PLANT CELL CULTURE

Subject: Plant Biotechnology
Sub. Code-MBT-401
Class- M.sc Biotechnology Semester-IV

Dr. Shiv Kumar Giri
Professor
Department of Biotech & Microbiology

Historical Development of Plant tissue Culture

1) Gottlieb Haberlandt (1902) - 1st attempt invitro Cultuture of isolate plant Cell. Concept of Totipotency.
2) Hanning (1904) - Embryo culture in Cruciferous Species
3) Kotte & Robbins (1922) - In vitro Culture of Root tip & Shoot tip (Meristematic tissue)
4) Prof. White (1934-39) Succesful Culture of tomato root tip. Develop PTC medium with three vitamin (Pyridoxin, thiamine, Nicotinic acid) Importance of Vitamin B.
5) Gautheret, White & Nobecourt (1939) - Establishment of Callus Culture.
6) Van Overbeek (1941) - Nutritional value of liquid endosperm of Cocomut (Embryo Culture)
7) Ball (1946) - Stem tip Culture & organ → whole plant.
8) Muir (1953-54) - Plant from Single cell (Cell Suspension culture)
9) Miller & Skoog (1957) - Hormonal Control (Auxin & cytokinin) of organ formation.
10) Reinert & Steward (1959) - Somatic Embryogenesis (carrot suspension culture)
11) Cocking (1960) - Protoplast Isolation. Bergman - Single Cell cloning
12) Murashige & Skoog (1962) - Develop medium for Callus.
13) Guha & Maheshwari (1964) - 1st haploid plant (anther Culture)
14) Power (1970) - Protoplast fusion (Inter & Intra specific fusion)
15) Takabe (1971) - Plant from Protoplast culture.
16) Carlson (1972) - Interspecific Somatic hybrid by protoplast fusion (Nicotiana
17) Chilton et al (1977) - Integrated Ti Plasmid in plant. Horsh (1984) - Transgenic Tobacco Plant

Nutritional Requirement of Plant tissue Culture

Composition of Nutrient media depend on → Particular species of Plant
→ Part used

↓
Determine Succesful establishment & growth of Plant Cell invitro.

1) **Inorganic Nutrient** – Inorganic Salts

- Macro Nutrients (>30 ppm / >0.5 mmol/L): N, P, K, Ca, Mg, S
  - Mg → Part of Chlorophyll Cofactor
- Micro nutrients (<30 ppm): Fe, Mn, I, B, Mo, Ni, Cu, Zn
  - Part of Certain Enzyme (Ni – urease Synthesis)

2) **Carbon Source** – + in form of Carbohydrate. Sucrose, Glucose frequently used. Other – Lactose, Maltose, galactose, Starch (Scarcely used)

3) **Organic Nutrient** –

a) Vitamin – Normal growth & development, as Catalyst. Thiamine (B_1) required by all Cell for growth. Nicotinic acid (B_3), pyridoxin (B_6) Calcium pentothanate (vit B_5). Biotin, riboflavin ascorbic acid, vit E folic acid.

b) Amino acid – Substitution or augmentation of Nitrogen Supply Glycine, arginine, cystein, Glutamine tyrosine.

c) other organic supplements – Natural Substance or Extract. Coconut milk, Corn milk, Yeast & malt extract, Organ juice & tomato juice.

5) **Plant Growth Regulator**

Auxin – Cell division & growth, root formation (IAA, IBA, NAA, 2,4-D)
Cytokinin – Cell division. Commonly used. Benzylamino purine, kinetin, Zeatin) Shoo formation
Gibberellin, Abscissic acid, Ethylene

6) **Solidifying agent** – Agar (0.5-1

Optimum pH – 5.6-5.8

Types of Plant Tissue Culture

A) **on the basis of Explant used**

1) Seed Culture
2) Meristem / Shoot tip Culture
3) Root tip Culture
4) Leaf / Leaf primordia Culture
5) Complete flower Culture
6) Bud Culture
7) Embryo Culture
8) Anther and pollen
9) Ovary Culture
10) Protoplast Culture
11) Hairy root Culture

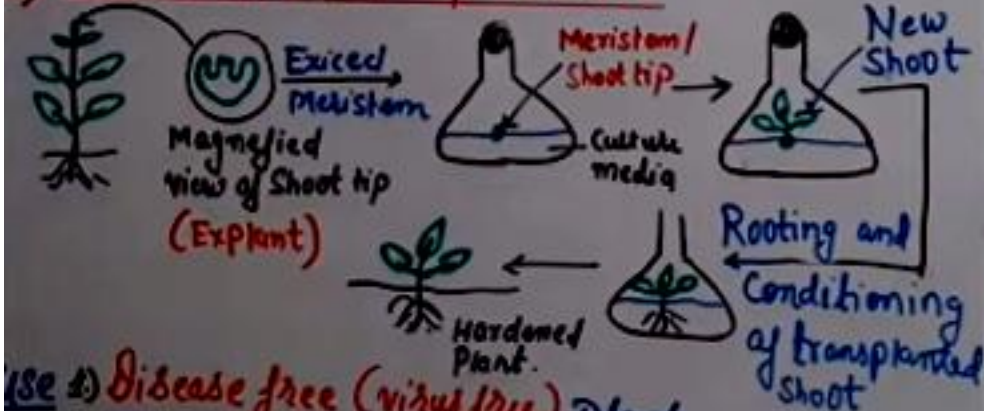
B) **on the basis of type of invitro growth**

1) Callus Culture 2) Suspension Culture

1) Seed Culture :-

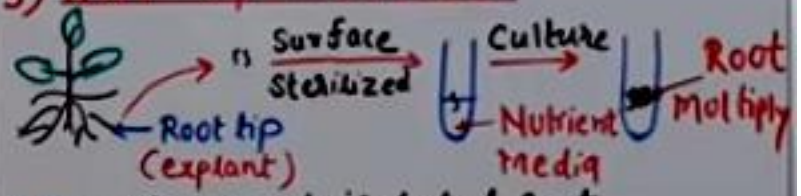
use 1) ↑ Efficiency of germination of Seed.
2) Clean Seedling for Explant / Meristem Culture

2) Meristem / Shoot tip Culture



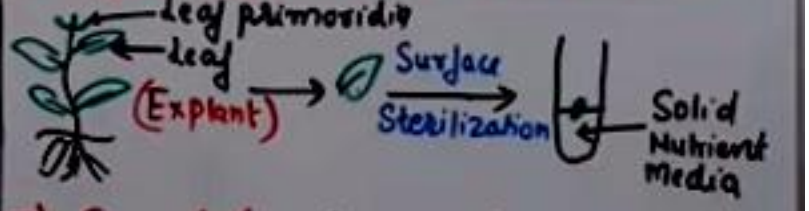
use 1) Disease free (virus free) Plant.
2) Micropropagation
3) Germ plasm Conservation.

