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MICROPROPAGATION

Micropropagation is the artificial process of producing plants vegetatively through tissue culture or cell culture techniques. In this artificial process of propagation, plants are produced invitro by asexual means of reproduction or by vegetative propagation.

Plants can be produced both asexually i.e, via vegetative parts' multiplication or sexually i.e., seed production. One of the means of asexual reproduction is by multiplying genetic replicas of plants that are referred to as clonal propagation wherein plants can be populated from a single individual through asexual means of reproduction.

For the in vivo propagation of specific plants, **asexual reproduction** via multiplication of vegetative parts is the only resort since they do not generate functional seeds as seen in figs, grapes, bananas etc. Successful application of clonal propagation to the following is observed: potato, apple and many other ornamental plants.

Stages of Micropropagation

1. **Selection and Sterilization of Explant:**

Suitable explant is selected and is then excised from the donor plant. Explant is then sterilized using disinfectants.

2. **Preparation and Sterilisation of Culture Medium:**

A suitable culture medium is prepared with special attention towards the objectives of culture and type of explant to be cultured. Prepared culture medium is transferred into sterilized vessels and then sterilized in autoclave.

3. **Inoculation:**

Sterilized explant is inoculated (transferred) on the culture medium under aseptic conditions.

4. **Incubation:**

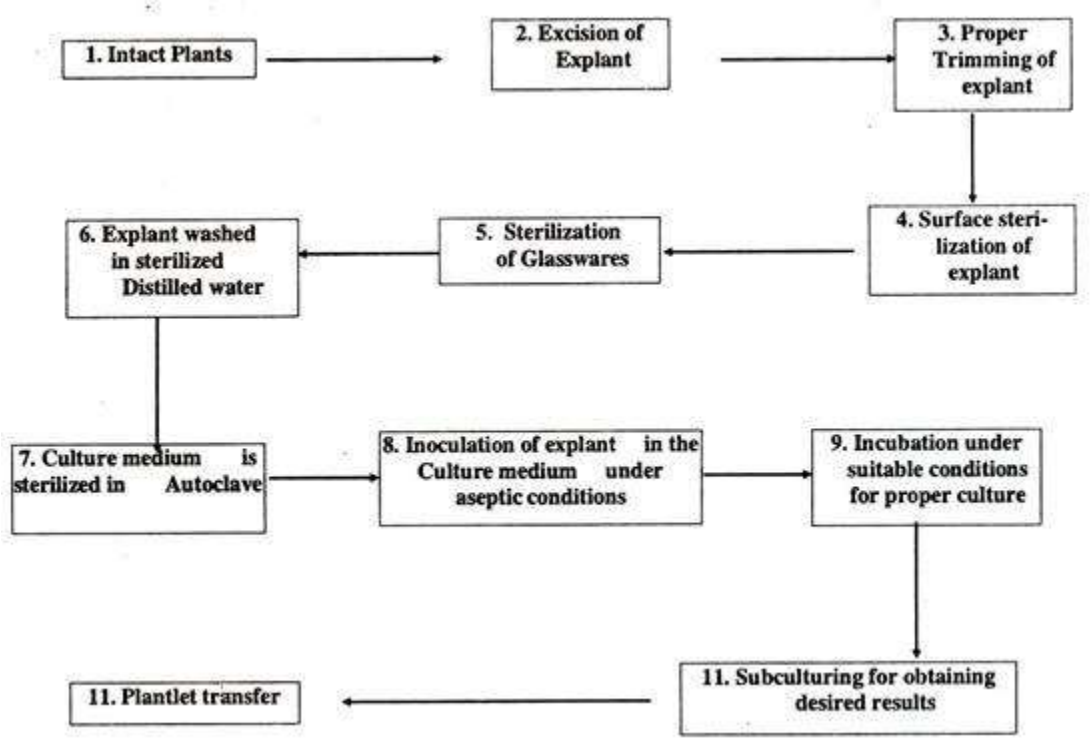
Cultures are then incubated in the culture room where appropriate conditions of light, temperature and humidity are provided for successful culturing.

