Tissue Culture

Introduction :-

Def.: "The technique of growing (culturing) plant cells or tissues in an artificial medium to regenerate the entire plant under aseptic conditions is called as tissue culture". The artificial medium contains macro- and micro-elements, sugar, vitamins and growth hormones. This technique has helped to produce large number of plants varieties of plants within a short time.

Haberlandt (1902) was a pioneer of tissue culture. He successfully cultured somatic cells of higher plants in simple nutrients solutions. Tissue culture technique has wide range of applications in agriculture, floriculture, horticulture, industries, forestry, plant breeding, etc. Tissue culture means 'in vitro' culture of plant cells.

For about 32 years after Haberlandt (upto 1934), little further progress in cell culture research was made, although culture of embryos, roots and other tissues was achieved in this period. During 1934-1939, due to discovery of importance of auxins & B-vitamins, the foundation of plant tissue culture was laid down by three scientists. ie. Gautheret, White & Nobecourt. During next 20 years (1940-1960) a variety of chemicals (hormones, vitamins, etc.) were identified for their effect on cell division, growth and differentiation. In India, the research in plant tissue culture was started in 1960 at the Botany Department of Delhi University.

Consequently, media and culture techniques for a variety of plant materials became known and extensively utilized. Plant tissue culture research has become a thrust area during the last decade.

★ Basic aspects of tissue culture laboratory :-

Culture of plant cells and tissues in vitro is not an easy task. A good laboratory is required which must have following facilities :-

(1) Washing & storage facilities :- A separate area is required which should have large sink with provision for hot & cold running water, distillation apparatus,

(1) Media preparation room :- In this room, there should be provision of for bench space for chemicals, labware, culture vessels, and equipments required for media preparation. This lab. also contains oven, autoclave, hot plates & stirrer, pH meter, balance, waterbath, burners, refrigerator, etc.

(2) Culture room :- In culture room, temp. & humidity are maintained & cultures are grown. The culture room should have racks for placing the culture tubes or conical flasks. Shaking machine for growing suspension culture should also be present.

(3) Washing & Sterilization :- A separate area is required for sterilization washing the glassware. The culture media are sterilized

in autoclave at 121 °C for 20 minutes. The instruments such as forceps, scalpels, needles, spatula, etc. are sterilized by dipping in 95% alcohol. The glasswares are sterilized in hot air oven at 160-180 °C for 2-4 hours.

The plant material is surface sterilized using sodium hypochloride (1% (0.1%)) (NaOCl) or mercuric chloride ($HgCl_2$) (0.2%) & washed 6-10 times with sterilized distilled water.

✱ Technique of tissue culture (Steps) :-

(1) Preparation of suitable culture medium :-

(a) Inorganic nutrients :- The inorganic nutrients like carbon, hydrogen, nitrogen, calcium, potassium, phosphorus, sulphur & magnesium are required in large amount & behave as macronutrients, while boron, molybdenum, copper, zinc, manganese, iron & chloride are required in small amount & behave as micronutrients.

(b) Organic nutrients :- Sucrose & D-glucose are commonly used, but glycerol & myoinsitol are also the principal source of carbon. Other org. compounds are peptone, malt extract, tomato juice, yeast extract, malt extract; etc. coconut milk, corn milk, etc. are also used.

(c) Growth hormones :- The growth hormones included in culture media involve auxins, cytokinines & gibberellins. The auxins facilitate cell-division & root differentiation. Commonly used auxins are IBA (Indole-3-Butyric Acid), & NAA (Naphthalene Acetic Acid). IAA & IBA are used for rooting & for shoot elongation. cytokinines facilitate cell-div^{n} & differentiation.

The most widely used cytokinines are kinetin, zeatin, etc. Gibberellins are of less importance.

(d) Agar :- It is polysaccharide obtained from red alga (ie. a sea weed Gelidium amansii). It is used to solidify the medium. Generally 0.5 - 1% agar is used. In liquid medium, due to submerged condition, the tissue may die. So, it is solidified.

Media preparation :- Dry powdered media of different constituents are available commercially. This powder is dissolved in small quantity of distilled water. Other desired supplements are added and final volume is made using distilled water. pH is adjusted & agar is added. The pH is adjusted bet^{n} 5 to 6 by adding 0.1 N NaOH or 0.1 N HCl. The culture medium is sterilized in an autoclave at 121 °C / 20 min.

Generally, MS medium is used for tissue culture.

(2) Selection & sterilization of explants :-

The explants are any part of plant to be used in tissue culture. (eg :- axillary bud, leaf & stem segments, root tip, shoot tip, anther, ovary, endosperm etc.). The explants are sterilized by 1% Sodium hypochloride (NaOCl) or 0.2% mercuric chloride ($HgCl_2$) & washed 6 - 10 times with sterile distilled water.

(3) Inoculation :- The sterile plant part (explant) is inoculated on the surface of solidified sterile nutrient medium under aseptic conditions.

(4) Incubation :- The cultures are incubated in the tissue culture room at 25 ± 2°C temp; 50 - 60% relative humidity & 16 hours of photoperiod. After the defined period, callus develops on the medium & shoots / roots develop from the explant.

(5) Regeneration :- A portion of callus is transferred to another medium. There is induction of roots and shoots from it. This medium contains different grown hormones in it. Plantlets are produced in this medium.