3) Root tip Culture :

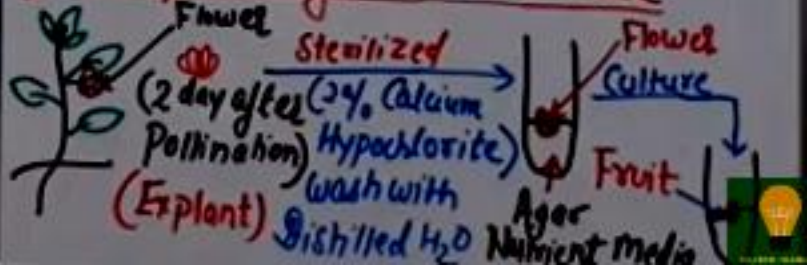


use - Clones of isolated root
Production of virus free Germplasm

4) Leaf / Leaf Primordia Culture

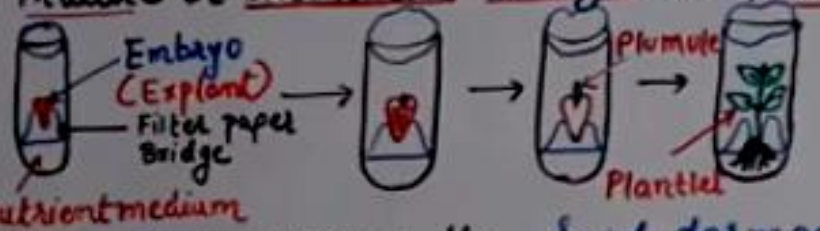


5) Complete flower Culture



Types of Plant Tissue Culture

6) **Embryo Culture** – Culture of Excised Mature or immature embryo into plantlet

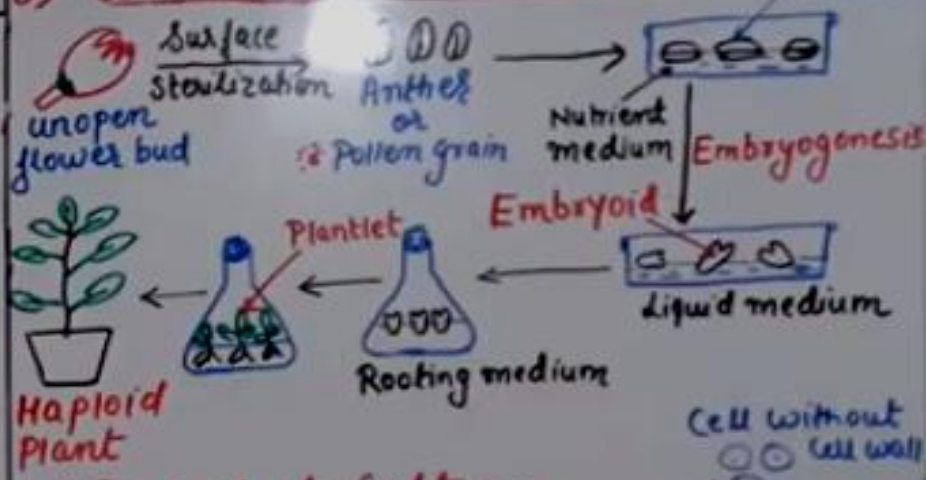


uses – 1) Overcome the Seed dormancy
2) Embryo Rescue (overcome the Embryo immaturity.
3) Disease free plant.

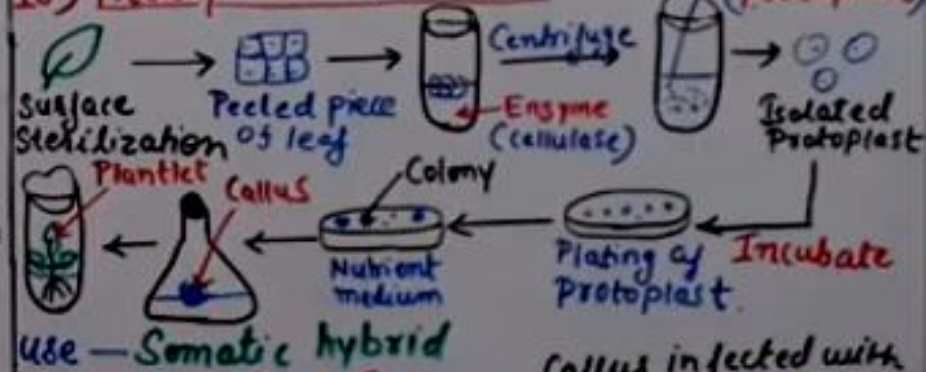
7) **Bud Culture** – Axillary bud (contain active meristem) develop in plantlet.

8) **Ovary Culture** – Culture of unfertilized ovary to obtain haploid plant.
Uses – 1) Production of Haploid plant.
2) Studies on fruit Development
3) Preferred Explant – Somatic Embryogenesis

9) **Anther / Pollen Culture**

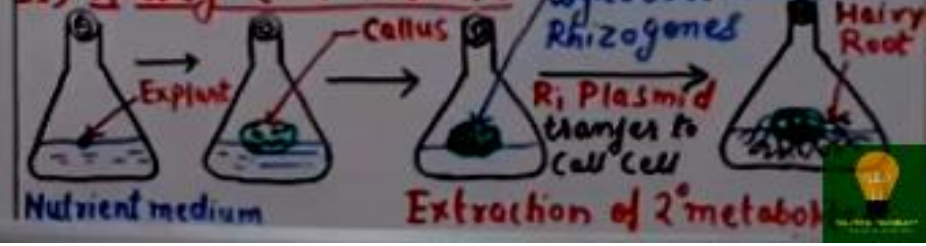


10) **Protoplast Culture** –



use – Somatic hybrid

11) **Hairy Root Culture** – Callus infected with Agrobacterium Rhizogenes



Types of Plant Tissue Culture

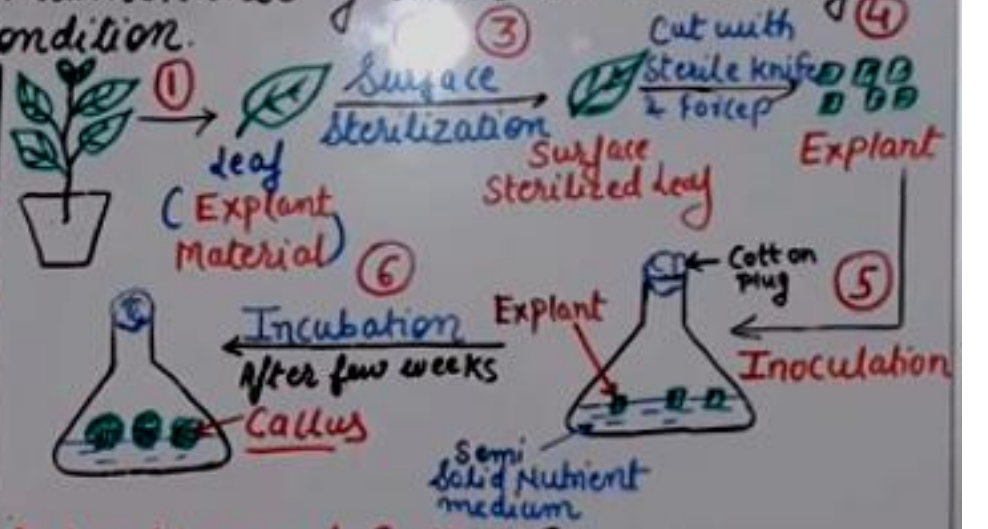
Callus Culture - Production and maintenance of Callus on a Solidified Nutrient medium under aseptic condition.