5. Sub culturing:

Cultured cells are transferred to a fresh nutrient medium to obtain the plantlets.

6. Transfer of Plantlets:

After the hardening process (i.e., acclimatization of plantlet to the environment), the plantlets are transferred to green house or in pots.



Indirect Organogenesis

At the cut edges of the explants callus is formed that is known as dedifferentiation callus. Organization of such a callus is done to give a new shoot. Manipulation of phytohormones is required for indirect organogenesis.

Direct Organogenesis

In direct organogenesis, the explant cells produce plantlets by directly undergoing organogenesis without formation of a callus. When axillary or apical meristems are difficult to obtain in culture, the direct organogenesis method may prove to be useful for micropropagation. Any tissue of explants in this method will produce shoots directly, e.g., root, cotyledon, and leaf may be used as the explants and these produce plantlets on inoculation.

CALLUS CULTURE

A callus is an undifferentiated mass of tissue. In vitro, the callus formation is induced by placing a piece of tissue in a growth culture media under favorable conditions. Then, the callus is transferred to another fresh media containing a high concentration of auxin or auxin and cytokinin (plant growth regulators) for organ development.

Some ***limitations of callus culture include high biochemical variability and slow growth rate.*** These limitations hinder the utilization of the callus culture techniques at a higher level by the culturists.

Brief Past History of Callus Culture:

R. J. Gautheret (France) (1934-1937):

He first succeeded in promoting the development of callus tissue from excised cambial tissue of Salix capraea and other woody species. He was able to promote the growth of the callus tissue using simple nutrient medium supplemented with three vitamins (thiamine, pyridoxine and nicotinic acid) and indole-3-acetic acid (IAA) newly discovered by F W Went and K V Thimann (1937).

P. Nobecourt (France) (1939):

He first established the callus culture capable of potentially unlimited growth on semisolid agar medium. He started his work using the tap root explant of Daucus carota. He was also to maintain the culture by simply transferring portions of the callus to fresh medium at regular interval of four to six weeks.

J. Van Overbeck, M. E. Conklin and A. F. Blakeslee (1941):

They first reported the importance of coconut milk in callus culture.

S. M. Caplin and F. C. Steward (1948):

They first succeeded in obtaining the growth of differentiated non-cambial cells isolated from Daucus carota using coconut milk in medium. Later, they .used coconut milk in combination with synthetic auxin such as 2, 4-dichlorophenoxy acetic acid in medium and were able to induce the division of cells in species which had previously been difficult to grow.

F. Skoog (1954-1955):

He was able to produce the callus culture from the cut piece of stem of Nicotiana tabacum in nutrient medium containing auxins. The callus remained active for some time but failed to grow. Later, he discovered kinetin from old sample of herring sperm DNA. After the addition of kinetin in culture medium, it was possible to renew the growth of tobacco stem callus tissue.

F. Skoog and C. O. Miller (1957):

They first put forward the concept of hormonal control of organ formation from callus tissue. They also suggested that equal concentration of auxin and kinetin induced the continuous growth of callus tissue. The inclusion of kinetin in culture media has made it possible to produce callus culture from a large number of plant species.

What is the Meaning of Callus Tissue?

Callus tissue means an unorganised proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions.

Principles of Callus Culture:

For successful initiation of callus culture, three important criteria should be accomplished:

(i) Aseptic preparation of plant material,

(ii) Selection of suitable nutrient medium supplemented with appropriate ratio of auxins and cytokinins or only appropriate auxin, and

(iii) Incubation of culture under controlled physical condition.

Different plant parts carry a number of surface borne micro-organisms-like bacteria, fungus etc. So, before attempting to initiate a callus culture, it is necessary to surface sterilize the plant parts which are to be cultured. Typical plant parts may be segments of root or stem, pieces of leaf lamina, flower petals etc. The excised plant parts called explants are at first washed with liquid detergent (generally 5% v/v 'Teepol').

