTRIENTS:

- Iron- synthesis of cytochrome p4 50
- Cobalt-vit B12
- Copper-co-enzymes

2. ITAMINS:

These are used to growth promotes thiamine, pyridoxine, nicotinic acid and inositol
this it are mainly used in culture media.

3.GROWTH HORMONES:

In which axin, cytokine, giberlin, zentine Oxin-requried elongation of cell Cytokine-for rooting purpous Gibertin-shouting purpous

Zentine-for embryo devlopements

.complex extract

Use a growth promotes these are yeast extract tomato and coconut milk extract.

5. Solidified media

Solidified maintain humidity and water supply to culture media

Ex:- agar, gelatine, starch , etc.

Agar → defuse→ provide sustain release to medium

6.distiled water

Double distilled water will be used to prepare media

7 . pH

pH for maintain of prepare culture medium at about pH:-5 to 6. In addition charcoal for
isolation of toxins .

media preparation

1) Dissolve macronutrient in 200 ml distilled water .
2) Dissolve micronutrient in 200 ml.
3) Dissolve Harmon’s in 100 ml and stored in refrigerator to avoid any contamination.
4) Dissolve vitamins in 100 ml water and stored in refrigerators to avoid growth of micro- organism and contamination to culture media.
5) Then add 2 gm agar and add amino acid, sugar source etc.
6) Prepare 1 lit. of culture media it the final volume of media with pH 5.5
7) Put conical flask in autoclave.

hardening:

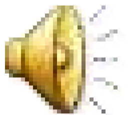
This is a method in which the tissue culture plants developed in artificial media are habituated to grow in natural environment. Firstly these plants are taken out from nutrient media and washed thoroughly with water. Then these plants are grown in netted plastic pots filled with liquid nutrient medium and kept in green house for 6 – 8 weeks.

This is called Primary hardening. Afterwards the plants are transferred to polybags filled with potting mixture and grown under shaded house for 6 – 8 weeks. This is called Secondary hardening. After secondary hardening the plants are suitable for growing in farmer's fields.

Aseptic Techniques in Plant Tissue Culture

- ✓ **Need for maintenance of aseptic conditions**
- ✓ **Sources of contaminations**
- ✓ **Prevention of contamination**
- ✓ **Conclusion**
- ✓ **Suggested reading**

- **Plant tissue culture medium very rich in nutrition**
- **Microbes can grow very fast on it as compared to plants**
- **This is harmful for the plants**
- **Therefore aseptic conditions are to be maintained in the culture vessel**



Possible sources of contamination :-

- culture vessel
- nutrient medium
- Instruments used during various operation
- Explant
- operator
- environment of the tissue culture laboratory

Glassware, plasticware and Medium sterilization:-

a)Dry heat

- Used for glassware, metal instruments etc. dry-heating in an oven at 160-180^{0}C for 3 hours approx. (1 hr heat up period to reach to temp and cooling period)
- Wrapping in aluminum foil
- Cannot be used for plastic ware, however, certain plastic wares can also be heat sterilized (Instructions of the manufacturer must be read before doing this)

b) Wet heat

- **Autoclaving (steam under pressure) or a home pressure cooker steam pressure of 1.05kg/cm^2 (temperature 121 ^{0} C) for 15- 45 minutes**
- **Time required for autoclaving varies with the volume of liquid to be sterilized**
- **Do not close the escape valve until a steady steam comes out of autoclave**
- **Actual time of autoclaving should be started when proper temperature is reached**
- **Over autoclaving should be avoided**
- **Once autoclaving is over, pressure must not lost rapidly, it should be allowed to return to atmospheric pressure slowly as rapid loss of pressure will lead to vigorous boiling of liquids inside the culture vessels**
- **It should be opened when the pressure is zero as this might cause accidents**

➢ **Now a days pre-sterilized ready-to-use plastic ware is available, which can be used directly to pour medium etc.**

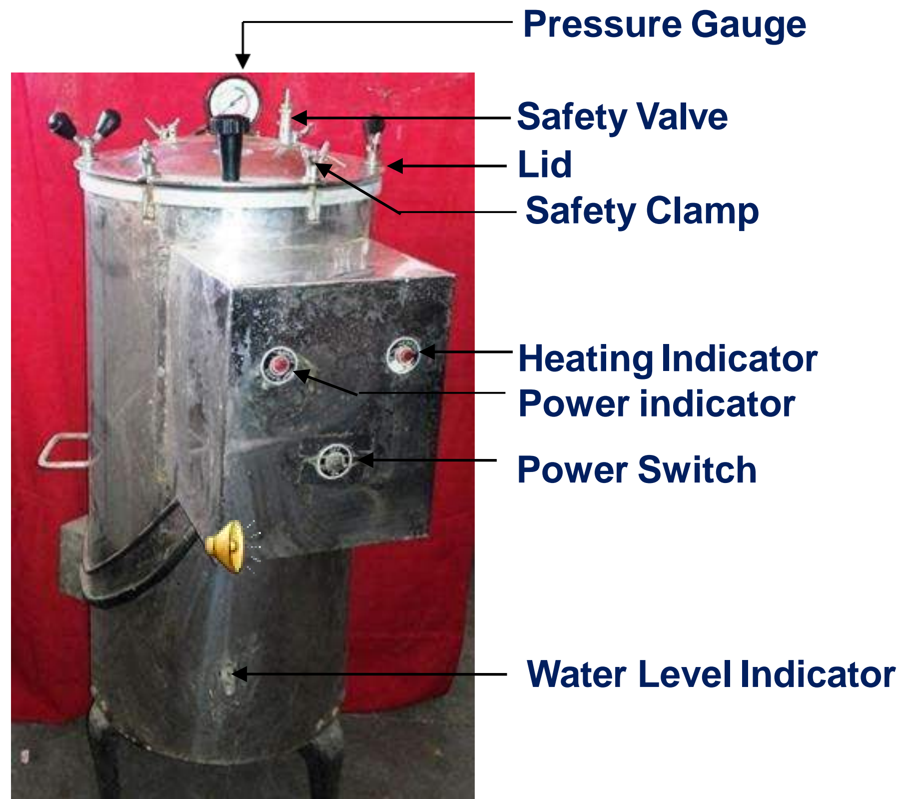


Figure. Autoclave unit used for wet sterilization of nutrient media

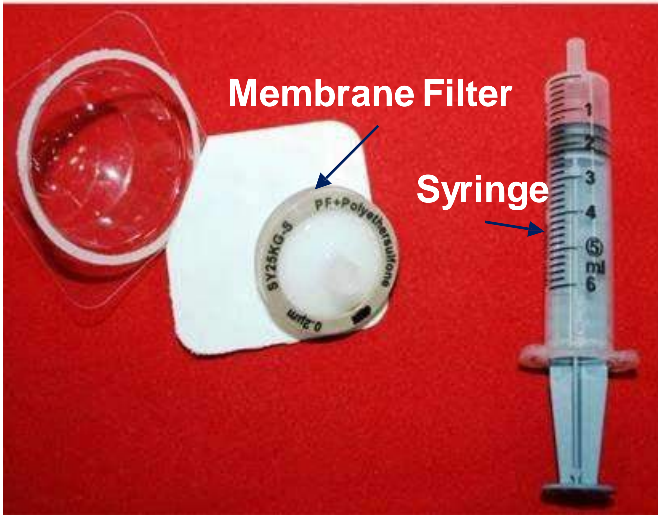
- **Certain components of medium like Zeatin, GA3, pantothenic acid, antibiotics etc. are thermolabile and cannot be autoclaved**

- **These can be sterilized by membrane filtration and added to autoclaved medium once it has cooled down to ~ 40^0C**

c) Filter sterilization:

- **filter membranes of pore size 0.45 µm or less are used**
- **Filter assemblies of different sizes are available**
- **Once the component is filter sterilized, it is collected in a sterile container which can be used immediately or dispensed in smaller amounts to be used later**

- **These filter sterilized components can be stored at 4^0C or -20^0C depending on the frequency of their usage**



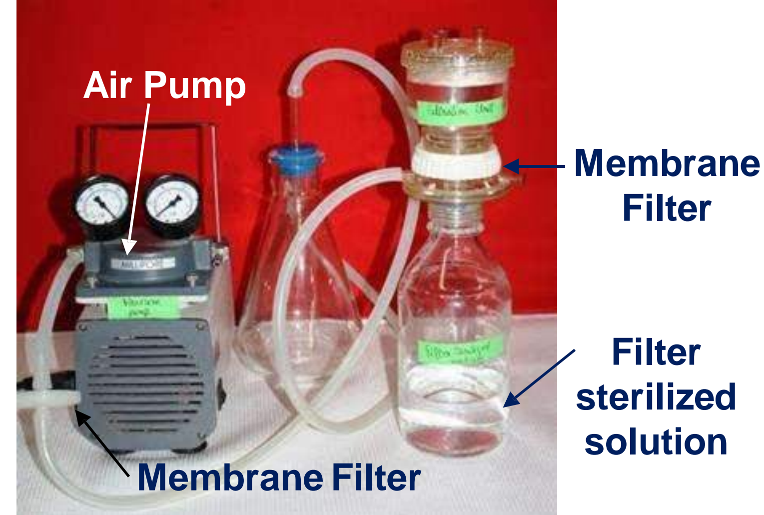


Figure. Filter sterilization techniques

Instruments :-

- **Sterilized by dipping in 95% ethanol followed by flaming and cooling**
- **Glass bead sterilizer and infra-red sterilizer are available commercially operated by electricity these instruments are safer and not a fire hazard**
- **Glass bead sterilizer has glass bead in a heated cavity where a temperature of nearly 250^{0}C is maintained instruments are pushed into the cavity for 5-7s**
- **Infrared sterilizer also has a cavity where a temperature of nearly 700^{0}C can be achieved by infra-red wave heating. Exposure of 2-5 s is effective for sterilization of instruments**

Scalpel

Scissor

Scalpel holder

Forceps (Small, Medium & Large)

Figure: Commonly used instruments in plant tissue culture

Plant material

- Growing in nature and exposed to a variety of microbes so it is a very rich source of contaminants and needs to be surface sterilized before inoculation into the medium
- Variety of surface sterilizing agents are available which vary in their efficacy and toxicity
- Too strong treatment can kill the explants whereas too mild treatment may not yield any sterile explant
- So sterilizing treatment is selected on the basis of the state of explants
- explants are hardy and apparently contaminated a strong treatment can be given if explants are juvenile and soft, a mild treatment should be preferred
- In certain cases surface sterilization of the actual explant may not be required. e.g. for culturing the immature ovule the whole ovary is surface sterilized and the ovule is dissected out under aseptic conditions

- Generally adding few drops of surfactant in the sterilizing solution enhances its efficiency

- **environment of the tissue culture laboratory**

Media room

- Maintain cleanliness, removal of contaminated culture, restricted entry

Washing room

- Contaminated cultured should be autoclaved and discarded with utmost care, maintain general cleanliness

Transfer area

- A sterile area is required for performing various aseptic manipulations during tissue culture. This ensures that contaminants do not gain entry into the culture vial

- Laminar air flow cabinets of various shapes and sizes are available commercially. These cabinets allows the tissue culturists to work in the sterile environment for long stretch of time. It provides a covered enclosed area for working

HEPA Filter

Acrylic Sheet

Gas Burner

Working surface

Pre-Filter

Figure: Laminar air flow cabinet in use

Transfer area

- There are UV lights inside the chambers which are switched on for 10-15 minutes before using the laminar air flow cabinet

- It has small motors at the base for blowing the air which is first passed through coarse filter. This step ensures removal of large contaminants. Then the air passes through fine filters called HEPA filters. HEPA stands for 'High Efficiency Particulate Air' . These filters removes impurities which are larger than 0.3 µm therefore the air coming out of these is clean.

- Air coming out of these filter comes with some force which prevent entry of contaminants from the worker or environment into the working area

- A gas burner or spirit lamp facility is also available for flaming the instruments

Growth room

- Maintain cleanliness, removal of contaminated culture, restricted entry

Figure: Regular inspection of culture and removal of contaminated samples reduces the chances of contaminations

Operator:

- **Clean hand and forearm properly before starting the work**
- **Hands may be cleaned with dilute solution of alcohol or commercial hand sanitizers available in the market**
- **Wearing a surgical mask and head gear and lab coat while working in the laminar will reduce the chances of contamination**
- **Presence or other persons in or near the transfer area should be minimized .**

Cellular totipotency in plants

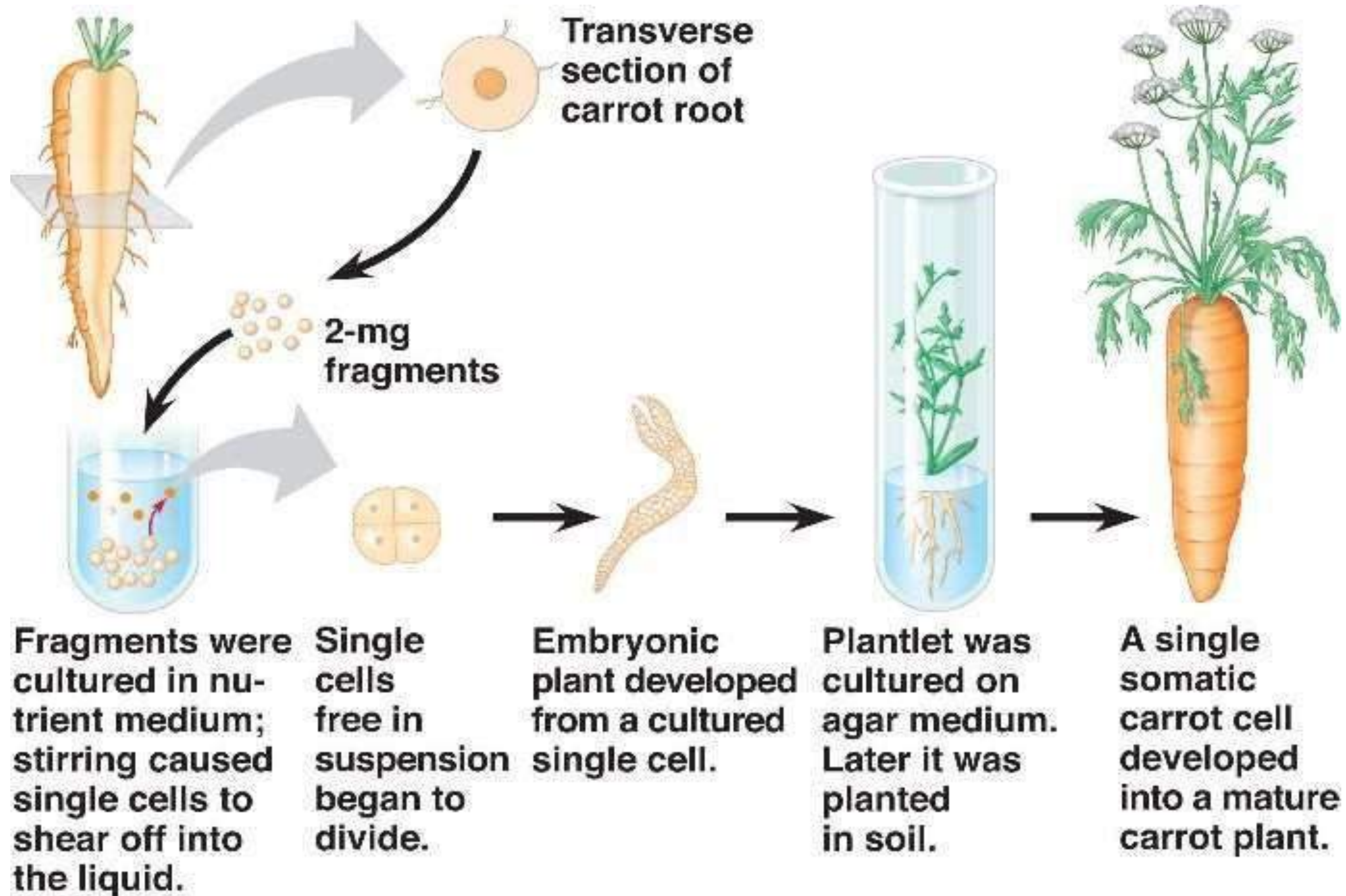


Fig 1: Expression of Totipotency in Culture

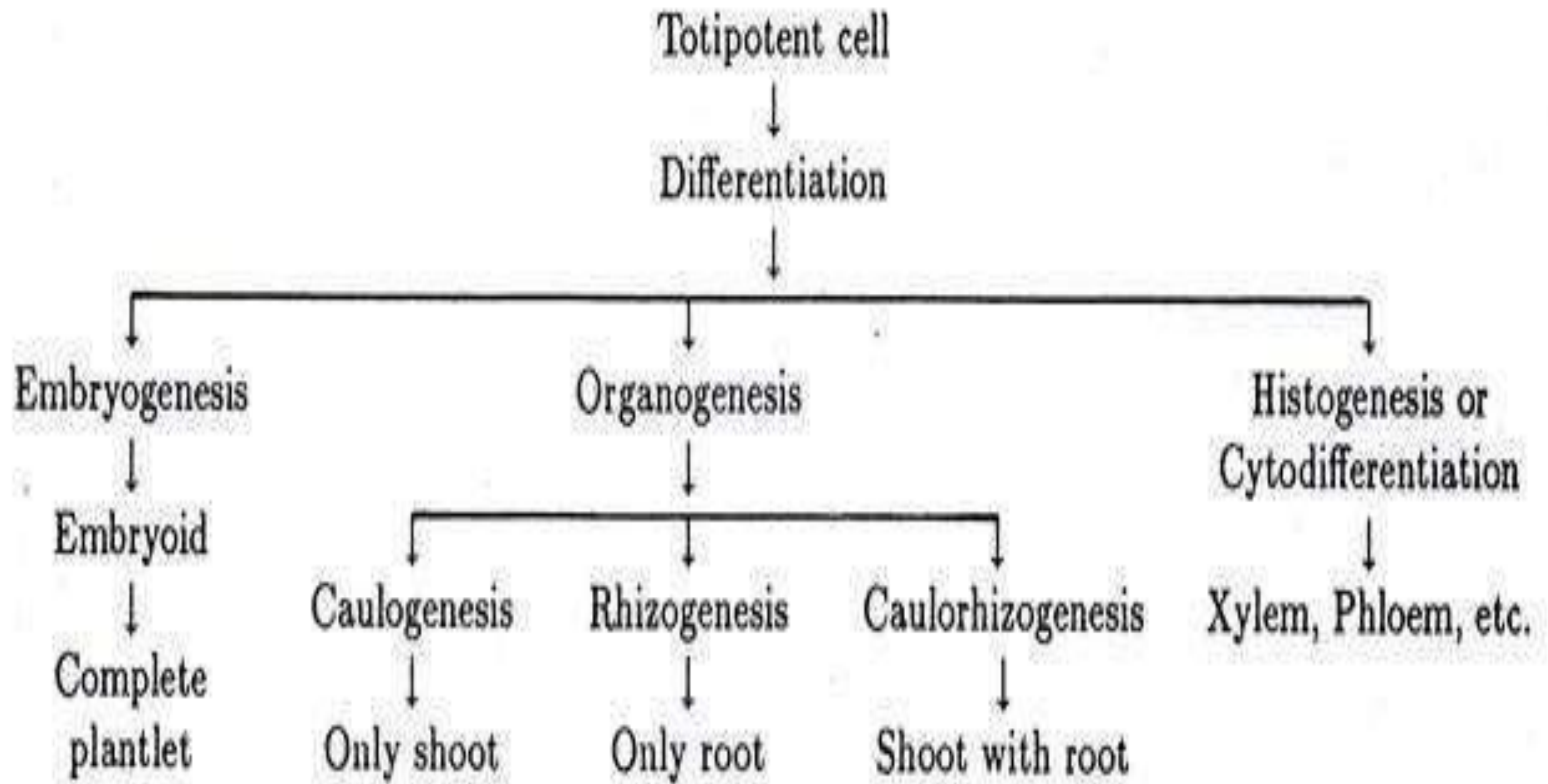


Fig 2 : Differentiation of totipotent plant cell

CULTURE SYSTEMS

Callus culture :

unspecialized, unorganized, growing and dividing mass of cells.

- Can be maintained indefinitely.
- Suitable for cytodifferentiation.
- No photosynthesis and grows in dark.
- Can be used to isolate single totipotent cells.
- Many cultures lose their potential for differentiation duting continual subculture due to epigenetic changes.
- Difficult to follow many cellular events during its growth and developmental phases.

Suspension culture :

- **Type of culture in which single cells or small aggregates of cells**

multiply while suspended in agitated liquid medium.

- **Receive more homogeneous stimuli in a defined medium**

supplemented with the requisite amount of inducers such as

sugar or auxin.

- **Can be studied for the production of secondary metabolites**

such as alkaloids.

- **Produce mutant cell clones from which mutant plants can be raised.**
- **Possible to analyse biochemical pathways related to**

differentiation of cells.

Single cell culture :

- Method of growing isolated single cell aseptically on a nutrient medium under controlled conditions.
- Can be isolated from a variety of tissue and organ of green plant as well as from callus tissue and cell suspension either mechanically or enzymatically.
- Could be used successfully to obtain single cell clones.
- Plants could be regenerated from the callus tissue derived from the single cell clones.
- Isolated single cells can be handled as a microbial system for the treatment of mutagens and for mutant selection.
- Single cell culture is an ideal system for the study of biotransformation.

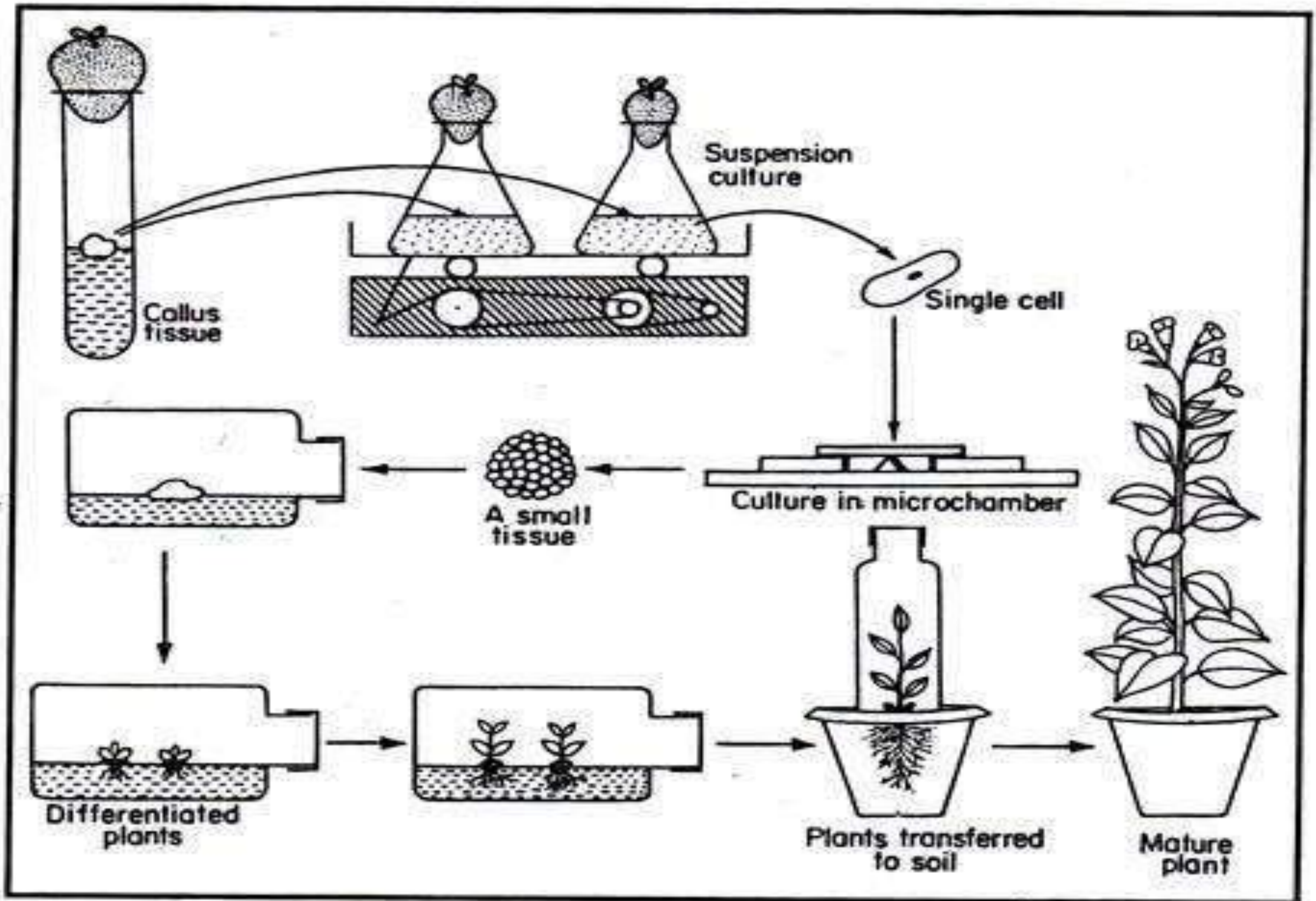


Fig 9.6

Development of a tobacco plant from a single cell

Protoplasts culture :

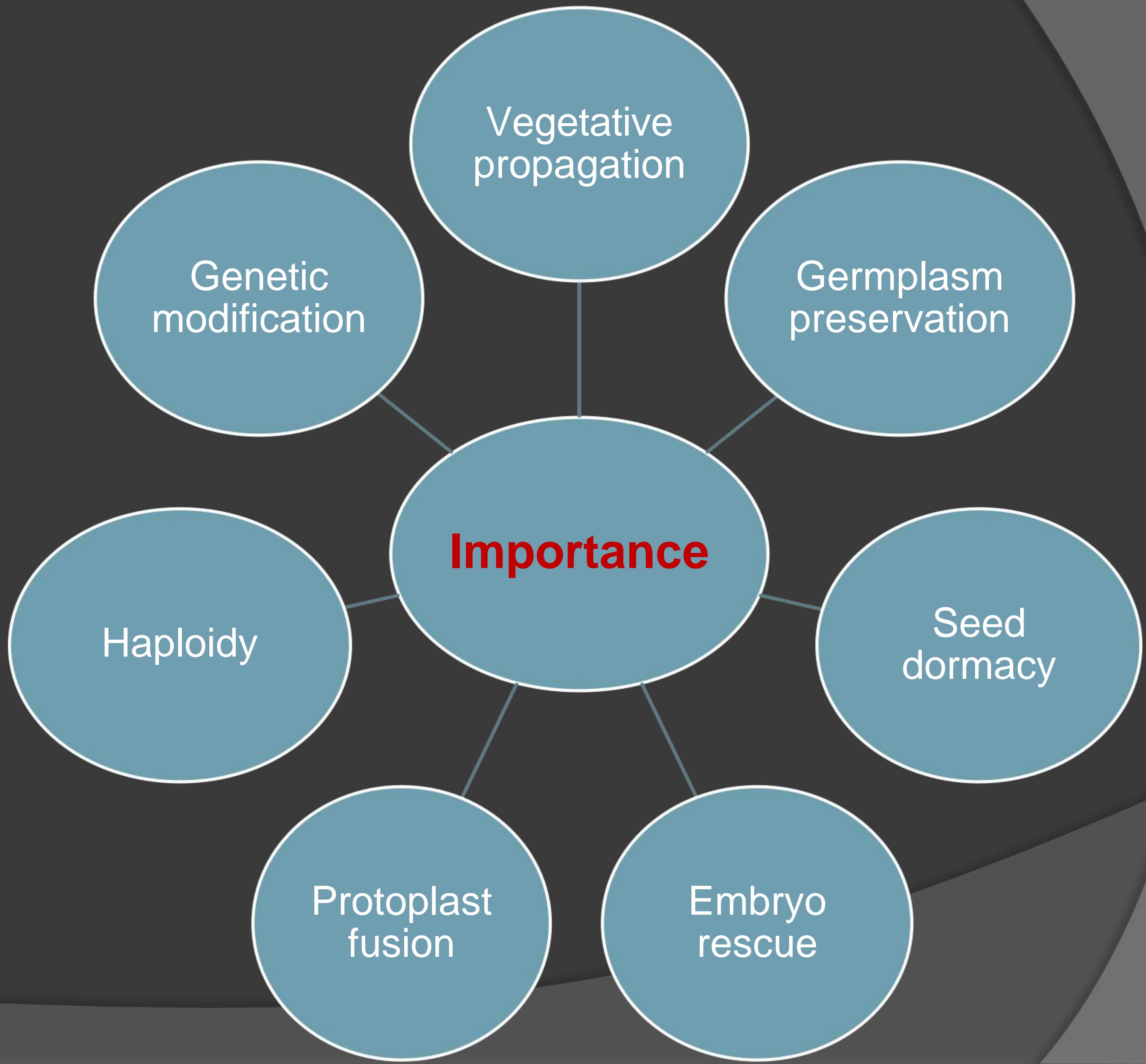
- Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components.
- Isolated most frequently from mesophyll tissue of fully expanded leaves of young plants or new shoots, either mechanically or enzymatically.
- The protoplast in culture can be regenerated into a whole plant.
- Hybrids can be developed from protoplast fusion.
- It is easy to perform single cell cloning with protoplasts.
- Genetic transformations can be achieved through genetic engineering of protoplast DNA.
- Protoplasts are excellent materials for ultra-structural studies.
- Isolation of cell organelles and chromosomes is easy from protoplasts.
- Protoplasts are useful for membrane studies (transport and uptake processes).

Some factors that affect cellular totipotency are :

I. **Source of explant :**

II. **Nutrient media and constituents :**

III. **Culture environment (Physical form of medium) :**

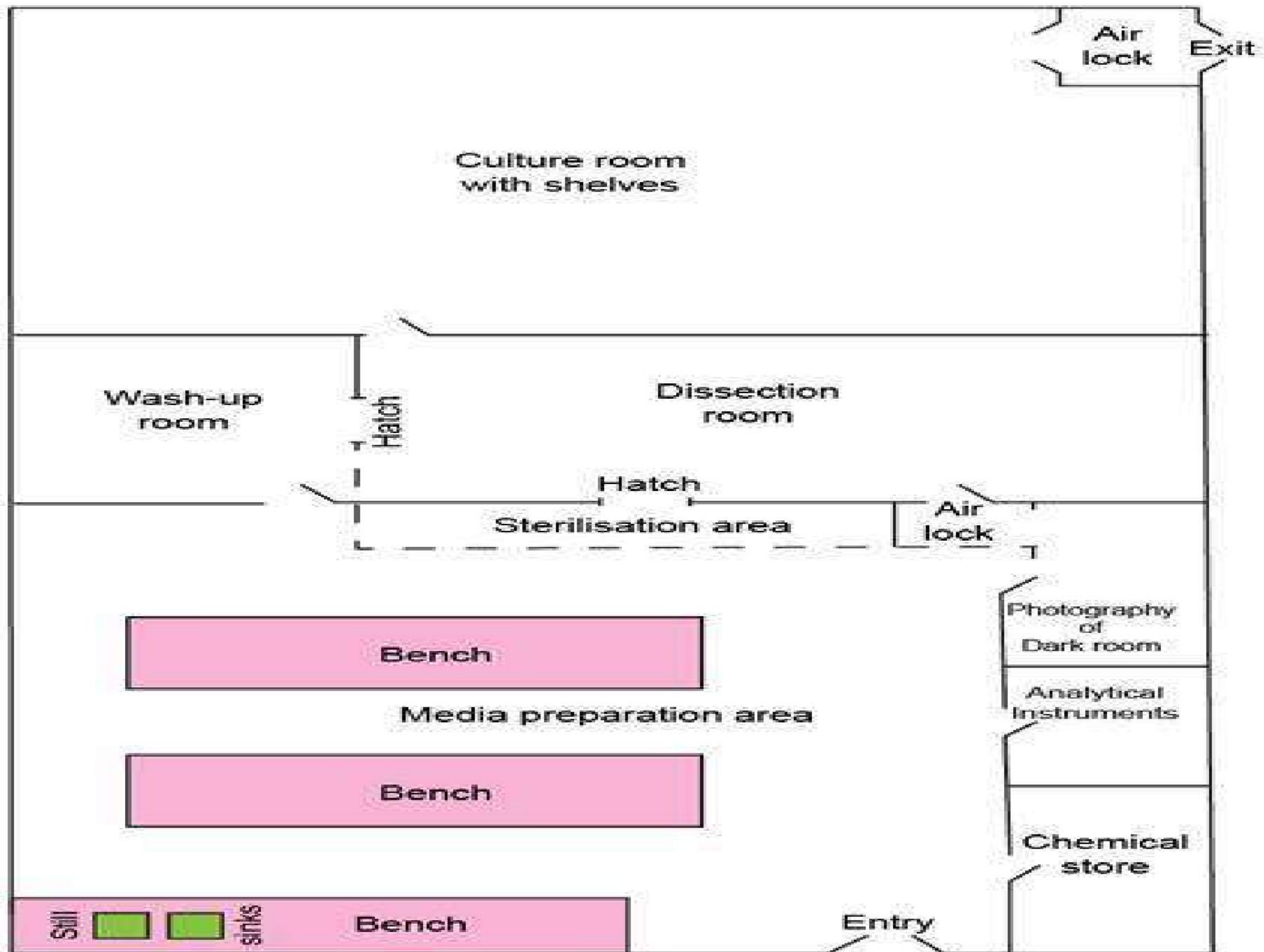

Vegetative propagation
Genetic modification
Germplasm preservation
Importance
Haploidy
Seed dormacy
Protoplast fusion
Embryo rescue

PLANT TISSUE CULTURE LABORATORY

NEED OF LOW CAST LAB.

- Low cost options should lower the cost of production without compromising the quality of the micro-propagation and plants.
- Micro propagation technique is too much costly.
- Potential increasing interest in agricultural crop production increased.
- Low-cost tissue-culture technology will stay a high priority in agriculture, horticulture, forestry, and floriculture of many developing countries for the production of suitably priced high quality planting material.

GENERAL REQUIREMENT
FOR
SET UP
A LOW COST LABORATIRY



Air lock
Exit
Culture room with shelves
Wash-up room
Hatch
Dissection room
Hatch
Sterilisation area
Air lock
Photography of Dark room
Bench
Analytical Instruments
Media preparation area
Bench
Chemical store
Still
sinks
Bench
Entry

LOCATION

- The location shall confirm to the land use plan of the area.
- The building should be located away from sources of contamination such as soil mixing area, storage etc.
- The site shall be accessible to service roads, water supply and electric lines.
- The site shall be well drained.

STRUCTURAL REQUIREMENT

- The roof structure should be treated timber or steel with anti-rust paint. Roof vents, when provided, shall be properly screened.
- Ceilings shall be at least 2.4 m from the finished floor line and painted with latex paint and should be prevent from dust.
- The wall shall be concreted, smooth finished and properly painted with anti-fungal- epoxic-paints and slope shall be at 45^{0c}
- All windows shall be properly installed with 16-mesh screen and at least 1 m from the finish floor line.
- Main door ways shall be at least 1.5 m wide and moisture and rust-resistant material..
- Floor shall be concreted, smooth, non-skid, slope 2% -4% towards the drain.

THE BASIC FACILITIES OF LABORATORES

- One Room With Computer With Locker For Keeping Apron
- Working Place
- Layout Of Plant Tissue Culture -
- Washing Room
- Sterilization Room
- Media Preparation Room
- Inoculation Room
- Incubation Room.
- Skilled Labour

WASHING ROOM

- The glassware washing area shall be located near the sterilization and media preparation areas.
- Washing sink(45cm x 60cm) can be fitted in one side of Room and should be acidic and alkali resistant.
- Room also provided with Bucket, detergent , mug and also Plastic tray to kept washed Media bottles and sterilized media.
- Room provided with water line and electricity line.

STERILIZATION ROOM

- The room should have One autoclave(vertical or horizontal) or cooker with well ventilated exhaust fan.
- Also having tools for support while placing the media bottles in autoclave or cooker for sterilization.
- The bottles should sterilized At 121^{0C} at 15 lb. for 1 hrs.
- It contains wrapping paper to wrap and the rubber to tight the inoculating dishes for sterilization.

MEDIA PREPARATION ROOM

- The area shall be provided with working tables which should be up to 1.2 m wide and the height should be 850 mm - 900 mm.
- Table top shall be covered with materials which are easily cleaned and which will stand disinfectant solution.
- Water source and glassware storage area shall be provided.
- Room contains double distilled water unit , pH meter, buffer solution, electronic balance , cooling freeze, measuring cylinder, magnetic stirrer, pipette, hot plate, media bottle.
- Room should contains the stock A,B,C,D, E and F solution with required chemicals such as sucrose, adenine sulphate , innositol , agar-agar(solidifying agent) etc.

INOCULATION ROOM

- The floor of room covered with tiles to facilitate proper cleaning.
- Room contains the laminar air flow cabinet with installation of a HEPA (high efficiency particulate air) filter for filtered air supply.
- It also requires sodium hypochlorite, mercuric chloride and 70% alcohol for surface sterilization .
- It requires forceps , scalpel, sterile bottles, sterile dishes, spirit lamp , cotton and hand care etc.