Steps of Callus Culture

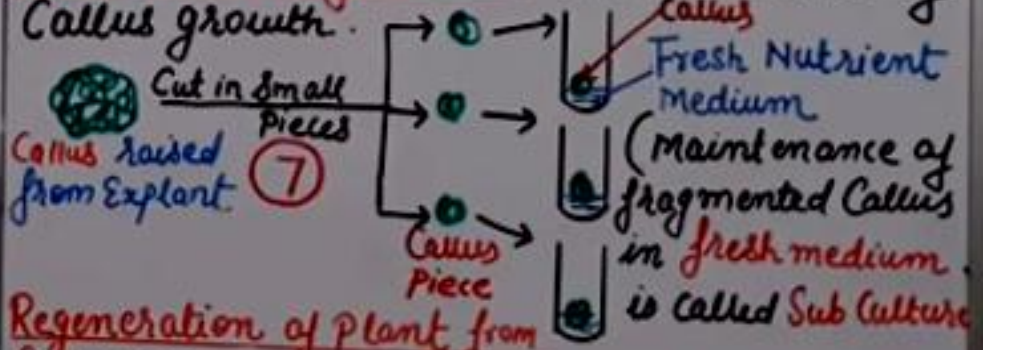
1) Selection and Collection of Explant material
2) Selection and preparation of Culture media.
3) Surface Steritization of Explant material.
4) Preparation of Explant.
5) Inoculation (transfer) of Explant in Culture medium.
6) Incubation of Culture
7) Subculture of Callus.
8) Regeneration of Plant from Callus.
   a) Organogenetic Method
   b) Embryogenesis

Stage of Development of Callus

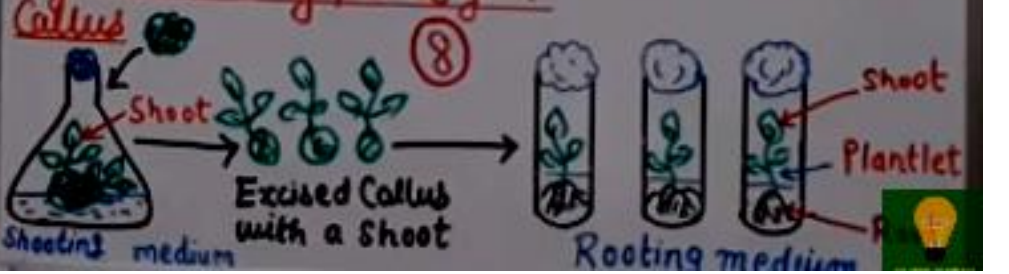
Induction → Cell division → Cell Differentiation



Subculture of Callus (Maintenance of Callus growth.



Regeneration of Plant from Callus



Types of Plant tissue Culture

Suspension Culture – Single cell or small aggregates of cells multiply while suspended in agitated liquid medium. Uniform suspension of seperate cells in liquid medium.

Preparation of Suspension Culture

Isolation of Single Cell → ① Callus Culture
② Plant organ

Plant part → Explant
② Mechanical (grinding) / Enzymatic method (Macerozyme) → Plant cell Suspension

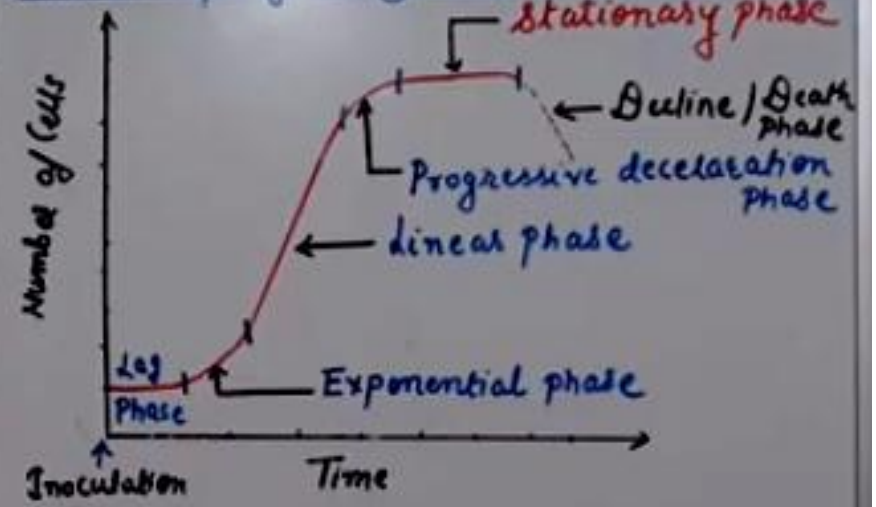
① Explant → Agar medium → Callus → Liquid Nutrient medium → Shaker → Plant cell Suspension

Plant cell Suspension → Filtration → Centrifugation / Resuspended in to fresh medium

Centrifugation / Resuspended in to fresh medium → Subculturing with fresh liquid medium → Agar medium → Plating of Cell Suspension → Cell colonies → Selection of More productive Clone → Plant regeneration

Bioreactor → Extraction of 2° metabolite

Growth Phase of Suspension Culture



Maintenance of Suspension Culture

1) **Batch Suspension Culture** – More amount of Nutrient medium is added to original Culture of Cell are transfered to fresh medium (close System.)

2) **Semicontinuous Suspension Culture** – Periodic removal of Culture & addition of fresh medium.

3) **Continuous Suspension Culture** – Volume of Culture remain constant. Fresh medium and Culture are continuesly added and withdrawn respectively.