Then the explants are surface sterilized by the most commonly used chemicals such as 0.1% w/v mercuric chloride ($HgCl_2$) or Sodium hypochlorite (0.8% to 1.6% available chlorine) for a limited time (generally 10-15 minutes). After surface sterilization, the explants are repeatedly rinsed with autoclaved distilled water.
The surface sterilized plant material is cut aseptically into small segments (a few millimeters in size). Size of explants is a critical factor for the induction of callus tissue. The explants are finally transferred aseptically on a suitable nutrient medium solidified with agar.

Agar solidified or semi-solid nutrient medium after its preparation and sterilization by autoclave at 15 lbs. pressure for 15 minutes is used for the induction of callus tissue. In most cases successful callus culture depends upon the inclusion of plant growth hormones in the nutrient medium and for healthy callus growth usually both an auxin and a cytokinin are required.

Incubation of culture under controlled physical conditions such as temperature, light, and humidity is indispensible for the proper initiation of callus tissue. The suitable temperature for in vitro callus initiation and growth is usually 25 ±2°C. In some plant materials initiation and growth of the callus tissue take place in totally dark condition.

However, in case of other plant materials, a particular photoperiod (16 hrs. light and 8 hrs. dark) is necessary for the initiation and growth of callus tissue. Approximately 2,000 to 3,000 lux artificial light intensity is needed. Cool, white fluorescent lamps (4 ft. 2 No.) are generally used for providing light. Generally 55% to 60% relative humidity is maintained in the culture room.

Once the growth of the callus tissue is well established, portions of the callus tissue can be removed and transferred directly onto fresh nutrient medium to continue growth. In this manner, callus cultures can be continuously maintained by serial subcultures.

Protocol of Callus Culture:

Callus tissue can be induced from different plant tissues of many plant species. Carrot is a highly standardized material.

So the callus culture from excised tap root of carrot is described here by the following procedure:

(1) A fresh tap root of carrot is taken and washed thoroughly under running tap water to remove all surface detritus

(2) The tap root is then dipped into 5% 'Teepol' for 10 minutes and then the root is washed.

The carrot root, sterilized forceps, scalpels, other instruments, autoclaved nutrient medium petridishes are then transferred to laminar air flow or inoculation chamber. Throughout the manipulation sequence forceps, scalpels must be kept in 95% ethanol and flamed thoroughly before use.

(3) The tap root is surface sterilized by immersing in 70% v/v ethanol for 60 seconds, followed by 20-25 minutes in sodium hypochlorite (0.8% available chlorine).

(4) The root is washed three times with sterile distilled water to remove completely the hypochlorite.

(5) The carrot is then transferred to a sterilized petridish containing a filter paper. A series of transverse slice 1 mm in thickness is cut from the tap root using a sharp scalpel.

(6) Each piece is transferred to another sterile petridish. Each piece contains a whitish circular ring of cambium around the pith. An area of $4mm^2$ across the cambium is cut from each piece so that each small piece contains part of the phloem, cambium and xylem. Size and thickness of the explants should be uniform.

(7) Always the lid of petridish is replaced after each manipulation.

(8) The closure (cotton plug) from a culture tube is removed and flamed the uppermost 20 mm of the open end. While holding the tube at an angle of 45°, an explants is transferred using forceps onto the surface of the agarified nutrient medium. Nutrient medium is Gamborg's B_5 or MS medium supplemented with 0.5 mg/L 2, 4-D.

(9) The closure is immediately placed on the open mouth of each tube. The forceps are always flamed before and after use. Date, medium and name of the plant are written on the culture tube by a glass marking pen or pencil.

(10) Culture tubes after inoculation are taken to the culture room where they are placed in the racks. Cultures are incubated in dark at 25°C.

(11) Usually, after 4 weeks in culture the ex- plants incubated on medium with 2, 4-D will form a substantial callus. The whole callus mass is taken out aseptically on a sterile petridish and should be divided into two or three pieces.

(12) Each piece of callus tissue is transferred to a tube containing fresh same medium.

(13) Prolonged culture of carrot tissue produces large calluses.

