B.Sc. III (2021-22)

Unit IV: Plant Tissue Culture

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Sterilization Techniques

Sterilization is done for killing or removal of all micro-organisms, including bacterial spores. In PTC, sterilization is achieved by one of the following:

i) Dry heat treatment

ii) Flame sterilization

iii) Autoclaving

iv) Filter sterilization

v) Wiping with 70% ethanol

vi) Surface sterilization

i) Dry heat treatment:

Dry heat sterilization is done in hot air oven. Generally, used for glass equipments used in PTC. Dry heating generally achieved in 15 minutes at a temperature 160°C.

ii) Flame sterilization:

Metallic instruments *viz.* forceps, scalpel, needle, spatula and scissors are dipped in 70% ethanol followed by flaming with a burner (Bunsen burner or spirit lamp) and cooling.

(**Note:** Ethanol is volatile and inflammable. So this procedure involves the risk of fire during sterilizing the equipments.)

Figure: Commonly used instruments in plant tissue culture

iii) Autoclaving:

In autoclaving, pressurized steam is used to heat the material to be sterilized. Plant tissue culture media are generally sterilized by autoclaving at a temperature 121°C and 15 lbs pressure for 15 min.

Figure. Autoclave unit used for wet sterilization of nutrient media

iv) Filter sterilization:

Some growth regulators (GA3, urea, certain vitamins and enzymes) are heat labile. Such compounds are filter sterilized by passing their solution through a membrane filter of 0.45 micron or lower pore size.

Laminar air flow cabinets are used to create an aseptic working space by blowing filter-sterilized air through HEPA (High Efficiency Particulate Air) filter. This filter can remove 99.97% of dust, pollen, mold, bacteria and any airborne particles with a size of 0.3 μm.

iv) Wiping with 70% ethanol :

70% ethanol is effective to kill microbes, bacteria and other micro-organisms on the surfaces. Worker's hands and laminar flow cabinet occasionally sterilized by wiping with 70% ethanol.

v) Surface sterilization:

Surface sterilization is used for explant sterilization. For this, sodium hypochloride, hydrogen peroxide, mercuric chloride etc. are used in low concentration. Dipping the explant in 0.1% mercuric chloride for 5 min. followed by three times wash in distilled water is commonly used method.

The sterilizing agent used for surface disinfection are-

- Calcium Hypochloride (9-10%)
- Sodium Hypochloride (2%)
- Mercuric chloride (0.1-1%)

} Commonly used

- Silver nitrate (1%)
- Bromine water (1-2%)
- Hydrogen Peroxide (10-12%)

Explant sterilization in Laminar Air Flow
(Dipping in 0.1% $HgCl_2$ followed by 3 times washings in distilled water)

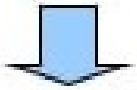
Figure: Laminar air flow cabinet in use

Basic requirements of PTC

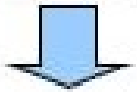
- Plant material
- Equipments and Glasswares
- Aseptic conditions
- Washing and storage facilities
- Media preparation room
- Sterilization room
- Nutrient medium
- Culture room or incubators

Important things: (In PTC, use Borosilicate or Pyrex glasswares)

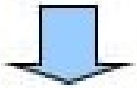
Soak glassware in 10% soap water (teepol) for 1 hour.



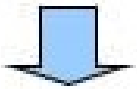
Transfer glassware to conc. HCl and keep for 2 hours.



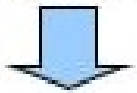
Rinse glassware in tap water.



Wash the glassware at least twice with distilled water.



Keep glassware for drying in oven at 100 °C for 1 hour.



Autoclave/ keep glassware in oven at 140-160 °C for 2 hours.

Incubation of culture

- Cultures are incubated in a culture room where light, temperature and humidity are controlled.
- For some tissues dark is essential while for some both dark and light conditions are required.
- Humidity has also some effect.
- The cultures are incubated on culture rack at 25-28 oC constant temperature. Culture tubes are placed at 35-40o inclined position.
- Culture to give a light intensity of 4-10 X 103 lux for 16 hrs.

Botany Paper- VIII
Section-I
Unit IV: Plant Tissue Culture

4a.: Principle and Totipotency

4b.: Components of tissue culture media, Sterilization techniques

4c.: Techniques in Tissue culture (Callus culture and Cell suspension)

4d.: Organogenesis and Embryogenesis

4e.: Anther culture

4f.: Applications of Plant Tissue Culture

Terminology used in PTC

- **Totipotency:**

Latin word- 'Totipotentia' means ability for all things.

"A living, nucleated plant cell which is capable of dividing, contain a complete information for the development of new plant in its genome. Under ideal conditions such cell can regenerate and develop into a complete new plant. This property of the cell is known as totipotency."

- **Callus:** It is an unorganized mass of thin walled cells or a tissue in which the cells are loosely arranged and undifferentiated.
- **Clone:** A group of plants multiplied vegetatively from a single plant is a clone.
- **Cybrid:** A hybrid which is produced by the fusion of isolated protoplast in culture media belonging to different genera or species is called as cybrid.
- **Cultivar:** The plant which is selected for the tissue culture is called as cultivar.
- **Explant:** The protoplast, cell, tissue or organ of the plant used for tissue culture is called explant.
- **Organogenesis:** Formation of organs such as root, shoot from the callus in PTC is called organogenesis.

- **Sub-culture:** Transfer of a fragment of the parent culture to a new medium is called as sub-culture.

- **Inoculation:** The sterilized material (explant) carefully transferred to the test tubes or suitable container and put on the culture medium with the help of sterilized forceps. This process of transformation of explant on nutrient medium under asepetic conditions is known as inoculation.

- **Incubation:** The process of keeping the inoculum under ideal conditions (proper temperature, light etc.) or Conditions required for the development of explant into callus the time period is known as incubation.

- **Plantlet:** The plant obtained through the tissue culture technique is called plantlet.

- **Hardening:** The introduction of tissue-cultured plantlets into the soil by gradual acclimatization technique called as hardening.

Tissue Culture / *In-vitro* culture:

"The technique of separation of cells or tissues or organs of a plant and growing them aseptically in suitable glass container on a sterile nutrient medium under controlled conditions of temperature and light is called as tissue culture."

HISTORY

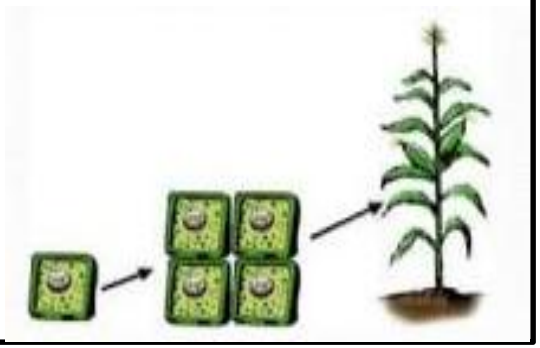
1902	The idea of the totipotency of plant cell was given by Haberlandt
1937	White first time established successful root culture of tomato
1941	Vanoverbeek used coconut milk for growth and development of young Datura embryos
1957	Skoog and Miller demonstrated the role of auxin and cytokinin on root and shoot formation in tobacco – tissue
1962	Murashige and Skoog introduced the medium for tobacco culture
1987	Isolation of Bt. gene form bacterium *Bacillus thuringiensis*