INCUBATION ROOM

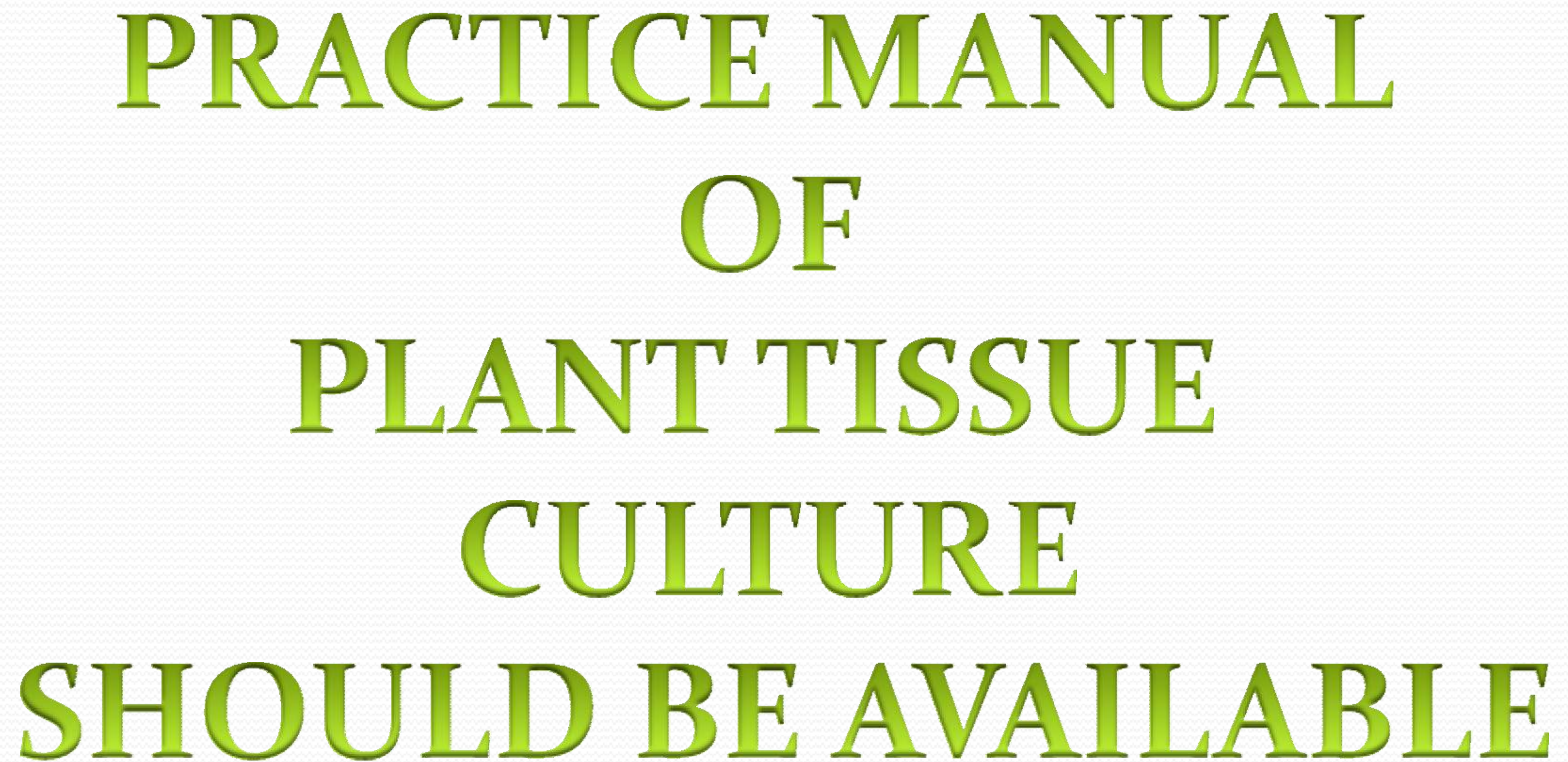
- **The width of the racks should be 405 mm to 1 m. The distance between each layer of the racks should be not less then 455 mm.**
- **Photoperiod racks with culture bottles shall be provided. we mostly operate light intensity with a standard day length of 16 hours and 8 hour dark regime.**
- **Temperature is maintained at a standard 25 + 2 o C with 1.5 ton window A.C. to creating the controlled environment condition.**
- **Light intensity required about 200 lux to 1600 lux for growth of culture.**

INSTRUMENT ROOM

- Bottle washing machine and autoclave.
- Freezer and Rotary shaker.
- Multipurpose Magic stove.
- Weighing balance, thermometer .
- Magnetic stirrer and Digital pH meter.
- Laminar air flow with U.V. tube .
- Racks-photoperiodic culture Glass bed steriliser .
- Air conditioner with Stabiliser and Micro air oven etc.

Glassware and Other requirement

- Glass bottle, Petridis, burette, funnel.
- Beakers and pipettes.
- conical flask with screw cap.
- Graduated measuring cylinder
- Spatula, scalpel and forceps
- Wrapping paper and bucket, gloves etc.


PRACTICE MANUAL
OF
PLANT TISSUE
CULTURE
SHOULD BE AVAILABLE

Total Cost For Low cast Lab. Set up

General Chemical Cost

- Glasswares - 10,000/-
- All chemicals – 20,000/-
- Aluminium racks – 5,000/-
- Other requirement for laboratories – 25,000/-

Instrumentation

- Water Purifier System – 15,000/-
- Electronic Balance – 1,200/-
- pH Meter – 10,000/-
- Hot Plate – 1,000/-
- Refrigerator – 15,000/-
- Laminar Air Flow – 25,000/-
- Pipette – 10,000/-
- Autoclave – 20,000/-
- Computer – 12,000/-

OTHER FACILITIES

- Hand washing facilities
- Fire extinguishing system
- Waste disposal area should be provided

Plant Tissue Culture medium

Plant Tissue Culture medium

- Media used in plant tissue culture contain nutritional components.
- Nutritional components are essential for growth and development of cultured tissue
- The success of the tissue culture depends very much on the type of culture media used.

- Each plant tissue culture medium must contain the following essential components to support invitro plant growth.
- These are as follows.

1) Macro inorganic nutrients.
2) Micro inorganic nutrients
3) Iron (as chelating agent)
4) Vitamins
5) Carbon sources
6) Organic nitrogen
7) Plant growth regulators
8) Agar (as gelling substance)

Nutrients

Inorganic nutrients

Organic nutrients

macro nutrients

micro nutrients

1. vitamins
2. hexitols
3. amino acids
4. carbohydrate

Nitrogen
Phosphorus

1. **Macro nutrients**

- Need of macro nutrients is higher.
- It is present in milli molar (mM) quantities (more than 30 ppm/1 or mg/1)
- Macro nutrients provide both anions and cations for the plant cells.

- Macro nutrients are nitrogen (as NO3 and NH4),phosphorus(PO4),potassium(K),sulphur(as SO4),magnesium(Mg),and calcium(Ca).

- Macro nutrients have structural and functional role in protein synthesis, cell wall synthesis enzyme Co-factors and membrane integrity.

Nitrogen

- In organic form used as amino acids, different organic acids and casein hydrolysate.
- In inorganic form used as Nitrate or ammonia.
- Nitrogen is major component of all plant tissue culture media.
- Nitrogen helps to synthesis complex organic molecule.

Potassium

- K ion is present in high concentration in the cytoplasm (100-200 mM) and in chloroplast(20-200 mM).
- K+ is essential for mentaining the ion balancing, activation of many enzymes. Maintaining osmotic pressure and osmotic regulation of cells.

Calcium

- Calcium functions with different enzymes as Co-factor and bound to the cell wall and cell membrane.
- It gives strength to cell wall.
- It helps in the regulation of the the cell membrane structure.
- Deficiency causes disintegration of the membrane and shoot tip necrosis.
- Important in cell and root multiplication.
- Supplied as calcium chloride and calcium nitrate.

Phosphorus

- Phosphorus
- Very important for energy metabolism.
- Essential element for DNA & RNA.
- Deficiency may cause delayed growth and dark green coloure of leaves.
- Supplied as sodium hydrogen phosphate or potassium hydrogen phosphate.

Magnesium

- Essential for enzymatic reactions, energy metabolism(ATP synthesis).
- Supplied as magnesium sulphate.

- Sulphur
- Important substance.
- Deficiency of Sulphur inhibits protein synthesis and decreases Chlorophyll in leaves.
- Supplied as magnesium Sulphate and Potassium Sulphate.

- **Micro Nutrients**
- Boron(B), Manganese(Mn),Zinc(Zn),Molybdenum(Mo),Copper(Cu),Cobalt(Co).
- Used in less amount less than 30ppm.(mg/l).
- Concentration is always in uM.

- Zinc
- Zn plays an active role in protein synthesis and in the synthesis of tryptophan.
- Supplied as Zinc Sulphate.

Manganes

- Plays an important role in the Hill reaction of photasynthesis.
- Required in many enzymatic activities.
- Supplied as Manganese Sulphate.

Copper

- Copper plays important role in photosynthesis.
- Intermediate of the electron transport chain between photo system 1 & 2
- Deficiency leads to decrease in photosynthesis.
- Supplied as Copper Sulphate.

Molybdenum

- Essential for conversation of Nitrate to Ammonium.
- Supplied as Sodium molybdate.

Boron

- Involves in different enzymatic activities.
- Supplied as Boric acid.

Iron

- Important Enzyme Co-factor
- Supplied in uM quantities.
- It is supplemented with chelators and Complex compounds due to its solubility problem.
- Supplied as Na2FeEDTA
- Iron deficiency have severe effects on the growth and development plant cells.

Organic Nutrients

1. Vitamins

- Plant synthesis required vitamins.
- Essential for many biochemical reaction.
- Cultured cell are capable to produle vitamins at some level.
- They require an exogenous supply of different vitamins for optimum growth.
- Most usable vitamins are Thiamine, Pyridoxine nicotinic acid Vitamin B Complex.

Hexitols

- Most tissue culture media have this compound.
- Essential for seed germination, sugar transport, carbohydrate metabolism, membrane structure and cell wall formation.
- Mannitol and sorbital are hexitols.

Amino Acids

- Glycine is the most common Amino Acid used in different culture media.
- It is not essential but Nitrogen containing Amino Acid enhance growth and plant regeneration.

Carbohydrate

- Cells and tissue reduires exogeneous supply of carbohydrates to replace the carbon which the plant normally fixes from the atmosphere by photosyntheis.
- Supplied by adding sucrose.
- Concentration is 20-30 gm/l.

Gelling Agent

- Agar – Agar
- Agar is a natural product of seaweeds.
- Since 1658 agar-agar is obtain from red algae (Gelidium Gracilaria)
- With water it melts at 100’C and solidify at 45’C

Agarose

- It is highly purified agar prepared from Gelidium sp. Of seaweed.
- Agarose melt and gel at temperatures below 30'C and dissolve through boiling.
- Agarose is much more expensive agar-agar

Gelrite or Phytagel

- Gelrite is a naturally derived polymer and produced by the microbial termentation of a bacterium Pseudomonas elodea.
- It is low cost gelling agent.
- 0.1-0.2 % concentration pen litre required.

Natural Media

- Endosperm fluid / coconut milk
- Fruit materials
- Potato extract
- Extracts of malts, yeast
- Animal extracts
- Protein hydrolysates

- Coconut fruit
- Orange juice,
  Tomato juice
  Banana pulp
- potato
- Malt, Yeast
- Fish emulsion
- Casein hydrolysate
  Peptone

Plant growth regulators

- A plant hormone can be defined as a small organic molecule that elicits a physiological response at very low conc.
- PGS plays an important role in the phenotype.
- Act as messenger between environment and the genome.

Auxins

- Essential for cell division, cell elongation, cell differentiation, organogenesis and embryogenesis, callus formation
- Natural form auxins are IAA, IBA, PAA
- Synthetic form of auxins are NAA, 2, 4-D.

Cytokinins

- Cytokinins promote cell division, shoot proliferation and influence the cell cycle.
- Embryogenesis and inhibit roof formation.
- Synthetic form is 2-ip which is most active cytokinins.
- Natural forms are BAP and kinetin.

Gibberellins

- It promotes stem elongation, bulb corm formation and embryo maturation but can inhibit callus growth and root induction.
- GA3 is most common gibberellins.

Abscisic acid

- It inhibits shoot growth and germination of embryo.
- It is thermostable but light sensitive

Ph of tissue culturemedia

- Ph is adjusted between 5 & 5.8 before gelling and sterilization with the help of dilute NaOH, KOH or HCL.
- Ph below 5 will not gel properly.
- Ph above 6 may be too hard.

Sterilization techniques used in Plant Tissue Culture ,MEDIA,PREPARATION

- **Technique Materials sterilized**
- **Steam sterilization/Autoclaving**
- (121°C at 15 psi for 20-40 min) Nutrient media, culture vesels, glasswares and plasticwares
- **Dry heat (160-180°C for 3h) Instruments (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares**
- **Flame sterilization Instruments (scalpel, forceps, needles etc.), mouth of culture vessel**
- **Filter sterilization (membrane filter**
- made of cellulose nitrate or cellulose acetate of 0.45- 0.22μm pore size) Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
- **Alcohol sterilization Worker's hands, laminar flow cabinet**
- **Surface sterilization (Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc) Explants**

- **Preparation of Media: This is a very crucial step for the experiment to be successful. While making the media taking individual constituents, each ingredient is separately weighed and dissolved before putting them together. After making up volume by water, pH is adjusted and then medium is autoclaved. Preferably, following four stock solutions are prepared:**
- • Major salts (20X concentration)
- • Minor salts (200X concentration)
- • Iron (200X concentration)
- • Organic nutrients (200X concentration)

- Separate stock solution for each growth regulator is prepared. Appropriate quantities are taken from stocks and mixed to constitute basal medium. Required quantity of agar, sucrose and organic supplements if needed are added separately.

- ***5.Solidifying agents: are used for preparing semisolid tissue culture media to enable explant to be placed in right contact with the media (not submerged but on surface or slightly embedded) to provide aeration. Agar is high molecular weight polysaccharide obtained from seaweeds and can bind water. It is added to the medium in concentration ranging from 0.5% to 1% (w/v). Agar is preferred over other gelling agents because it is inert, neither does it react with media constituents nor digested by plant enzymes.***

(unlike agar which is translucent) and hence

support for cell or tissue growth can also be

polyurethane foam etc.

- **pH: pH affects absorption of ions and also solidification of gelling agent. Optimum pH for culture media is 5.8 before sterilization. Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development** ***in vitro*****.** ***The pH of culture media generally drops by 0.3 to 0.5 units after autoclaving and keeps changing through the period of culture due to oxidation and also differential uptake and secretion of substances by growing tissue.***

Topic : sterilization

Sterilization

- Sterilization is the process of killing all forms of microbial life in or on the given object or preparation
- Articles having direct application on humans and animals are subjected to sterilization
- These materials include gloves, drugs, laboratory equipments, surgical equipment, etc

Methods of sterilization

Sterilization is done by 2 main methods

- **Physical methods**

- Heat
- Radiation
- Filtration

- **Chemical methods**

- gaseous sterilants
- Liquid sterliants

Physical methods

- **Heat**

- It is the most reliable method. The application of heat is a simple, cheap and effective method of killing pathogens. Methods of heat application vary according to the specific application.
- There are two types of heat i. moist heat ii. Dry heat.

Radiations

- UV, x-rays and gamma rays are used
- X-rays and gamma rays are ionizing and far more penetrating
- UV has limited penetration , kill DNA of microbes

Filtration

- Filters are used to sterilize heat sensitive materials
- enzymes, vaccines,drugs and vitamins
- Filter microbes

Chemical methods

➢ **Propylene oxide**

- In industry for sterilizing culture media, powdered and flaked foods, barley seeds and dried fruits

➢ **Beta- Propiolactone**

- Used in the pharmaceutical industry to sterilize plasma and vaccines and to fumigate houses

Ethylene Oxide:

- Ethylene oxide gas treatment is one of the common methods used to sterilize because of its wide range of material compatibility.
- It is also used to process items that are sensitive to processing with other methods, such as radiation

Aldehyde

- Glutaraldehyde and formaldehyde solutions are liquid sterilizing agents.
- Glutaraldehyde and formaldehyde are volatile, and toxic by both skin contact and inhalation.
- The mechanism of action of formaldehyde is based on protein denaturation.

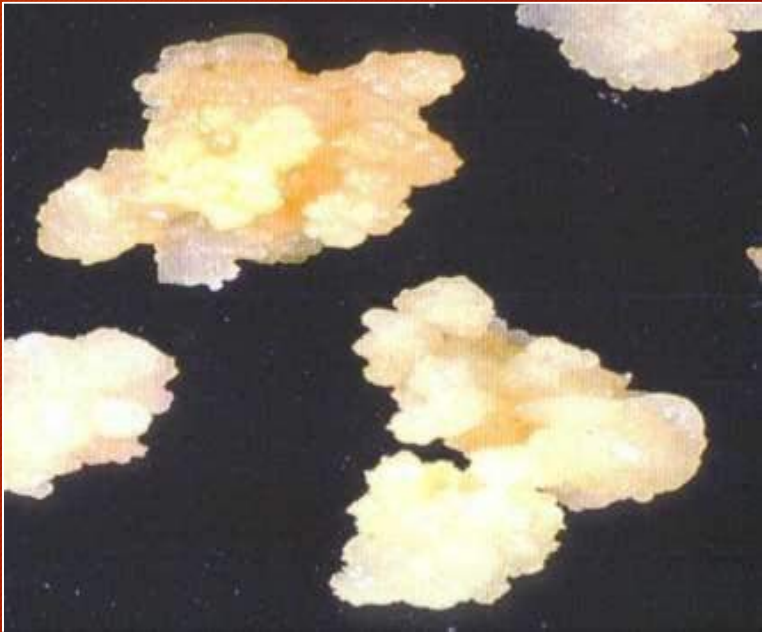
Phenol:

- Phenolics are phenol (carbolic acid) derivatives. These biocides act through membrane damage and are effective against enveloped viruses, fungi and vegetative bacteria.

Alcohols

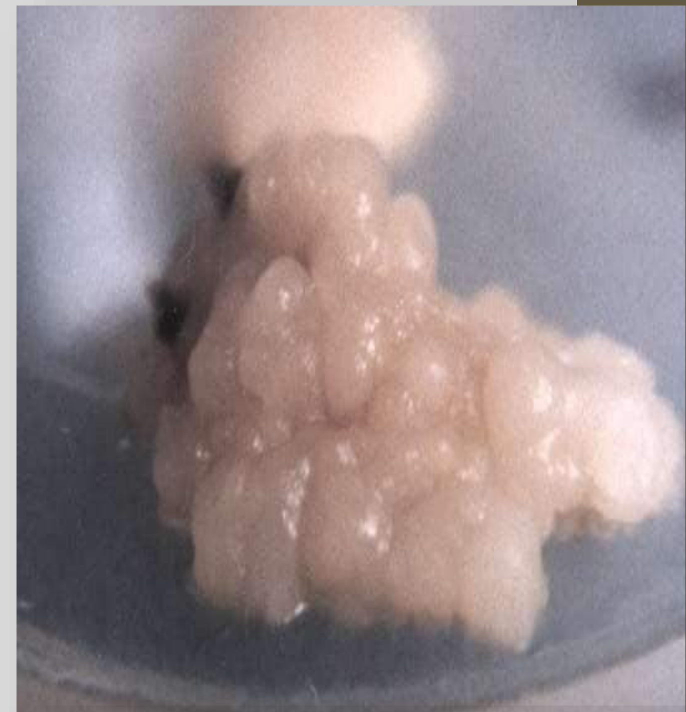
- Ethanol or 2-propanol is used to disinfect skin and decontaminate clean surfaces.
- These are effective against fungi, vegetative bacteria, Mycobacterium species and some viruses.
- Alcohols work through the disruption of cellular membranes, solubilization of lipids, and denaturation of proteins by acting directly on S-H functional groups.

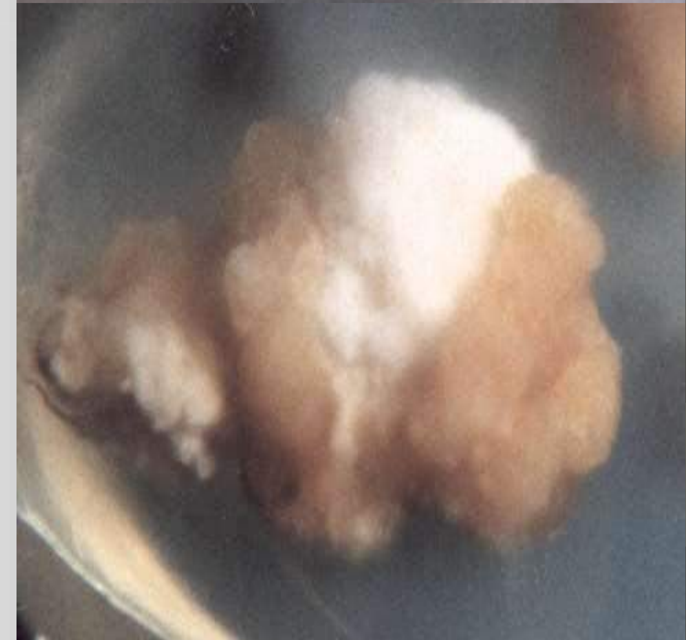
Callus culture



Callus

- It is an **unspecialized** , **unorganized**, **growing** and **dividing mass** of cells.
- It produced when explants are cultured on the appropriate solid medium, with both an **auxin** and a **cytokinin** in a correct conditions. 2,4-D are commonly used.
- During callus formation there is some degree of **dedifferentiation** both in morphology and metabolism, resulting in the lose the





- A callus is a blob of tissue – (mostly undifferentiated cells)
- A callus is naturally developed on a plant as a result of a wound
- This callus can be left to develop or can be further divided

Callus

Callus cultures may be compact or friable.

- ✓ **Compact callus** shows densely agg
- ✓ **Friable callus** shows loosely associated cells and the callus becomes soft and breaks apart easily.

- **Habituation**: it reduce the requirement of auxin and/or cytokinin by the culture during long-term culture.

Properties of callus culture

- It Often comes from shoot in early culturing
- It Can be maintained indefinitely
- **No- photosynthesis** and grows in dark
- It Can be used to isolate single cells with stem cell like properties (totipotent)

Three stages of callus culture

1.Induction:

Cells in explant dedifferentiate and begin to divide

2.Proliferative Stage:

Rapid cell division

3.Morphogenesis stage:

Differentiation and formation of organized structures; specifically processes that lead to plant regeneration from somatic cells

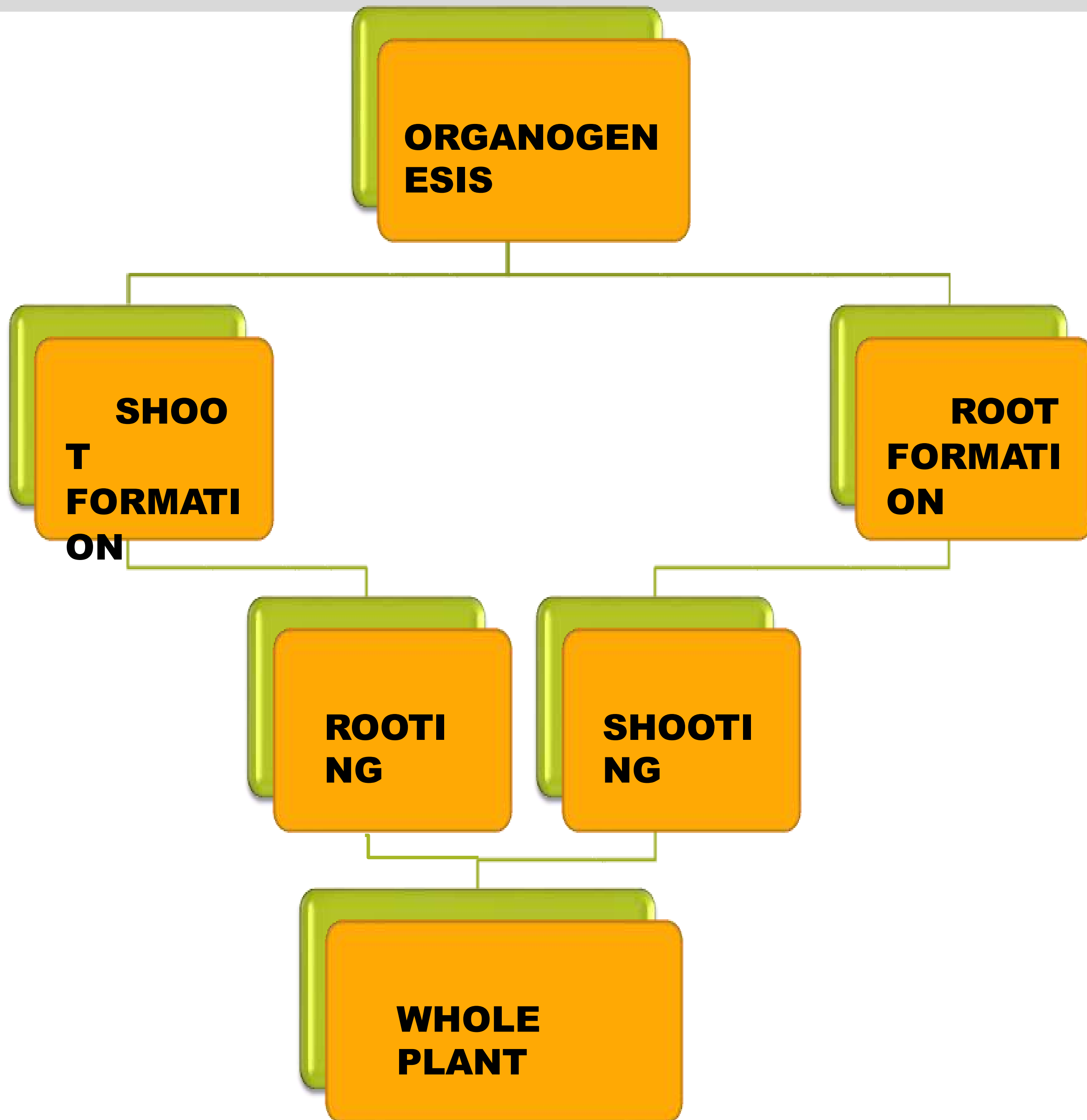
1.Induct

2.Di

Growth of callus over time — t
8 weeks

3.Differ entiatio

- Organogenesis
- Somatic embryogenesis


ORGANOGEN
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WHOLE
PLANT

Plant morphogenesis

1. Organogenesis

The formation of organs (such as leaves, shoots, roots) on a plant organ, usually of a different kind

1. Enhancement of axillary bud proliferation/ development
2. Adventitious shoot formation
3. Adventitious root formation

2. Somatic embryogenesis

Embryo initiation and development from somatic cells

3.1 Organogenesis

- **Enhancement of axillary bud proliferation and development** *in vitro* **-** proliferation of lateral buds stimulate the apical meristem that
- **Adventitious shoot formation -** dedifferentiation and/or differentiation and development of shoots from non-meristematic cells (one or more than one) either directly or indirectly
- **Adventitious root formation -** roots are initiated adventitiously at the base of the shoot apex and a vascular continuum is established to complete plant regeneration.

3.2 Somatic Embryogenesis

- ✓ **Dedifferentiation is typically minimal but a meristemoid -like tissue can be formed in the latter case**
- ✓ **Histogenesis of somatic embryogenesis is characterized by the formation of a bipolar structure, in contrast to adventitious organogenesis**
- ✓ **Single cell origin of somatic embryos makes chimerism infrequent; adventitious shoots**

MORPHOLOGY and characteristics of callus

- **Callus tissue proliferate as amorphous mass of cells having no regular shape .**
- **So it difficult to describes its external morphology.**
- **But they can be distinguished on the basis of other characteristics such as Texture , Colouration , Hormone requirements etc.**
- **On that basis , even callus tissue initiated**

textu
re

- On the basis of texture callus tissue can be two categories

  such as ,

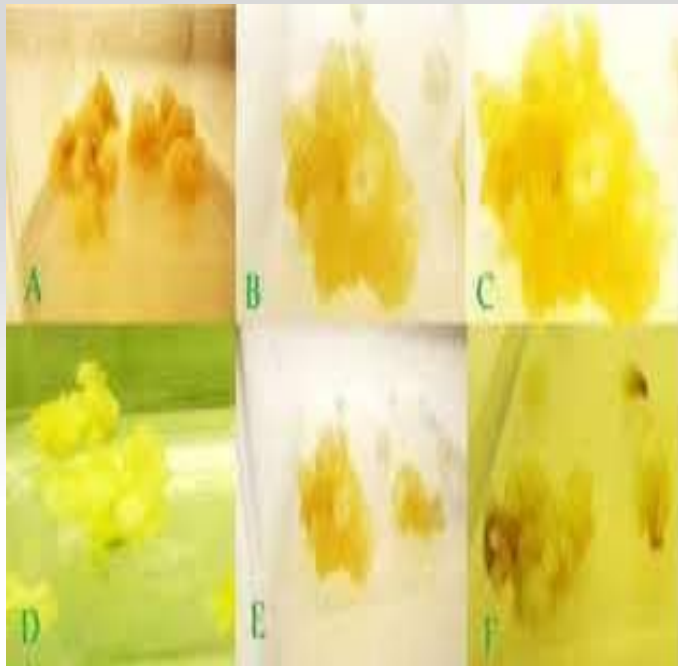
1. Soft Callus

2. Hard callus

- **1. Soft callus** is friable in nature and is made of Heterogenous mass of cell having minimal contact.
- **2. Hard callus** consists of giant cells , tracheid like cells and closely packed cells i.e. Compact in

colouration

- Generally callus tissue is creamish yellow or white in colour.
- Sometime it may be Pigmented.
- Pigmentation may be uniform or patchy.
- It may be green in colour.
- Sometime white callus tissue grown under dark condition turn it into green colour after transferring in light condition.
- Some may be yellow in colour due to synthesis of Carotenoid pigments.
- In some cauliflower culture it is in purple colour due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B
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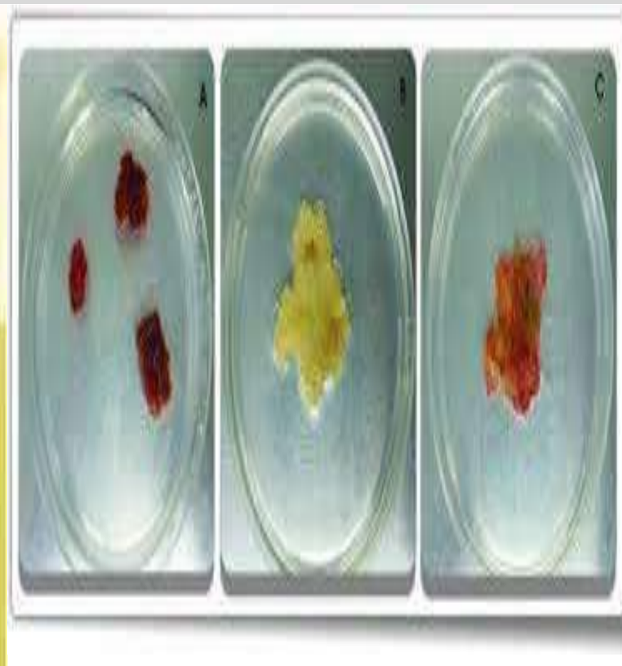

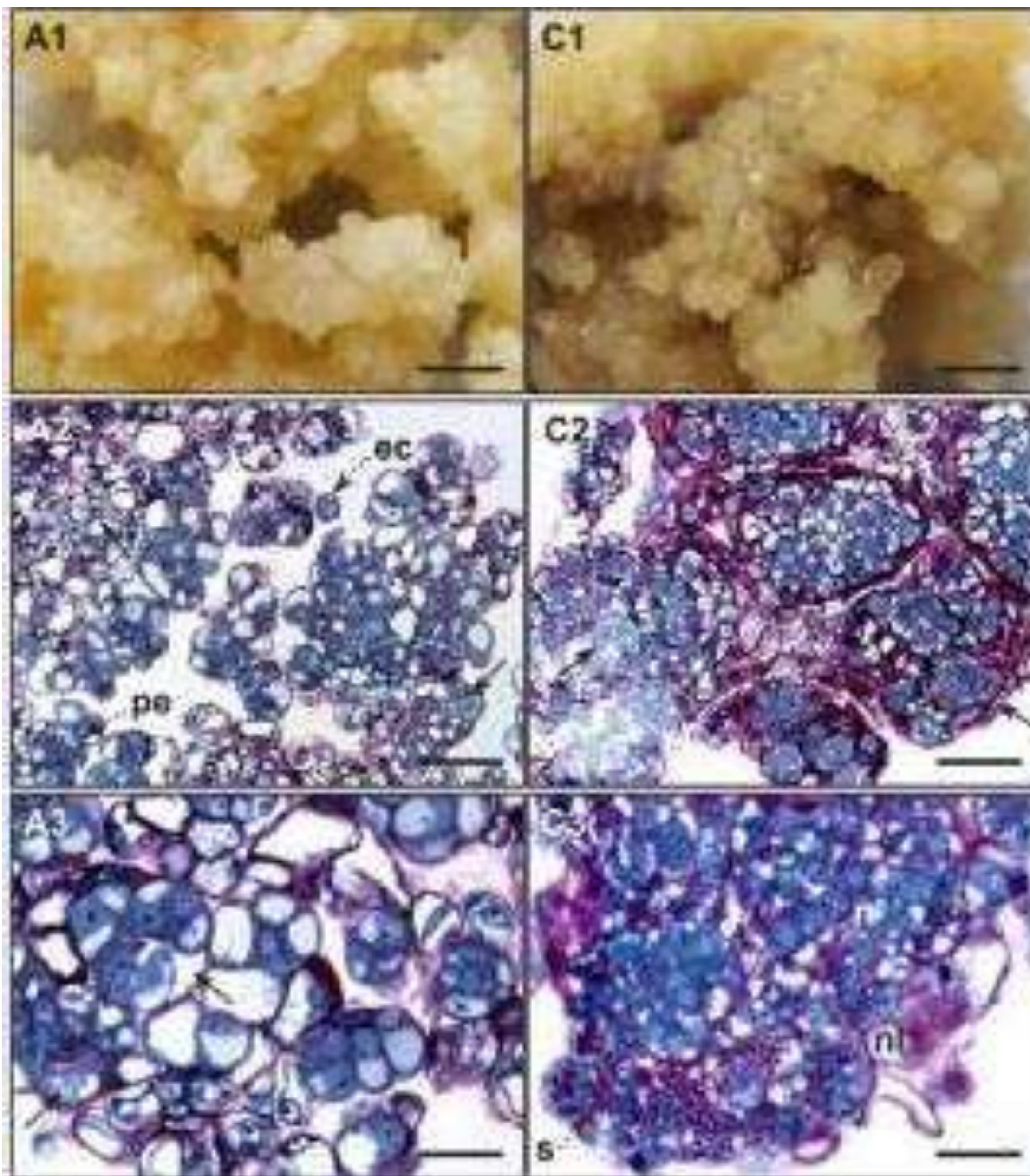
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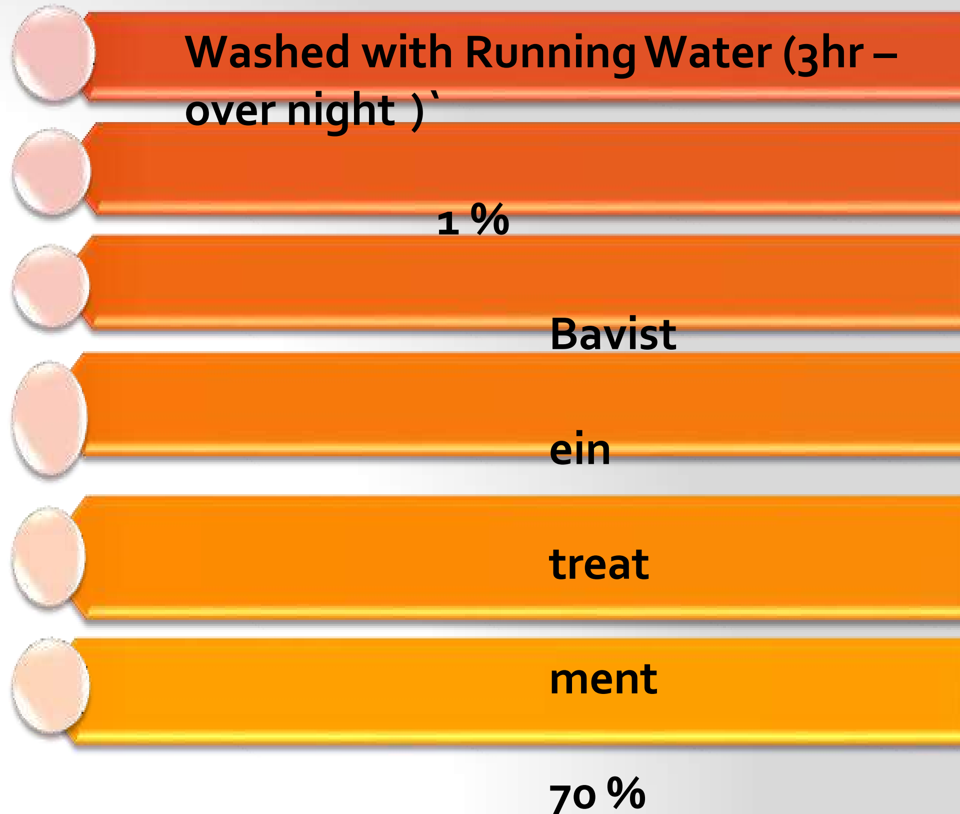
Principle / procedure of callus culture

- There are Three criteria for callus culture are,

1. Aseptic preparation of plant material
2. Selection of suitable nutrient medium

1.Aseptic preparation of plant material

- Surface sterilization :-



LA
F

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2.Selection of suitable nutrient medium

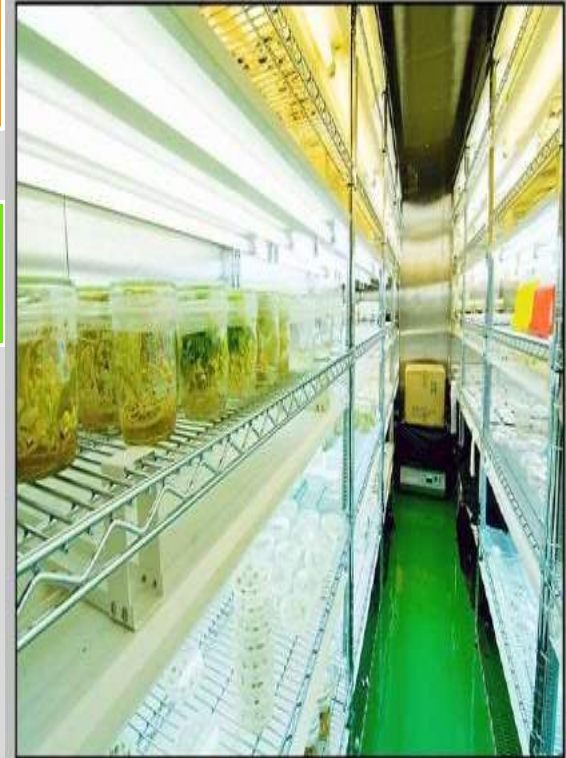
- Auxin/cytokinin 10:1-100:1 induces roots.
- 1:10-1:100 induces shoots
- Intermediate ratios around 1:1 favor callus growth.
- Agar solidified or semi – solid nutrient medium are used.
- That media are autoclaved at 15

3.Incubation of culture under controlled physical

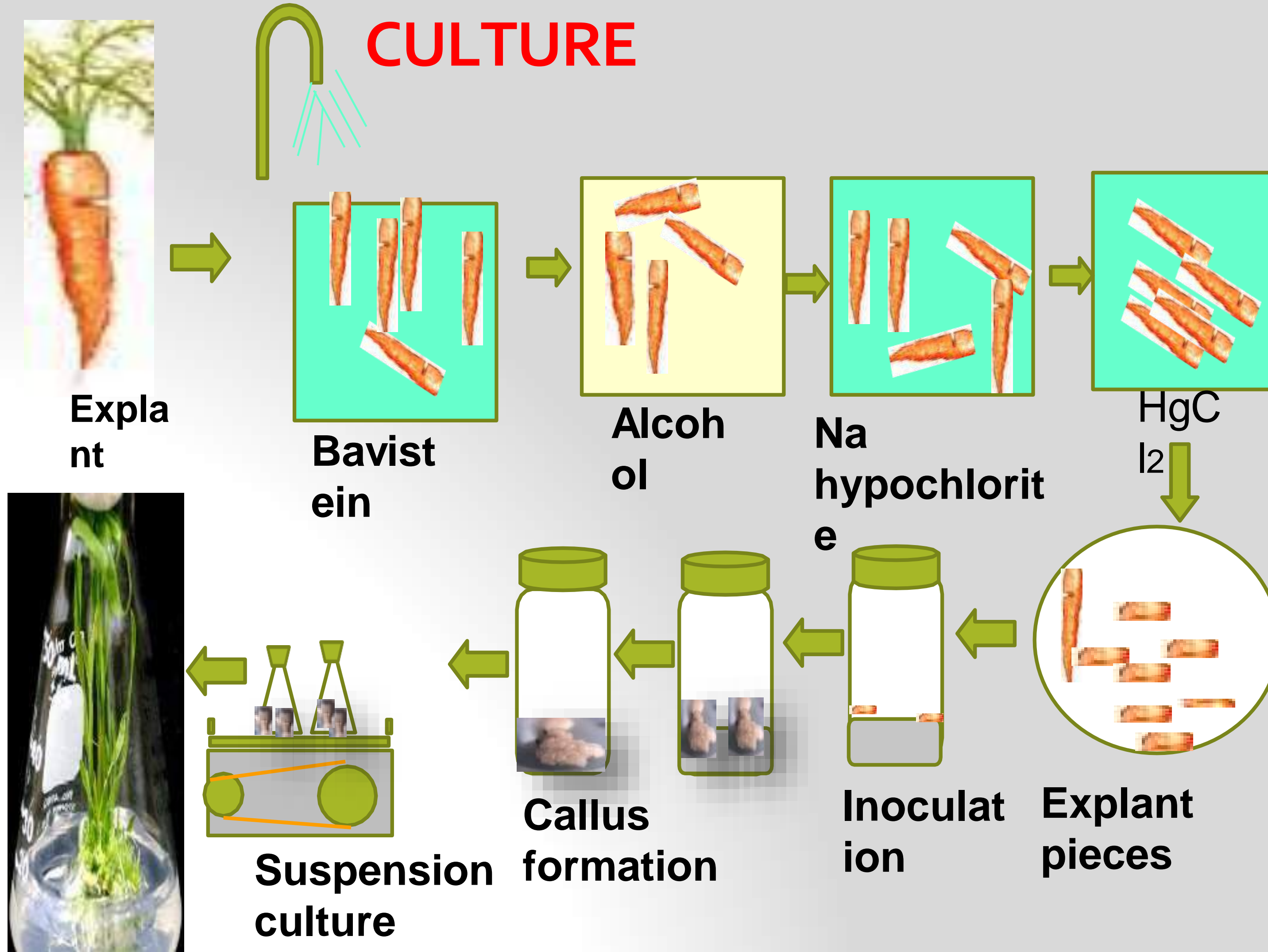
Temperature : 25 ± 2 °C

Photoperiod : 16 hr Light , 8 hr Dark

Light intensity : 2000 – 3000 lux Relative



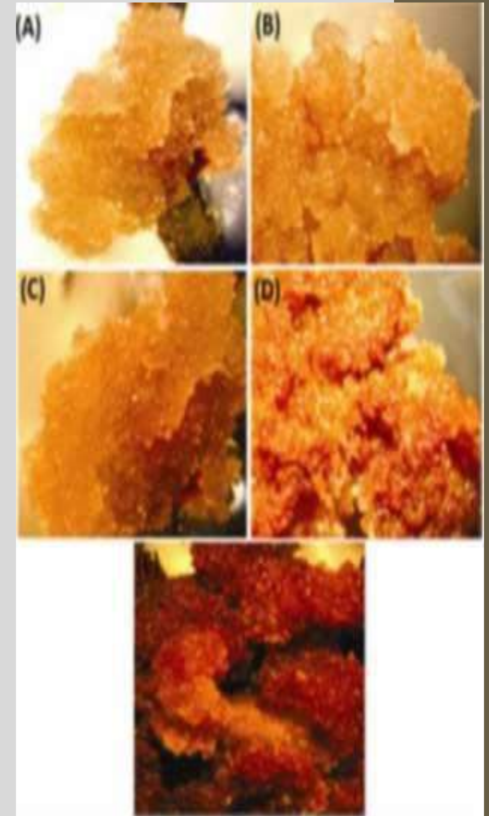
STEPS INVOLVED IN CALLUS CULTURE



New plant

Callus multiplication

Actively growing callus can be initiated on culture media with an even physiological balance of cytokinin and auxin.
After callus biomass increases two to four times (after 2–4 weeks of growth), callus can be divided and placed on fresh media.



