RAMA UNIVERSITY

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FACULTY OF MEDICAL SCIENCES

WHAT IS SINGLE CELL CULTURE METHOD???

Single cell culture is a method of growing isolated single cell aseptically on a nutrient medium under controlled condition

PRINCIPLE

- The basic principle of single cell culture is the isolation of large number of intact living cells and cultures them on a suitable nutrient medium for their requisite growth and development.
- Single cells can be isolated from a variety of tissue and organ of green plant as well as from callus tissue and cell suspension.
- Single cells from the intact plant tissue (leaf, stem, root cladode etc.) are isolated either mechanically or enzymatically.

- Single cell culture could be used successfully to obtain single cell clones.
- Plants could be regenerated from the callus tissue derived from the single cell clones.
- Single cell culture are very important in relation to crop improvement programmes.
- Single cell culture is an ideal system for the study of biotransformation.
- single cell culture in large-scale could become a valuable technique for industrial production of such important natural compound.

ISOLATION OF SINGLE CELLS

This can be achieved from:

1. Intact organs

2. Callus

From intact organ: Mechanical and enzymatic methods are used to isolate the cells from intact organs.

- ✓ The plant part preferably leaf tissue is subjected for grinding in a mortar with pestle along with addition of grinding medium consisting of sucrose 20 µmol . MgCl2 10 µmol, Tris Hydrochloride buffer 20 µmol (pH 7-8).The resulting mixture is subjected for centrifugation and cells are taken up into a liquid media.

- ✓ The plant tissue is treated with an enzyme known as macerozyme along with 1% potassium dextran sulphate, which causes the digestion of middle lamella there by liberating the cells.

- ✓ The enzyme also causes the weakening of cell wall thus creating the problems associated with osmosis. Hence the cells are provided with osmotic protection with a substance known as osmoticum.

- ✓ Commonly used osmoticums are sorbitol 450-800 mmol/l, KCl 335 mmol/l, Magnesium sulphate 40 mmol/l.

From Callus:

- ✓ Callus preferably friable callus upon agitation in suitable liquid media results in the dissociation of the cells from callus.

- ✓ The agitation will also helps in uniform distribution, breaking up of lumps and gaseous exchange between culture air and atmosphere.

FACTORS AFFECTING SINGLE CELL CULTURE

1. The composition of the medium for the growth of single cell culture is generally more complex than callus and cell suspen-sion culture. For example, Convolvulus cells require a cytokinin and amino acids that are not necessary for the callus culture of that species.

2. Induction of division of single cells using pa-per raft technique indicates that isolated cells get the exact essential nutrient from the callus mass. It has been suggested that the callus mass leaches out the essential nu-trient through plasma membrane of the cells.

3. In case of petri dish plating technique the initial plating cell density is very critical.

IMPORTANCE OF SINGLE CELL CULTURE

Single cell culture technique is very impor-tant for the fundamental and mutation studies and it has a wide industrial application.

1. Single cell culture could be used successfully to obtain single cell clones.

2. Plants could be regenerated from the cal-lus tissue derived from the single cell clones.

3. The occurrence of high degree of spontaneous variability in the cultured tissue and their exploitation through single cell culture are very important in relation to crop im-provement programmes.

4. One of the major problems of mutation breeding in higher plants is the formation of chimeras following the mutagenic treat-ment of multi-cellular organism. In this re-spect single cell culture method are more ef-ficient. Isolated single cells can be handled as a microbial system for the treatment of mutagens and for mutant selection.

In prac-tice, single cells are grown on a medium con-taining the mutagenic compounds and the proliferating cell lines are isolated. The mu-tant nature of the selected cell lines can be confirmed by regenerating the plants and comparing their phenotypes with a normal plant. Many cell lines resistant to ammo acid analogues, antibiotics, herbicides, fun-gal toxins etc. have been selected by this simplest method.

5. Many plants synthesize various important natural compounds in the form of alkaloids, steroids etc. Some of these natural com-pounds are highly medicinally important. Several workers have reported the synthesis of several times higher amounts of alka-loid by cell culture than the alkaloid con-tent in the intact plant. So, from the com-mercial point of view, single cell culture in large-scale could become a valuable tech-nique for industrial production of such im-portant natural compound.

6. Biotransformation means the cellular con-version of an exogenously supplied subs-trate compound not available in the cell or the precursor of a particular cellular com-pound to a new compound or the known compounds in higher amounts.

DIFFERENT METHODS OF CULTURING SINGLE CELLS

1. The Paper Raft Nurse Technique
2. The Petri Dish Platting Technique
3. The Micro Chamber Technique
4. The Nurse Callus Technique
5. The Micro Droplet Technique

PAPER RAFT NURSE TECHNIQUE

1. Single cells are isolated from suspension cultures or a friable callus with the help of a micropipette or micro-spatula.
2. Few days before cell isolation, sterile 8 mm x 8 mm squares of filter paper are placed aseptically on the upper surface of the actively growing callus tissue of the same or different species.
3. The filter paper will be wetted by soaking the water and nutrient from the callus tissue.

4. The isolated single cell is placed aseptically on the wet filter paper raft.

5. The whole culture system is incubated under 16 hrs. cool white light (3,000 lux) or under continuous darkness at 25° C.

6. The single cell divides and re-divides and ultimately forms a small cell colony. When the cell colony reaches a suitable size, it is transferred to fresh medium where it gives rise to the single cell clone

Summary

The callus tissue, on which the single cell is growing, is called the nurse tissue. Actually the callus tissue supplies the cell with not only the nutrients from the culture medium but some-thing more that is critical for cell division. The single cell absorbs nutrients through filter paper. The nutrients actually diffuse upward from cul-ture medium through callus tissue and filter pa-per to the single cell. A callus tissue originating from a single cell is known as a single cell clone.

