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B.Pharm

Semestern III

Subject Name: Pharmacognosy and Phytochemistry I
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Chapter 3 – Plant Tissue Culture

Historical development of plant tissue culture, types of cultures, Nutritional requirements, growth and their maintenance.
Applications of plant tissue culture in pharmacognosy. Edible vaccines

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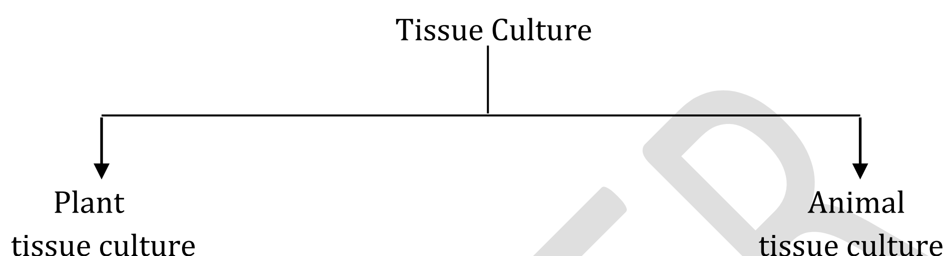
Chapter 3 – Plant Tissue Culture

1. Introduction

Tissue Culture

Definition

It is an experimental technique in which growth of tissue cells takes place in a laboratory medium. It is been broadly classified into two types as below:



Plant tissue culture

Definition

Plant-tissue culture is *in-vitro* cultivation of plant cell or tissue under aseptic and controlled environment conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate a plant.

In other words it is an experimental technique through which a mass of cells (callus) is produced from a portion of plant body (explant) tissue. The callus produced through this process can be utilized directly to regenerate plantlets or to extract or manipulate some primary and secondary metabolites. The plant tissue culture refers to the cultivation of a plant cell which normally forms a multicellular tissue. When grown on agar medium, the tissue forms a callus or a mass of undifferentiated cells. The technique of cell culture is convenient for starting and maintaining cell lines, as well as, for studies pertaining to organogenesis and meristem culture.

Brief Historical development of plant tissue culture

It was only in last two decades importance of plant tissue culture was appreciated. Various scientists have contributed for plant tissue culture which can be described in brief as follows:

1756, Henri-Louis Duhamel du Monceau's pioneered an experiment on would healing in plants and demonstrated spontaneous callus formation on the deocrticated region of the Elm plant.

1838-1839, Schleiden and Schwann proposed cell theory in plants and animals.

1902, Haberlandt's first aseptically culture attempt in a nutrient solution.

1904, Hannig cultured embryos from several cruciferous species.

1908, Simon obtained more promising results as he achieved success in regeneration of bulky callus, buds and roots from popular stem segments and thus he succeeded in establishing the basis for Callus culture to some extent.
1922, Kolte and Robbins successfully cultured root and stem tips respectively.
1926, Went discovered fist plant growth hormone-Indole acetic acid.
1934, White introduced vitamin B as growth supplement in tissue culture media for tomato root tip.
1939, Gautheret, White and Nobecourt established endless proliferation of callus cultures.
1941, Overbeek was first to add coconut milk for cell division in datura.
1946, Ball found the exact part of the shoot meristem, which give rise to whole plant.
1953-54, Muir was first to break callus tissues into single cells.
1957, Skoog and Miller proposed the concept of hormonal control of organ formation.
1960, Bergmann filtered cell susupension and isolated single cells by planting.
1960, Cocking introduced protoplasmic plant tissue culture.
1960, Moral initiated micropropagation technique and produced virus free orhcid.
1962, Murashige and Skoog developed MS medium with higher salt concentration.
1962, Kanta and Maheshwari developed test tube fertilization technique.
1966, Steward and co-worker demonstrated totipotency by raising large number of plantlets from carrot root suspension culture via somatic embryogenesis.
1970, Power and co-workers demonstrated the intra and interspecific fusion between the prototplasts of different plant roots.
1974, Nitsch demonstrated microspore culture.
1978, Melchers demonstated protoplast fusion.

The technique of *in-vitro* cultivation of plant cells or organs is primarily devoted to solve two basic problems as follows:

1. To keep the plant cells or organs free from microbes
2. To ensure the desired development in cells and organs by providing suitable nutrient media and other environmental condition.

Advantages of tissue culture

1. Availability of raw material

Some plants are difficult to cultivate and are also not available in abundance and tissue culture technique is considered a better source for regular and uniform supply of raw material for medicinal plant industry for production of phytopharmaceuticals or active constituents.

2. Fluctuation in supplies and quality

Quality of plant varies due to changes in climate, crop diseases and seasons. All these problems can be overcome by plant tissue culture.

3. Novel methods for isolation

It is possible to obtain new methods for isolation and newer compounds from plant by this technique and for which patent rights can be obtained.

4. Biotransformation

It is process through which the functional group of organic compound are modified by living cells reactions are feasible using plant-cell cultures.

5. Disease free

Large scale production of plant with disease free could be stored and maintained without any damage during transportation for subsequent plantation.

6. Biosynthetic pathway

Plant tissue culture can be used for tracing the biosynthetic pathways of secondary metabolites using labelled precursor in the culture medium.

7. Immobilization of cells

Tissue culture can be used for plants preservation by immobilization (entrapment) of cell further facilitating transportation and biotransformation.

8. Continuous, uniform biomass is obtained.
9. Medicinally important compound can be synthesized, which can't be synthesized chemically.
10. Useful natural compounds can be produced, independent of soil condition and change in climatic conditions.
11. Improvement of medicinal plant species.
12. Propogation of plant without seeds in defined and controlled condition.

Disadvantages of tissue culture

1. High level of expertise is required.
2. A small error may lead to complete collapse of product/plant.
3. Lots of chemicals are required for plant tissue culture which must contain high purity.
4. There is no chance for evaluation of mutation.
5. Culture on artificial medium may lead to the depression of unusual metabolic pathways, which may not be beneficial to biotechnologist.
6. In majority cases amount of secondary metabolites produced is negligible.
7. The protocols for individual plants differ very widely and change in the medium constitution and environmental parameters affect the rate of cell growth and accumulation of secondary metabolites.
8. To maximize on the cell mass produced the cell suspension culture eventually becomes very dense and these presents problems of even aeration.
9. Instability
10. Slow growth

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11. Expensive process
12. Aseptic conditions are to be maintained throughout the growth of plant.

Broadly two fields are there were plant tissue culture is applied which are as follows:

Agronomical

With the help of plant tissue culture agronomically rapid multiplication of selected plants identical to original plants can be done. New plant obtained is different from original and more efficient according to certain defined criteria. Production of high-yielding, herbicide, drought, insect, salt resistant crops are obtained.

Industrial

On industrial scale production of known molecule, using biosynthetic capacities of plant cells breed in a bioreactor. It is an innovative aspect, employing the new source of variability accessible *in-vitro* to obtain new molecules.

2. Basic requirements

Basic requirements for plant tissue culture are as follows:
a. Plant material
b. Equipments and Glassware's
c. Aseptic Condition
d. Washing and storage facilities
e. Media preparation room
f. Sterilization room
g. Nutrient medium
h. Transfer room
i. Culture room or incubators
j. Proper and optimum aeration
k. Well equipped observation or recording area

a. Plant material

The plant material should be disease free and should not be too old. Authentic species/variety/genotype should be used. Generally *in-vitro* germinated seedlings are frequently chosen as seed is often also much more readily sterilized than softer plant tissues. When plants are healthy and at the desired stage for use, it is often the case that only a specific part of these plants will give the best explants. E.g. internode, the youngest fully expanded leaf etc.

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b. Equipments and Glassware's

It includes various equipments and glassware's as follows:

Incubating chamber or laminar airflow cabinet with UV light fitting for aseptic transfer Incubator with temperature control ±0.5°C generally temperature recommended for most tissue culture studies is 36°C.
Autoclave for sterilization of glassware, media etc.
Refrigerators and freezers for storage of reagents, tissue culture stock solutions, chemicals etc.
Hot air oven for dry sterilization of glassware, media etc.
Simple and special microscope with a provision to take camera is required. The stage of this microscope should be large enough to accommodate large roller bottles in specific cases.
pH meter for adjusting the pH of the medium
Spirit burner or gas micro burner for flame sterilization of instruments
Washing up equipments for washing glassware, pipette etc. in deep soaking baths or washing sinks of stainless steel or polypropylene are suitable for manual washing and rinsing of almost all types of glassware except pipettes. Standard siphon type pipette washers are suitable for washing the pipettes soaked in detergent for overnight. The washed pipettes should be rinsed with deionised water and dried in a stainless steel pippette dryer.
Water purifier as pure water is required at most of the plant tissue culture study.
Centrifuge to increase the concentration of cell suspension culture
Shakers to maintain cell suspension culture
Balance to weigh various nutrients of the preparation of the medium
Shelves build from rigid wire mesh to allow maximum air movement and minimum shading should be used in the culture room.
Scissors, scalpels and forceps for explant preparation from excise plant parts are for their transfer
Culture vessels usually of borosilicate glass vessels are preferred, it includes test tubes, conical flasks, bottles, special flat tubes etc. Now, the common vessels are 100 ml conical flasks or large test tubes of 25 × 150 mm size.
Glassware's like measuring cylinders, beakers, funnels, petri dishes, graduated pipette, conical flask etc. are required for preparation of nutrient media.
Miscellaneous includes non absorbent cotton plug, screw cap or polyurethane foam is required to close the mouth of the culture vessel. Aluminium foil is required to cover the exposed part of plug from becoming wet when autoclaved. Labels, marking pencils, hand lens, plastic disposables like syringes, plastic bottles, hot plate, stirrer etc.
Microwave is not essential but it melts the solidified media for pouring in culture vessels like petri dishes etc.

c. Aseptic Condition

The plant materials (tissues), equipments, culture media and the room should be free from microorganisms. Usually dry heat, wet heat, ultrafiltration and chemicals are used for the sterilisation process. Surface sterilisation of plant materials such as seed, fruit, stem, leaf etc. by agents like 9-10% calcium hypochlorite for 5-30 min., 2% sodium hypochlorite solution for 5-30 min., the materials need to be washed thoroughly in double-distilled water, after sterilising in these solutions, 10-12% of hydrogen peroxide solution for 5-15 min., 1-2% bromine water, for 2-10 min.
1% solution of chlorine water, mercuric chloride, silver nitrate or antibiotics etc. can also be used and absolute alcohol is used for hard tissues.
Dry heat method is used for sterilisation of equipments in hot air oven. Sterilisation of equipment with chromic acid-sulphuric acid mixture, hydrochloric acid, nitric acid strong detergent solution, alcohol, incubator or autoclaves etc. is use for this purpose. Wet heat method is used for sterilisation of glassware, culture media in autoclave at 121°C and 15 lb pressure for 15 min. Ultrafiltration is used for sterilisation of liquid media which are unstable at high temperature. Antibiotics are added to medium to prevent the growth of the microorganisms e.g. Potassium benzyl penicillin, streptomycin sulphate, gentamycin etc. Chemicals like alcohol are used for sterilisation of working area and the instruments. Sterilisation of the environment is done by fumigation method, the inoculation chamber is generally laminar airflow cabinet is widely used these days.

d. Washing and storage facilities

Fresh water supply and disposal of waste water facility should be available. Space for distillation unit for the supply of distilled and double distilled water and de-ionized water should be available. Working table, sink or wash basin for apparatus/equipment washing should be acid and alkali resistant. Sufficient space is required for lacing hot air oven, washing machine, pipette washers etc. For storage of dried glassware separate dust proof cupboards or cabined should be provided.

e. Media preparation room

It should be spacious to accommodate lab ware, culture vessels, equipments, chemicals etc. The preparation room should also be well equipped with refrigerator, freezer etc. for storage of media and stock solutions.

f. Sterilization room

In the tissue culture laboratory it is desirable to have separate sterilization room for sterilization of culture media, glassware, metallic equipments like scissors, scalp etc. Generally sterilisation is done in autoclave or hot air oven.

g. Nutrient medium

Media is composed of following nutrients:
Inorganic nutrients includes macronutrients like nitrogen, phosphorous, potassium, calcium etc. and micronutrients like boron, copper, iron, manganese, zinc etc.
Organic nutrients include Vitamins like Vitamin B_1, B_6, B_3, B_5 etc. Amino acids like L-arginine, L-asparagine, L-cysteine HCL, L-glutamine etc, Carbon source like glucose or maltose, Growth hormones/regulators like auxin, cytokinins and gibberellins, ethylene, abscisic acid.
Others media substances like protein hydrolysates, yeast extracts, fruit (e.g. banana) extracts, coconut milk, solidifying agents like agar, alginate, gelatin etc., Iron source e.g.EDTA
pH of the medium should be in a range of 5.6-6.0 before autoclaving the culture medium.

h. Transfer room

It is provided with the laminar flow hood where most of the work of culture initiation and subsequent sub culturing is performed. Culture re-plantation, transfer or re-initiation in a clean media, harvesting of 'ripe' cultures is also performed in this area.

i. Culture room or incubators

Cultures are incubated on shelves or in incubators under specific condition of temperature, humidity, air circulation and light. Incubation chamber or area should have both light and temperature controlled devices managed for 24 hours period. Generally high output, cool, white fluorescent light is preferred for a photo-period duration (specified period for total darkness as well as for higher intensity light) with a temperature range of 25 ± 2°C (range 18-25°C). The rooms are required to be maintained at a relative humidity up to 70-75% (range of 20-90% controllable to ±3%) and uniform forced air circulation.

j. Proper and optimum aeration

Adequate aeration is required for cell to grow. Tissues which are cultured on semisolid media do not require any special method for aeration, but tissues which are grown in suspension cultures, require special devices for aeration. The culture vessels are closed with non-adsorbent cotton covered in cheese cloth. This process

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allows proper aeration but prevents the entry of microorganisms. Aeration for submerged cultures can be provided by following methods:

1. Placing the culture vessel with the liquid medium on an automatic shaker.
2. The two ends of the filter paper are dipped in a medium and the middle horizontal portion on which the tissue is placed remains above the level of the medium this method is called as filter paper bridge method.
3. Passing sterilised air through the medium and by stirring the medium.

k. Well equipped observation or recording area

At regular intervals growth and maintenance of the tissue culture in the incubators should be recorded. All observations should be done in aseptic environment. For microscopic examination separate dust free space should be marked for microscopic work.