(6) Hardening and Acclimatization :- Hardening is the gradual exposure of plantlets for acclimatization to environmental conditions. This is the most important stage in tissue culture. The plants grown in tissue culture grow in luxury and become adapted to such controlled environment. Sucrose is provided as a source of carbon and hence negligible photosynthesis takes place. Leaves may lack cuticle. Temperature and all essential conditions are controlled in tissue culture plants. Such plants, when transferred to open environment, they can not face it and may die. In order to face this problem, such plants after their removal from rooting medium, are transferred in culture bottles containing soil which is moistened with nutrients. This is called hardening. Such plants develop photosynthetic activity & become autotrophic. Now, these plants are shifted to greenhouse and transferred to polybags containing normal garden soil. Such plants in polybags adapt to greenhouse environment and become suitable for transfer to nursery or field. This is called as acclimatization.

(7) Plantlet transfer :- After hardening process, the plantlets are transferred to field conditions.

* Concept of totipotency of cell. :-

Each living cell of a multicellular organism, is capable of independent development when provided with suitable conditions. In 1901, T. H. Morgan coined the term 'totipotency' to denote this capacity of cell to develop into an organism by regeneration. In other words, totipotency is the ability of a somatic cell of a plant to produce a new complete plant." Haberlandt claimed that, every living cell of a plant is totipotent. The concept of totipotency is important in tissue culture.

However, for the first time, somatic embryos could be produced in carrot cell suspensions & the results were presented independently by F. C. Steward (USA) & Jacob Reinert (Germany) in 1958. In their expt., carrot root tissues were inoculated on media supplemented with auxin & coconut milk. The cells divided profusely & gave rise to embryogenic cultures, which later converted into somatic embryos. The embryos successfully gave rise, to carrot plants.

* Callus culture :-

Cell from any plant species can be cultured asceptically on a nutrient medium. After planting a sterilized explant on an agar medium, within 2-4 weeks, depending upon the plant species, an undifferentiated (unorganized) mass of cells is produced. It is called as callus. Such a callus can be sub-cultured by transferring a small piece on to the fresh agar medium.

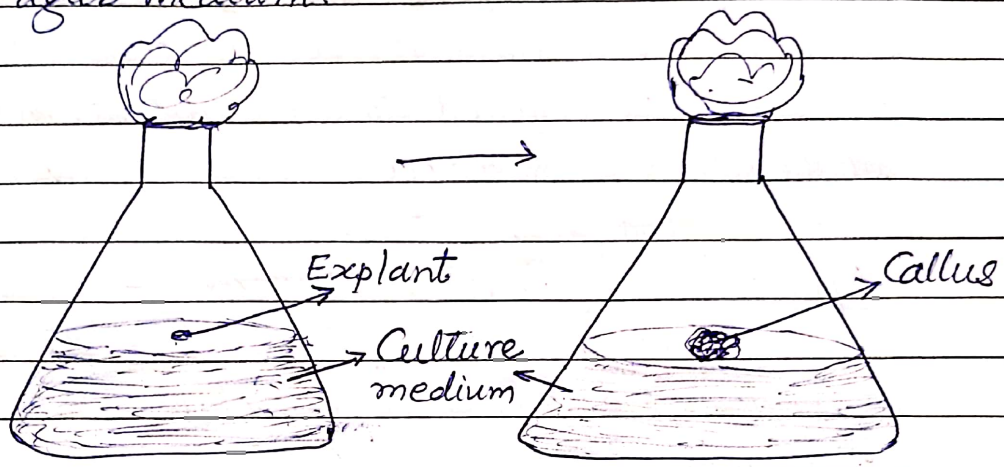


Fig. : Formation of callus.

A suitable-sized and sterilized explant is transferred asceptibally under a laminar air flow bench on to an appropriate nutrient medium (for example, MS medium or B5 medium) containing appropriate combination of plant growth regulators. It is incubated at 25-28 °C in an alternate light & dark period as required. Within 2-4 weeks, the upper surface of explant is covered with an undifferentiated mass of cells, called callus. The callus is produced from outer layers of cortical cells in a stem explant by repetative

divisions of cells. These dividing cells generate a pressure on epidermis, which ultimately ruptures exposing the newly formed callus. This callus is divided into small segments & each segment is transferred to fresh culture medium. This is called sub-culturing.

Callus cultures are slow growing system as compared to cell ~~culture~~ suspension cultures. Cells grow as clumps or masses in callus cultures & only lower cells are in contact with the medium, whereas, cells in upper layers get their nutrients from cells in lower layers. Cells are in close association as compared to cell cultures, in which all the cells are in direct contact with the medium and dissociated.

Applications of callus cultures :-

Callus cultures may be used to study the following aspects of plant metabolism and differentiation :-

(i) Nutrition of plants.
(ii) Cell & organ differentiation & morphogenesis.
(iii) Developing cell-suspension cultures and protoplast cultures.
(iv) In the production of secondary metabolites.