- An isolated cell which generally fails to divide when plated directly on the medium used for callus cultures is able to divide under the nursing effect of the callus.

- Apparently, the callus supplies the cell with not only the nutrients from the culture medium but something more that is critical for cell division.The cell division factor(s) can diffuse through the filter paper.

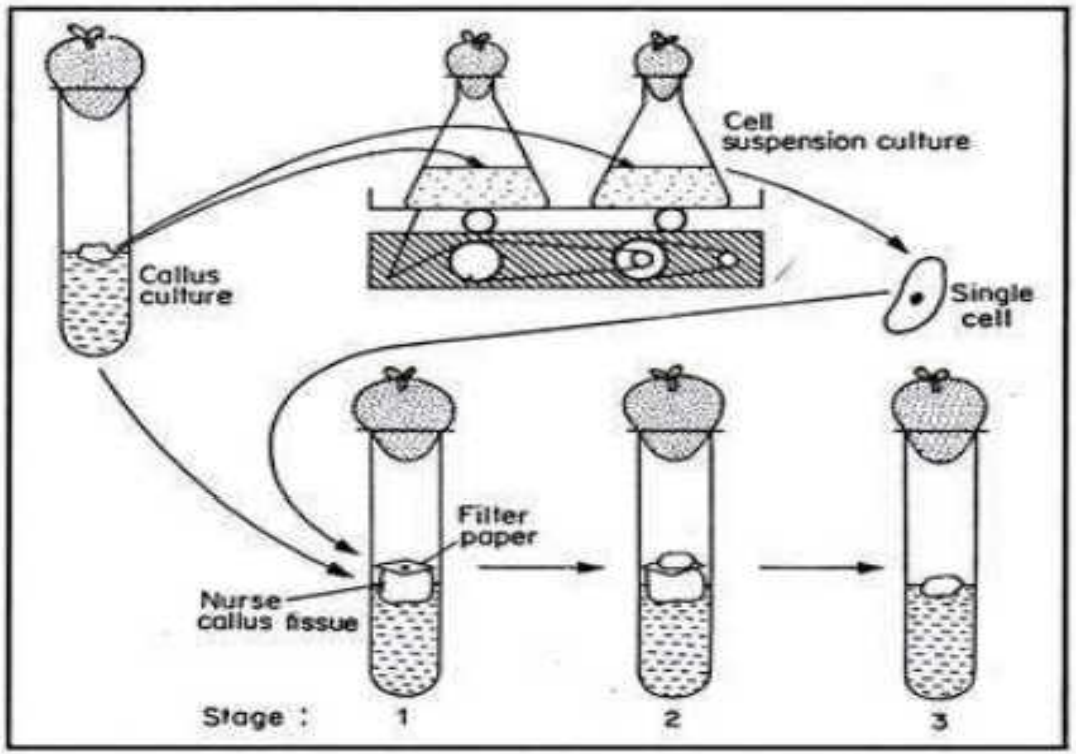

Fig 9.1

Growth of single cells using a 'nurse' technique. Stage 1 : a single cell taken from a friable callus is placed on upper surface of filter paper which is in contact with nurse callus. Stage 2 : the single cell divides and daughter cells proliferate to form colony. Stage 3 : when colony reaches a suitable size it is transferred to fresh medium where it gives rise to a single cell clone.

https://www.biologydiscussion.com/plant-tissues/single-cell-culture/single-cell-culture-5-methods-with-diagram-plant-tissue-culture/14699

PETRI DISH PLATING TECHNIQUE:

1. A suspension of purely single cells is pre-pared aseptically from the stock cell suspen-sion culture by filtering and centrifugation requisite cell density in the single cell suspension is adjusted by adding or reduc-ing the liquid medium.

2. The solid medium (1.6% 'Difco' agar added) is melted in water bath.

3. In front of laminar air flow, the tight lid of falcon plastic petri dish is opened With the help of sterilized Pasteur pipette 1 5 ml of single cell suspension is put an equal amount of melted agar medium when it cools down at 35°C, is added in the single cell suspension

4. The lid is quickly replaced and the whole dish is swirled gently to disperse the cell and medium mixture uniformly throughout the lower half of the petri dish.

5. The medium is allowed to solidify and the petri dish is kept at the inverted position.

6. The cultures are incubated under 16hrs light (3,000 lux, cool white) or under con-tinuous dark at 25°C.

7. The petri dishes are observed at regular in-tervals under inverted microscope to see whether the cells have divided or not.

8. After certain days of incubation, when the cells start to divide, a grid is drawn on the undersurface of the petri dish to facilitate counting the number of dividing cells.