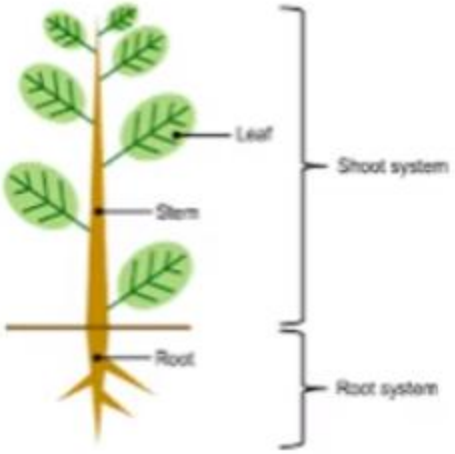
Culture of isolated single cell

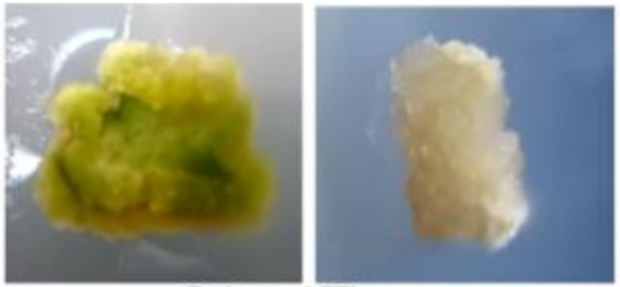
- Single cells can be isolated from plant organ or from callus.
- They can be cultured by the following methods:

1. Filter paper raft / Nurse tissue technique
2. Microchamber
3. Microdrop Method
4. Bergman's plating technique

Isolation Of Single Cells

- **From Plant Organs**
- The most suitable material for the isolation of single cells is the leaf tissue, since a more or less homogenous population of cells in the leaves offer good material for raising defined and controlled large scale cell cultures.
- **From Cultured Tissues**
- The most widely applied approach is to obtain a single cell system from cultured tissues.
- Two methods to isolate single cells from leaf are:
- **Mechanical Method**
- **Enzymatic Method**





Cultured Tissues

Isolation of single cells from plant organs

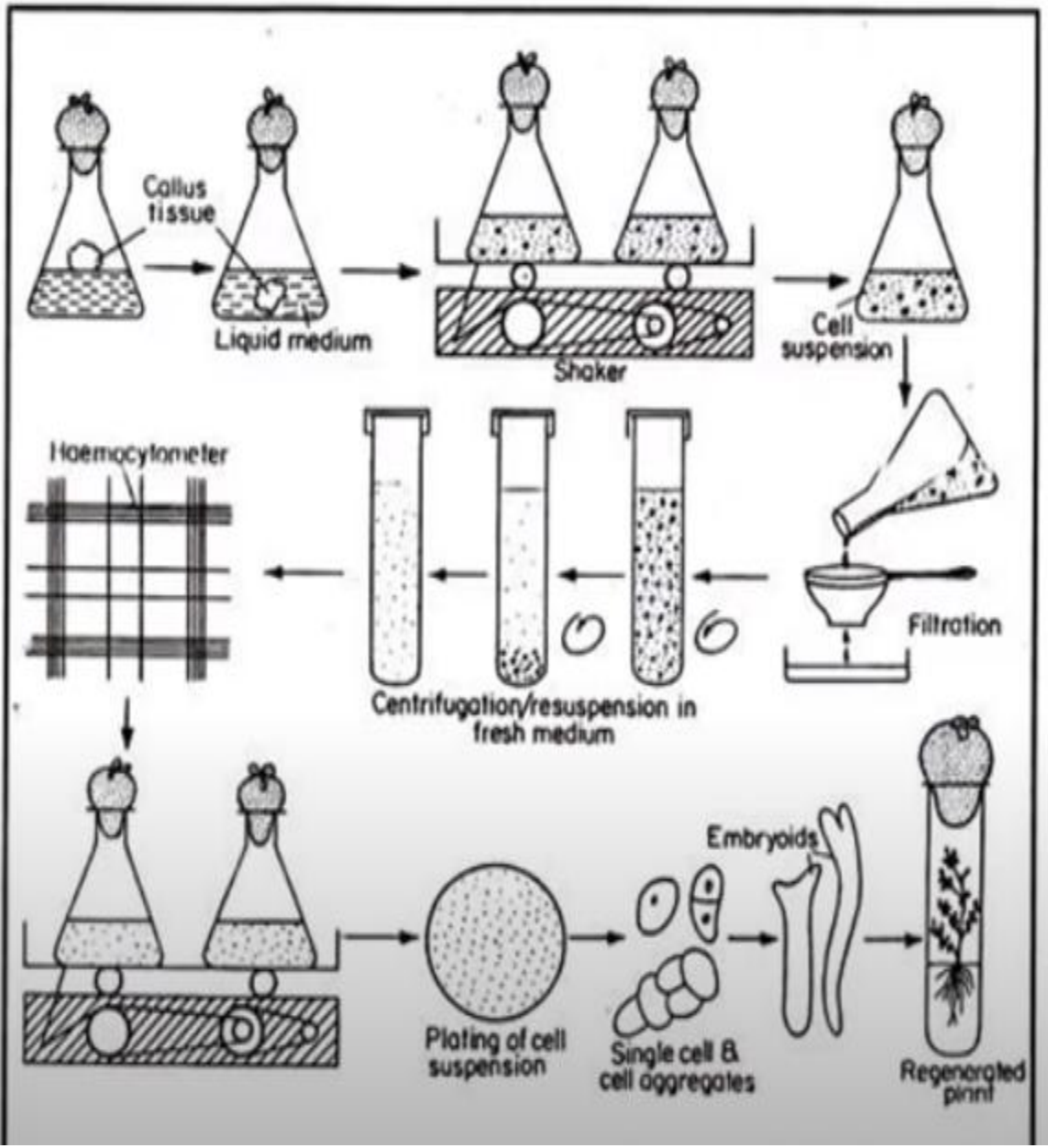
- **Mechanical Method**
- Gnanam and Kulandaivelu (1969) developed a procedure to isolate mesophyll cells active in photosynthesis and respiration, from mature leaves of several species of dicots and monocots including the grasses.
- The procedure involves:
- Mild maceration of 10g leaves in 40ml of the grinding medium (20µ mol. Sucrose, 10µ mol $MgCl_2$, 20µ mol tris HCl buffer, pH 7.8) with a mortor and pestle.
- The homogenate obtained is passed through two layers of muslin cloth and the cells thus released are washed by centrifugation at low speed using the medium.
- The mechanical isolation of free parenchymatous cells can also be achieved on a large scale.

