RIZVI COLLEGE OF ARTS SCIENCE AND COMMERCE

TYBSc USBO503
Unit IV PAPER 3

PLANT TISSUE CULTURE

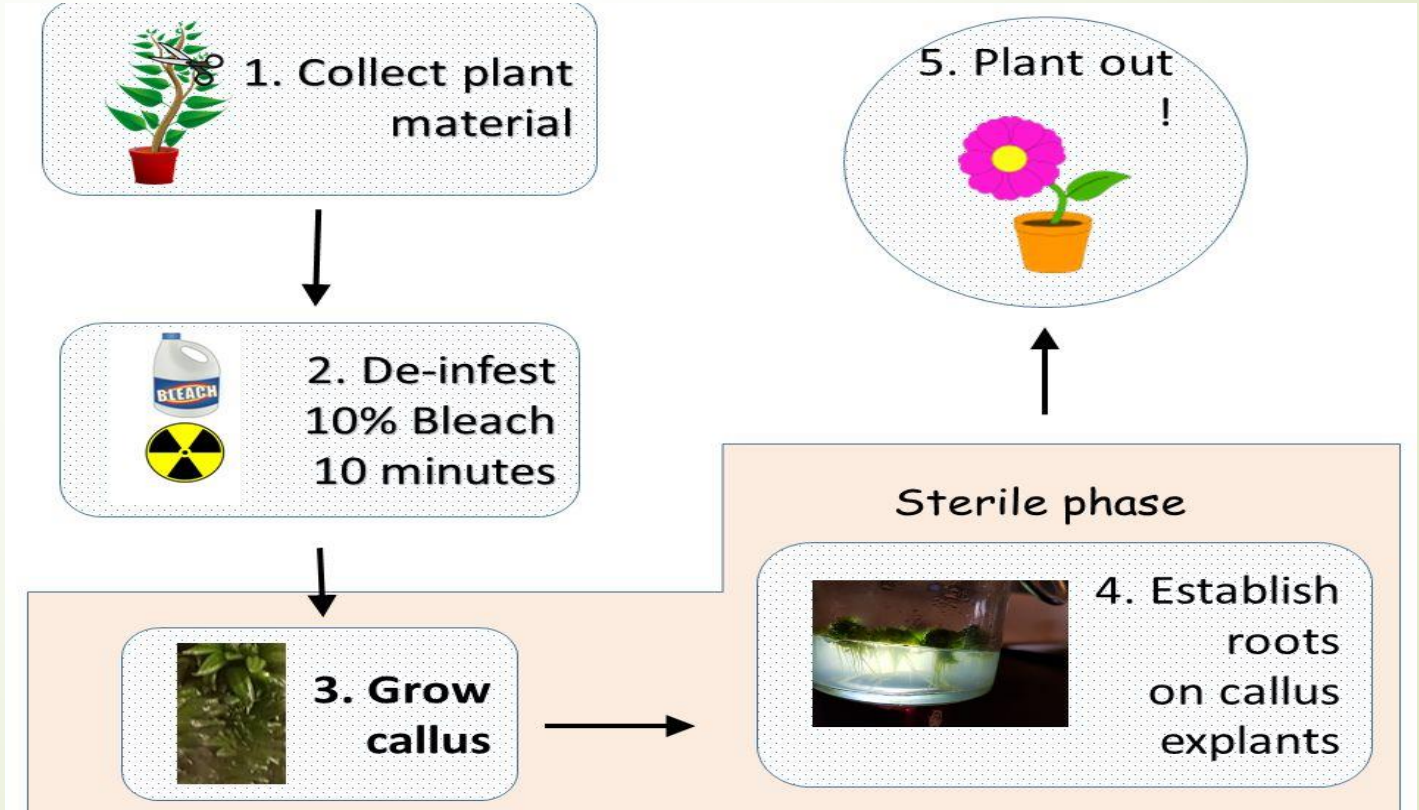
- MRS NEHA NANGIA
- ASSOCIATE PROF IN BOTANY
- BOTANY DEPT

FORM AND FUNCTIONS – II
Unit IV

- **Aspects of micro propagation with reference to floriculture** –Detailed study of orchid cultivation
- **Plant cell suspension cultures for the production of secondary metabolites** – with special reference to shikonin production
- Somatic embryogenesis and artificial seeds
- **Protoplast fusion and somatic hybridization**
  - Concept, definition and various methods of protoplast fusion
  - Applications of somatic hybridization in agriculture

DEFINITION

- Tissue culture is the in vitro aseptic cultures of cells, tissues, organs or entire plants under controlled conditions
- Conditions include proper supply of nutrients, pH medium, adequate temperature
- The resultant clones are true – to – type of the selected genotype



TISSUE CULTURE TECHNIQUES

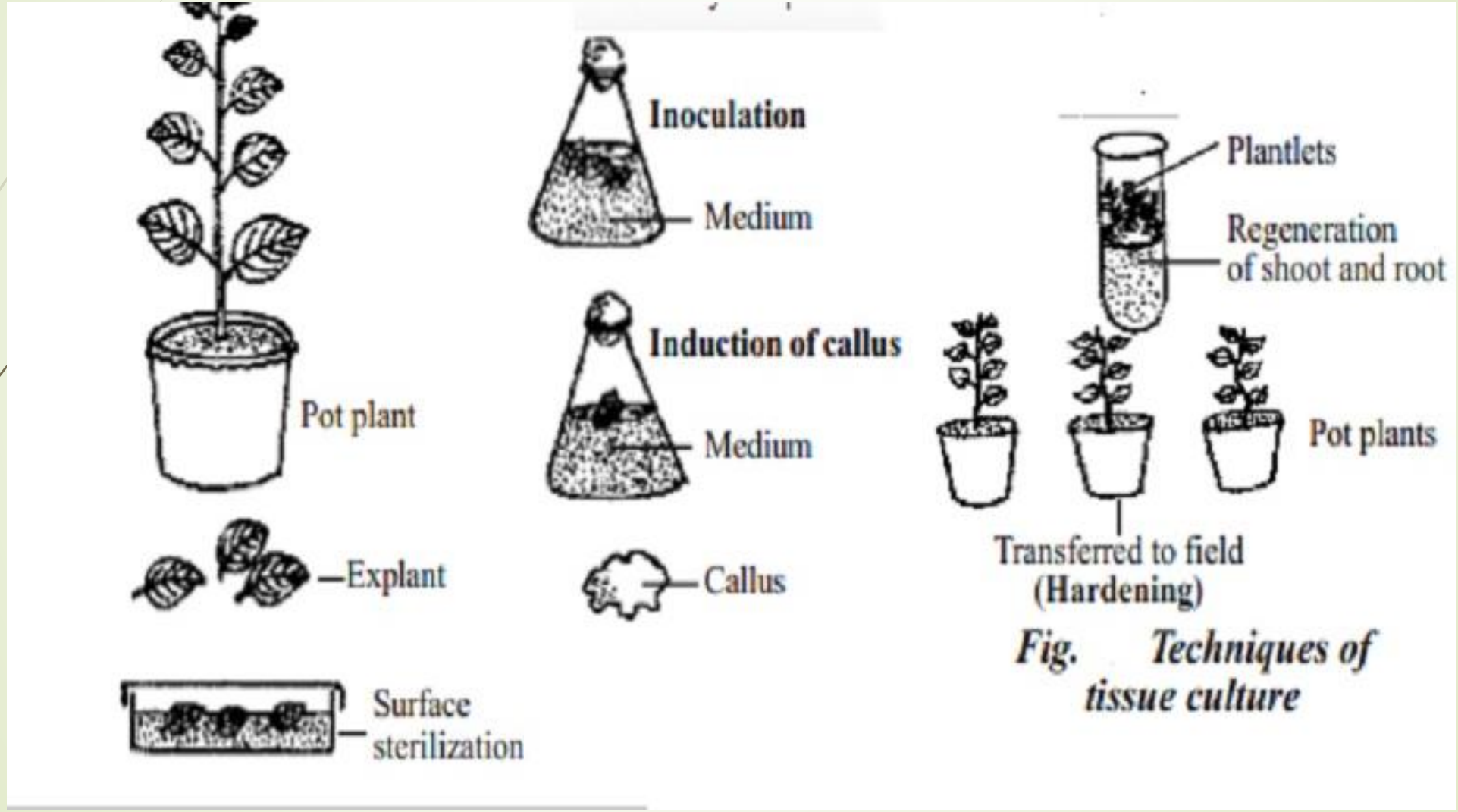
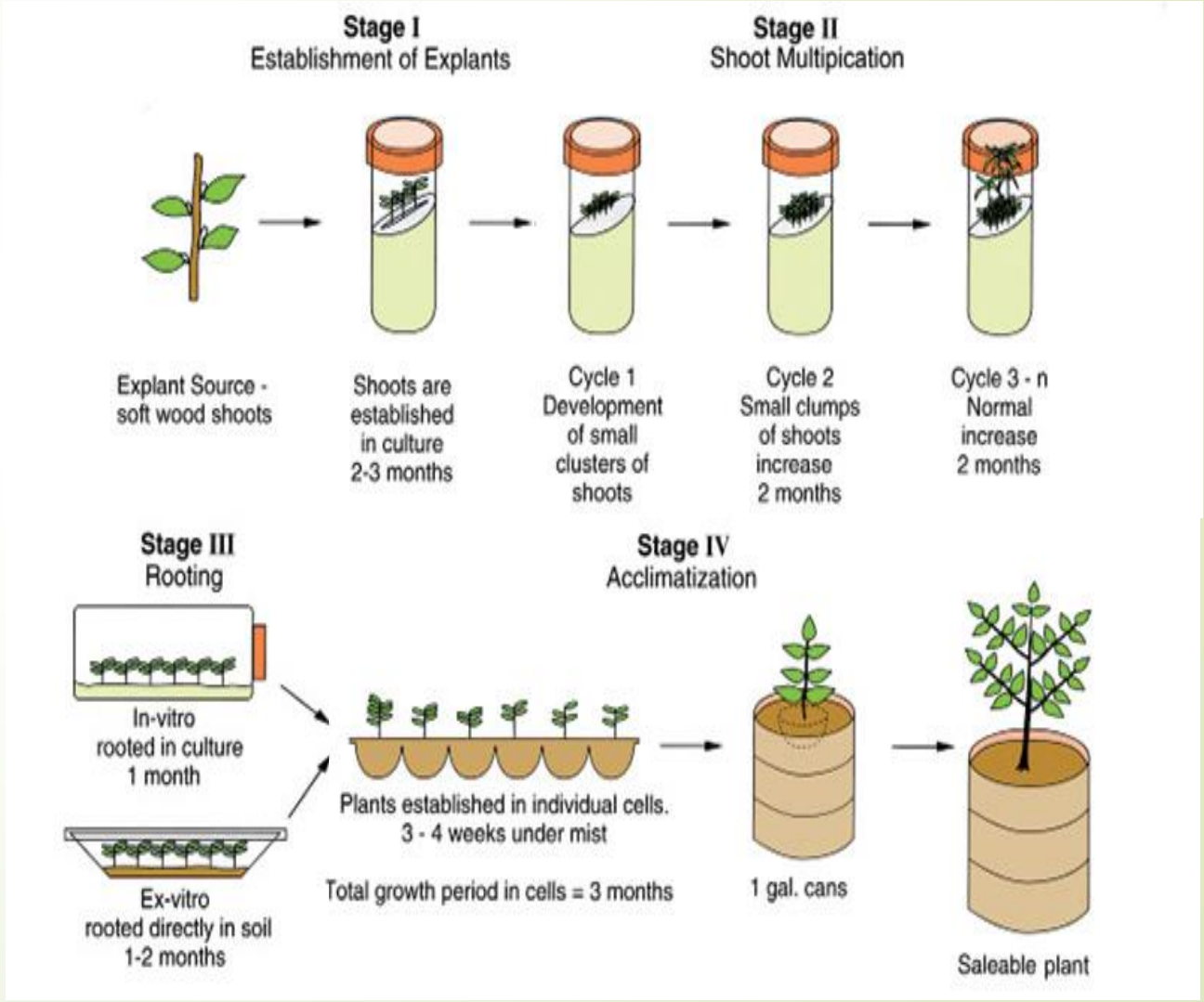