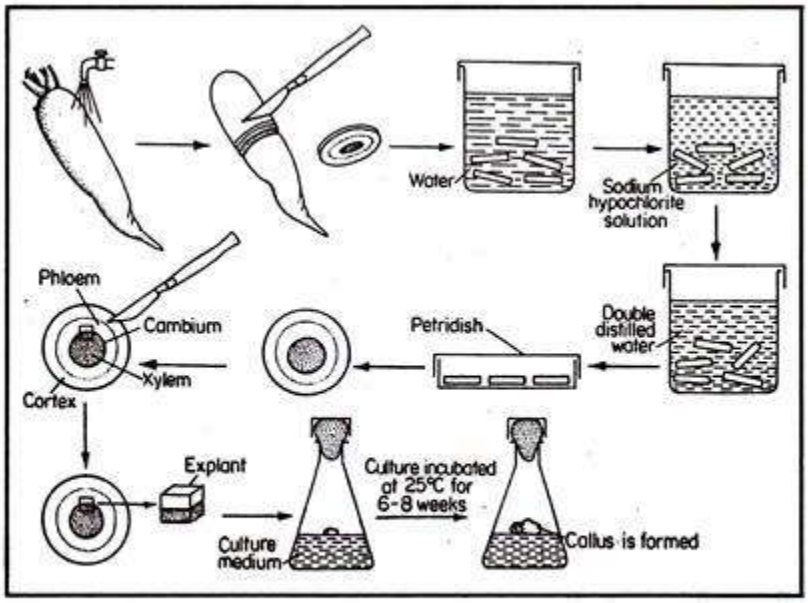


Fig 3.1

Procedure for the callus culture from carrot root

Significance of Callus Culture:

Callus culture as such has no major importance unless and until it is used for other experimental objectives.

Still, callus culture has got some importance:

(i) The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium. This phenomenon is known as plant regeneration or organogenesis or morphogenesis.

Similarly, by manipulation of nutrient and hormonal constituents, cluster of embryos can be achieved directly from the somatic cells of callus tissue. These embryos are called somatic embryos. This phenomenon is known as somatic embryogenesis. Somatic embryo directly gives rise the whole plant.

(ii) Callus tissue is good source of genetic or karyotype variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

(iii) Cell suspension culture in moving liquid medium can be initiated from callus culture.

(iv) Callus culture is very useful to obtain commercially important secondary metabolites. If a bit of tissue from a medicinally important plant is grown in vitro and produced callus culture, then secondary metabolites or drug can be directly extracted from the callus tissue without sacrificing the whole plant. So, this alternative technique helps the conservation of medicinal plants in nature.

(v) Several biochemical assays can be performed from callus culture.