Suspension cultures

- Can be initiated from any part of the plant.
- Usually initiated from friable callus already growing in culture.
- Transferred into liquid medium

Agitation

- **Breakdown of cell aggregates into smaller clumps of cells**
- **Maintains a uniform distribution of cells and cell clumps in the medium**
- **Provides gas exchange**

A rice plant growing from callus with nutrient rich agar

Significances of callus culture

- Callus culture as such has no major importance unless and until it is used for experimental objectives . Still ,callus culture got some importance for other

1. ' The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium . This phenomenon is known as plant regeneration or organogenesis or morphogenesis .
2. Callus tissue is good source of genetic variability.
3. Cell suspension culture in moving liquid medium can be initiated from callus culture.
4. Callus culture is very useful to obtain commercially important secondary metabolites.
5. Several biochemical assay can be performed from callus

Conclusion:

- **Sterilization, Hormone concentration , And Proper handling is an important step in callus culture for successful results….**

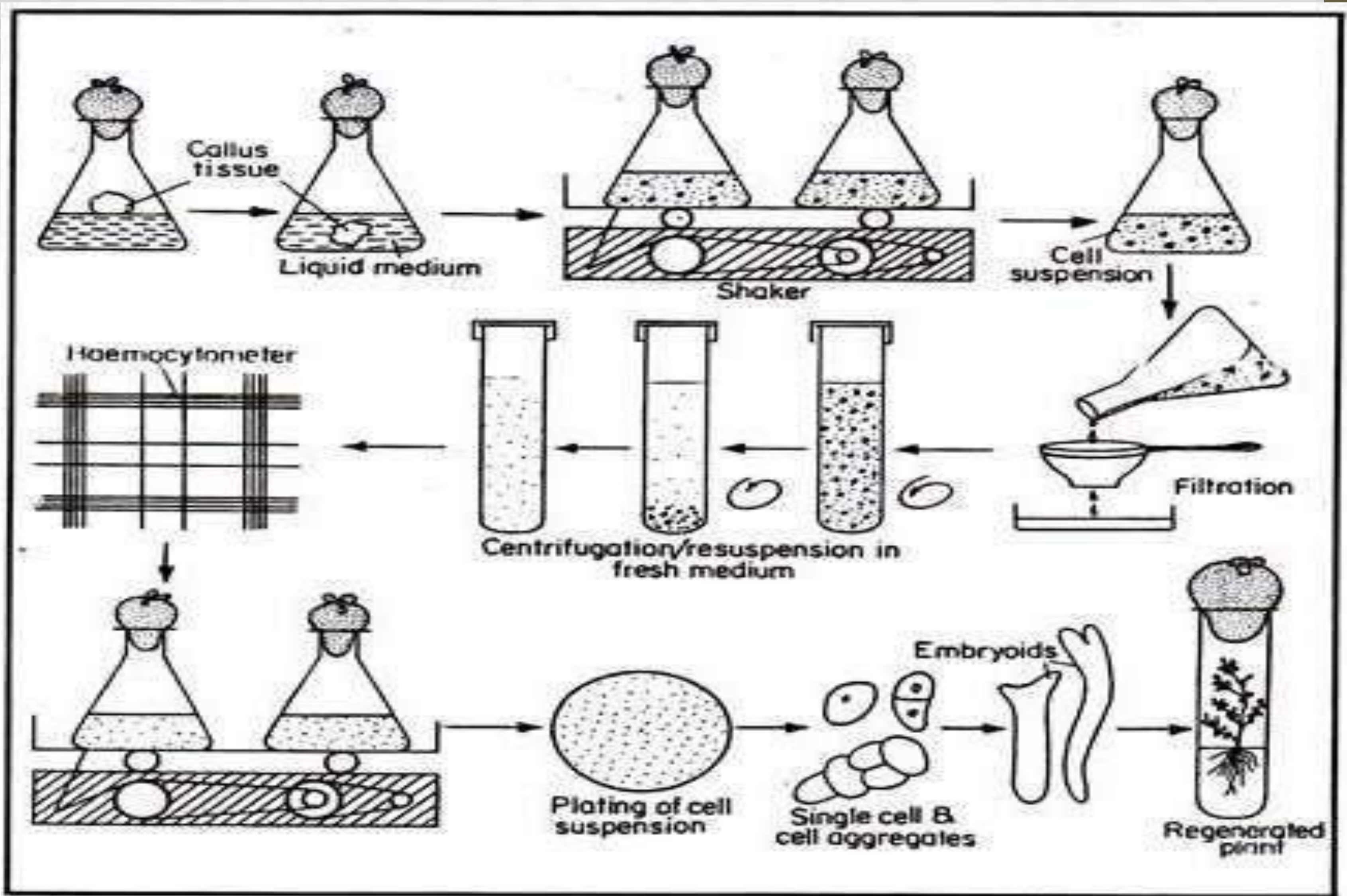
CELL
SUSPENSION
CULTURE

Introductio n

- **Plant Tissue Culture (PTC)** is defined as a collection of experimental methods of growing plant cells, tissues and organs in an artificially prepared nutrient medium static or liquid, under aseptic conditions.
- It is also referred to as micropropagation.
- It was introduced by G. Haberlandt.
- The basic key used in plant tissue culture is the totipotency of plant cells, meaning that each plant cell has the potential to regenerate into a complete plant.
- With this characteristic, plant tissue culture is used to produce genetically identical plants (clones) in the absence of fertilization, pollination or seeds.

Cell suspension Culture

- The **cell suspension culture** also called as the **plant cell culture** is a system for production of fine chemicals.
- It can be defined as “The culture of tissue and cells cultured in liquid nutrient medium, producing a suspension of single cells and cell clumps.”
- Cell suspension culture is the primary route for studying plant cell secondary metabolism.
- The cell suspension culture requires optimization of the cell line, the cultivation media, and the bioreactor system.


Callus tissue
Liquid medium
Shaker
Cell suspension
Filtration
Haemocytometer
Centrifugation/resuspension in fresh medium
Embryoids
Plating of cell suspension
Single cell & cell aggregates
Regenerated plant

Types Of Cell Suspension Cultures

- There are two types of cell suspension cultures :

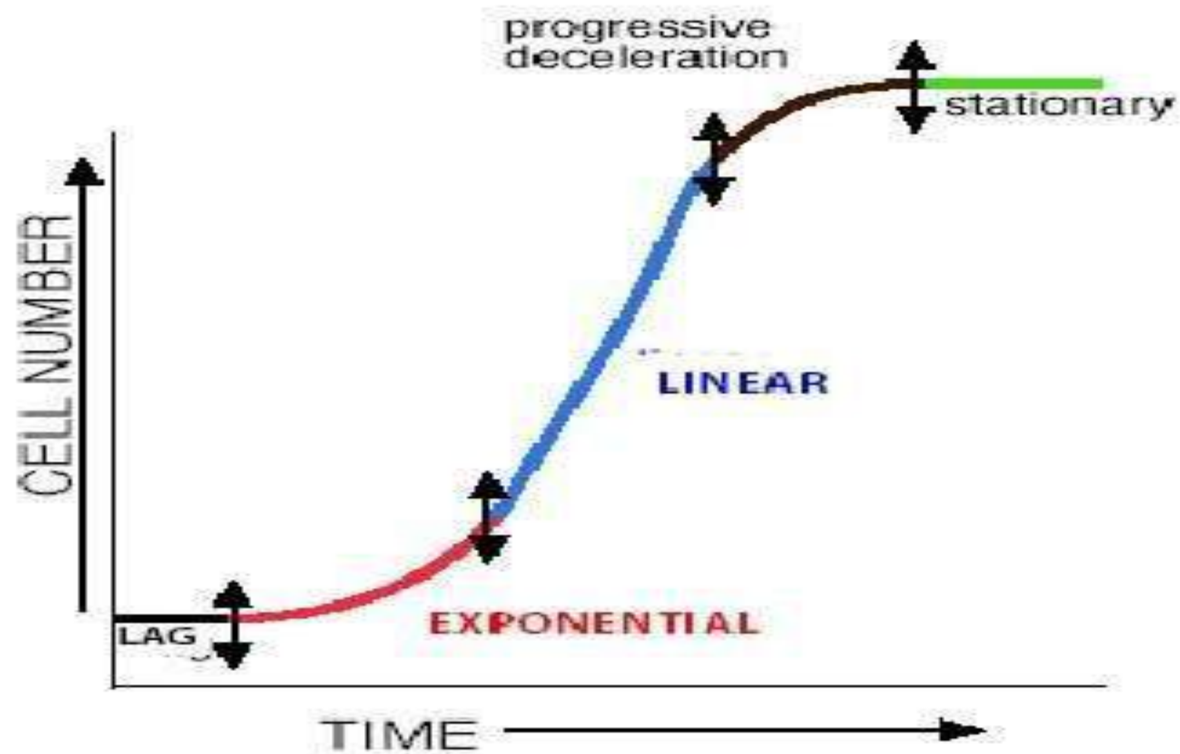
A. **Batch culture**
B. **Continuous culture**

- Each of these cultures have its own advantage and all types are being used in practice.

A. Batch Culture

- Batch culture is a type of cell suspension where the cell material grows in a finite volume of agitated liquid medium.
- These cultures are maintained continuously by sub culturing.
- Batch cultures are most commonly maintained in conical flasks incubated on orbital platform shakers at the speed of 80-120 rpm.
- It is a closed system, with no additions or removal of nutrient and waste products during the period of incubation.

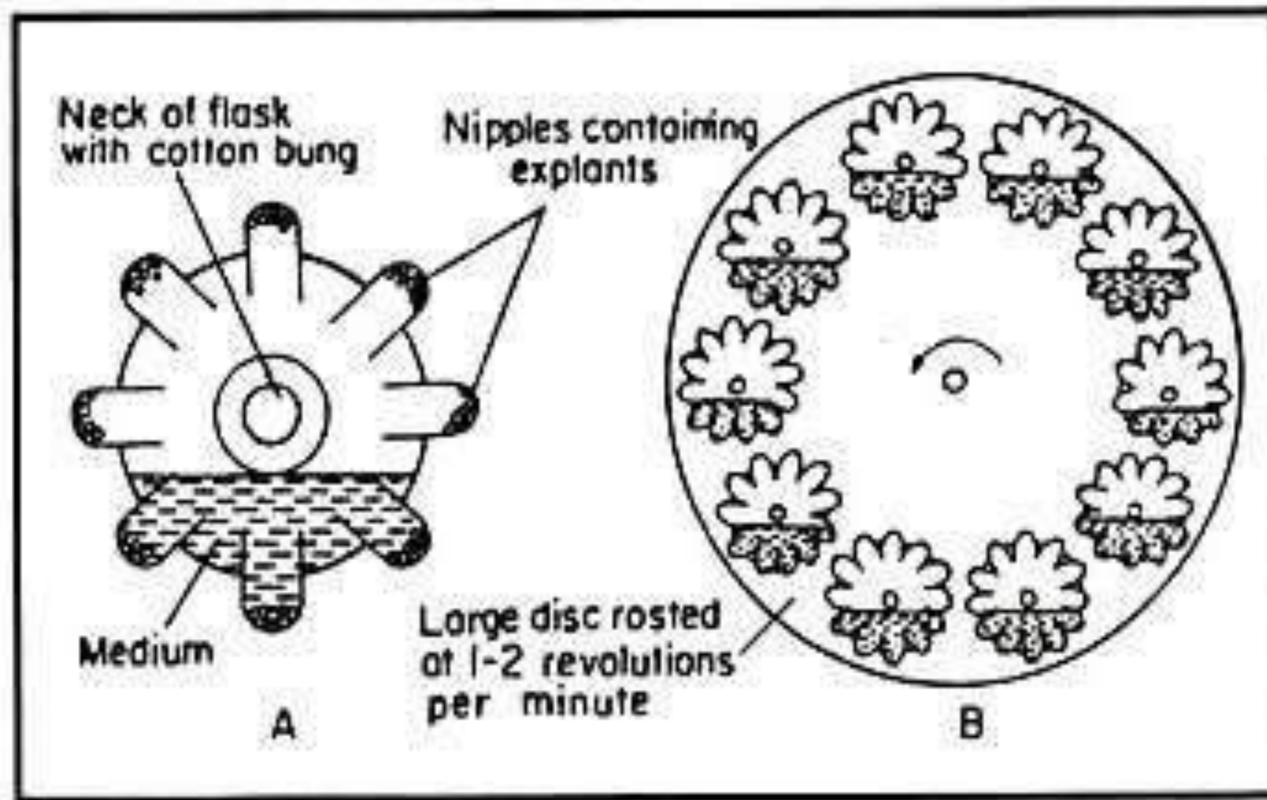
Growth curve in batch culture



Types

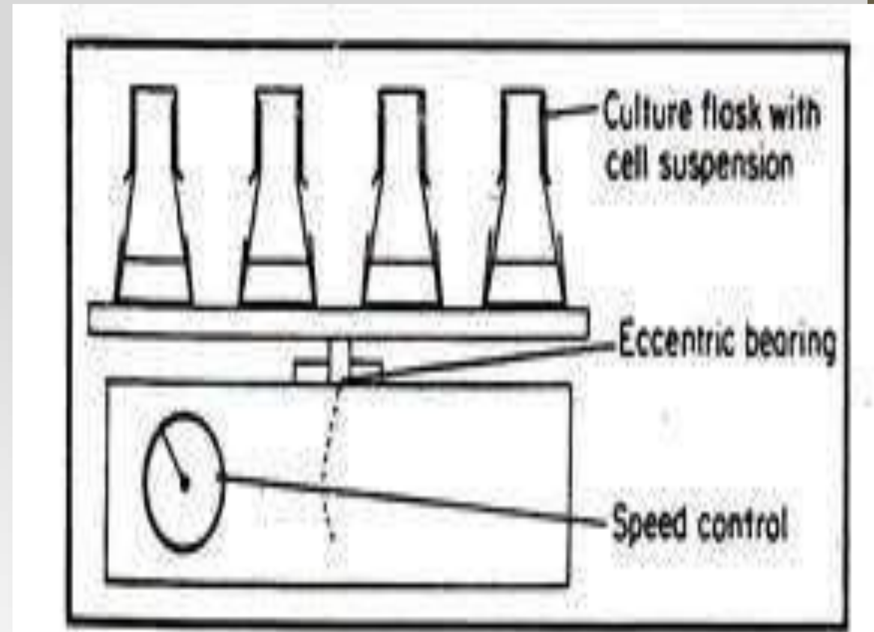
1. **Slow rotating cultures :**

- In this culture, single cells and cell aggregates are grown in a specially designed flask, *the nipple flask*.
- Each nipple flask possesses eight nipple like projections, having a capacity of 250ml.
- They are loaded in a circular manner on the large flat disc of vertical shaker.
- When the flat disc rotates at a speed of 1-2rpm, the cells within each nipple of the flask are alternatively bathed in the culture medium and exposed to air.


Neck of flask
with cotton bung
Nipples containing
explants
Medium
A
Large disc rosted
at 1-2 revolutions
per minute
B

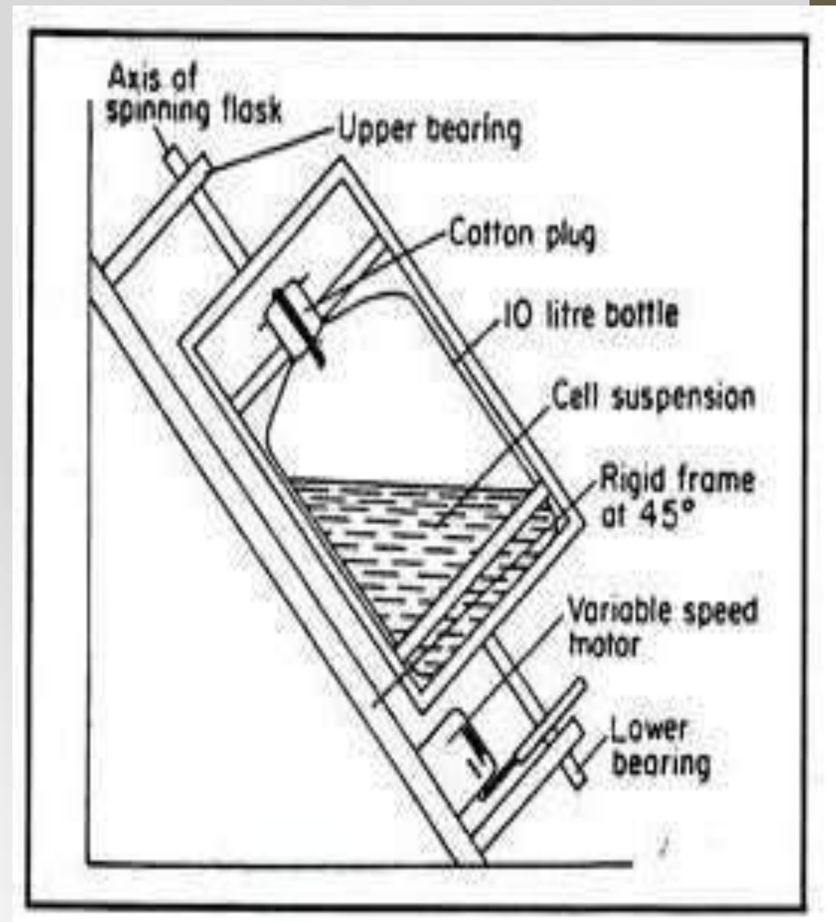
2. Shaker cultures :

- It is very and effective system.
- In this method, single cells and cell aggregates in fixed volume of liquid medium are placed in conical flasks.
- These flasks are then mounted with the help of clips on a horizontal large square plate of an orbital platform shaker.
- The square plate moves in a circular motion at the speed of 60-180 rpm.



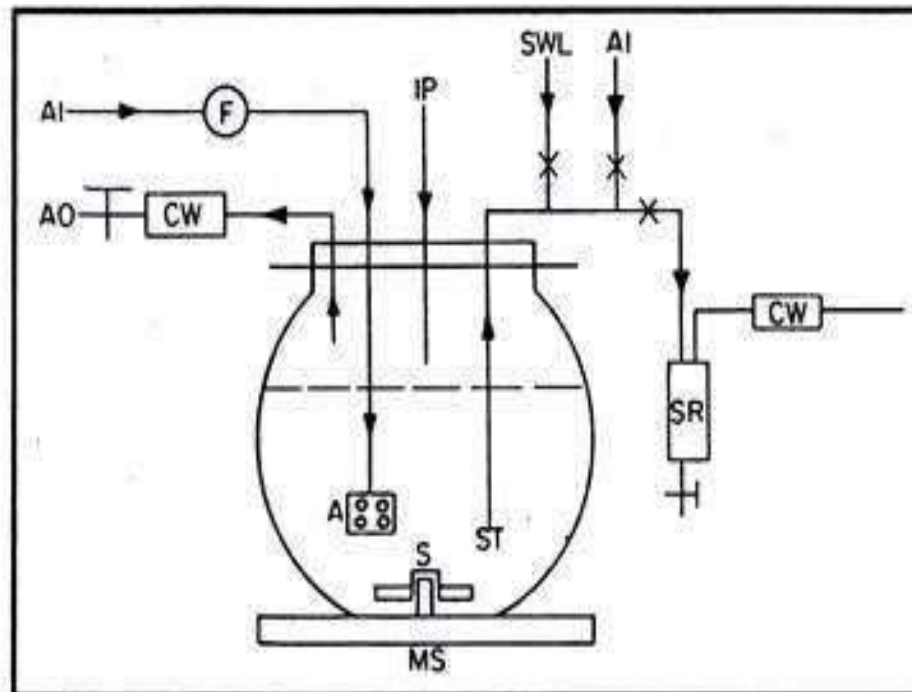
3. Spinning Cultures :

- In this culture system, large bottles are used, usually bottles with the capacity of 10L.
- Large volumes of cell suspension is cultured in 10L bottles, with the bottles spinning in a spinner at 120 rpm at an angle of 45° .



4. Stirred Culture :

- This system is used for large scale batch culture.
- In this method, the large culture vessel (round-bottom flask) is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through the culture medium.
- Internal magnetic stirrer is used to agitate the culture medium safely.
- The magnetic stirrer revolves at 200-600 rpm.



❐ Fig 4.6

Stirred batch culture unit. Arrow indicate direction of flow of air; AI = air input; F = sterilizing glass-fibre air filter; AO = air outlet; CW = cotton wool; IP = inoculation port; A = aerator; S = stirrer magnet; ST = sample tube; MS = magnetic stirrer; SWL = sterile water line; SR = sample receiver; (Diagram after Dr. P. King)

B. Continuous Culture

- In continuous culture system, the old liquid medium is replaced continuously by the fresh liquid medium to stabilize the physiological states of the growing cells.
- In this system, nutrient depletion does not occur due to the continuous flow of nutrients and the cells always remain in the steady growth phase.
- Continuous culture is further divided into two types :

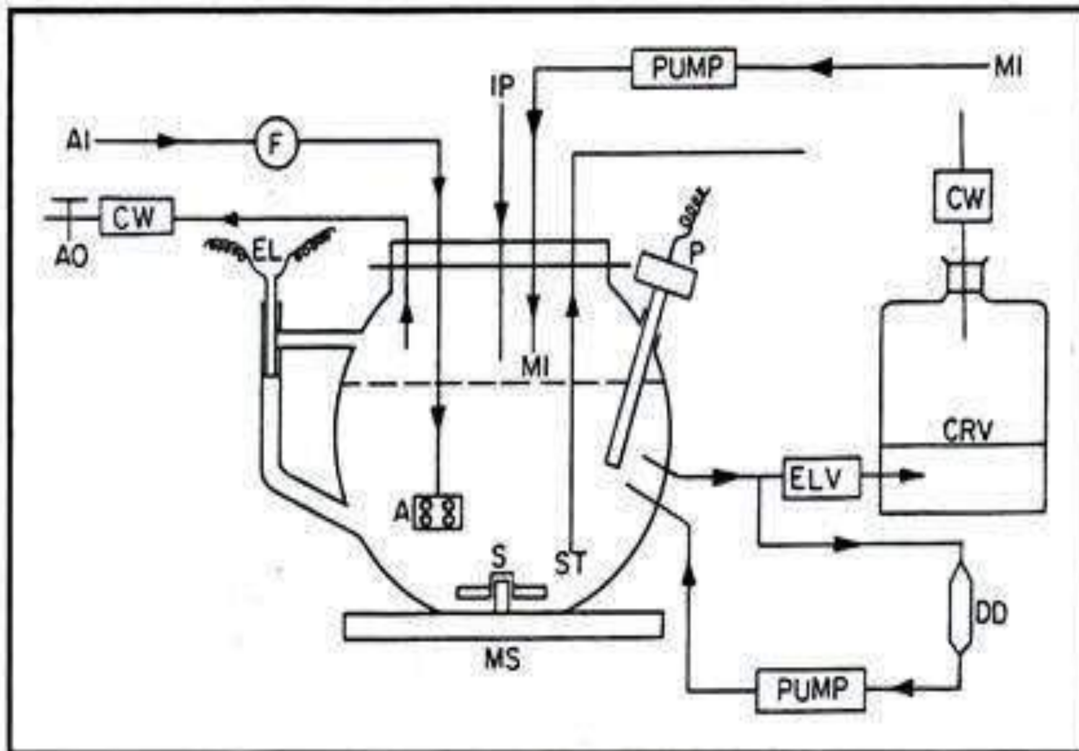
1. In closed type, the used medium is replaced with the fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

2. In open type, both the cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate.

- Open continuous cell suspension culture is of two types :

i. **Chemostat :**

- In this system, culture vessels are usually cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction and removal of cells and medium.
- Such a system are maintained in a steady state.
- Thus in a steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant.
- Such continuous cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants.



- Fig 4.7

Chemostat culture. Arrows indicate direction of flow of liquid; AI = air input; F = sterilizing glass-fibre; AO = air output; CW = cottol wool; EL = volume-sensing electrodes; ELV = volume controlling outlet valve; MI = medium input; S = stirrer magnet; ST = sample tube; P = probe for oxygen tension; DD = density detector, CRV = culture receiving vessel; MS = magnetic stirrer; IP = inoculation port (Diagram after Dr. P. King)

ii. **Turbidostats** :

- A turbidostat is a continuous culturing method where the turbidity of the culture is held constant by manipulating the rate at which medium is fed.
- In this system, the cells are allowed to grow upto a certain turbidity, when the predetermined volume of culture is replaced by fresh culture.
- The turbidity is measured by the changes of optical density of medium
- An automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way as to maintain the optical density or PH at chosen, present level.

Importan c e o f c e l l s u

- Such systems are capable of contributing significant information about cell physiology, biochemistry, metabolic events, etc.
- It is important to build up an understanding of an organ/embryoid formation starting from a single cell.
- Mutagenesis studies maybe facilitated by cell suspension culture to produce mutant cell clone from which mutant plants can be raised.

Advantages :

- The nutrients can be continually adjusted.
- This system can be scaled for large scale production of the cells.
- A whole plant can be regenerated from a single plant cell.

Disadvantages :

- The productivity of suspension cultures decreases over extended subculture periods.
- Slow growth and low productivity of plant cells.
- Cells may get damaged by shear conditions.

Explant Preparation

Explant

The explant is a piece of plant tissue placed into tissue culture. Explant isolated from the tissues of higher plants and brought into culture. Like excised organs, require a nutrient medium consisting of mineral salts mixture, a carbon source, (usually sucrose) and vitamins. In addition phytohormons (auxins and cytokinins), or their synthetic counterparts, are required to initiate and maintain cell division; occasionally other organic supplements, for instance amino acids or hexitols, are necessary to ensure the prolonged growth of the excised tissue to give an established callus.

To selection explant there are some factors that must be considered are as follows:

1 Physiological or ontogenic age of the organ that is to serve the explant source.

2Season in which the explant is obtained. 3- Size and location of the explant.

4- Quality of the source plant.

5- Ultimate goal of cell culture.

6- Plant genotype.

1 Explant age:

The age of the explant is very important. Physiologically younger tissue is more responsive in vitro, usually the newest formed and is easier to surface disinfect and establish clean cultures. While, older tissue will not form callus that is capable of regeneration.

2 Season:

The season of the year can be effects on contamination and response in culture. For example, during the spring of the year buds or shoots taken are more responsive.

3 Explant size:

The size of the explant has an effect on the response of the tissue. The smaller explant harder to cultuer wherese the medium of culture has to have additional components. Subsequently, the large explants probably contain more nutrient reserves and plant growth regulators to sustain the culture.

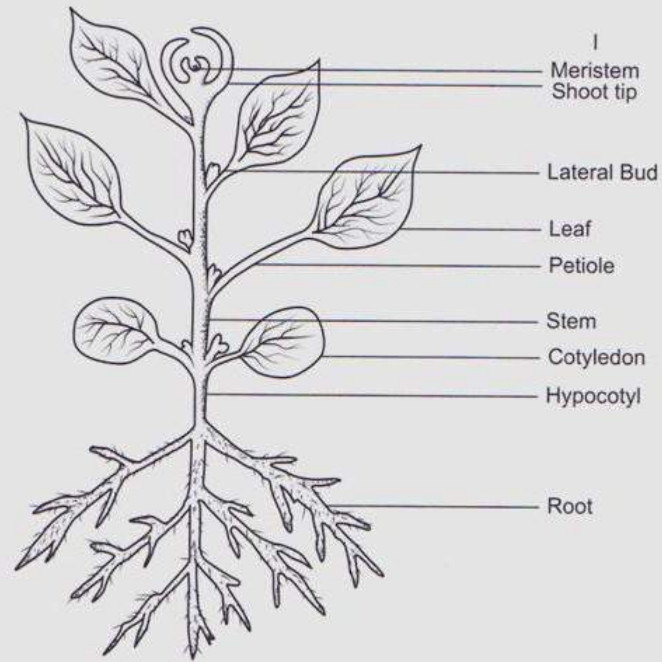
4 Plant Quality:

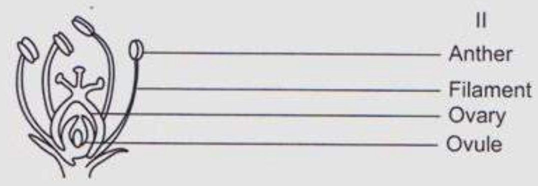
It is best to obtain explants from healthy plants compared to plants under nutritional or water stress or plants which are exhibiting disease symptoms.

5 Goal:

The choice of explant tissue will vary depending on what type of a response is desired from the cell culture. For instance:

(a) if the goal is clonal propagation, then the explant will be a lateral or terminal shoot or bud.
(b) If callus induction is the goal, then pieces of the cotyledon, hypocotyl, stem, leaf, or embryo are used.
(c) For protoplast isolation, leaf tissue from aseptically germinated seed is a good source.





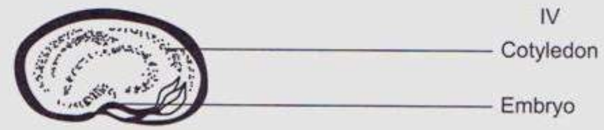


FIGURE 4.1 Schematic drawings (from top to bottom) of a plant, a flower, and monocotyledenous and dicotyldenous seeds indicate potential explant tissues.

Explant: 1- Embryo and Organ Culture Embryos of maize (Zea mays)

The removal and culture of embryos of higher plants was one of the earliest successful techniques in plant tissue and organ culture.

Embryos removed from the seeds of Shepherd's Purse (Capsella bursa-pastoris) can be cultured under completely aseptic conditions.

Over the years the excised embryos of many species have been brought into culture using relatively simple nutrient media.

The advantages of growing

The advantages of growing an embryo isolated from the rest of the seed, apart from the intrinsic interest in doing so, are to remove the immature plant from the endosperm and/or cotyledon(s) which may in particular cases prevent or modify the development of the plant.

In certain instances the excised embryo can also be used as a means of propagating species which resist attempts to use standard methods of vegetative propagation. In this experiment the subject, an embryo of maize, is large, easy to remove from the grain, and can be brought into sterile culture easily and successfully.