Principle of PTC

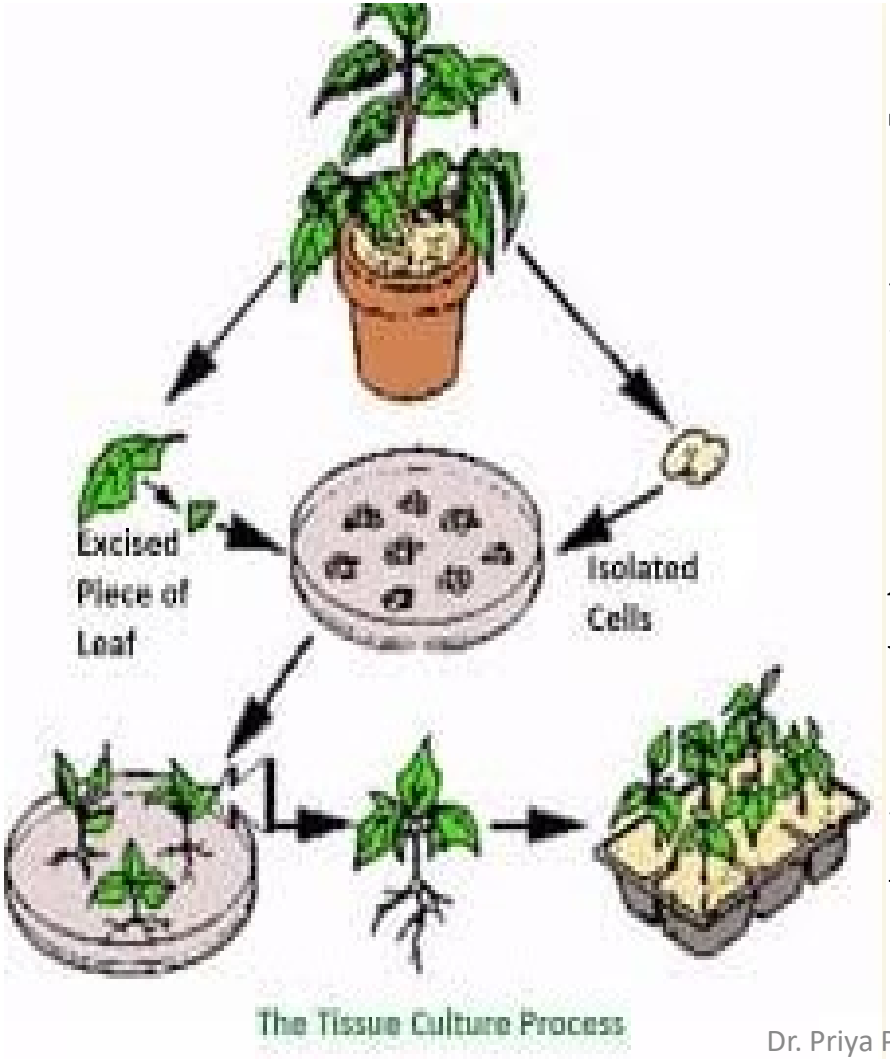
- PTC depends on-

1) **Totipotency:** It is ability of plant cells to regenerate into a whole plant.

2) **Plasticity:** It is the ability of plants to alter their metabolism, growth and development to best suit their environment.



Steps involved in PTC



- **Selection of explant:**

The explant which is selected it may either haploid or diploid explant.

- **Sterilization:**

All thermomaterials e.g. vessels, instruments, medium, plant material etc. used in culture work must be free from microbes.

Steps in PTC

Selection of explant

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Isolation of explant

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Sterilization of explant

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Inoculation of explant

↓

Incubation

↓

Initiation of callus

↓

Sub-culturing

↓

Regeneration

↓

Hardening

↓

Transfer of plantlets to Green house or open field

Factors Affecting Tissue Culture Efficiency

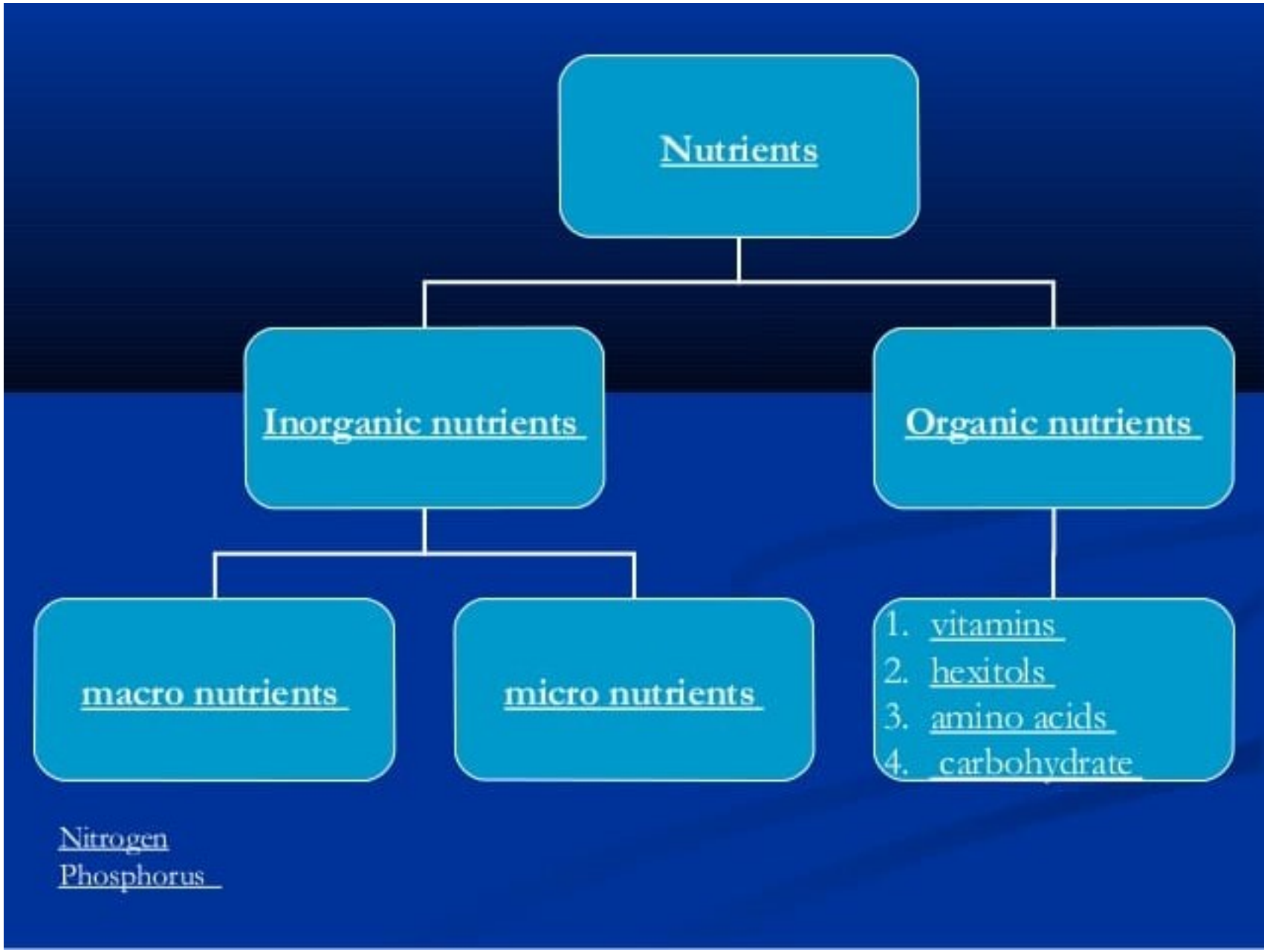
Plant regeneration from tissue culture varies with the following parameters:

- plant species,
- genotype within the species,
- source of the cultured tissue,
- age and health of the donor plant,
- nutrient medium, other factor

Plant Tissue Culture medium

• Media used in PTC contain nutritional components.
• Nutritional components are essential for growth and development of cultured tissue.
• The success of the tissue culture depends on the type of culture media used.
• Each PTC medium must contain the following essential components to support *in vitro* plant growth.
•PTC medium consists of :

i) Macro inorganic nutrients
ii) Micro inorganic nutrients
iii) Iron (as a chelating agent)
iv) Vitamins
v) Carbon source
vi) Organic nitrogen
vii) Plant growth regulators
viii) Agar (as a gelling substance)


Nutrients
Inorganic nutrients
Organic nutrients
macro nutrients
micro nutrients
1. vitamins
2. hexitols
3. amino acids
4. carbohydrate
Nitrogen
Phosphorus

i) Macro nutrients:

- Required in higher concentration.
- It is present in medium in milli molar (mM) quantities
- Macro nutrients provide both anions and cations for the plant cells.
- e.g. Nitrogen (as NO_3 and NH_4), Phosphorus (PO_4), Potassium (K), Sulphur (SO_4), Magnessium (Mg) and Calcium (Ca).
- These 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 caesin hydrolysate.
- In inorganic form used as Nitrate or ammonia.
- Nitrogen is a major component of all PTC media
- Nitrogen helps to synthesis complex organic molecule.

❖ Potassium:

- K ion present in high concentration in the cytoplasm (100-200 mM) and in chloroplast (20-200mM) .
- It is essential for maintaining 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 the cell wall.
- It helps in regulation of 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:

- Very important for energy metabolism.
- Essential element for DNA and RNA.
- Deficiency may cause delayed growth.
- Supplied as sodium hydrogen phosphate or potassium hydrogen phosphate.

❖ Magnessium:

- Essential for enzymatic reactions, energy metabolism (ATP Synthesis).
- Supplied as magnessium sulphate.