Enzymatic Method

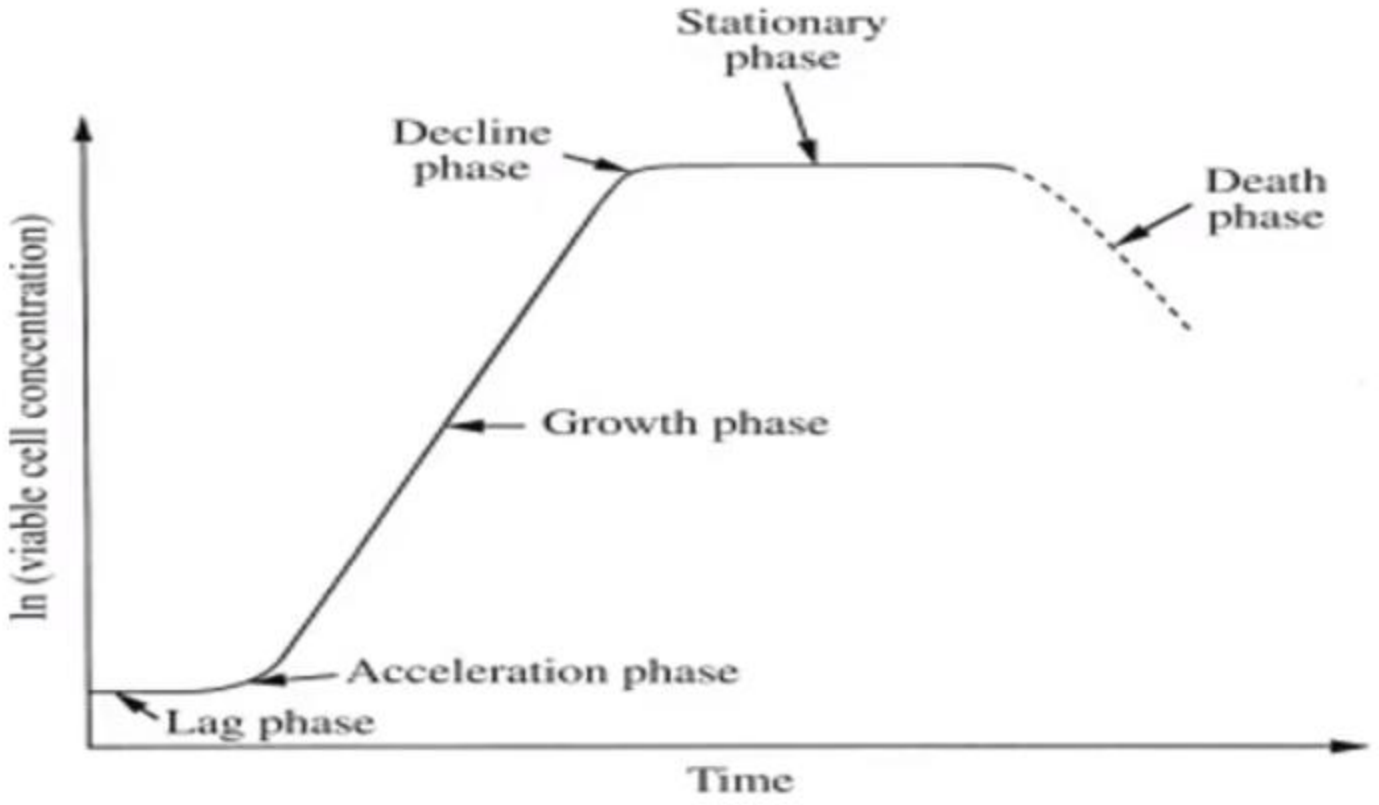
- In 1968 Takabe et al., treated tobacco leaf tissue with the enzyme **pectinase** and obtained a large number of metabolically active cells.
- **Macerozyme** degrades the middle lamella and cell wall of the parenchymatous tissue.
- The process is convenient.
- High yields can be obtained.
- With minimum damage or injury to the cells.
- This can be accomplished by providing **osmotic protection** to the cells which is obtained by adding mannitol, sorbitol etc.
- Single cells can also be obtained by suitably filtering out cell clumps and harvesting the cells by centrifugation.

FROM CULTURED TISSUES

- The pieces of undifferentiated and **friable callus** are transferred in a continuously agitated liquid medium.
- **Agitation** is done by placing the flasks on orbital platform shaker or suitable device.
- Movement of the culture medium exerts mild pressure on small pieces of tissues breaking them into free cells and small aggregates. Further it augments the gaseous exchange and also ensures uniform distribution of cells in the medium.
- The period of incubation during which the suspension cultured is developed from callus tissue is usually called as the initiation passage.
- The concentration of **auxins and cytokinins** is often critical for the growth of cell suspension culture.
- The cells in the cell suspension may vary in shapes and sizes. They may be oval, round, elongated or coiled, but they are thin walled.


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

Sigmoid Growth Curve



Measurement of Growth

- Cell Number: Counting cell number is the most reliable method, only with the condition that the suspension does not have cell aggregates and all the cells are isolated and free.
- PCV: Packed Cell volume is determined by pippeting a known volume into 1 ml graduated centrifuge tube, spinning at 200g for min and reading the volume of cell pellet, which is expressed as mlcells/1ml of culture.
- Fresh Weight: The cell mass is placed on a preweighed filter paper or nylon filter , washed with distilled water, excess water is removed under vaccum and weighed along with filter. The filter is preweighed in wet condition too.
- Dry Weight: the cells and filter are dried in an oven at 60C for 12 h and weighed. The filter is preweighed in dry condition.

Synchronous Cell SuspensionCultures

- For studying cell cycle or cell metabolism in suspension cultures, it is desirable to use synchronous or partially synchronous cultures. A cell culture in which the majority of cells proceed through each cell cycle phase (G1, S, G2 and M) simultaneously is regarded as synchronous.
- Synchronization in cell suspension cultures could be achieved by starvation or inhibition. In the former, the cells are starved of a nutrient (phosphate, nitrogen) or hormonal factor required for cell division to arrest them in G1 or G2 phase of the cell cycle. When these cells are transferred to complete medium they enter division synchronously.
- Synchronization in cell suspension cultures can also be achieved by using the inhibitors of DNA synthesis, such as 5-aminouracil, FudR, hydroxy urea, and thymidine. In the presence of these inhibitors, the cell cycle proceeds only up to the G1 phase and the cells get collected at G1/S interphase. Removal of the inhibitor from the culture medium is followed by synchronous division of cells.

Viability Check

- Tests for Viability of CulturedCells

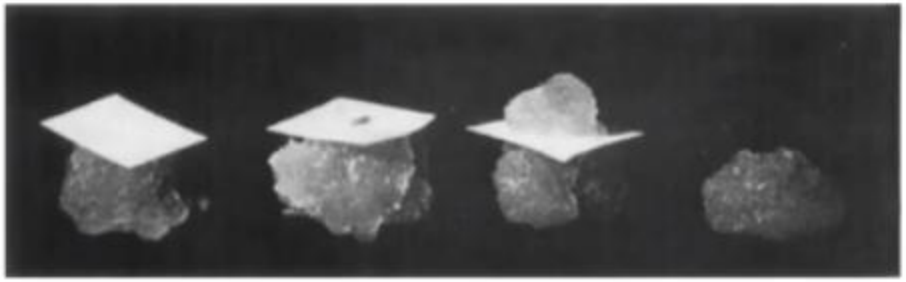
  (i) Phase contrast microscopy. Microscopic assessment of cell viability is based oncytoplasmic streaming and the presence of a healthy nucleus. While the phase contrast microscopy gives a better picture of these features, it is often not difficult to observe them under bright field microscopy.
  (ii) Tetrazolium test. In this test, the respiratory efficiency of cells is measured by reduction of2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazan which can be extracted and measured spectrophotometrically. Although this method allows quantification of observations, it alone may not always give a reliable picture of the cell viability.