3. Types of Culture

The plant culture can be broadly classified into two types as follows:

A. Based on the solid/liquid state of culture medium

B. Based on the plant organ/protoplast/bacteria used for the regeneration of the plant

A. Based on the solid/liquid state of culture medium

It is classified as below:

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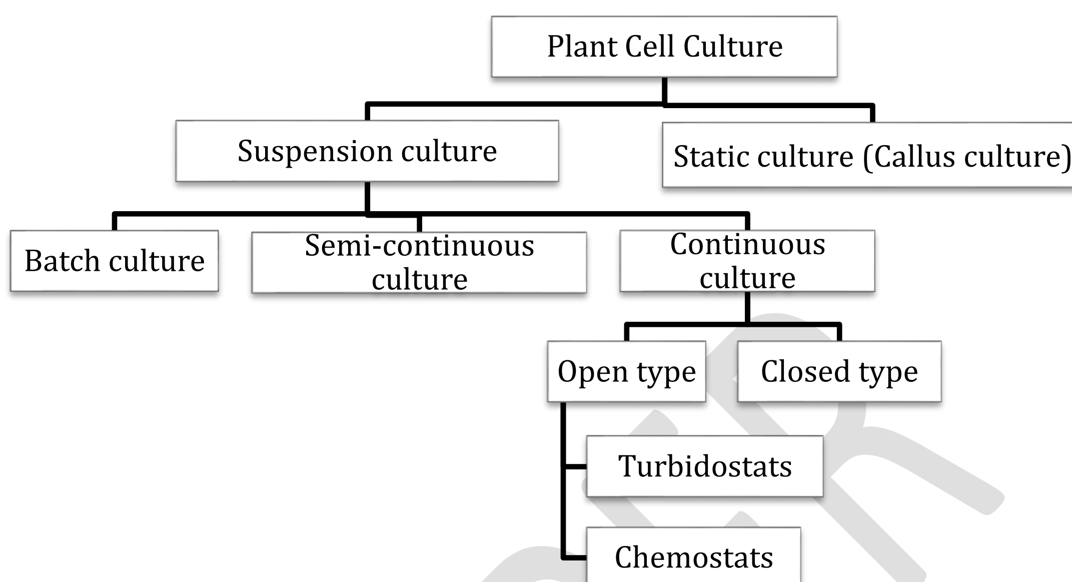

C. **Based on the plant organ/protoplast/bacteria used for the regeneration of the plant**

It is classified as below:

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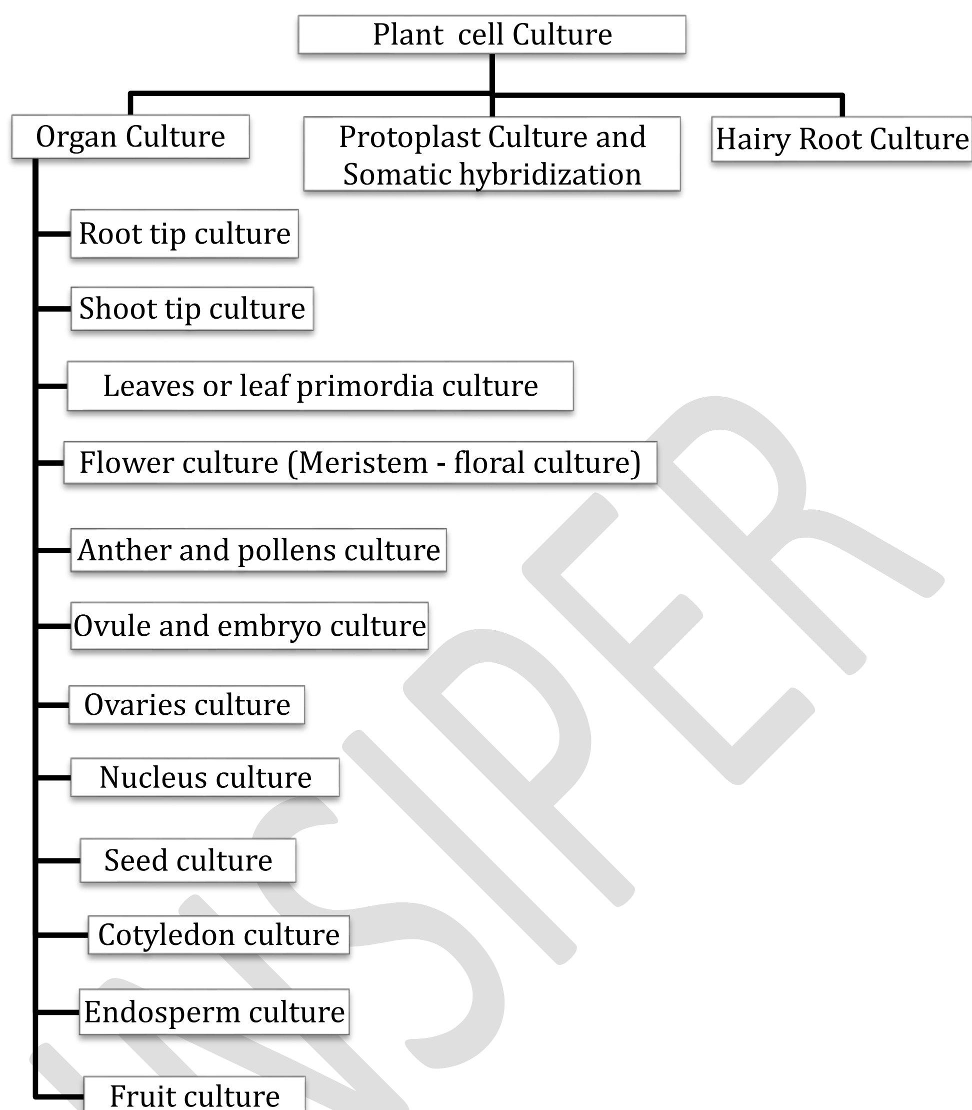

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A. Based on the solid/liquid state of culture medium

Plant cell culture

Culture of isolated cells or very small aggregates remaining dispersed in solid/liquid medium. A. Based on the state of culture medium mainly two types of culture are there as mentioned below:

I. Callus Culture (Static Culture)

Callus a mass of undifferentiated plant cells grown on solid media from plant part/explant. It may initiate from explants of any multi-cellular plant. The organs such as root, stem tips, leaves, flowers and fruit are grown on solid media. The cell groups are initiated from Explant/Segments (of root, stem or leaf either from the

mature or embryogenic plant) or Explant/Excised fragments (of parenchyma or mixed tissue containing cambium or endosperm). The longer the tissue explant the more complex the range of cell types and greater the possibilities of initiating a culture of mixed cells. Callus can be induced to undergo organogenesis and/or embryogenesis and eventually whole plant by providing suitable nutrient medium. It is useful to study the biosynthetic pathway of various metabolic processes by using tracer elements in callus culture. It is also useful for the production of secondary metabolites.

II. Suspension Culture

It involves active proliferation of callus as undivided unit suspended or submerged in a liquid medium. The nutrient medium in this case is in constant agitation so as to prevent the cells from settling or aggregating in to clumps. Suspension cultures are normally initiated by transferring pieces of undifferentiated callus to a liquid medium which is agitated during incubation. It is further divided into three types as mentioned below:

i. Batch Suspension Culture

It means 'Culture in a fixed volume of culture medium'. In general, a nutrient medium and cellular inoculum are mixed, aerated and allowed to grow. In Batch cultures, as the cells grow, the medium is depleted of nutrients and metabolic byproducts from the cells accumulate. Batch cultures are characterized by:

– Continuous changes in the medium
– Continuous internal changes in cellular composition
– Accumulation of metabolic products

The system is "closed" with respect to additions or removal of culture, except for circulation of air.

ii. Semicontinuous Suspension Culture

Here, the system is 'open'. There is periodic removal of culture and the addition of fresh medium, due to which growth of the culture is continuously maintained.

iii. Continuous Suspension Culture

The system is 'open' here also. In this system volume of culture remains constant and fresh medium is added continuously to a fixed volume of growing culture and withdrawn. Cell proliferation takes place under constant condition. This system allows:

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- Establishment of steady states of growth and metabolism.
- Study of the changes which occur in transitions from one steady state to another.
- Identification of the controlling factors.

Continuous suspension culture mainly of two types as mentioned below:

1. Open type

In this type of continuous suspension culture regulation of new medium and balancing the harvest of equal volume of culture is done. It is mainly of two types as follows:

a. Chemostat

Continuous new medium input is set at a predetermined rate and determine the nature of the resulting equilibrium. It is useful to study the steady states. Desired rate of growth is maintained by adjusting the level of concentration of nutrient by constant inflow of fresh medium.

b. Turbidostat

Cell density is set at a predetermined level (as monitored by the optical properties of the culture) and new medium is added to maintain cell density within limits. It is particularly valuable for work at low cell densities. It offers a simplified system for study of effects of physical factors and growth regulating substance in growth rate and associated metabolism.

2. Closed type

In this type the used medium is replaced with fresh medium, hence, the cells from used medium are mechanically separated and added back to the culture and thus, the cell biomass keeps increasing.

B. Based on the plant organ/protoplast/bacteria used for the regeneration of the plant

Various types of culture based on the plant part/protoplast/organism used for the regeneration of the plant are mentioned below:

I. Organ Culture

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Various organ parts of the plant are used for regeneration of whole plant from an organ/organ part. It can be classified as follows:

i. Root tip culture (Meristem – root tip culture)

Root apical meristem is mainly responsible for the growth of roots by cell division, cell enlargement and cellular differentiation. Tips of the lateral roots are sterilised, excised and transferred to fresh medium. The lateral roots continue to grow and provide several roots, which after seven days, are used to experimental cultures. By this culture method it is possible to study the nutritional requirements of roots, shoot and root growth, conditions required for the development of secondary vascular tissues, lateral root and bud formation, nodulation etc.

ii. Shoot-tip culture (Meristem – shoot tip culture)

The shoot apex or shoot-tip (100-1000 μm long) consists of the apical meristem and one to three adjacent leaf primordia. Actively growing shoot-tip is surface sterilized and is placed on a defined culture medium under sterile conditions.
Shoot Node culture is simplified form of shoot culture, which is yet another method for production from pre-existing meristem. It is the simplest method but is associated with the least genetic variation. Some of the crop species that have been freed of viruses by this technique, they include soyabean, sweet potato, sugar cane and rhubarb. This method is used with both monocot and dicot species.

iii. Leaves or leaf primordia culture

The growth of leaves on the culture medium depends upon the stage of the leaves during excision. It is observed that explants from immature young leaves grow better than explants from older leaves. Leaves (800 μm long) may be detached from shoots, surface sterilized and placed on a solidified medium where they will remain in a healthy conditions for a long period. It is believed that leaf culture depends upon the physiological state and the age of the leaf. The shoot forming potentials differ in the leaf cultures as per the derivation of the explant and the hormonal factors involved.

iv. Flower culture (Meristem - floral culture)

Flowers two days after pollination are excised, sterilized by immersion in 5% calcium hypochlorite, repeatedly washed with sterilized water and transferred to culture tubes containing an agar medium. When cultured, such flowers produce fruits. Larger fruits are obtained on medium supplemented with growth hormones. Flowers excised before pollination do not produce fruits.