Cell Suspension Culture :-

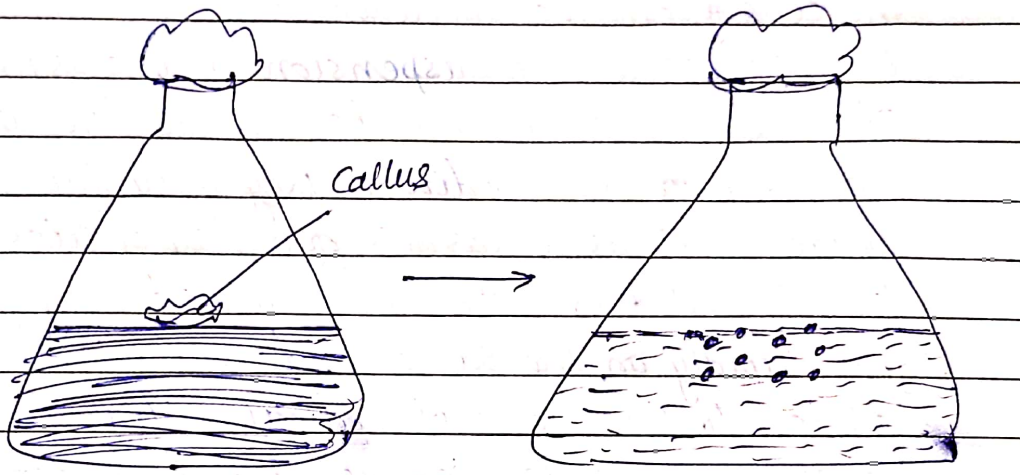
Cell suspension cultures are initiated by transferring the friable callus to liquid nutrient medium. In liquid medium, plant tissues remain submerged which leads to anaerobic conditions and ultimately death of the cells. Therefore, such cultures are agitated on rotary shaker at 80-150 rpm. Agitation serves both to aerate the cultures and to disperse the cells. The cells from the inoculum are separated during this process and a suspension of cells is produced. If relatively small number of cells are transferred to a new medium, they may fail to divide, whereas a larger quantity of tissue transferred from the same culture may proliferate rapidly on the same medium. This observation has led to the concept of critical initial cell density. This is defined as, "the smallest inoculum per volume of medium, from which a new culture can be reproducibly grown." The critical initial cell density is determined by following factors -

(a) The culture's physiological characteristics.
(b) The length of time and conditions under which the culture was previously maintained.
(c) The composition of the fresh medium.

The cell cultures are sub-cultured by dilution of the stock culture, 5-10 times V/V (volume by volume), depending upon the growth of cells. Growth of cell suspension culture is always higher than callus cultures & therefore, require rapid sub-culture (7-21 days) as compared to callus cultures (4-8 weeks).

Applications of cell suspension culture :-

(i) It offers opportunities in the study of single cells & group of cells.
(ii) Protoplast can be isolated.
(iii) Nutrition can be studied.
(iv) Cell-differentiation can be studied.
(v) Secondary metabolites can be obtained.
(vi) The process of cell-division & factors affecting cell-division can be studied.



Callus culture Cell suspension culture

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* Differentiation and morphogenesis :-

The callus cells are parenchymatous. The differentiation of those cells is required for the formation of various tissues and organs. It has been observed that, auxins play important role in vascular differentiation & cytokinines and gibberellins promote differentiation into xylem tissue.

For the regeneration of whole plant from callus mass, cellular differentiation is not sufficient & there is differentiation leading to organ formation (eg.: stem, roots, etc.). Such differentiation into various plant organs is called as morphogenesis.

* Applications of tissue culture :-

(1) Micropropagation :- (Diagram P.No. 24)

The plant tissue culture made possible large scale cloning of plant species. The clonal propagation through tissue culture technique is known as micropropagation. It is vegetative propagation as it involves only mitotic cell divisions. Progeny obtained by vegetative propagation (or asexual reproduction) of a single plant constitute a clone. Micro-propagation is also called as clonal propagation.

~~Regeneration of plantlets~~

The most common technique of micro-propagation is to place excised meristematic tissue on a medium which encourages axillary bud development. The new shoots can be separated and sub-cultured to produce more axillary shoots, or placed on a medium that encourages rooting & planted out. Alternatively tissue can be used to establish callus cultures, which may be induced to form roots & shoots.

Micropropagation can also be an effective way to eliminate viruses & other pathogens & produce commercial quantities of pathogen-free propagules. The technique has been useful for potato, lilies, tulips & other species that are normally propagated vegetatively. There is large scale micropropagation of important trees yielding fuel, pulp, timber, oils or fruits. However, the most important advantage of micropropagation is the rapid multiplication of medicinal plants & trees which grow slowly or which can not be stored by conventional methods.

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Micropropagation :-

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Asexual multiplication of plants in a small area of glass vessels (in vitro) under controlled conditions is called micropropagation. OR. Mulliplication of plants by tissue culture method is called as micropropagation. It is also called clonal propagation.

Method of micropropagation :-

The following stages are involved in microprop^n :

a) Management of donor plants (Source of explant): The donor plants should be maintained in clean environment or greenhouse & explant obtained from them are comparitively better than those obtained from open field conditions.

b) Establishment of aseptic cultures :- The explant should be sterilized to get aseptic culture.

c) Multiplication of shoots :- The shoots obtained by culturing the explant are multiplied by repeated sub-culturing.

d) Induction of roots. : Shoots of suitable size are excised from shoot cultures & inoculated on a medium containing root-inducing hormones. The auxins like IBA (Indole butyric acid), NAA (Naphthalene acetic acid) & IAA (Indole acetic acid) are mostly used for induction of roots.

e) Hardening, acclimatization & transfer of plants in soil: This is most important stage in micropropagation. The plants grown in tissue culture live in luxury & get adapted to such controlled envt. Such plants when transferred to open envt., they can't face it & die. In order to solve this problem, after their removal from rooting medium, the plants are transferred to culture bottles containing soil, which is moistened with nutrients. This is called hardening. Such plants develop photosynthetic activity & become autotrophic.

Now, these plants are shifted to greenhouse and finally to polybags containing normal garden soil. Such plants in polybags adapt to greenhouse envt. & become suitable for transfer to their field or nursery. This is called acclimatization. Adopting the above procedures, a large number of tissue culture plants can be established in soil.