Fig. Techniques of tissue culture

MICROPROPAGATION

- It is the rapid vegetative propagation of plants under in vitro conditions of light, temperature and in a defined medium
- It can be applied to a substantial number of commercial vegetatively propagated plant species



- Micropropagation can be divided into four stages:
- **INITIATION STAGE** – explant is cut, disinfested, washed and placed on a medium containing mineral salts, sucrose and agar. An aseptic culture is achieved
- **MULTIPLICATION STAGE** – a growing explant can be induced to produce vegetative shoots by including cytokinin in the medium (cytokinin promotes shoot formation)
- **ROOTING or PREPLANT STAGE** – auxins are added in the medium to produce adventitious roots. May or may not be needed
- **ACCLIMATIZATION** – the growing rooted shoot can be removed from tissue culture and placed in soil. Humidity is reduced over time to prevent wilting

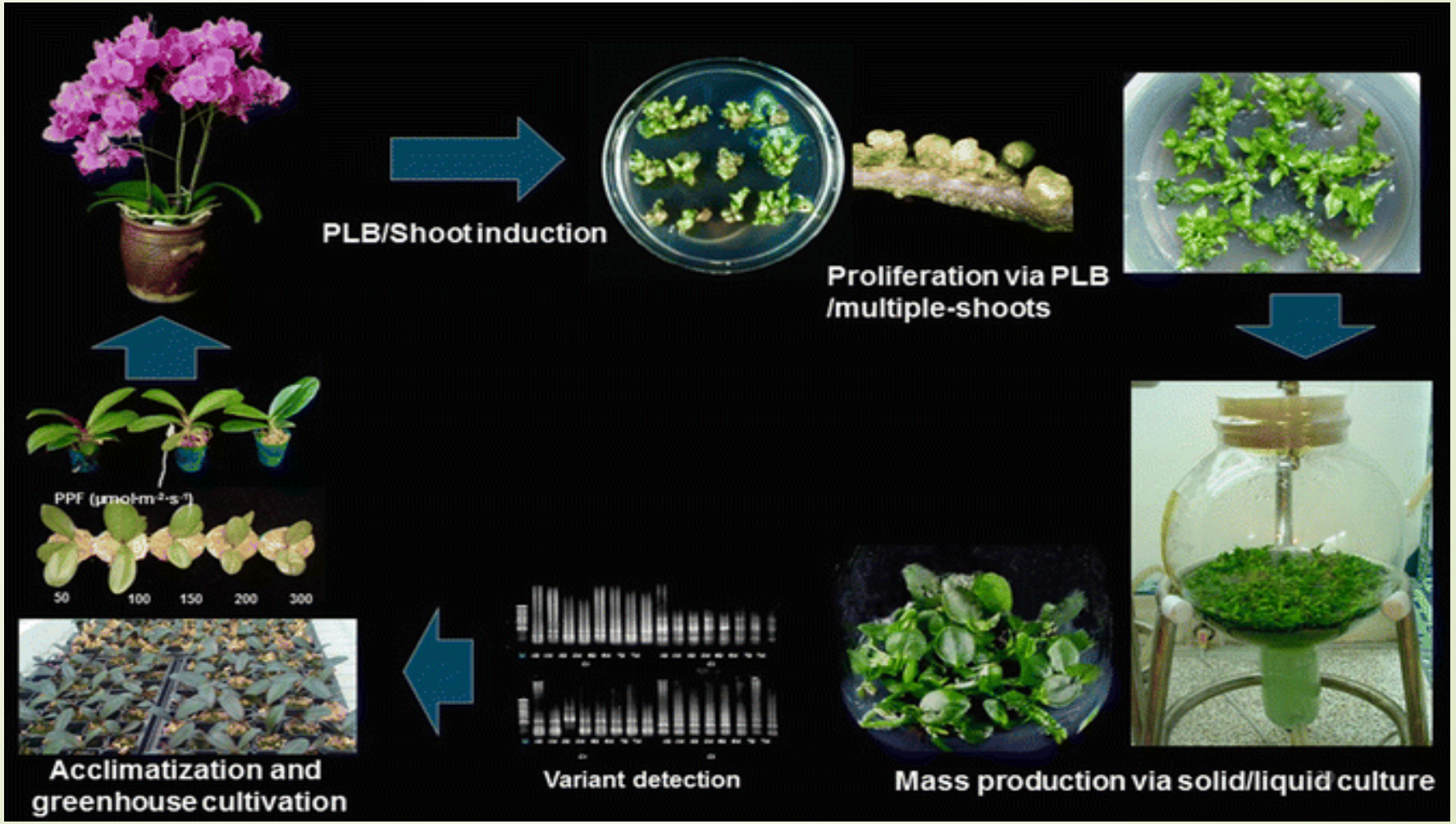
MICROPROPAGATION OF *Orchids*

- LARGE SCALE PRODUCTION…… WHY?
- Orchids are in demand for their large, beautiful long lasting flowers
- They exhibit an incredible range in color, size and shape
- For floriculturists it is an international business
- Large scale production with the help of tissue culture has made it one of the top ten cut flowers
- It is today a million dollar industry globally

- **REQUIREMENTS** –
- **Light** – it is necessary for flowering. Under suitable conditions of light the plants show strong upright growth
- **Air** - Orchid potting media should be open with good drainage and moisture holding capacity
- **Water** – Quantity and frequency of water is important. Water should run out freely from the drainage holes. Frequency of watering depends on the pot size
- **Fertiliser** – The urea content required is less and application is to be done once a week or every two weeks

