TOPIC

Regeneration of plants and micropropagation

PLANT REGENERATION

The process of growing an entire plant from a single cell or group of cells. Regeneration is possible because plant cells can be made totipotent using hormones. Differentiated tissue: stems, leaves, roots, etc. Undifferentiated (embryonic) cells are totipotent: can become a whole new plant by differentiating into a whole new plant.

Basic concepts of plant tissue culture (PTC) or plant regeneration .Two concepts are central to understanding plant cell, tissue, organ culture and regeneration

1) **Plasticity:-**Ability to initiate cell division from almost any tissue of the plant. Ability to regenerate lost organs or undergo developmental pathways in response to particular stimuli.
2) **Totipotency:-**each cell has the capacity to regenerate the entire plant.

Cells lines differentiate to form specialized tissues and organs. Unlike animal cells, living plant cells re-differentiate. Therefore, tissue can be regenerated from explants such as cotyledons, hypocotyls, leaf, ovary, protoplast, petiole, root, anthers, etc.

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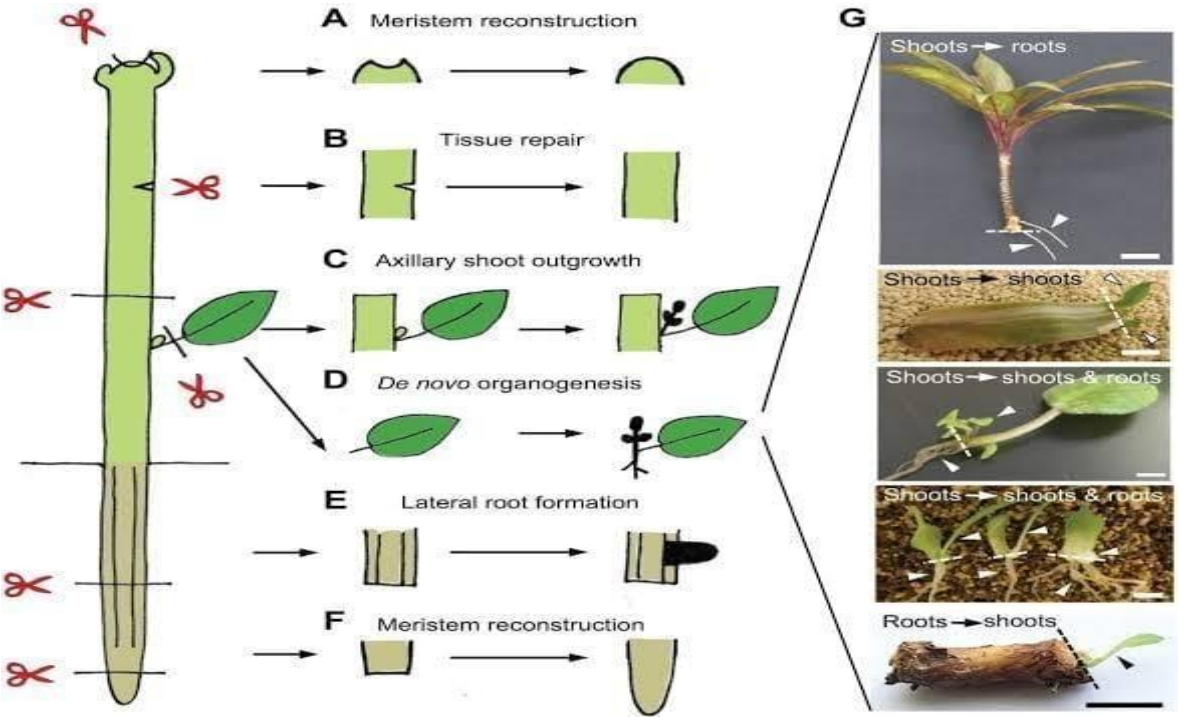
A German plant physiologist Gottlieb Haberlandt (1902) is regarded as the father of plant tissue culture.

Plant Regeneration Pathways

There are two methods of whole plant regeneration: organogenesis and somatic embryogenesis.

Organogenesis:-

. Plant regeneration through the formation of shoots and roots is known as plant regeneration through organogenesis. Organogenesis can occur directly from the explants depending on the hormonal combination of the medium and the physiological state of the explants. Miller and Skoog demonstrated that the initial formation of roots or shoots on the cultured callus or explant tissue depends on the relative concentration of auxins and cytokine ns in the culture media. Medium supplemented with relatively high auxin concentration will promote root formation on the explants and high cytokine in concentration will promote shoot differentiation. In tissue culture practices there may be three types of medium in relative combinations of auxins and cytokine which promote either the shoot formation or root formation or both simultaneously. In the latter case, you can get the complete plantlets, having both shoot and roots, which can be directly transferred to the pots in the greenhouse. Whereas in other cases, after the formation of shoots, individual shoots are transferred to the rooting medium, which promote root formation. The rooted plantlets can be transferred to a greenhouse for acclimatization. Plant regeneration through organogenesis is commonly used for mass multiplication, for micropropagation, and for conservation of germplasm at either normal or sub-zero temperatures (cryopreservation)



Somatic Embryogenesis:-

This is another major path of regeneration and development of plantlets for micropropagation or mass multiplication of specific plants. The cells, under a particular hormonal combination, change into the physiological state similar to zygotes (somatic zygotes) and follow an embryonic path of development to form somatic embryos. These somatic embryos are similar to normal embryos (seed embryos) developed from zygotes formed by sexual fertilization. The somatic embryos can develop into a complete plant. Since somatic embryos can germinate into a complete plant, these can be used for the production of artificial seeds. Somatic embryos developed by tissue or cell cultures can be entrapped in certain inert polymers such as calcium alginate and used as artificial seeds. Since the production of artificial seed is amenable to mechanization and for bioreactors, it can be produced in large numbers.