❖ Sulphur:

- Important substance.
- Deficiency inhibits protein synthesis and decreases chlorophyll in leaves.
- Supplied as magnessium sulphate and Potassium sulphate.

ii) Micro nutrients:

- Required in less concentration.
- It is present in medium in micro molar (µM) quantities
- e.g. Boron (B), Manganese (Mn), Zinc (Zn), Molybdenum (Mo), Copper (Cu), Cobalt (Co) etc.
- Used in less amount less than 30 ppm (mg/l).

❖ Zinc:

• Zn plays an active role in protein synthesis and in the synthesis of tryptophan.

• Supplied as zinc sulphate.

❖ Manganese:

- Plays an important role in the Hill reaction of Photosynthesis.
- Required in many enzymatic activities.

❖ Boron:

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

❖ Copper:

- Intermediate of the electron transport chain between photo system I and II.
- Deficiency leads to decrease in photosynthesis.
- Supplied as Copper sulphate.

❖ Molybdenum:

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

❖Iron:

- Important enzyme co-factor. Supplied in μM quantities.
- It is supplemented with chelators and complex compounds due to its solubility problem.
- Iron deficiency have severe effects on the growth and development plant cells.
- Supplied as $Na_2FeEDTA$.

Other Nutrients

1) Vitamins:

- Plant synthesis required vitamins.
- Essential for many biochemical reactions.
- Cultured cells are capable to produce 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 etc.

2) Hexitols:

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

3) 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.

4) Carbohydrate:

• Cells and tissue requires exogeneous supply of carbohydrates to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis.

• Supplied by adding sucrose.

•Concentration is 20-30 gm/l.

5) Gelling agent:

• Agar-agar is used as a gelling agent.

• Agar is a natural product of seaweeds.

• Since 1658, agar-agar is obtained from red algae (*Gelidium* & *Gracillaria*).

•With water it melts at 100ºC and solidify at 45ºC.

6) Plant Growth Regulators:

- A plant hormone can be defined as a small organic molecule that elicits a physiological response at very low concentration.
- PGR's 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 callus formation.
- Natural forms are IAA, IBA, PAA etc.
- Synthetic forms are NAA, 2,4-D.

• **Cytokinins:**

•Cytokinins promote cell division, shoot proliferation and influence the cell cycle.

• Promotes embryogenesis and inhibit root formation.

• Synthetic form is 2-ip, which is most active cytokinin.

• 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 culture media:

• pH is adjusted between 5.5 to 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.

TECHNIQUES IN TISSUE CULTURE

❖ Callus culture:

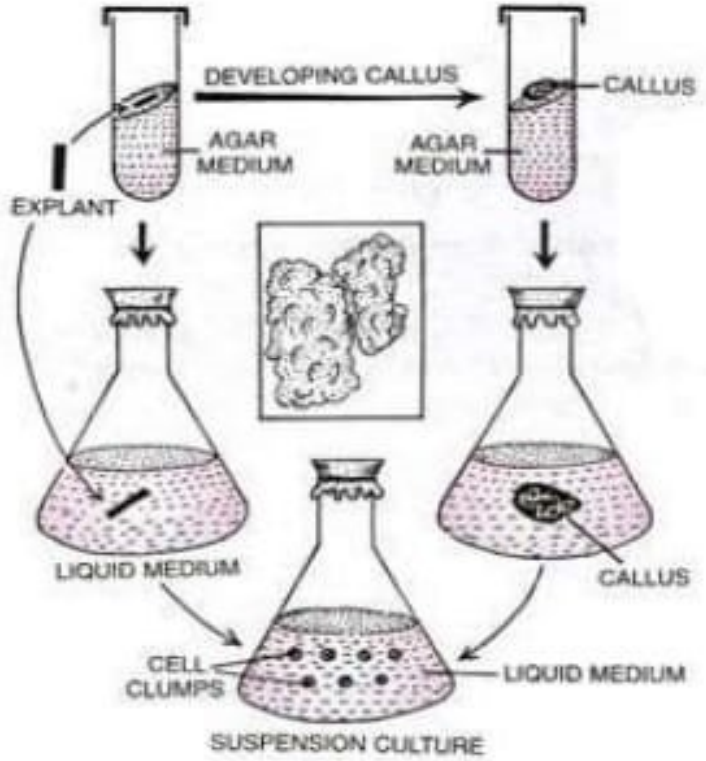
- Callus is formed by the proliferation of the parent tissue.
- Cells are parenchymatous, amorphous and unorganized.
- When tissue on culture produce unorganized mass of callus with no regular form then it is called callus culture.
- First observed by Rechinger in 1893.
- These culture need to be sub-cultured every 3-5 weeks.
- Risk free, easy to handle.

Callus Culture

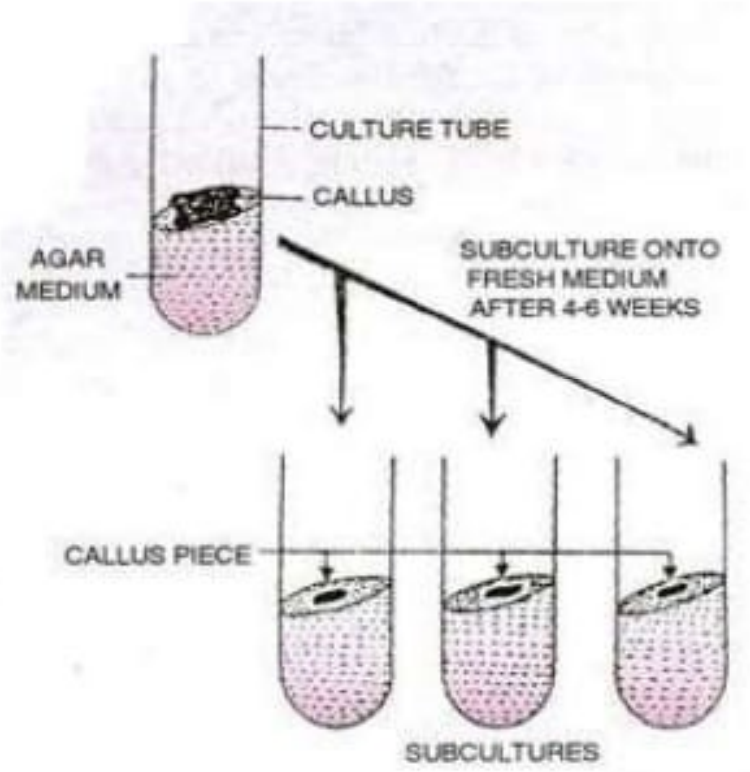
- In Callus culture, cell division in explant forms a callus.
- Callus is irregular unorganized and undifferentiated mass of actively dividing cells.
- Darkness & solid medium gelled by agar stimulates callus formation.
- The medium contains the auxins and BAP (Benzyl amino purines). Both are growth regulators (Hormones).
- This stimulates cell division in explant.
- Callus is obtained within 2-3 weeks.

Callus culture and Sub culture

1



Initiation of callus and suspension cultures.



Schematic representation of subculturing.

Maintenance

- After sufficient time of callus growth on the same medium following changes will occur :

❑ Depletion of nutrient in the medium

❑ Gradual loss of water

❑ Accumulation of metabolic toxins

- Hence for maintenance of growth in callus it is necessary to **subculture the callus.**
- Subculture should be repeated after 4-5 weeks

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)

Explant —**dedifferentiation**→ Callus —**redifferentiation**→ Organ / Embryo

Three stages of callus culture

1.Induction:

Cells in explant dedifferentiate and begin to divide

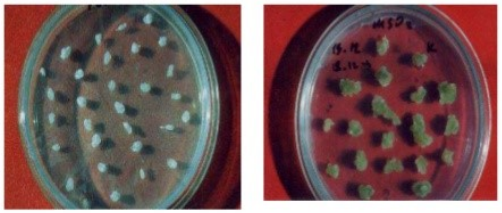
2.Proliferative Stage:

Rapid cell division

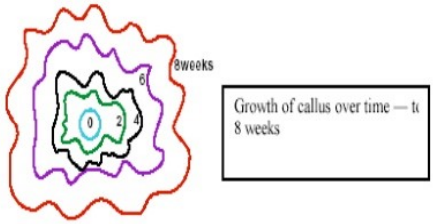
3.Morphogenesis stage:

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

1.Induction



2.Division

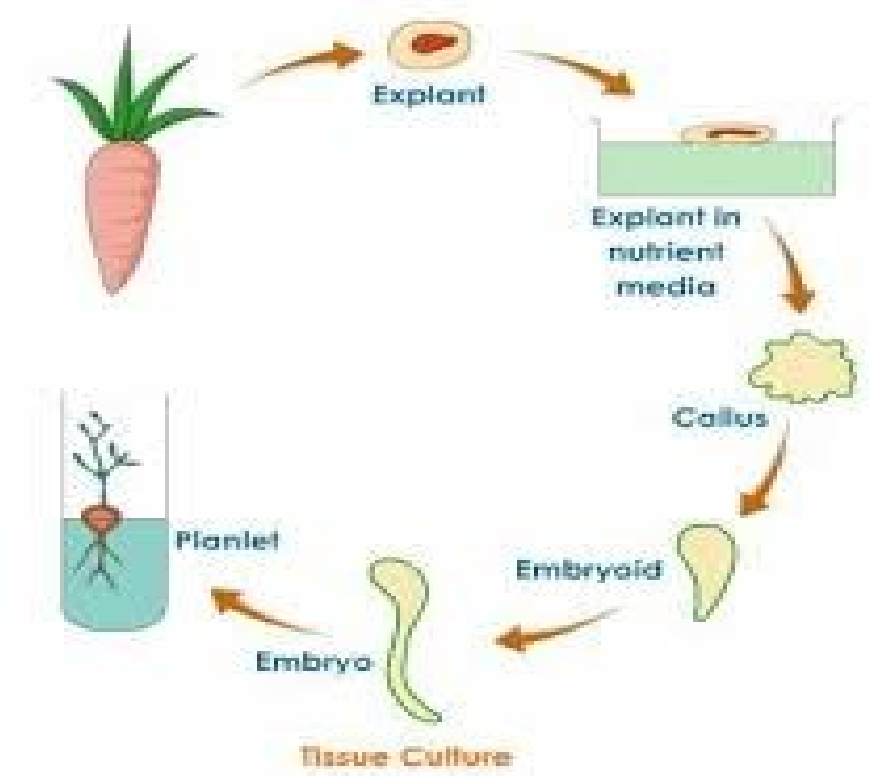


Growth of callus over time — t
8 weeks

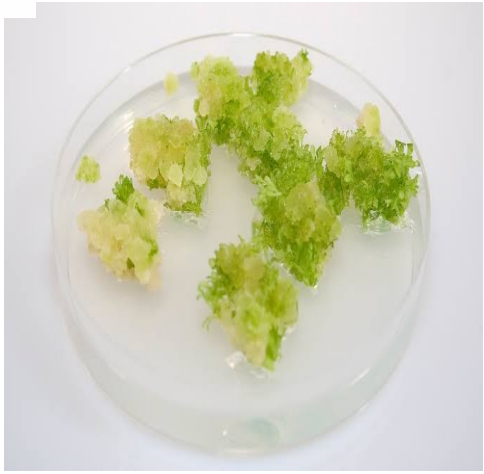
3.Differentiation

Organogenesis

Somatic embryogenesis



Callus Culture

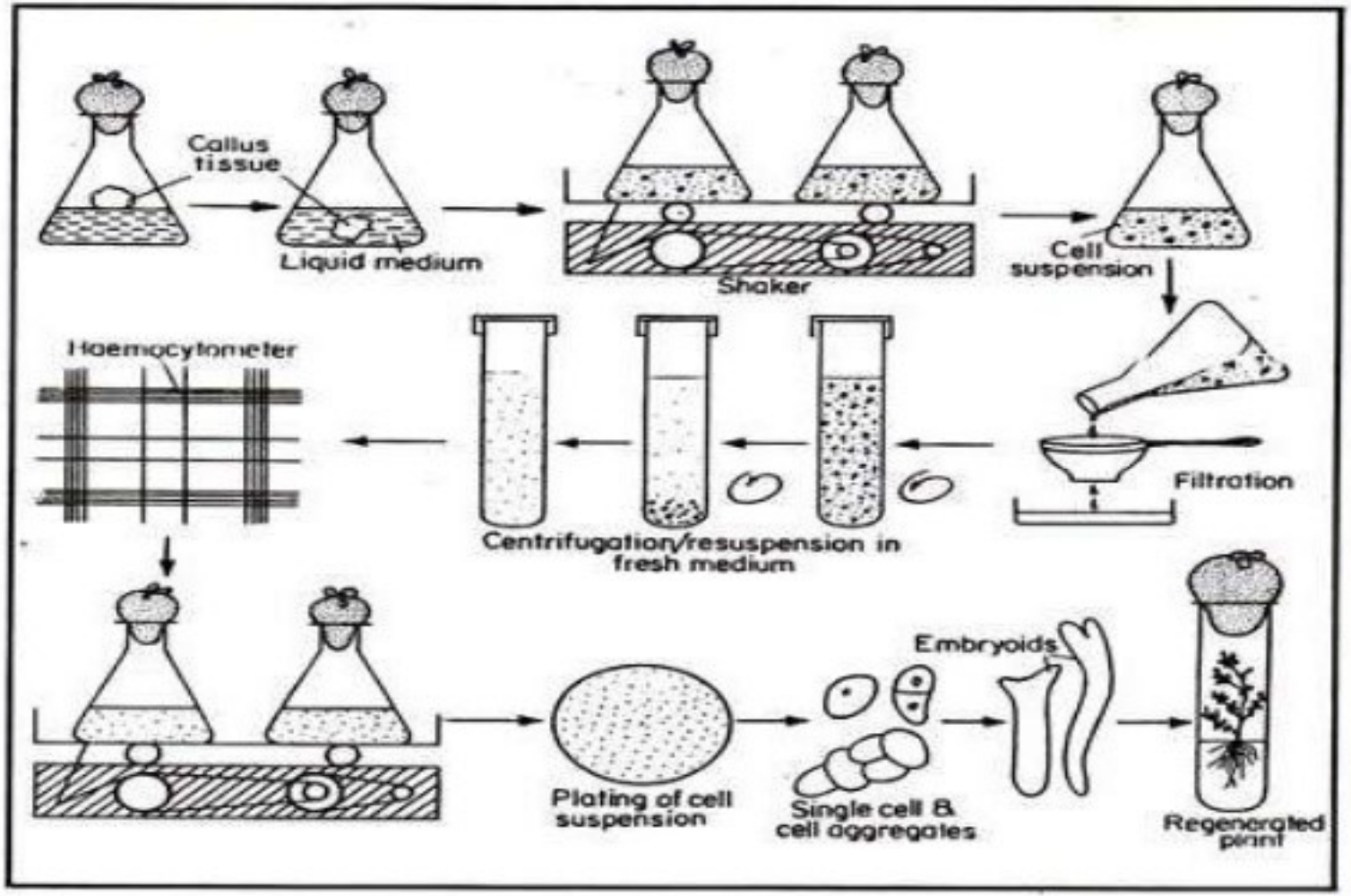


Cell suspension Culture

- 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.

SHORT HISTORY

- ***W H Muir 1953***---First Reported Fragments of Callus could be cultured in the form of Cell Suspension.
- ***Nickell – 1956***---First report of a continuously maintained cell suspension culture, *Phaseolus vulgaris*
- ***F C Steward & E M Shantz 1956***--- Suspension Culture from Root & obtained very large number of Plantlets.