NODAL CULTURE / AXILLARY BUD CULTURE

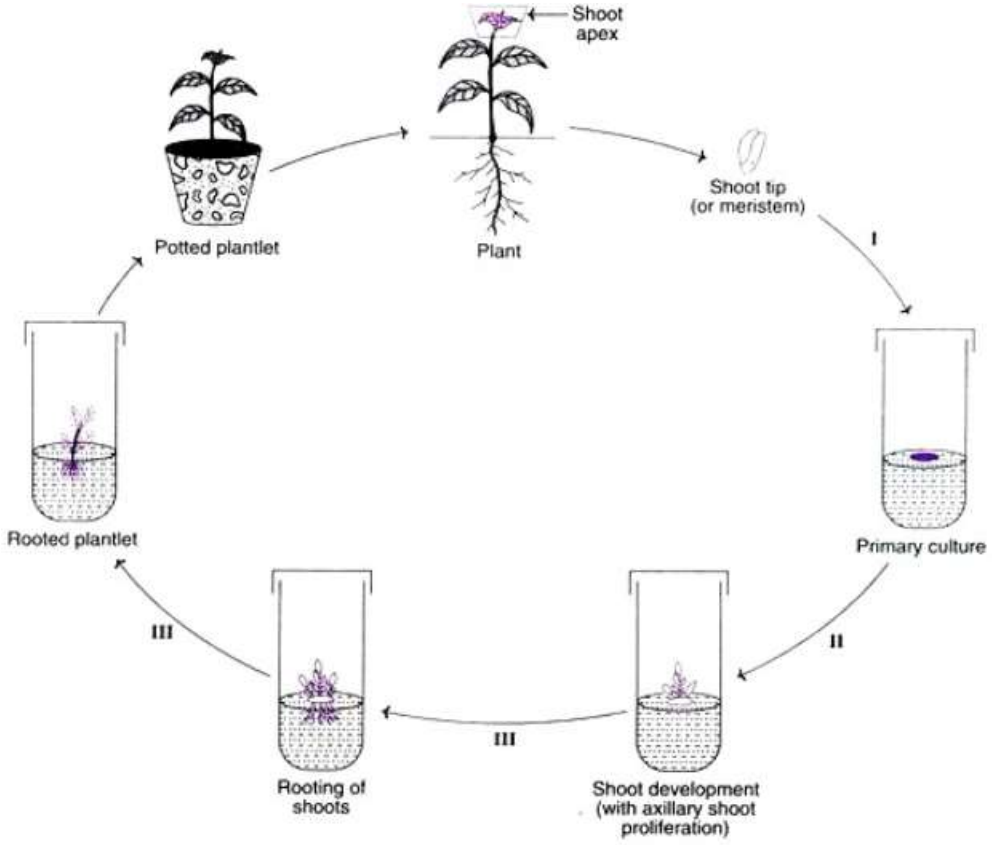
Micropropagation through proliferation of axillary buds is a common technique in in vitro multiplication of forest trees. Axillary bud proliferation approach typically results in many fold increase in shoot number. Each culture passage makes feasible to obtain as many as possible propagules from a single explant and they are true-to-type. The induction of axillary bud proliferation seems to be applicable as a means of micropropagation in many woody forest trees.

MERISTEM CULTURE

Meristem (type of tissue) consists of an undifferentiated group of cells capable of dividing throughout their life. These cells produce new cells that can stretch, differentiate, and enlarge as they mature. Meristematic tissue is present at several locations in the plants. In roots and shoots, meristem is present at the tip and called root apical meristem and shoot apical meristem. Meristem culture is used for shoot apical meristem culture in vitro. Meristem culture was developed by Morel and Martin in 1952 for rivers eliminating from Dahlia. Orchid Cymbidium was micropropagated using meristem culture by Morel in 1965. An already existing shoot meristem grows in the meristem culture and adventitious roots regenerate from these shoots. In the shoot tip beyond the youngest leaf lies the primordium meristem. It measures up to 250 mm in length and 100 mm in diameter. In addition to the apical meristem one or three leaf primordia would be present in a shoot tip of 100–500 nm. When virus elimination is the objective, to obtain disease-free plants, shoot tips of up to 10 mm are used. For rapid clonal propagation, a shoot-tip culture is followed in which (5–10 mm) explants are used. Hence, the majority of meristem culture are essentially shoot-tip cultures. Various sized nodal explants are also employed for rapid clonal propagation. The size of the shoot tip used for culture is not important when the main objective is micropropagation. But when the objective is to obtain virus-free stock (or stock free from other pathogens) it is necessary for the excision of the apical meristem to be done with a minimum of the surrounding tissue. The shoot tip may be cut into fine pieces in order to obtain more than one plantlet from each shoot tip. Pieces of curd (the inflorescence) are used in some species such as cauliflower. Shoot tips or tissue pieces bearing buds of such stems may be used for those plants having an underground stem. Explants taken from actively growing plants early in the growing season are the most suitable. Explants of meristem are then placed on Murashige and Skoog's (MS) medium, which is considered to be an effective medium for the majority of species. Lower salt concentration is suitable for some species. Fungicides (bavistin) or antibiotics (chloramphenicol/streptomycin) can be added to the medium during growth to remove the endophytic contamination. Similarly, meristem culture follows steps similar to micropropagation: (i) initiation of culture, (ii) shoot multiplication, (iii) rooting of the developed shoots, and (iv) transfer of the plantlets to the pots or soil.