Plant Tissue Culture

Plant-tissue culture is a technique that can be used for the mass multiplication of exotic plants. Plant-tissue culture is a tool which is used extensively in the nursery business and in plant biotechnology for the rapid production of many genetically-identical plants using relatively small amounts of space, supplies, and time. Basically, the technique consists of taking a piece of a plant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile, (usually gelbased) nutrient medium where it multiplies. The formulation of the growth medium is changed depending on whether one is trying to produce somatic undifferentiated callus tissue, multiply the number of plantlets, grow roots, or multiply embryos for 'artificial seed.'

The following are the major steps involved in plant-tissue culture:

1. Selection of explant or tissue.

2. Surface sterilization of the explant to remove the microflora present on the surface using disinfectants such as sodium hypochlorite or mercuric chloride, followed by repeated washing with sterile distilled water.

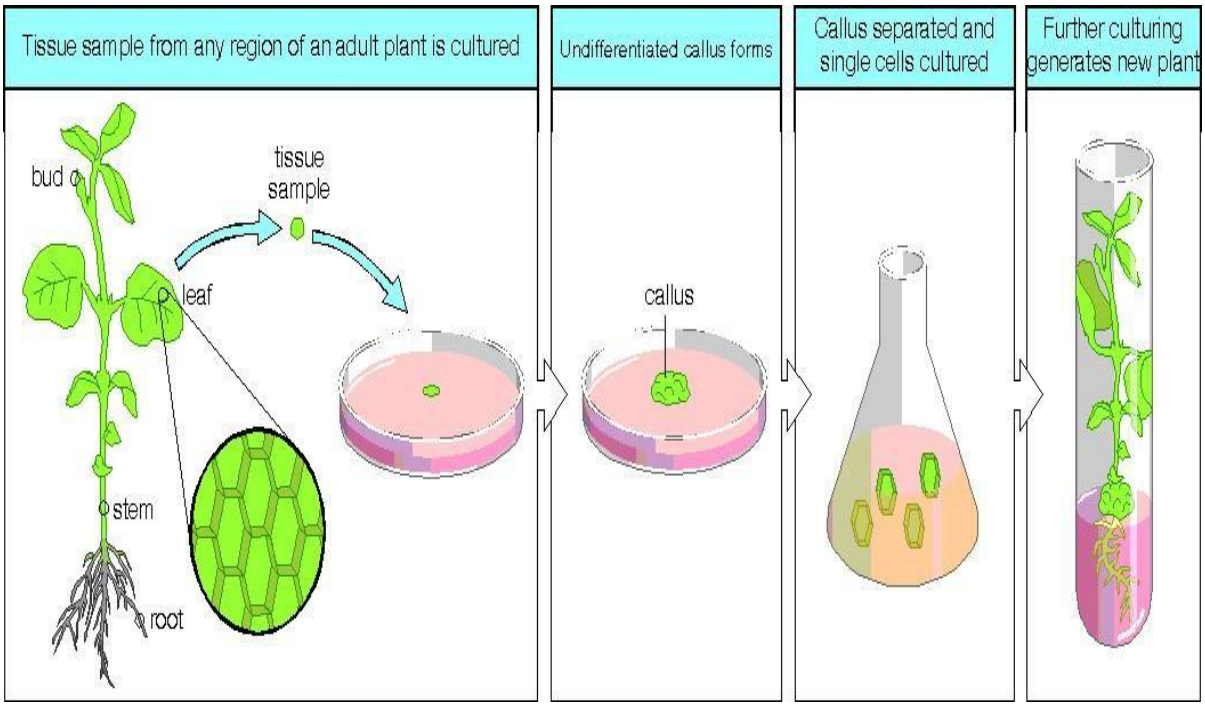
3. Inoculation of the surface sterilized explant of suitable size into the culture media under aseptic conditions inside a laminar air flow chamber.

4. The culture media should contain suitable nutrient components in right composition including the growth regulators.

5. Inoculated cultures are transferred to growth chambers or tissue-culture rooms having appropriate physical conditions such as the right temperature (26 to 28°C), relative humidity (50 to 60%), and fluorescent light (16 h photoperiod).

6. Plantlets are regenerated from the cultured tissues and cells.

7. Fully regenerated plants (with shoot and root) are transferred to the greenhouse and then to fields after proper acclimatization.



INITIATING TISSUE CULTURES:-

Explants

Tissue cultures are started from pieces of whole plants. The small organs or pieces of tissue that are used are called explants. The part of the plant (the stock plant or mother plant) from which explants are obtained, depends on:

• The kind of culture to be initiated;

• The purpose of the proposed culture;

• The plant species to be used.

Explants can therefore be of many different kinds. The correct choice of explant material can have an important effect on the success of tissue culture. Plants growing in the external environment are invariably contaminated with micro-organisms and pests. These contaminants are mainly confined to the outer surfaces of the plant, although, some microbes and viruses may be systemic within the tissues (Cassells, 1997). Because they are started from small explants and must be grown on nutritive media that are also favorable for the growth of microorganisms, plant tissue cultures must usually be established and maintained in aseptic conditions. Most kinds of microbial organism, and in particular bacteria and fungi, compete adversely with plant material growing in vitro. Therefore, as far as possible, explants must be free from microbial contaminants when they are first placed on a nutrient medium. This usually involves growing stock plants in ways that will minimize infection, treating the plant material with disinfecting chemicals to kill superficial microbes, and sterilizing the tools used for dissection and the vessels and media in which cultures are grown. Some kinds of plants can, however, be micro propagated in non-sterile environments.

Isolation and incubation:-

The work of isolating and transferring cultured plant material is usually performed in special rooms or inside hoods or cabinets from which microorganisms can be excluded. Cabinets used for isolation can be placed in a draught-free part of a general laboratory, but are much better situated in a special inoculation or transfer room reserved for the purpose. The accommodation, equipment and methods that are required for successful inoculation and transfer are described in.Cultures, once initiated, are placed in incubators or growth rooms where lighting, temperature and humidity can be controlled. The rate of growth of a culture will depend on the temperature (and sometimes the lighting) regime adopted.