This culture system is useful in studying microclimates or nutritional effects on the vegetative and reproductive processes of the plant.

v. Anther and pollens culture

Young flower buds are removed from the plant and surface sterilized. Immature stage of anther or late stage of anther containing starch filled pollen usually grow abnormally or the development is generally ineffective and hence for better response always select mature anther or pollen. The anthers are then carefully excised and transferred to an appropriate nutrient medium. The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period. Anther or pollen grains of different species have been successfully culture to obtain large number of haploid plants.

Pollen grains removed from the anther either mechanically or by naturally dehiscence. Anthers placed in 5 ml of liquid medium in a petri dish containing pollen grains in the culture media are sealed with parafilm and incubated. After incubation haploid plantlets are developed. The homozygous diploid plants obtained by doubling the chromosomes of haploids have great importance in plant breeding and crop improvement.

vi. Ovule and embryo culture

Mature embryos are excised from ripened ovule/seeds and cultured mainly to avoid inhibition in the seed for germination. Very small globular embryos require a delicate balance of the hormones. Embryo is dissected from the ovule/seed and put into culture media. This type of culture is relatively easy as the embryos require a simple nutrient medium containing mineral salts, sugar and agar for growth and development. Also, multicellular immature embryos are dissected out and cultured aseptically to obtain viable hybrids. Once the embryo is rescued, two genomes are needed to be combined together to produce a fertile plant.
By this method dormancy period of seeds can be shortened, as well as haploids can be produced. By ovule culture, it is possible to grow, study various nutritional requirements and stages young embryos or zygote.

vii. Ovaries culture

Ovaries excised after pollination can produce fruits on a simple medium containing mineral salts, sugar and vitamins. Ovaries taken from unpollinated flowers fail to produce fruits on such a simple medium but can develop into seedless fruits on a medium supplemented with hormones.

By this culturing method physiology of fruit development can be studied. Haploids can be produced. Rare hybrids can also be produced by ovary culture. Dormancy period of seeds can be reduced.

viii. Nucellus culture

Nucellar tissues excised from unfertilized ovules have no adventitive embryoids. If cultured on a medium containing malt extract and adenine, embryoids are formed but they are unable to germinate. When such embryoids are excised and cultured on a medium containing gibberellins, plantlets may be formed which can then be transplanted to the field. Disease-free clones can be obtained by nucellus culture.

ix. Seed culture

The seeds are treated with 70% alcohol for about two min., washed with sterile distilled water, treated with surface sterilizing agent for specific period. Once again rinsed with sterilized distilled water and kept for germination by placing them on double layers of pre-sterilized filter paper, placed in petri-dish moistened with sterilized distilled water or placed on moistened cotton swab in petri-dish.
The seeds are germinated in dark at 25-28°C and small part of the seedling is utilized for the initiation of callus.

x. Cotyledon culture

Immature cotyledon develops into somatic embryos, shoot buds and complete plants if cultured on a suitable nutrient medium.

xi. Endosperm culture

If endosperm is cultured on a proper nutrient medium continued proliferation of callus mass takes place and subsequently plant is regenerated. It is useful for plant breeding and horticultures. It is useful in the production of plantlets as an alternative to the conventional methods of crossing i.e. tetraploid with diploids or triploids induction which is applicable to fruit trees.

xii. Fruit culture

The culture of fruit tissues as whole organ or isolated tissue section such as ovary has been successfully cultured to give rise to mature fruits e.g. strawberry. Usually when an isolated portion of the fruit tissue is introduced into a sterile environment it immediately loses structural integrity and degenerates into a rapidly dividing callus

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mass. Loss of structural integrity is associated correspondingly with an alteration of physiology that is subsequently reflected in the production of an altered metabolism.
So it is not possible to make a meaningful study of fruits developing using callus derived culture. At present, the use of fruit culture is to serve as a bioassay system to study fruits maturatiodn events within a controlled environment.

II. Hairy/Transformed roots (Transinfection)

Certain soil bacteria of the genus *Agrobacterium* (Gram negative bacteria) infects a wide range of plant species and causes the infection in plant termed as "Hairy root" disease. The disease is transformed by their genome t-DNA from a bacterial plasmid to plant hairy root cells. A large number of small fine hairy roots covered with root hairs originated directly from the explant in response to *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. The hairy roots are produced by inoculating the host plant when grown in a hormone-free medium give rise to copious roots referred to as 'transformed roots' or 'hairy roots'. These are fast growing, highly branched adventitious roots at the site of infection. They are genetically stable and affect a wide range of dicotyledonous plants and have same metabolic features.

Establishment/Methodology of Hairy root culture

The explant material is inoculated with a suspension of *Agrobacterium rhizogenes* generated by growing bacteria in Yeast Mannitol Broth (YMB) medium for two days at 25°C with gyratory (round circle) shaking, pelleting by centrifugation (5×10 rpm; 20min) and suspending the bacteria in YMB medium to form a thick suspension. Transformation may be induced as aseptic plants grown from seeds, of on detached leaves, leaf discs and petioles of stem segments from green house plants following sterilization of excised tissue with 10% (v/v) domestos for 20 min.
Scratching the midrib of a leaf or the stem of a plantlet, with the needle of a hypodermic syringe containing the thick bacterial suspension allows inoculation with small (about 5-10µl) droplets containing *Agrobacterium rhizogenes*. In some species a profusion of roots may appear directly at the site of inoculation, but in other a callus will form initially and roots emerge subsequently from it. In either case hair rot normally appear within 1-4 weeks although the susceptibility of species to infection is variable. Table no. 1.1 describes some plants and active constituents obtained by Hairy root culture.

Applications of Hairy root culture

1. Growth kinetic and Genetic stability over prolonged period of growth *in vitro.*
2. Ease of culture *in vitro* using simple media lacking phytohormones.

3. Many plant cell culture systems, which did not produce adequate amount of desired compounds is being reinvestigated using hairy root culture methods.
4. A diversified range of plant species has been transformed using various bacterial strains.
5. Plant regeneration, plant improvement and genetic manipulation in plant can be done.
6. Reproducible and predictable levels of product synthesis.
7. Capability to synthesize (novel) secondary metabolites specific to that plant species from which they have developed in equal or even higher amount compared to field grown plants.
8. More accumulation of secondary metabolites. E.g. Levels of steroidal alkaloid solasodine is significantly higher in hairy root cultures than callus or suspension cultures.
9. It is applied for phytoremediation of elemental and organic pollutants.
10. Production of protein and modifying plant architecture.

Problems associated with Hairy root culture

1. No transformation.
2. Loss of gene effects.
3. Excess bacterial growth.
4. Slow regeneration.
5. Decreased cell division and transformation due to stress.

Table no. 1.1 Plants with active constituents obtained by Hairy root culture

Sr. No.	Plant	Active constituents
1	*Artemisia annua*	Artimisinin
2	*Hyoscyamus muticus*	Hyoscyamine
3	*Nicotiana tabacum*	Nicotine, Anatabine
4	*Podophyllum spp.*	Lignans
5	*Panax ginseng*	Polyacetylene analogues
6	*Solanum aviculare*	Solasodine
7	*Withania somnifera*	Withanolides
8	*Valeriana wallichi*	Valpotriates

III (a) Protoplast culture

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Protoplast are plant cells with a plasma membrane but without cell wall, because of this the protoplast provide the starting point for many of the technique of genetic manipulator of plants, in particular the induction of somaclonal variation, somatic hybridization and genetic transfer.

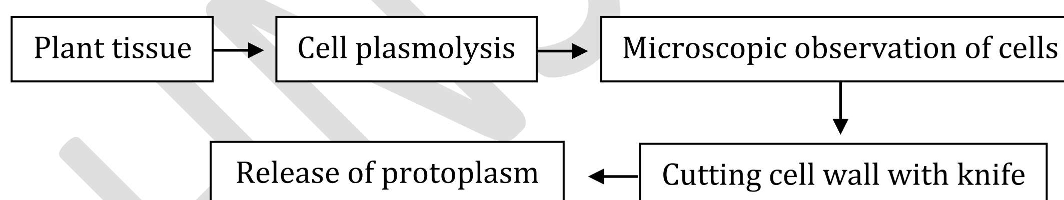
Isolation of Protoplasts

Protoplasts can be isolated from almost all plant parts i.e., roots, leaves, fruits, tubers, root nodules, endosperm, pollen cells and cells of callus tissue. It can be done by two methods as follows:

1. **Mechanical method and**
2. **Enzymatic method**

1. Mechanical method

The cells were kept in a suitable plasmolyticum (lysis of plasma membrane) and cut with a fine knife. Cells were cut only through the cell wall, releasing intact protoplast. This mechanical procedure gave low yield of protoplasts and could be utilized for only highly vacuolated and non meristematic cells. The method is laborious and tedious. Mechanical method for isolation of protoplast is shown as below (Flow chart 1.2):



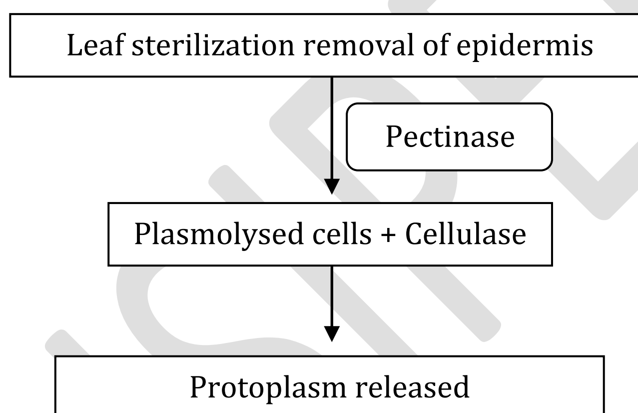
Flow chart 1.2 Mechanical method of protoplast isolation

2. Enzymatic method

Commercial preparations of purified cell wall degrading enzymes such as macerozyme, cellulase and hemicellulose became available that gave further progress to enzymatic isolation of protoplasts. By this method very large number of protoplast are obtained compared to mechanical method. Cells are not damaged or broken. Osmotic shrinkage of protoplast is much less. Enzymatic method of protoplast isolation can be classified into two types as follows:

a. Sequential enzymatic method

It involves two steps where first macerated plant tissues are incubated with pectinase to get single cells followed by cellulase treatment to get protoplast.
The leaf segments with a mixture of 0.5% pectinase (maceroenzyme), 0.3% potassium dextran sulphate in 13% mannitol at pH 5.8 are vacuum filtered for 5-7 min., transferred to a water bath at 25°C and subject to slow shaking. After 20 min. the enzyme mixture is replaced by fresh enzyme mixture and leaf segments are incubated for another one hour. The mixture is filtered using nylon mesh, centrifuged 100g for 1 min. and washed 3 times with 13% mannitol to get a pure sample of isolated cells. These cells are then, incubated with 2% cellulase in a 13% solution of mannitol at pH 5.4 for about 90 min. at 30°C. After incubation, the mixture is centrifuged at 100g for 1 min. so that protoplasts form a pellet, which is cleaned 3 times to get a cleaned protoplast. Enzymatic method for isolation of protoplast by sequential method is shown below (Flow chart 1.3):

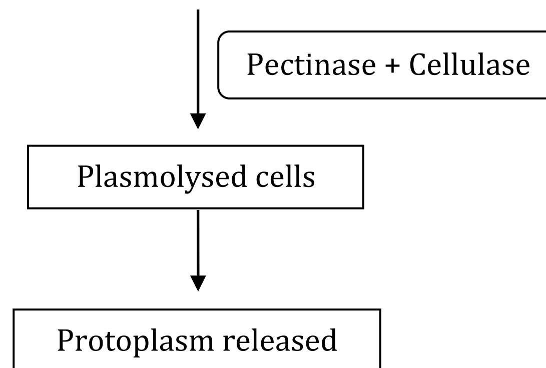


Flow chart 1.3 Sequential enzymatic method of protoplast isolation

b. Mixed enzymatic method

This involves simultaneous separation of cells and degradation of their walls to convert protoplast by immersing plant tissues in mixture of pectinases and cellulases.
The leaf segments are incubated overnight with enzyme mixture of 0.5% pectinase (maceroenzyme), 2% cellulose in 13% sorbitol or mannitol at pH 5.4 at 25°C and treated gently to liberate the protoplasts. The mixture is filtered with wire guaze to remove leaf debris, transferred to screwcap tubes and centrifuged for 1 min. The protoplast form a pellet and supernatant removed. The process is repeated for 3 times and protoplasts are washed with 13% sorbitol solution, which is later replaced by 20% sucrose solution and centrifuged for 1 min, the cleaned protoplast, which will float can be pipette out.
Enzymatic method for isolation of protoplast by mixed method is shown below (Flow chart 1.4):