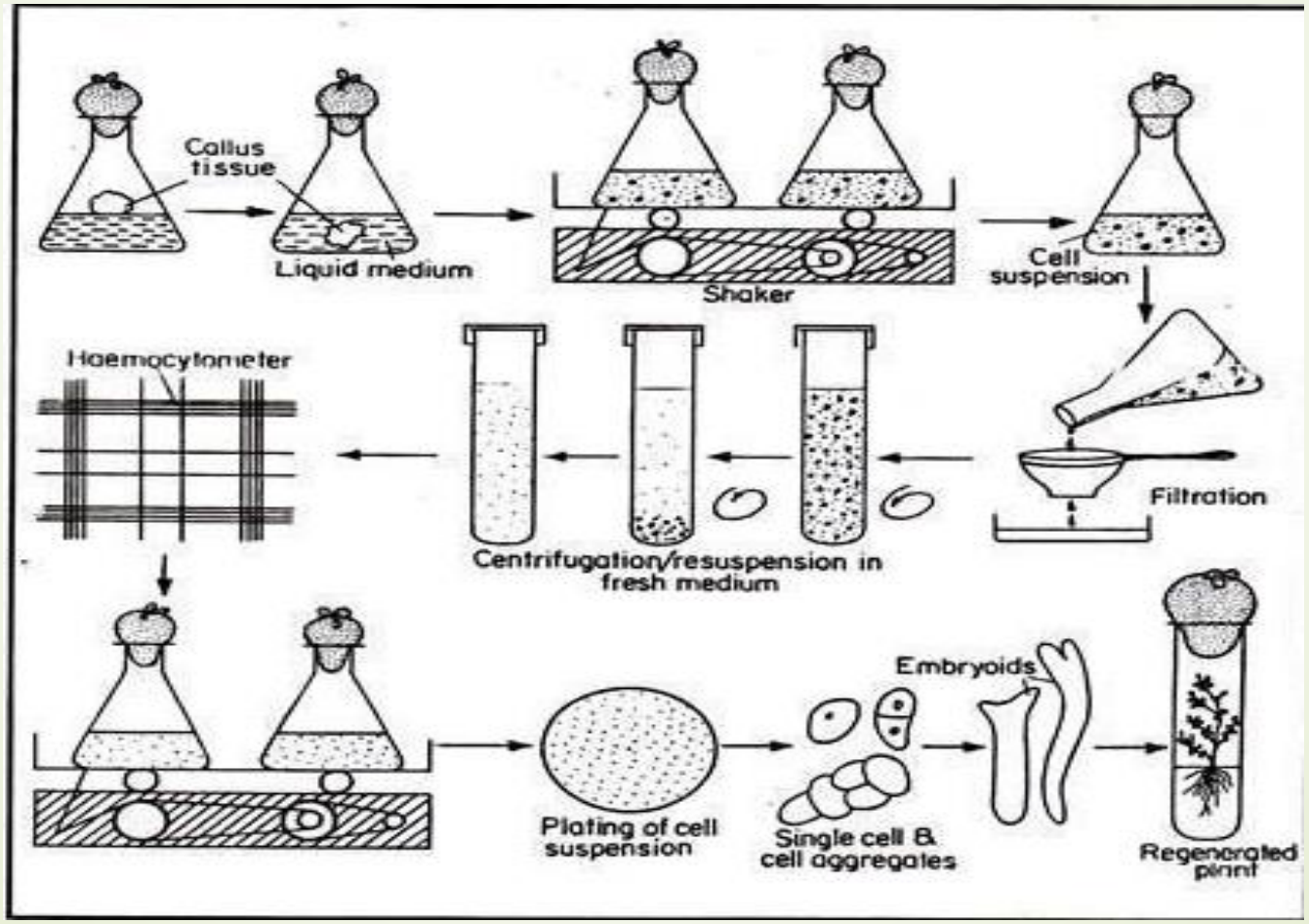
- **SHOOT TIP CULTURE FOR *in vitro* PROPAGATION** –
- Two media are used –MS +1mg/l BAP + 3% sucrose in which the explants are initiated and sub-cultured for 3 to 4 months in a four week cycle
- - N69 (Nitsch basal media)+ 0.5mg/l BAP +0.5mg/l biotin + 0.5mg/l folic acid + 2% sucrose in which proliferating shoots are obtained
- **INFLORESCENCE AXIS AND FLOWER BUD CULTURE** – effective donor organs for micropropagation of orchids
- Mass production for moth orchids which is today one of the most popular orchids
- The in vitro propagation involves exposing the buds to high auxin levels, then high cytokinin levels and finally to high anti auxin levels
- Media used are MS and N69

- Protocol outline -



CELL SUSPENSION CULTURE

- It is a type of culture in which single cells or cell aggregates multiply while suspended in agitated liquid medium

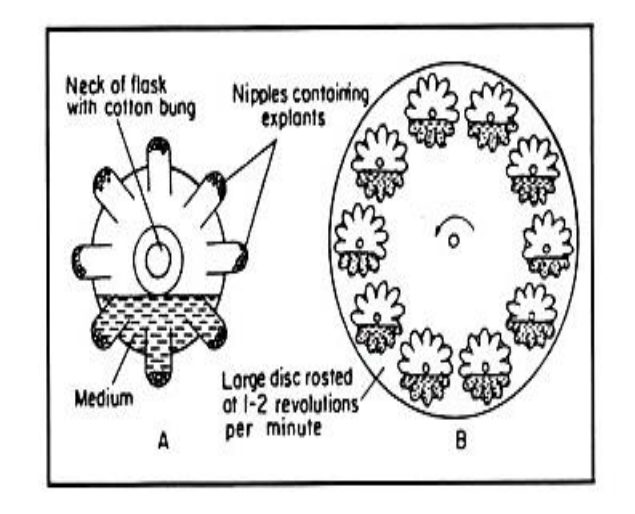


- **PROTOCOL** –
  - 150 – 250 ml autoclaved liquid medium
  - Pre established callus tissue transferred to it
  - Mouth of flask covered with cotton plug and paper
  - Kept on a shaker moving at 80 – 120 rpm/minute
  - After 7 days contents sieved through sterilized sieve and filtrate collected – it contains only free cells and cell aggregates
  - Filtrate is centrifuged at 5000 rpm and supernatant decanted and residue is resuspended in fresh liquid medium
  - 3 -4 such sub culturings are done with fewer cells
  - Plating and regeneration is done
  - (increasing the concentration of auxins or cellulose is effective for cell dispersion)

CATEGORIES OF SUSPENSION CULTURE

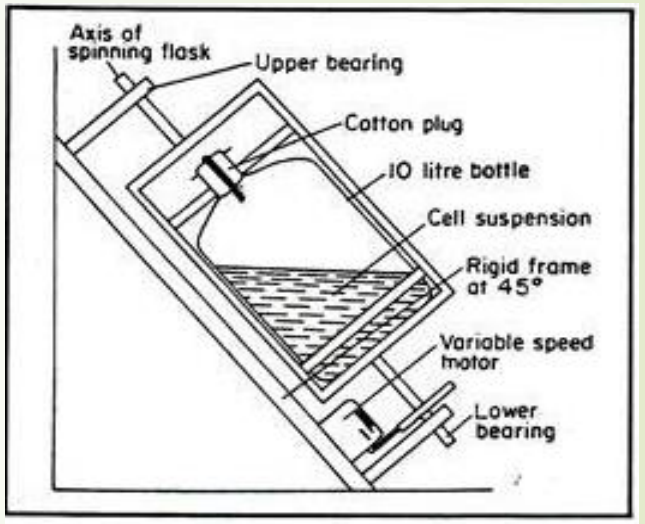
- **BATCH CULTURE** – cell material grows in a finite volume of agitated liquid medium. May be of the following types –
  - 1. Slowly rotating cultures
  - 2. Shake cultures
  - 3. Spinning cultures and stirred cultures
- **CONTINUOUS CULTURES** – the old liquid medium is continuously replaced by fresh liquid medium to stabilize the physiological states of growing cells
  - 1. Use of chemostats
  - 2. Use of turbidostats

