INTRODUCTION:

PLANT TISSUE CULTURE

Plant tissue is a collection of experimental methods of growing large number of isolated cells or tissues under sterile and controlled conditions. The cells or tissues are obtained from any part of the plant like stem, root, leaf etc. Which are encouraged to produce more cells in culture and to express their totipotency. Cells or tissues are grown in different types of glass vials containing a medium with mineral nutrients, vitamins and phytohormones.

1.1 CELL SUSPENSION CULTURE

Suspension culture is a type of culture in which single cells or small aggregates of cells multiply while suspended in agitated liquid medium. It is also referred to as cell culture or cell suspension culture. Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Such systems contribute to our understanding of the interrelationships and complementary influences of cells in multicellular organisms. Many plant biotechnologists recognized the merits of applying cell cultures over an intact organ or whole plant cultures to synthesize natural products.

1.2 BRIEF HISTORY

- The pioneering attempts made by **Haberlandt** failed to achieve divisons in free cells, but his detailed paper in 1902 stimulated further studies in this area.
- **H. Muir** (1953) – First reported that the fragments of callus of ***Tagetes erecta*** and ***Nicotiana tabacum*** could be cultured in the form of cell suspension
- **Nickel** (1956) – Described the continues growth of a variety of ***Phaseolus vulgaris***
- **C. Steward** and **E. M. Shantz** (1956) – Reported the suspension cultures from carrot root explants and obtained very large number of plantlets from the culture.

1.3 PRINCIPLES OF CELL SUSPENSION CULTURE

1. 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.

2. Callus proliferates as an unorganized mass of cells. So, it is very difficult to follow many cellular events during its growth and developmental phases. To overcome such limitations, the cultivation of free cells as well as small cell aggregates in a chemically defined liquid medium as a suspension was initiated.

3. In culture, the single cells divide and redivide to form a callus tissue. Such callus tissue also retains the capacity to regenerate the plantlets through organogenesis and embryogenesis.

4. To achieve an ideal cell suspension, most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses.

5. After eliminating the large callus pieces, only single cells and small cell aggregates are again transferred to fresh medium and after two or three weeks a suspension of actively growing cells is produced.

6. This suspension can then be propagated by regular sub-culture of an aliquot to fresh medium.

7. Ideally suspension culture should consist of only single cells which are physiologically and biochemically uniform.
8. Movement of cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of the cells due to gravity and eliminates the nutrient gradients within the medium and at the surface of the cells.

1.4 METHODOLOGY

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2 ISOLATION OF SINGLE CELLS

2.1 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.

Two important methods to isolate single cells from leaf are:

1. Mechanical Method
2. Enzymatic Method

2.2 FROM CULTURED TISSUES

The most widely applied approach is to obtain a single cell system from cultured tissues.

2.2.1 Mechanical Method

Gnanam and Kulandaivelu (1969) developed a procedure which has since been successfully used 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:

1. 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.
2. 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.

2.2.2 Enzymatic Method

In 1968 Takabe et al treated tobacco leaf tissue with the enzyme pectinase and obtained a large number of metabolically active cells. A point to note is that potassium dextran sulphate in the enzyme mixture improved the yield of free cells.

Isolation of single cells by the enzymatic method has been found convenient as it is possible to obtain high yields from preparations of spongy parenchyma with minimum damage or injury to the cells. This can be accomplished by providing osmotic protection to the cells while providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of the parenchymatous tissue. Applying the enzymatic method to cereals has proven difficult since the mesophyll cells of these plants are apparently elongated with a number of interlocking constrictions, thereby preventing their isolation.