The cultural environment:-

Plant cultures are commenced by placing one or more explants into a pre-sterilised container of sterile nutrient medium. Some explants may fail to grow, or may die, due to microbial contamination: to ensure the survival of an adequate number, it therefore is usual to initiate several cultures at the same time, each being started from an identical organ or piece of tissue. Explants taken from stock plants at different times of the year may not give reproducible results in tissue culture. This may be due to variation in the level of external contaminants or because of seasonal changes in endogenous (internal) growth regulator levels in the stock plant.

Media:-

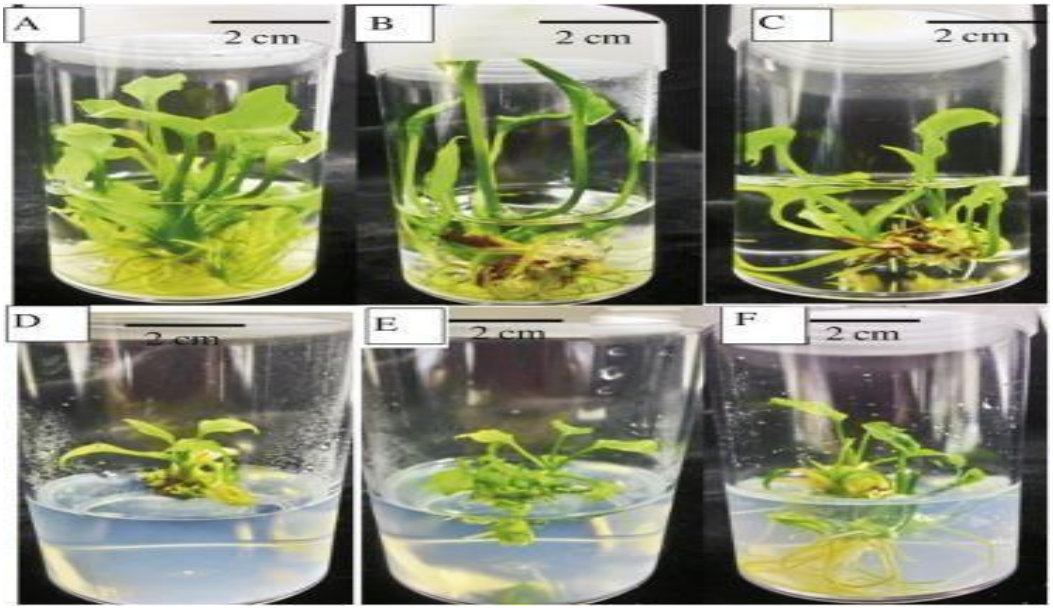
Plant material will only grow in vitro when provided with specialised media. A medium usually consists of a solution of salts supplying the major and minor elements necessary for the growth of whole plants, together with: various vitamins (optional); various amino acids (optional); an energy source (usually sucrose).Growth and development of plant cultures usually also depends on the addition of plant growth regulators to the medium.Plant growth regulators are compounds, which, at very low concentration, are capable of modifying growth or plant morphogenesis. Many workers define a medium as a completed mixture of nutrients and growth regulators. This is a rather inflexible method, as growth regulators frequently need to be altered according to the variety of plant, or at different stages of culture, whilst the basic medium can stay unchanged. It is therefore recommended that nutritional and regulatory components should be listed separately. Plant material can be cultured either in a liquid medium or on a medium that has been partially solidified with a gelling agent.The method employed will depend on the type of culture and its objective.

Solidified media

Media which have had a gelling agent added to them, so that they have become semi-solid, are widely used for explant establishment; they are also employed for much routine culture of callus or plant organs (including micropropagation), and for the long-term maintenance of cultures. Agar is the most common solidifying agent, but a gellan gum is also widely used. Cultures grown on solid media are kept static. They require only simple containers of glass or plastic, which occupy little space. Only the lower surface of the explants, organ or tissue is in contact with the medium. This means that as growth proceeds there may be gradients in nutrients, growth factors and the waste products of metabolism, between the medium and the tissues. Gaseous diffusion into and out of the cells at the base of the organ or tissue may also be restricted by the surrounding medium.

Liquid media

Liquid media are essential for suspension cultures, and are preferred for critical experiments on the nutrition, growth and cell differentiation in callus tissues. They are also used in some micropropagation work. Very small organs (e.g. anthers) are often floated on the top of liquid medium and plant cells orprotoplasts can be cultured in very shallow layers ofstatic liquid, providing there is sufficient gaseous diffusion. Larger organs such as shoots (e.g. proliferating shoots of shoot cultures) can also often be grown satisfactorily in a shallow layer of nonagitated liquid where part of the organ protrudes above the surface. However, some method of support is necessary for small organs or small pieces of tissue, which would otherwise sink below the surface of a static liquid medium, or they will die for lack of aeration.



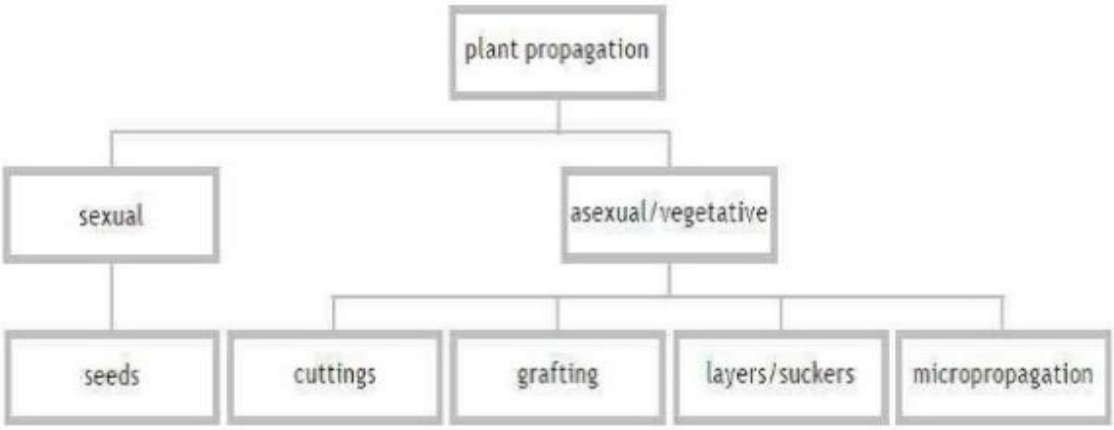
Once the plant is placed in tissue culture, proliferation of lateral buds and adventitious shoots or the differentiation of shoots directly from callus (unorganized mass of cells) results in the tremendous increase in the number of shoots available for rooting. Rooted plantlets of many species have been established in production situations and have been successfully grown either in containers or in field plantings. The two most important points that we have to bear in mind is that this methodology is a means of accelerated asexual propagation and that plants produced by these techniques respond similarly to any vegetatively propagated plant.