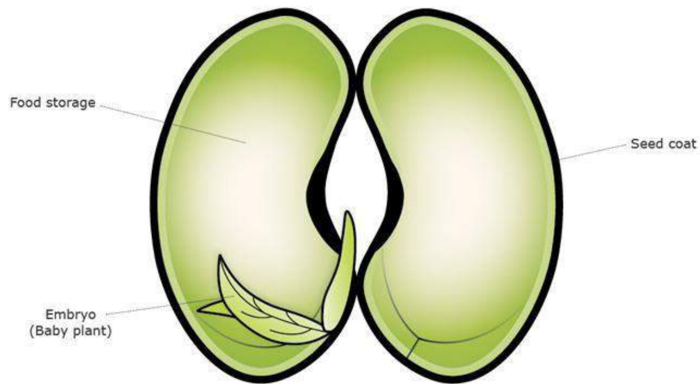
Cotyledon

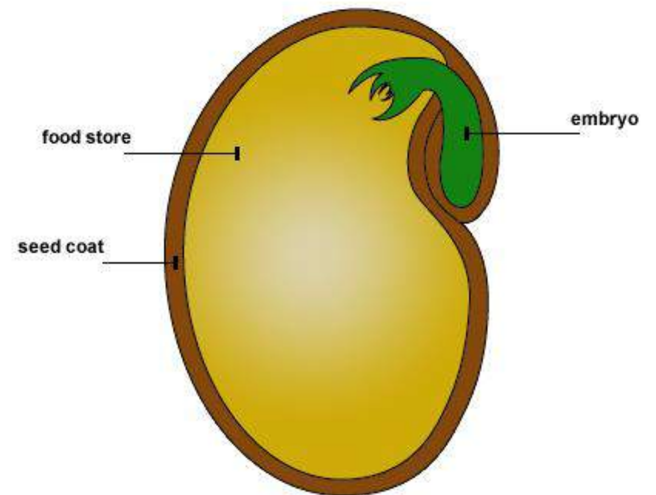
Plumule

Radicle

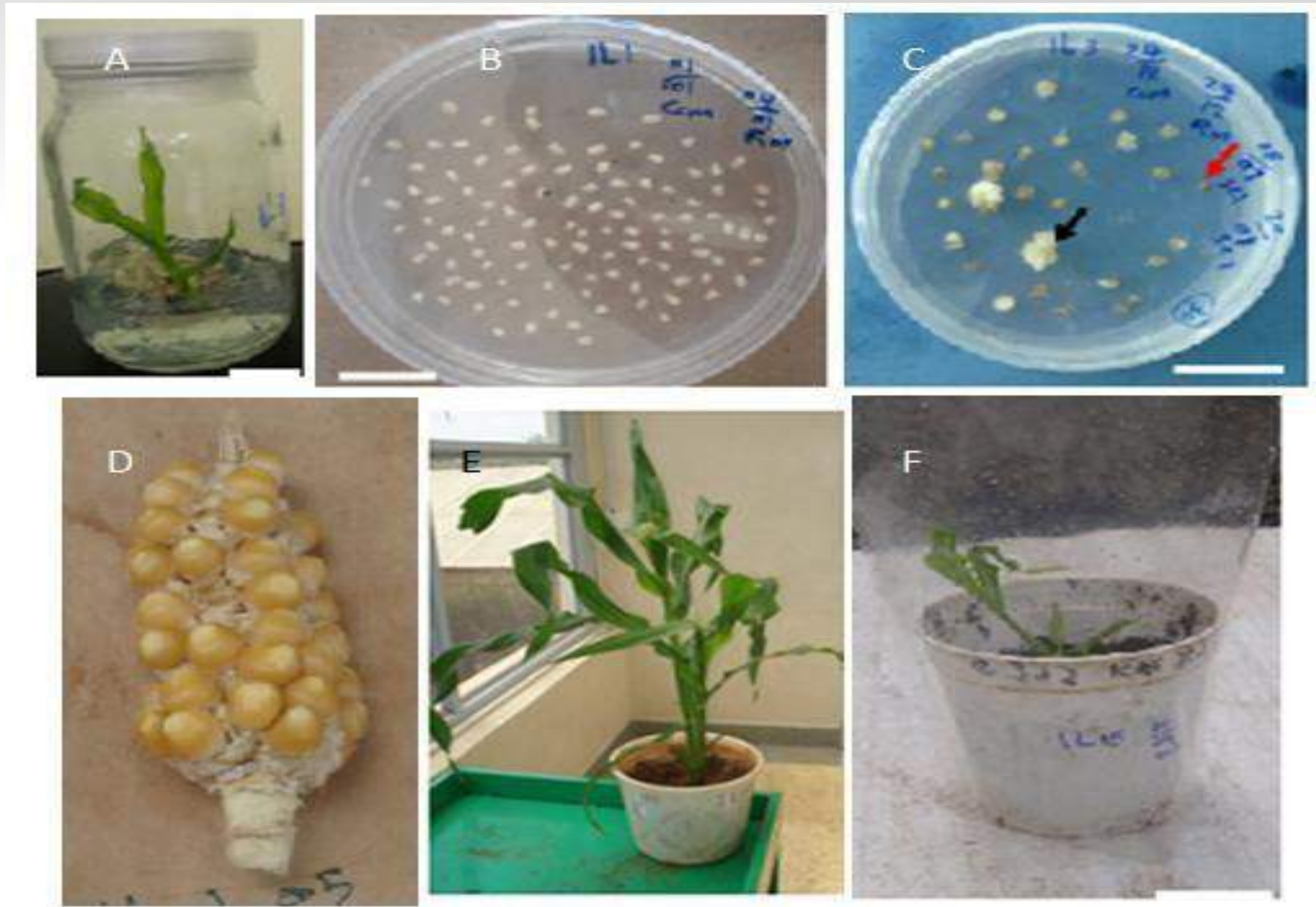
Seed coat or testa

Micropyle



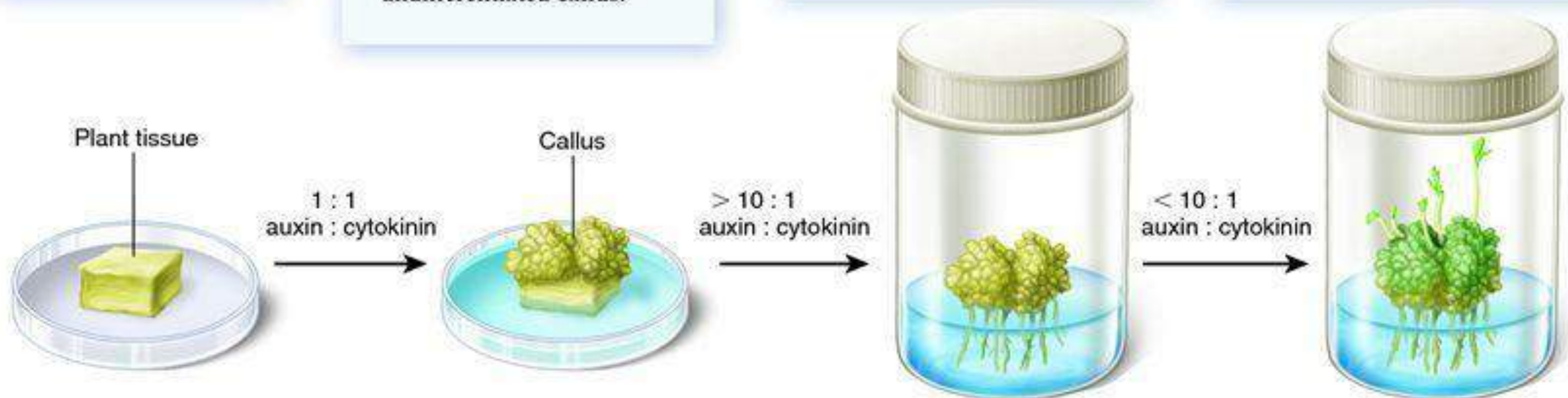


Embryo and Organ Culture; Embryos of maize (Zea mays)



2- Isolation of Explants, Establishmentand Maintenance of Callus (*Daucus carota*)

- An explant can develop a callus as a wound response that consists of unorganized, friable, large, vacuolated, dividing cells that are highly differentiated.
- Callus also can be produced without wounding by germinating of seeds on a plant medium containing growth regulator.The cells of callus are vary in size, shape, pigmentation and in genetic expression.



Plant tissue culture illustrates the impact of different proportions of auxin and cytokinin on plant organ development.

Purpose: To gain experience in aseptic technique and callus induction from varied explants (seedling, fruit, root).

Materials and Equipments:

-MS medium
- Cotton
- glass petri dishes
- Stirile distilled water contained in Erlenmeyer conical flasks
- sheets of aluminium foil
- Forceps
- Scalpels

Non sterile items

-Tap root of carrot at least 200 mm in length and 40 mm in diameter.
- racks, preferably plastic or metal to hold 12 culture tubes
-1000 ml of a solution of sodium hypochlorite approximately 20% (v/v)
- waterproof marking pen
- Glass beaker
- analytical balance
- bunsen or ethanol burner
- Erlenmeyer flask containing 100ml 95% ethanol
- roll parafilm

Experiments Procedures

1Reject all diseased, damaged, or irregularly shaped individuals. Scrub the carrots under running tap water to remove all surface detritus using a brush.

2Cut the root into ½- inch sections and surface sterilization in 15% chlorine bleach for 10-15 min.

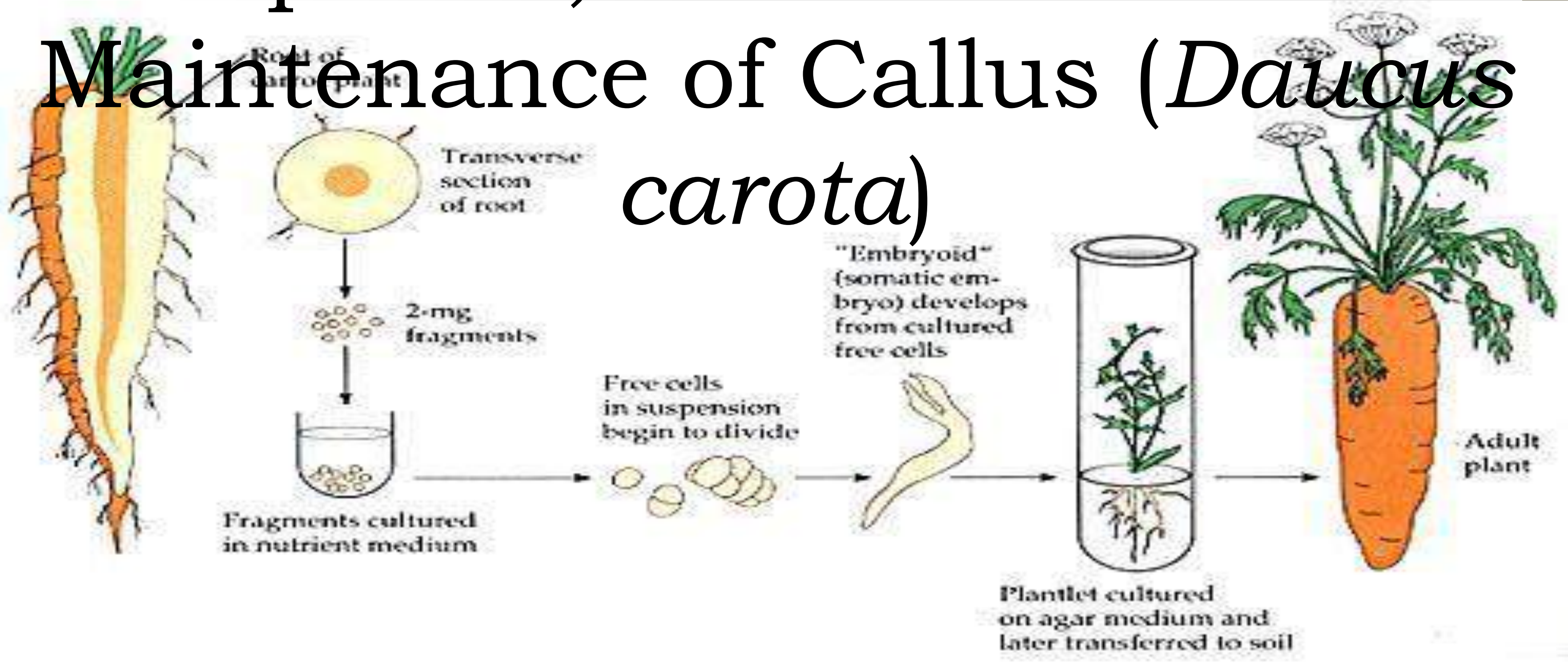
3Transfer sterilized carrots to the sterile room marking sure the UV lights are switched off before entering. Powerful UV rays are harmful to the eyes and skin! After wiping it clean with 70% ethanol set out the working.

4 Rinse three times in sterile distilled water to completely remove the hypochlorite.

4 Cut off tissue burned by chlorine bleach and culture.

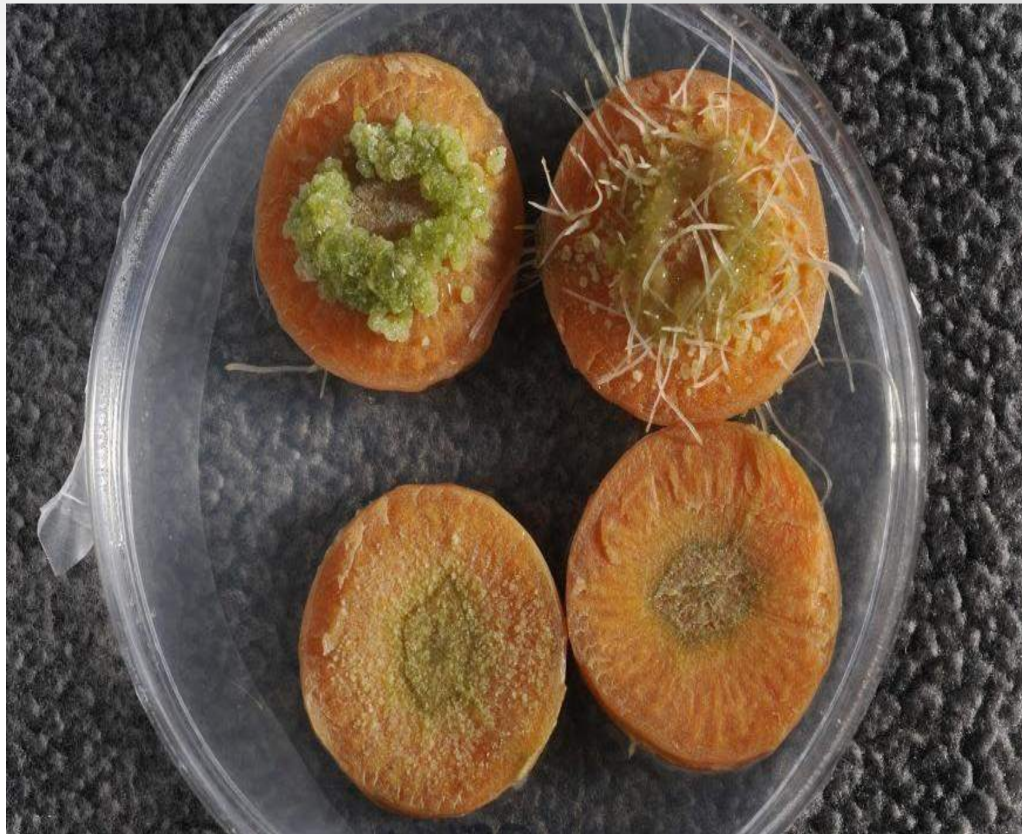
5 Place all cultures in the dark and incubate in the dark at 27 – 30° C.

Isolated of Plant Material and Studies on Growth and Cell Division Experiment: Isolation of Explants, Establishment and Maintenance of Callus (*Daucus carota*)



Observations:

Record cultures observations once a week over a 6-week period. It is include notes on culture conditions, callus formation, and contamination.



Isolation, Culture and Fusion of Protoplasts from Higher Plants

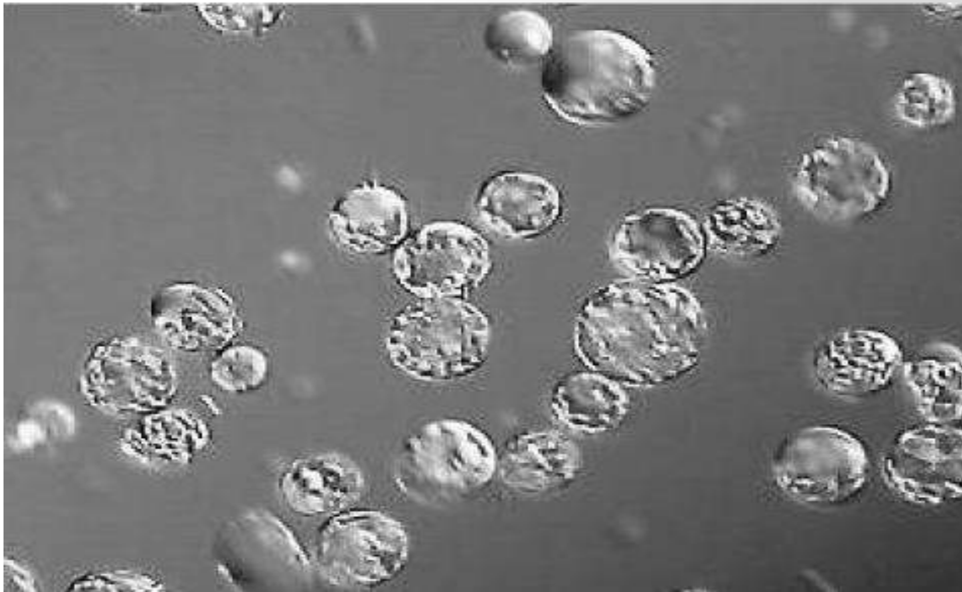
** Isolation and Culture of Mesophyll Protoplasts from Tobacco Leaves

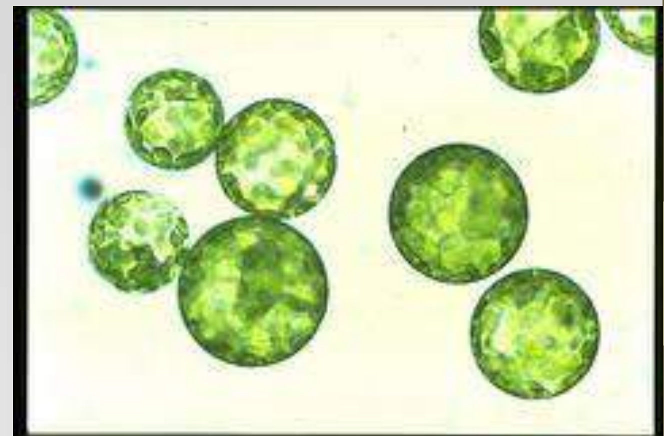
-Isolated plant protoplasts are cells from which the wall has been removed either mechanically or enzymically.

Protoplast preparation was at first done in two steps:

1. At first was the middle lamella dissolved by pectinases.
2. then was the cell wall broken down by cellulase .

Isolation, Culture and Fusion of Protoplasts from Higher Plants (Isolation and Culture of Mesophyll Protoplasts from Tobacco Leaves)

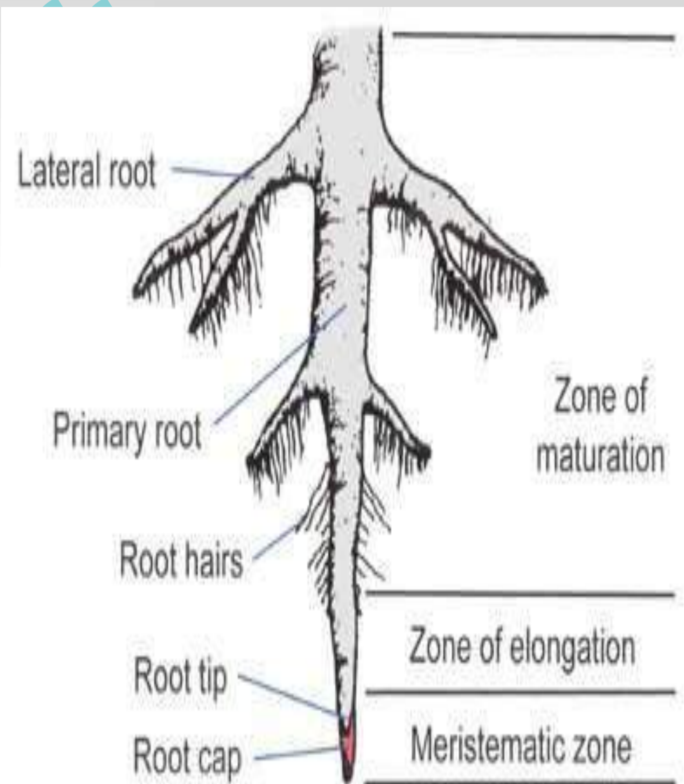


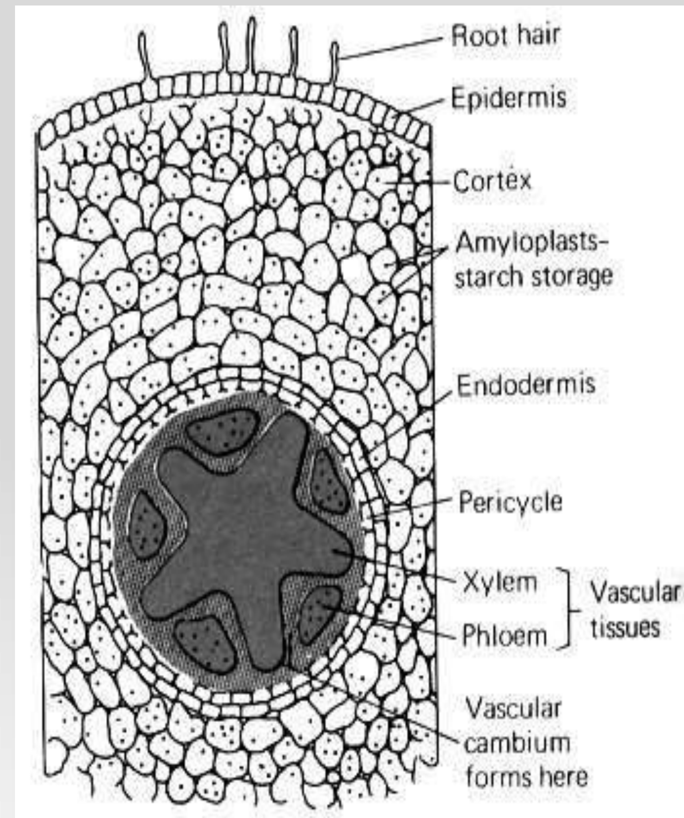


Root culture.

ROOT CULTURE:

Root culture is culturing of excised radical tip of aseptically germinated seed in the [illegible] medium to grow independently [illegible]olled conditions.





and its

Important parts and function of plant root system:

- **Root hair** - extension of specialized root epidermal cells increasing the surface area for adsorption of water and minerals.
- **Epidermis** - outer layer of root.
- **...tex** - region between epidermis and vascular
- ...igin for secondary roots.
- ...ter and minerals throughout the
- ...ved sugar and organic

Principle:

- The root culture is initiated from aseptically germinated seeds, which is maintained in an moving liquid medium, which induce the growth of roots system as in the natural condition.
- clone of the excised roots can be established root culture by repeatedly cutting and main root tips or of lateral tips into ery subculture at the interval of
- d roots can be expressed in eight, increase in length of ergent laterals and total re.

Protocol:

- Initiation of isolated root cultures:

Seeds are first surface sterilized by

proper method Seeds are germinated on

al medium at 25°C in dark

0 to 40 mm in length, 10 mm apical

xercised and

o the liquid medium.

- <u>Initiation of clones</u>:

The root culture is established and maintained for 10 days.

↓

Then the main axis of the root is cut into number of pieces, each bearing four or five young laterals.

↓

sector inoculum is transferred into
medium and
incubated.

↓

e, the growing sector is cut
sectors to
or culture again.

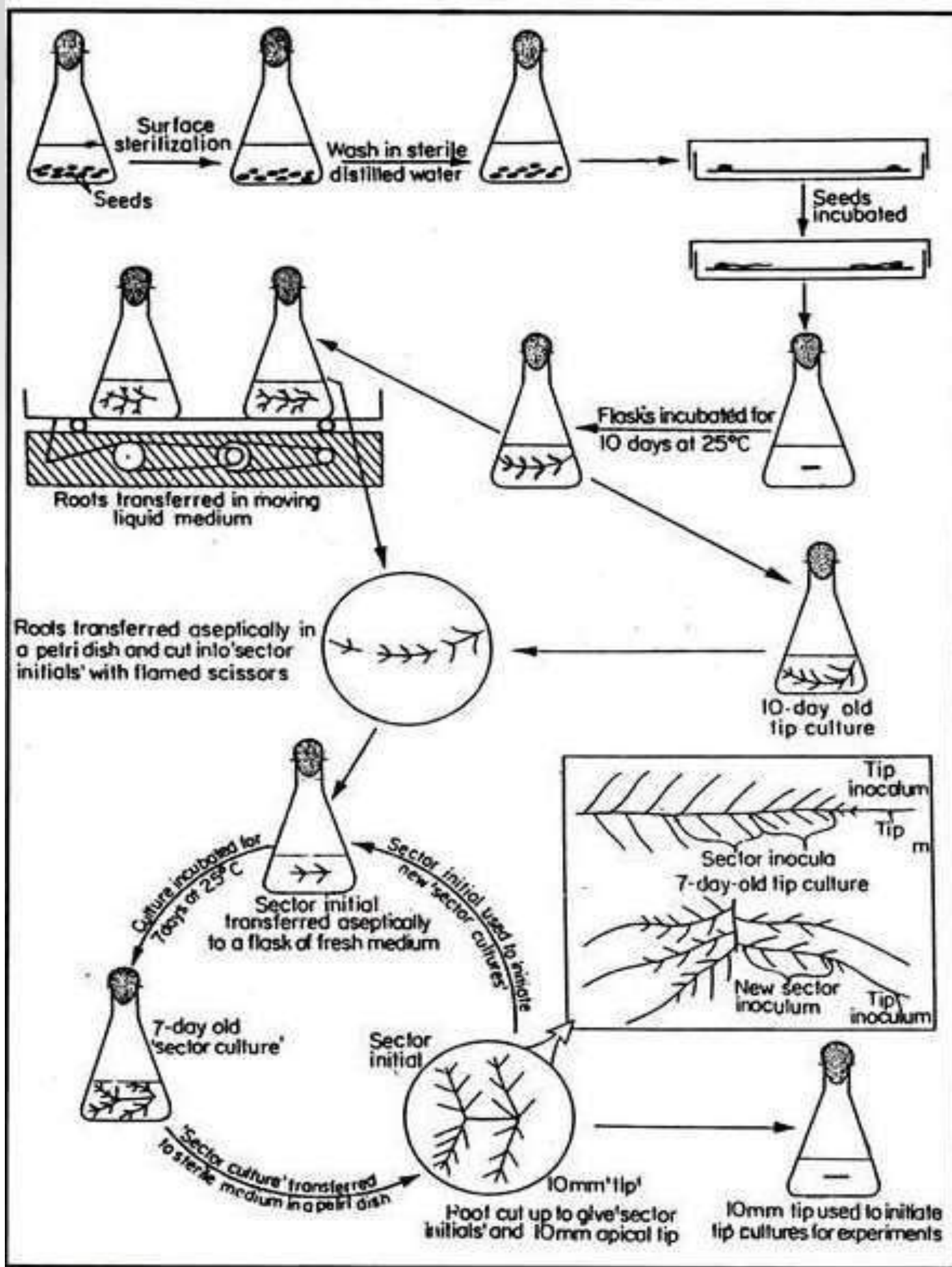


Figure: Procedure for root culture

Importance of root culture:

1. Give the knowledge of carbohydrate metabolism and role of minerals, ions and vitamins etc in root growth.
2. Provided the basic information regarding the dependence of roots on shoots for growth hormones.
3. Root clones are used in study of effect of various compounds on root growth.
4. Study of nodulation of leguminous roots in culture.
5. For regeneration of shoots on roots.
6. Study of synthesis of secondary metabolites from root culture.

Organogenesis

- The process of initiation and development of an organ is called organogenesis.
- In plant tissue culture, Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation.
- Organogenesis in plant tissue culture involves two distinct phases: dedifferentiation and redifferentiation.
- Dedifferentiation begins shortly after the isolation of the explant tissues with an acceleration of cell division and a consequent formation of a mass of undifferentiated cells (called callus).
- Redifferentiation, also called budding in plant tissue culture, may begin any time after the first callus cell forms. In this process of tissue called organ primordia

Process

Explant

Callus

Meristem

oids

Shoot bud

root primordia

Factors effecting organogenesis

Genetic or a physiological change

- In a callus tissue the changes of chromosome structure or number such as aneuploidy, polyploidy, cryptic chromosomal rearrangements etc. Such chromosomal changes may lead to loss of totipotency of the cells.
- At the early stage of culture, the callus tissue exhibits maximum number of diploid cells. Eventually at the later stage of culture, the cells of callus tissue become mixaploid due to alteration of chromosome number and organogenesis could not be induced in such callus tissue, Occasionally, rooting occurs but shoot bud does not develop.

Phytoharmones

- For organogenesis the required balance of phytohormones by an exogenous supply of auxin, cytokinin or gibberellin either separately or in combination is essential .
- Generally high concentration of cytokinin brings about shoot bud initiation, whereas high levels of auxin favours rooting.
- Therefore, to obtain organogenesis, different permutation and combination of hormones as well as various concentrations of hormones are supplemented in the culture medium.

Other Chemicals

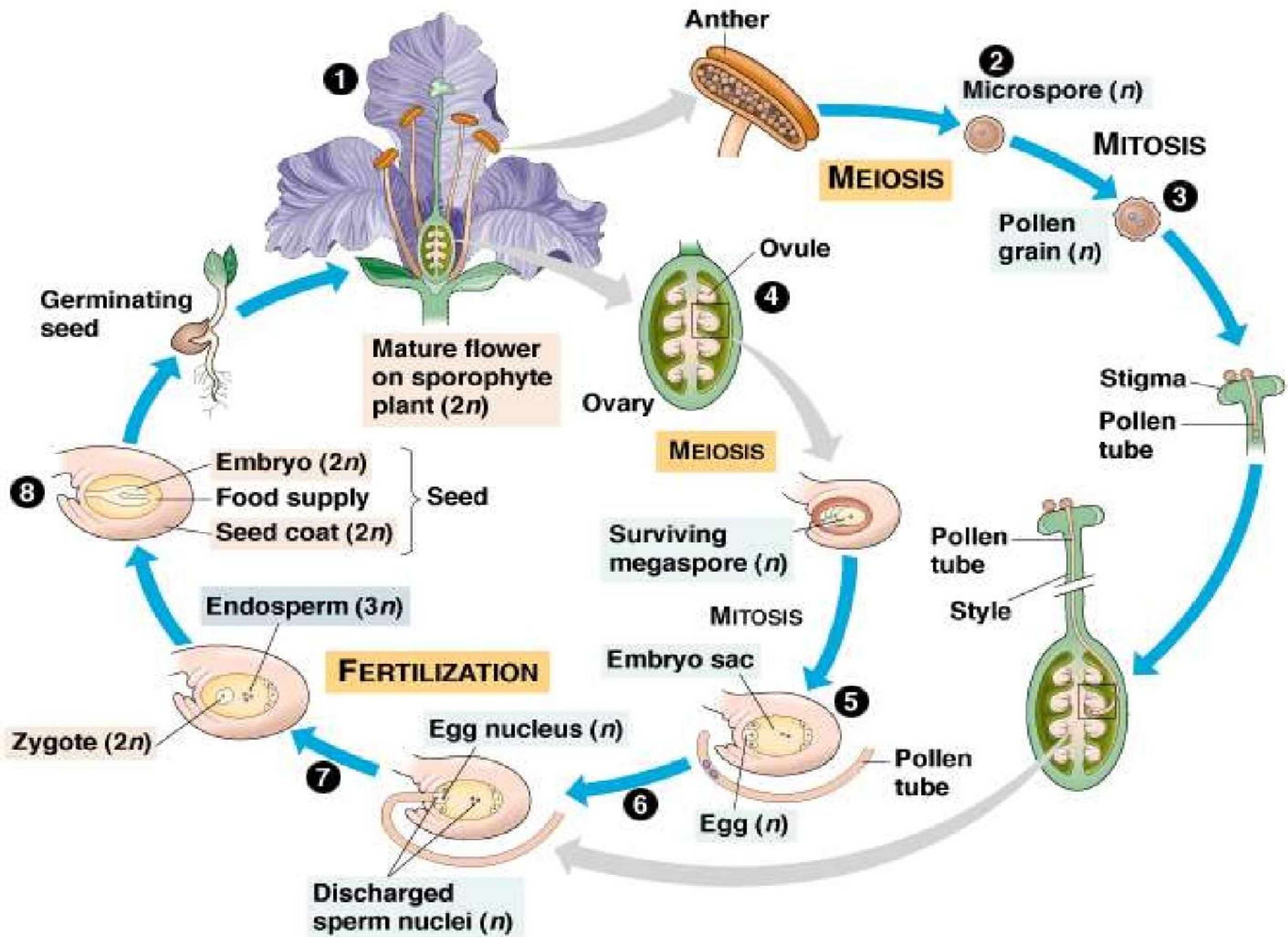
- Certain **phenolic compounds** also induce shoot initiation in tobacco callus- Phenolic compounds actually inactivate the auxins and conse- quently rise in the physiologically effective level of cytokinins which ultimately bring about the initiation of shoot buds.
- The use of **auxin inhibitor or auxin antagonist** via culture medium also causes the induction of shoot bud.
- Additions of **adenine** in the culture medium also induce shoot bud in the callus tissue.
- **Chelating agent like 1, 3 diamino-2-hydoxypropane-**N.N.N'.N' **tetra- acetic acid** initiates Shoot bud in haploid tobacco cultures.
- **Abscisic acid** in place of cytokinin also induces

Enzymes

- **Peroxidase**- One of the most important functions of peroxidase is involvement in the metabolism of auxin.
- **Enzymes involving in carbohydrate metabolism-** Gibberellic acid, which represses starch accumulation by mobilising high amylase synthesis/activity, also inhibits shoot formation.
- **Embden Meyerhof-Parnas (EMP) and Pentose Phosphate (PP)** Pathway enzymes namely phosphoglucose isomerase, aldolase, pyruvate kinase, glucose-6- phosphate dehydrogenase, 6- phosphogluconate dehydrogenase etc. also involving in the shoot formation

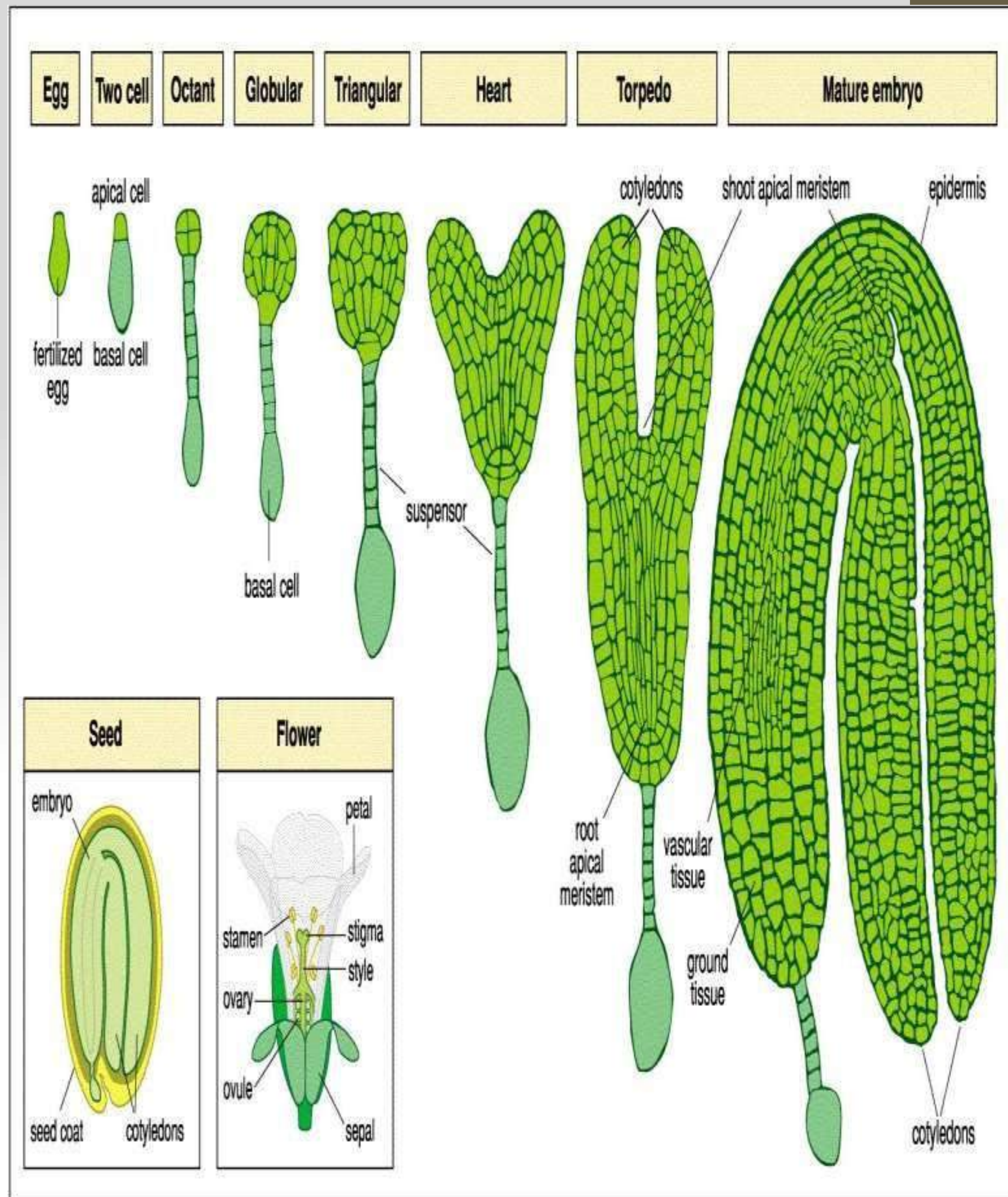
Embryo genesis

- The process of formation of an embryo is called embryogenesis. Embryogenesis starts from a single embryogenic cell, that can be a zygote or an undifferentiated callus cell.
- Embryos developing from zygotes are called zygotic embryos, while those derived from somatic cells are called somatic embryos.
- In plant tissue culture, the developmental pathway of numerous well- organised, small embryoids resembling the zygotic embryos from the embryo genic potential somatic plant cell of the callus tissue or cells of suspension culture is known as somatic embryogenesis.
- Embryoid is a small, well-organised structure



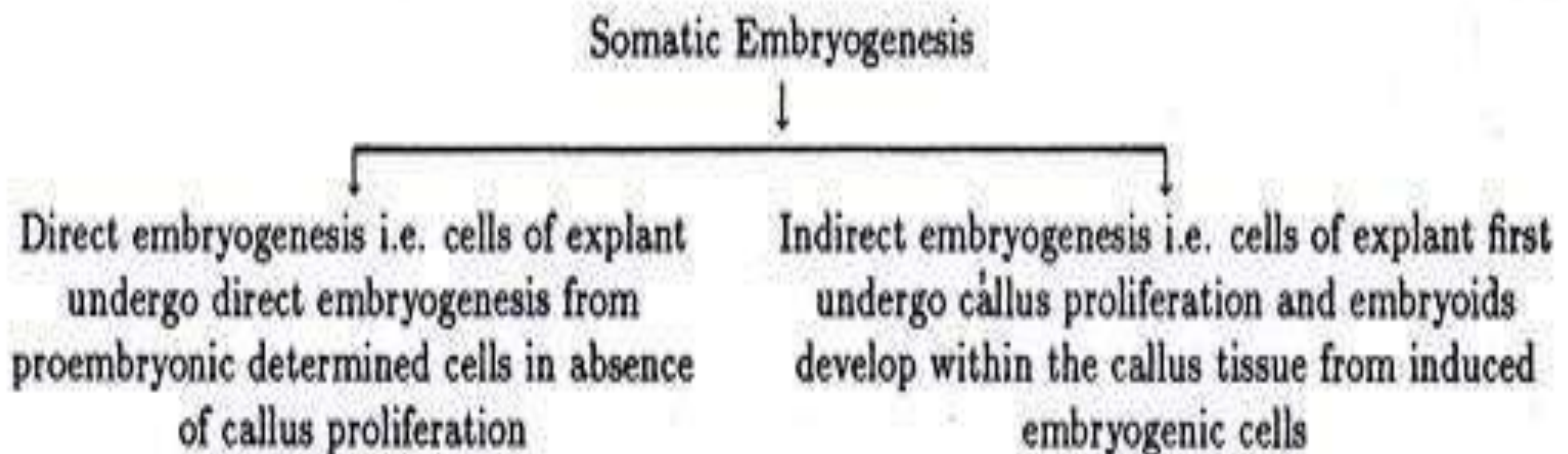
Anther
1
2
Microspore (n)
Mitosis
Meiosis
3
Pollen grain (n)
Ovule
4
Germinating seed
Mature flower on sporophyte plant (2n)
Ovary
Stigma
Pollen tube
Meiosis
Embryo (2n)
8
Food supply
Seed
Seed coat (2n)
Surviving megaspore (n)
Pollen tube
Style
Endosperm (3n)
Mitosis
Fertilization
Embryo sac
5
Zygote (2n)
Egg nucleus (n)
Pollen tube
7
6
Egg (n)
Discharged sperm nuclei (n)

- Zygotic and somatic embryos share the same gross pattern of development.
- Both types of embryos develop as typical passing through globular, heart, torpedo and cotyledonary stages for dicots and conifers, such as globular, scutellar, and coleoptilar stages, for monocots or stages.
- Embryo development is bipolar, having a shoot and a radicular pole at



- Somatic embryogenesis appears to occur when the progenitor cell undergoes an unequal division, resulting in a larger vacuolate cell and a small, densely cytoplasmic (embryogenic) cell .
- The embryogenic cell then either continues to divide irregularly to form a proembryonal complex or divides in a highly organized manner to form a somatic embryo.
- Later root apex and shoot apex were developed and transformed to globular stage.
- Morphological changes during the transition from the globular stage to the heart stage are the first visible sign of the formation of the two embryonic organ systems: the cotyledons and the axis.