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

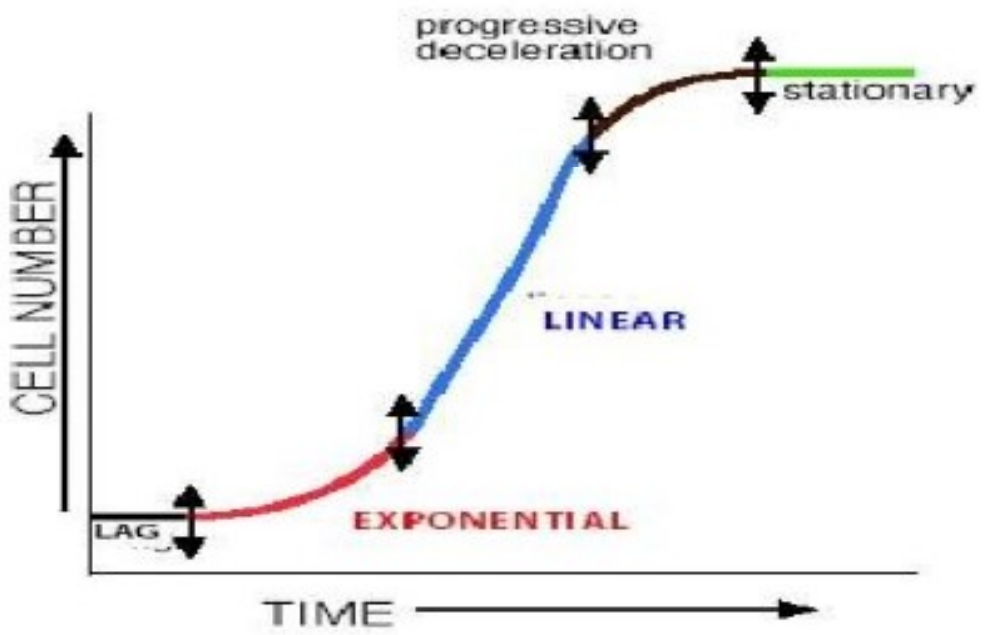
BATCH CULTURE

- A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium.
- Cell suspension increases in biomass by cell division and cell growth until a factor in the culture environnent (nutrient or oxygen availability) becomes limiting and the growth ceases.

The cells in culture exhibit the following five phases of a growth cycle

1. Lag phase : where cells prepare to divide.
2. Exponential phase : where the rate of cell division is highest.
3. Linear phase : where cell division slows but the rate of cells expansion increases.
4. Deceleration phase : where the rates of cell division and elongation decreases.
5. Stationary phase : where the number and size of cells remain constant.

Growth curve in batch culture



CONTINUOUS CULTURE

- A culture is continuously supplied with nutrients by the inflow of fresh medium but the culture volume is normally constant.
- Continuous culture is further divided into two types :

1. Close continuous culture
2. Open continuous culture

Continuous culture - culture which is replenished with medium

Closed continuous culture - fresh medium is supplied concomitant with medium harvest, however, cells are not harvested

Culture has a very extended exponential, linear and stationary growth phases

Cell viability is maintained in stationary phase; may be useful for active synthesis of secondary products, **example**

Open continuous cultures - medium input is balanced with culture (cells + medium) harvest

Growth may be maintained at any growth phase

Open continuous culture

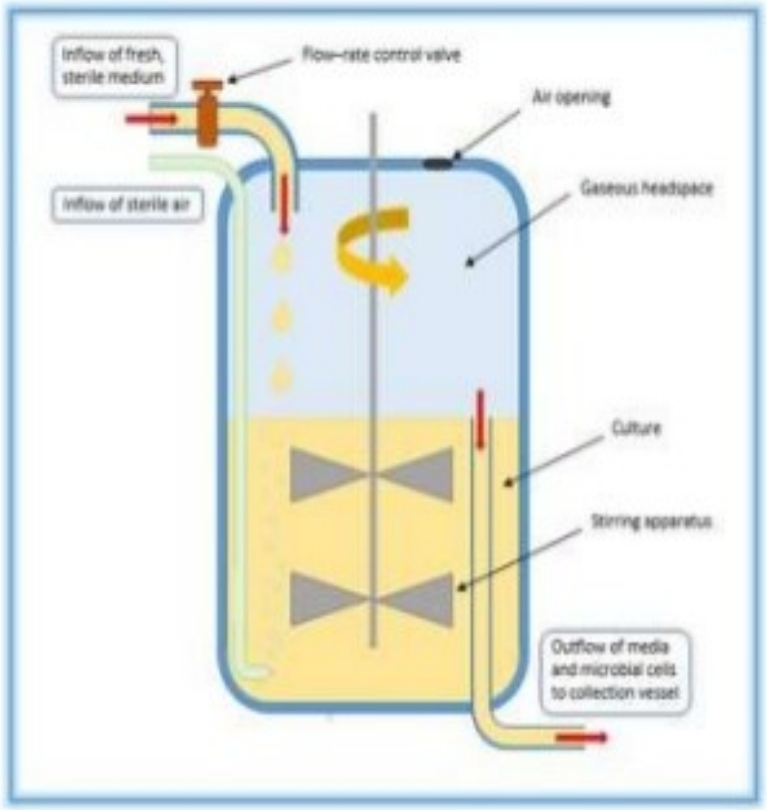
. In open continuous culture both the cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate.

. Open continuous cell suspension culture is of two types

1. Chemostat
2. Turbidostats

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



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 predetermine d volume of culture is replaced by fresh culture.
- the turbidity is measured by the changes of optical density of medium.

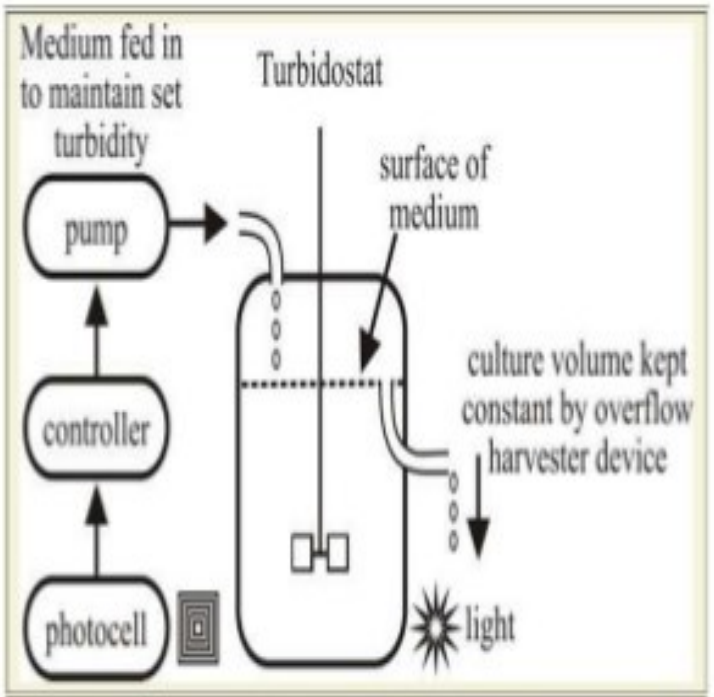
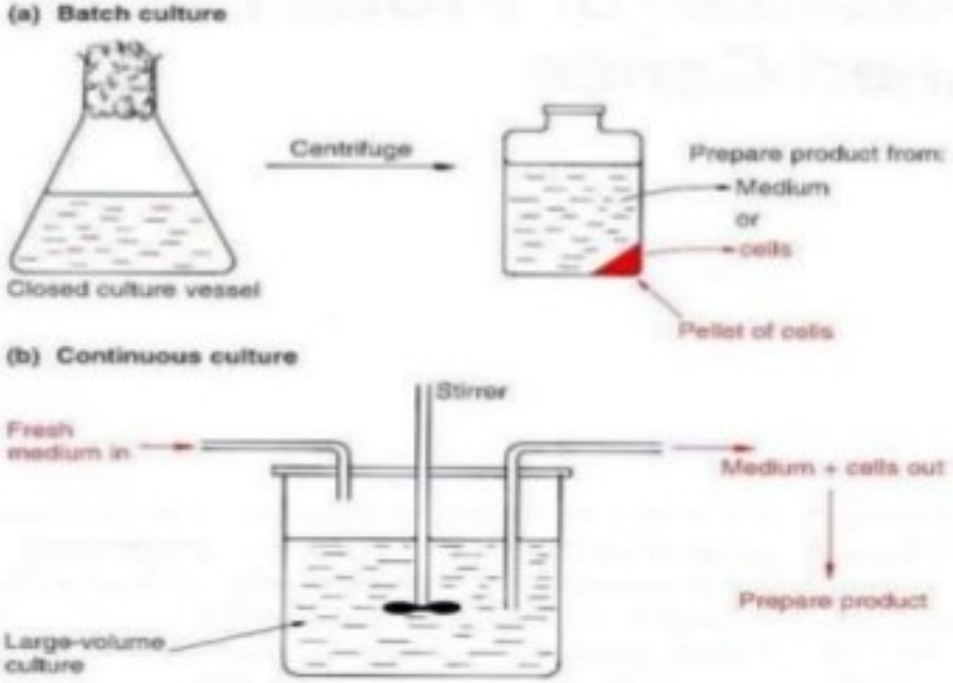


Fig. 26. Schematic of a turbidostat. Photocells measure the light transmitted through the turbid culture; when turbidity (that is, transmitted light detected) reaches a certain level, a medium pump is switched on to return the turbidity to the required level.