Plant Propagation

Propagation of plant is defined as production of new individuals from a selected plant having all the characters of the original one. New plants are created from a variety of sources: seeds, cuttings, bulbs, tissue culture etc. Plant propagation in done sexually and asexually as well as on artificial platform using TISSUE CULTURE TECHNEQUIES.

PRINCIPLE:

The basis of plant propagation is totipotency, the capability of cells to regenerate missing parts and, subsequently, an entire organism.



Type of plant propagation

1) **Sexual propagation:-**It is with the use of seed or spore that is separated from the parent plant. This method is so termed "sexual" because there is the involvement of the sexes, referring to the contribution of both the male and female gametes in the production of new plants. Pollen is transferred from anther to stigma. Fertilization occurs and seeds are produce.
2) **Asexual propagation:-**It is also called vegetative propagation, is with the use of planting materials which are vegetative parts of any plant rather than seeds or spores which are reproductive parts. E.g. cutting, layering, grafting etc. Micropropagation is the type of asexual propagation.

Micropropagation

Micro-propagation is the propagation of plants through tissue culture. It is a proven means of producing millions of identical plants under a controlled and aseptic condition independent of

seasonal constraints. Small pieces of plant tissues (Ex plants) use for regeneration (shoot system rooting and growing into full size plant)

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation. In nature, clonal propagation occurs by apomixes (seed development without meiosis and fertilization) and/or vegetative propagation (regeneration of new plants from vegetative parts). Tissue culture has become popular method for vegetative propagation of plants. Aseptic method of clonal propagation is called as Micropropagation and it offer the advantage of large number of true-to-type plantlets can be produced with relatively short time and space from a single individual. It is the fact that micropropagation is the only commercially viable method of clonal propagation of most of the horticultural crops. E.g Orchids

Explants used in micropropagation:-

Different kinds of explants were used in micropropagation. For example, in case of orchids, shoot tip (Anacamptispyramidalis, Aranthera, Calanthe, Dendrobium), axillary bud (Aranda, Brassocattleya, Cattleya, Laelia), inflorescence segment (Aranda, Ascofinetia, Neostylis, Vascostylis), lateral bud (Cattleya, Rhynocostylis gigantean), leaf base (Cattleya), leaf tip (Cattleya, Epidendrum), shoot tip (Cymbidium, Dendrobium, Odontioda, Odontonia), nodal segment (Dendrobium), flower stalk segment (Dendrobium, Phalaenopsis) and root tips (Neottia, Vanilla) are being used in micropropagation. Different explants Differentiation of shoots directly from callus.

Stages in micropropagation:-

Micropropagation generally involves five stages. Each stage has its own requirements.

Stage 0:Preparative stage

This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in stage 1. To reduce the contamination problem in the subsequent stages, mother plant should be grown in a glasshouse and watered so as to avoid overhead irrigation. This will also reduce the need for a harsh sterilization treatment. Stage 0also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. For example, red-light treated plants of Petunia provided leaf explants which produced up to three times as many shoots as did the explants from untreated plants.

Stage 1:Selection of the Leaves

Leaves, stems, or any other suitable plant part may be cut from healthy plants. The explants should be cut to suitable sizes. Old tissue parts should be avoided. Wash the dust off the tissue in a beaker of distilled water, which should be surface sterilized.

Stage 2:Surface Sterilization and Preparation of the Explants

This part of the procedure should be carried out in a sterile working area, or with a meticulous aseptic technique. The leaf or any other tissue should be immersed in 70% ethanol for 30 seconds, and then transferred to a sterile petri dish. Sterile scissors and forceps are then used to cut the tissues into small suitable sizes of explants. The explants are transferred into a 10% hypochlorite bleach solution or mercuric chloride for five minutes, gently agitating once or twice during this time. They are then washed thoroughly by immersing in four successive beakers of sterile distilled water, leaving them for two to three minutes in each.

Stage3.Initiation of Culture

A suitable explant such as terminal meristem or auxiliary is used for initiating the culture. The surface sterilized explant is implanted into a suitable nutrient medium with the correct type of growth hormones in appropriate concentrations. Depending on the nutrient conditions and hormone combination, the explant either starts developing callus or directly produces shoot buds and roots to form a large number of plantlets. If callus is formed, it is transferred to another medium having the hormone combination favoring multiple shoot formation. The shoots are then transferred to another medium, which favors the root initiation. The rooted shoots are now ready for transfer to the pots for hardening under greenhouse conditions.

Shoot formation. If the cultures develop multiple shoots, they are excised and transferred to a rooting medium.

Root formation. Shoots that are transferred to the rooting medium develop roots and are ready for being transferred to the greenhouse for hardening**.**

Stage 4: Transplantation and Hardening

The plantlets that develop roots can be transferred to special pots with sterilized sand for hardening under greenhouse conditions. Hardening is a process by which the in vitro generated plants are acclimatized to the greenhouse conditions. Then they can be transferred to the field. Recently, the mass multiplication of plants through micropropagation has been automated and robotized to increase the efficiency and speed of the methods of micropropagation of agricultural and horticultural plants. Micropropagation methods for a large number of useful plants including forest trees and other medicinal herbs are now available.