- **Batch culture – rotating, shaking and spinning**



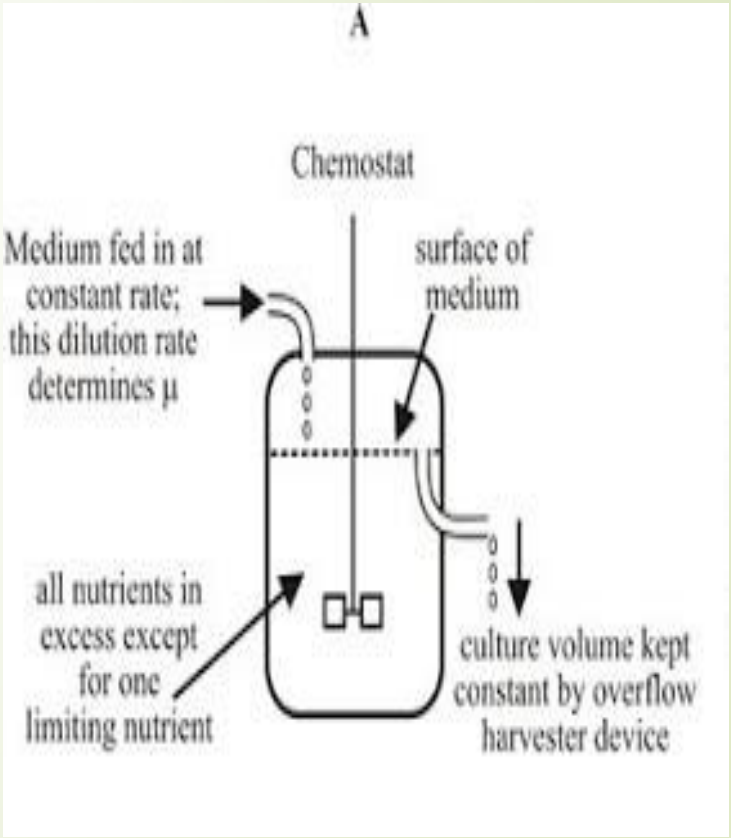
Rotating

Shaking

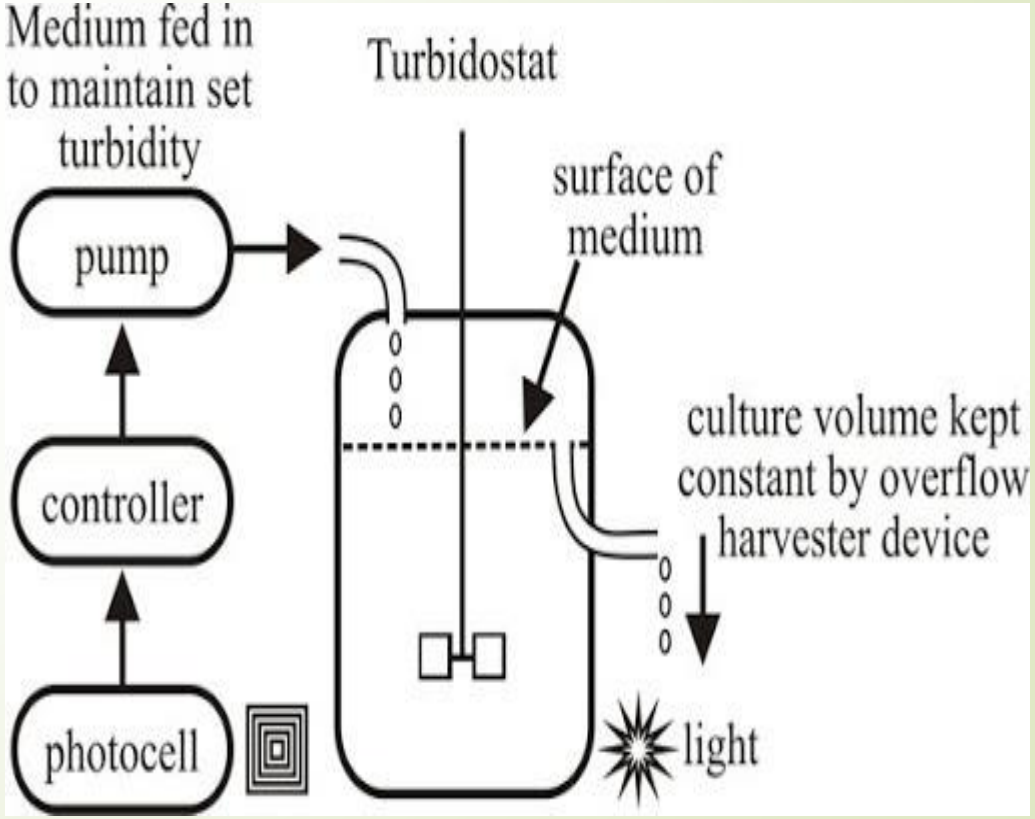


Spinning

- **CONTINUOUS CULTURE – Chemostats** –
- Culture vessels cylindrical or circular
- Inlet and outlet for aeration and inoculation and removal of cells
- Fresh sterile media is pumped in at constant rate an equal volume of old media is removed
- This steady state helps in maintaining the growth rate, chemical composition and metabolic activity constant



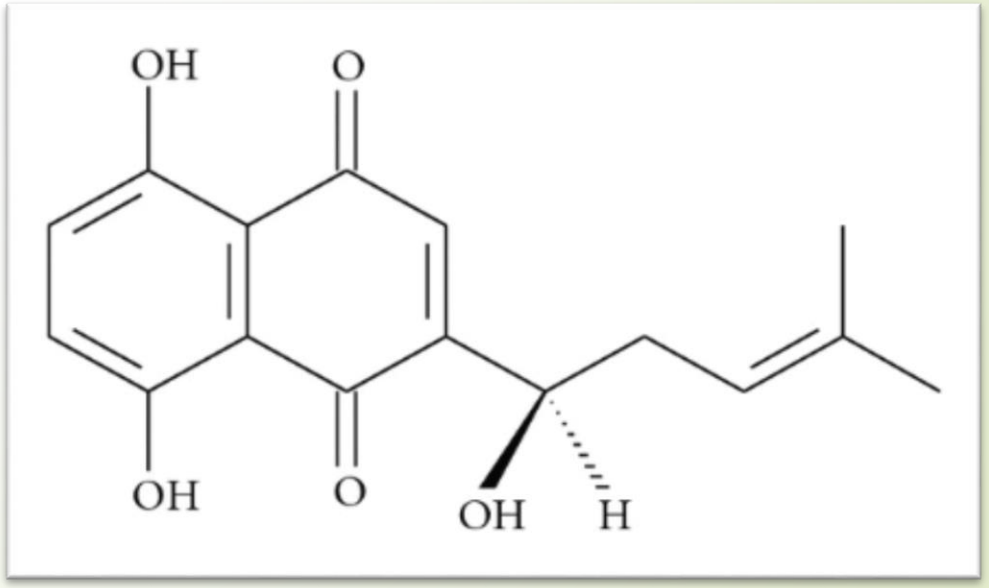
- **Turbidostats** –
- When cells increase in number turbidity changes
- Measured by the changes of optical density of medium
- pH also changes
- Automatic monitoring unit is attached to maintain optical density or pH at a preset level



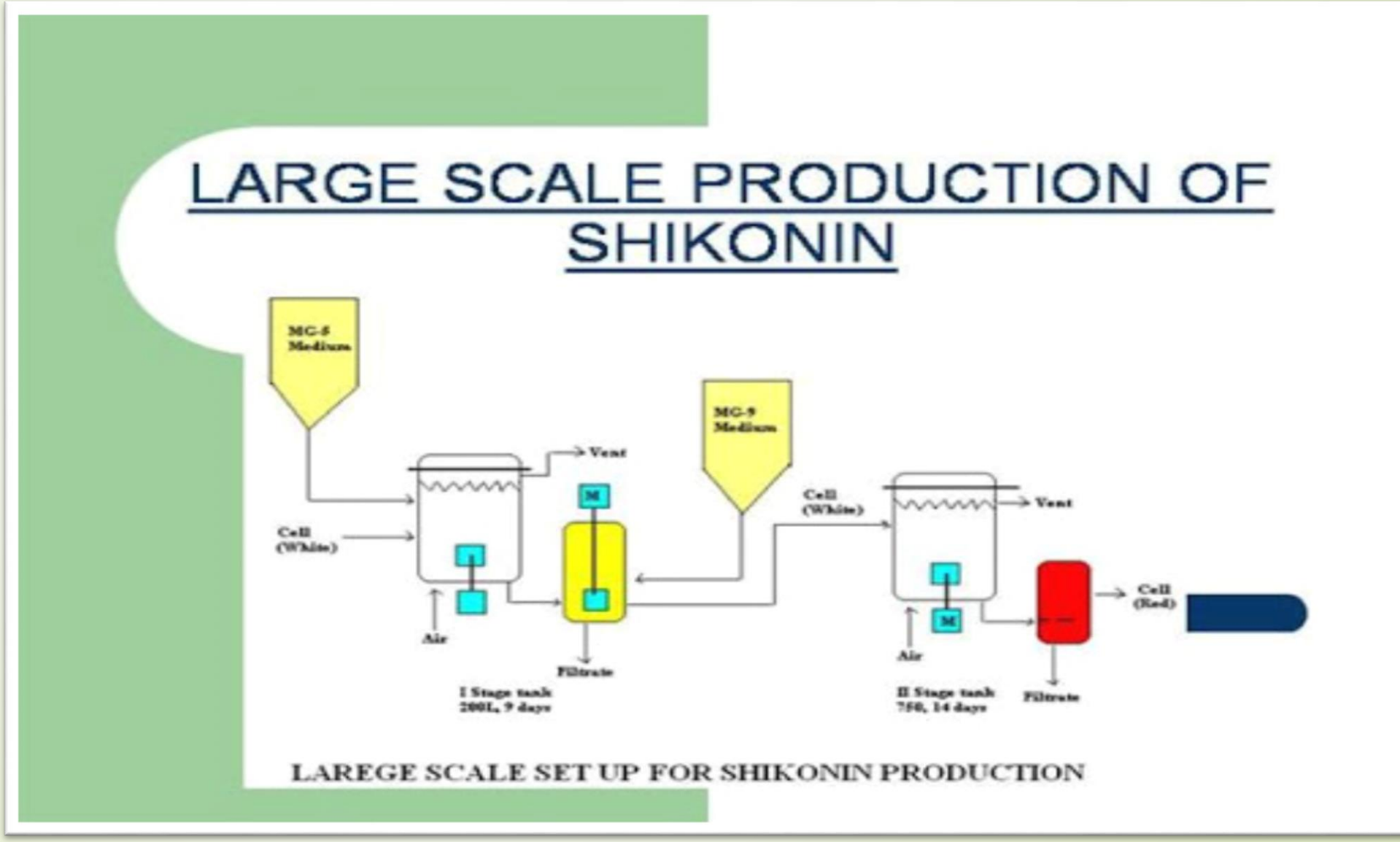
SECONDARY METABOLITES AND SHIKONIN PRODUCTION

- Secondary metabolites are organic compounds produced by bacteria, fungi or plants
- They ae not directly involved in the normal growth, development or reproduction
- Play an important role in plant defense against herbivory
- Humans use secondary metabolites as medicines, flavourings,etc
- Can be divided into four major classes – terpenes, phenolics, glycosides and alkaloids