9. The dividing cells ultimately form pin-head shaped cell colonies within 21 days of incu-bation.

10. The plating efficiency (PE) can be calculated from the counting of cell colonies by the following formula:

PE = Number of colonies per plate/Number of total cell per plate x 100

11. Pin-head shaped colonies, when they reach a suitable size, are transferred to fresh me-dium for further growth.

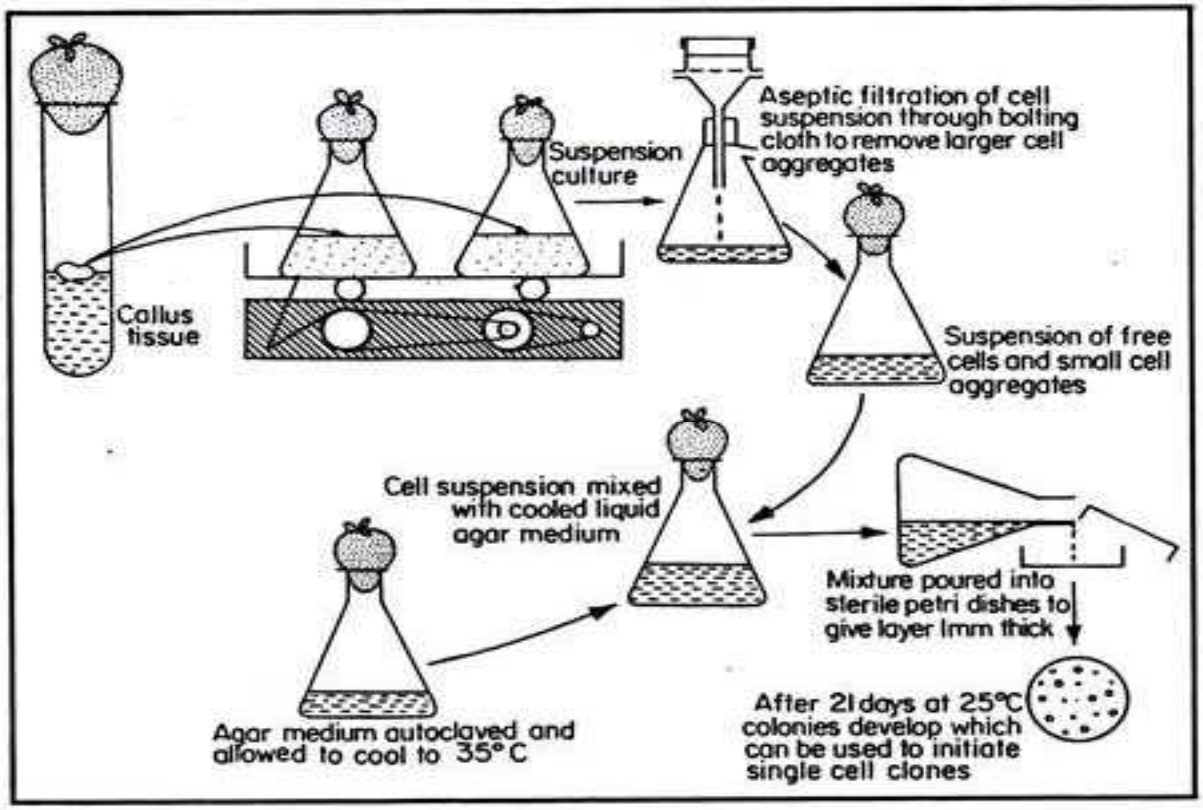

Fig 9.2

Procedure for obtaining single cell clones using a petri dish plating technique

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MICRO-CHAMBER TECHNIQUE

1. In this method 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.

2. A drop of oil is placed on either side of the culture drop and a coverglass placed on each drop.

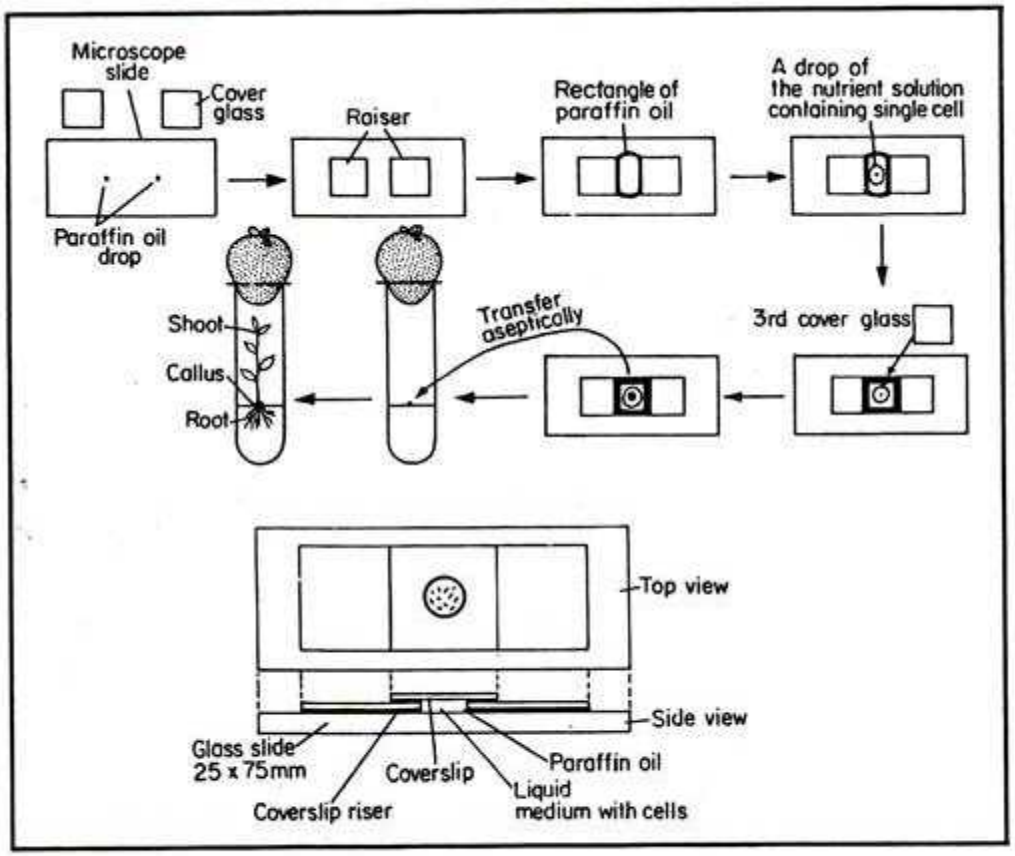
3. A third coverglass is then placed on the culture drop bridging the two coverglasses and forming a microchamber to enclose the single cell aseptically within the mineral oil.

4. The oil prevents water loss from the chamber but permits gaseous exchange.

5. The whole microchamber slide is placed in a petri-dish and incubated.

6. When the cell colony becomes sufficiently large the coverglass is removed and the tissue is transferred to fresh liquid or semi-solid medium.