Importance of Micropropagation :—

1) Very small amount of explant is needed for generation of millions of clonal plants in a year.
2) Valuable germplasm can be stored for long time.
3) Multiplication rate is very high.
4) Plants are easily exported due to their required size and quality.
5) Micropropagation can be carried out throughout the year.
6) It is used to multiply threatened & ~~extict~~ extinct plant species.

(2) Production of disease-free plants :—

(Diagram P. No. 240)

Many plant species which propagate vegetatively are infected by viruses, bacteria & fungi. In order to ensure highest possible yield & quality, it is necessary to provide disease-free plants to growers.

The process of tissue culture is carried under aseptic conditions. The choice of explant is done carefully from shoot apex which is not infected by viruses or other disease-causing pathogens & therefore the new plants obtained by tissue culture are disease-free.

The disease-free plants can be easily obtained by tissue culture technique because explants chosen are disease-free & all steps of tissue culture are carried under aseptic conditions.

Though such disease-free plants can be obtained by tissue culture, they are not disease-resistant & pathogens can attack them when they are planted in the open environment.

(3) Production of secondary metabolites :-

The plant cells contain sugars, phosphates, amino acids, lipids, proteins & nucleic acids. These comprise the basic machinery of the cell & called primary metabolites. However, the cells may also synthesize variety of other organic molecules which are not essential for their survival. These molecules are called secondary metabolites. These include alkaloids, glycosides, steroids, terpenoids & variety of flavours.

It is found that, many secondary metabolites have significant role in pharma-ceutical, agrochemical & cosmetic industries.

Secondary metabolites can be commer-cially obtained through callus culture. The cells secrete their metabolites in the medium which can be harvested & used in industries.

(4) Anther culture and production of haploids :-

The most popular method of production of haploid (n) plants is through culturing of anthers or microspores on artificial culture medium. This leads to the growth of microspores (pollen grains) into gametophytic (haploid) plants. In 1964, Guha & Maheshwari successfully produced haploid Datura plant by anther culture technique. Haploids of more than 150 plant species belonging to 23 families have been obtained by this technique.

During anther culture, flower buds are brought to the laminar air flow chamber & sterilized using appropriate chemical treatment

The anthers are removed & cultured on solid medium where they directly give rise to embryoids or may lead to callus formation. The embryoids develop into haploid plantlets, which are colchicine-treated to get diploid homozygous plants.

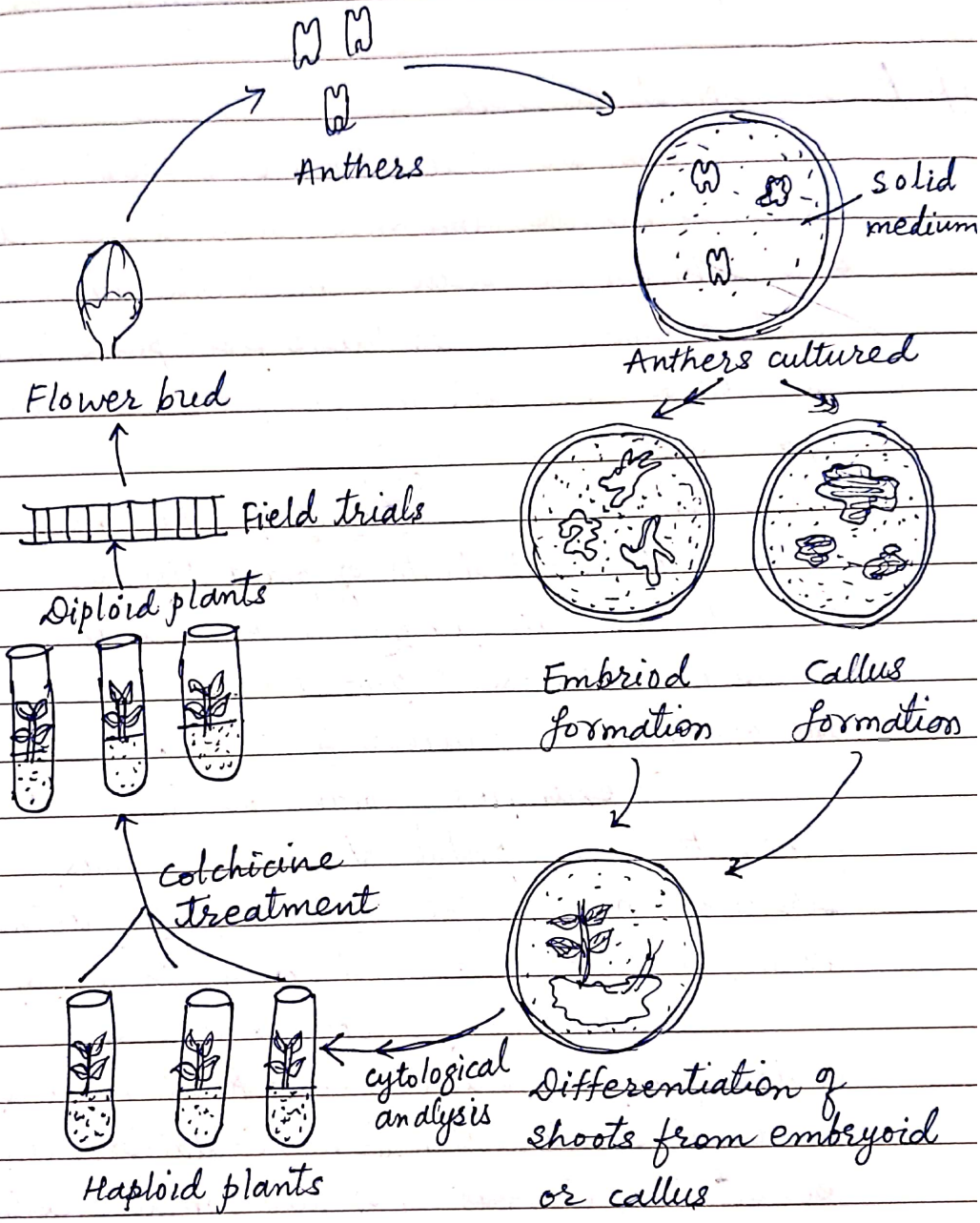


Fig. : Steps in anther culture.