- **SHIKONIN** – main active principle of **Zicao**, a traditional Chinese herbal medicine
- Made from dried root of *Lithospermum erythrorhizon*
- Has diverse properties like antioxidant, anti inflammatory, antimicrobial and wound healing



Shikonin production



- Cells of high producing strain M18 are propagated in growth medium **MG 5**
- No shikonin derivatives are produced
- Cells from MG5 or modified LS medium are transferred to **M9** medium
- Initial cell density and medium concentration result in increased yield
- Concentration of dissolved oxygen optimum is 6.4 to 6.0ppm for first and second culture stage
- Stock culture cells are inoculated in first stage tank containing 200L of MG5 medium and incubated for 9 days
- Filtered

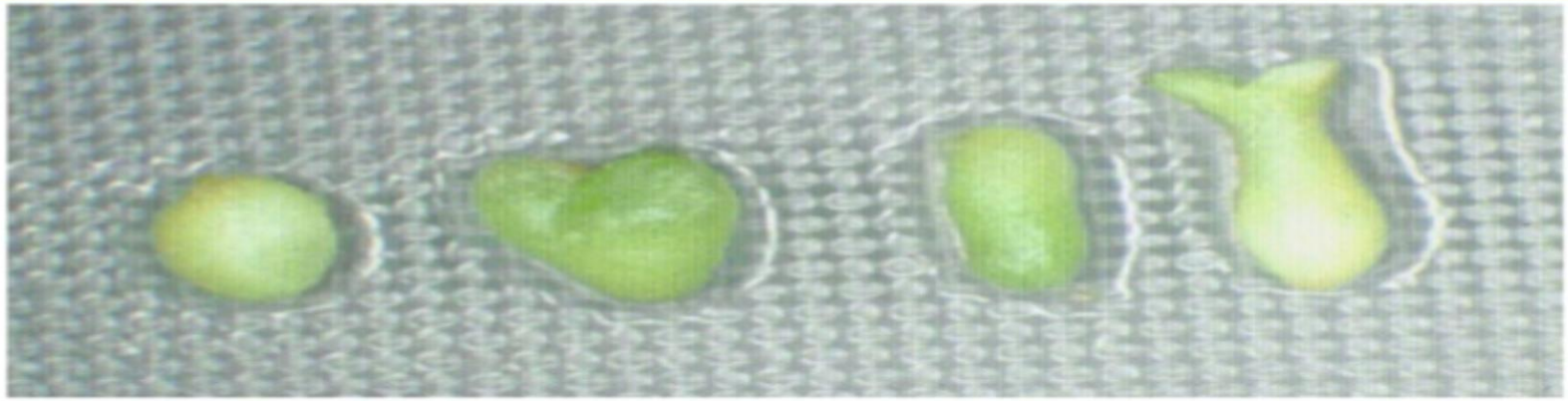
- 750L od M9 medium is pumped into second tank
- Incubated for 14 days
- Pigmented cells are harvested by filtering
- Extraction and purification is carried out with n hexane and then hydrolysis with 2% KOH
- Recrystallised to give pure shikonin

- **FACTORS AFFECTING SHIKONIN PRODUCTION** –
  - **Temperature** – between 20 – 25 C in the dark
  - **Growth hormones** - IAA, a natural auxin, promotes growth and induces shikonin synthesis
  - **Carbon source** – 5% sucrose in LS medium is best for maintaining callus culture
  - **Nitrogen source** – 20m NH4 and 40mM No3 along with L – phenylalanine increases level of products
  - **Rosmarinic acid** – endogenous level important in cellular control
  - **Agar** powder in LS medium induces production
  - High concentrations of Ca, NO3. Fe2, SO4 increase shikonin yield

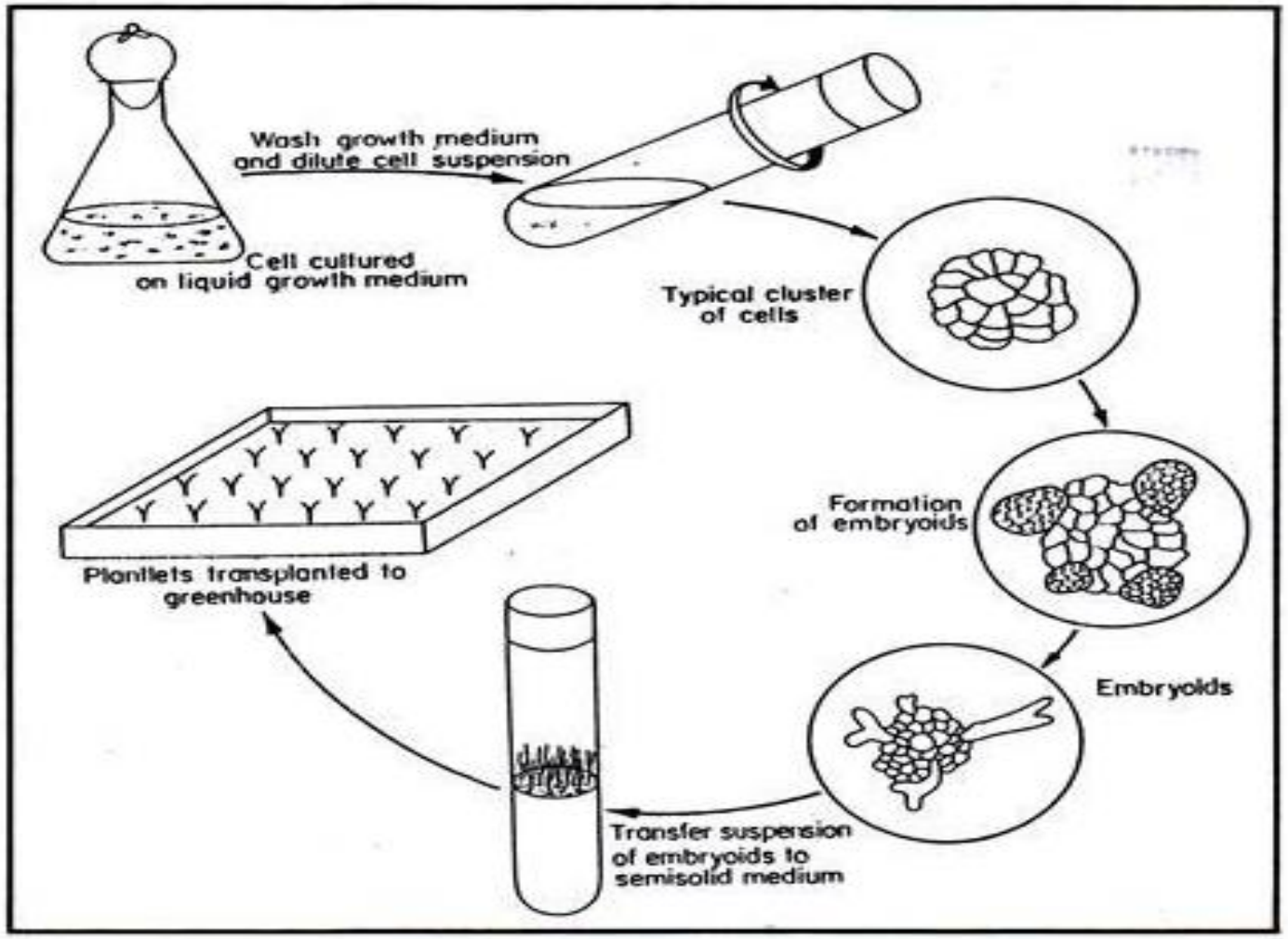
SOMATIC EMBRYOGENESIS

Somatic Embryo Culture

- A somatic embryo develops from a somatic cell.
- The pattern of development of a somatic embryo is comparable to that of a zygotic embryo.
- Somatic embryo culture is induced by a high concentration of an auxin, such as 2,4-D.
- These embryos develop into mature embryos.
- Mature somatic embryos or embryoids germinate to give complete plantlets.