SHOOT-TIP CULTURE

Explants/shoot tips consist of shoot apical meristem, unexpanded leaves at various development stages, and a number of leaf primordia about 1 cm in length. In shoot-tip culture the explants are inoculated in cytokinin-supplemented media. Suppression of apical dominance is caused by cytokinin and a highly branched shoot system formation is facilitated. Then manipulation of the shootlets is done in the rooting medium to develop plantlets



SOMACLONAL VARIATION

Somaclonal variation is defined as genetic or epigenetic changes that arise *in vitro* between clonal regenerants and their corresponding donor plants. The genetic changes are cytogenetic abnormalities and alterations to specific sequences of DNA; epigenetic changes are alterations of gene expression without changes to DNA sequences. Somaclonal variation, independent from the mechanisms involved, has been reported for a number of plant species. The occurrence of somaclonal variation in tissue culture has a negative effect on the rapid production of clonal plants of elite cultivars, but may promote the production of novel horticultural

crop genotypes. An improved understanding of the mechanisms of variation in tissue culture, specifically epigenetic variation, may be a potential tool in producing cultivars adapted to meet the growing demand for food.

The term somaclonal variation by **Larkin** and **Scowcroft (1981**) was given for the variability generated by the use of a tissue culture cycle. Somaclonal variation is defined as genetic variation observed among progeny plants obtained after somatic tissue culture in vitro. Theoretically all progeny plants regenerated from somatic cells should be identical clones. However, variations might occur in number of progeny which are known as somaclones and they are genetically variable from their explant. The initiating explant for a tissue culture cycle may come virtually from any plant organ or cell type including embryos, microspores, roots, leaves and protoplasts. So, all somatic tissue culture can result in somaclonal variation. Somaclonal variation is a phenotypic changes as a result of chromosomal rearrangement during tissue culture.

Basis of somaclonal variation:

- Following are some of the basis of chromosomal rearrangement which results in somaclonal variation

    1. **Karyotype changes**
    2. **Changes in chromosome structure**
    3. **Single gene mutations**
    4. **Cytoplasmic genetic changes**
    5. **Mitotic crossing over**
    6. **Gene amplification and nuclear changes**
    7. **Transposable elements**
    8. **DNA methylation**

Somaclonal variation is highly exploited in plant breeding programs

- Production of agronomically useful plants
- Resistance to diseases
- Resistance to abiotic stresses
- Resistance to herbicides
- Improved seed quality.

SUSPENSION CULTURE

The suspension culture method involves the formation of suspension by the multiplication of single cells or aggregates when agitated in an aerated and sterile liquid medium. The two types of suspension culture include batch culture and continuous culture.

Principle:

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

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

This suspension can then be propagated by regular sub-culture of an aliquot to fresh medium. Ideally suspension culture should consist of only single cells which are physiologically and biochemically uniform. Although this ideal culture has yet to be achieved, but it can be achieved if it is possible to synchronize the process of cell division, enlargement and differentiation within the cell population.

The culture of single cells and cell aggregates in moving liquid medium can be handled as the culture of microbes. The suspension culture eliminates many of the disadvantages ascribed to the callus culture on agar medium. Movement of the 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.

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 alluminium 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 haemocytometer 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.

Importance of Cell Suspension Culture:

The culture of single cells and small aggregates in moving liquid medium is an important experimental technique for a lot of studies that are not correctly possible to do from the callus culture. Such a system is capable of contributing many significant information's about cell physiology, biochemistry, metabolic events at the level of individual cells and small cell aggregates.