- In zygotic embryogenesis, maturation is characterized by attainment of mature embryo morphology, accumulation of storage carbohydrates, lipids and proteins, reduction in water content and a gradual decline or cessation of metabolism.
- Somatic embryos usually do not mature properly. Instead, due to environmental factors such as keeping a constant contact with inducing medium, somatic embryos often deviate from the normal



Importance of Somatic Embryogenesis:

- The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system.
- The adventitious embryo is a bipolar structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture.
- Somatic embryo has no food reserves, but suitable nutrients could be packaged by coating or encapsulation to form some kind of artificial seeds. Such artificial seeds produce the plantlets directly into the field.
- Unlike organogenesis, somatic embryos may arise from single cells and so it is of special significance in mutagenic studies.
- Plants derived from asexual embryos may in some cases be free of viral and other pathogens. So it is an

Synthetic Seeds

- Synthetic seeds are encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing as a seed or that possesses the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that can retain this potential also after storage.
- Synthetic seeds are produced by encapsulating a plant propagule in a matrix which will allow it to grow into a plant. Plant propagules consist of shoot buds or somatic embryos that have been grown aseptically in tissue culture.
- In the production of Synthetic seeds , an artificial endosperm is created within the encapsulation matrix. The encapsulation matrix is a hydrogel made of natural extracts from seaweed (agar, carageenan or alginate), plants (arabic or tragacanth), seed gums (guar, locust bean gum or tamarind) or microrganisms (dextran, gellan or xanthan gum.).
- These compounds will gel when mixed with or dropped into an appropriate electrolyte (copper sulphate, calcium chloride or ammonium chloride). Ionic bonds are formed to produce stable complexes. Useful adjuvants such as nutrients, plant

Types of

Uncoated, Desiccated Somatic Seeds:

- The embryos are desiccated to contain 8-15% moisture contents

Coated, Desiccated Somatic

- The somatic embryos are coated with polyoxyethylene and then desiccated.

Coated, Hydrated Somatic Seeds:

- Use of hydrated gels (like sodium alginate) with embryo.

Steps of Synthetic Seeds Production

Establishment of somatic embryogenesis

↓

Maturation of somatic

↓

embryos Synchronization and

↓

simulations of somatic embryos

↓

Mass production of

↓

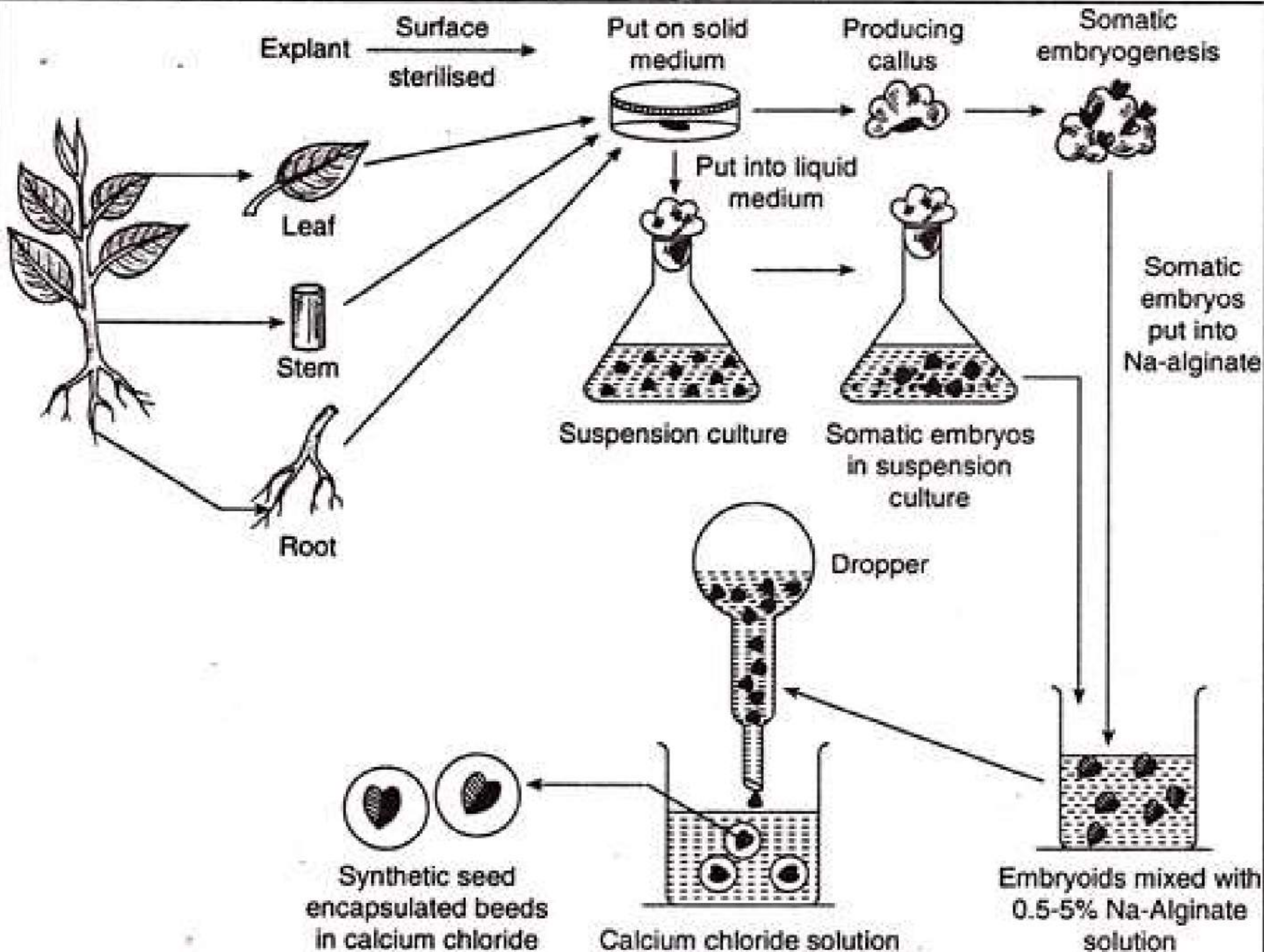
somatic embryos

↓

Standardization of

↓

encapsulation


Explant
Surface
sterilised
Put on solid
medium
Producing
callus
Somatic
embryogenesis
Leaf
Stem
Root
Put into liquid
medium
Suspension culture
Somatic embryos
in suspension
culture
Somatic
embryos
put into
Na-alginate
Dropper
Synthetic seed
encapsulated beeds
in calcium chloride
Calcium chloride solution
Embryoids mixed with
0.5-5% Na-Alginate
solution

Encapsulation

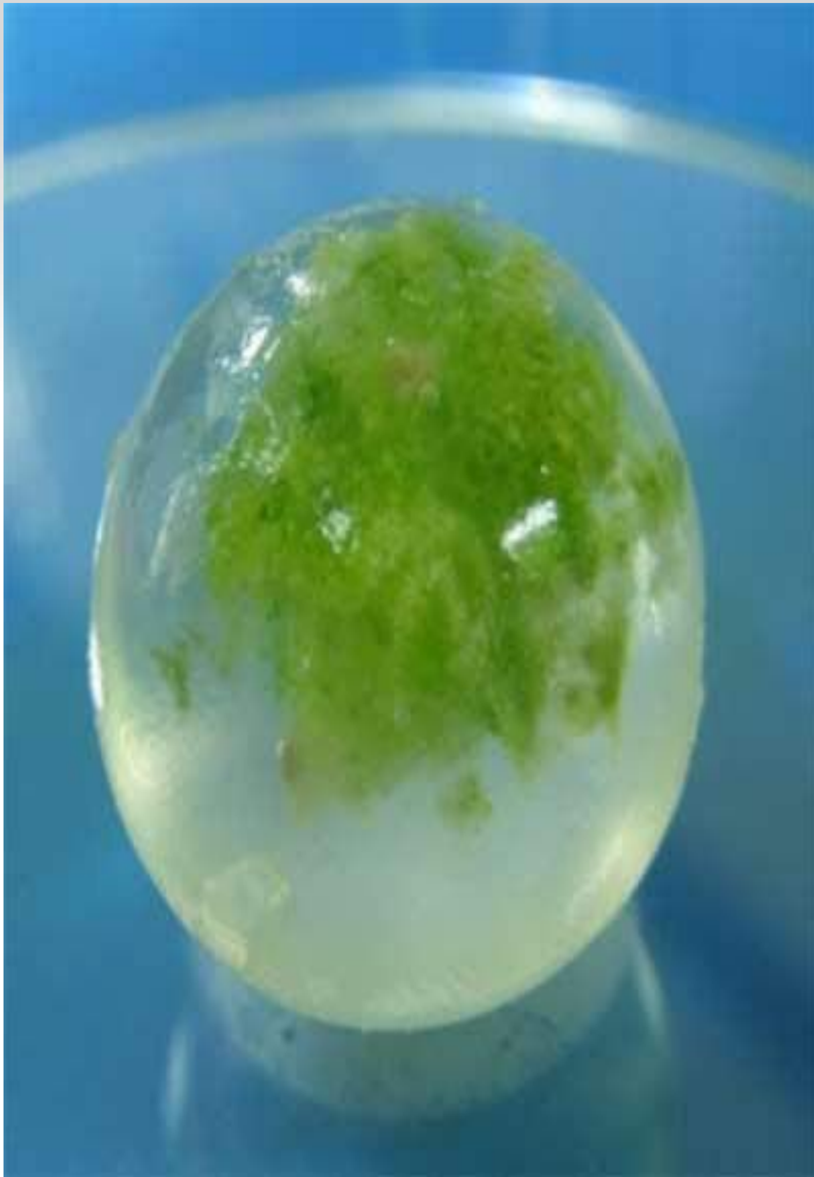
- Encapsulation is necessary to produce and to protect synthetic seeds. the encapsulation is done by various types of hydrogels which are water soluble. the gel has a complexing agent which is used in varied c

Gelling agent (% w/v)	Complexing agent (µM)
Sodium alginate (0.5 – 5.0)*	Calcium salts (30 –100)
Sodium alginate (2.0) with Gelatin (5.0)*	Calcium chloride (30 –100)
Carragenan (0.2 – 0.8)	Potassium chloride
Locust beam gum (0.4-1.0)	Ammonium chloride (500)
Gelrite (0.25)	temperature lowered

- Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its moderate viscosity and low spinnability of solution, low toxicity for somatic embryos and quick gellation, low cost and biocompatibility characteristics.

Principle and Conditions for Encapsulation with Alginate Matrix

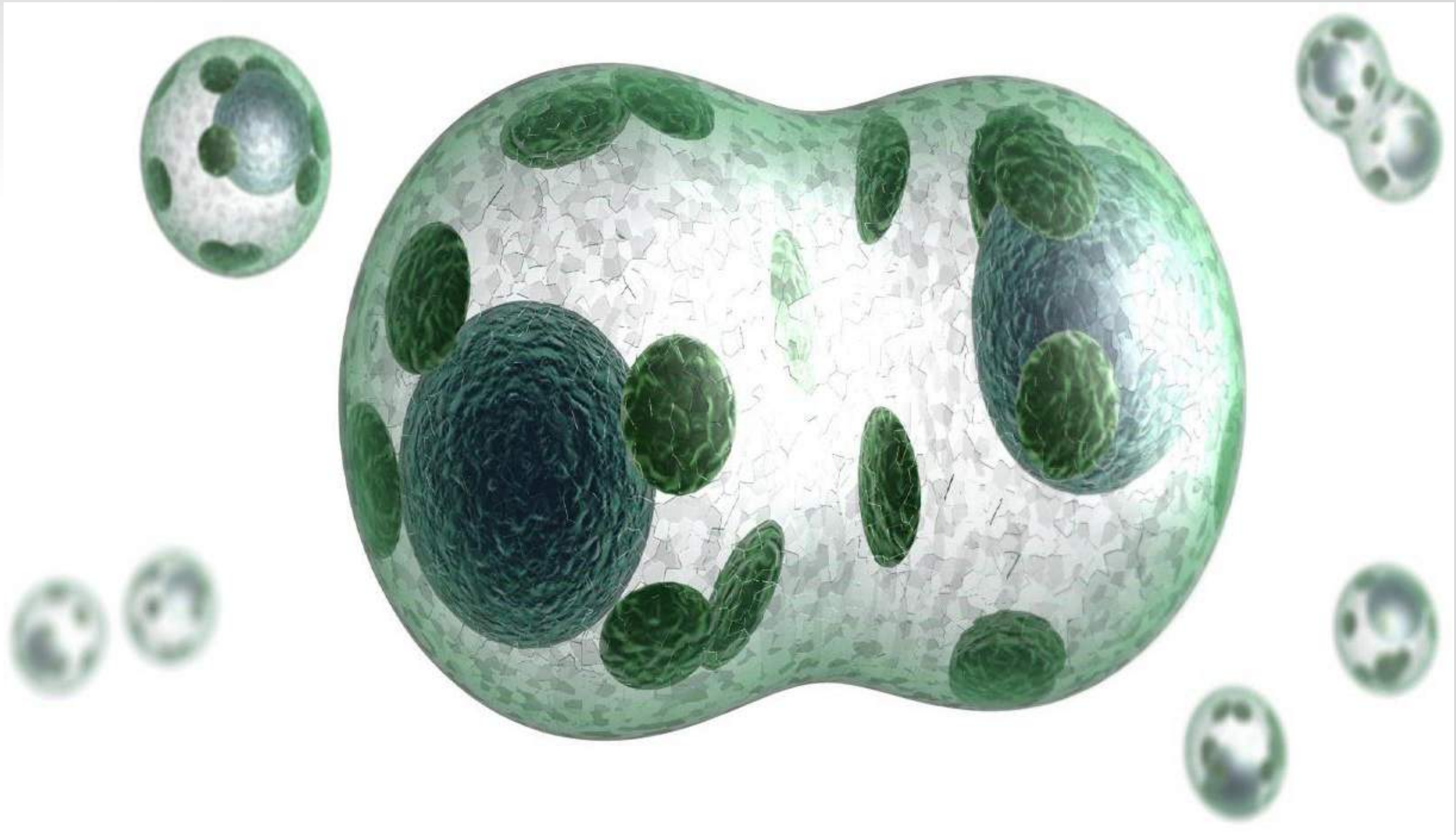
- The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the somatic embryos when dropped into the $CaCl_2.2H_2O$ solution form round and firm beads due to ion exchange between the Na+ in sodium alginate with Ca_2+ in the $CaCl_2$. $2H_2O$ solution.
- The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with calcium ions. Hence the concentration of the two gelling agents i.e., sodium alginate and calcium $CaCl_2.2H_3O$ and the complexing time should be optimized for the formation of the capsule with optimum bead



Application of Synthetic Seed

- Artificial seed provides low price production.
- It's going to act as distinctive delivery system.
- It plays a task of reproductive structure in embryo development.
- Artificial seed technology has evolved as another and probably economical technique for mass propagation of various plant varieties.
- By the employment of artificial seed technology species may be propagated.
- Cereals, fruits and healthful plants may be studied with the assistance of artificial seed development at any place in the world.
- Artificial seeds area unit terribly little therefore, they're straightforward to handle.
- Artificial seed may be transported from one country to a different while not any obligations from the quarantine department.
- Direct inexperienced house and field delivery of elite(seeds) chosen genotypes, genetically built plants area unit doable.
- Artificial seed crop area unit sometimes straightforward to handle attributable to
  uniform genetic constituent.

Protoplast Culture



Contents

- Introduction
- Protoplast isolation
  - Mechanical Method
  - Enzymatic Method
- Protoplast fusion
- Culture of Protoplast
- Importance
- Application
- Advantages and Disadvantages
- Factors affecting protoplast culture
- Future of protoplasts

Introduction - The Protoplast

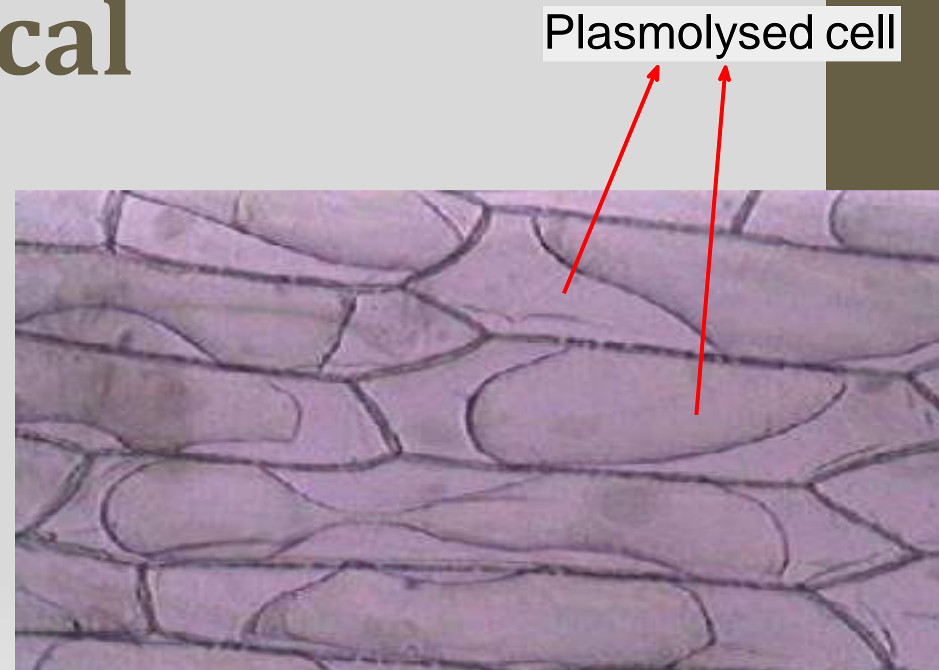
- Is a naked cell surrounded by a plasma membrane. It can regenerate cell wall, grow and divide.
- Is fragile but can be cultured and grow into a whole plant.
- Cells can originate from any type of tissue.
- Is important for crop improvement.
- Via somatic hybridisation, new hybrids are produced
- Is used by biotechnologists, microbiologists, pathologists,...
- Somatic Hybridization was firstly introduced by Carlson in *Nicotiana* '**glauea'**
- In 1960, E.C Cocking contributed to the enzymatic isolation and culture of protoplast

Protoplast isolation

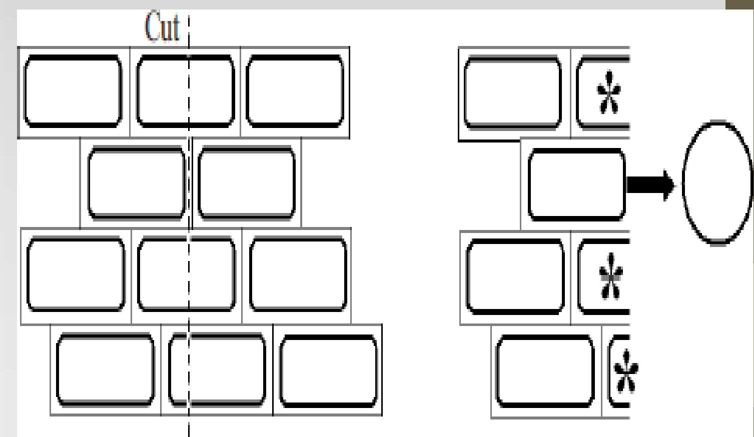
- Refers to the separation of protoplast from plant tissue
- Important to isolate viable and uninjured protoplast as gently and as quickly as possible
- Involves two methods:
    - Mechanical
    - Enzymatic

Mechanical method

- Tissue is immersed in 1.0 M sucrose until protoplasm shrunk away from their enclosing cell wall (Plasmolysis)



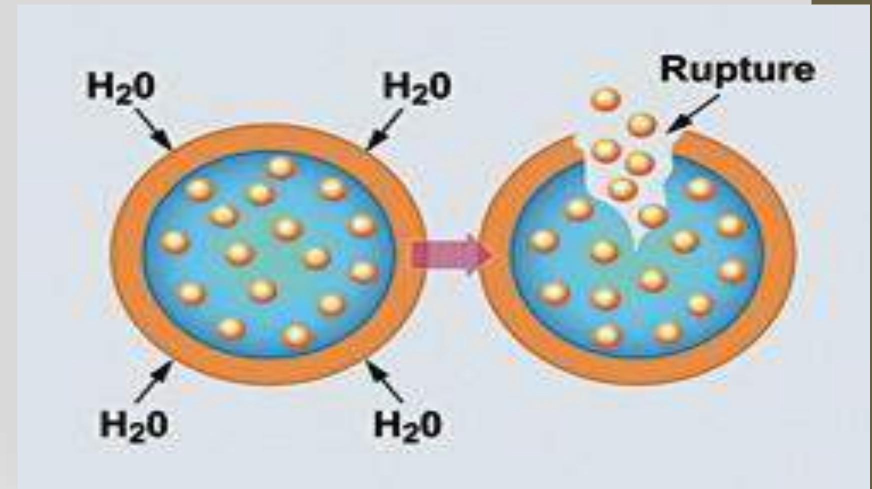
- Plasmolysed tissue is cut with a sharp knife at such a thickness that only cell walls are cut



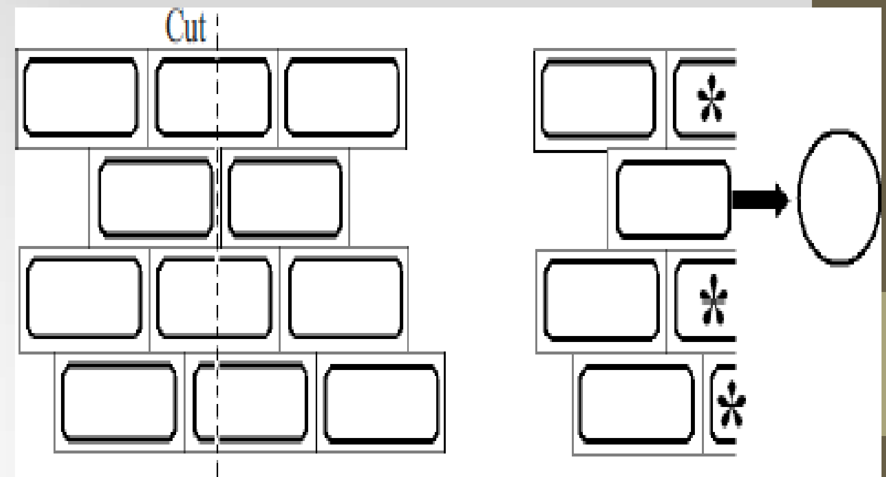
Mechanical method

- Undamaged protoplast in strips are released by osmotic swelling when placed in a low concentration of sucrose solution

Low [sucrose soln]



- <u>Problem encountered:</u> some cells release uncut complete protoplast while the rest produces broken dead protoplasts.



Enzymatic method

- Refers to the use of enzymes to dissolve the cell wall for releasing protoplasts

- **Advantages:**
  - Used for variety of tissues and organs such as fruits, roots, petioles, leaves…
  - Osmotic shrinkage is minimum
  - Cells remain intact and not injured
  - Protoplast readily obtained

- Involves two methods:
  - Direct method (One step only)
  - Sequential method (Two step method)

1. Direct method

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

2. Sequential method

- Two enzyme mixtures(mixture A and mixture B) are used one after the other
- Leaf segments with mixture A (Macerozyme in manifold at pH 5.8) are vacuumed infiltrated for 5 mins, transferred to a water bath at 25°C and subjected to slow shaking
- The enzyme mixture is then replaced by fresh 'enzyme mixture A' and leaf segments are incubated for another hour

2. Sequential method

- The mixture is filtered using nylon mesh and centrifuged for 1 min
- Washed 3 times with 13% mannitol
- Cells are then incubated with ‘enzyme mixture B’ (Cellulase in mannitol solution at pH 5.4) for above 90 mins at 30°C
- The mixture is centrifuged for 1 min so that protoplast form a pellet and clean 3 times with sorbitol

Purification of protoplast

- Protoplasts are purified by removing:
  - Undigested material (debris)
  - Bursts protoplasts
  - Enzymes

- **Debris** are removed by filtering the preparation through a nylon mesh

- **Enzymes** are removed by centrifugation whereby the protoplasts settle to the bottom of the tube and the supernatant removed with the help of a pipette

- Intact protoplasts are separated from **broken protoplasts** through centrifugation and removed by a pipette as they are collected at the top of tube

Protoplast Culture

- Isolated protoplast can be cultured in an appropriate medium to reform **cell wall** and generate **callus**

- Optimal culture conditions:
  1. Optimal density to the culture.
  2. Optimal auxin to cytokinin ratio, glucose and sucrose.
  3. Maintain osmoprotectant in the medium
  4. Temperature: 20-28°C

     pH: 5.5-5.9

     0.25% Casein hydrolysate

     BAP and NAA

Culture of protoplasts

- Protoplasts cultured in suitable nutrient media first generate a new cell wall
- The formation of a complete cell with a wall is followed by an increase in size, number of cell organelles, and induction of cell division
- The first cell division may occur within 2 to 7 days of culture
- Resulting in small clumps of cell, also known as micro colony, within 1 to 3 weeks

Culture of protoplasts (continued)

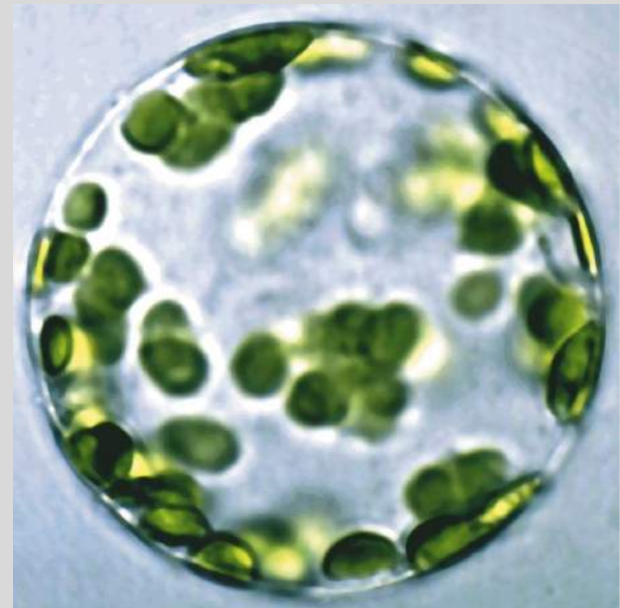
- From such clumps, there are two routes to generate a complete plant (depending on the species)

1. Plants are regenerated through organogenesis from callus masses

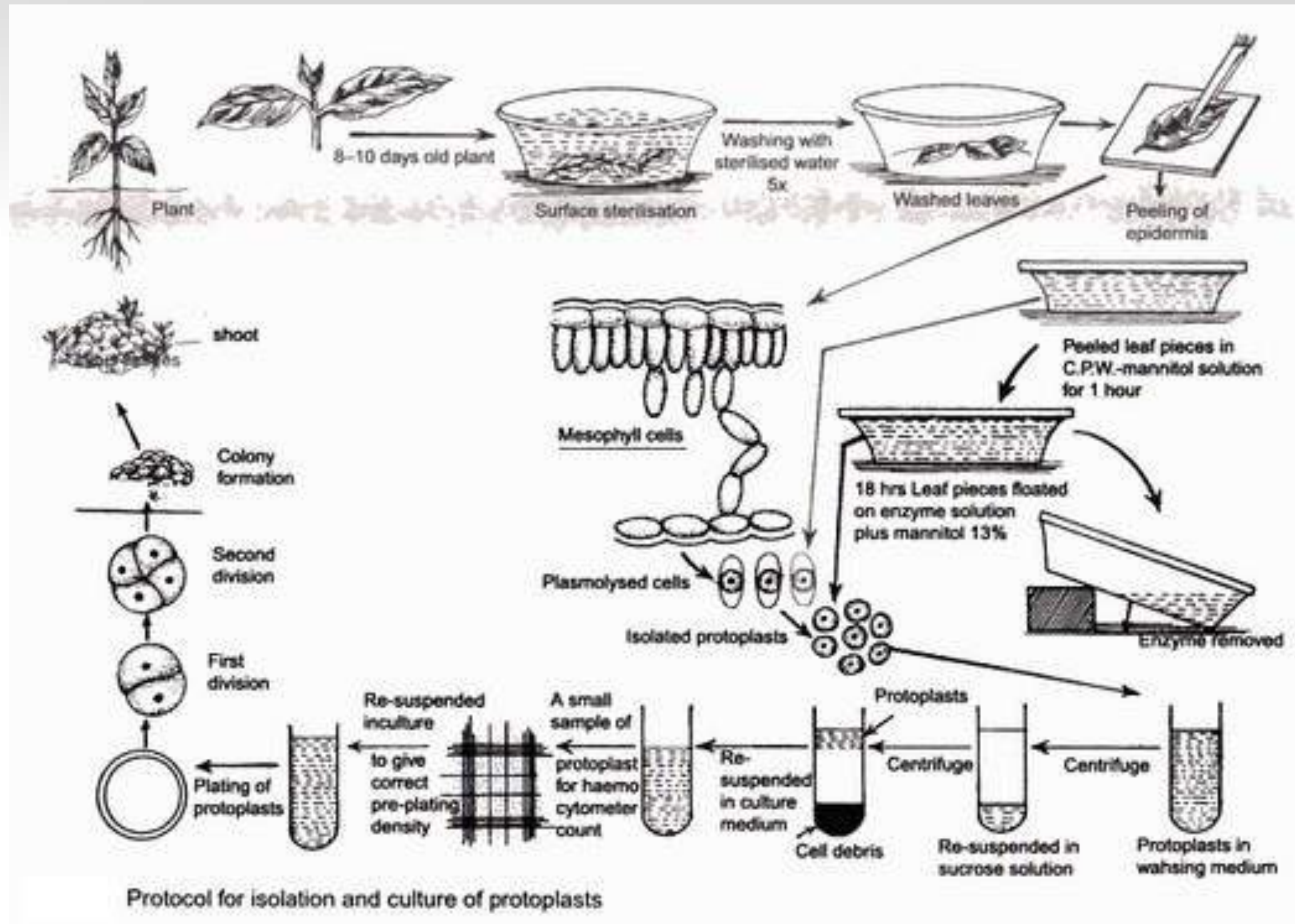
2. The micro calli can be made to develop into somatic embryos, which are then converted into whole plant through germination

Importance of Protoplast Culture

- Study of Osmotic behavior
- Study of IAA action
- Study of Plasmalemma
- Study of Cell wall formation
- Organelle isolation
- Study of Morphogenesis
- Virus uptake and replication
- Study of photosynthesis
- Gene Transfer



Summary of isolation and culture of protoplasts



Protocol for isolation and culture of protoplasts

Factors affecting protoplast culture

1. Plant species and varieties
   - Small genetic difference leads to varying protoplast responses to culture conditions

2. Plant age and organ
   - Age of donor plant and its developmental stage
     - Stages are germinating embryos, plantlets, leaves…

3. Pre-culture conditions
   - Protoplast culture are highly influenced by climatic factors and different culture of seedlings yields protoplast having different responses when cultured

Factors affecting protoplast culture

4. Pre-treatment to the tissue,before isolating protoplasts
   - Cold treatment,plasmolysis and hormone increases the chance of recovery of viable protoplasts and their plating efficiency

5. Density
   - Crucial as it influences plating efficiency and surviving of protoplasts. At higher density, protoplasts compete with one another while at lower density losses of metabolites from protoplasts is more.

Protoplast Fusion

Protoplast fusion techniques:

1. Electrofusion
2. Polyethene glycol - induced fusion (PEG)
3. High Ca2+ , high pH

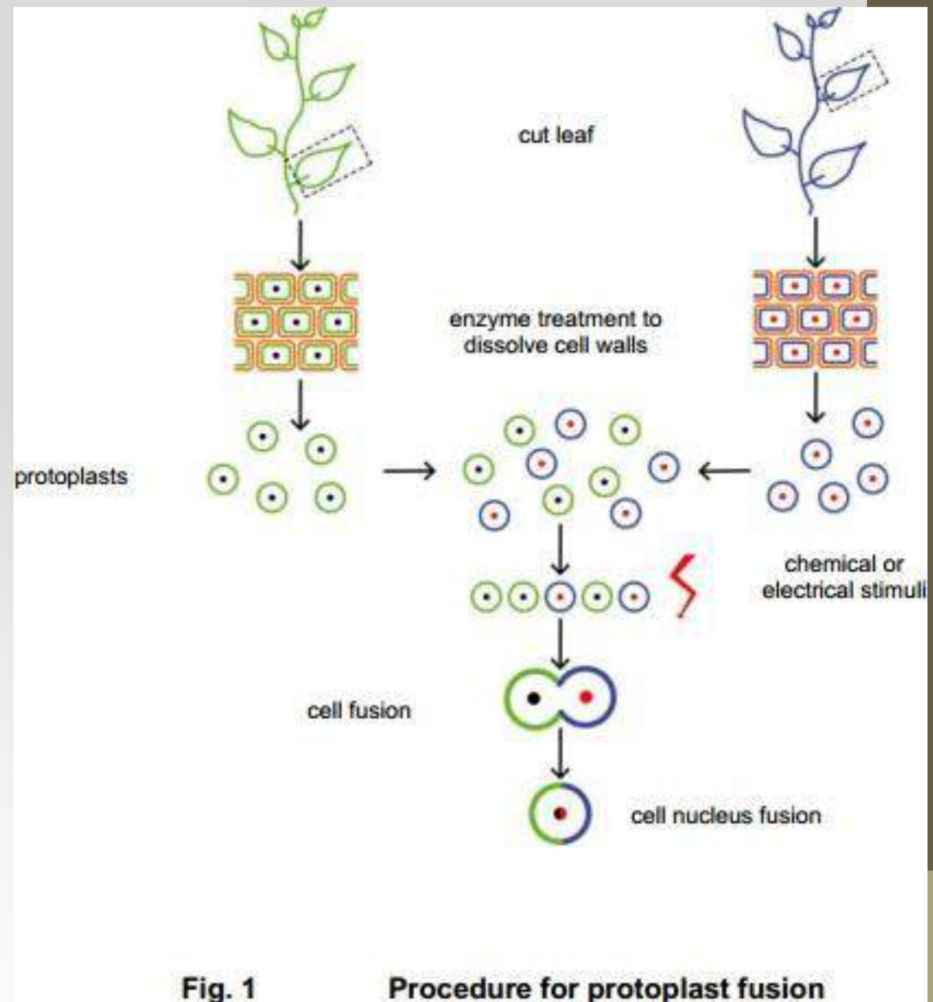


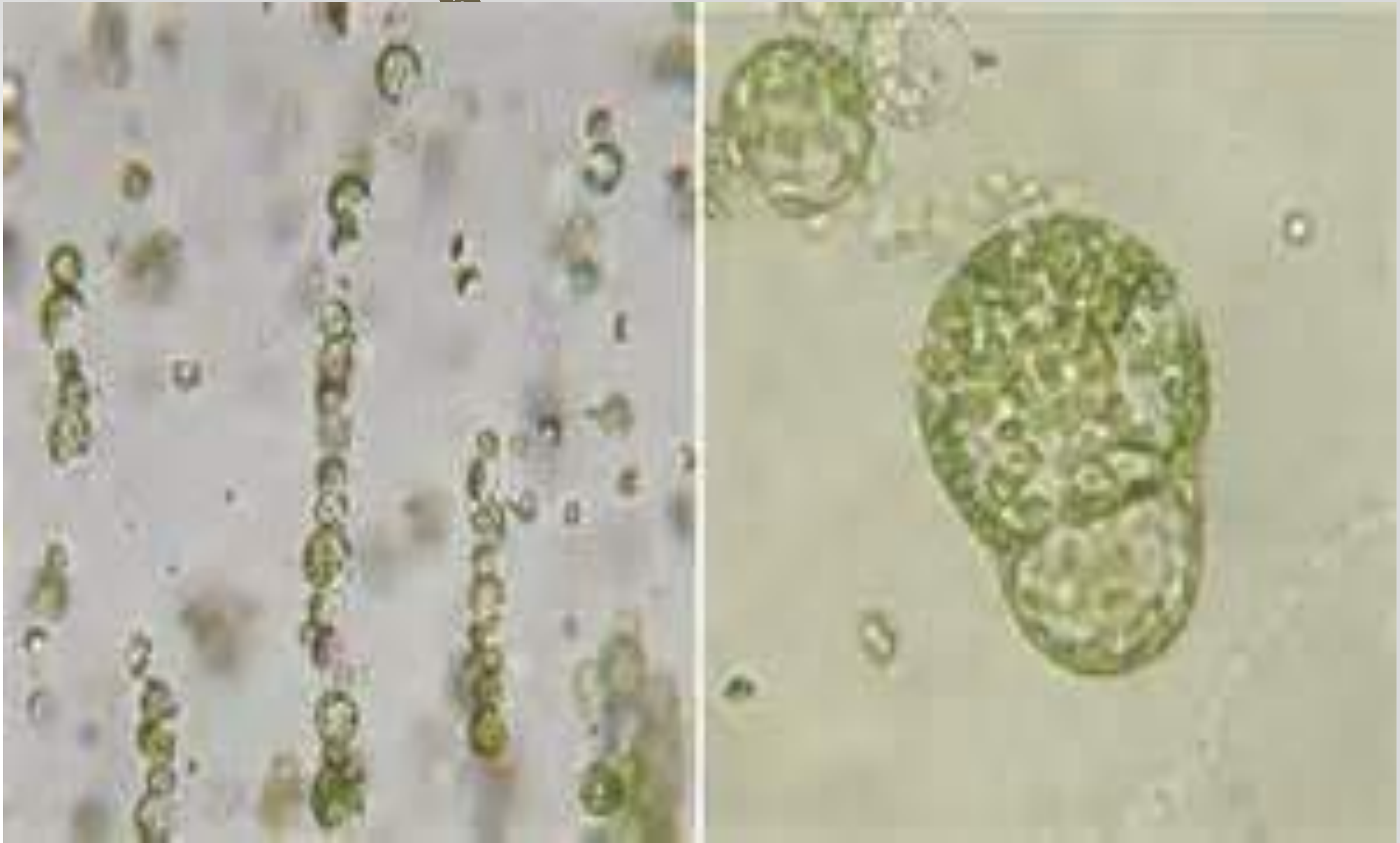
Fig. 1 Procedure for protoplast fusion

Electrofusion

- Mild electrical stimulation is being used to fuse protoplasts.
- Two glass capillary microelectrode are placed in contact with the protoplasts.
- An electric field of low strength give rise to dielectrophoretic pole generation within the protoplast suspension.
- This leads to pearl chain arrangement of protoplasts.
- No of protoplasts within a pearl chain depends upon the population density of the protoplast and the distance between the electrodes.
- Subsequent application of high intensity electric impulse for some microseconds results in the electric breakdown of membrane and subsequent fusion.
- There are two types of current that are used here:
    - A.C current (fq=0.5-5.0 MHz and field strength=50-300V/cm)
    - D.C current (t=5-50 us and field strength=500-1000V/cm)