Use of haploid plants :—

i) Use of haploid plants in plant breeding is most significant. The haploids are made diploid by

colchicine treatment. These diploids are genetically very pure & used for hybridization.

ii) The haploids are also useful in mutation research, cytogenetic research, evolutionary studies, genetic studies, etc.

(5) Protoplast culture & Somatic hybridization :-

(A) Protoplast culture :-

Protoplasts (cell minus cell-wall) is the biologically active and most significant material of cell. When cell-wall is mechanically or enzymatically removed, the isolated protoplast is called as "naked plant cell". The protoplasts are cultured on appropriate culture media.

In 1960, E. C. Cocking for the first time isolated the protoplasts of plant tissues by using cell-wall degrading enzymes. viz: cellulase, hemi-cellulase, pectinase & protease. Later on, the protoplasts were cultured in vitro.

The examples of plant species that have generated from protoplasts are Cucumis sativus (ककड़ी), Capsicum annum (मिरची), Beta vulgaris (बीट), Helianthus annus (सूर्यफूल), Glycine max (सोयाबीन), Rosa sp. (गुलाब), Chrysanthemum sp. (सुवर्णफूल), etc.

The isolated protoplasts are used for various purposes, such as – (i) Biochemical & metabolic studies (ii) genetic manipulation, (iii) drug sensitivity, (iv) fusion of two somatic cells to get somatic hybrids, (v) fusion of nucleated and enucleated cells to produce cybrids.

(a) Isolation of protoplast :-

(i) Surface sterilization of leaves :- Fully expanded leaves are obtained from the healthy plant & are sterilized by dipping them into 70% ethyl alcohol for about a minute & then treating them with 2% solution of sodium hypo-chlorite for about 20-30 minutes. The leaves are then rinsed three times with sterile distilled water & the subsequent operations are carried out under aseptic conditions (under laminar air flow chamber).

(ii) Peeling of lower epidermis :- The lower epidermis of sterilized leaves is carefully peeled off & the leaves are cut into small pieces. Mesophyll protoplasts can be obtained from these peeled leaf segments. In cereals, where it is difficult to peel off epidermis, leaves are cut into long strips & used with enzyme mixture. When the starting material is an *in vitro* shoot culture, the sterilization step can be omitted & leaves can be cut into pieces & used directly for enzymatic treatment.

(iii) Isolation of protoplast :- The peeled leaf segments are transferred to the enzyme mixture (cellulase & pectinase). This leads to penetration of the enzyme into the tissue within 12-18 hours at 25°C. The protoplasts isolated as above are present alongwith variety of cell-debries and broken cell-organelles. So, the protoplast should be purified.

(iv) Purification of protoplasts :- Crude protoplast suspension is centrifuged at low speed (500-100 g for 5 min.). The protoplasts form a pellet & supernatant containing cell-debries can be pipetted off. The pellet is gently resuspended in fresh culture medium plus mannitol and rewashed. The process is repeated 2-3 times to get clean protoplast suspension.

(b) Protoplast culture & regeneration :- One ml protoplast suspension is poured on sterile & cooled culture medium in petriplates & incubated at 25°C in a dim white light. The protoplasts regenerated cell-wall, undergoes cell-division and form a callus. The callus can be sub-cultured. When callus is transferred on suitable medium, it develops into seedling.