Viability Check

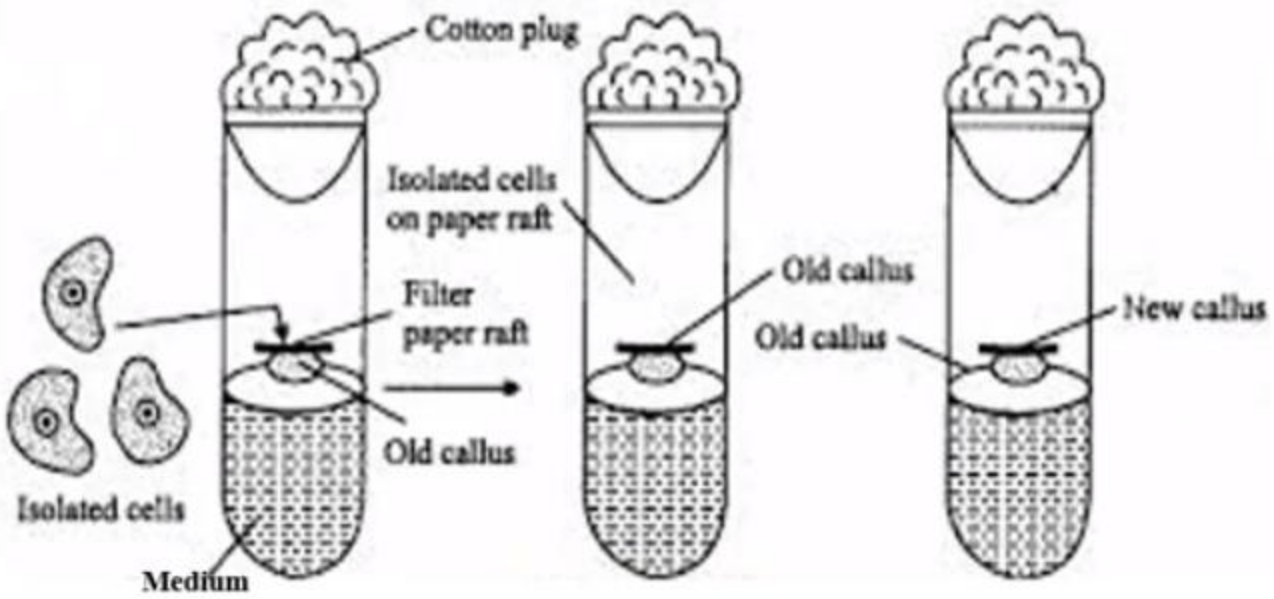
- (iii) **Fluorescein diacetate (FDA) test.** This technique offers a quick visual assessment of percentage viability of cells. Stock solution of FDA at a concentration of 0.5 % is prepared in acetone and stored at 0 C. To test viability, the solution is added to the cell or protoplast suspension (for protoplasts, an appropriate osmotic stabilizer is added to the FDA solution) at a final concentration of 0.01 %. After about 5 min of incubation the cells are examined under an epifluorescent microscope. FDA is nonfluorescent and being nonpolar, freely permeates across the plasma membrane. Inside the living cell it is cleaved by the activity of esterase enzyme, releasing the fluorescent polar portion, fluorescein, which fluoresces green under UV. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, but in dead and broken cells it is lost.
- (iv) **Evan's blue staining.** The stain can be used as complementary to FDA. When the cells are treated with a dilute (0.025 %) solution of Evan's blue the damaged cells take up the stain but the intact and viable cells exclude it, and thus remain unstained.

Special Methods to culture single cells

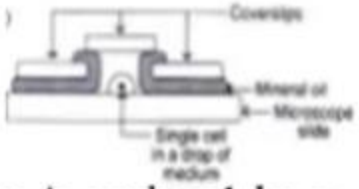
- **Filter paper raft-nurse technique**
- This method was developed by Muir et al. (1954). Individual cells were isolated from suspension cultures or a friable callus with the aid of a micropipette.
- A sterile filter paper is placed aseptically on the top of the established callus of the same or a different species. The filter paper is wetted with liquid and nutrients from the nurse tissue.
- Isolated single cell is placed on the wet filter paper raft. After a visible callus develops from the cell on the filter paper raft, it is transferred to agar medium for further growth and maintenance of the cell clone.
- The nurse-callus supplies the cells with the nutrients from the culture medium for multiplication and growth.