PROTOCOL OF SOMATIC EMBRYOGNESIS



- **SOMATIC EMBRYOGENESIS-** is a process by which asexual or somatic cells are induced to form embryos in culture. It is a multistep regeneration process
- **EMBRYOGENIC POTENTIAL** – capacity of the somatic cell to produce embryoids
- **EMBRYOIDS**- embryo like structure originating from somatic cells
- Somatic embryogenesis may be of two types:
  - Direct embryogenesis
  - Indirect embryogenesis

- **DIRECT EMBRYOGENESIS** - no callus production
  - Developed from pro embryogenic determined cells

- **INDIRECT EMBRYOGENESIS** – first callus proliferation
  - Embryos develop from within callus tissue from induced embryogenic cells
  - Two media may or may not required – first or induction media must contain auxin
  - Second media no auxin or low concentration of auxin

- **PROTOCOL** – leaf petiole or root segments obtained from aseptically grown seedlings
  - Explants placed individually on semi - solid MS medium containing auxin and sucrose
  - Incubated in the dark
  - Callus produced
  - Callus cells transferred to suspension media and placed on a shaker
  - Sub culturing done every 4 weeks
  - For more uniform embryo population suspension culture passed through sieve

- To induce somatic embryogenesis cells inoculated in media containing no auxin
- Cultures incubated in dark
- After 3 to 4 weeks – embryos in globular first then heart shaped and then torpedo stage seen
- Placed in agar medium without auxin for plantlet development
- Plantlets transferred to vermiculite for further development

FACTORS AFFECTING SOMATIC EMBRYOGENESIS

- **AUXIN** – minimal level of auxin essential for induction of embryogenic potential cells but for organization and maturation of embryoids auxin plays no role
- **CYTOKININ** – zeatin stimulates embryogenesis when cells are cultured in auxin free medium but kinetin inhibits
- **GIBBERELLIN** – no positive effect
- **REDUCED NITROGEN** – addition of NH_4Cl to medium containing KNO_3 produces optimal embryoids. Glutamine, urea and alanine may partially replace NH_4Cl
- Optimal level of oxygen and high potassium level are important factors

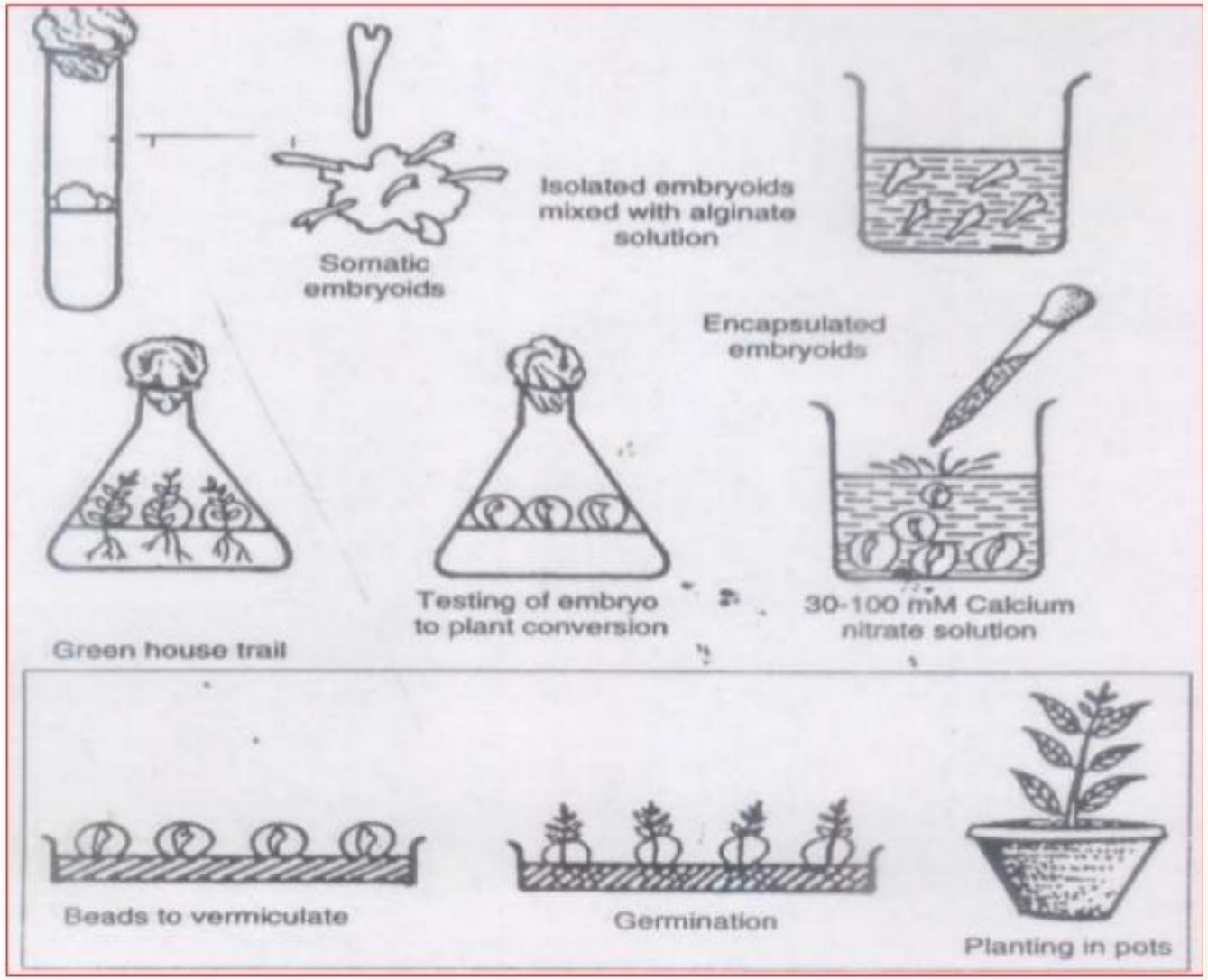
IMPORTANCE -

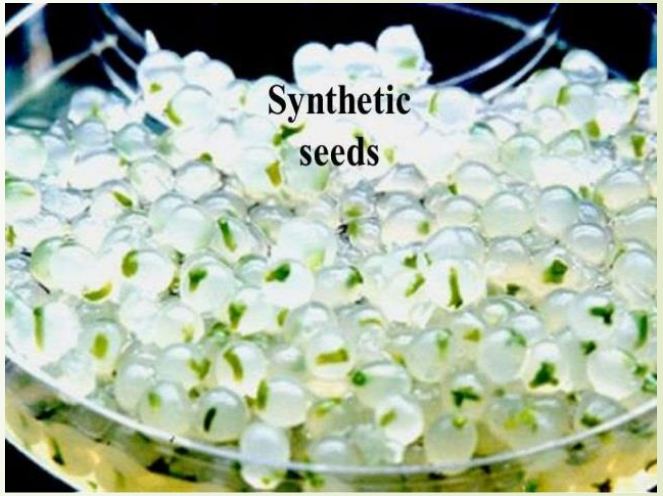
- Ideal propagation system
- No need for separate rooting phase
- May be free of virus and pathogens
- Used to produce artificial seeds

ARTIFICIAL SEEDS

- Somatic embryoids not enclosed by seed coat
- Cannot survive in field due to microbial contamination and dessication
- Hence artificial seeds – **embryoids encapsulated by a hydrogel**
- **Steps** – establishment of callus
  - Induction of somatic embryogenesis
  - Formation of somatic embryos
  - Encapsulation
  - Test for embryoid to plant conversion
  - Green house and field planting

PROTOCOL


Somatic embryoids
Isolated embryoids mixed with alginate solution
Encapsulated embryoids
Testing of embryo to plant conversion
30-100 mM Calcium nitrate solution
Green house trail
Beads to vermiculate
Germination
Planting in pots


Synthetic seeds

- Many hydrogels have been standardized for making artificial seeds

Gels	Conc. % (w/v)	Complexing agent	Concentr-ation (mM)
Sodium alginate	0.5 - 5.0	Calcium salts	30 - 100
Sodium alginate with gelatin	2.0	Calcium chloride	30 - 100
Carrageenan with	0.2 - 0.8	Potassium or	
Locust Bean Gum	0.4 - 1.0	Ammonium chloride	500
Gelrite	0.25	Low Temperature	

- **METHOD 1** – isolated embryoids mixed with 0.5 to 5% W/Vsodium alginate
  - Dropped into 30 to 100 micromolar Calcium nitrate solution
  - Surface complexation immediately and then gel formation within 30 minutes
- **METHOD 2** – isolated embryoids in Gel –rite
  - Placed in microtitier plate
  - Gel formation on cooling