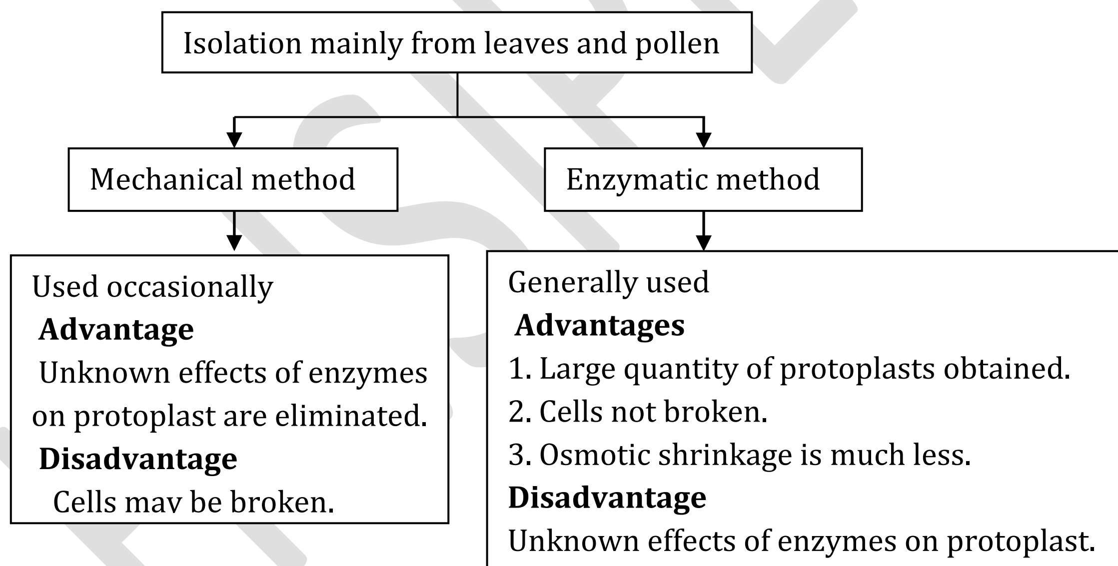
Leaf sterilization removal of epidermis



Flow chart 1.4 Mixed enzymatic method of protoplast isolation

Advantages and Disadvantages of Mechanical and Enzymatic methods for isolation of protoplast

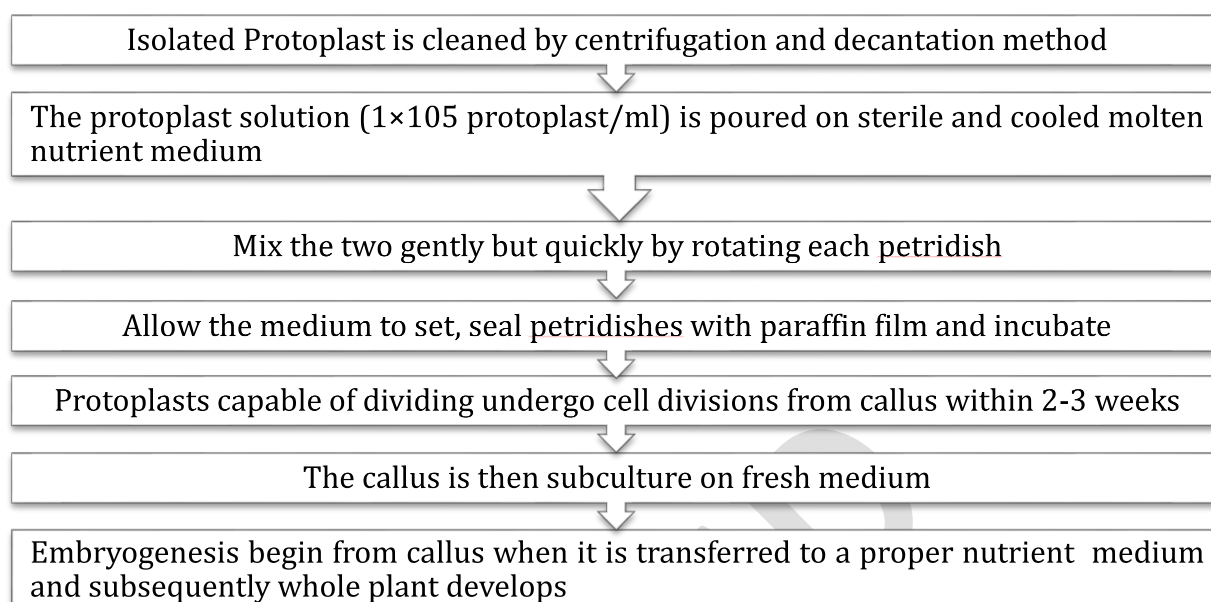
It is shown below in Flow chart 1.5



Flow chart 1.5 Method of protoplast isolation and their advantages and disadvantages

Methodology of Protoplast Culture

Isolated protoplasts are usually cultured in either liquid or semisolid agar media plates. They require somatic protection in culture medium until they generate a strong cell wall. Methodology of Protoplast culture is described below (Flow chart 1.6):



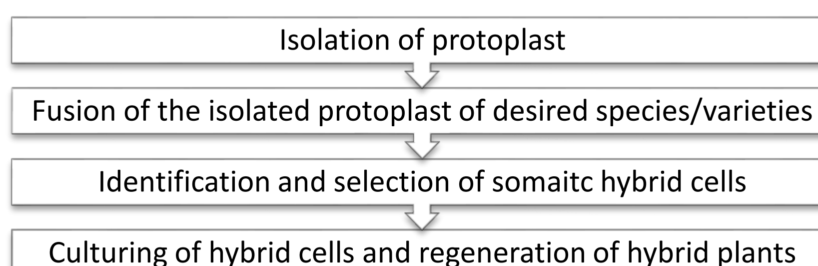
Flow chart 1.6 Methodology of Protoplast culture

Applications of protoplast culture

1. To develop Novel hybrid plant through protoplast fusion.
2. In single cell derived colony, isolation of mutants through mutagens is easier.
3. Single cell cloning can be easily performed with protoplasts.
4. Regeneration of entire plant or plant improvement through protoplast culture.
5. Genetic transformation through DNA uptake can be achieved.
6. Reproducible protoplasts to plant systems are now available for many plants of agronomic value.

III (b) Somatic Hybridization

Somatic hybridization-It is the technique of hybrid production through the fusion of isolated somatic protoplast under *in-vitro* conditions and subsequent development of their product to a hybrid plant is called somatic hybridization. Various stages of somatic hybridization are as follows (Flow chart 1.1):



Flow chart 1.1 Stages of Somatic hybridization

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i. Isolation of protoplast

Already discussed in protoplast culture

ii. Protoplast fusion

It is the technique in which two or more protoplasts are fused into a single cell protoplast. The protoplast fusion allows us to bring desirable plant traits in combinations that are not possible by sexual means. It may occur between same or different plants and mutinucleate protoplasm is obtained after fusion. Three main phases are there for fusion:

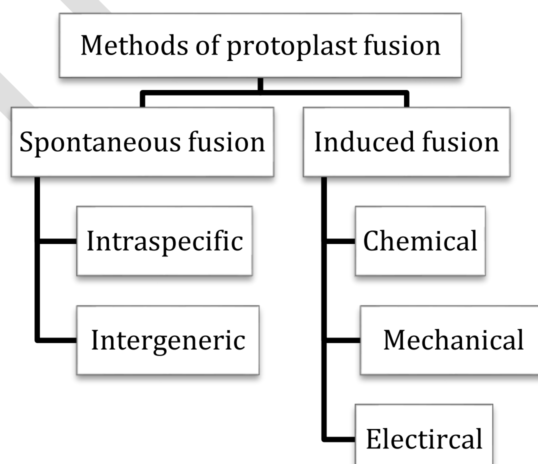
Agglutination: Plasma membranes of two or more protoplast are brought into close proximity as shown in Figure 1.3 (A and B).

Formation of cytoplasmic bridge: Membranes of protoplasts agglutinated by fusogen get fused at the point of adhesion. This results in the formation of cytoplasmic bridge between the protoplast.

Rounding off: The fused protoplast is rounded off due to the expansion of cytoplasmic bridge forming a spherical heterokaryon (A-B) or homokaryons (A-A or B-B). Binulceate heterokaryons. The fusion of the nuclei results in a tetraploid hybrid cell. Also cybrid cell is formed with a selective chromosome loss.

Techniques/Methods of Protoplast fusion

Fusion of protoplast of two different genomes can be done by mainly two different methods as follows:



1. Spontaneous Fusion

Protoplasts fuse spontaneously during isolation process mainly due to physical contact among similar parental protoplast. Protoplasts from their adjoining cells fuse through their plasmodesmata to form a multinucleate protoplast. Spontaneous fusion is of two types as below:

a. **Intraspecific** produce homokaryones
b. **Intergeneric** have no importance

2. Induced Fusion

Freely isolated protoplasts from different sources are fused with the help of fusion inducing agents. Fusion needs an inducing agent that actually reduces electro negativity and allows the isolated protoplast to fuse. Fusion is induced by fusogens which are categorized into three types as below:

a. **Chemical fusion**
b. **Mechanical fusion**
c. **Electrical fusion**

a. Chemical Fusion

Chemical fusion is induced by various types of fusogens like polyethylene glycol (PEG), Sodium nitrate ($NaNO_3$), Calcium (Ca^{2+}) ions, polyvinyl alcohol (PVA) etc. The cell membrane posses negative charge and after treatment with such chemical agents when cell membrane are brought into close physical contact they fuse.

b. Mechanical Fusion

It is not dependent upon the presence of fusion inducing agent. Physical fusion of protoplasts is done under microscope by using micromanipulator and perfusion micropipette.

c. Electrical Fusion

In this type of fusion protoplast are placed into a small culture cell containing electrodes and a potential difference (10kv m^{-1}) is applied then the protoplast will line up (form chain) between the electrodes. Fusion of protoplasts of chain is induced by the application of high strength electric field (100kv m^{-1}) for few microseconds.

iii. Identification and selection of somatic hybrid cells

The protoplasts of two parents may be labelled by different fluorescent compounds, which will then enable for the identification and selection of hybrids.
Identification is based on difference between the parental cells and hybrid cell with respect to pigmentation, cytoplasmic markers, presence of chloroplast, nuclear staining etc.
In the mixture of both fused and unfused protoplast Selection of hybrid is done on the basis of several procedures like Genetic complementation, antibiotic-resistant cell lines, isoenzyme analysis, phytotoxins, Specific amino acid, auxin autotrophy, auxotrophic, metabolic mutants, chromosomal analysis by hybrid cell, herbicides etc.

iv. Culturing of hybrid cells and regeneration of hybrid plants

Hybrid cells are cultured on suitable medium provided with the appropriate culturing conditions. Plants are induced to regenerate from hybrid cells. These hybrid plants must be at least partially fertile, in addition to having some useful property, to be of any use in breeding schemes.