- Apply A.C-->Pearl chain-->D.C-->Pores form-->Fusion-->A.C

Electrofusion



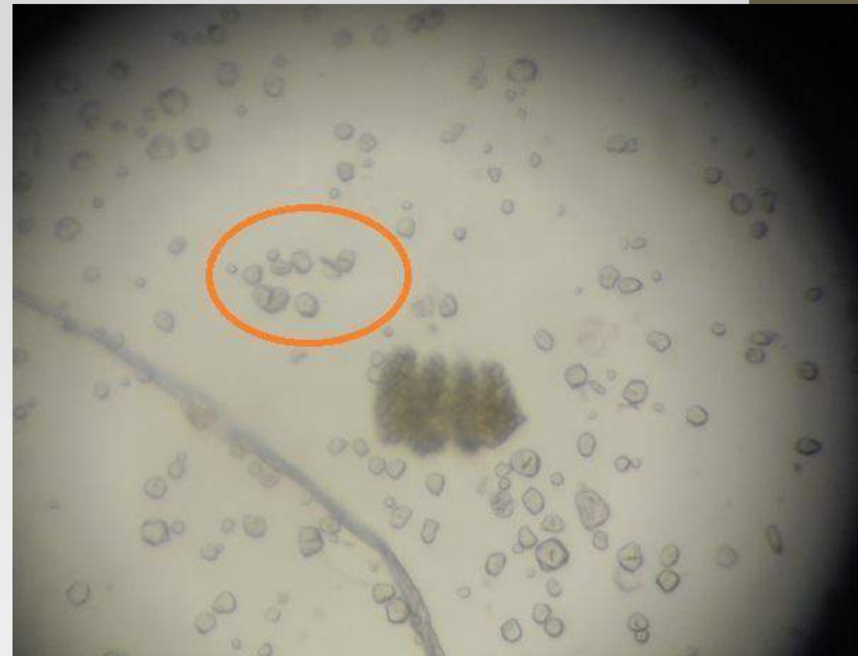
PEG (Polyethylene glycol) Fusion

- This chemical has been identified as a possible/potent fusogen.
- It has a high molecular weight about 1500-6000.
- Usually a PEG solution of about 28-50% is used for protoplast fusion.
- This polymer binds to the lipid membrane of cells and thus induces fusion
- Fusion takes place for 45 min in

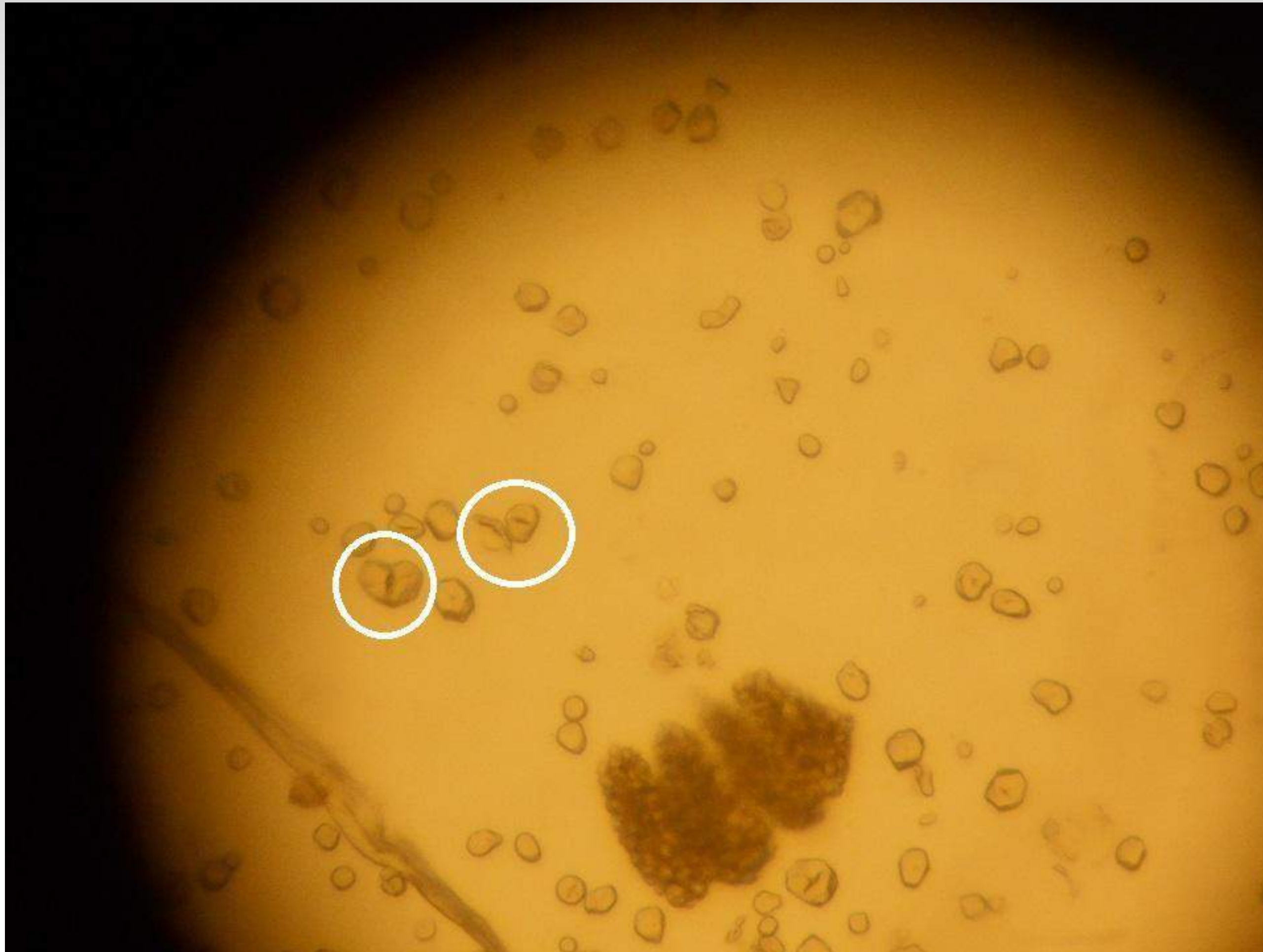
incubation at r.t.p

- Fusion takes place slowly during

elusion of PEG with liquid culture medium.



Class practical results: Protoplast fusion



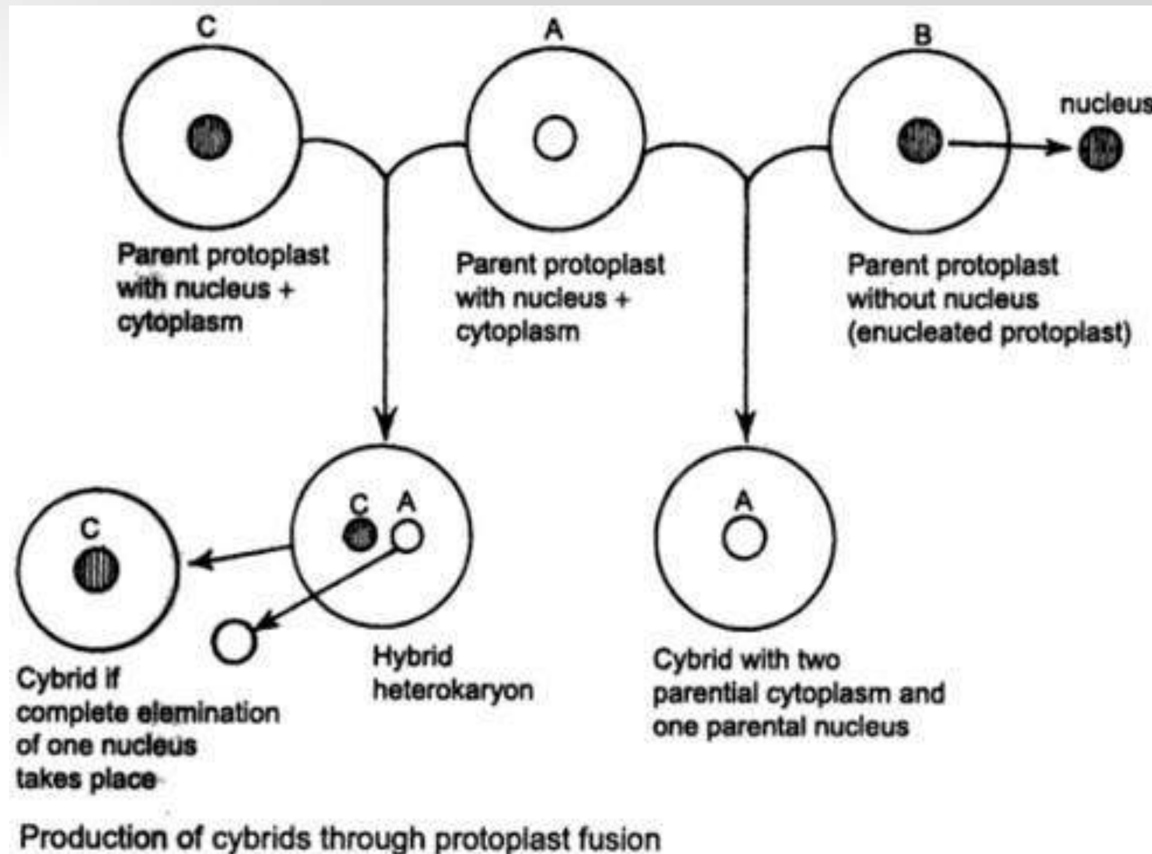
Application of Protoplast

Protoplasts can be used:

- In the production of Cybrid
- For Somatic Hybridization to overcome sexually incompatible species
- Ingesting “Foreign” material into cytoplasm
- For DNA transformation
- Used to study wall synthesis and decomposition
- Studied as Single Cell System

- Production of Cybrid

Cybrid contain nuclear and cytoplasmic genome of one parent and only the cytoplasmic genome of the second.



Production of cybrids through protoplast fusion

- Somatic Hybridization

Fusion of protoplast that facilitates the mixing of 2 whole genomes and could be exploited in crosses at:

intergeneric, interkingdom and interspecific levels

Somatic hybridization is used to produce hybrids from sexually incompatible species.

This method could also be used to study selection procedures.

Somatic hybridization

Limitations of Somatic Hybridization

1. Intergeneric crosses between widely related plants which are not compatible sexually are not possible.
2. In certain wide crosses, elimination of chromosomes from hybrid cell is another limitation of somatic hybridisation.
3. In protoplast fusion experiments, the percentage of fusion between two different parental protoplast is very low.
4. For hybrid identification, selection and isolation at the culture level, there is no standardized method which is applicable for all material.

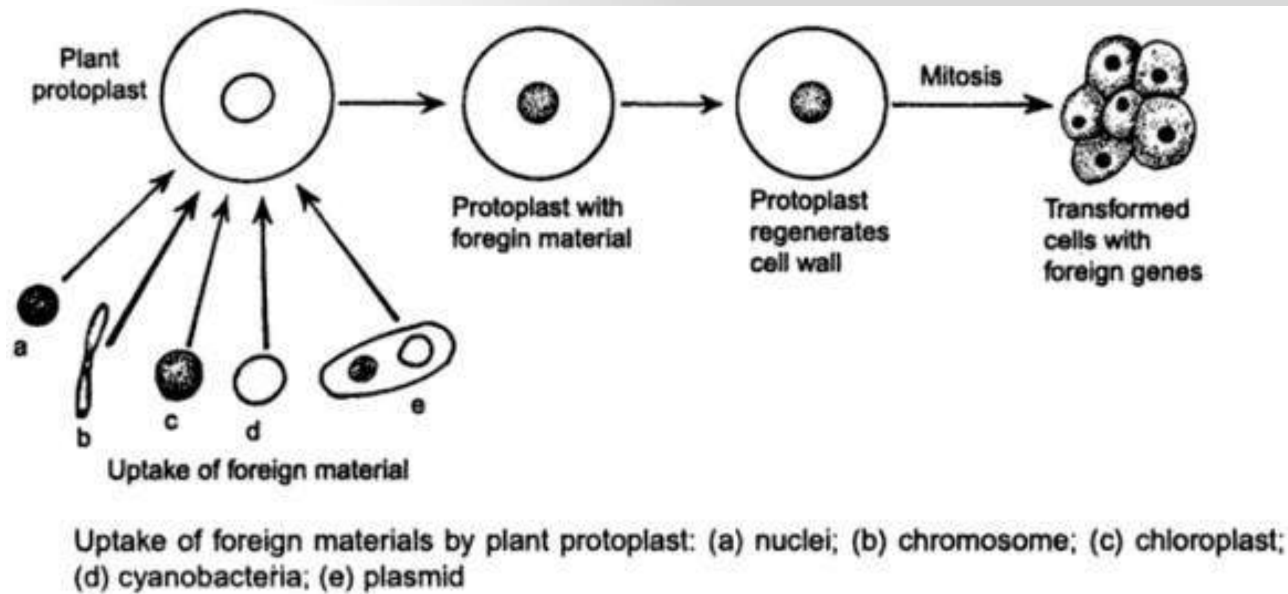
Ingestion of foreign material

Protoplast being wall-less show high pinocytic activity and can ingest biological active foreign bodies such as

DNA, plasmids, bacteria , viruses etc.

- results into modified cells.

Advantageous to plant breeder in getting more efficient crop varieties in near future.



Uptake of foreign materials by plant protoplast: (a) nuclei; (b) chromosome; (c) chloroplast; (d) cyanobacteria; (e) plasmid

Advantages of Protoplast fusion

1. It facilitates the mixing of two genomes and can be used in crosses at interspecific, intergeneric or even intraspecific levels

2. To create new strains with desired properties and for strain improvement

3. Mixing two genomes opens the door to gene transfer and a study of gene expression, stability of several traits and cell genetic changes

Disadvantages of Protoplast Fusion

During the mechanical method of isolation of protoplasts:

1. It yields a very small amount of protoplasts after a rather tedious procedure

2. It is not suitable for isolating protoplasts from meristematic and less vacuolated cells

- During and subsequent to digestion of the cell wall, the protoplast becomes very sensitive to osmotic stress. Thus, cell wall and protoplast storage must be done in an isotonic solution to prevent rupture of the plasma membrane

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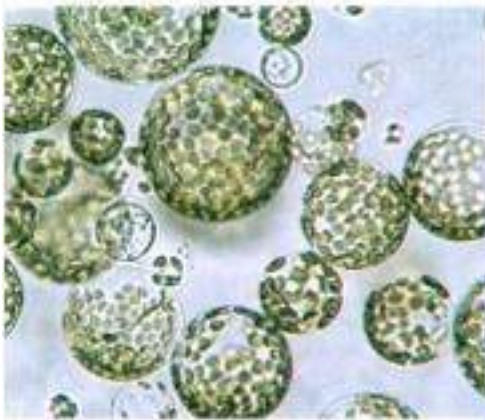

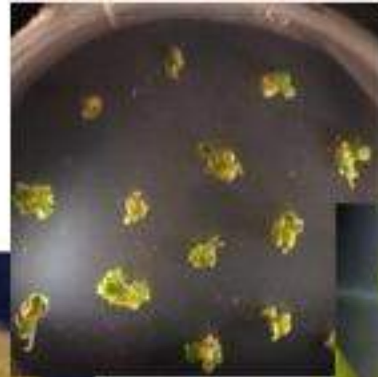

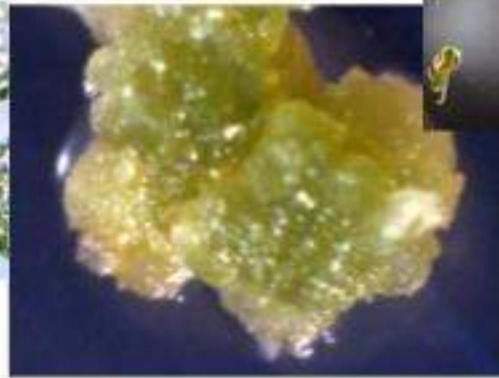

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Protoplast - Up to now

- Transfer of useful genes from one species to another.
- Technique used in strain improvement for bringing genetic recombinations.
- Technique used in microbial engineering; new strains produced for industrial purposes.
- Protoplast fusion used by:
    - Molecular biologists
    - Biotechnologists
    - Tissue Culturists
    - Industries

Alcohol Production

- Creation of newstrain of *Saccharomyces* from *S.cerevisiae* and *T.reesei*
  - Alcohol production- up to 80% from cellulose material
  - Less dependent on Fossil Fuels

- Strains from *S.uvarum*
  - Can produce low carbohydrate beer of acceptable flavour
  - Production of Better beer

Crop Production

- Palm Oil production (*Elaeis guineensis*, tenera oil palm)
  - Largest source of edible oil before soybean oil in the world, contributes 31.8% of the world's production of oils and fats
  - Takes 8 years to fully grow and produce fruits.
  - Protoplast culture helps to grow the trees faster to satisfy the increasing demands. Plantlets obtained within 2 years!

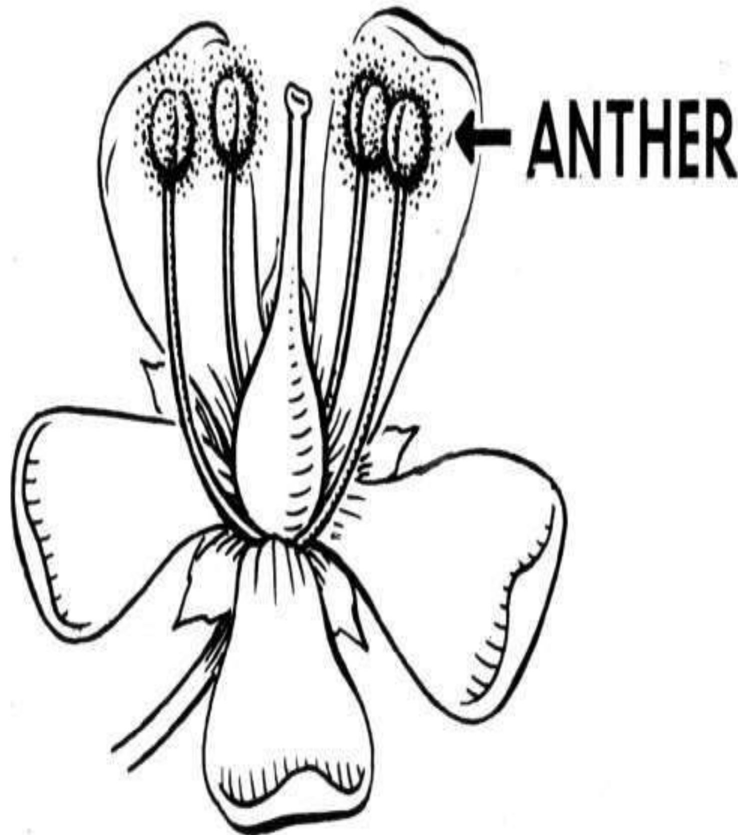
- *Citrus*
  - Subtropical fruit crop in the world
  - *C. sinensis & C. reticulata* = New Seedless citrus fruit (Cybrids) Faster than c.breeding

Crop Production (cntd)

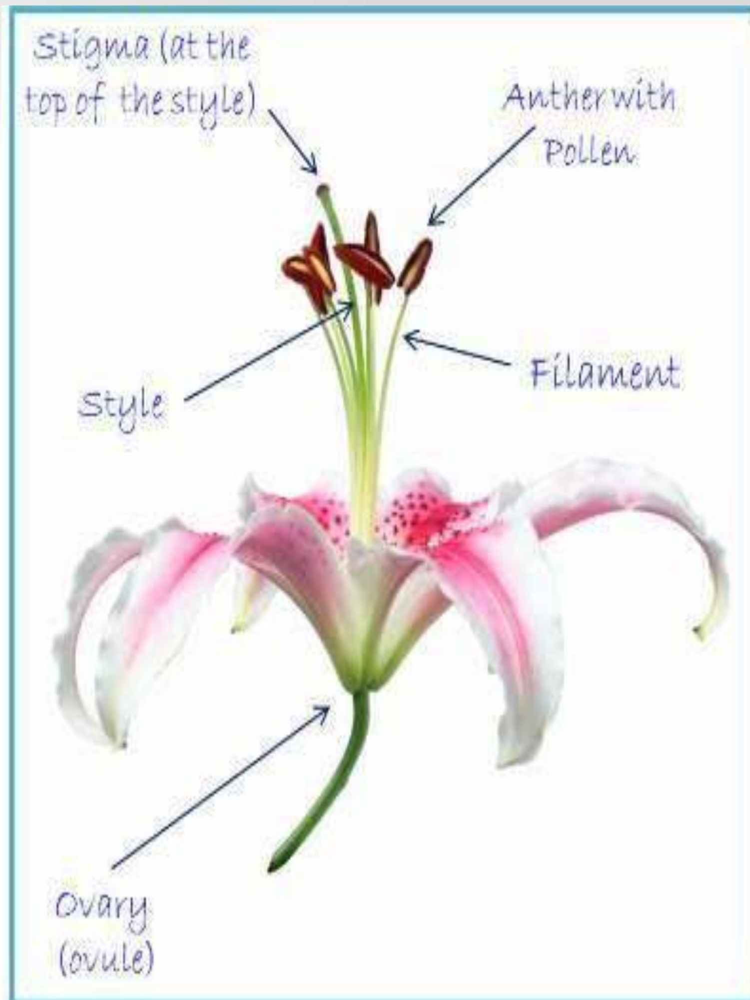
- Date fruit production (*Phoenix dactylifera* L.)
  - Deglet nour and Takerboucht (2 varieties)
  - Deglet nour-excellent fruit quality
  - Takerboucht-resistant against “bayoud” disease
  - Combination to give new strain- Protoplast fusion

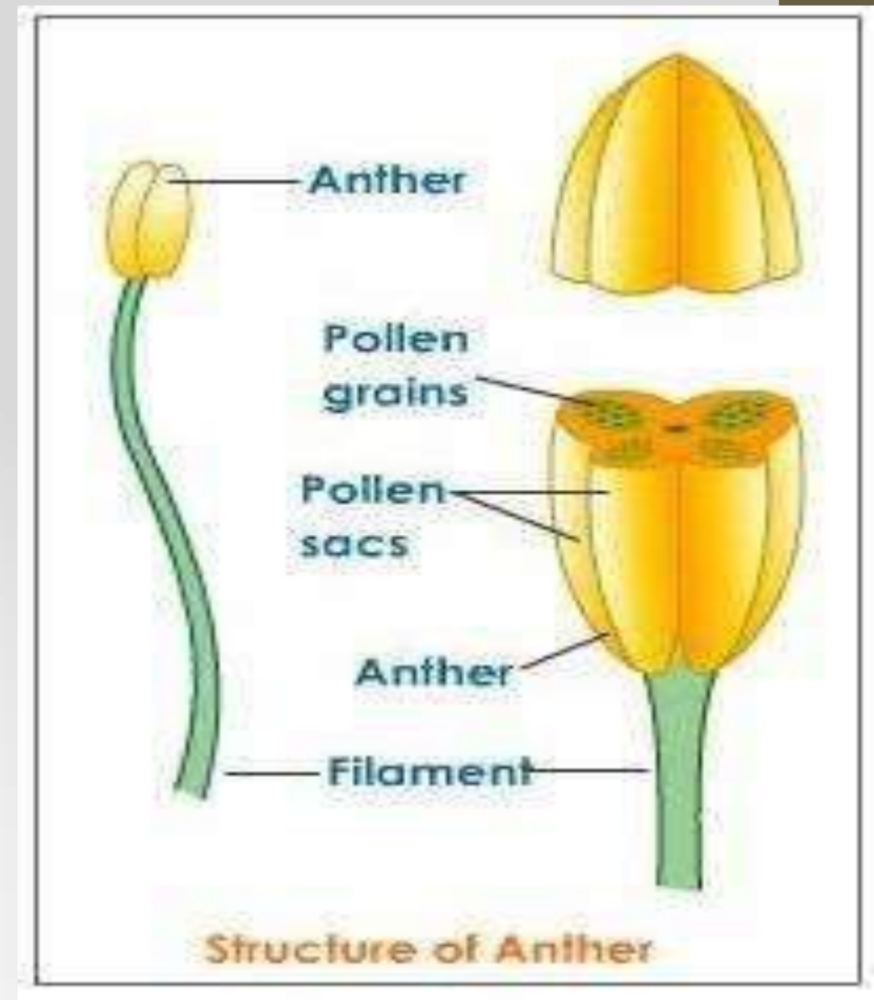
- Potato production
  - *Solanum tuberosum & S.brevidens* (incompatible)
  - Protoplast fusion to introduce resistance to potato leaf roll virus.
  - Hybrids showed resistance and were female fertile.

ANTHER AND POLLEN CULTURE



- **ANTHER**: A part of stamen containing pollen.
- **POLLEN**: A fertilizing powder discharged from flowers anther.





HISTORY

- **W.TULECKE(1953)**

First observed that mature pollen grains of *Ginkgo biloba* (gymnosperm) can be induced to proliferate in
culture

to form haploid callus.

- **S.GUHA AND S.C MAHESWARI(1964)**

First reported the direct development of embryos from
microspores

of

Anther culture

- Culturing of anther obtained from unopened flower bud in the nutrient medium under aseptic condition. callus tissue or embryoids from anther, that give rise to haploid plantlets either though organogenesis or embryogenesis.

Pollen culture

- Pollen or microspore culture is an in vitro technique by which the pollen grains preferably at the uninucleated stage, are squeezed out aseptically from the intact anther and then cultured on nutrient medium.
- The microspores develop into haploid embryoids or callus tissue that give rise to haploid plantlets by embryogenesis or organogenesis.

Androgenesis

- Androgenesis is the in vitro development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation.

It is of two

1)Direct androgeneis:- The microspores behaves like a zygote and undergoes change to form enbryoid which ultimately give rise to a plantlet.

2) Indirect Androgenesis:

The microspores divide repeatedly to form a callus tissue which differentiates into haploid plantlets.

Principle of anther and pollen culture

- The production of haploid plants is to exploit the totipotency of microspore .

- In this process the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcely to a new metabolic pathway for vegetative cell division .

Method of anther culture

1)Collection of unopened flower buds.

2)Surface sterilized with 70% ethanol.

3)Anthers excised from flower buds and kept separately. 4)Anthers in first meiotic division is selected by acetocarmine test.

5)Inoculated in the medium containing glutamine, L-

6). Incubated the culture at 25°C for 15 days. Here, anthers grow in to embryoids.

7)Embryoids transfer to rooting medium under 3000 lux illumination after 4-5 weeks the embryoids became plantlets.

8)For acclimatization, transfer to green house.

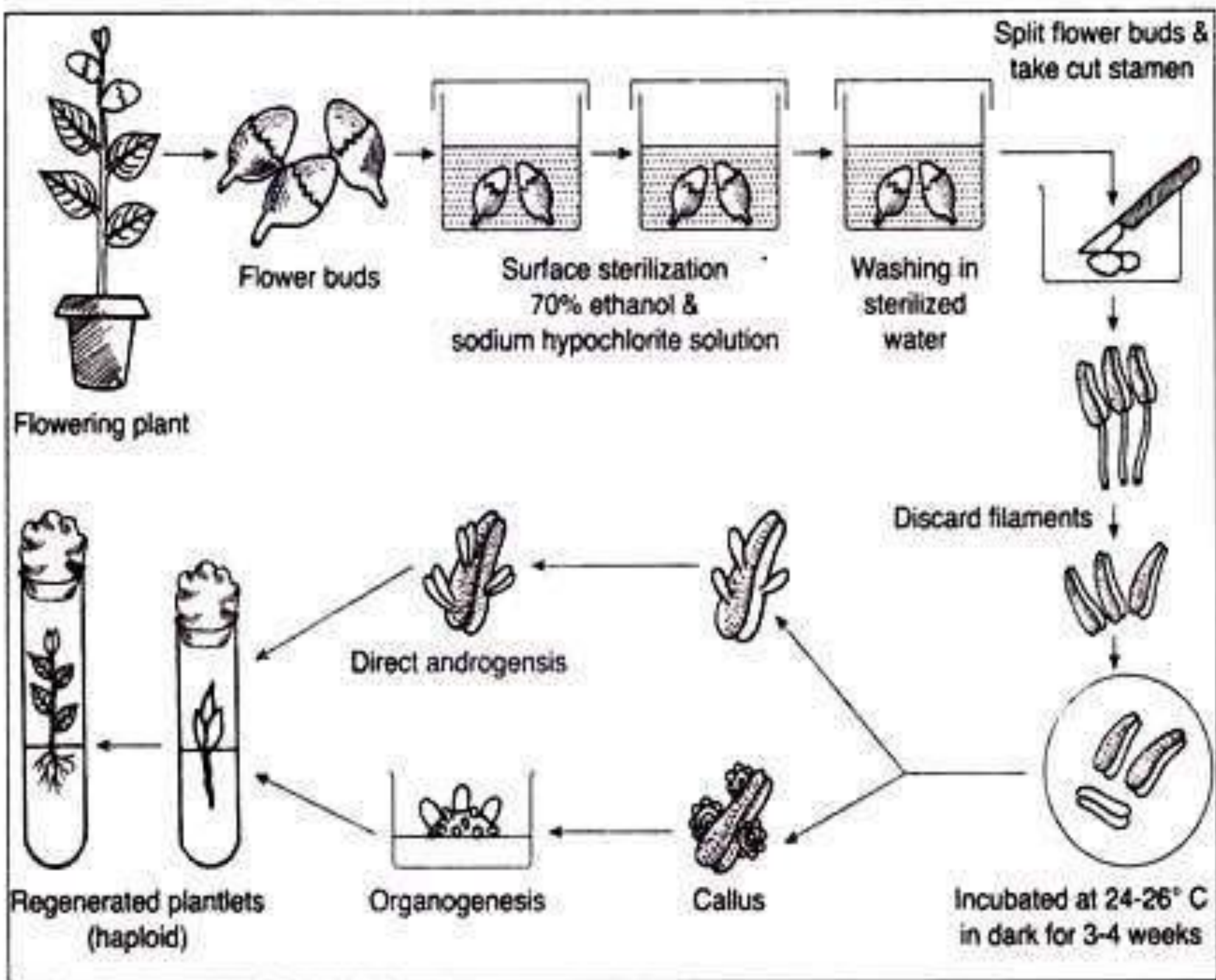


Fig. 21.2A: Anther culture

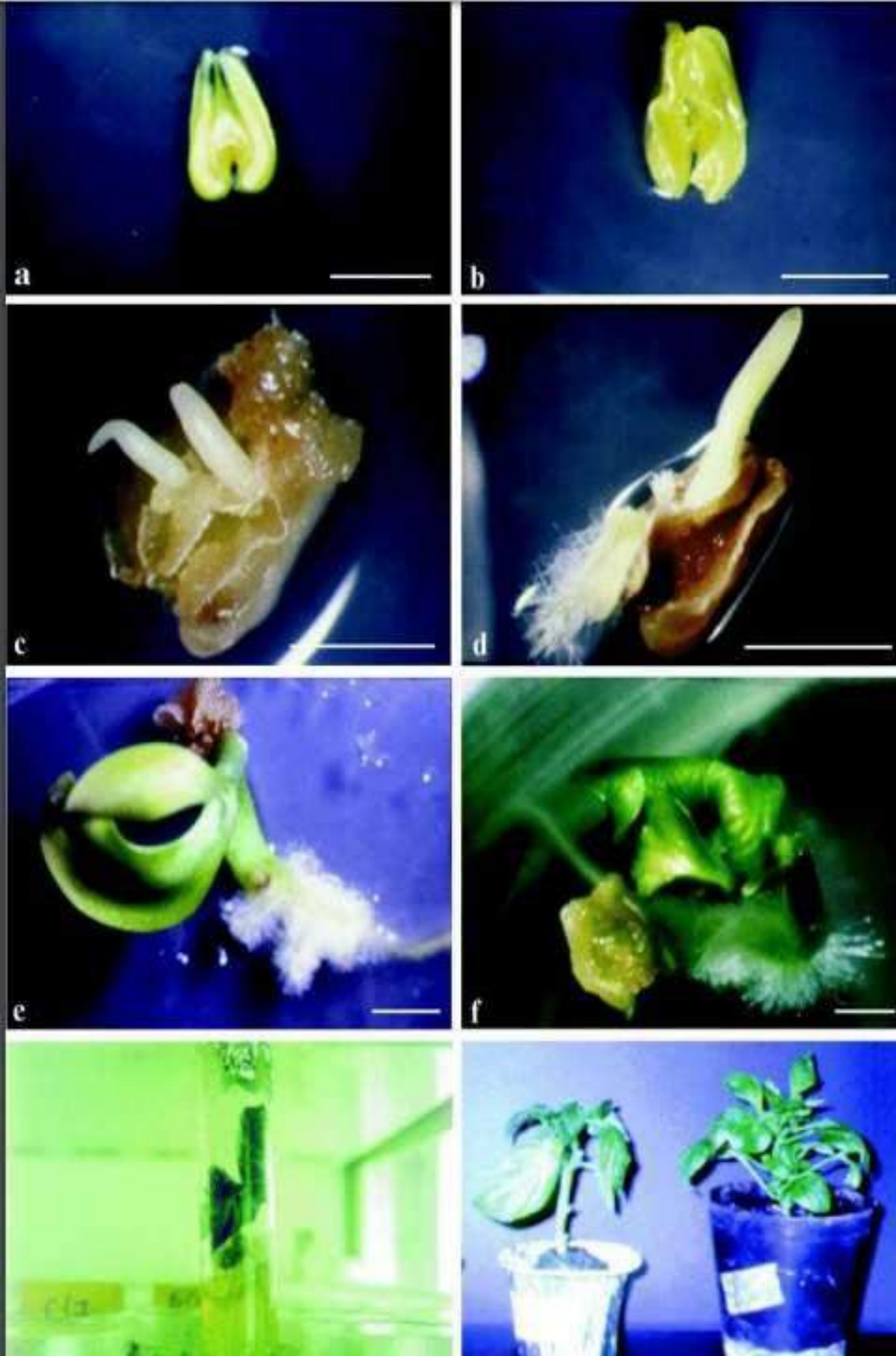


figure : Anther culture and haploid plants regeneration.

(a) Anther at the onset of the culture. (**b**) Anther after 6 days in culture. (**c**, **d**) Embryos emerging from the anthers after 30 days in culture, showing roots (**c**) and shoots (**d**). (**e–g**) Plantlets with cotyledons (**e**) and with leaves (**f**, **g**) subcultured in growing medium. (**h**) 80-day-old regenerated haploid plant from anther culture (left-hand side).

METHOD OF POLLEN CULTURE

1)Anther collected from flower buds and pollen grains are isolated and about 50 anthers are placed in small sterile beaker containing 20 ml of liquid basal medium (MS or White or Nitsch)

2)Anthers are then pressed against the side of beaker with the sterile glass rod to squeeze out the pollens.

3)The homogenized anthers are then filtered through a nylon sieve to remove that the anther tissue debris.

4) The filtrate or pollen suspension is then centrifuged at low speed (500-800 rpm/min) for five minutes. The supernatant containing fine debris is discarded and pellet of pollen is suspended in fresh liquid

5)A 2.5 ml of pollen suspension(usually 10^3 to10 4/ml)
is pipetted off and is spread in 5 cm petridish. Pollens are best grown in liquid medium but, if necessary, they can be grown by plating very soft agar added medium.

6)Petridishes are incubated at 27-30°C under low intensity of white cool light (500 lux,16 hr).

7)Young embryoids can be observed after 30 days. The embryoids ultimately give rise to haploid plantlets.