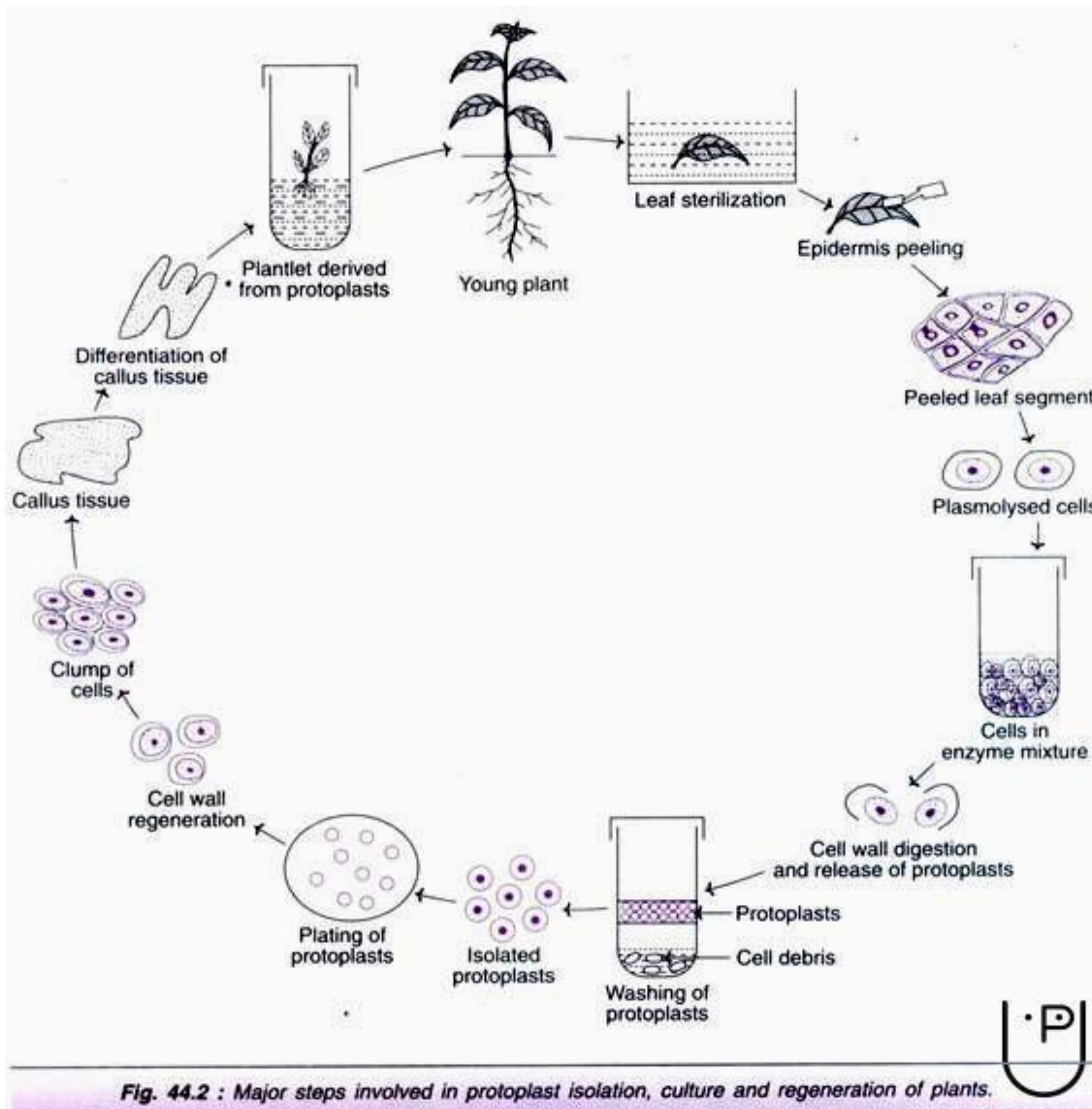


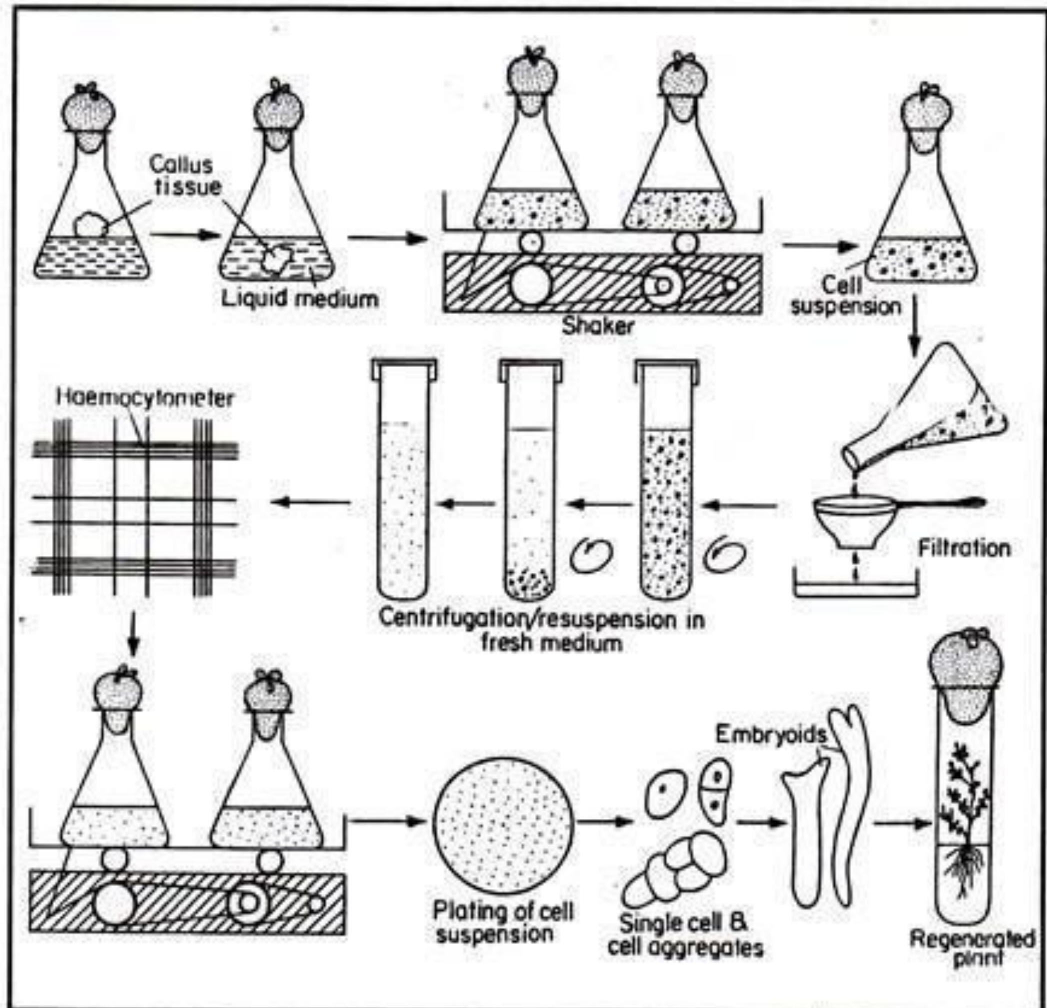
Fig. 44.2 : Major steps involved in protoplast isolation, culture and regeneration of plants.

2.3 FROM CULTURED TISSUES

1. Raise sterile tissue culture plants and obtain callus from them.
2. The callus is separated from an explant and transferred to a fresh medium of the same composition to enable it to build up a mass of tissue.
3. Repeated subculture on an agar medium improves the friablity of a callus, a pre requisite for raising the fine cell suspension in a liquid medium.
4. The pieces of undifferentiated and friable callus are transferred in a continuously agitated liquid medium dispersed in autoclaved flasks or other suitable vials.
5. Agitation is done by placing the flasks on orbital platform shaker or suitable device.
6. 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 between the culture medium and the culture air and also ensures uniform distribution of cells in the medium.
7. The period of incubation during which the suspension cultured is developed from callus tissue is usually called as the initiation passage.
8. The concentration of auxins and cytokinins is often critical for the growth of cell suspension and the concentration of auxin and cytokinins used for callus culture is generally reduced for suspension culture.
9. The cells in the cell suspension may vary in shapes and sizes. They may be oval, round, elongated or coiled, but thery are thin walled, even with the presence of other lignified, trachieds like elements.