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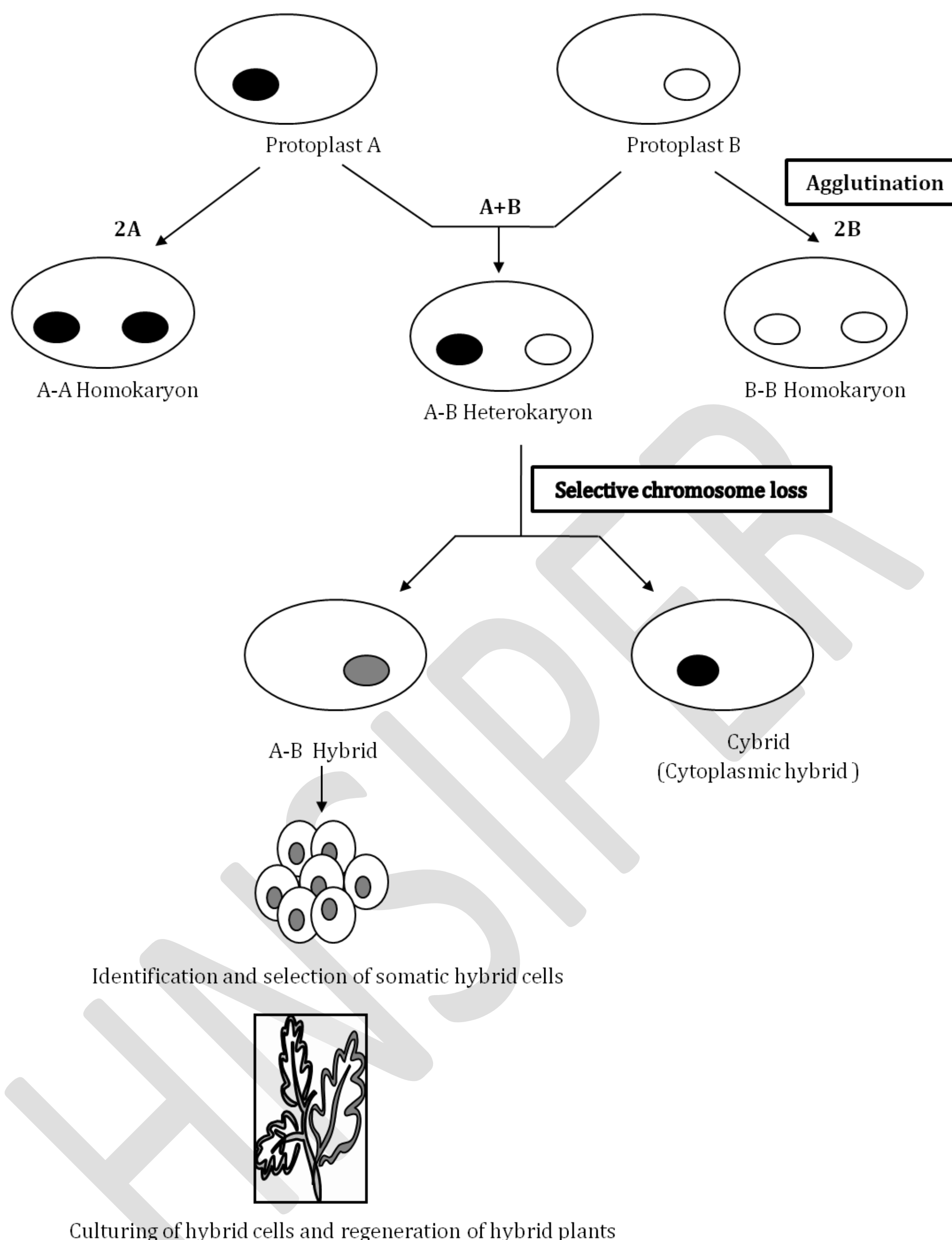

Figure 1.3 Stages of protoplast fusion and somatic hybridization

Advantages of somatic hybridization

1. Novel approach for introducing or increasing genetic variability at nuclear or extra nuclear organelle genome level, in higher plants.
2. Production of novel interspecific and intergenic hybrid e.g. Pomato (Hybrid of potato and tomato).

3. Somatic hybridization is significant in improvement of plants such as banana, potato, sugarcane and yam.
4. Production of fertile diploids and polyploidy from sexually sterile haploids, triploids and aneuploids.
5. Transfer gene for disease resistance, abiotic stress resistance, herbicide resistance and many other quality characters.
6. Production of heterozygous lines in the single species which cannot be propagated by vegetative means.
7. Studies on the fate of plasma genes.
8. Production of unique hybrids of nucleus and cytoplasm.

Limitations of Somatic hybridization

1. Poor regeneration of hybrid plants
2. Non-viability of fused products
3. Not successful in all plants.
4. Production of unfavorable hybrids
5. Lack of an efficient method for selection of hybrids
6. No confirmation of expression of particular trait in somatic hybrids.
7. Elimination of chromosomes from the hybrid cell

Plant regeneration (General methodology of plant tissue culture)

Whole plant can be regenerated from the above mentioned cultures. Establishment of any plant tissue culture follows a standard methodology. General methodology (Figure 1.1) used in the isolation and growth of plant tissue is described as follows:

1. *Preparation of suitable nutrient medium*: As per the selection of plant medium is autoclaved.
2. *Selection of explant*: Any excised part of health plant to be used e.g. Bud, leaf, root, seed etc.
3. *Sterilisation of explants*: by sodium hypochlorite, mercuric chloride etc. and washed aseptically for 6-10 times with sterilised water.
4. *Inoculation (Transfer)*: The sterile explant is inoculated on solidified nutrient medium under aseptic condition.
5. *Incubation*: Cultures are incubated at of 25±2°C and at a relative humidity up to 50-70% for 16 hrs of photo period. The callus formed after incubation under goes subculturing.
6. *Regeneration*: Plantlets regenerated after transferring a portion of callus into another medium and induction of roots and shoots or directly from explants.
7. *Hardening*: It is the gradual exposure of plantlets for acclimatisation to environment condition.

8. *Plantlet transfer*: Plantlet are transferred to green house or field conditions.

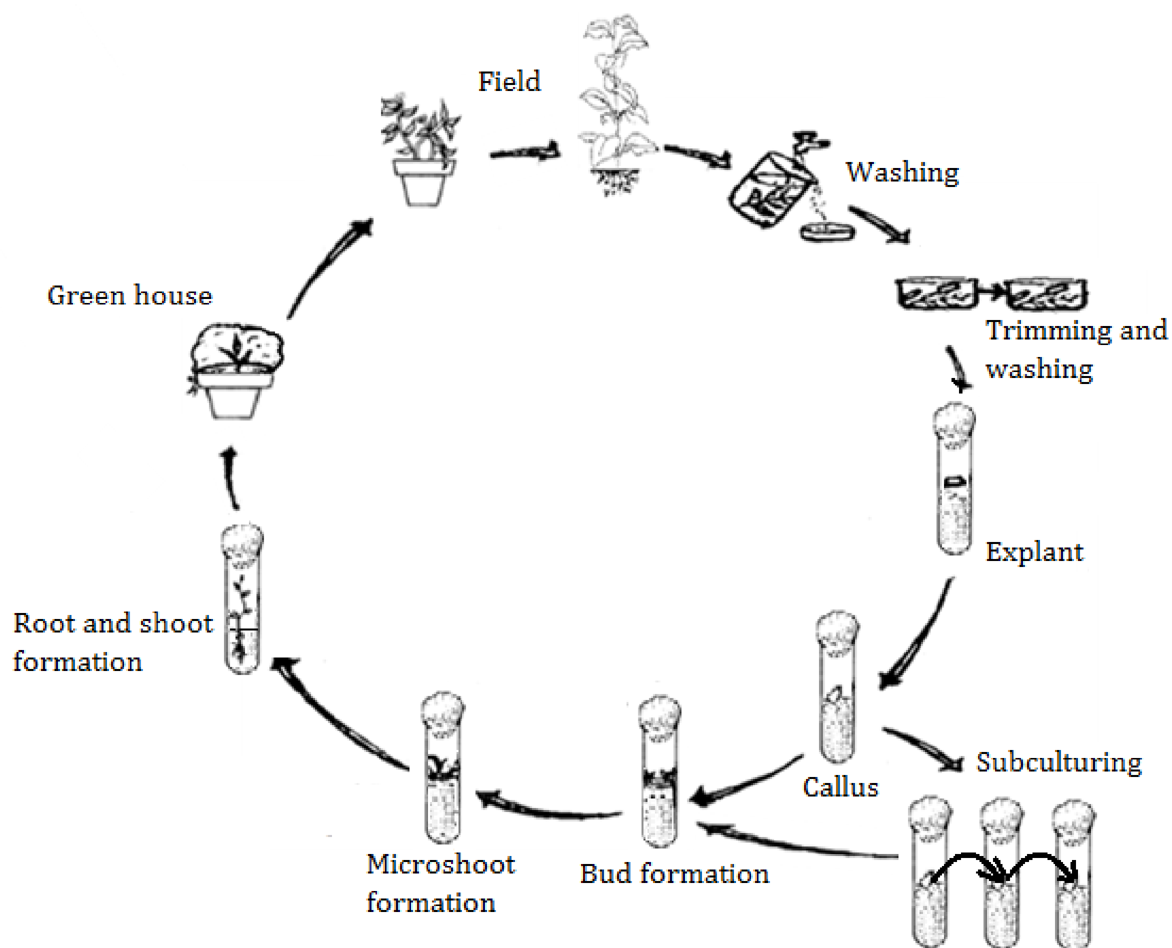

Figure 1.1 General Methodology of plant tissue culture

In broad terms, two methods of plant regeneration are widely used in plant transformation studies: organogenesis and embryogenesis

Organogenesis

Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds or root or even whole plantlets. In short, the formation of organs is called organogenesis. In plants, development is the process that results in a functional mature organism which includes all the events during the life of the plant that produce the body of the organism and provides the capacity to obtain food, to reproduce and deal with the hazards of its environment. Therefore, organogenesis is a process that is in some way unique to plants.
The shoot buds are monopolar structures which in turn give rise to leaf primordial and the apical meristem. The stimulation of shoot bud differentiation in plants depends on many factors which differ for different plant species. Other factors affecting organogenesis are size and source of the explant. Light intensity plays an

important role in organogenesis. High light intensity has been shown to be inhibitory for shoot bud formation in tobacco. Even the quality of light has effect as blue light has been shown to induce shoot formation while red light stimulates rooting in tobacco. The optimum temperature required may vary with plant species. A medium solidified with agar favours bud formation although there are some reports about the development of leafy shoot buds on cultures grown as suspension.

Embryogenesis

Embryo is defined as the earliest recognisable multicellular stage of an individual that occurs before the development of characteristic organs of the given species. Production of embryo like structure from callus is known as embryogenesis. In higher plants such embryos usually arise from zygote formation and are termed as zygotic embryos.

Various types of cells and tissues can be used as source of embryogenic cells. It may be microspores (1n), zygote (2n), somatic cells (2n) or somatic hybrids (4n). Embryogenesis can be induces in such cells by non-proliferative or proliferative direct embryogenesis or by growing embryogenic callus which in turn germinates and develops into whole plant. Embryogenesis can be initiated in an explant only from the more juvenile or meristematic tissues. Immature zygotic embryos, cotyledons and hypocotyl dissected from ungerminated seeds are common explants. Isolated somatic cells can develop into embryos. Embryo development occurs through an organised sequence of cell division, enlargement and differentiation. The final stages of development towards maturation are distinguished by overall enlargement and matured embryo morphology.

Somatic embryogenesis involves three distinct phases which are absent in organogenesis as below:

Induction-It is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called embryogenic clumps (ECs). These ECs develop into initial stages of somatic embryo i.e. globular stage.

Maturation-In this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped and the mature embryo here undergoes biochemical changes to acquire hardness.

Conversion-In this phase embryos germinate to produce seedlings.

Figure 1.2 describes plant regeneration from an explant by organogenesis and embryogenesis.