7. The microchamber technique permits regular observation of the growing and dividing cell



❒ Fig 9.3

Microchamber used to observe the growth of single cells

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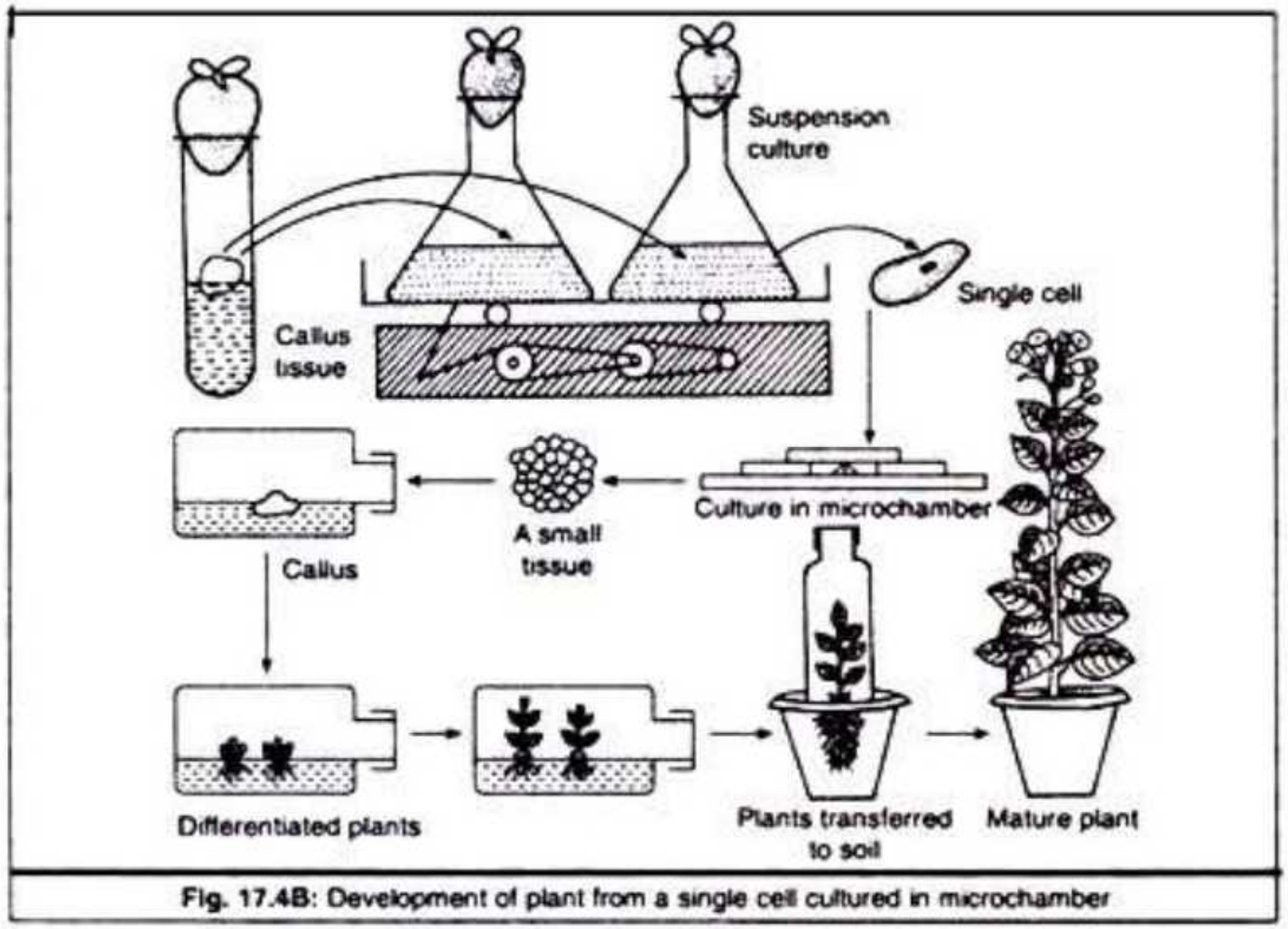


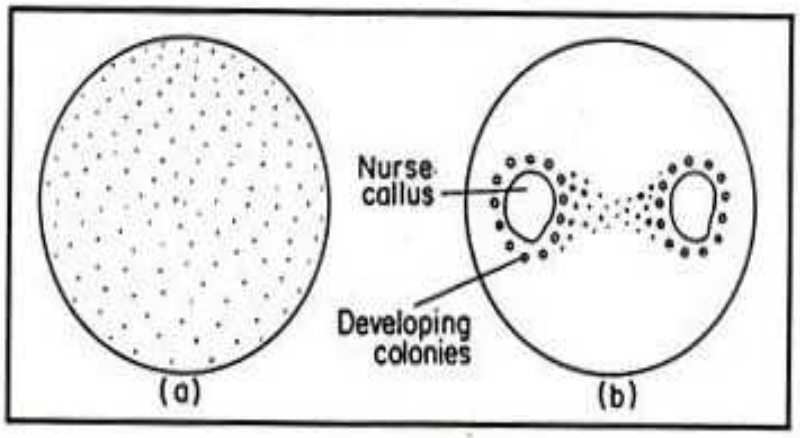
Fig. 17.4B: Development of plant from a single cell cultured in microchamber

https://www.slideshare.net/DrSureshSolleti/cell-culture-78526106

NURSE CALLUS TECHNIQUE

This method is actually a modification of petridish plating method and the paper raft nur-se culture method. In this method, single cells are plated on to agar medium in a petridish as described ear-lier Two or three callus masses (Nurse tissue) derived from the same plant tissue are also em-bedded directly along with the single cells in the same medium.

Here the paper barrier between single cells and the nurse tissue is re-moved. Cells first begin to divide in the regions near the nurse callus indicating that the single cells closer to nurse callus in the solid medium gets the essential growth factors that are lib-erated from the callus mass. The developing colonies growing near to nurse callus also stim-ulate the division and colony formation of other cells.