Advantages of micropropagation

1. Clonal mass propagation - extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting in vegetative propagation, one can obtain more than 1,000,000 plants per year from one initial explant through micropropagation.

2.Culture is initialized from small parts of plants – so no need of much space: from 1 m2 space in culture room, 20000 - 100000 plants can be produced per year.

3. Production of disease and virus free plantlets. This leads to simplification of international exchange of plants

4. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as Narcissus and other bulbous crops.

5. Introduction of disease free new cultivars is possible through micropropagation.

6. Vegetative propagation of sterile hybrids can be used as parent plants for seed production. E.g. Cabbage

7. One of the rapid methods for cloning of disease free trees.

8. In vitro cultures can be stored for long time through cryopreservation.

9. Breeding cycle can be shortened.

Disadvantages of micropropagation

1. Expensive laboratory equipment and service

2. No possibility of using mechanization

3. Plants are not autotrophic

4. Poor Acclimatization to the field is a common problem (hyperhydricity)

5. Risk of genetic changes if 'de novo' regeneration is used

6. Mass propagation cannot be done with all crops to date. In cereals much less success is achieved

7. Regeneration is often not possible, especially with adult woody plant material.

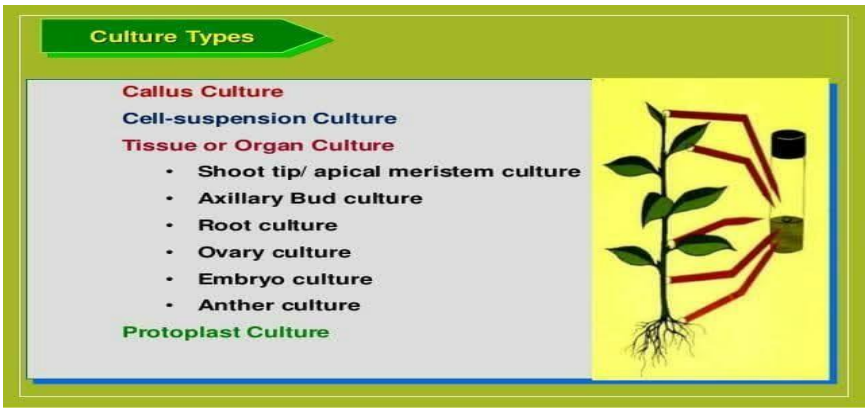
8. More problems in inducing rooting

9. May not get uniform growth of original plant from tissue culture. Each explant has different in vitro growth rates and maturation. Thus cannot be used for floriculture crop production where uniformity is critical.

Types of Cultures

Organ cultures

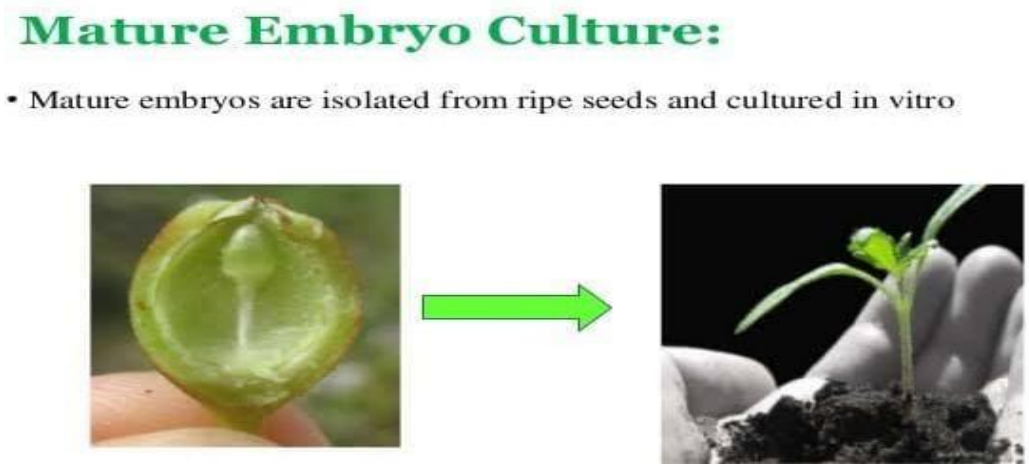
Culturing isolated organs or tissues such as roots, stem, or leaf in an artificial media under controlled conditions are known as organ culture. Depending on the type of organs or tissue used for establishing the culture, organ cultures are named accordingly.



The following are the various types of organ culture and its specific purpose:

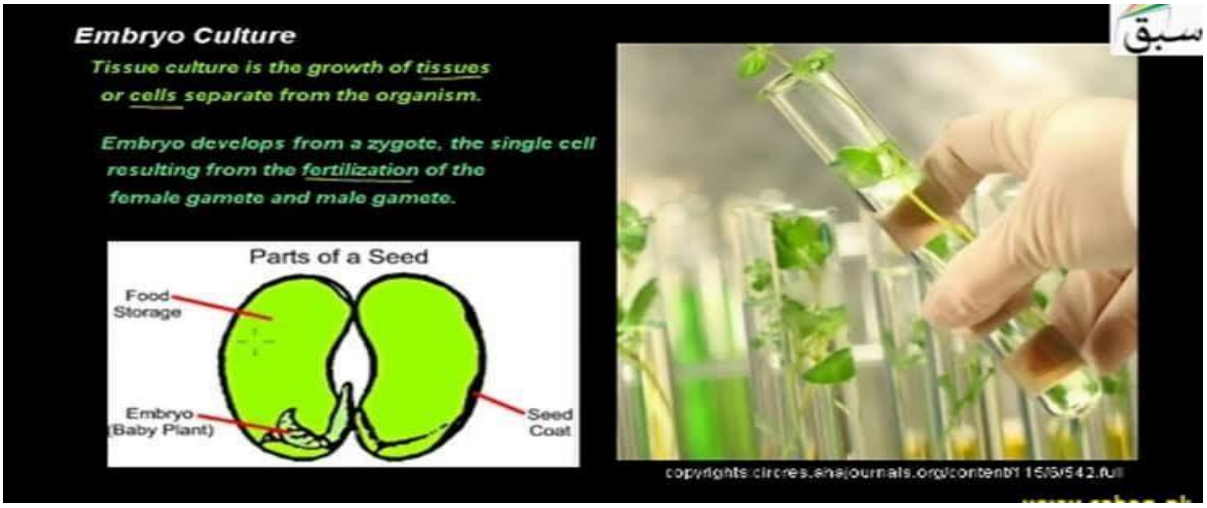
Seed culture

Increasing the efficiency of germination of seeds that are difficult to germinate in vivo precocious germination by application of plant-growth regulators, and production of clean seedlings for explants or meristem culture.