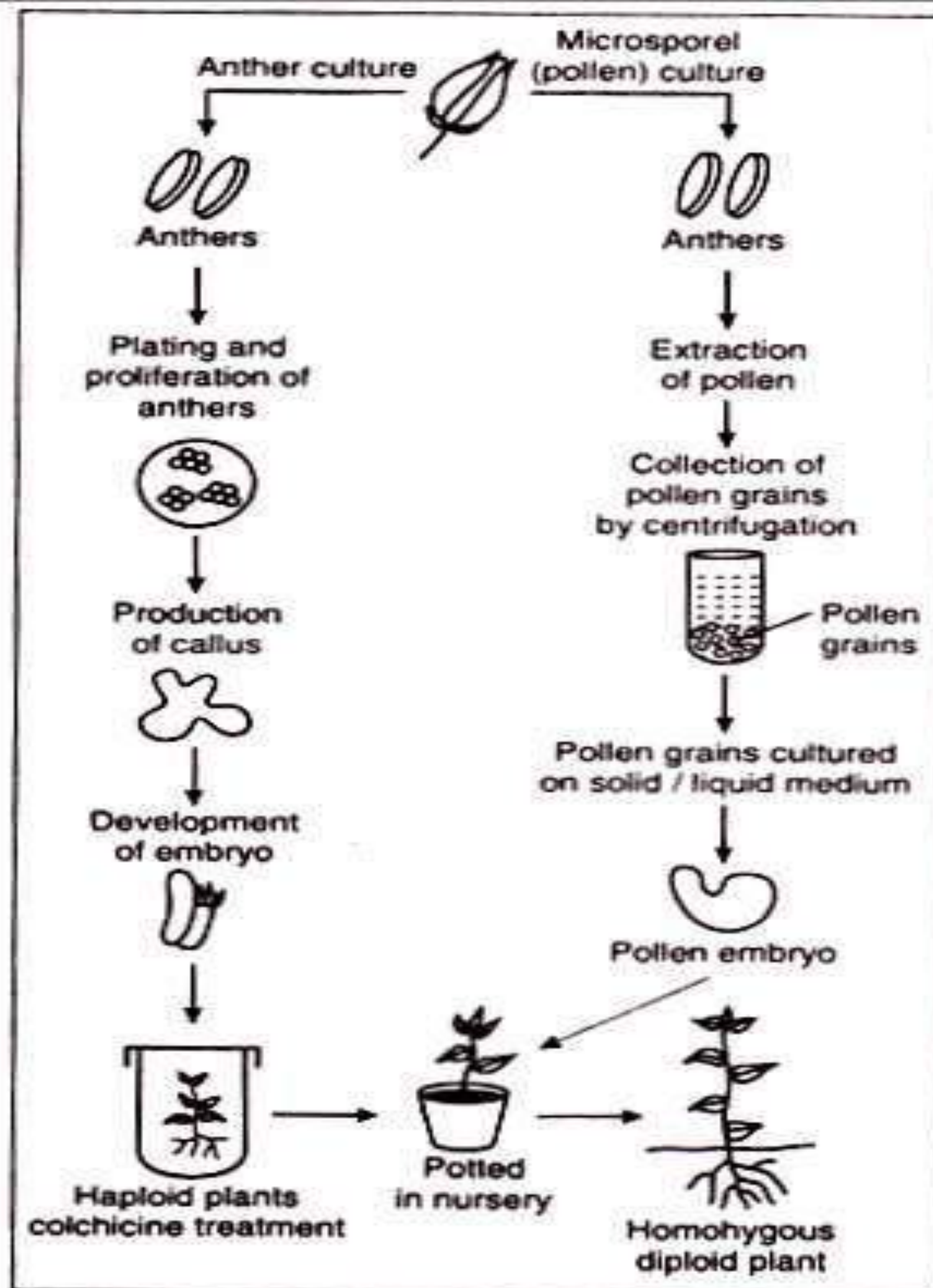


Fig. 10.14: Anther and microspore culture for the generation of haploid plants

Factors influencing anther and pollen culture

1) Genotype of donor plants:

The genotype of the donor plant plays a significant role in determining the frequency of pollen production.

Example :- *Hordeum* of each genotype differs with respect to androgenic response in anther culture.

2)STAGE OF THE MICROSPORE

Anthers are more productive when cultured at uninucleate microspore stage.

Example : Barley, Wheat, Rice, etc.

3) CULTURE MEDIUM:

- For anther culture, medium requirements vary with genotype and the age of the anther as well as condition under which donor plants are grown.
- Incorporation of activated charcoal into the medium has stimulated the induction of androgenesis.
- The iron in the medium plays a very important role for the induction of haploids .
- Potato extracts, coconut milk and growth regulators like auxin and cytokinin are used for anther and pollen culture.

4)Temperature:

- Temperature enhance the induction frequency of microspore androgensis.
- The low temperature treatment to anther or flower bud enhance the haploid formation.
- The low temperature effects the number of factors such as dissolution of microtubules, lowering of absscisic acid, maintenance of higher ratio of viable pollen capable of embryogenesis.

5)Physiological status of donor plant

- Physiological status of donor plant such as water stress nitrogen requirement and age of donor plant highly affect the pollen embryogenesis.
- Plants starved of nitrogen may give more responsive anthers compared to those that are well fed with nitrogenous fertilizers.

Advantages of antherculture

- simple.
- Less time consuming.
- Responsive

Disadvantages

- Requires skill to remove anthers without causing damage.
- Not much successful in case of cereal crop.
- Risk of chimera and callus formation from anther wall.

APPLICATIONS:

1) For mutation study.

2) For plant breeding and crop improvement.

3) To obtain the secondary metabolites.

Eg. *Hyoscyamus niger* obtain by anther culture having

higher alkaloid content.

4) Haploids are used in molecular biology and genetic engineering.

Example:- Haploid tissue of *Arabidopsis* and *Lycopersicon* have been used for the transfer and

SOMACLONAL AND GAMETOCLONAL VARIANT SELECTION

- **Somaclonal variation:** is one of the aspects of tissue culture technology. Is defind as genetic variation observed among progeny of plant regenerated from somatic cell cultured *in vitro*.
- **It was introduced by Larkin and Scowcroft(1981).**
- **Gametoclonal variation:** can be developed by ing male and female gametic cell (anther culture).
- **Application:-**

1. ding.
2. t.
3. oxin resistance.
4. high temperatures, ph).
5. bacteria,….)

Take from any tissue

- ❖ Meristemic, leave, internod or root

Explant

Mutagenic agent:

- ❖ Ultra violet light and radiation
- ❖ Change in the culture condition (PH,Tem)
- ❖ Or genetic disorder in the initial explant

This will cause:

- ❖ Change in the structure and number of chromosomes
- ❖ Single gene mutation (transposable element)
- ❖ Spontaneous mutation

s formation

ultiplication

Tolerant cell

Selection and isolation of the variant cell by retested and subcultured this cell on the herbicide or antibiotic supplement media.

The survival cell

Micropropigation

Plant shooting

e whole plant

somoclonal variant

- ❖ Must to make detection:

1. Morphological trait (size, coior, hight)
2. Biochemical and chromosomal basis variation.
3. Generation of variation.

Somaclonal Variations

Somaclonal Variation

Genetic variations in plants that have been produced by plant tissue culture and can be detected as genetic or phenotypic traits.

Basic Features of Somaclonal Variations

* Variations in number and structure of chromosomes are commonly observed.

* Regenerated plants with altered chromosomal changes often show changes in leaf shape and colour, growth rate and habit, and sexual fertility.

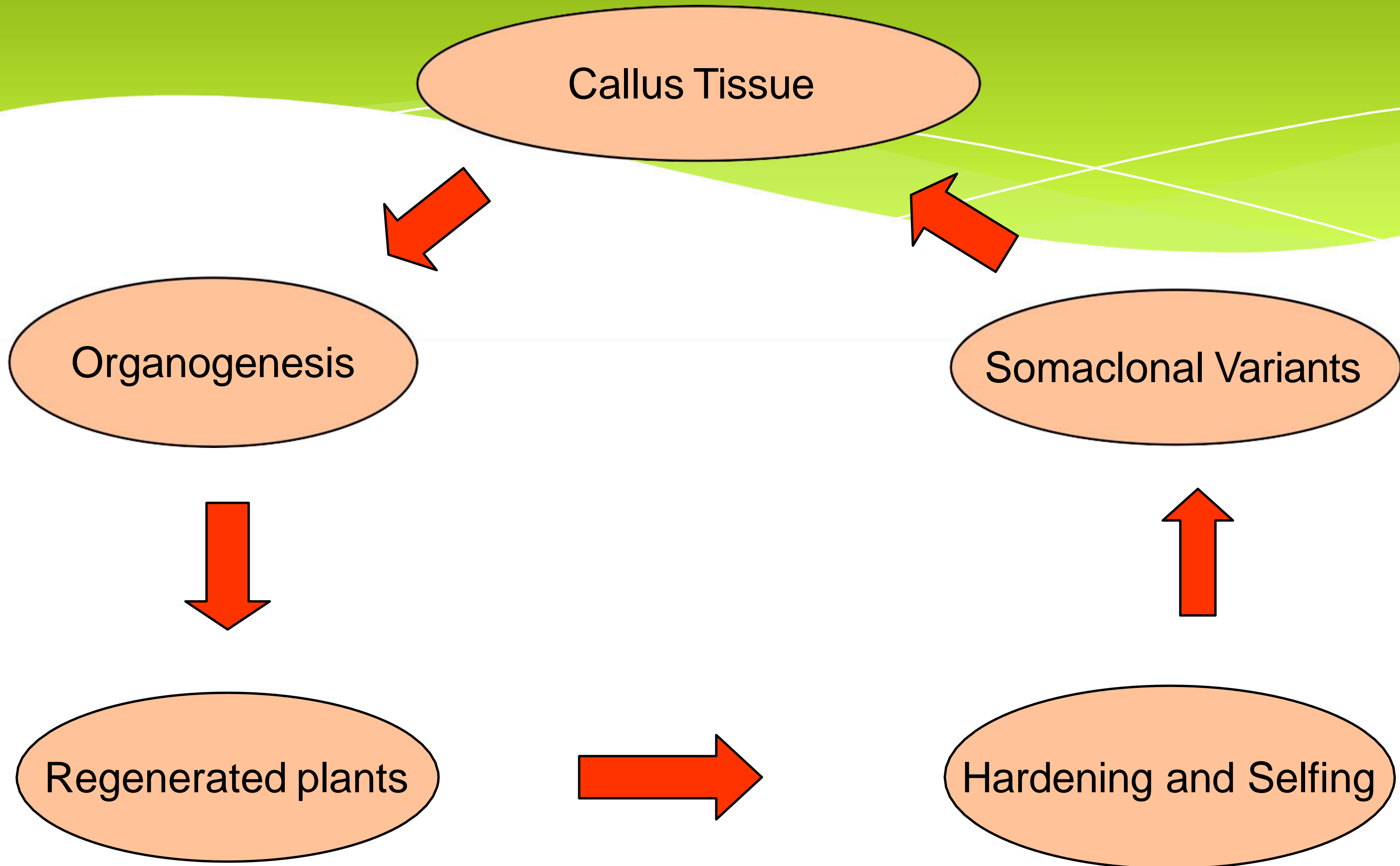
* It is generally heritable mutations and persist in plant population even after plantation into the field.

Mechanism of Somaclonal Variations

1. **Genetic (Heritable Variations)**
   - Pre-existing variations in the somatic cells of explant
   - Caused by mutations and other DNA changes
   - Occur at high frequency

2. **Epigenetic (Non-heritable Variations)**
   - Variations generated during tissue culture
   - Caused by temporary phenotypic changes
   - Occur at low frequency

Steps involved in induction and selection of Somaclonal Variations



Causes of Somaclonal Variations

Physiological Cause

Genetic Cause

Biochemical Cause

Physiological Cause

- Exposure of culture to plant growth regulators.
- Culture conditions

Genetic Cause

1. **Change in chromosome number**
   * **aneuploidy – gain or loss of 1 or more chromosomes**
   * **polyploidy – gain or loss of an entire genome**
   * **translocation – arms of chromosomes switched**
   * **inversion – piece of chromosome inverted**

2. **Change in chromosome structure**
   * **Deletion**
   * **Inversion**
   * **Duplication**
   * **Translocation**

Genetic Cause

3. Gene Mutation
   - Transition
   - Transversion
   - Insertion
   - Deletion
4. Plasmagene Mutation

5. Transposable element activation

Genetic Cause

6. DNA sequence

- Change in DNA
  - Detection of altered fragment size by using Restriction enzyme
- Change in Protein
  - Loss or gain in protein band
  - Alteration in level of specific protein
- Methylation of DNA
  - Methylation inactivates transcription process.

Biochemical Cause

* **Lack of photosynthetic ability due to alteration in carbon metabolism**
* **Biosynthesis of starch via carotenoid pathway**
* **Nitrogen metabolism**
* **Antibiotic resistance.**

Detection and Isolation of Somaclonal Variants

1. **Analysis of morphological characters**
   * Qualitative characters: Plant height, maturity date, flowering date and leaf size
   * Quantitative characters: yield of flower, seeds and wax contents in different plant parts

2. **Variant detection by cytological Studies**
   * Staining of meristematic tissues like root tip, leaf tip with feulgen and acetocarmine provide the number and morphology of chromosomes.

3. **Variant detection by DNA contents**
   * Cytophotometer detection of feulgen stained nuclei can be used to measure the DNA contents

Detection and Isolation of Somaclonal Variants

4. Variant detection by gel electrophoresis

- Change in concentration of enzymes, proteins and hemical products like pigments, alkaloids and amino acids can be detected by their electrophoretic pattern

5. Detection of disease resistance variant

- Pathogen or toxin responsible for disease resistance can be used as selection agent during culture.

6. Detection of herbicide resistance variant

- Plantlets generated by the addition of herbicide to the cell culture system can be used as herbicide resistance plant.

Detection and Isolation of Somaclonal Variants

7. Detection of environmental stress tolerant variant

* Selection of high salt tolerant cell lines in tobacco
* Selection of water-logging and drought resistance cell lines in tomato
* Selection of temperature stress tolerant in cell lines in pear.
* Selection of mineral toxicities tolerant in sorghum plant (mainly for aluminium toxicity)

Advantages of Somaclonal Variations

* Help in crop improvement
* Creation of additional genetic varitions
* Increased and improved production of secondary metabolites
* Selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity
* Suitable for breeding of tree species

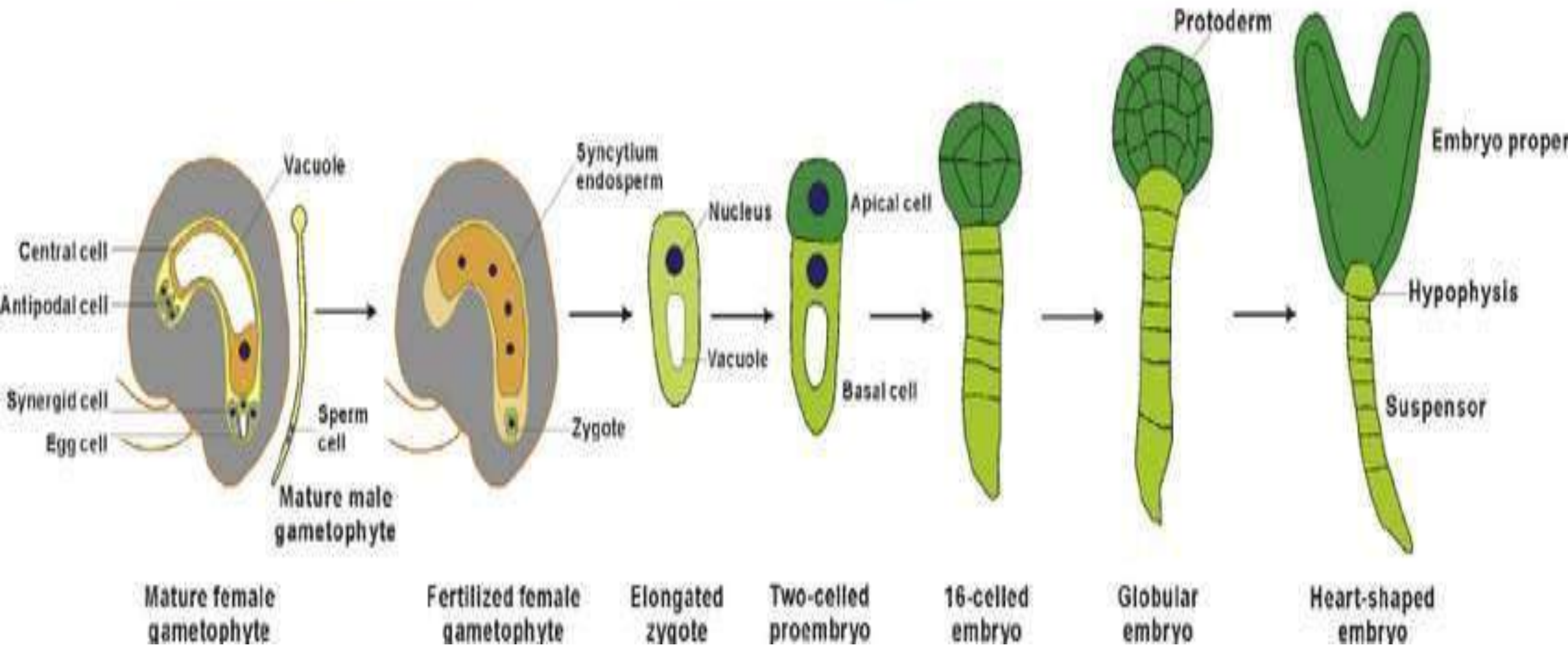
Applications to crop improvement

- Improvement of existing clonal cultures
  - sugarcane – selections for higher yield & disease resistance
  - potatoes – yield & disease resistance
  - improved geraniums (esp. scented varieties)
  - woody ornamentals (e.g., Paulownia – selection for leaf variegation

Disadvantages of Somaclonal Variations

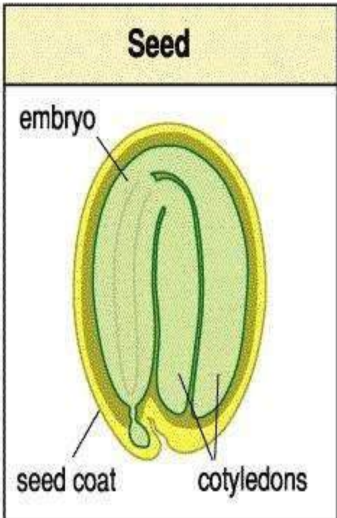
- **A serious disadvantage occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes**
- **Sometime leads to undesirable results**
- **Selected variants are random and genetically unstable**
- **Require extensive and extended field trials**
- **Not suitable for complex agronomic traits like yield, quality etc.**
- **May develop variants with pleiotropic effects which are not true.**

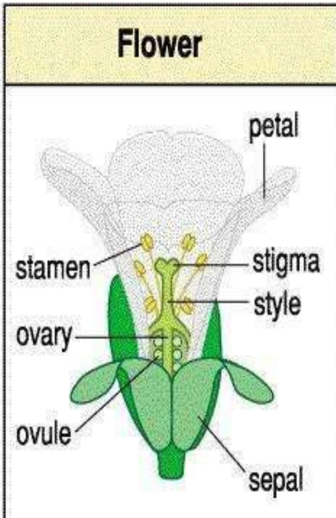
Somatic embryogenesis



Egg | Two cell | Octant | Globular | Triangular | Heart | Torpedo | Mature embryo

apical cell
fertilized egg
basal cell
basal cell
suspensor
cotyledons
shoot apical meristem
epidermis
root apical meristem
vascular tissue
ground tissue
cotyledons





Somatic Embryogenesis:

- Somatic embryogenesis is a process by which somatic cells or tissues, including haploid cells develops into differentiated embryos and to regenerate plants.
- Stewart et al., (1958): First induced embryo through suspension culture in carrot.
- Reinert (1959): Produce embryo from callus in carrot through suspension culture.

Types of Embryos:

1. Zygotic Embryos:

- These formed by fertilized egg or the zygote.

2. Non-Zygotic Embryos:

a) Somatic Embryos:

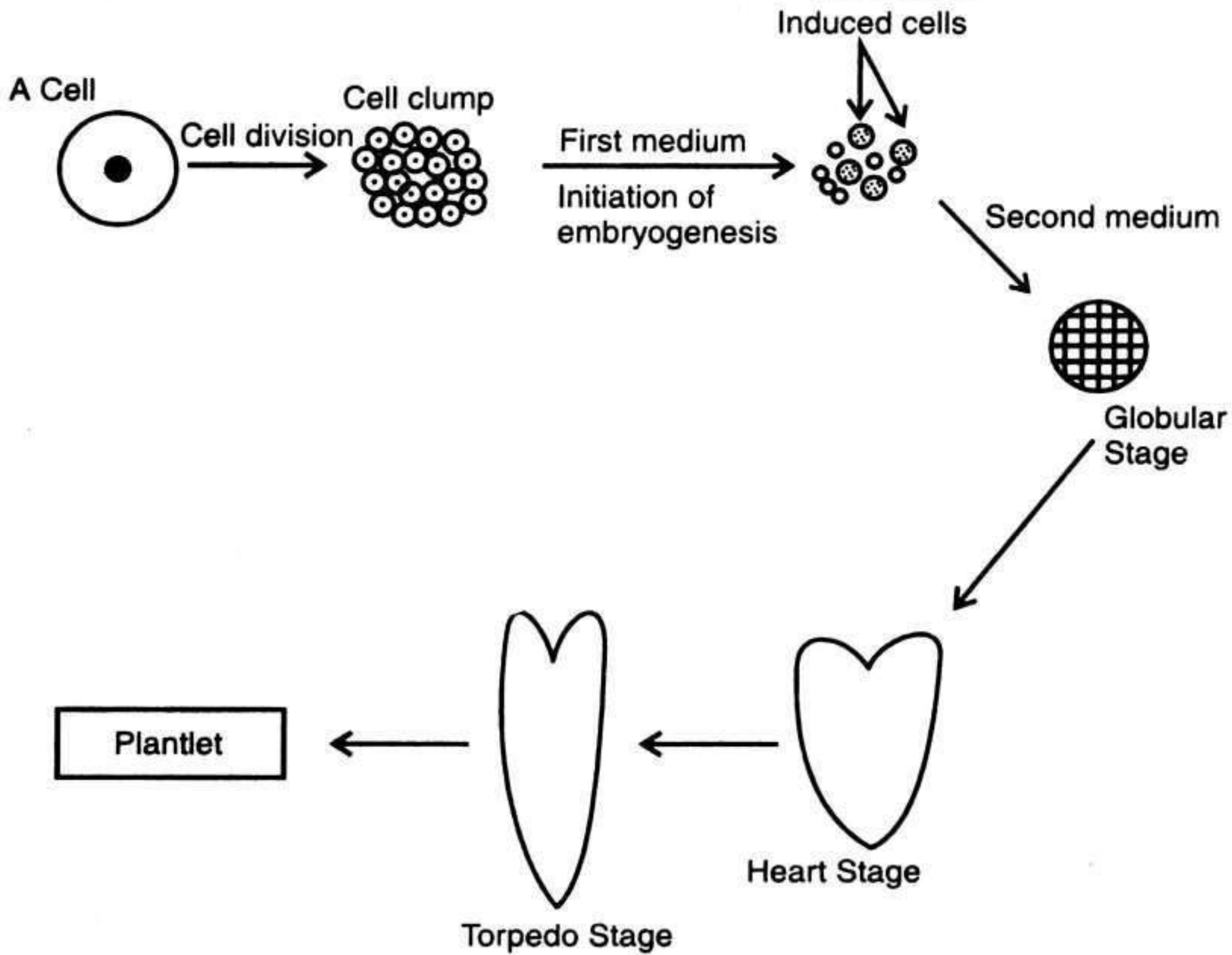
- Those formed by Sporophytic cells in in-vitro condition. Such somatic embryos arsing directly from other embryos or organs are termed adventive embryos.

b) Parthenocapic Embryos:

- Those formed by unfertilized egg.

c) Androgenic Embryos:

- Those formed by the male gametophyte.


Induced cells
A Cell
Cell clump
Cell division
First medium
Initiation of
embryogenesis
Second medium
Globular
Stage
Plantlet
Heart Stage
Torpedo Stage

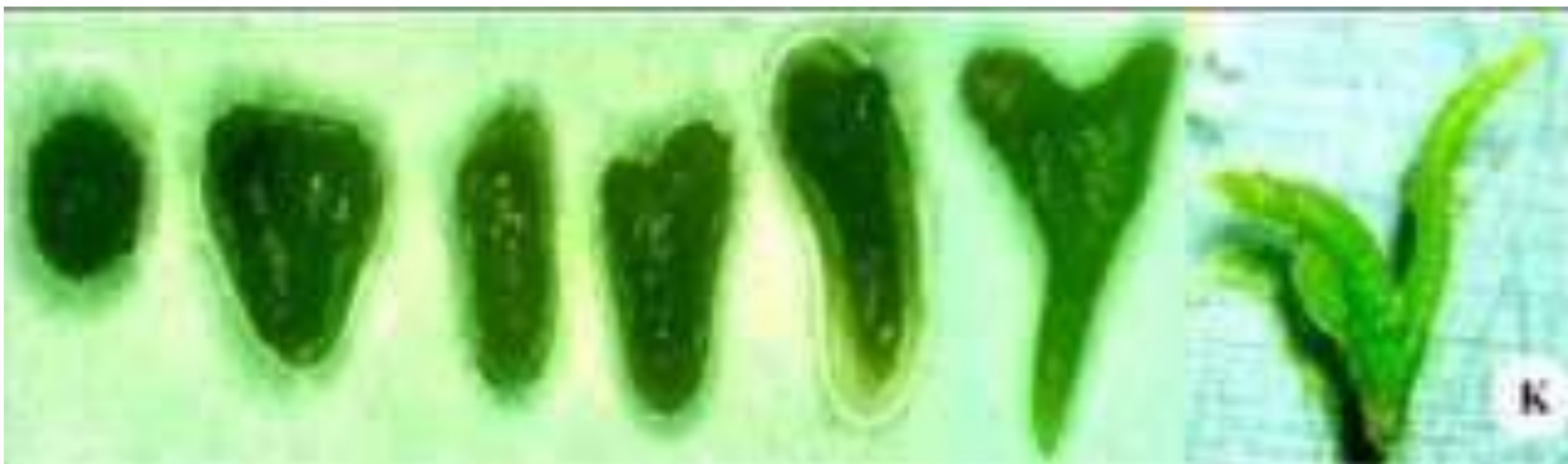
Importance of Somatic Embryogenesis:

- Higher propagation rate.
- Suitable in Suspension culture.
- Artificial seed production.
- Labour savings.

STAGES OF SOMATIC EMBRYOGENESIS

- Somatic embryogenesis encompasses various stages such as
- 1. Callus initiation
- 2. Embryo development and maturation
- 3. Plantlet formation

Meristematic cell
Cuticle
Globular Somatic embryo
Heart-shaped Somatic embryo
Initiation of somatic embryo development
Early Proembryo
Callus/Explant
Callus/Explant
Cotyledonary stage Somatic embryo
Torpedo-shaped Somatic embryo



Factors effecting somatic embryogenesis

1. Characters of explants:

- Even though a variety of explants can be utilized, the correct developmental stage of the explants are also crucial for the initiation of embryogenic callus.
- Young or juvenile explants produced more somatic embryos than older explants
- As a further complexity, different explants tissue from the same mother plant produced embryogenic callus at different frequencies.

- **2. Growth regulators:**
- **Auxins:** Auxin alone or in combination with cytokinin appear essential for the onset of growth and the induction of embryogenesis of all the auxins, 2,4-D followed by NAA have proven to be extremely useful.
- Effective concentration ranges are 0.5 – 27.6 µ M for 2,4-D and 0.5 – 10.7 µ M for NAA.
- **Cytokinins:** CKs have been used in the primary medium invariably during embryogenesis of crop plants.
- Effective concentrations for kinetin0.5 – 50.0µ M. CKs are important in fastening somatic embryo maturation and especially cotyledon development.

Clip slide

- **ABA:** It is added at inhibitory levels @0.1- 1 µM promotes somatic embryo development and maturation and at same time inhibits abnormal proliferation and initiation of accessory embryos.
- **3. Nitrogen source:** Form of nitrogen has marked influenced on somatic embryogenesis. In carrot NH_4^+ form has a promotive effect.
- Somatic embryos development occurs on a medium containing NO_3^- as the sole nitrogen source.
- **4. Other factors:** High K^+ levels and low dissolved O_2 levels prevents somatic embryo regeneration.

Somatic Embryo Germination Media:-

- MS medium: BAP (0, 1, 2, 3, 4and 5 mg/l),
  NAA (0, 0.5, 1.0, 1.5, 2.5 and 4.0 mgL-1)

- Media were kept in the incubation room 25±2°C with 16 hrs of light provided by fluorescent bulbs and a light intensity of 16.75 µmolm^{-2}s^{-1} for eight weeks.

- Calculation: Callus induction frequency(%)
  Regeneration frequency(%).

Types of Somatic Embryogenesis:-

- Two types of somatic embryogenesis

- Direct somatic embryogenesis
  - The embryos initiate directly from explants in the absence of callus formation. Embryos are formed due to PEDCs cell.

- Indirect somatic embryogenesis
  - Callus from explants takes place from which embryos are developed. Embryos are formed due to IEDCs cells.

Direct Somatic Embryogenesis

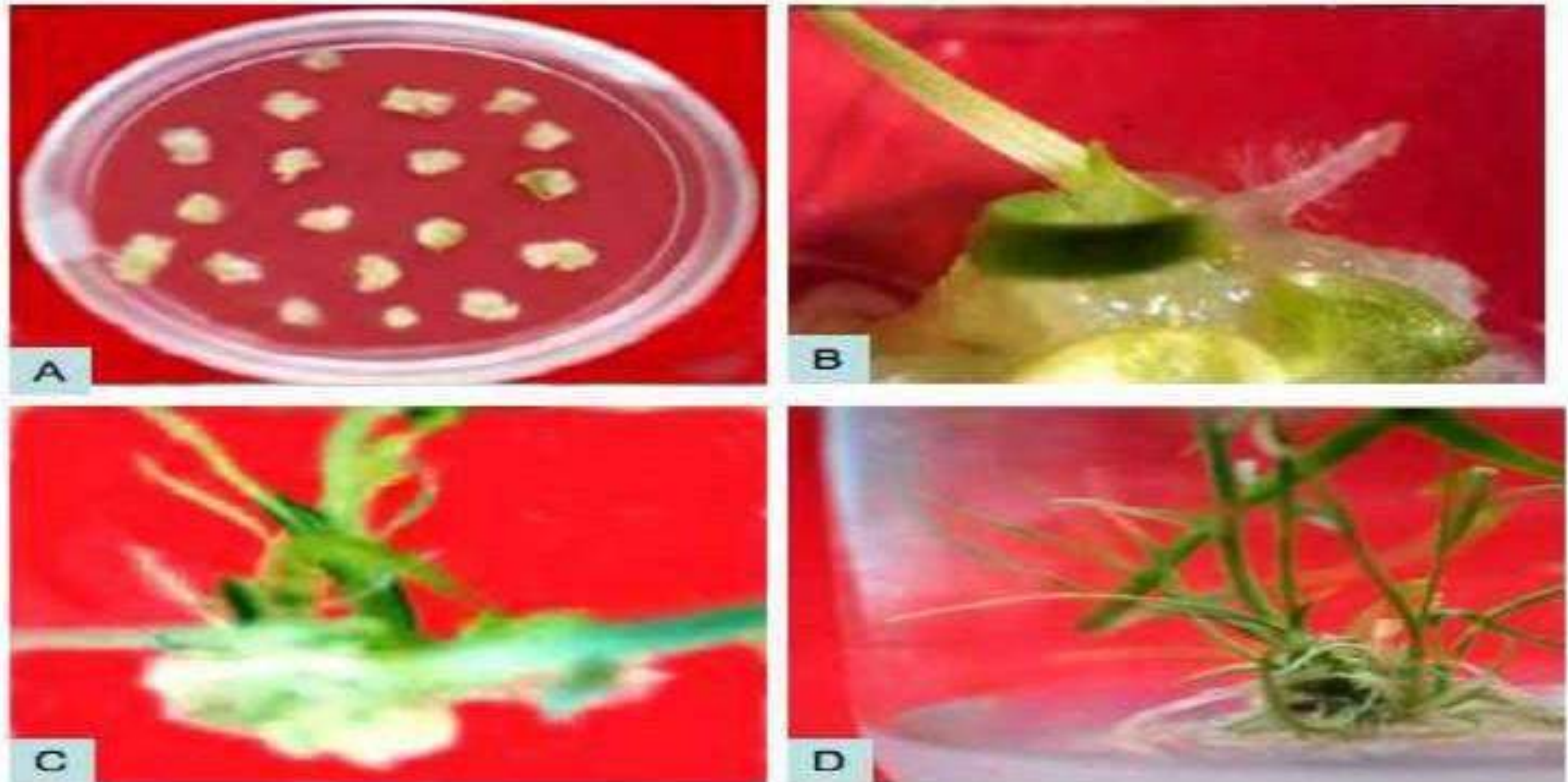


Fig 1 (A) Callusing in Indian wheat genotypesof *in vitro* selected lines using mature embryo cultures (B) Initiation of shoot and root primodia (C) Multiple shoot induction and elongation (D) Multiple shoot regeneration of in vitro selected lines

Yadav *et al.*, 2014

Indirect Somatic Embryogenesis

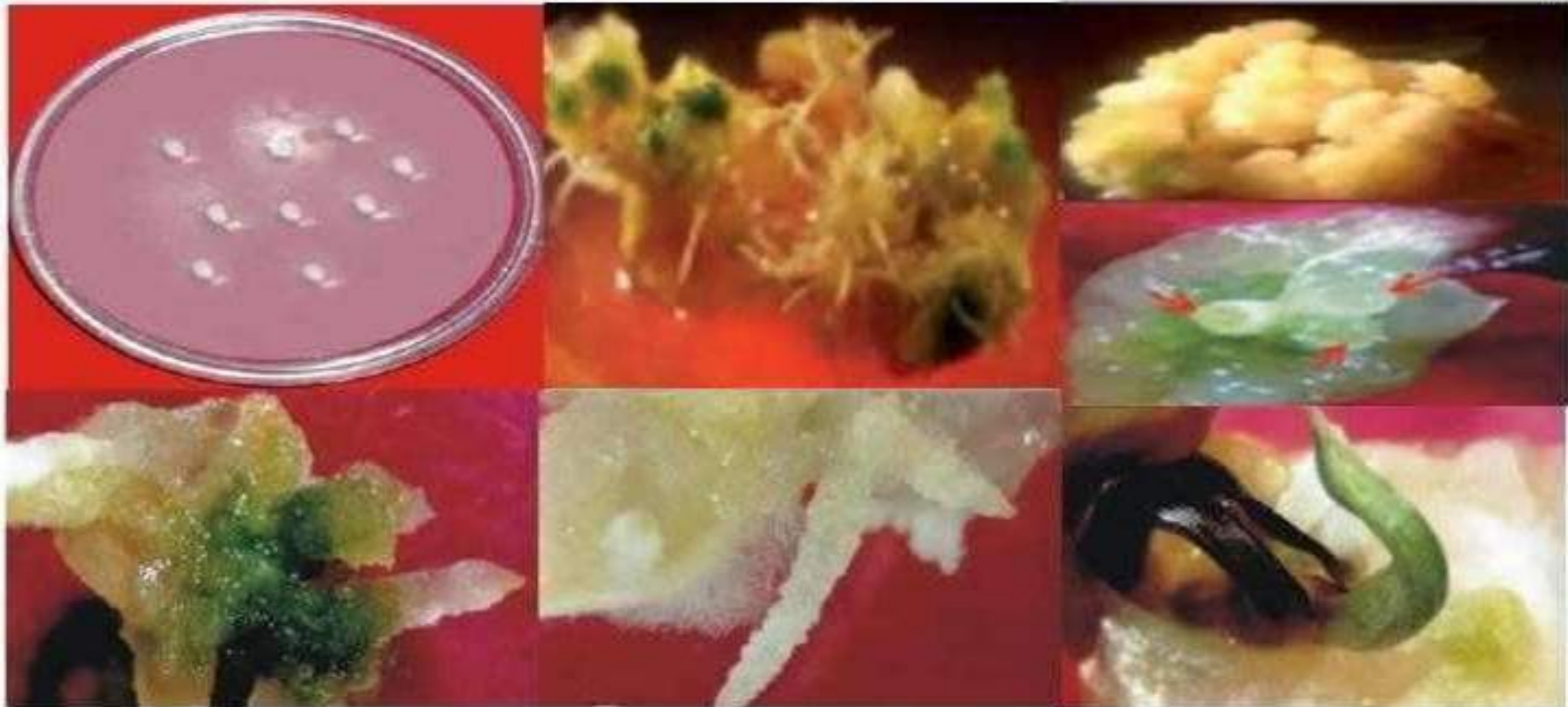


Figure 1 - Primary callus induction, somatic embryogenesis and plant regeneration from immature embryos of maize cv. Gaurav (A) Inoculation of immature embryos in MS with 5 mg/L 2,4-D and 2 mg/l NAA + 1 mg/l BAP, (B) Callus proliferation in MS with 5 mg/L 2,4-D and 2 mg/l NAA + 1 mg/l BAP, (C-D) Globular shape observed during somatic embryogenesis (arrows) (E) Regenerating calli in MS medium with 5.0 mg/L 2,4-D. Culture showing mature green somatic embryos (arrow) (F) Root induction in MS medium with 5.0 mg/L 2,4-D (Joshi et al., 2010).

Indirect Somatic Embryogenesis in Cereals:

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Research Article Open Access

High Frequency Embryogenic Callus Induction and Whole Plant Regeneration in *Japonica* Rice Cv. Kitaake

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Crop: Japonica Rice cv. Kitaake seeds

Callus induction media

CHO source- maltose (40g/l)

Agar (0-8, 1 and 1.2%)

Hormones- 2,4-D and BAP (3.0 mg/l)

Proline (0.6 g/l) and Phytagel (0.3%)

Regeneration Media:

Hormones- NAA (0.2 mg/l) and BAP (3.0 mg/l),

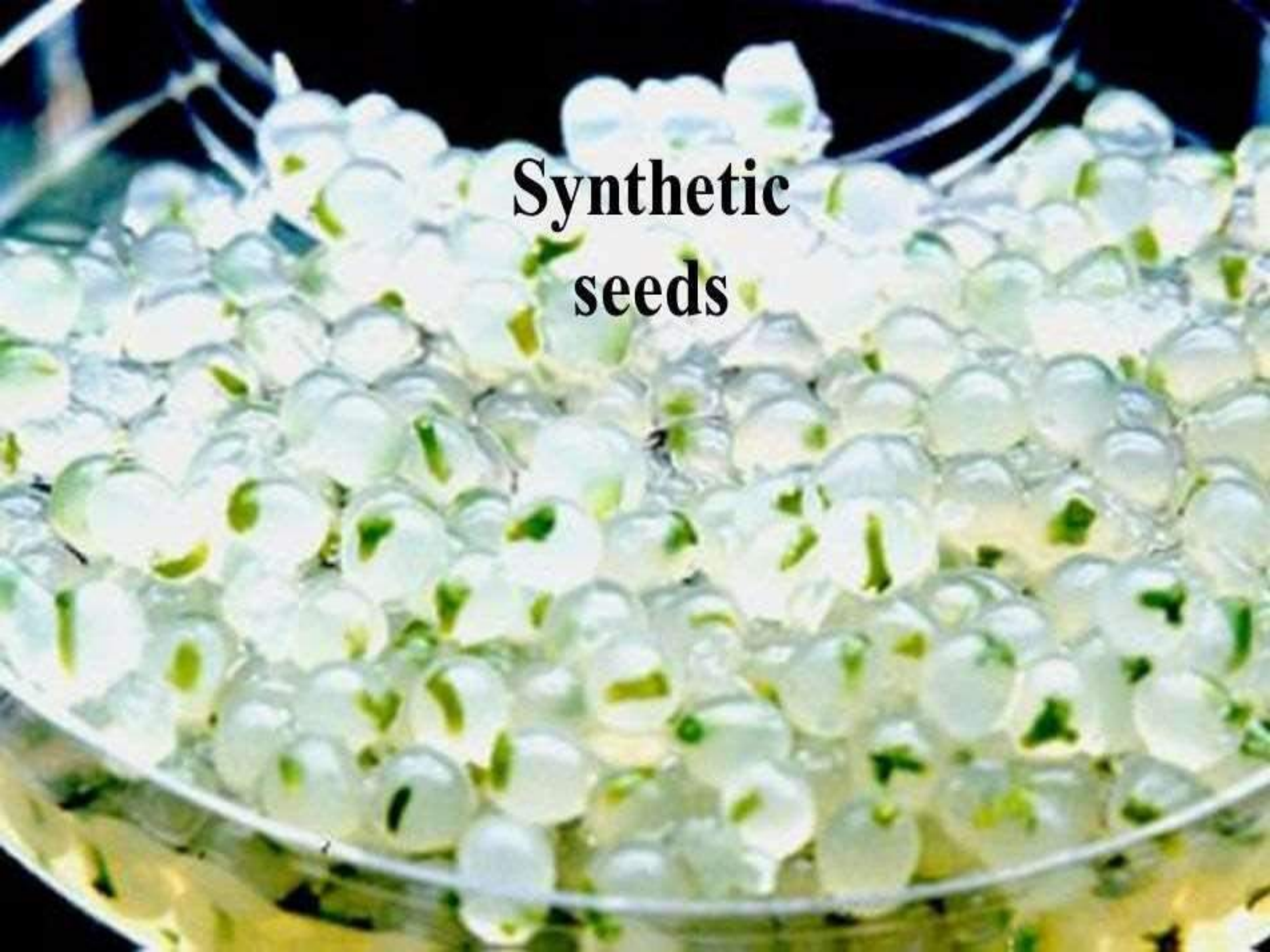
Treatments: Either alone or in combination of Hormones, gelling agents, proline and maltose supplemented with basic MS media.