❒ Fig 9.4

Growth of colonies from a low density cell suspension in the presence of callus tissue. A. Petri dish inoculated with low density suspension of cells—no colonies develop. B. Petri dish inoculated with low density suspension plus nurse callus—colonies grow near to nurse calluses only

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MICRO DROPLET TECHNIQUE

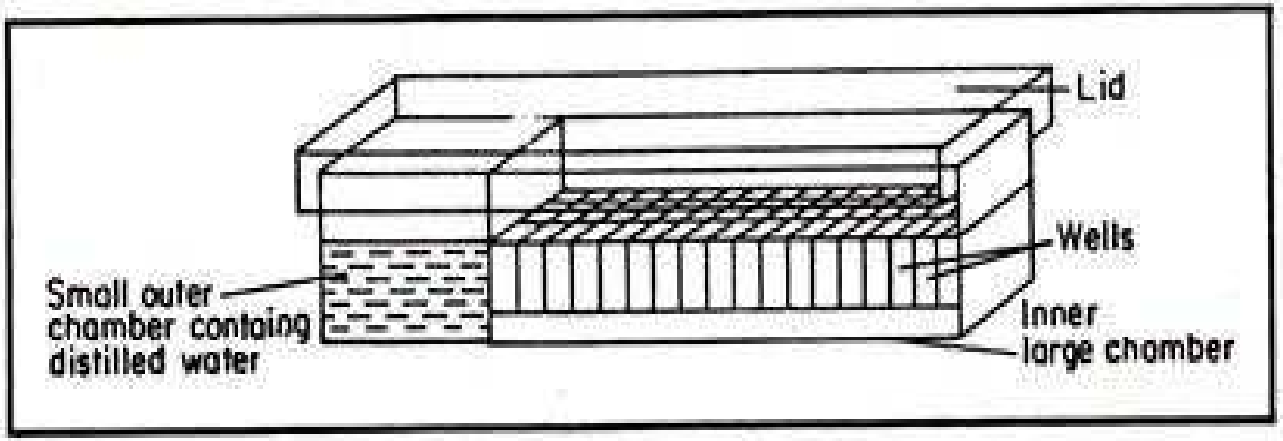
- The technique requires a specially designed cuprak dishes which have smaller outer chamber and larger inner chamber.

- The inner chamber carries numerous numbered wells, each with a capacity for 0.25 to 25 µl droplet of nutrient medium.

- Individual cell is transferred to each well of the chamber along with conditioned medium.
- The outer chamber is filled with sterile water to maintain humidity inside the dish.

- After covering with lid, the dish is sealed with parafilm and maintained at optimal light and temperature conditions.

- When cell group develops it is transferred to medium to form callus



❒ Fig 9.5

Diagrammatic view of Cuprak dish used for the microdroplet technique of single cell culture

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CULTURE CELL VIABILITY TEST

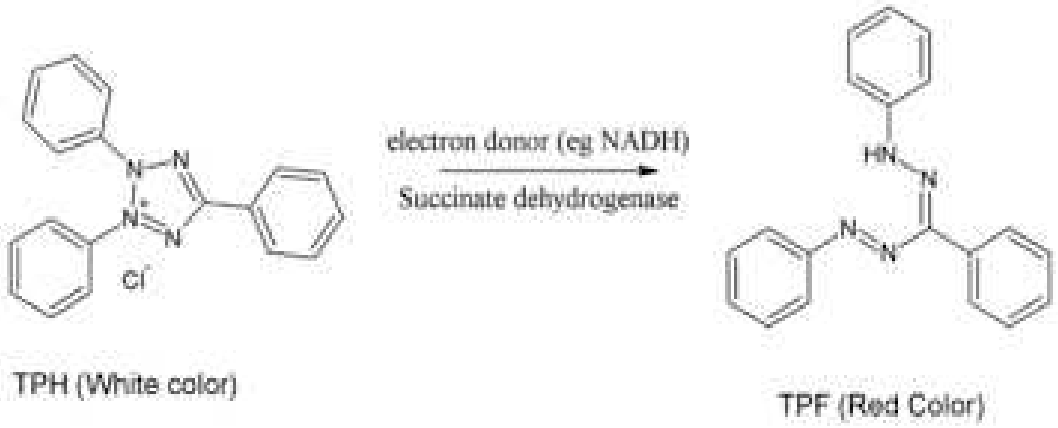
Phase contrast microscopy:

Using phase contrast microscopy, the cytoplastic streaming and the presence of healthy nucleus will be observed.

Thus the viable cells are counted for the given volume of culture.

Reduction of tetrazolium salts:

When the cell cultures are incubated with 2,3,5 triphenyl tetrazolium chloride (TTC), the viable cells converts theTTC into a red colored substance known as 1,3,5 triphenyl Formazan, which is estimated spectrophotometrically.



TPH (White color)