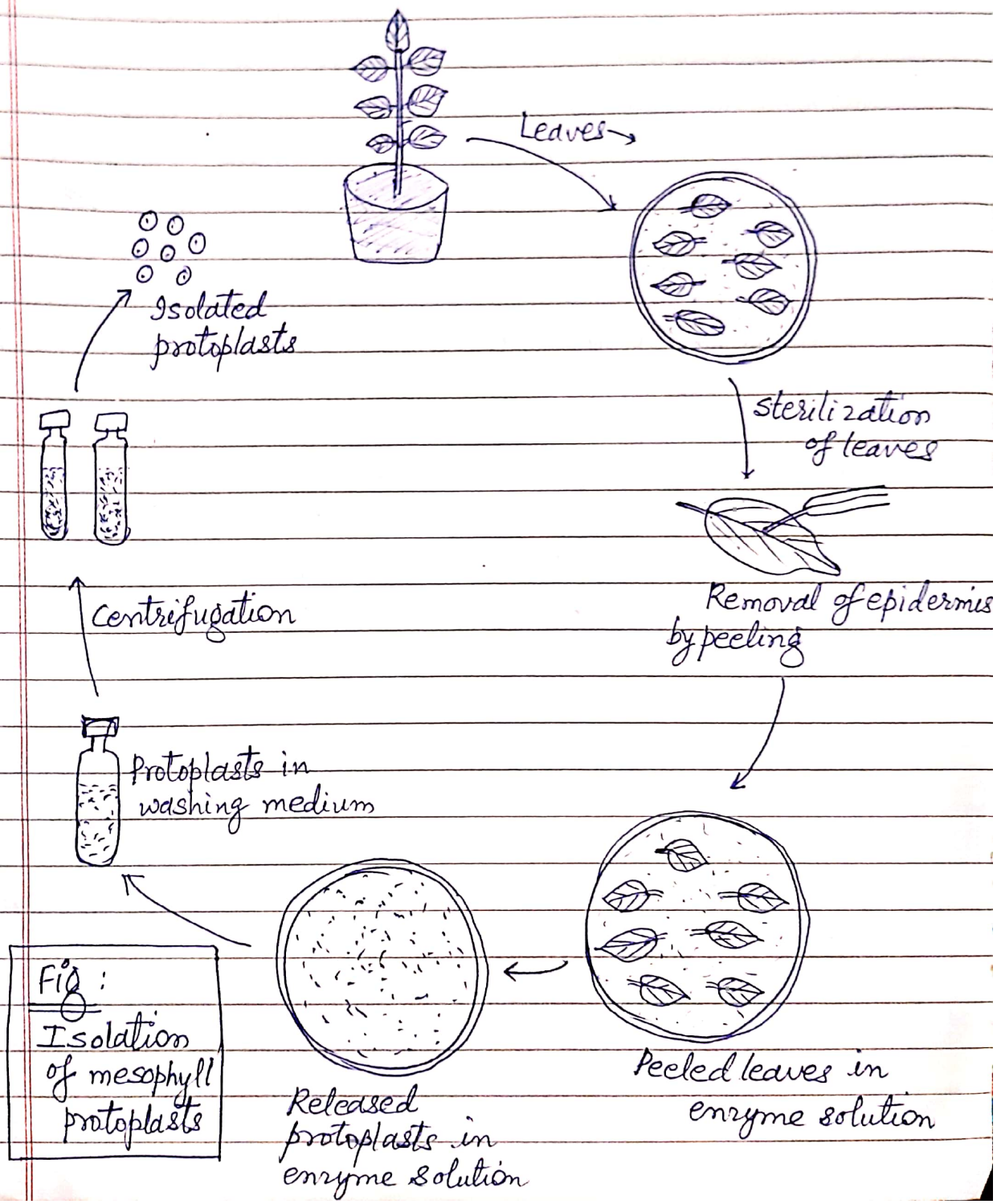


Fig : Isolation of mesophyll protoplasts

(B)

Protoplast fusion & Somatic hybridization :-

Protoplast fusion is a new tool of genetic manipulation of plants. The fusion of protoplasts of genetically different lines or species has also been possible. Somatic hybridization of crop plants is a new challenge to plant breeding & crop improvement. Somatic hybridization allows the production of hybrids between the lines and species, that can not be produced normally by means of sexual hybridization.

Procedure for somatic hybridization :-

(i) Isolation of protoplasts from suitable plants.
(ii) Mixing of protoplasts in centrifuge tube containing fusigenic chemicals, ie. chemicals promoting protoplast fusion, such as polyethylene glycol (PEG) / Sodium nitrate ($NaNO_3$), maintenance of high pH 10.5 & temp. 37°C. As a result of the protoplast fusion, heterokaryons are produced.
(iii) Wall regeneration by heterokaryotic cells.
(iv) Fusion of nuclei of heterokaryons to produce hybrid cells.
(iv) Plating & production of colonies of hybrid cells.
(v) Sub-culturing & induction of organogenesis.
(vi) Transfer of mature plants from the regenerated callus.

Fusion of cytoplasm of two protoplasts results in coalescence of cytoplasm. The nuclei of two protoplasts may or may not fuse together even after fusion of cytoplasms. The binucleate cells are known as heterokaryons. When nuclei are fused, the cells are known as hybrids., and when only cytoplasms fuse & genetic information from one of the two nuclei is lost, the cells are called cybrid. ie. cytoplasmic hybrid.

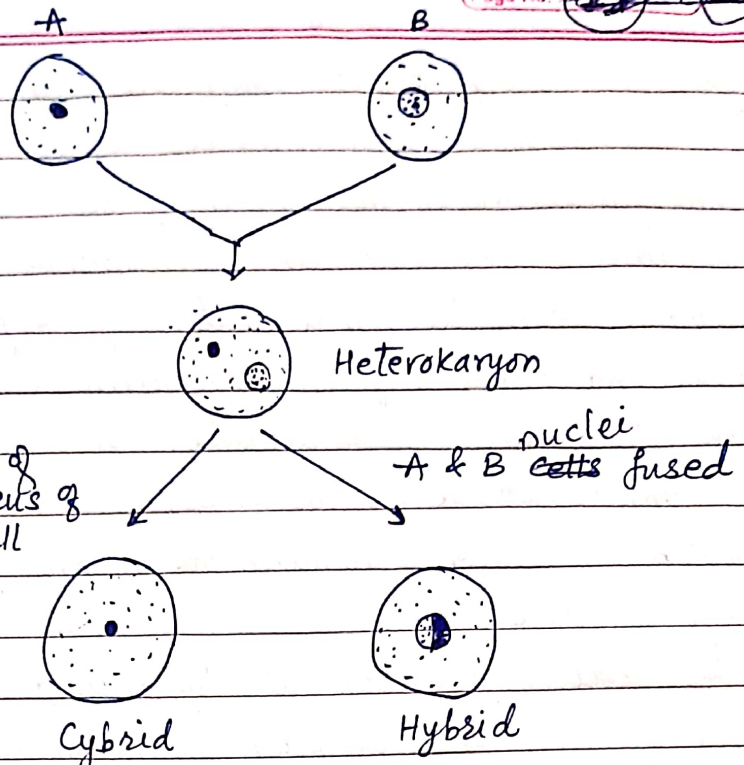


Fig: Hybrid/Cybrid prodⁿ thro' proto. fusion.