Embryo culture

Overcoming embryo abortion due to incompatibility barriers, overcoming seed dormancy and self-sterility of seeds, and embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor, shortening of breeding cycle, etc.



Ovary or ovule culture

A common explant for the initiation of somatic embryogenic cultures, for the production of haploid plants, overcomingabortion of embryos of wide hybrids at very early stages of development due to incompatibility barriers, and in vitro fertilization for the production of distant hybrids avoiding style and stigmatic incompatibility that inhibits pollen germination and pollen tube growth.

Anther or pollen culture

Production of haploid plants, production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce inbred lines, and for uncovering mutations or recessive phenotypes.

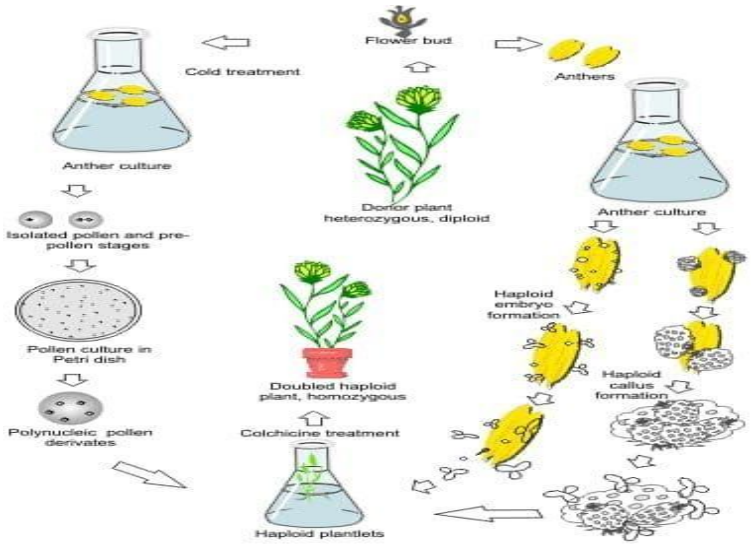
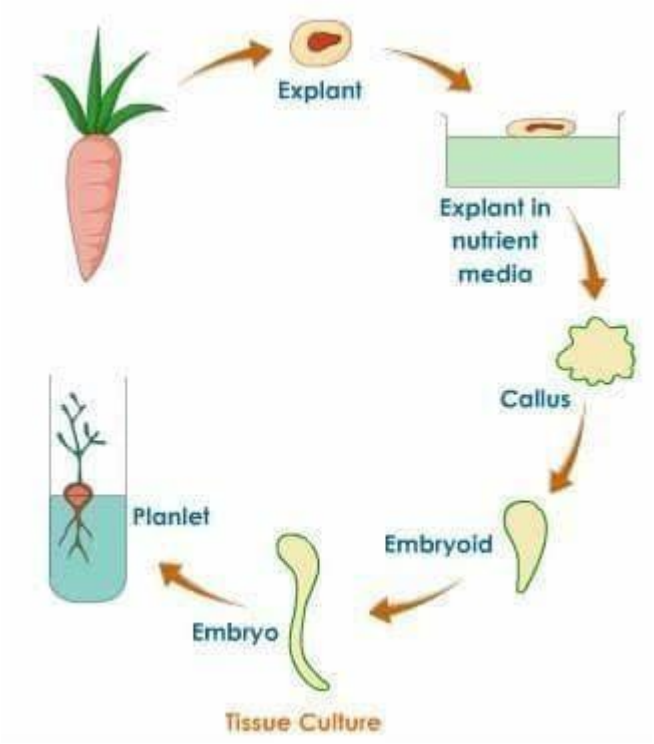


FIGURE 85: Schematic drawing of haploid production by androgenesis (anther and pollen culture