Batch Culture	Continuous Culture
cells are grown in a fixed volume of liquid medium in a closed vessel	nutrients are added and cells harvested at a constant rate
No microorganisms, fluid or nutrients are added or removed from the culture during the incubation period	Volume of suspension is kept constant
Used for producing secondary metabolites, such as penicillin and other antibiotics, which are relatively unstable and not essential for the growth of the culture	Fermenter does not have to be emptied, cleaned and refilled very often
Secondary metabolites can be extracted economically only when they reach a high concentration in the culture	Production is almost continuous
	Continuous cultivation needs sophisticated equipment to maintain constant conditions. Highly trained staff need to operate the equipment. Therefore this process can be expensive

Batch vs. Continuous culture



Importance of cell suspension culture

- 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.

APPLICATIONS OF PLANT TISSUE CULTURE

❖ Micropropagation

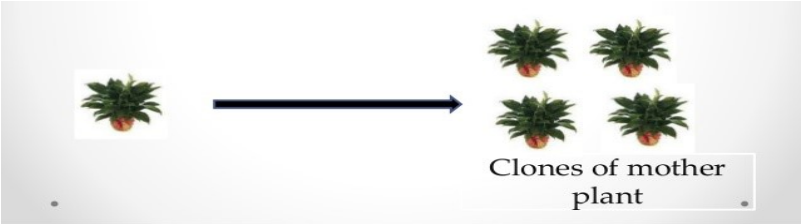
Synonyms: Mass propagation, clonning, In vitro clonal propagation

•**Definition:** Micropropagation is the rapid vegetative multiplication of valuable plant material to produce a large number of progeny plants by tissue culture.

• Use of this technique first time by Morel (1960) for orchids.

• The technique is widely used for orchids, ferns, many interior foliage plants etc.

• It is the multiplication of genetically identical individuals by asexual reproduction so called as clonal propagation.



Features of Micropropagation

- Clonal reprodution
- Multiplication stage can be recycled many times to produce an unlimited number of clones
- Easy to manipulate production cycles.
- Disease-free plants can bee produced.

Selection of plant material

- Part of plant
- Genotype
- Physiological condition
- Season
- Position on plant
- Size of explants

Steps of Micropropagation

Step 0-1: Establishment

Selection of explant.
Sterilization of the plant tissue.
Establishment in growth medium.

Step 2: Proliferation

Transfer to proliferation media.
Shoots can be constantly divided.

Step 3: Rooting & Hardening

Explants transferred to root media.
Explants returned to soil.

ADVANTAGES

- From one to many propagules rapidly.
- Multiplication in controlled lab conditions.
- Continuous propagation year round.
- Potential for disease-free propagules.
- Inexpensive per plant once established.
- Orchids have a slow rate of growth and multiplication. 10-12 years are required to develop an adult orchid from seed. By the method of micropropagation, these orchids can be propagated within short time.

- The rare, endemic and endangered plant species which are on the verge of extinction, which cannot be propagated by any other method, can be multiplied and saved by micropropagation.
- The plants which lost their ability of sexual reproduction or sterile hybrid plants can be multiplied easily by this method.
- Heterozygous hybrids which can not maintain their characters by sexual reproduction can be multiplied by the micropropagation to maintain the desirable characters.
- The plants which produces sterile seeds or non-viable seeds can be multiplied by this method.
- Large scale production of plants throughout the year n a small place. The commercial laboratory of tissue culture can produce 1 to 3 million plants per year. This is the most important advantage of this technique.

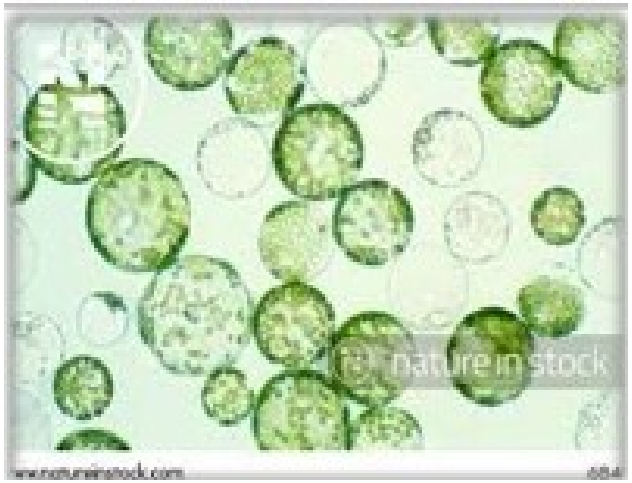
Disadvantages

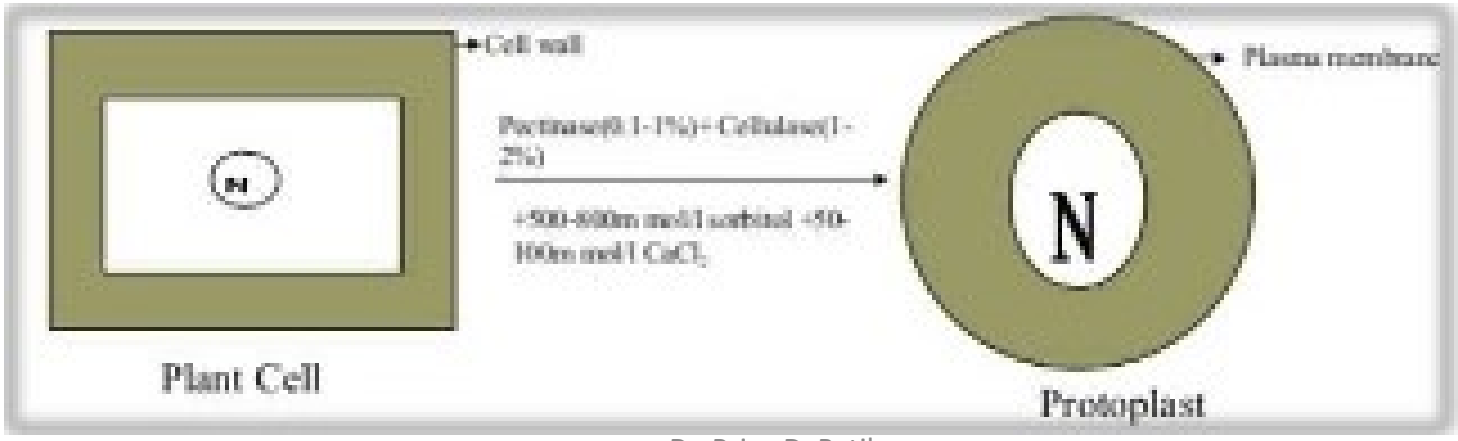
- Specialized equipment/facilities required.
- More technical expertise required.
- Protocols not optimized for all species.
- Plants produced may not fit industry standards.
- Relatively expensive to set up.

❖ Protoplast Culture

WHAT IS PROTOPLAST?

Protoplast is a plant cell that **cell wall is completely removed** by using either mechanical or enzymatic means.



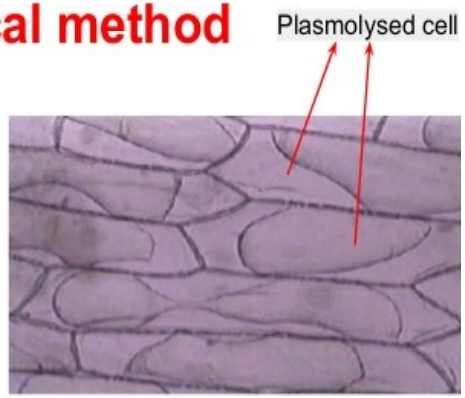


Protoplast isolation

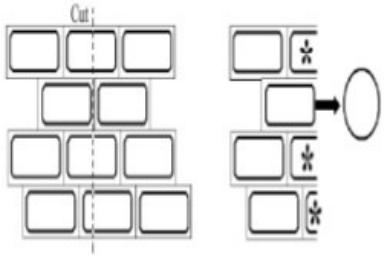
- Refers to the separation of protoplast from plant tissue
- Important to isolate viable and uninjured protoplast as gently and as quickly as possible
- Involves two methods:
  - Mechanical
  - Enzymatic

Mechanical method

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

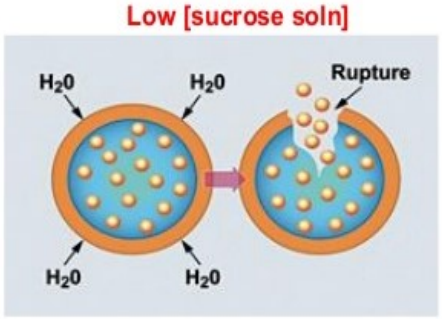


- Plasmolysed tissue is cut with a sharp knife at such a thickness that only cell walls are cut