The examples of somatic hybrids are pomato (potato + tomato), Datuzotropa (Datura + Atropa), barley + wheat, wheat + oat, sugarcane + Sorghum, etc.

* Applications of protoplast fusion/Somatic hybridⁿ

(i) Production of fertile somatic hybrids of sexually incompatible species is achieved.

(ii) Transfer of only a part of genetic information from one species to another using the phenomenon of chromosome elimination.

(iii) Transfer of cytoplasmic genetic information from one to the second line or species.

(iv) It has been possible to transfer useful genes (nif genes, disease resistance genes) from one species to other.

(6) Synthetic Seeds / Artificial seeds:-

(Diagram – P. No. 25)

- The concept of synthetic seed was firstly given by T. Murashige (USA) in 1977.
- The synthetic seed has been defined as, "A somatic embryo encapsulated inside a protecting gel (coating)."
- In these ~~ee~~ seeds, gel acts as seed-coat and also provides nutrient as endosperm in the seed.
- The gel used to coat is water-soluble.
- Usually Na/Ca alginate (a product of algae) is used to encapsulate, because it is less toxic to embryo.
- In the conventional plant tissue culture for clonal propagation, storage & transportation of propagules for transplantation is a major problem.
- To overcome this problem, in recent years, the concept of synthetic/artificial seeds has become popular.
- In India, this technique of synthetic seeds is being standardized & practiced for sandlewood & mulberry at BARC (Mumbai) under the leadership of Dr. P.S. Rao.

Advantages of synthetic seeds :-

1) They can be stored upto a year without loss of viability.
2) They are easy to handle.
3) They can be directly sown in soil like natural seeds & do not need hardening in greenhouse.

The only limitation of synthetic seeds is high cost of ~~their~~ production.

Micropropagation :-

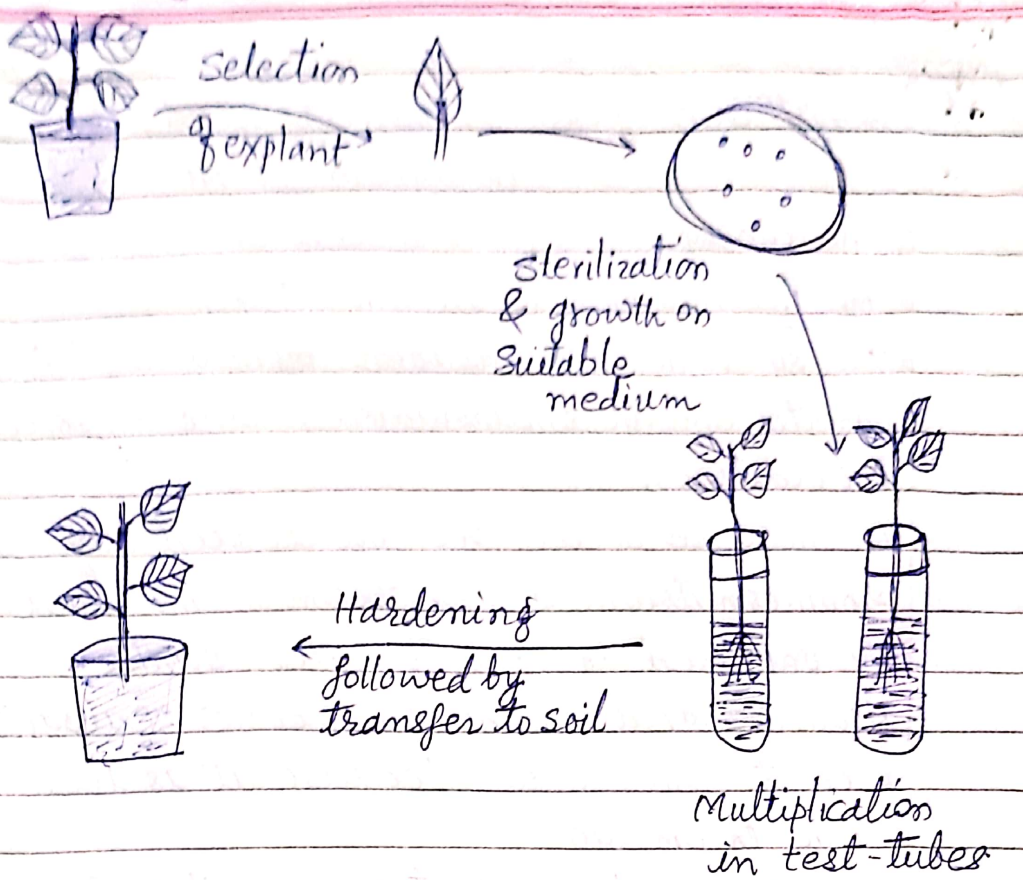


Fig.: Steps involved in micropropagation.