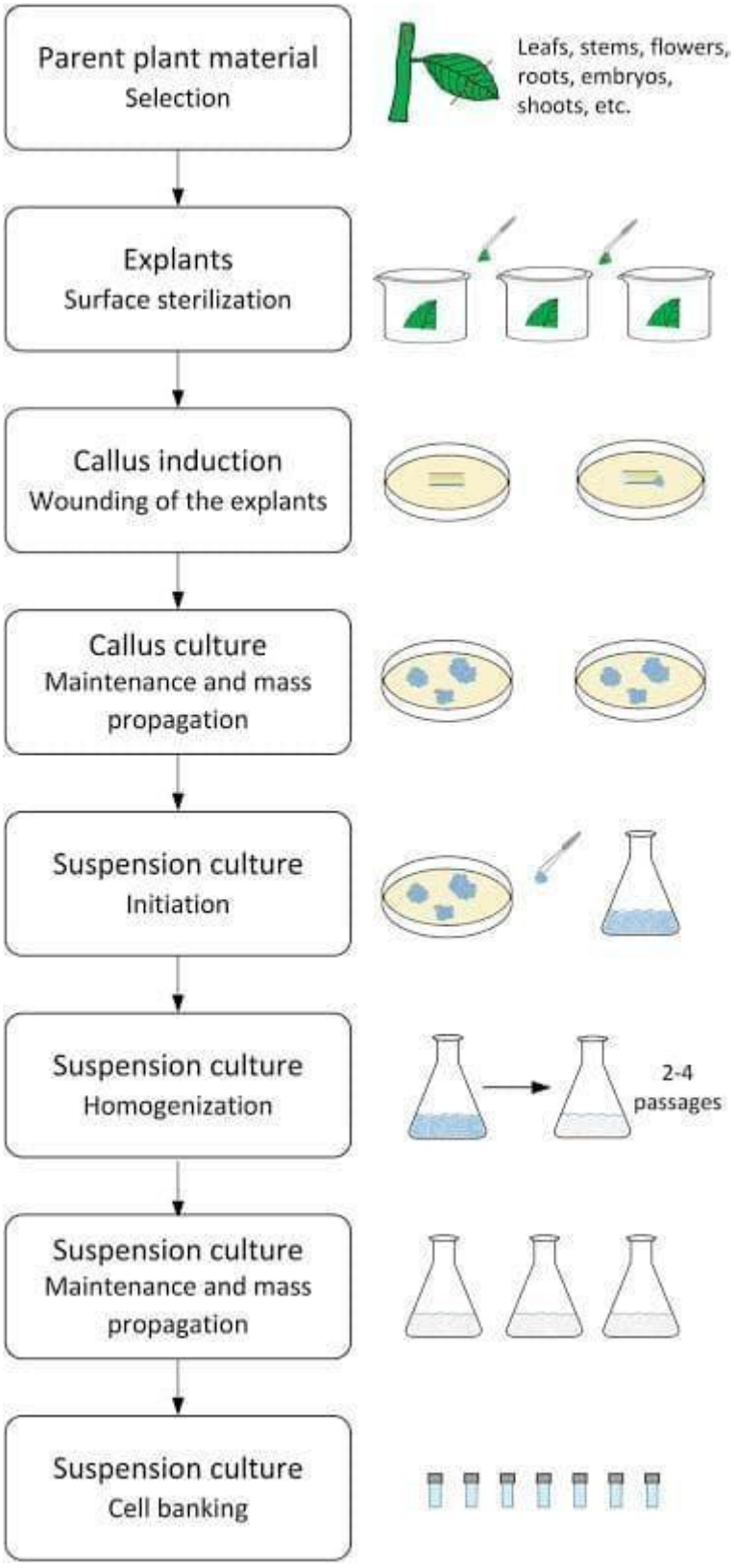
Explants Culture

Explant culture is actually the tissue culture. Culturing of any excised tissue or plant parts such as leaf tissue, stem parts, cotyledon, hypocotyls, root parts, etc., is called explant culture. The primary purpose of explant culturing is to induce callus cultures or to regenerate whole plantlets directly from it without the formation of callus. Shoot apical meristem culture is an example, and its important uses are the following: Production of virus-free germplasm or plantlets, mass production of desirable genotypes, facilitation of exchange between locations (production of clean material), and cryopreservation (cold storage) or in vitro conservation of germplasm, etc., are the main purposes of meristem or shoot apex culture.



Callus Culture

Callus represents an unorganized or undifferentiated mass of cells. They are generally composed of parenchymatous cells and usually undergo division. When an explant is cultured in a medium supplemented with sufficient amount of auxins, it starts producing mass of cells from the surface of the explant (Figure 19.3). The concentration of auxins required for each type of explant will be different and is mainly dependent on the physiological state of the explant tissue. Callus cultures can be maintained for a very long time by intermittent sub-culturing to a fresh medium. The callus cultures can be manipulated for different purposes by changing the hormone concentrations in the media. Callus cultures can be used for regeneration of plantlets, preparation of single cells or suspension cultures, or for protoplasts preparation. Callus cultures can also be used for genetic transformation studies. In some instances, it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis. Callus cultures are suitable for the generation of useful somaclonal variants (genetic or epigenetic) and can be used for in vitro selection of cells and tissue variants.



Cell-Suspension Cultures

The cell suspension culture also called as the plant cell culture is a system for production of fine chemicals.

It can be defined as The culture of tissues and cells cultured in liquid nutrient medium, producing a suspension of single cells and cell clumps.

Enzymatic methods can also be adopted for establishing a fine cell-suspension culture. This is based on the use of certain pectin digesting enzymes in the culture medium, such as pectinase or macerozyme. These enzymes act on the pectin, which joins two adjacent cells in plant tissues, so that the cells become independent and grow freely as single cells.

Protoplast Cultures

Protoplasts are plant cells without cell walls. The cell wall can be removed with an enzymatic method. The cells may be from the leaf tissue or from any other part of the plant or may be the cells from the suspension cultures. These cells are incubated in an enzyme mixture consisting of cellulase, hemicellulase, and pectinase for a specific period of time. The enzyme mixture acts on the cell wall and is completely digested, so that the underlying cell membrane is exposed. This protoplast on culturing in a proper medium will regenerate its cell wall and become a normal cell and then can regenerate into a whole plant.

Applications of plant tissue culture

1. Plant tissue culture Horticulture and Forestry:

Micropropagation method is used for rapid multiplication of ornamental plants as well as important trees yielding high fuel, pulp, fruits or oil at a large scale. Applications of plant tissue culture Today's high yielding oil palms on plantations in Malaysia were generated in the 1960s by tissue culture methods. These palms produce 30% more oil than normally cultured palms. Improvement of economically important forest trees is being done through genetic transformation and rapid Micropropagation .e.g in vitro regeneration and genetic transformation of conifers.