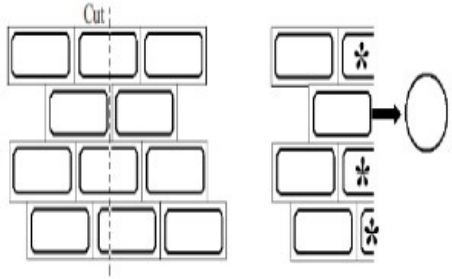


Mechanical method

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

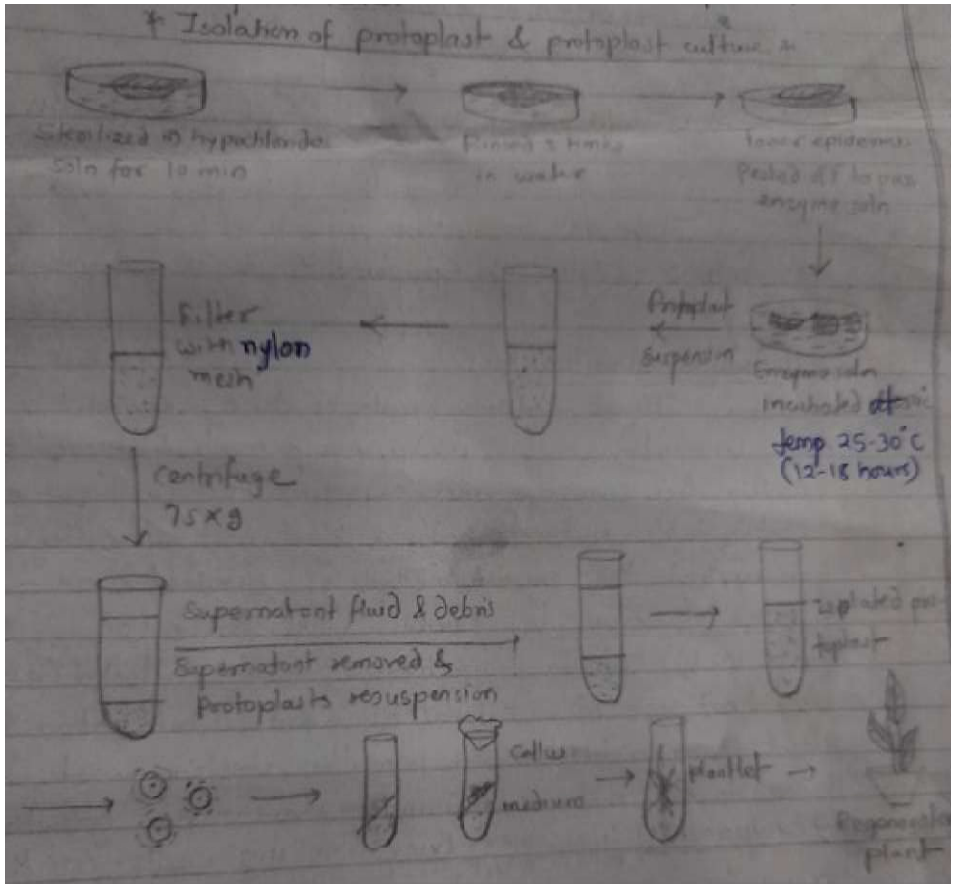


- Problem encountered: some cells release uncut complete protoplast while the rest produces broken dead protoplasts.



❖ Protoplast Culture

"Removal of cell-wall from a plant cell yields protoplast which can be regenerated into an entire plant on a suitable medium is called as protoplast culture."



Procedure of Protoplast Culture

1) Mature leaves of any suitable plant are taken and rinsed in tap water and then immersed in sodium hypochloride solution for 10 minutes.
2) After 10 min., the leaves are repeatedly washed in distilled water.
3) Lower epidermis of leaves is peeled off so that enzyme solution enters into them.
4) The leaves are then cut into small pieces and placed in a petridish containing enzyme solution having a composition of 13% mannitol, 2% cellulase and 0.5% macerozyme. Petridishes are sealed and incubated at 25ºC for 12-18 hours.
5) By aegitation the protoplasts are released in enzyme solution. Then this solution was filtered to remove cell wall debris and undigested tissues.

6) Then the filtrate is centrifuged. Due to this, protoplasts are settled at the bottom of the tube. The supernatant is discarded.

7) Isolated protoplasts are resuspended in the culture medium and the process is repeated trice. Then protoplasts are ready to culture.

8) Protoplast just after isolation is more or less spherical. It can be cultured in a MS medium supplemented with cytokinins and auxins which helps the growth of protoplasts.

9) Cultures are incubated at 25ºC in dim white light. For wall formation ammonium nitrate and calcium chloride are added to the medium. Cell wall is formed within 5-7 days.

10) The cell is then allowed to produced a callus from which new plantlets and plants can be grown.

ADVANTAGES

- The rapid multiplication of economically important plants, as single protoplast of single cell forms one entire plant. The plants obtained by this method exactly resemble their parent plant and hence best varieties obtained. E.g. Tobacco, Potato & Tomato.

- Protoplast derived from different sources can be induced to fuse and then a hybrid plant can be generated.

- Protoplasts are aminable to manipulation. Hence, they can be used for inducing and isolating mutant cell lines.

- Protoplast culture is a potent tool in the hands of Genetic engineer for further valuable researches.

- It provides cell modification experiments.

- It helps to solve many plant and cell physiological problems.

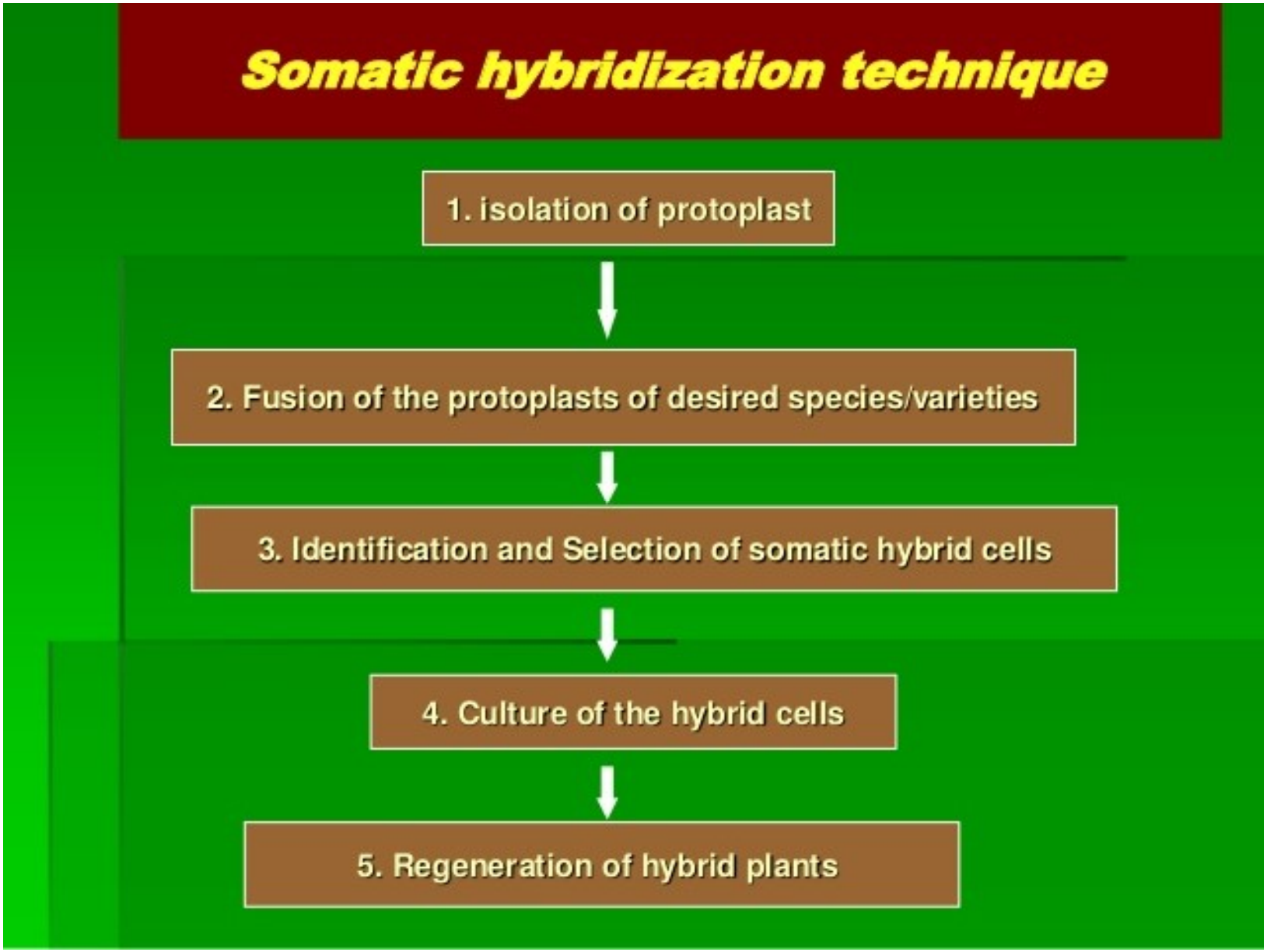
DISADVANTAGES

1) **Needed specialized expertise.**
2) **Special equipped laboratory is required.**
3) **More expensive technique.**