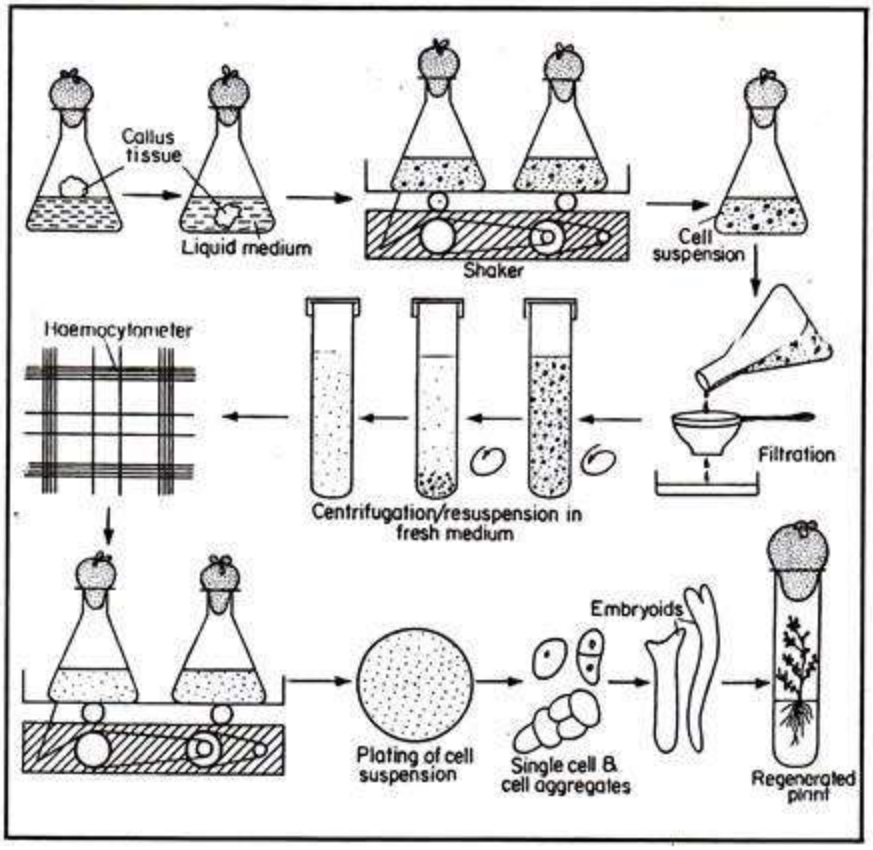
It is also important to build up an understanding of an organ formation or embryoid formation starting from single cell or small cell aggregates. The technique of plating out cell suspension on agar plates is of particular value where attempts are being made to obtain single cell clones.

Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids and a considerable amount of industrial effort is being placed on the exploitation and expansion of this area.

Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised. Cell population in a suspension can be subjected to a range of mutagenic chemicals e.g. ethyl methane-sulphonate (EMS), N-nitroso-N-methyl urea etc.

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 hope is that permanent changes in the DNA patterns of some of the cells would be achieved by such treatments.

Plants could be raised from the mutant cell clones and the mutant plants are selected from the population either by morphological differences or by metabolic/biochemical differences. The selected plants can then be grown on and propagated further to produce a mutant population for evaluation studies.



❒ Fig 4.1

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

APPLICATIONS OF MICROPROPAGATION

• Plant tissue in small amounts is sufficient for the production of millions of clones in a year using micropropagation. It would take a great deal of time to produce an equal number of plants using conventional methods.

• The technique of micropropagation provides a good alternative for those plant species that show resistance to practices of conventional bulk propagation.

• An alternative method of vegetative propagation for mass propagation is offered through micropropagation. Plants in large numbers can be produced in a short period. Any particular variety may be produced in large quantities and the time to develop new varieties is reduced by 50%.

• Large amounts of plants can be maintained in small spaces. This helps to save endangered species and the storage of germplasm.

• The micropropagation method produces plants free of diseases. Hence, disease-free varieties are obtained through this technique by using meristem tip culture.

• Proliferation of in vitro stocks can be done at any time of the year. Also, a nursery can produce fruit, ornamental, and tree species throughout the year.

• Increased yield of plants and increased vigor in floriculture species are achieved.

• Fast international exchange of plant material without the risk of disease introduction is provided. The time required for quarantine is lessened by this method.

• The micropropagation technique is also useful for seed production in certain crops as the requirement of genetic conservation to a high degree is important for seed production.

• Through somatic embryogenesis production of synthetic artificial seeds is becoming popular nowadays.