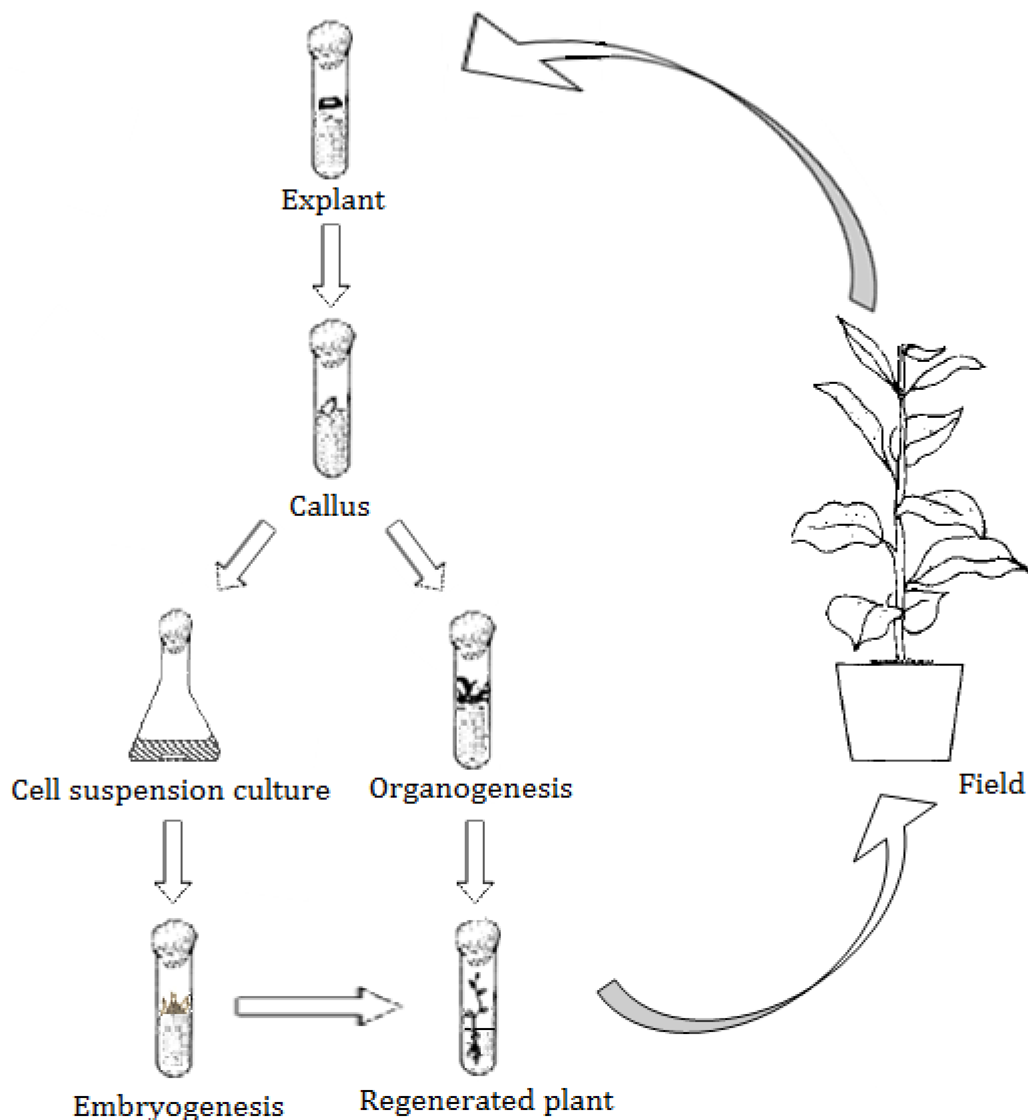

Figure 1.2 Regeneration of plant from an explant

Advantages of Organogenesis and Embryogenesis

1. Efficiency of process i.e. reduction in labour cost and time and the formation of plantlets is fewer steps.
2. The potential for the production of much higher number of plantlets and morphological and cytological uniformity of the plantlets.
3. Production of several plants on commercial scale includes food crops, vegetables, spices, and fruits, medicinal and aromatic plants.

4. Nutrient Media for plant tissue cultures

Nutrient medium is necessary growth of the plant. Functions of the nutrient medium include:

1. Provide water
2. Provide mineral nutritional needs
3. Provide vitamins
4. Provide growth regulators
5. Access to atmosphere for gas exchange
6. Removal of plant metabolite waste

Composition of Culture media

In tissue culture literature the concentrations of inorganic and organic constituents of the medium are generally expressed in mass values mg l^{-1}. This has been followed in Table no. 1.2. Culture Media is mainly composed of three nutrients as below:

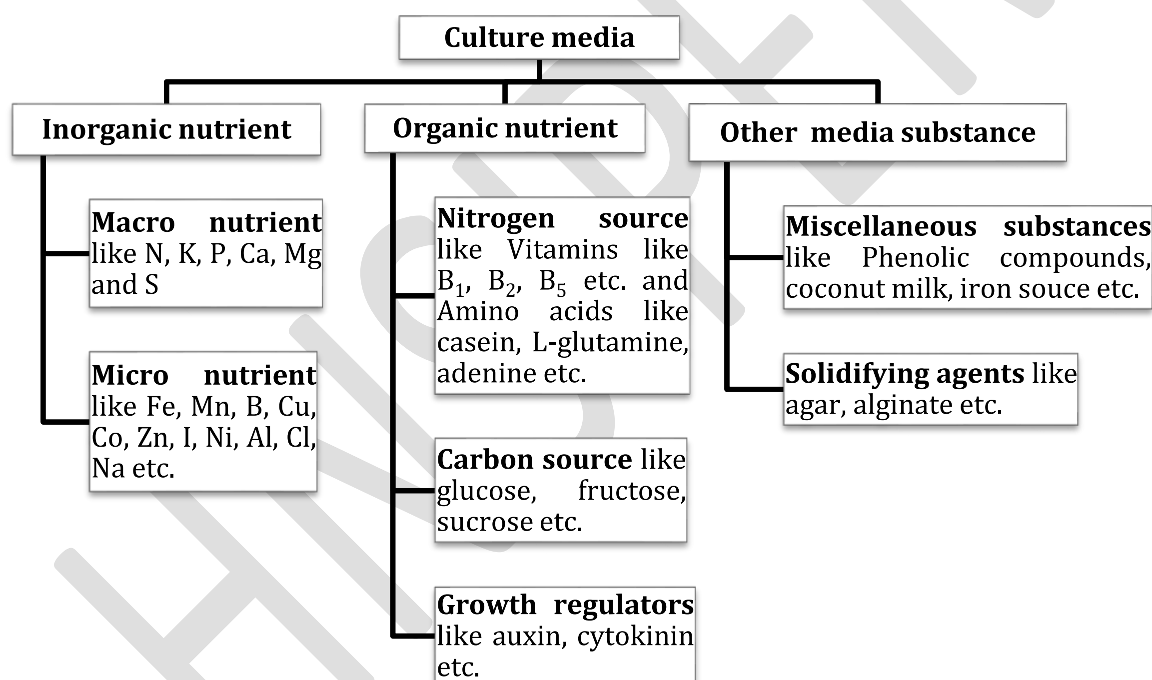

Table no. 1.2 Compositions of some plant tissue culture media

Nutrient medium	Media (amount in mg l^{-1})				
	Heller (1953)	MS (1962)	White (1963)	B_5 (1968)	Nitsch (1969)
Inorganic nutrient					
Macronutrient					
KH_2PO_4	-	170	-	-	68.0
$CaCl_2 \cdot 2H_2O$	75	440	-	150	-
$CaCl_2$	-	-	-	-	166
NH_4NO_3	-	1650	-	-	720

HNO_3	-	1900	80	2527.5	950
$NaNO_3$	600	-	-	-	-
$Ca(NO_3)\cdot 4H_2O$		-	300	-	-
$Na\ H_2PO_3$	125	-	19	150	-
$MgSO_4\cdot 6H_2O$	250	370	750	246.5	185
Micronutrient					
$AlCl_3$	0.03	-	-	-	-
$Fe_2(SO_4)_3$	-	-	2.5	-	-
$Fe_2(SO_4)\cdot 7H_2O$	-	27.8	-	-	27.8
$MnSO_4\cdot 4H_2O$	0.1	22.3	5	-	25
KCl	750	-	65	-	-
$NiCl_2\cdot 6H_2O$	0.03	-	-	-	-
$MnSO_4\cdot H_2O$	-	-	-	10	-
$ZnSO_4\cdot 7H_2O$	1	8.6	3	2	10
$CuSO_4\cdot 5H_2O$	0.03	0.00025	0.01	0.025	0.025
$FeCl_3\cdot 6H_2O$	1.00	-	-	-	-
$MnSO_4\cdot H_2O$	-	-	-	10	-
$FeSO_4\cdot 7H_2O$	-	27.8	-	-	27.8
KI	0.01	0.83	0.75	0.75	-
H_3PO_3	1	6.2	1.5	3	10
$Na_2MoO_4\cdot 2H_2O$	-	0.25	-	0.25	0.25
MoO_3	-	-	0.001	-	-
$COCl_2\cdot 6H_2O$	-	0.025	-	0.025	-
Organic nutrient					
Vitamin					
Glycine	-	2.0	3.0	-	2.0
Folic acid	-	-	-	-	0.5
Nicotinic acid	-	0.5	0.05	1	5
Thiamine HCl	-	0.10	0.01	10	0.5
Pyridoxine HCl	-	0.5	0.01	1	0.5
Carbohydrate					
Sucrose	-	3%	2%	2%	2%
Growth regulators					
Inositol	-	100	-	100	100
2,4-D	-	0.1	-	-	-
IAA	-	1.0	-	-	-
Kinetin	-	0.04	-	0.02	0.1
NAA	-	-	-	-	-

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Myo-inositol	-	100	-	100.0	-
Other Media Substance					
$Na_2EDTA \cdot 2H_2O$	-	37.3	-	-	37.3
pH	-	5.7	5.5	-	-

Note: Growth regulators and complex nutrient mixtures described by various authors are not included. Concentration of sucrose is expressed in percentage.
MS- Murashige and Skoog
B5- Gamborg et al.

I. Inorganic nutrients

Mineral elements play very important role in the growth of plant function of nutrients in plant growth. Essentially about 15 elements found important for whole plant growth have also been proved necessary for the growth of tissue(s) in culture. It mainly includes macro nutrient and micro nutrient as follows:

i. Macronutrient

Elements required in the life of a plant greater than 0.5 mmol/lit are referred as macronutrients. The macronutrients include six major elements are as follows:

Nitrogen (2-20mmol/lit)–It Influences plant growth rate, essential in plant nucleic acids (DNA), proteins, chlorophyll, amino acids, and hormones.
Phosphorus (1-3mmol/lit)–It is abundant in meristematic and fast growing tissue, essential in photosynthesis and respiration.
Potassium (20-30mmol/lit)–It is necessary for cell division, meristematic tissue, helps in the pathways for carbohydrate, protein and chlorophyll synthesis.
Calcium (1-3mmol/lit)–It is Involved in formation of cell walls and root and leaf development. It participates in translocation of sugars, amino acids, and ties up oxalic acid (toxin).
Magnesium (1-3mmol/lit)–It is involved in photosynthetic and respiration system. It is active in uptake of phosphate and translocation of phosphate and starches.
Sulfur (1-3mmol/lit)–It is involved in formation of nodules and chlorophyll synthesis, structural component of amino acids and enzymes.

ii. Micronutrient

Elements required in the life of a plant less than 0.5 mmol/lit are referred as macronutrients. Overall the plant thrives on seventeen elements out of which four like carbon, hydrogen, oxygen and nitrogen are derived from the atmosphere and the

rest thirteen microelements like boron, copper, iron, manganese, zinc, cobalt, molybdenum, nickel, aluminium, iodine, ferrous, sodium, chlorine. A media lacking in these micronutrient does not support healthy and wholesome growth and the plant exhibits deficiency symptoms such as pigmentation, absence of vessels, presence of narrow cambial zone, cellular hypertrophy and symptoms of chlorosis due to absence of ferrous and sulphur. The micronutrients include following elements:

Iron (Fe-1μM) is involved in cell division, respiration, chlorophyll synthesis and photosynthesis. E.g. FeNaEDTA-sodium salt of EDTA. *Manganese* (Mn-5-30μM) is involved in cell elongation, regulation of enzymes and growth hormones. It assists in photosynthesis and respiration. *Boron* (B) is responsible for cell division and cell elongation. *Copper* (Cu-0.1μM), *Molybdenum* (Mo-1μM), *Cobalt* (Co-0.1μM), *Zinc* (Zn), *Iodine* (I), *Nickel* (Ni), *Aluminum* (Al), *Chlorine* (Cl) and *Sodium* (Na) are also required.

II. Organic nutrients

It includes nitrogen substances, vitamins, amino acids, carbon source, growth hormones/regulators

i. Nitrogen source

Most cultured plant cells are capable of synthesizing essential vitamins but not in sufficient amount. To achieve best growth it is essential to supplement the tissue culture medium with one or more vitamins and amino acid.

a. Vitamins

Thiamine (Vitamin B_1) is essential as a coenzyme in the citric acid cycle. It is required mostly in tissue culture and is considered to be essential. Thiamine hydrochloride in 0.1-1mg/lit concentration is required. Nicotinic acid (Niacin-Vitamin B_3-0.5 mg/lit), Pyridoxine (Vitamin B_6-0.5 mg/lit), Calcium pentothenate (Vitamin B_5-0.1 mg/lit) are known to improve growth of the tissue culture material. Myo-inositol part of the B complex, in phosphate form is part of cell membranes, organelles and is not essential to growth but beneficial and have important role in many biosynthetic pathways. Cynocobalamin (Vitamin B_{12}), Riboflavin (Vitamin B_2,), Folic acid (Vitamin M-0.5 mg/lit), Biotin (Vitamin H), p-amino benzoic acid (PABA), Ascorbic acid (Vitamin C), α-tocopherol (vitamin E) are added in special cases but their exact role is not yet well established.

b. Amino Acids

Some cultured plant-cells can synthesize all amino acids, none are considered essential. The most common sources of organic nitrogen used in culture media are amino acid mixtures, (e.g., casein hydrolysateb), L-glutamine, L-asparagine, orginine, methionine and adenine. When amino acids are added alone, they can be inhibitory to cell growth. Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. L-tyrosine - stimulates shoot formation. Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.

ii. Carbon source

Carbohydrates are used in tissue culture media as an energy source of carbon. Most plant tissue culture is nonautotropic and is therefore entirely dependent on an external source of carbon. The most commonly used carbon source is Sucrose (2-5% or 20-30g/lit). Glucose and Fructose are used for good growth. Maltose and raffinose are used in some cases. In general excised dicotyledonous roots grow better with sucrose where as monocots do best with dextrose (glucose). Other carbohydrates like mannose, sorbitol, pentoses, sugar alcohol, glycols, hexoses, uronic acid, lactose, galactose, potato starch, grain starch and even glycosides can be used depending on the experimental conditions.

iii. Growth hormones/regulators/Modulators

The success of plant tissue, cell and organ culture will depends on the amount of plant hormones and growth substance added into nutrient medium. Auxins, ethylene, abscisic acid, cytokinins and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones. The requirement of these hormones varies considerable with their endogenous levels. Other plant hormones like polyamines, jasmonates, salicylates are also used depending on the experimental conditions and plants to be cultured.