❖ Somatic Hybridization

Definition: "Development of hybrid plants through the fusion of somatic protoplasts of two different plant species or varieties is called somatic hybridization."

History: Kunster (1909) reported random fusion between mechanically isolated protoplasts.
Takebe (1971) obtained entire plants from hybrid protoplasts of tobacco. Later, entire plantlets were obtained by this method in citrus, carrot, Solanum & Petunia.


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

Spontaneous Fusion

- Protoplast fuse spontaneously during isolation process mainly due to physical contact
  - Intraspecific produce homokaryons
  - Intergeneric have no importance

When two or more protoplasts fuse then their cytoplasm always fuse but their nuclei may or may not fuse. Therefore, after cytoplasmic fusion the cell having dissimilar nuclei is called **heterokaryon** or **heterokaryocyte**.

If a binucleate heterokaryon one of the nuclei disappears then it is called as **cybrid** or **cytoplasmic hybrid** or **heteroplast.**

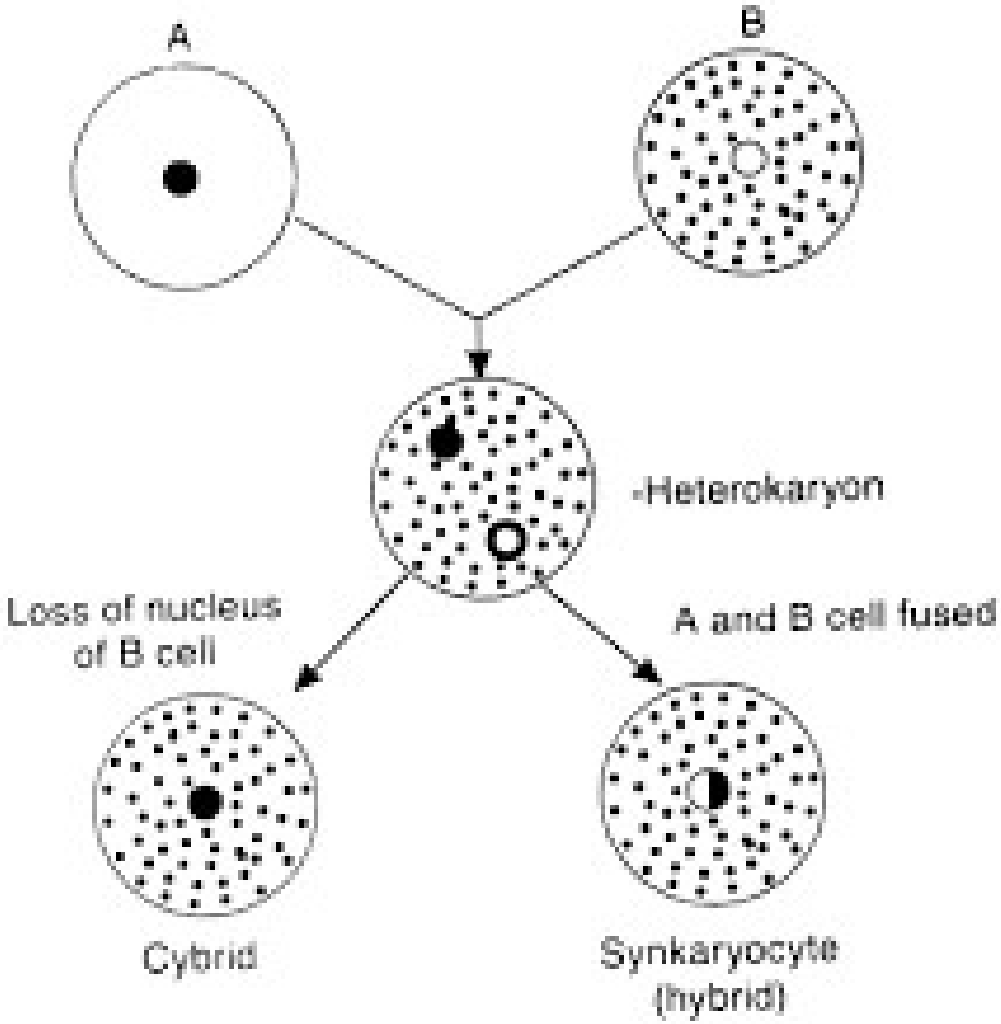


Fig.: Fusion products of two different protoplasts

Intraspecific protoplast fusion

- Intraspecific protoplast fusion is the cross between the same species
- This technique offers the only way of carrying out crosses and genetic analysis.

Interspecific protoplast fusion

- Interspecific protoplast fusion is the crosses between two different species.
- Interspecific protoplast fusions are of much importance in the area where new products are to be produced.
- Due to new genetic set up many noval secondary metabolites such as, antibiotics may be produced.

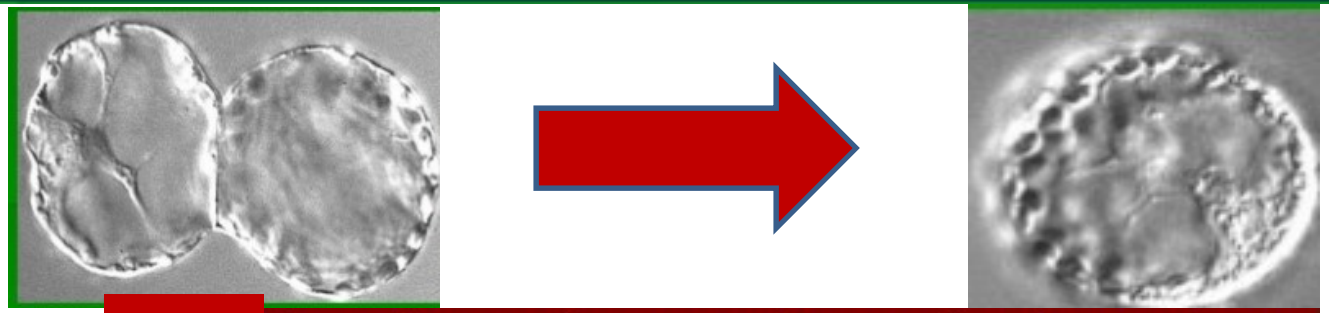


Fig.: Two tobacco plant protoplast are fused to produce a cell that acquires some of the characteristics of both parents

Advantages

1) Protoplast fusion has great significance in hybridization and plant breeding.
2) By protoplast fusion cytoplasmic male sterility can be transferred from one plant to another within the species or between two species.
3) By this method of tissue culture, development of disease resistant species is possible by induction of genome from disease resistant plants to disease susceptible plants.
4) This technique is useful in the development of heterozygous plants in vegetatively propagated species.
5) By somatic hybridization, cytoplasmically encoded characters can be transferred directly.
6) It has been considered as possible approach to modify non-legume plant to fix nitrogen.

Limitations of Somatic hybridization

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

APPLICATIONS OF PLANT TISSUE CULTURE

1.It helps in rapid multiplication of plants.

2. A large number of plantlets are obtained within a short period.

3. Plants are obtained throughout the year under controlled conditions, independent of seasons.

4.. It is an easy, safe and economical method for plant propagation.

5. In case of ornamentals, tissue culture plants give better growth, more flowers and less fall-out.

6. Genetically similar plants are formed by this method.

7. The rare plant and species are multiplied by this method and such plants are saved.

THANK YOU