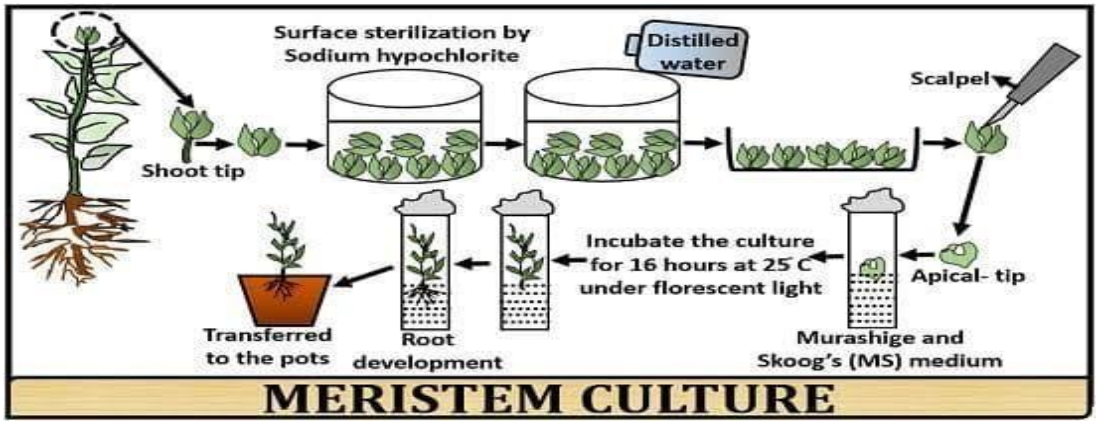
2. Plant tissue culture Industries:

Plant cell culture is used for biotransformation (modification of functional groups of organic compounds by living cells). Food and agricultural biotechnologists are involved in using tools of molecular biology to enhance the quality and quantity of foods and economic crops. For example, Golden Rice was genetically enhanced with added beta carotene, which is a precursor to Vitamin A in the human body.

3. Virus-Free Plants

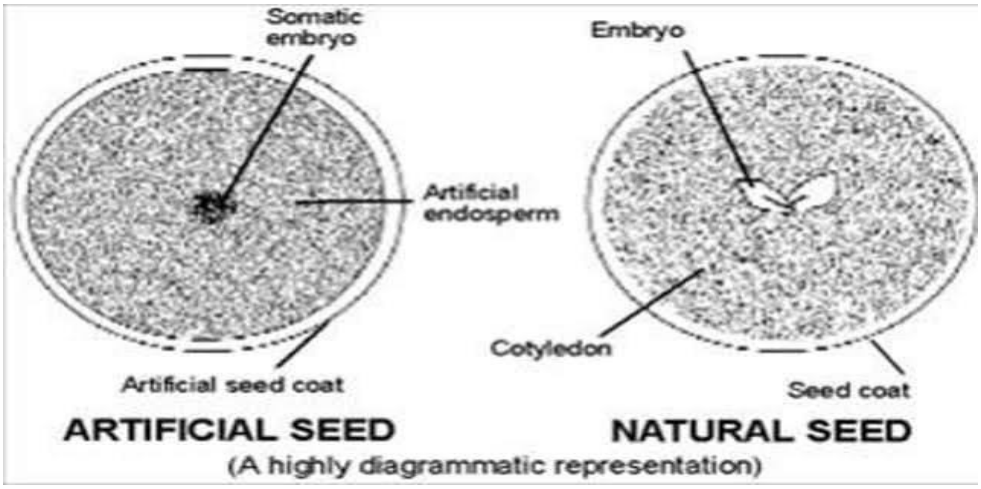
One of the main purposes of propagation through tissue culture is to produce virusfree plants. This is very important to increase yield and quality. Since meristems such as apical meristem

and auxiliary meristem are free from viral infections these tissues can be used as explants for mass multiplication to get a large number of virus-free plants. Agricultural plants, which have a high risk of damage due to viral diseases include plants such as bananas, sugarcane, potatoes, tapioca, apples, etc. Meristem culture is usually practiced to eliminate viruses and to make virusfree plants. Tissue culture using meristem is sometimes referred to as ‘meristem culture’ or ‘meristemming,’ and the plantlets produced are known as ‘mericlones.’



Artificial Seeds

Artificial seeds are the somatic embryos encapsulated by certain inert polymeric compounds such as alginate. They are also very useful in the mass propagation of agricultural and hybrid varieties.



In Vitro Plant Germplasm Conservation

Germplasm collection and conservation is one of the major tasks of many research establishments as a means for the conservation of biological diversity. Tissue-culture

methods are used for the conservation of plant varieties for future studies and plant-breeding purposes. Germplasm is the plant genotype representing whole genetic materials and that of its related species. Primitive crops and their wild varieties are very important as they may have unique and valuable genes, which are agriculturally very important. Because of the extensive use of modern agricultural varieties and hybrids, primitive crops and their wild relatives are onthe path to extinction. The best method of protecting them and to prevent the erosion of plant genetic resources is to conserve them through tissue culture. Germplasm conservation can be carried out by collecting and preserving genetic resources by conventional methods in the form of seeds, vegetative propagules, etc. This collection of plant seeds and propagules is done in in vivo gene banks. Collection and conservation of any plant parts or tissue through tissue culture is done in in vitro gene banks. The conventional method of germplasm conservation has several limitations. Germplasm cannot be conserved for a very long time. The seeds are short lived and have demerits of seed dormancy, seed-borne diseases, and germplasm needs high input of labor, space, and cost. The in vitro approaches through cell and tissue-culture can overcome many of these inconveniences and problems, with the following tissue-culture-mediated conservation methods:

Cryopreservation

This method is the free storage of plant tissues and cells under frozen conditions. It is a long-term preservation of selected cells and tissues at very low temperature, equal to the temperature of liquid nitrogen (– 196°C). The tissues can be stored at this temperature indefinitely with the help of cryoprotectants such as glycerol, mannitol, dimethylsulfoxide, proline, etc.

Cryopreservation has proved to be an efficient conservation method for the long-term storage of genetic stocks without any loss of viability and mutations. Cold Storage. This method of storage of plant parts and tissues for conservation is suited for short term or medium term. The germplasm can be stored in the form of meristems such as shoot tips or auxiliary buds or other similar parts under low temperatures ranging from 4 to 15°C under nutrient limitations. The disadvantage is the tissue cannot be stored indefinitely.

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