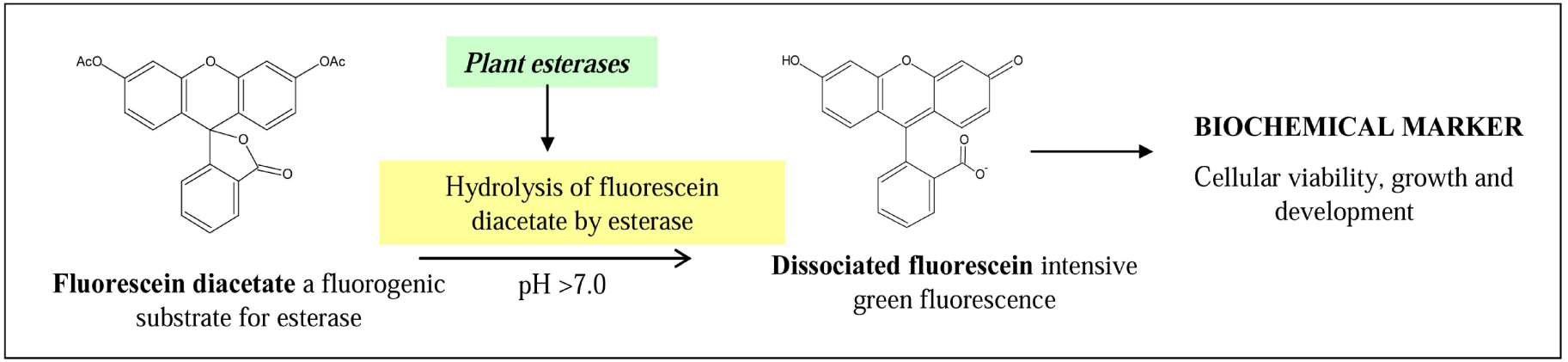
TPF (Red Color)

Fluorescein diacetate (FDA) method:

Cell cultures are incubated with 0.5% FDA for 5 min.

FDA being non polar and non fluorescing, enters the cells and is cleaved by esterase activity in the living cell resulting into polar Fluorescein.

Since, fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, thus those cells exhibit green colour fluorescence.



Evans Blue method:

Cell cultures are incubated with 0.025% Evans blue for 5 min.

Evans blue dye has been used as a viability assay on the basis of its penetration into non-viable cells i.e dead and damaged cells.

The dead or damaged cell takes the blue colour.

Cryopreservation

Cryopreservation (Greek,krayos-frost)literally means in the frozen state. The principle involved in cryopreservation **to bring the plnt cells and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presences of cryoprotectants** (DMSO (dimethyl sulfoxide), glycerol, ethylene, propylene, sucrose, mannose, glucose, praline, acetamide etc).

CRYOPRESERVATION broadly means the storage of germplasm at very low temperature using :-

- ✓ Over solid carbon dioxide(at 79°C)
- ✓ Low temperature deep freezer(at -80°C)
- ✓ Using vapour nitrogen (at- 150°C)
- ✓ In liquid nitrogen(at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen(at -196°C), the cell stay in a completely inactive state and thus can be conserved for longer period. Infact cryopreservation has been successfully applied for germplasm conservation of some plant species e.g rice, wheat, peanut, sugarcane, coconut.

MECHANISM OF CRYOPRESERVATION

The technique of freeze preservation is based on the **transfer of water present in the cells from a liquid to solid state.** Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze(even up to -68°C) compared to the freezing point of pure water(around 0°C). **When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to standstill.**

TECHNIQUE OF CRYOPRESERVATION

The cryopreservation of plant cell culture followed the regeneration of plants broadly involves the following stages.

1. Development of sterile tissue culture.
2. Addition of cryoprotectant and pretreatment.
3. Freezing
4. Storage
5. Thawing
6. Reculture
7. Measurement of survival/viability
8. Plant regeneration

1.DEVELOPMENT OF STERILE TISSUE CULTURE

The selection of plant species and the tissue with particular references to the morphological and physiological characters largely influences the ability of the explants to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperm, ovules, seeds, culture plants.

2.ADDITION OF CRYOPROTECTANT

Cryoprotectant are the compound that can **prevent the damage caused to cells by freezing or thawing**. There are several cryoprotectant which include:

(DMSO, GLYCEROL, ETHYLENE, PROPYLENE, SUCROSE, MANNOSE, GLUCOSE…..)

3.FREEZING

The sensitivity of the cells to low temperature is visible and largely depends on the plant species.Four different types of freezing are used. Slow freezing method Rapid freezing method Stepwise freezing method Dry freezing method

4.STORAGE

Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperature in the range of -72 to-196°C. **Storage is ideally done in liquid nitrogen refrigerator at - 150°C in the vapour phase, or at -196°C in the liquid phase.** The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage temperature at -196°C in liquid nitrogen is ideal.

5.THAWING

Thawing is usually carried out by plunging the frozen sample in ampoules into the warm water (temp 35- 45°C) bath with vigorous swirling. By this approach,rapid thawing(at the rate of 500-750°Cmin-1)occurs, and this protects the cell from the damaging effects ice crystal formation. As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm(35-45°C) water bath.

6.RECULTURE

In general thawed germplasm is washed several times to remove cryoprotectant. The material is then cultured in a fresh media.

7.PLANT REGENERATION

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cell/tissue have to be carefully nursed and grown. Addition of certain growth promoting substances ,besides maintenance of appropriate environmental condition is often necessary for successful plant regeneration.

SLOW GROWTH CULTURES

- Slow-growth of plantlets in-vitro provides an attractive alternative to freeze preservation of germplasm as it is simpler, cheaper and very effective. **Slow growth may be achieved by maintaining the plantlets either at a low temperature (4-9°C or Ca. l5°C) or on a medium having high osmotic concentration (e.g., 20% sorbitol or sucrose) or both.**
- **In addition, the nutritional status of the medium may be lowered to restrict the growth of plantlets.** Under the conditions of slow-growth, cultures may be attended to only once in several months. Its subculture may, be necessary only after long periods, once every 236 months.

- The slow-growth approach is being utilized for germplasm conservation of specified root, tuber and tree species by the NBPGR, New Delhi.

- A National Facility for Plant Tissue Culture Repository has been created for this purpose. It has so far developed the slow-growth protocols for ginger, garlic, banana, sweet potato, etc.