Production of disease-free plants :-

Meristem as explant

↓ Hot water treatment & transfer to MS-medium

Callus formation

↓ Multiply & transfer to rooting medium

Root initiation (3 weeks)

↓

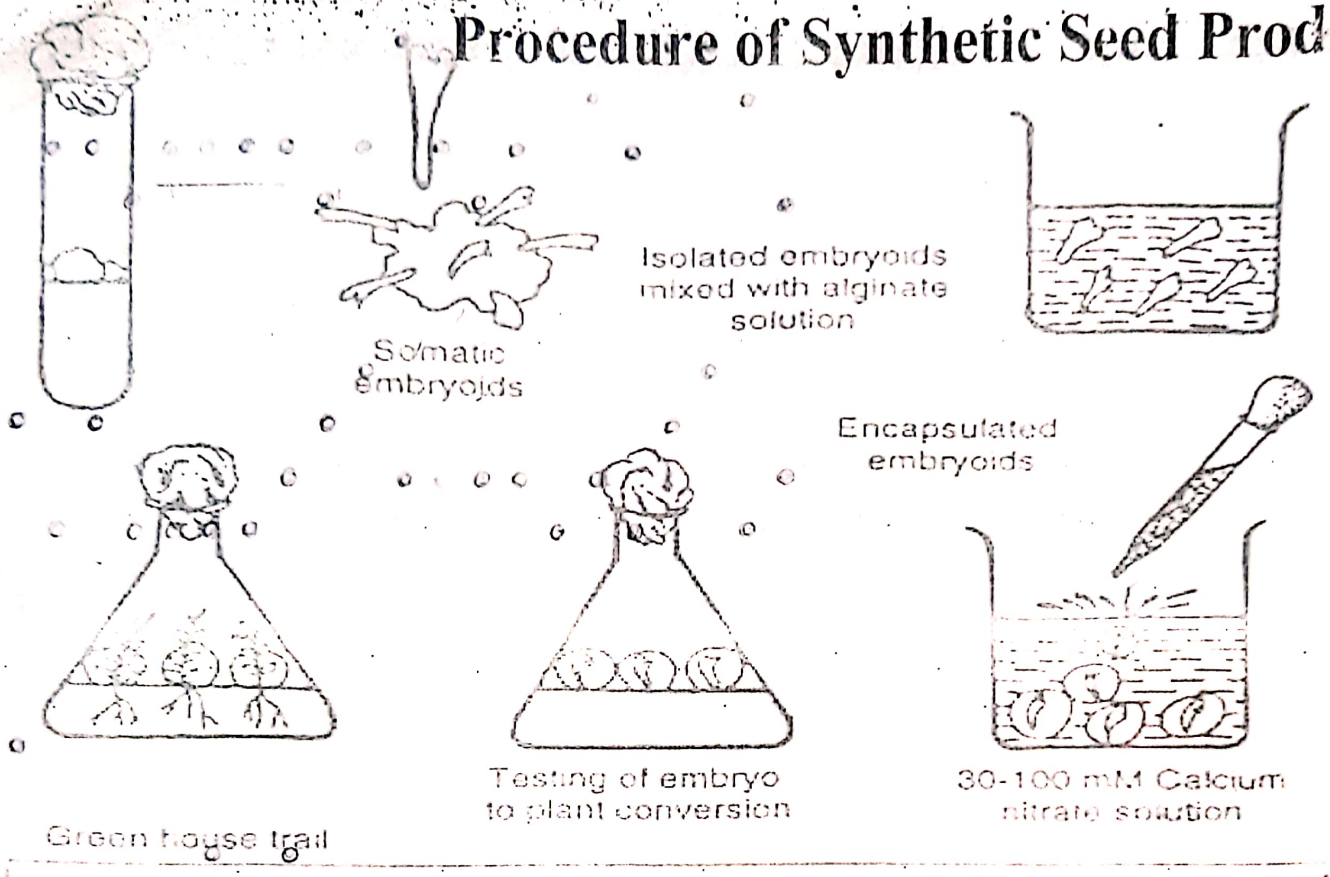
Plants in pots

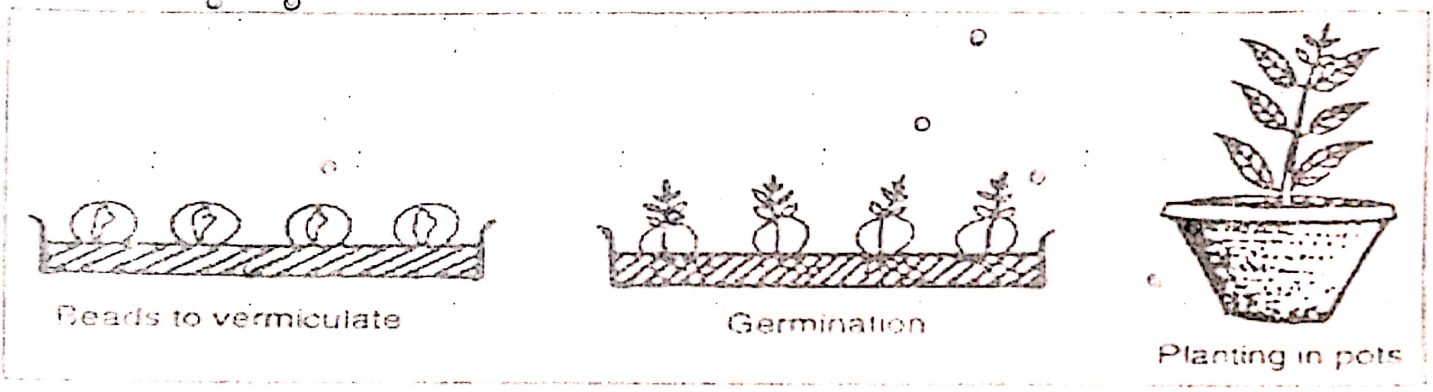
↓ Hardening & transferring to soil

Disease-free plants

Fig.: Outline of production of disease-free plants.

Procedure of Synthetic Seed Production





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