2.3.1 Protocol

1. Take 150/250 ml conical flask containing autoclaved 40/60 ml liquid medium
2. Transfer 3-4 pieces of pre-established callus tissue (approx. Wt. 1 gm. Each) from the culture tube using the spoon headed spatula to conical flasks.
3. Flame the neck of conical flask, close the mouth of the flask with a piece of aluminum foil or a cotton plug. Cover the closure with a piece of brown paper.
4. Place the flasks within the clamps of a rotary shaker moving at the 80-120 rpm (revolution per minute)
5. After 7 days, pour the contents of each flask through the sterilized sieve pore diameter -60μ-100μ and collect the filtrate in a big sterilized container. The filtrate contains only free cells and cell aggregates.
6. Allow the filtrate to settle for 10-15 min. Or centrifuge the filtrate at 500 to 1,000 rpm and finally pour off the supernatant.
7. Re-suspend the residue cells in a requisite volume of fresh liquid medium and dispense the cell suspension equally in several sterilized flasks (150/250 ml). Place the flasks on shaker and allow the free cells and cell aggregates to grow.
8. At the next subculture, repeat the previous steps but take only one-fifth of the residual cells as the inoculum and dispense equally in flasks and again place them on shaker.
9. After 3-4 subcultures, transfer 10 ml of cell suspension from each flask into new flask containing 30 ml fresh liquid medium.
10. To prepare a growth curve of cells in suspension, transfer a definite number of cells measured accurately by a hemocytometer to a definite volume of liquid medium and incubates on shaker. Pipette out very little aliquot of cell suspension at short intervals of time (1- or 2-days interval) and count the cell number. Plot the cell count data of a passage on a graph paper and the curve will indicate the growth pattern of suspension culture.



❐ Fig 4.1

Flow diagram illustrating the method of cell suspension culture and regeneration of plant hro embryogenesis

2.4 DIFFERENT CATEGORIES OF CELL SUSPENSION CULTURE

Broadly speaking there are two types of suspension cultures

1. Batch Culture
2. Continuous Culture

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

2.4.1 Batch culture

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

Types of batch culture

2.4.1.1 Slow rotating cultures:

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

2.4.1.2 Shaker culture

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

2.4.1.3 Spinning culture

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

2.4.1.4 Stirred culture

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

2.4.2 Continuous culture

A technique used to grow microorganisms or cells continually in a particular phase of growth.

In this technique the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological state of the growing cells.in this state nutrients depletion does not occur due to the continuous supply of nutrients and cells are remain in steady growth phase.

For example, if a constant supply of cells is required, a cell culture maintained in the log phase is best; the conditions must therefore be continually monitored and adjusted accordingly so that the cells do not enter the stationary phase (see bacterial growth curve). Growth may also have to be maintained in a particular growth phase if an enzyme or chemical product is produced only during that phase.

Continuous culture growth phase future divided into two types

1. Open continuous culture
2. Close continuous culture

2.4.2.1 Close continuous culture

The used medium is replaced by the fresh medium hence the cells from the medium mechanically retrieve and added back to the cell culture and thus the cell biomass keeps increasing. Culture has very extended exponential, linear and stationary growth phases.

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

2.4.2.2 Open continuous culture

Both the cells and medium are taken out from open continuously cultures and replaced by equal volume of the fresh medium. The replacement volume is so adjusted that culture remain in sub-maximal growth phase indefinitely. Growth may be maintained at any growth phase.

Open continuous cell suspension culture is of two types

1. Chemostat
2. Turbid stats

2.4.2.2.1 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 remain constant's continuous cultures are ideal for studying growth kinetics and regulation of metabolic activity in higher plants.

2.4.2.2.2 Turbidostat

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 up to a certain turbidity, when the predetermined volume of culture is replaced by fresh culture. The turbidity is measured by the changes of optical density of medium. An automatic monitoring unit is comended with the culture vessel and such unit adjust the medium flow in such a way as to maintain the optical density or PH at the chosen present level.

2.5 IMPORTANCE

- 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 of cell suspension culture.

2.6 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.

2.7 DISADVANTAGES OF CELL SUSPENSION CULTURE:

1. Maintaining the sterile aseptic condition is the most difficult part of cell culture.
2. The productivity of **suspension cultures** decreases over extended subculture periods.
3. Slow growth and low productivity of plant **cells**.
4. **Cells** may get damaged by shear conditions.
5. Chances of chemical and microbial contamination are very high in *in vitro* methods.
6. High possibility of cross contamination of different types of cells in culture.
7. Experience and expertise are required for an effective maintenance for most of the cells.
8. Due to the rapid growth rate of cells artificial culture, there is a high chance of genetic variation within in a cell population.