APPLICATION OF PLANT TISSUE CULTURE IN TRNSGENIC PLANTS

- ✓ Improved Nutritional Quality
- ✓ Insect resistance
- ✓ Disease resistance
- ✓ Herbicide resistance
- ✓ Salt tolerance
- ✓ Delayed Fruit Ripening
- ✓ Biopharmaceuticals and Vaccines

Golden Rice

- ✓ 124 million children worldwide are deficient in vitamin A, which leads to death and blindness
- ✓ b-carotene is precursor to vitamin A & consuming milled rice leads to vitamin A deficiency
- ✓ Mammals make vitamin A from b-carotene, a common carotenoid pigment normally found in plant photosynthetic membranes

Rainbow cauliflower

- ✓ Produced by traditional breeding –Non Transgenic
- ✓ The Orange cauliflower has higher than normal levels of b-carotene that encourages healthy skin
- ✓ Purple colour comes from Anthocyanin which may prevent Heart disease by slowing blood clotting
- ✓ Tests of the orange cauliflowers in America found that they contained25 times the concentrations of beta carotene in normal cauliflowers

Insect Resistant Plants

- ✓ Bt gene of a bacterium Bacillus thuringiensis has found to encode endotoxin which pose cidal effect on certain insect pests
- ✓ The cry gene found to express the proteinaceous toxin, when specific pest ingest the toxin, they are killed.
- ✓ The toxin affect specific group of insects and do not harm silkworm, butterflies and other beneficial insects
- ✓ Using biotechnological approaches many transgenic plants with cry gene have been developed eg., Bt-brinjal, cauliflower, cabbage, canola, corn, maize, tobacco, rice, soyabean

Herbicide resistant plants

- ✓ Plants that can tolerate herbicides
- ✓ The herbicide disturb the metabolic activity of photosynthesis or synthesis of amino acid
- ✓ For the development of herbicide resistant plants two main strategies are being applied

– Modification of target molecules that may be insensitive to herbicides

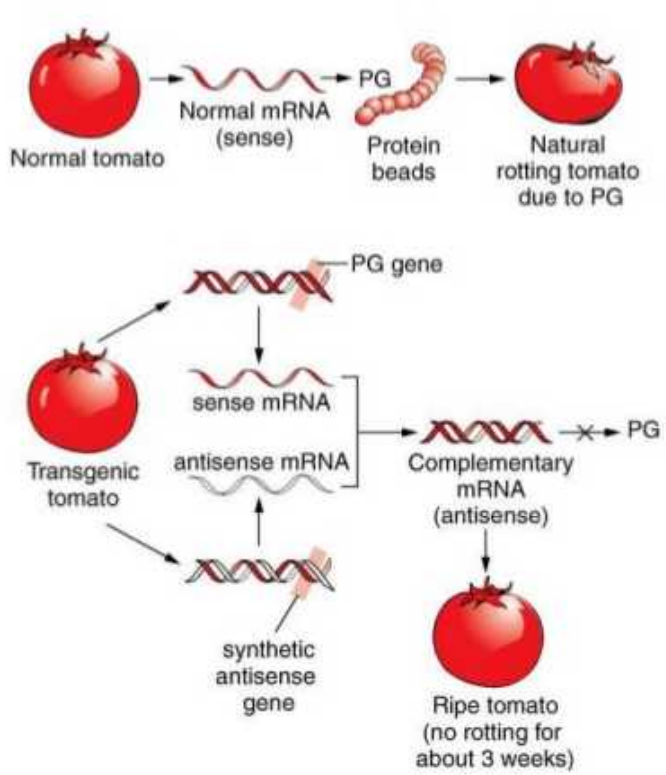
– Degradation of herbicides

Salt Tolerance

- ✓ A large fraction of world's irrigated land cannot be used to grow most important crops due to increased salinity in soil
- ✓ Researcher's have created transgenic tomatoes that grew well in saline soils
- ✓ The transgene introduced was sodium/proton antiport pump that sequestered excess sodium in vacuole of leaf cells

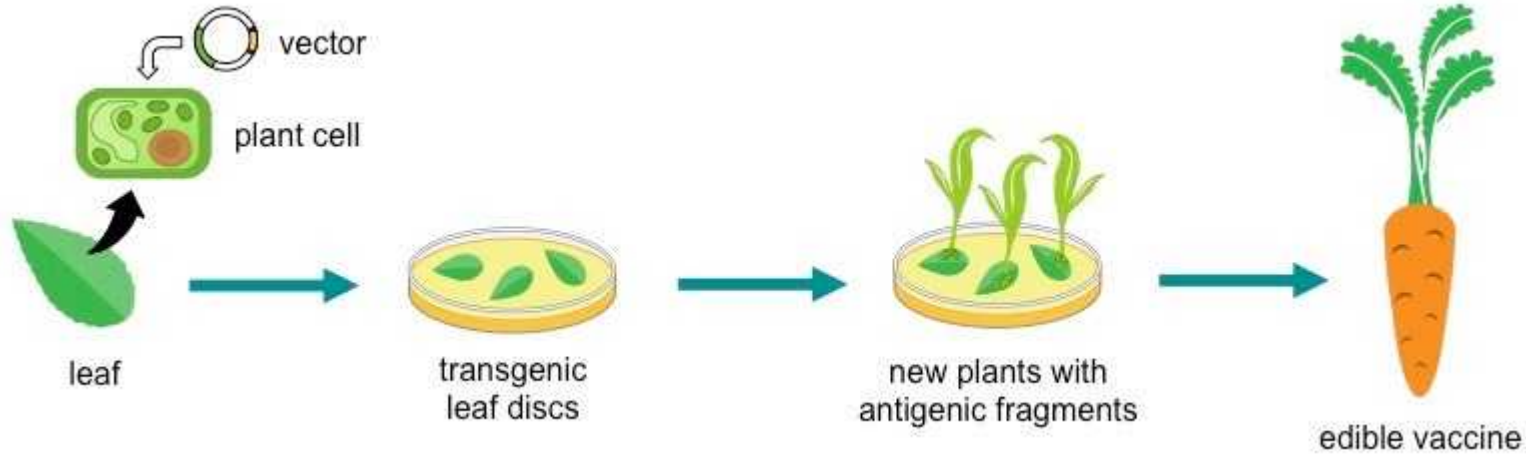
Delayed ripening

- ✓ Antisense technology is used produce the Flavr-Savr tomato in 1994.
- ✓ Enzyme polygalacturonase breaks down structural polysaccharide pectin in wall of a plant.
- ✓ This is part of the natural decay process in a plant
- ✓ Monsanto identified the gene than encodes the enzyme and made another gene that blocked the production of the enzyme.