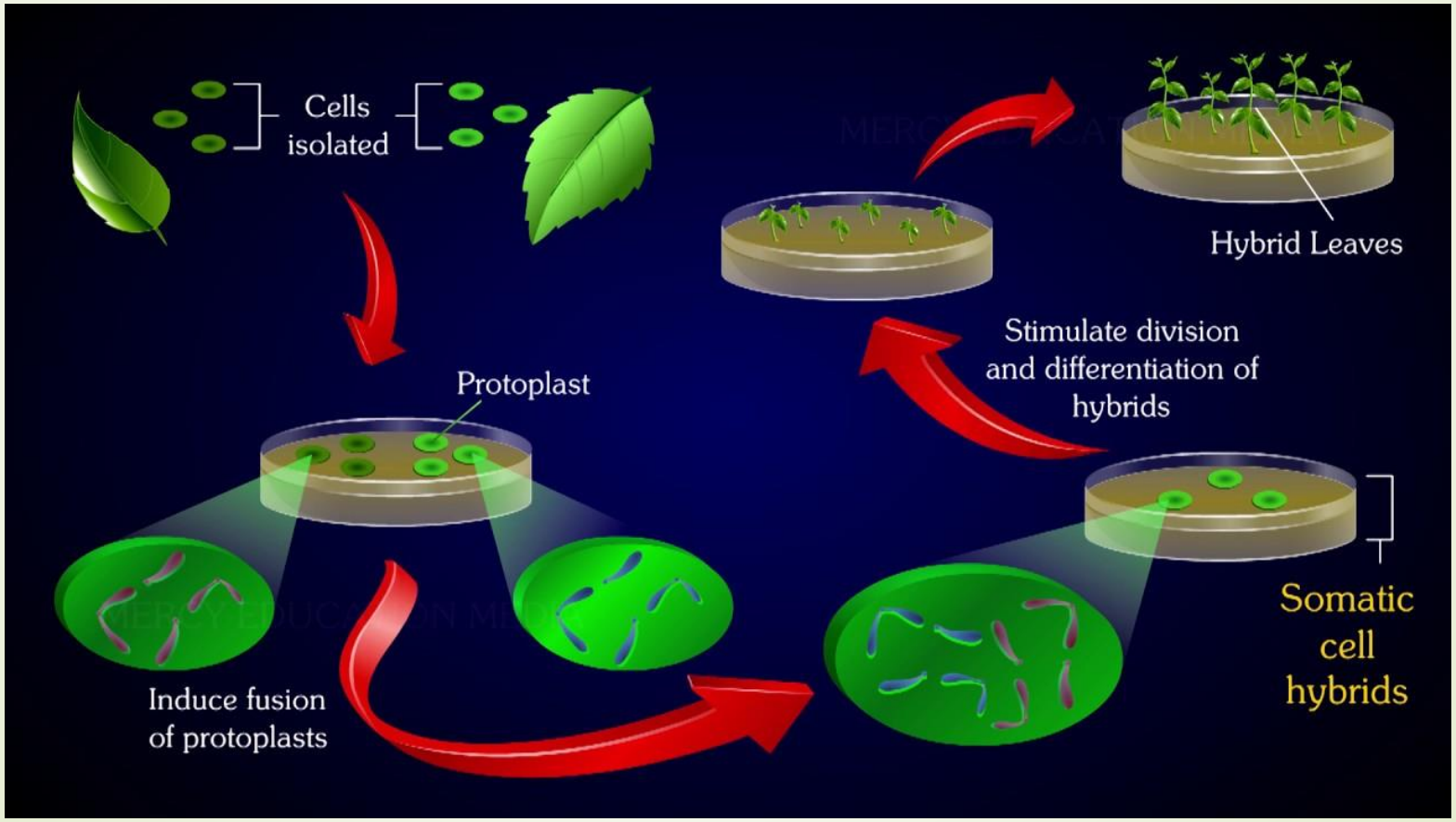
- **EMBRYO TO PLANT CONVERSION** –
  - Encapsulated embryoids placed on agar medium with minimal nutrients
  - Germination and development of root and shoot system
  - Formation of true leaves
  - Green plant with normal phenotype
- **STORAGE** - low temperature
  - Limited storage time

IMPORTANCE

- Available within a month
- Not time or season dependent
- Life cycle can be shortened
- Applicable for large scale monocultures and mixed genotype plantations
- Help to study role of endosperm and seed coat formation

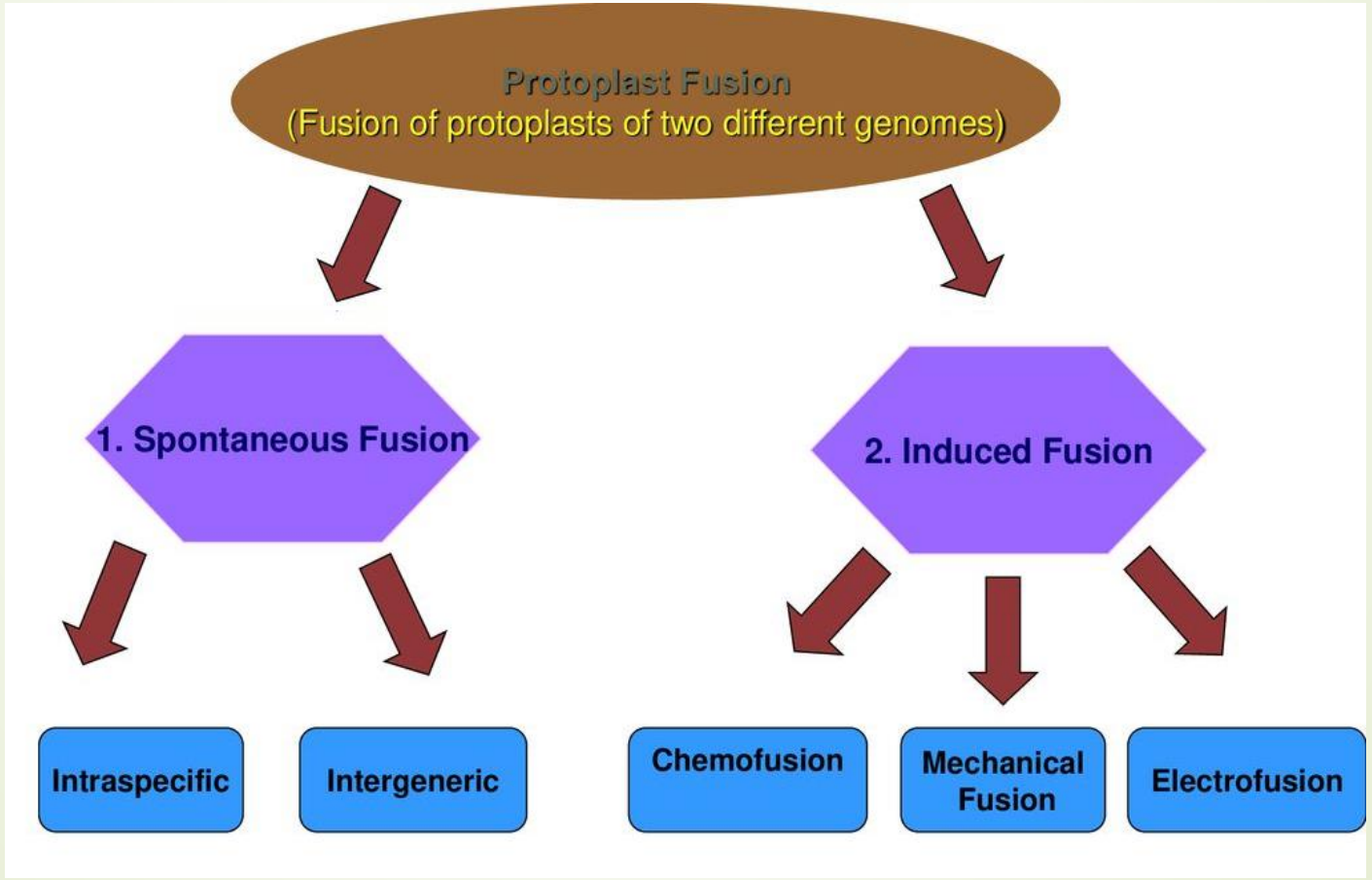
PROTOPLAST FUSION

- Protoplast from two genetically different plants isolated from somatic cells and fused experimentally to form hybrid protoplasts



- **Protoplast fusion and somatic hybridisation** is based on:
  - Isolation of protoplast
  - Fusion to form viable heterkaryons
  - Cell wall regeneration
  - Fusion of nuclei
  - Division of hybrid calls and then their growth
  - Selection of hybrid cell
  - Induction of organogenesis and
  - Formation of mature plants