Auxin

Auxin show a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division and organization of meristems giving rise to either callus or defined organs. In organized tissue, auxin cause root formation, delaying leaf senescence, fruit ripening and used in embryogenesis. Commonly used natural auxin is indole-3-acetic acid (IAA-1-50 mg/lit), but depending on the species, other natural auxins are 4-chloroindole-3-acetic acid, indole-3-butyric acid (IBA). Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA-0.1-10 mg/lit) and 2, 4 dichlorophenoxyacetic acid (2, 4-D-0.05-0.5 mg/lit).

Cytokinin

Cytokinins are useful in culture for stimulation of cell division (cytokinesis), release of lateral bud dormancy and induce adventitious bud formation. Cell division is regulated by the joint actions of auxin and cytokinin. Auxin affect DNA replication where as cytokinin seems to exert some control over the events leading to mitosis. In intact plants, cytokinin promotes lateral bud growth and leaf expansion, promote chlorophyll synthesis and enhance chloroplast development. The most commonly used cytokinins are the substituted purines such as synthetic derived kinetin (0.1-10 mg/lit), 6-benzyladenine (BA). Zeatin and 6-γ-γ-dimethylamino purine (2-iP) are naturally occurring cytokinins. Other cytokinins are adenosine and adenylic acid.

Gibberellin

Gibberellins will promote flowering, seed germination and stem or shoot elongation. There are over 20 known Gibberellins. Gibberellin (GA_3) is usually used to increase the shoot elongation. Gibberellins are used rarely compared to auxin and cytokinin. Cultured callus cells synthesize their own Gibberellins.

Abscisic acid (ABA)

Abscisic acid (ABA) is naturally produced in plant tissues. ABA and other structurally related natural compounds are most likely produced by the cleavage of xanthophyll. ABA is often regarded as being an inhibitor, as it maintains bud and seed dormancy, inhibits auxin-promoted cell wall acidification loosening and slows cell elongation. ABA plays a key role in closing of stomatal apertures (reducing transpiration) and abscission of leaves. ABA also control of water and ion uptake by roots.

Ethylene

It is a gaseous hormone synthesized in cultured cell, fungi and bacteria. Ethylene gas promotes fruit ripening, senescence, and leaf abscission. At higher concentrations the gas decreased cell elongation but increased cell expansion. The role of ethylene can be difficult to understand because its effects vary with development stage and because low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect. Ethylene is synthesized from methionine. Auxin stimulates the production of ethylene but the physiological significance of ethylene in tissue culture is quite obscure.

III. Others media substances

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It includes various protein hydrolysates (e.g., soy-protein hydrolyzates), yeast extracts, fruit (e.g. banana) extracts, demineralised water and Natural complexes like coconut milk fresh/pasteurized, fish emulsion, tomato juice which promotes growth of the tissue culture.

Phenolic compounds like Phloroglucinol stimulates rooting of shoot sections.

Activated charcoal is used as a detoxifying agent, it detoxifies wastes from plant tissues and impurities. Adsorption quality vary, concentration normally used is 0.3 % or lower. It adsorbs the secondary products secreted by the culture tissue.

Solidifying agent various solid media are often preferred because its improved oxygen supply and support culture growth. Therefore, substance with strong gelling capacity is added into the liquid media. The most commonly used substance is agar and others are alginate, carrageenan, gelatin, starch, silica gel, hydroxy ethyl cellulose and polyacrylamide. Agar is extraordinary resistant to enzymatic hydrolysis at incubation temperature and only few bacteria exist which are capable of producing degrading enzyme.

EDTA can be used as a *iron source*.

pH of the medium should be in a range of 5.6-6.0 before autoclaving the culture medium.

Antibiotics are used for prevention of excessive contamination in the culture medium. Generally fungicides and bactericides are used in culture medium but are not been very useful because they can be toxic to the explant and the contaminant sometimes reappears as soon as they are removed. Commonly used antibiotics are Carbonicillin (500mg/lit) and Augmentin (250 mg/lit)

5. Application of plant tissue culture

Plant tissue culture technology has been used in almost all the field of biosciences. Its applications include the following:

1. Production of phytopharmaceuticals and secondary metabolites.
    a. Biotransformation (Biochemical Conversion)
    b. Plant cell immobilization
    c. Genetic transformation (Transgenic plant)
    d. Elicitors
2. Micropropagation (Clonal Propagation)
3. Synthetic seed
4. Protoplast culture and somatic hybridization
5. Hairy root culture
6. Cryopreservation
7. Tracing the biosynthetic pathways of secondary metabolites
8. Generation novel compound from plant
9. Respiration, organ function and metabolism in plant tissue culture can be studied.

10. Plant improvement by studying diseases of plant and their elimination with the help of plant tissue culture.
11. Mutant cell selection is done by addition of toxic substance to cells followed by isolation of resistant cells.
12. Production of economical valuable chemicals by plant tissue culture which are not possible by other chemical methods.

Some application of plant tissue culture is discussed in brief as below:

I. Production of phytopharmaceuticals and secondary metabolites

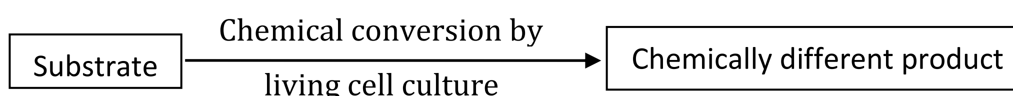
Secondary plant metabolites like alkaloids, terpenoids, flavonoids, lipids, oils, tannins, anthraquinones, flavones, napthaquinones, vitamins, proteins, anticancer agents, antiviral agents etc. are isolated from plant tissue culutre. Table no. 1.3 lists some phytopharmaceuticals derived from plant tissue culture of some plant species.

Table no. 1.3 Phytopharmaceuticals derived from plant tissue culture

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

a. Biotransformation (Biochemical Conversion)

It is a process through which the functional group of organic compounds are modified by living cells. This process can be done by using microorganism or plant cell suspension, hairy root culture and immobilized cell.



Biotransformation by plant cell cultures yield a wide range of reactions, such as glycosylation, glucosyleserification, hydroxylation, oxido-reductions, hydrolysis, epoxidation, isomerisation, methylation, demethylation and dehydrogenation etc. Table no. 1.4 describes some plants which have under gone biotransformation. It not only increases the yield but also very economical for commercial production.

Table no. 1.4 Biotransformed plants

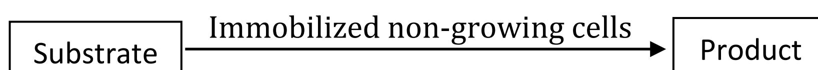
Class	Substrate	Product	Plant
Alkaloids	Codeinone	Codeine	*Papaver somniferum*
Phenolics	Resorcinol	Glucoside	*Datura innoxia*
Steroids	Digitoxin	Digoxin	*Digitalius Lanata*
Phenolic flavonoids	liquiritigenin	Glucoside	*Datura innoxia, Catharanthus roseus*
Phenolic anthraquinones	Rhein	Glucoside	*Perilla frutescens*
Phenolic courmarins	Umbelliferone	Umbelliferone-7-glucoside	*Datura innoxia, Catharanthus roseus*
Terpenoids	Citral	Citrol	*Lavandula angustifolia*

Advantages of Biotransformation reactions over non-biochemical reaction

1. Mild reaction conditions.
2. Environment friendly.
3. Selectivity, sterio-selectivity, region-selectivity.
4. Wide ranges of catalytic activities are available.

b. Plant cell Immobilization

It is defined as a technique, which confines to a catalytically active enzyme (or to a cell) with in a reactors system and prevents its entry into the mobile phase, which carries the substrate and product.



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It involves the entrapment of cells within a gel or passive adsorption on solid support materials, thus creating a situation for cell to imitate membership on a tissue of a whole plant. In immobilization technique the plant cells are entrapped in different polymerize matrices e.g. alginate, agar, agarose etc. and converted into hetrogeneous catalyst. Cells cease to grow and accumulate metabolites. It is a controlled process of agglutination, formation and adhesion on a matrix under controlled condition. It is a process in which highly reactive and sensitive enzyme is embedded in a matrix so that only substance and product can pass through matrix.

Types of immobilization

1. Adsorption-Direct intracellular binding due to natural affinity (adhesion or agglutination).
2. Covalent linkage-Covalent coupling on otherwise inert matrices.
3. Cross linking-Intracellular connection via bi or poly functional reagent.
4. Embedding-Mixing with suitable materials by changing their consistency and temperature.
5. Entrapment-Physical retention within the frame work of diverse pore size and permeability (Microencapsulation).

Methods of immobilization system

1. Entrapment

It is a method in which physical retention within the frame work of diverse pore size and permeability. It is mainly done by four methods as follows:

a. Entrapment in performed structures like hollow fibre reactors e.g. Polyurethane foam.
b. Gel entrapment by polymerization with polymers like polyacrylamide e.g. vinca
c. Gel entrapment by ionic network formation e.g. Entrapment of cell in calcium alginate.
d. Gel entrapment by formation by precipitation of some natural and synthetic polymer by changing one or more parameters such as pH, temperature, salinity etc.

2. Surface immobilization

It may occur on both natural and other matrices e.g. Cellulose, Nylon etc.

3. Immobilization by embedding

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It is done by fixing the substrate in a mass or matter like agar, agarose etc.

Applications of plant cell immobilization

i. Biotransformation.
ii. Rapid biosynthesis of secondary metabolites with increased yield over extended period of time.
iii. Synthesis from precursor.

Examples of plant cell Immobilization of various plants include capsicum, coffee, vinca, mentha, tobacco etc.

c. Genetic transformation (Transgenic plant)

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

Method of gene transfer

Transgenic plants are produced by two methods:

1. Vector mediated gene transfer (Indirect gene transfer)

a. Bacterial vectors e.g. Agrobacterium
b. Viral vectors e.g. *E. coli*

2. Direct gene transfer

a. Transmission of pollen.
b. Macro injection of DNA into plants.
c. Direct gene transfer to protoplasts with the help of chemical treatment (PEG), electrical treatment (Electroportion), DNA delivery (liposomes).
d. Direct gene transfer to cells and tissues with the help of microprojectile bombardment (Biolistic or DNA particle gun) and Microinjection of DNA into cells and protoplasts.
e. DNA uptake into imbibing zygotic embryos.
f. Fibre-mediated DNA delivery into plant cells.

Application of Genetic transformation

i. For the production of antigens, antibodies, proteins, alkaloids, enzymes and phenolics.
ii. Genes have been successfully transferred to many crops for resistance to various biotic stresses and abiotic stresses like herbicide resistance.
iii. It is applied to develop resistance against viral infection.
iv. Gene transfers to improve quality of food products.
v. Male sterility and fertility restoration in transgenic plants.
vi. Transgenic plants have both basic and applied role in crop improvement.

Examples of transgenic plant include tobacco, tomato, soybean, satavari, papaya, liquorice, neem etc.

d. Elicitors

Elicitors are organic/inorganic agents used in tissue culture to trigger rapid and increase production of secondary metabolites or rapid growth of plant cell culture/organs. Induction of stress in plant cultures in terms of specific environmental, physiological and biological conditions, to enhance the production of secondary metabolites, is known as Elicitation. The secondary compounds synthesized and accumulated in response to such conditions are called 'Phytoalexins', which act as defense agent to invading pathogens. The signals triggering the formation of phytoalexins are called elicitors. Table no. 1.5 describes influence of elicitors on production of secondary metabolites in some plants.