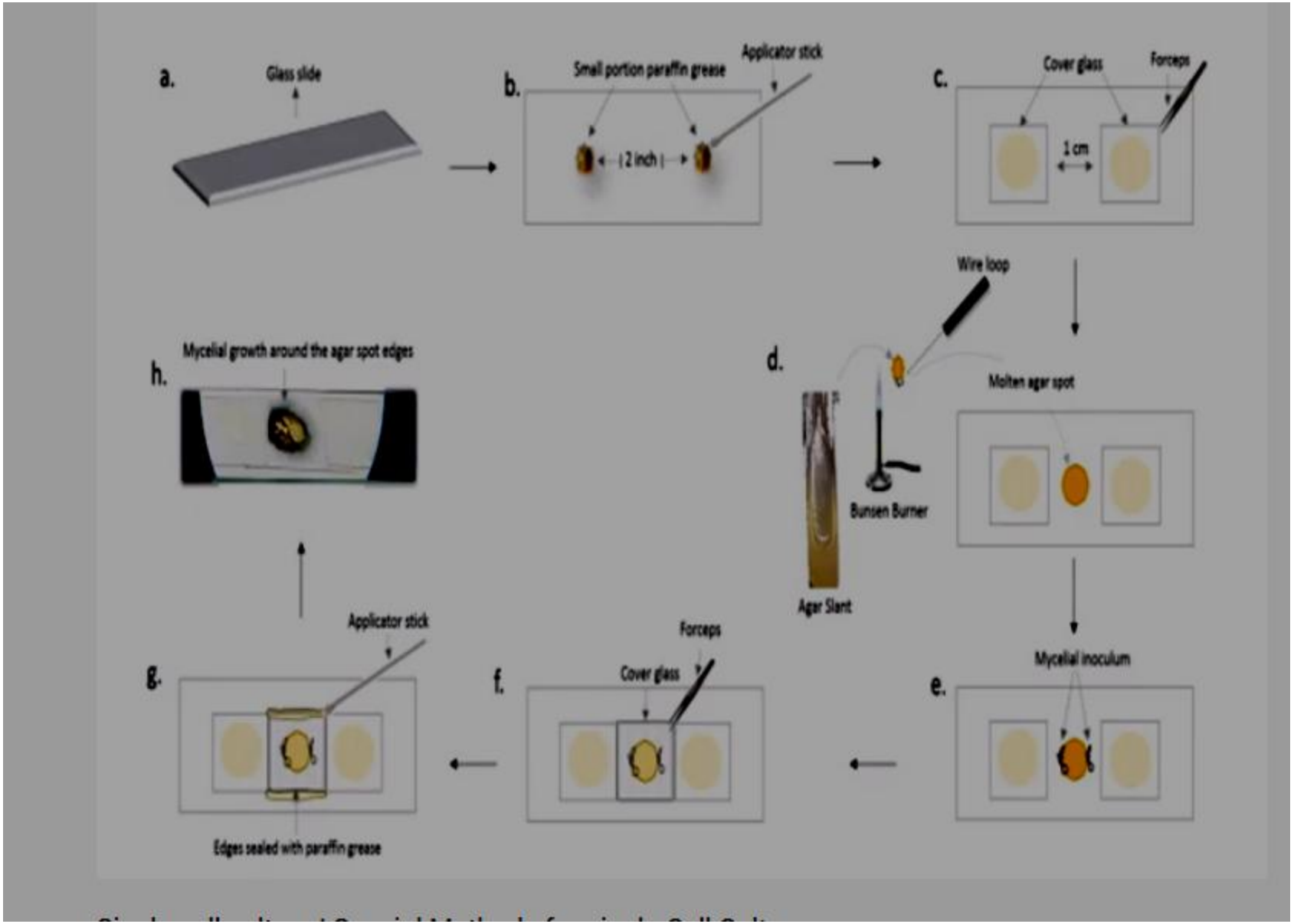
Filter paper raft-nurse technique



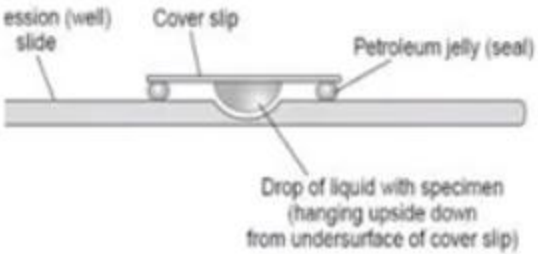
Micro chamber technique

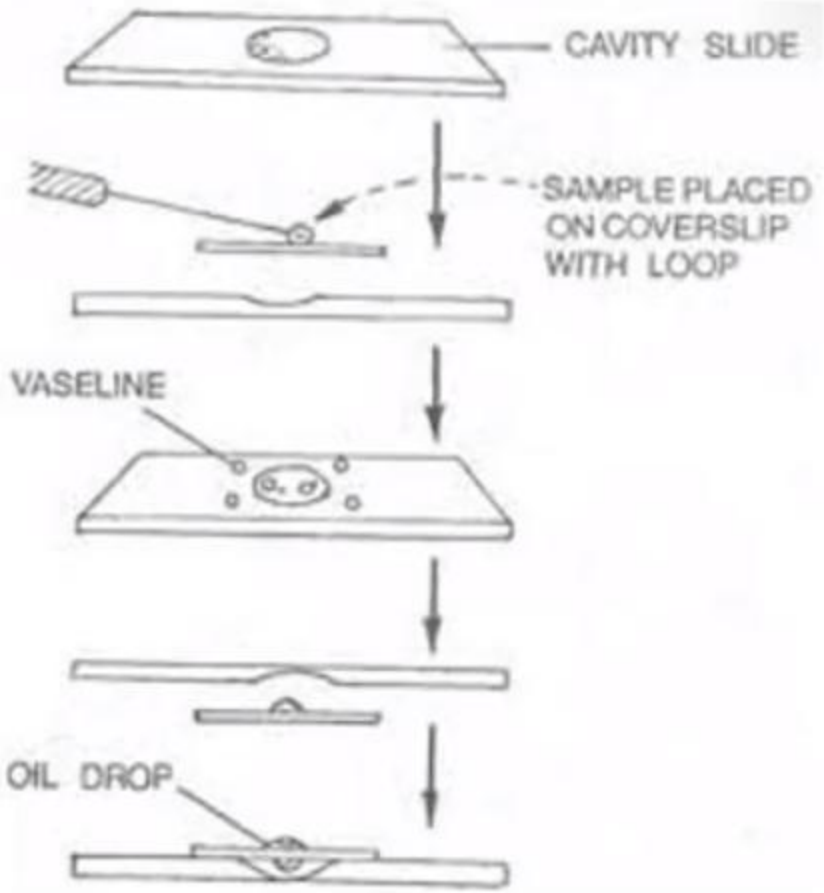


- It was devised by Jones et al. (1960), the nurse tissue is replaced by a conditioned medium, and single cells are grown in a microchamber. A drop of the medium carrying a single cell is isolated from suspension cultures, placed on a sterile microscope slide and ringed with sterile mineral oil. A drop of oil is placed on either side of the culture drop and a cover glass placed on each drop. A third cover glass is placed on the culture drop bridging the two cover glasses and forming a microchamber to enclose the single cell aseptically within the mineral oil.
- The oil prevents water loss from the chamber but permits gaseous exchange. The whole microchamber slide is placed in a Petri dish and incubated. When the cell colony becomes sufficiently large, the cover glass is removed and the tissue is transferred to fresh liquid or semi-solid medium.
- The microchamber technique permits regular observation of the growing and dividing cell.
- Using the microchamber technique Vasil and Hildebrandt (1965) demonstrated regeneration of complete flowering plants starting from an isolated single cell of tobacco.


a.
Glass slide
b.
Small portion paraffin grease
Applicator stick
(2 inch)
c.
Cover glass
Forceps
1 cm
d.
Wire loop
Molten agar spot
Bunsen Burner
Agar Slant
e.
Mycelial inoculum
f.
Forceps
Cover glass
g.
Applicator stick
Edges sealed with paraffin grease
h.
Mycelial growth around the agar spot edges