METHODS OF PROTPLAST FUSION



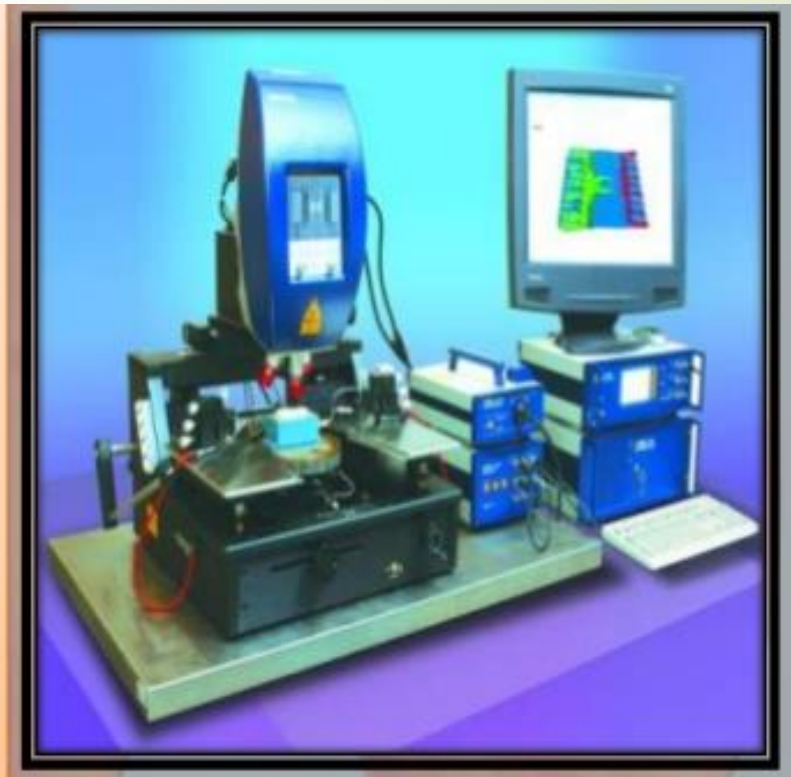
- **SPONTANEOUS FUSION**
  - Protoplast fuse automatically during isolation
  - Cell wall are enzymatically degraded
  - Plasmodesmata enlarges
  - Organelles enter adjacent cell
  - Complete fusion occurs
  - Spontaneous fusion is always intraspecific and gives rise to homokaryons
  - Resulting hybrids are of no practical use

- **INDUCED FUSION**
  - Isolated protoplasts do not fuse due to negative charge on protoplast surface
  - Fusion has to be induced
- Can be done by :
  - Mechanical fusion
  - Chemofusion
  - Electrofusion

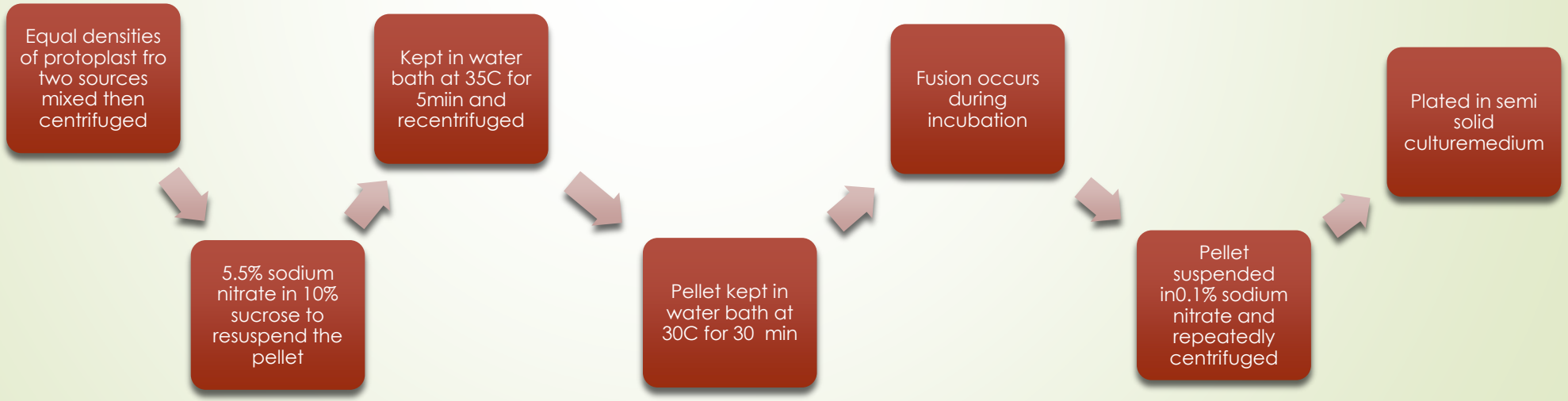
- MECHANICAL FUSION

1- Mechanical fusion:

- Isolated protoplast are brought into intimate physical contact mechanically
- under microscope
- using micromanipulator or perfusion micropipette.



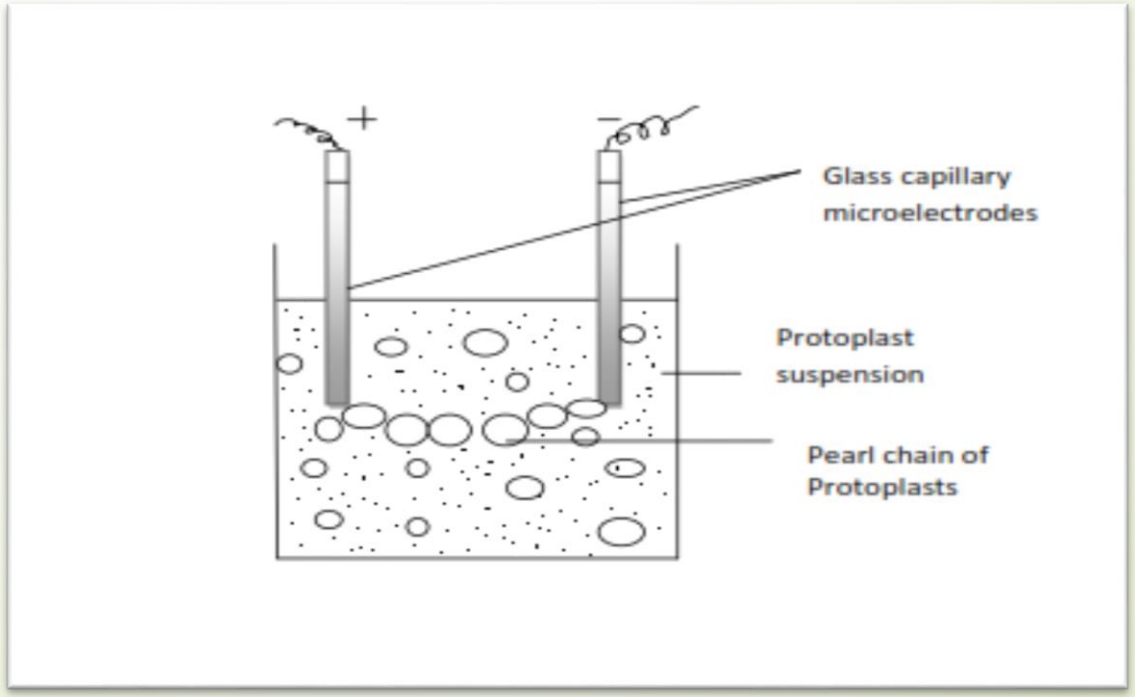
- **CHEMOFUSION**
- Chemical fusogens cause the isolated protoplast to adhere to one another, lea to tight agglutination and then fusion
- **FUSION INDUCED BY SODIUM OR POTASSIUM NITRATE**



FUSION INDUCED BY CALCIUM IONS AT HIGH pH

- **FUSION INDUCED BY POLYETHYLENE GLYCOL (PEG)**
- 37.5% PEG aggregates isolated protoplasts and fusion occurs when liquid culture media are slowly mixed with PEG
- PEG is more efficient when mixed with 10 to 15% dimethyl sulfoxide
- High pH and calcium are also beneficial

- **ELECTROFUSION**
- Two glass capillary microelectrodes are in contact with protoplasts
- Electric field of low strength forms a dielectric dipole
- Leads to pearl chain arrangement
- High electric impulse degenerates membrane and leads to fusion



- **SOMATIC HYBRIDISATION**

Somatic hybridization technique

- 1.Isolation of protoplast
- 2.Fusion of the protoplasts of desired species/varieties.
- 3.Identification and Selection of somatic hybrid cells
- 4.Culture of the hybrid cells
- 5.Regeneration of hybrid plants

- **APPLICATIONS OF SOMATIC HYBRIDISATION IN AGRICULTURE**
  - Development of new cultivated varieties
  - Interspecies hybrids used to transfer genes into cultivated crops
  - Several plant varieties express traits derived from wild species (*Solanum*, *Nicotiana*, barley, wheat, tomato)
  - One variety of tobacco is resistant to three diseases
  - Improved yield (oats and tobacco)

FOR THE CURIOUS……

- https://www.youtube.com/watch?v=zyfkN0QKoUA
- https://www.youtube.com/watch?v=HP_C_oe7MOk
- https://www.youtube.com/watch?v=Qv-Lo2bMjQc
- https://www.youtube.com/watch?v=utlKKvqxbWw
- "Plant Tissue Culture" by Kalyan Kumar De

Thank You
For Your Attention