ADVANTAGES

- It is observable, as its various culture conditions can be controlled.
- Lack of material is not a limiting factor for experimentation.
- High propagation rate.
- Somaclonal variations.
- Germplasm conservation.
- Labour saving.
- Elimination of diseases and viruses.

DISADVANTAGES

- Confined to few species.
- The somatic embryos show very poor germination because of their physiological and biochemical immaturity.
- Instability of cultured cells in long-term cultures is a major limitation in commercial exploitation and mass propagation of SEs.


Synthetic
seeds

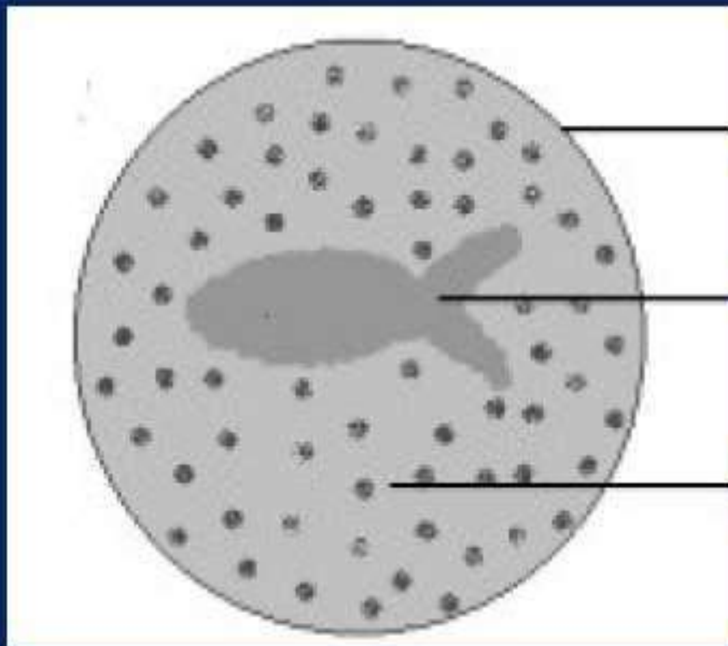
WHAT IS ARTIFICIAL SEED..?

- Artificial seed can be defined as artificial encapsulation of somatic embryos, shoot bud or aggregates of cell of any tissues which has the ability to form a plant in in-vitro or ex-vivo condition.
- Artificial seed have also been often referred to as synthetic seed.

HISTORY

- Artificial seeds were first introduced in **1970's** as a novel analogue to the plant seeds.
- The production of artificial seeds is useful for plants which do not produce viable seeds. It represents a method to propagate these plants.
- Artificial seeds are small sized and these provides further advantages in storage, handling and shipping.
- The term, "EMBLING" is used for the plants originated from synthetic seed.
- The use of synthetic varieties for commercial cultivation was first suggested in Maize (Hays & Garber, 1919).

The Concept of artificial seed



BASED ON THE TECHNIQUES TWO TYPES OF ARTIFICIAL SEEDS ARE PRODUCED

1. DESICCATED SYNTHETIC SEEDS- Desiccated synthetic seeds are produced nacked or polyoxyethylene glycol encapsulated somatic embryos. This type of synthetic seeds is produced in desiccation tolerant species plant.
2. HYDRATED SYNTHETIC SEEDS- Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogels like sodium alginate, potassium alginate, carrageenan, sodium pectate or sodium alginate with gelatine.

NEED FOR ARTIFICIAL PRODUCTION TECHNOLOGY

- Development of micro propagation technique will ensure abundant supply of desired plant species.
- Development of artificial seed production technology is currently considered as an effective and efficient method of propagation in several commercially important agronomic and horticultural crops.
- These artificial seed would also be a channel for new plant lines produced through biotechnological advances to be delivered directly to greenhouse and field.
- High volume propagation potential of somatic embryos combined with formation of synthetic seeds for low-cost delivery would open new vistas for clonal propagation in several commercially important crop species.

BASIC REQUIREMENT FOR THE PRODUCTION OF ARTIFICIAL SEEDS.

- **One pre-requisite for the application of synthetic seed technology in micropropagation is the production of high quality,**

1. **Vigorous Somatic Embryos that can produce plants with frequencies comparable to natural seeds.**
2. **Inexpensive production of large numbers of high quality somatic embryos with synchronous maturation.**
3. **Encapsulation and coating systems, though** of somatic embryos, are not the limiting factors for the development of synthetic seeds.

4. Commercialization of synthetic seeds.

PROCEDURE FOR PRODUCTION OF ARTIFICIAL SEEDS

Methods for artificial seed encapsulation

- **Dropping method**
- Somatic embryos are dipped in hydrogel, this step encapsulate SEs.
- Hydrogel used may be any of the following.
- Alginate – sodium alginate, agar from see weeds, seed gums like guar gum, locust bean gum.
- Sodium alginate solution (1 – 5%), prepared in MS basal medium solution.
- SEs are dipped in this solution.
- These coated beads are added one by one into a complexation solution flask kept on magnetic stirrer and kept such for around 20-30 minutes.

Contii.

- Embryos get covered by calcium alginate which is a stable complex due to ionic bond formation, become harder, Seeds become harder.
- Then gelled embryos are washed with water or MS basal medium.
- The synthetic seeds are ready.

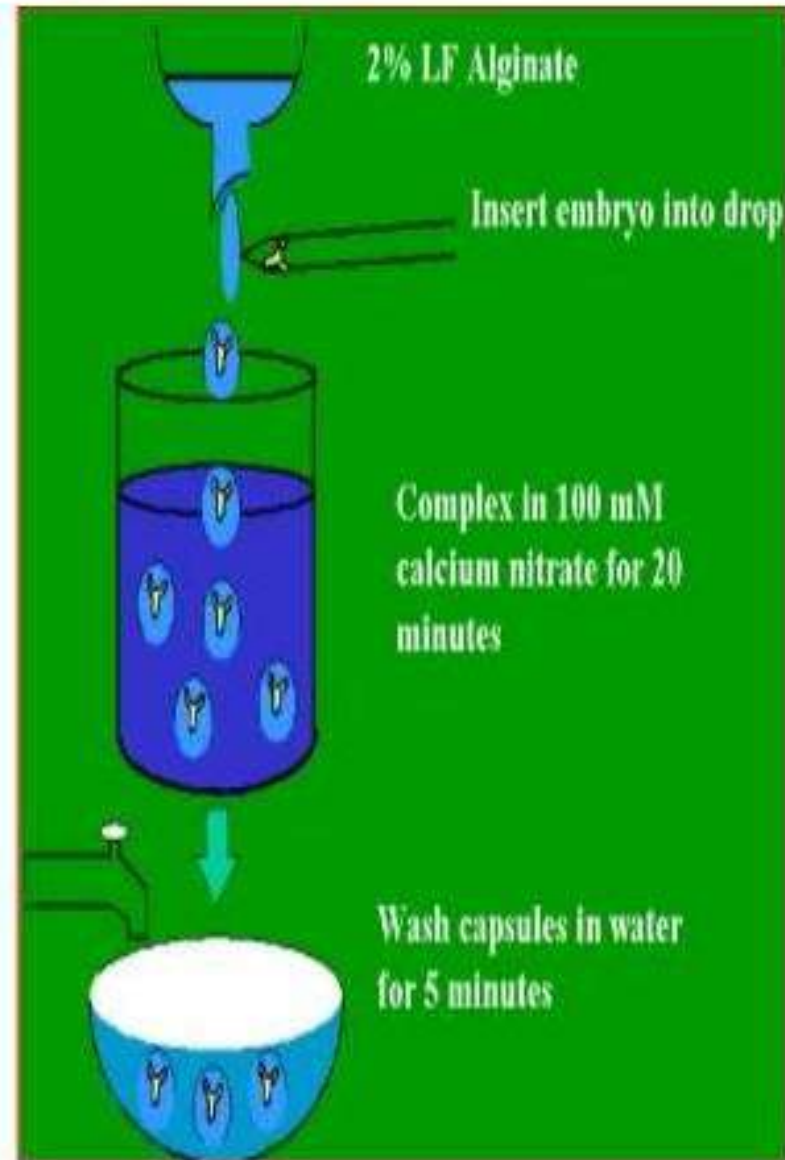
Encapsulation methods for synthetic seed

A) Dropping procedure

1) The most useful encapsulation system. Drip 2-3 % sodium alginate drops from at the tip of the funnel and the somatic embryos are inserted

2) Keep the encapsulated embryos complex in calcium salt for 20 min

3) Rinsed the capsules in water and then stored in a air tight container



Molding method

- This method follows simple procedure of mixing of embryos with temperature dependent gel (e.g. gel rite, agar).
- Cells get coated with the gel at lowering of the temperature.

ARTIFICIAL ENDOSPERM

- Somatic embryos lack seed coat (testa) and endosperm that provide protection and nutrition for zygotic embryos in developing seeds.
- To augment these deficiencies, addition of nutrients and growth regulators to the encapsulation matrix is desired, which serves as an artificial endosperm.
- These addition results in increase efficiency of germination and viability of encapsulated somatic embryos.
- These synthetic seeds can be stored for a longer period of time even upto 6 months without losing viabilty, especially when stored at 40°c.

ADDITION OF ADJUVANTS TO THE MATRIX

- To prevent the embryo from desiccation (state of extreme dryness) and mechanical injury, a number of useful materials such as nutrients, fungicides, pesticides, antibiotics and microorganisms (eg. rhizobia) may be incorporated into the encapsulation matrix.
- Incorporation of activated charcoal improves the conversion and vigour of the encapsulated somatic embryos and retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo.

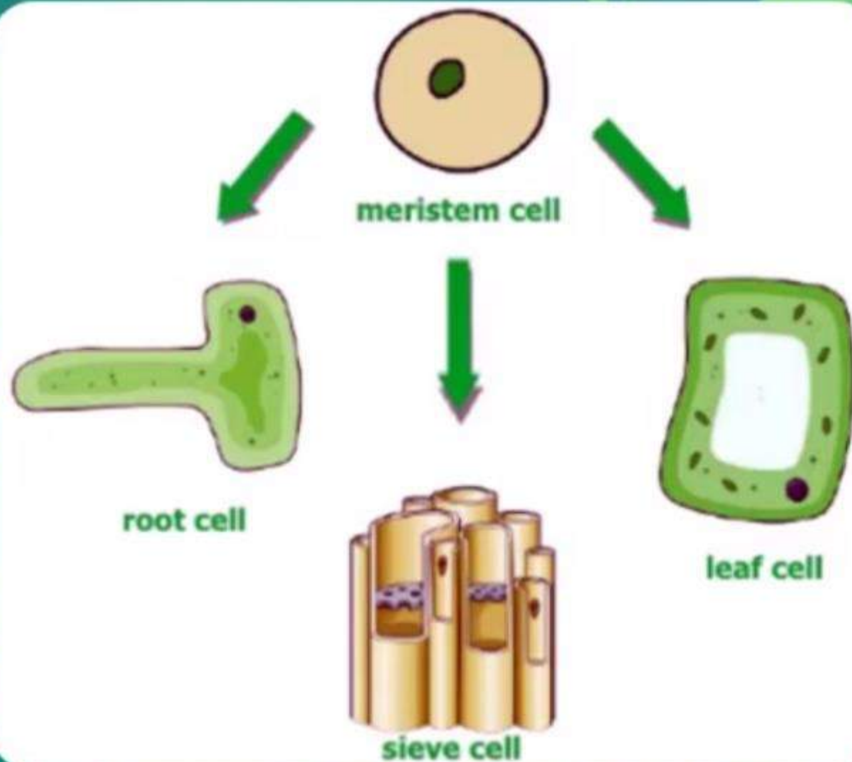
POTENTIAL USES OF ARTIFICIAL SEEDS

- Reduced costs of transplants(Cost effective)
- Direct greenhouse and field delivery of:
  - Elite, Select Genotypes
  - Large-scale mono cultures.
  - Carriers for adjuvant such as microorganisms, plant growth regulators, pesticides, fungicides, nutrients and antibiotics.

- **Can be conceivably handled as seed using conventional planting equipment.**
- **it can be produced throughout the year.**
- **Conservation of germplasm**
- **Large production of identical embryos in short period of time.**

- Differentiation
  - In this process, cells derived from root apical and shoot apical meristems and cambium differentiate and mature to perform specific functions.
- Dedifferentiation
  - Process in which living differentiated cells regain their capacity to divide
- Redifferentiation
  - Process in which differentiated cells that have lost their ability to divide are reformed from dedifferentiated cells and have the ability to perform specific functions.

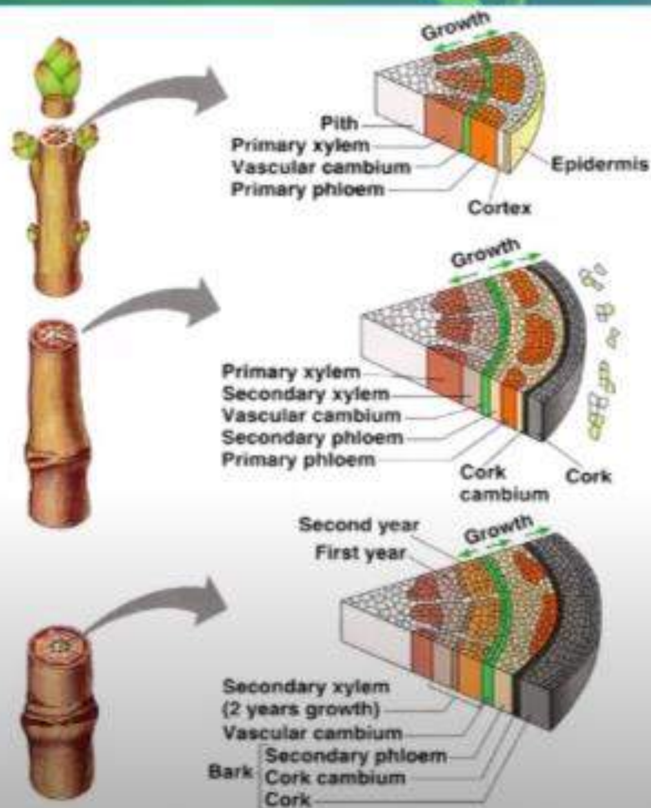
DIFFERENTIATION, DEDIFFERENTIATION & REDIFFERENTIATION



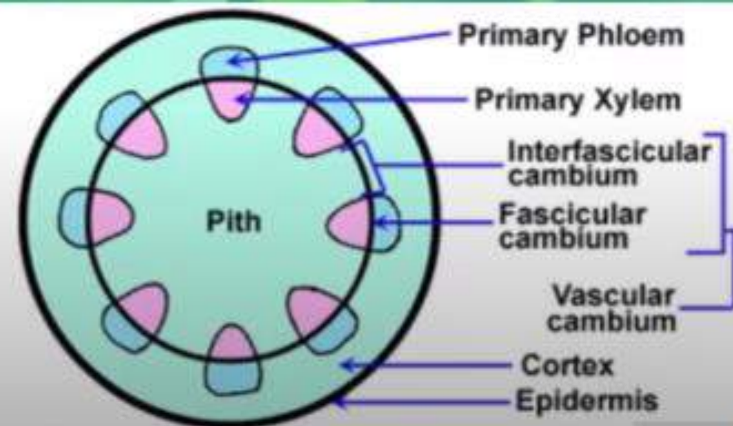
- **Differentiation** is a process in which the meristem cells (root apical & shoot-apical) and cambium differentiate and mature to perform specific functions.
- In this, cell walls & protoplasm undergo structural changes. Capacity of cell division is lost.
- E.g. Loss of protoplasm to form a tracheary element. They also develop very strong, elastic, lignocellulosic secondary cell walls, to carry water to long distances even under extreme tension.

Activate Windows

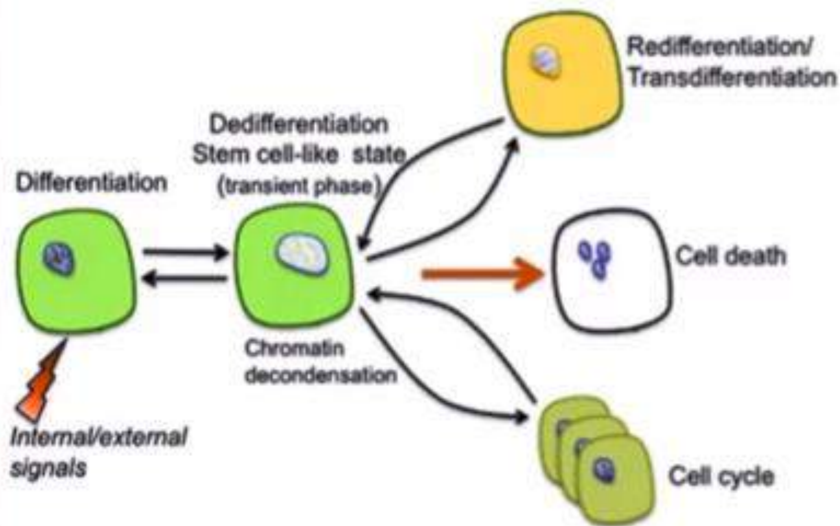
DIFFERENTIATION, DEDIFFERENTIATION & REDIFFERENTIATION



- Under certain conditions, living differentiated cells regain the capacity of division. This is called **dedifferentiation**.
- E.g. formation of meristems (interfascicular cambium & cork cambium) from differentiated parenchyma cells.



DIFFERENTIATION, DEDIFFERENTIATION & REDIFFERENTIATION



- Dedifferentiated cells can produce cells that again lose the capacity to divide but mature to perform specific functions. It is called **redifferentiation**.
- Plant growth is open, i.e., it can be indeterminate or determinate. Differentiation is also open, because cells/tissues arising out of the same meristem have different structures at maturity.
- Final structure at maturity of cell/tissue is also determined by the location of the cell.
- E.g. cells positioned away from **root apical meristems** differentiate as **root-cap cells**, while those pushed to **periphery** mature as **epidermis**.

Production of plant secondary metabolites

What are secondary metabolites?

- Secondary metabolites are generally defined as small organic molecules produced by an organism that are not essential for their growth, development and reproduction.
- They may include pharmaceuticals, flavours, fragrance, food additives, feedstock etc.

Why plant produce secondary metabolites?

- Plant hormones, which are secondary metabolites, are often used to regulate the metabolic activity within cells and oversee the overall development of the plant

- It protect plant against herbivores and microbial pathogens.

- It serves as attractants for pollination and seed dispersing animals.

Type of secondary metabolites

Type	Example	Uses
Alkaloids	Caffeine, Codeine, Quinine	Stimulant, Analgesic, Antimalarial
Cyanogenic glycoside	Diosgenin	Progesterone
Flavonoids	Quercetin, Procyanidins	Antibacterial, Antioxident, Anti-inflammatory
Phytic acid	-	Antioxident
Gossypol	Hypokalemic paralysis	-
Phytoestrogens	Resveratrol	Reduce risk of cardiovascular disease
Carotenoids	α-carotene, β-carotene and lycopene	Contribute to photosynthesis

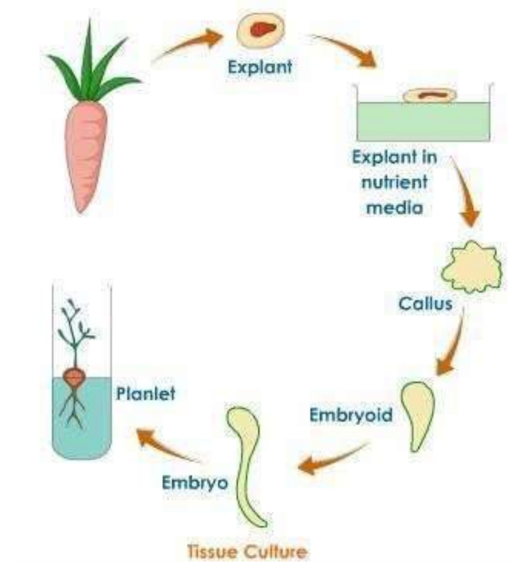
Hormone	Applications
Abscisic acid	Inhibit growth in response to change in temperature and light. Control closing of stomata in dry condition. ABA is applied on plants beefore shipping
Auxins	Stimulate cell elongated, differentiation of xylem and phloem, root initiation. Suppress growth of lateral buds Delay leaf senescence
Cytokinins	P r o m o te s c el l d i

Why in vivo production?

- According to WHO survey approximately 70-80% of world's total population depends on herbal drugs.
- Some compounds are difficult to synthesise chemically due to their structural complexity.
- Some novel compounds produced in cell cultures are not produced in intact plants. At least 85 novel compounds including 23 alkaloids, 19 terpenoids, 30 quinones and 11 phenyl compounds have been isolated from some 30 different plant culture systems.

Methods of in vivo culturing

- Callus Culture
- Suspension culture



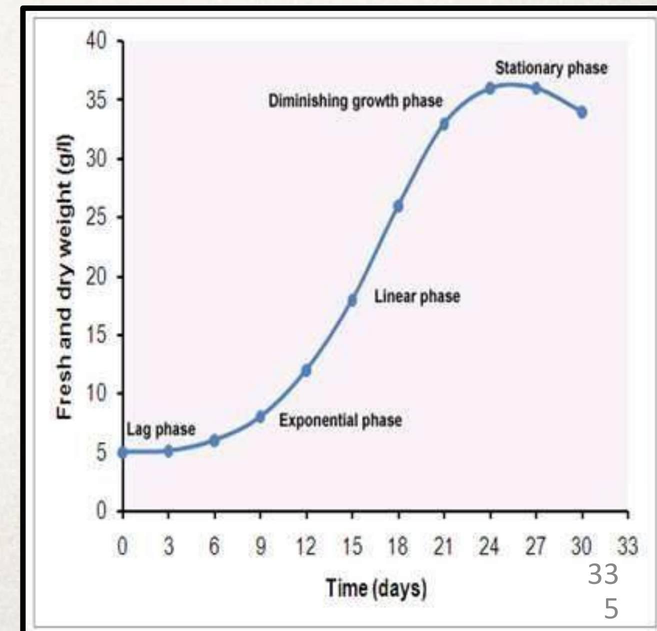
Process of suspension Culture

1) Callus culture

- The first step to establish cell suspension cultures is to raise callus from any explants of the plant. To maximise the production of a particular compound, it is desirable to initiate the callus from the plant part that is known to be high producer.
- Calli are generally grown on medium solidified with gelling agent.
- In morphological terms it can vary extensively, ranging from being very hard/compact and green or light green in color, where the cells have extensive and strong cell to cell contact. and friable where the callus consists of small, disintegrating aggregates of poorly-associated cells and has brownish or creamy appearance.

- Suspension culture could be run as batch culture or continuous culture.
- Predominantly batch culture is used because of:
  - Many secondary products are not growth associated.
  - Genetic instability of cultured cells.
  - Operability and reliability.
  - Economic considerations.

The growth of a cell suspension culture with respect to time is best described by the sigmoid curve. The growth rate is measured by the steepness of the curve, and it is the steepest when the population density reaches one-half of the carrying capacity.

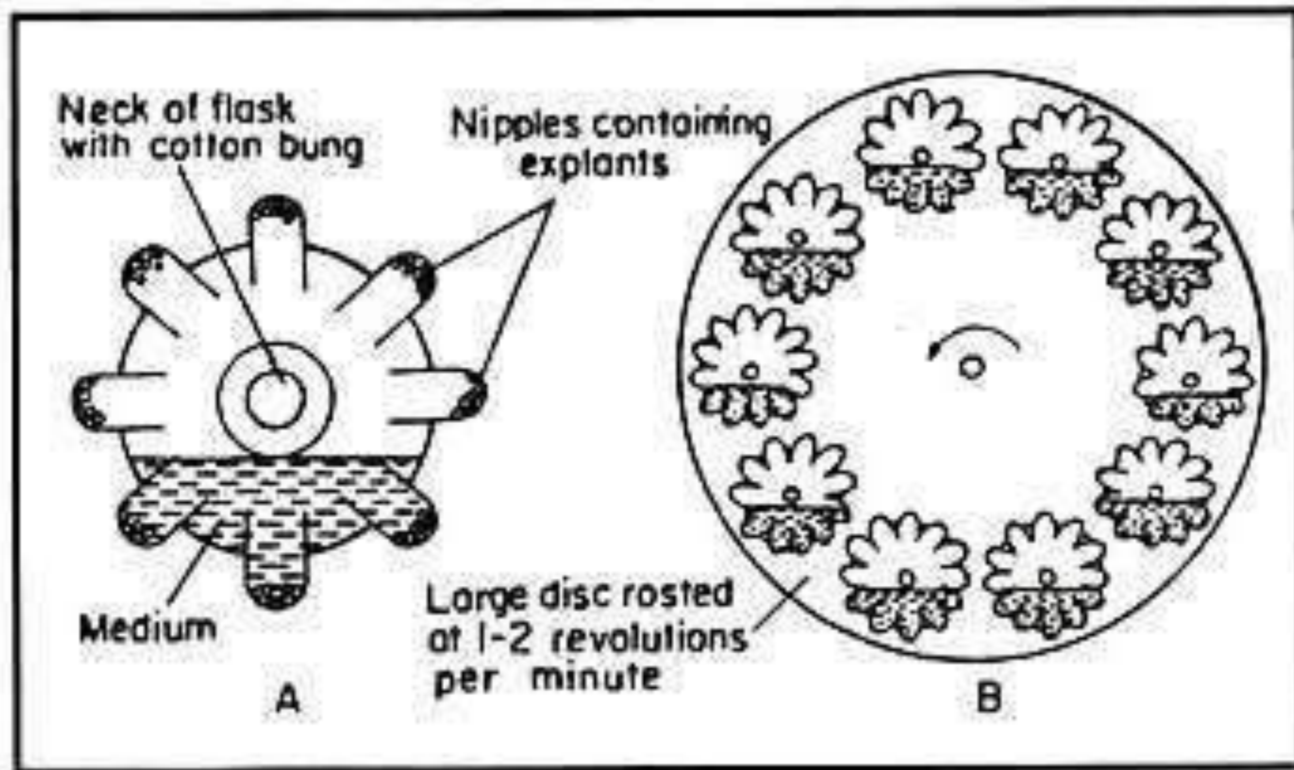


Types of Suspension culture

- **Batch culture**
  - Slowly rotating culture
  - Shake culture
  - Spinning culture
  - Stirred culture
- **Continuous culture**
  - Chemostats
  - Turbidostats

Slow motion rotating flask

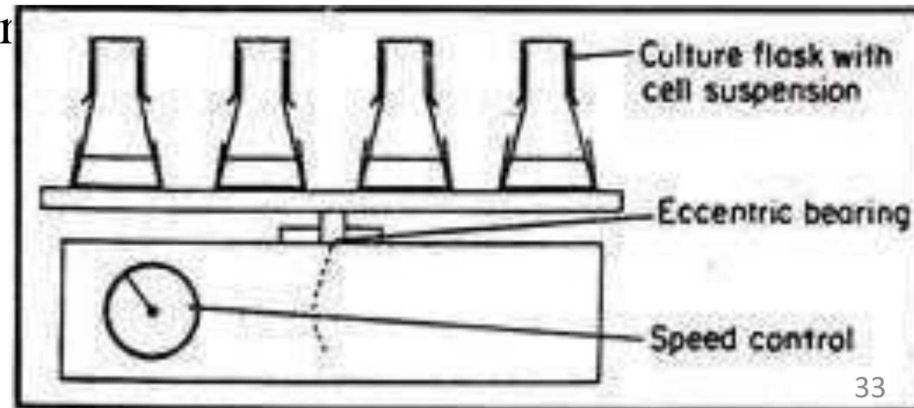
- In this culture, single cells and cell aggregates are grown in a specially designed flask, the nipple flask.
- Each nipple flask possesses eight nipple like projections, having a capacity of 250ml.
- They are loaded in a circular manner on the large flat disc of vertical shaker.
- When the flat disc rotates at a speed of 1-2rpm, the cells within each nipple of the flask are alternatively bathed in the culture medium and


Neck of flask
with cotton bung
Nipples containing
explants
Medium
A
Large disc rosted
at 1-2 revolutions
per minute
B

Shaker Culture

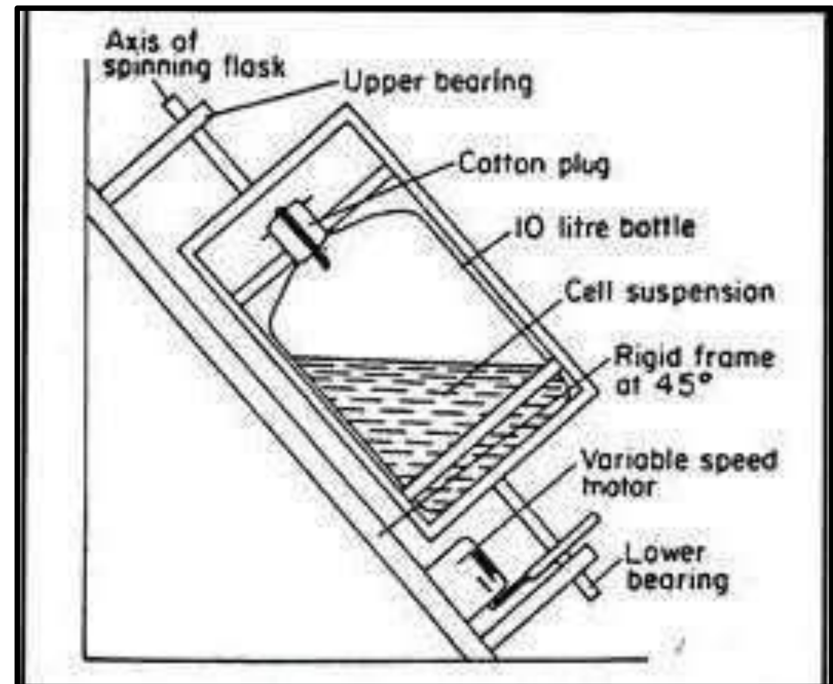
- It is very and effective system.

- In this method, single cells and cell aggregates in fixed volume of liquid medium are placed in conical flasks.
- These flasks are then mounted with the help of clips on a horizontal large square plate of an orbital platform shaker.
- The square plate moves in a circular speed of 60-180 rpm.



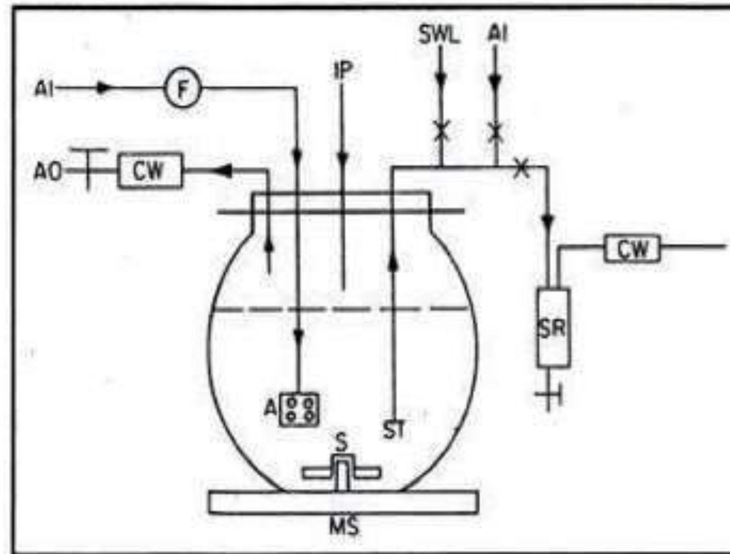
Spinning Culture

- In this culture system, large bottles are used, usually with a capacity of 10L.
- Large volumes of cell suspension is cultured in 10L bottles, with the bottles spinning in a spinner at 120 rpm at an angle of 45°.



Stirred culture

- This system is used for large scale batch culture.
- In this method, the large culture vessel (round-bottom flask) is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through the culture medium.
- Internal magnetic stirrer is used to agitate the culture medium safely.
- The magnetic stirrer revolves at 200-600 rpm



❒ Fig 4.6

Stirred batch culture unit. Arrow indicate direction of flow of air; AI = air input; F = sterilizing glass-fibre air filter; AO = air outlet; CW = cotton wool; IP = inoculation port; A = aerator; S = stirrer magnet; ST = sample tube; MS = magnetic stirrer; SWL = sterile water line; SR = sample receiver; (Diagram after Dr. P. King)

Continuous culture

- In continuous culture system, the old liquid medium is replaced continuously by the fresh liquid medium to stabilize the physiological states of the growing cells.

- In this system, nutrient depletion does not occur due to the continuous flow of nutrients and the cells always remain in the steady growth phase.

- Continuous culture is further divided into two types : In closed type, the used medium is replaced with the fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

- In open type, both the cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate.

- Open continuous cell suspension culture is of two types :

- **Chemostat :**

  - In this system, culture vessels are usually cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction and removal of cells and medium.

  - Such a system is maintained in steady state.

  - Thus in steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant.

  - Such continuous cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants.

Turbidostats :

- A turbidostat is a continuous culturing method where the turbidity of the culture is held constant by manipulating the rate at which medium is fed.

- In this system, the cells are allowed to grow upto a certain turbidity, when the predetermined volume of culture is replaced by fresh culture.

- The turbidity is measured by the changes of optical density of medium.

- An automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way, as to maintain the optical density or pH at chosen, present level.

Media Components

- Macronutrients
- Micronutrients
- Carbon and energy sources
- Vitamins and myoinositol
- Amino acids
- Growth regulators

MS - Murashige and Skoog

G5 - Gamborg et al

W - White

LM - Lloyd and

McCown VW - Vacin

and Went Km - Kudson

modified M - Mitra

NN - Nitsch and Nitsch media

Medium Components (mg.l⁻¹)	MS	G5	W	LM	VW	Km	M	NN
Macronutrients								
$Ca_3(PO_4)_2$					200.0			
NH_4NO_3	1650.0			400.0				720.0
KNO_3	1900.0	2500.0	80.0		525.0	180.0	180.0	950.0
$CaCl_2.2H_2O$	440.0	150.0		96.0				166.0
$MgSO_4.7H_2O$	370.0	250.0	720.0	370.0	250.0	250.0	250.0	185.0
KH_2PO_4	170.0			170.0	250.0	150.0	150.0	68.0
$(NH_4)_2SO_4$		134.0			500.0	100.0	100.0	
$NaH_2PO_4.H_2O$		150.0	16.5					
$CaNO_3.4H_2O$			300.0	556.0		200.0	200.0	
Na_2SO_4			200.0					
KCl			65.0					
K_2SO_4				990.0				
Micronutrients								
KI	0.83	0.75	0.75			80.0	0.03	
H_3BO_3	6.20	3.0	1.5	6.2		6.2	0.6	10.0
$MnSO_4.4H_2O$	22.30		7.0		0.75	0.075		25.0
$MnSO_4.H_2O$		10.0		29.43				
$ZnSO_4.7H_2O$	8.6	2.0	2.6	8.6			0.05	10.0
$Na_2MoO_4.2H_2O$	0.25	0.25		0.25		0.25	0.05	0.25
$CuSO_4.5H_2O$	0.025	0.025		0.25		0.025		0.025
$CoCl_2.6H_2O$	0.025	0.025				0.025		
$Co(NO_3)_2.6H_2O$							0.05	
Na_2EDTA	37.3	37.3		37.3		74.6	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8		27.8		25.0	27.8	27.8
$MnCl_2$						3.9	0.4	
$Fe(C_4H_4O_6)_3.2H_2O$					28.0			
Vitamins and other supplements								
Inositol	100.0	100.0		100.0				100.0
Glycine	2.0	2.0	3.0	2.0				2.0
Thiamine HCl	0.1	10.0	0.1	1.0		0.3	0.3	0.5
Pyridoxine HCl	0.5		0.1	0.5		0.3	0.3	0.5
Nicotinic acid	0.5		0.5	0.5			1.25	5.0
Ca-panthothenate			1.0					
Cysteine HCl			1.0					
Riboflavin						0.3	0.05	
Biotin							0.05	0.05
Folic acid							0.3	0.5

Advantages

- Compounds can be produced under controlled conditions as per market demands.

- Culture systems are independent of environmental factors, seasonal variation, pest and microbial diseases and geographical constrains.

- Cell growth can be controlled to facilitate improved formation.

Limitations

- The yield obtained in invivo production is lower when compared to intact plant.
- Cultured cells are genetically unstable and may undergo mutations. The production of secondary metabolites reduces drastically, as the culture ages.
- Strict aseptic conditions have to be maintained during culturing technique.