Hanging drop method

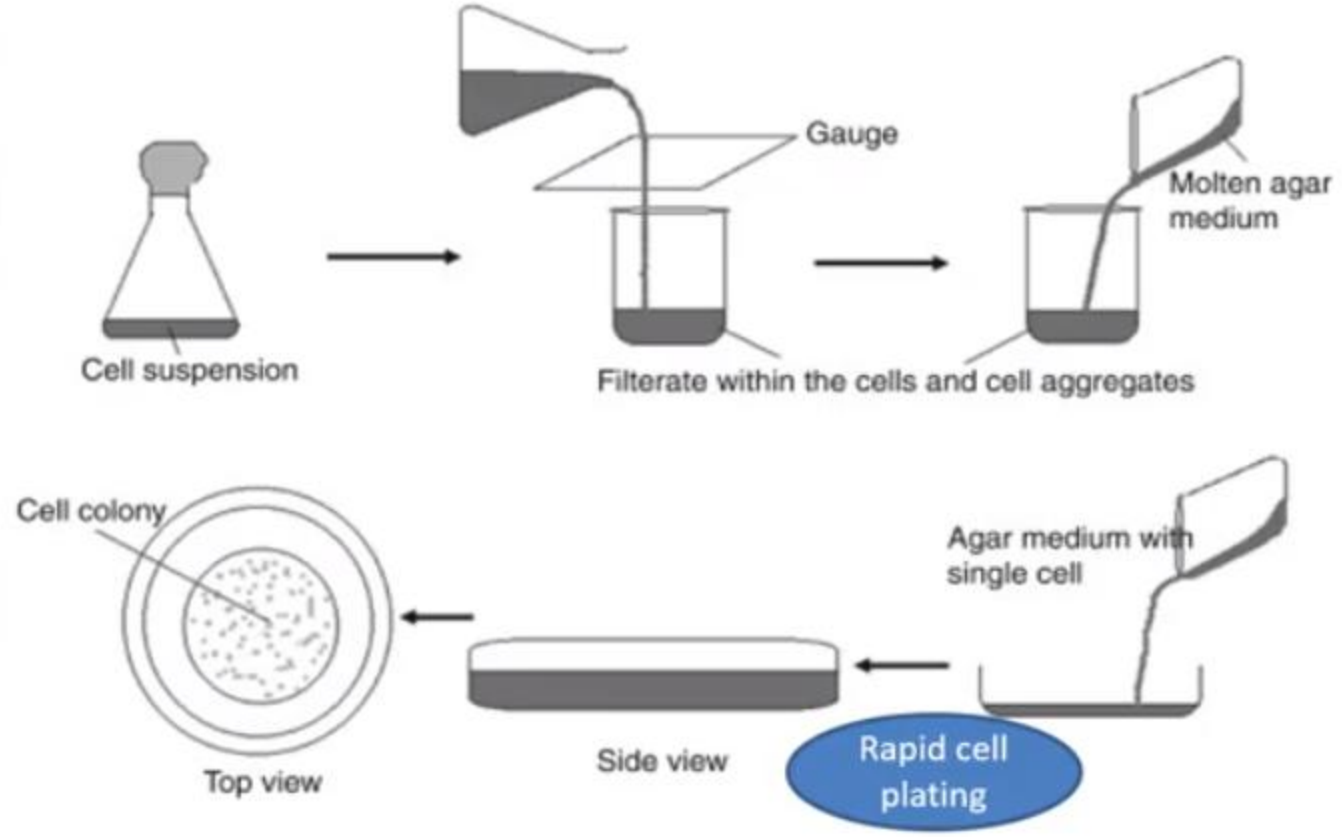




Bergmann's cell plating technique

- This is the most popular technique for single cell culture.
- Developed by Bergmann, in 1960.
- Free cells are suspended in a liquid medium at a density twice the finally desired plating cell density.
- In another vial molten agar (0.6–1 %) containing medium of otherwise the same composition as the liquid medium, is cooled and maintained at 40 C in a water bath. Equal volumes of the two media are mixed and rapidly spread out in Petri dishes in such a manner that the cells are evenly distributed and fixed in a thin layer (approximately 1 mm thick) of the medium after it has cooled and solidified.
- The Petri dishes are sealed with Parafilm.
- The culture plates are incubated at 25 °C in the dark.
- Plating efficiency : $\frac{\text{No of colonies/plate x 100}}{\text{No of cell units initially/plate}}$

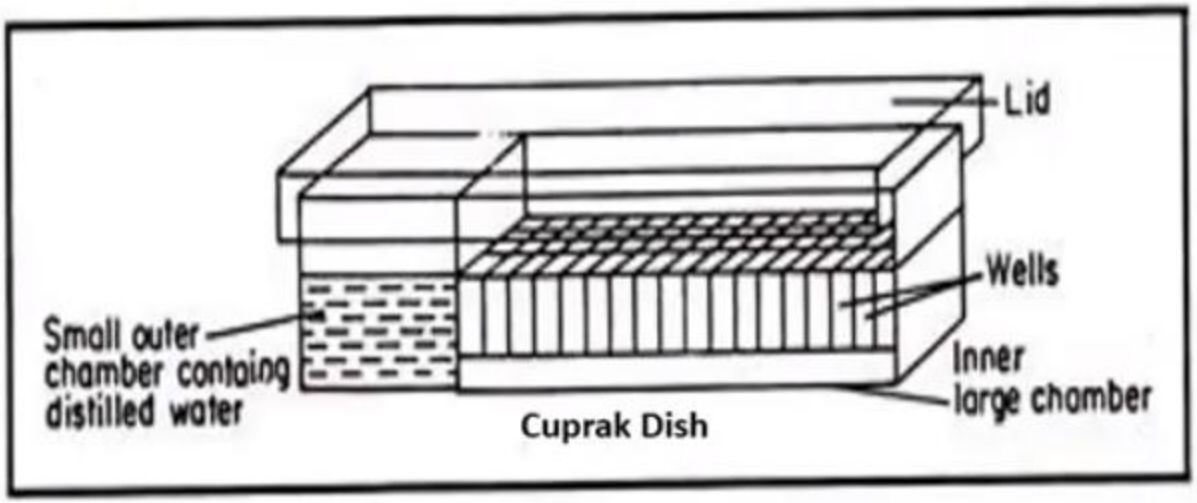
Bergmann's cell plating technique



Microdrop/Micro Well method

- This method has been especially useful for culturing individual protoplasts.
- It used a special disk called Cuprak disk which has a smaller outer chamber and larger inner chamber. The inner chamber has many wells which can have a volume ranging from 0.25 µl to 2.5 µl. It is filled with conditioned media and cell culture of specific cell density. In each well, only one cell is incubated. The outer chamber is small and filled with water to maintain humidity.

Micro drop/Micro Well Method



Factors Affecting Single Cell Culture

Composition of culture medium and the initial plating cell density are two important factors and are intricately linked.

- For the cells plated at high density a purely synthetic medium with a composition similar to that used for suspension cultures or callus cultures are generally satisfactory. At lower plating density, however, the medium needs to be supplemented with complex nutrient mixtures, such as coconut milk, casein hydrolysate or yeast extract.

Factors Affecting Single Cell Culture

- **The initial plating cell density**
- The cell density effect on cell division is based on that cells synthesize certain compounds necessary for their division.
- The endogenous concentration of these compounds should reach a threshold level before a cell step into division.
- The cells continue to lose their metabolites into the medium until equilibrium is reached between the cell and the medium. At high cell density the equilibrium is attained much earlier than at low density, and therefore the lag phase is short.
- Below a critical cell density the equilibrium is never reached and cells fail to divide.
- However, a conditioned medium, enriched with essential metabolites leached out of the cells, is able to support divisions at a fairly low cell density.

Importance Of Single Cell Culture

- To obtain single cell clones.
- Their use in embryogenesis studies.
- To study the morphological and biochemical changes during their growth and development phases.
- To understand the pathways of cellular metabolism.
- Single cell systems have a great potential for crop improvement.
- Free cells in cultures permit quick administration and withdrawal of diverse chemicals/ substances thereby making them easy targets for mutant selection.
- To produce high yielding cultures as well as plants with superior agronomic traits.

Importance Of Single Cell Culture

- Single cells derived from medicinally important plants can be studied for the production of secondary metabolites like alkaloids, glycosides.
- For mutagenesis study. The mutagens can be added directly in the liquid medium. After the mutagen treatment, cells are plated on agar medium for the selection of mutant cell clones.
- The production of recombinant proteins in plant cell suspension cultures can be achieved by transforming wild-type cells already in suspension and selecting those carrying a co-introduced marker gene, or by initiating cultures from transgenic plants.
- Cell suspensions have also proven to be excellent starting materials for the isolation of protoplasts to be used in a wide range of applications (cell fusion, genetic manipulation etc.).