Classification of Elicitors

It is classified on two main bases as below:

A. Based on Origin

The elicitors are inside or outside the plant cells and hence they are further classified as below:

i. Exogenous elicitors

They are originated outside the cell and induce immediate reactions. It includes enzymes, metal ions, U.V. light, chitosan etc.

ii. Endogenous elicitors

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They are formed via secondary reaction and are induced by signal of biotic or abiotic nature in the cell. It includes hepta-β-glucoside, dodeca β-1,4 D-galacturonide etc.

B. Based on nature

Depending on their nature they are further classified as below:

i. Biotic elicitors

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

ii. Abiotic elicitors

Product accumulation also occurs under stress caused by physical or chemical agents like U.V. light, low or high temperature, antibiotics, salts of heavy metals, freezing and thawing cycles, non-essential components of media (agarose and agaropectin), certain chemicals (methyl jasmonate, copper sulphate, silver nitrate etc.), and high salt concentration grouped under abiotic elicitors. Elicitors stimulated the accumulation of secondary metabolites in different plant culture like opium, dioscorea, datura, vinca, capsicum, carrot etc.

Table no. 1.5 Influence of elicitors on production of secondary metabolites in some plants

Plant	Secondary metabolite produced	Elicitor used
Catharanthus roseus	Monomeric alkaloids	Methyl jasmonate
Daucus carota	Anthocyanins	Metal ions (Ca^{+2}, Mn^{+2}, Zn^{+2})
Ruta graveolens	Alkaloid	Chitosan
Taxus sp.	Taxol	Arachidonic acid
Valeriana wallichii	Valepotriates	Colchicine

II. Micropropagation (Clonal propagation)

Micropropagation or Clonal propagation is a field dealing with the ability to regenerate plants directly from explants or from a single individual by asexual reproduction, constitute a clone. It is defined as True-to-type propagation of selected

genotypes using *in vitro* culture techniques. Vegetative method of propagating plant is termed as micropropagation or cloning tissue culture or growing *in vitro*.

Methods of Micropropagation

Various methods of micropropagation are as follows:

1. Micropropagation by proliferation of axillary buds.
2. Adventitious shoot proliferation.
3. Artificial seed.
4. Plant regeneration by organogenesis from callus and cell suspension cultures.
5. Plant regeneration from callus and cell suspension culture by somatic embryogenesis.
6. Direct (adventitious) somatic embryogenesis (Non-zygotic embryogenesis).

Advantages of Micropropagation

1. This method offers rapid multiplication of superior clones with maintenance of genetic uniformity.
2. Conservation of genetic resources of species and threatened medicinal plants.
3. The method is useful in improvement of the plant by developing virus-free, insect-resistant, disease–resistant, herbicide-resistant plant.
4. Multiplication of sexually derived sterile hybrids can be done.
5. High yielding crops of the desirable characters in a short period of time can be obtained.

Disadvantages of Micropropagation

1. Contamination is a serious threat.
2. It is an expensive method.
3. Specific condition of micropropagation, rooting and hardening may be required.
4. By this method plantlets take longer initial time to grow.
5. Technically skilled work and knowledge about material is required in the personnel.

Example of micropropagation include various plants like garlic, brahmi, vinca, eucalyptus, gymnema, liquorice etc. are propagated by this technique. Fennel is genetically heterozygous and produces wide variation in oil yield and composition.

III. Synthetic seed (Artificial seed or Somatic seed or Synerts or Synseed)

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Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage. Various plant species which are reported for artificial seed production are carrot, alfa alfa (somatic embryos), banana, cardamom (shoot buds or shoot tips), eucalyptus (Axillary buds) etc.

Classification of synthetic seed

They are classified as below:

1. Desiccated synthetic seed

a. Coated
b. Uncoated

It is produced from somatic embryos either naked or encapsulated in polyoxy ethylene glycol followed by their desiccation. E.g. Wheat, Soyabean (Uncoated), Carrot (Coated).

2. Hydrated synthetic seed

a. Encapsulated
b. Uncoated

It is produced in those plant species where somatic embryos are recalcitrant and sensitive to desiccation. Hydrated seeds are produced by encapsulating the somatic embryos or somatic propagules in hydrogel capsules. E.g. carrot, tomato (Uncoated), mango, alfa alfa (Encapsulated). Table no. 1.6 describes some plant species which have undergone artificial seed production.

Advantages of synthetic seeds

1. They can be produced within a short time where as natural seed production by plant is a time consuming and complex process.
2. By means of synthetic seeds dormancy period can be reduced to a great extent, there by shortening the life cycle of a plant.
3. They are useful in preserving germplasm.
4. They can be produced any time and in any season of a year.
5. They act as a carrier for beneficial supplements such as plant growth regulators, pesticides, fertilizers, microorganisms, and nutrients.

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6. They offer rapid and large scale multiplication with economy space, nutrients which lead to better cost-benefit ratio.
7. They are breed true propagules.

Limitations of synthetic seeds

1. By synthetic seed technique quality and quantity of somatic embryogenesis can be affected.
2. Limited production of viable micropropagules useful in synseeds production is produced.
3. Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synseeds.
4. Poor conversion of normally matured somatic embryos and other micropropagules into plantlets.

Applications of synthetic seeds

1. Micropropagation through artificial seeds.
2. For development of plants for breeding purpose
3. Propagation of variety of crop plants especially crops for which true seeds are not used or not readily available for multiplication or the true seeds are expensive. Hybrid plants may vegetatively propagated plants which are prone to infections e.g. garlic, potato, tomato, hybrid rice etc.
4. Transplanting improved or selected material in forestry to reduce the cost of breeding e.g. European larch, white spruce etc.
5. Germplasm conservation of endangered species through cryopreservation of synseeds in near future.

Table no. 1.6 Plants which have undergone artificial seed production

Plant	Propagules used for encapsulation
Eucalyptus	Axillary bud
Zingiber officiniale	Shoot bud or shoot tips
Daucus carota	Somatic embryos

IV. Cryopreservation

The preservation of cell, tissue and organs in liquid nitrogen is called cryopreservation and the science pertaining to this activity is known as cryobiology.
Cryopreservation is the non-lethal storage of biological material at ultra low temperature. At the temperature of liquid nitrogen (-196°C) almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods. However, only few biological materials can be frozen to (-196°C)

without affecting the cell viability. Cryopreservation of few endangered medicinal plants e.g. dioscorea, chirata, podophyllum etc. is done these days.

Edible vaccines

In the last decade, advancements in the field of medicine and healthcare have been possible because of the development of newer, safer and highly effective vaccines; recombinant vaccines, subunit vaccines, peptide vaccines and DNA vaccines to name a few. Although these vaccines have an undue advantage over traditional conventional vaccines, there is a flip side to them. Not only are these vaccines expensive, but their storage and transportation pose a problem as many of them require refrigeration. This is a disadvantage in many of the developing countries. So, as alternatives had to be thought of, it was envisaged that plants could be used as a cheap, safe and efficient production system for vaccines and thus the concept of edible vaccines was born.

DEFINITION Edible vaccines are nothing but transgenic plant and animal based production of or those that contain agents that trigger an animal's immune response. In simple terms, edible vaccines are plant or animal made pharmaceuticals. This essay highlights the importance of edible vaccines produced in plants.

INITIAL DEVELOPMENTS IN DESIGNING THE EDIBLE VACCINES The concept of edible vaccines was developed by Arntzen (www.genomenewsnetwork.org) in the 1990s. He currently heads the department of plant biology at the Arizona State University. He fell upon the idea after he attended a conference in New York, organized by the WHO. Although the idea seemed quite simple in the beginning, making it into a reality has required sophisticated science. The earliest demonstration of an edible vaccine was the expression of a surface antigen from the bacterium Streptococcus mutans in tobacco. As this bacterium causes dental caries, it was envisaged that the stimulation of a mucosal immune response would prevent the bacteria from colonizing the teeth and therefore protect against tooth decay.

Several plant derived vaccines for human use are approaching the market but it is likely that the first commercial Plant derived vaccine will be a veterinary vaccine. At least 30 such products have been expressed in plants, some providing protection against challenges with disease causing agents.The trial carried out by prodiGene Inc. showed for the first time that an oral vaccine produced in plants could protect live stock against virulence challenge.The first product to reach market could be a poultry vaccine developed by Dow AgroSciences, has been proposed for market release sometime in 2006.

HOW DO EDIBLE VACCINES WORK? Edible vaccines contain DNA fragments from the original pathogen. These fragments code for a protein that is usually a surface protein of the pathogen. This is responsible for eliciting the body's immune response.

SOME EXAMPLES OF EDIBLE VACCINES

• Transgenic Potatoes For Diarrhea The first human trial for an edible vaccine took place in 1997. Volunteers ate transgenic potatoes that contained the b-subunit of the

E. coli heat-labile toxin, which causes diarrhea. Ten of the 11 volunteers showed a 4-fold increase in serum antibodies. Researchers at the Boyce Thompson Institute at Cornell University conducted another clinical trial of an edible vaccine in 1999. Potatoes containing the Norwalk. virus (which causes vomiting and diarrhea) fed to volunteers elicited an immune response in 19 out of 20 volunteers. The disadvantage of using potato-based edible vaccine is that it has to be consumed raw; when cooked the protein may get denatured or in some cases less effective. Research has shown that by partial boiling at least half the vaccine remained alive.

Transgenic Tomatoes Against Diarrhea In the US at the Cornell University, researchers have developed transgenic tomatoes against the Norwalk virus, which causes severe diarrhea. The tomatoes produced a surface protein specific to the virus. Mice that ate these tomatoes developed an immune response to the virus. Recently, banana has been explored as an alternative source because not only does it eliminate the need for cooking but also it’s a locally grown plant. The expression of a protein in banana will depend on the identification of a tissue specific promoter. Other examples include rabies glycoprotein being expressed in viral vectors in spinach[8] and hepatitis B surface antigen in lettuce and potato.

ADVANTAGES OF EDIBLE VACCINES

1. They are cheap; therefore they can be mass-produced.
2. They can be ingested by eating the plant/part of the plant. So, the need to process and purify does not arise.
3. Extensive storage facilities like cold storage are not required.
4. If the local/native crop of a particular area is engineered to produce the vaccine, then the need for transportation and distribution can be eliminated.
5. Most importantly, they trigger the immunity at the mucosal surfaces such as those that line the mouth (mucosal immunity) which is the body’s first line of defense.

DISADVANTAGES OF EDIBLE VACCINES

1. Will the antigens be able to survive the hostile, acidic conditions of the stomach and even if they did, will they be able to trigger the immune system in the right way? Although initial trials have shown promising results in human subjects, it is not clear what will happen when the person comes in contact with the actual virus.
2. How can the vaccine dose be controlled? This remains the most difficult task. There seems to be a danger that too high a dose could provoke oral tolerance of an invading bacteria or virus, instead of an immune response. Also, the dosage requirements for children and adults will be different. So, research is on its way to find a solution to these problems.
3. Plants are living organisms that change, so the continuity of the vaccine production might not be guaranteed.
4. Glycosylation patterns in plants differ from those in humans and could affect the functionality of the vaccines.
5. People may develop an allergy to the fruit or vegetable expressing the foreign antigen.

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The first trial on humans in 1997 (using the heat labile B- toxin from E. coli) is a milestone on the road to creating inexpensive vaccines that might be particularly useful in immunizing people in developing countries, where high cost and logistical issues, such as transportation and the need for certain vaccines to be refrigerated, can thwart effective vaccination programs. The hope is that edible vaccines could be grown in many of the developing countries where they would actually be used. Whatever may be the current situation, a day is not far off when we will be able to pluck a fruit from the garden, eat it and be protected from diseases…making needles needles.

Study Questions

1. Define plant tissue culture.
2. Give Advantages and Disadvantages of Plant tissue culture.
3. Enumerate the basic nutritional requirements of plant tissue culture.
4. Enlist different types of plant tissue culture.
5. Discuss in brief about static or callus culture.
6. Discuss in brief about Suspension culture.
7. Discuss in brief about Hairy root culture.
8. Discuss in brief about Protoplast culture.
9. Discuss in brief about Somatic hybridization.
10. Write a note on Micropropogation and synthetic seed.
11. What is a plant cell immobilization? Describe methods & applications of plant cell immobilization.
12. Write a note on Elicitors, Biotransformation and Cryopreservation.
13. Discuss in brief about the applications of plant tissue culture.
14. Write a note on Edible vaccine

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