Pharmaceutical production in plants

- ✓ Genetically modified plants have been used as “bioreactors” to produce therapeutic proteins A recent contribution is the generation of edible vaccines.
- ✓ **Edible vaccines** are vaccines produced in plants that can be administered directly through the ingestion of plant materials containing the vaccine. Eating the plant would then confer immunity against diseases.
- ✓ Edible vaccines produced by transgenic plants are attractive for many reasons.
- ✓ The first human clinical trial took place in 1997. Vaccine against the toxin from the bacteria *E.coli* was produced in potato



https://ib.bioninja.com.au/options/untitled/b2-biotechnology-in-agricul/edible-vaccines.html

PRODUCTION OF SECONDARY METABOLITES & INDUSTRIAL PRODUCTS

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.

- Secondary plant metabolites like alkaloids, terpenoids, flavonoids, lipids, oils, tannins, anthraquinones, flavones, napthaquinones, vitamins, proteins, anticancer agents, antiviral agents etc. are isolated from plant tissue culutre.

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.

Production of phytopharmaceuticals and secondary metabolites.

a) Biotransformation (Biochemical Conversion)
b) Plant cell immobilization
c) Genetic transformation (Transgenic plant)
d) Elicitors

List of few secondary metabolites derived from plant tissue culture

Compound	Plant species	Culture type
Anthraquinones	*Cassia angustifolia*	Callus
Caffeine	*Coffee arabica*	Callus
Cardenolides	*Digitalis purpurea*	Suspension & Callus
Codeine	*Papaver somniferum*	Suspension
Diosgenin	*Dioscorea composita*	Callus
Glycyrrhizin	*Glycyrrhiza glabra*	Suspension
Papain	*Carica papaya*	Callus
Reserpine	*Rauwolfia serpentina*	Suspension
Rosmarinic acid	*Coleus blumei*	Callus & Suspension
Trigonelline	*Trigonella foenum-graecum*	Suspension
Vinblastine	*Catharanthus roseus*	Callus
Visnagin	*Ammi visnaga*	Suspension
Xanthotoxin	*Ruta graveolens*	Suspension

A. Biotransformation (Biochemical Conversion)

It is a process through which the functional group of organic compounds are modified by living cells.

This process can be done by using microorganism or plant cell suspension, hairy root culture and immobilized cell.

Biotransformation by plant cell cultures yield a wide range of reactions, such as glycosylation, glucosyleserification, hydroxylation, oxido-reductions, hydrolysis, epoxidation, isomerisation, methylation, demethylation and dehydrogenation etc.

It not only increases the yield but also very economical for commercial production.

B. Plant cell Immobilization: Discussed earlier.

C. Genetic transformation (Transgenic plant)

- The plants obtained through genetic engineering contain a gene usually from an unrelated organism, such genes are called transgenes, and the plants containing transgenes are called as transgenic plants.
- Genetic transformation can be defined as the transfer of foreign genes (DNA) or the recombinant DNA isolated from plants, viruses bacteria into a new genetic background.
- The targeted cells for gene transformation are cultured cells or protoplast, meristem cells from embryos, pollens, zygote and cells from immature embryos, shoots and flowers.

Application

- ✓ Genes have been successfully transferred to many crops for resistance to various biotic stresses.
- ✓ Genes resistant to abiotic stresses like herbicide resistance.
- ✓ Resistance against viral infection.
- ✓ Gene transfers to improve quality of food products.
- ✓ Male sterility and fertility restoration in transgenic plants.
- ✓ Transgenic plants have both basic and applied role in crop improvement. E.g. Tobacco, tomato, soybean, Satavari, papaya, liquorice, neem etc.

D. Elicitors

- Elicitors are organic/inorganic agents used in tissue culture to trigger rapid and increase production of secondary metabolites or rapid growth of plant cell culture/organs.
- Induction of stress in plant cultures in terms of specific environmental, physiological & biological conditions, to enhance the production of secondary metabolites, is known as Elicitation.
- The secondary compounds synthesized & accumulated in response to such conditions are called '**Phytoalexins'**, which act as defense agent to invading pathogens. The signals triggering the formation of phytoalexins are called **elicitors**.

Classification of Elicitors

i. Based on Origin

1. Exogenous elicitors: Enzymes, metal ions, U.V. Light, chitosan etc.
2. Endogenous elicitors: Hepta-β-glucoside, Dodeca β-1,4 D-galacturonide etc.

ii. Based on nature

1. Biotic elicitors
2. Abiotic elicitors

1. Biotic elicitors

- ✓ They are derived from microorganisms or produced within the plant cells by plant defensive process against microbial infection.
- ✓ They include mainly β-Linked glucans, chitosan, enzymes, cell wall derived polysaccharides like pectin, pectic acid, cellulose, etc.
- ✓ These elicitors when added to medium in low concentration (50- 250ng/l) enhance metabolite production.

2. Abiotic elicitors

- ✓ Product accumulation also occurs under stress caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, freezing and thawing cycles, non-essential components of media (agarose, tin, and agaropectin), certain chemicals (methyl jasmonate, copper sulphate, silver nigtrate etc.), and high salt concentration grouped under abiotic elicitors.

Elicitors stimulated the accumulation of secondary metabolites in different plant culture like Opium, Dioscorea, Datura, Vinca, Capsicum, Carrot etc.