inter nodal segments of stem, apical and axillary bud, leaf, leaf disc, petiole, anther, pollen, flower bud, petal, ovule, orary root, and even isolated epidermal peel, gland and trichome have been used as an explant. A suitable explant of a species is desirable for successful regeneration. Various types of cultures are discussed below:-

(i) Stem cultures- When stem segments are used to initiate the cultures, the cut ends are sealed with molten wax and then sterilized with any disinfectant and washed thoroughly with sterilized distilled water. Sterilized stem pieces are transferred in a pre-sterilized petridish or sterilized filter paper and ends are removed with the help of scalpel. The explants of suitable size consisting of node/nodes are prepared and transferred to the medium.

(ii) Anther cultures- The anthers may be taken from plants grown in the field or in pots but ideally these plants should be grown under controlled temperature, light and humidity.

Flower buds of the appropriate developmental stage are collected, surface sterilized and their anthers are excised and placed horizontally on culture medium. Flower buds with small anthers may themselves be cultured and in some cases the entire inflorescence has been cultured. Care should be taken to avoid injury to anthers since it may induce callus formation from anther walls.

(iii) Pollen cultures- Pollen cultures may be isolated either by squeezing or float-culturing the anthers. About 50 anthers may be placed in 20 ml of medium and squeezed with glass rod ; the solution is filtered through a nylon mesh of suitable pore size and centrifuged. The pollen pellet is collected, washed twice and suspended at a final density of 10^3 - 10^4 pollen\ml.

In float culture, excised anthers are floated on a shallow liquid medium in a petridish; the anthers dehisce in a few days releasing their pollen grains into the medium.

(iv) Embryo culture- For embryo culture, embryos are excised from immature seeds under laminar air flow cabinet. Sometimes the immature seeds are surface sterilized and soaked in water for few hours before the embryos are excised. The excised embryos are directly transferred to culture media.

(v) Ovule culture- Ovules after fertilization have been successfully cultured to obtain mature embryo / seeds. Depending upon when the embryo aborts, the ovules have to be excised any time soon after fertilization to almost developed fruits, which may sometimes be lost due to premature abscission. However, ovule culture is mainly tried only in those cases where embryo aborts very early and embryo culture is not possible due to difficulty of its excission at a very early stage. In some cases the medium may need to be supplemented with fruit/vegetable juice to accelerate initial growth.

(vi) Ovary culture- Ovary culture is often used when embryo culture and ovule culture either fail or are not feasible due to very small ovules. The ovaries are excised at the zygote stage or at the two celled proembryo stage and normal development is completed *in vitro*.

(vii) Leaves or leaf primordia culture- Leaves of 800 μm are separated from shoots, surface sterilized and are transferred to medium. Growth rate in culture depends on their stage of maturity of excission. Young leaves have more rate of growth as compared to mature leaves.

(viii) Shoot tip culture- The excised shoot tips of 100-1000 um long of various plant species are cultured on nutrient media. It forms adventitious roots and regenerate into entire plant.

Selected examples of regeneration from different explants and cultures-

A- Stem culture

Urginea indica
Tamarindus indica
Rose hybrida
Tecomella undulata
Camellia sinensis
Dalbergia latifolia
Ziziphus mauritiana

C- Flower culture

Arachis hypogaea
Phlox drumondii
Rannunculus scleratus
Tagetes erecta
Utricularia inflexa

E- Leaf culture

Artemisia annua
Azadirachta indica
Cicer arietinum
Curculigo orchioides
Dioscorea floribunda
Lycopersicon esculentum
Oryza sativa
Rauwolfia serpentina
Saccharum officinarum
Triticum aestivum
Zea mays

G- Root culture

Albizzia lebbeck
Aegle marmelos
Dalbergia sissoo
Vigna aconitifolia

I- Endosperm culture

Dendrophthoe falcata
Oryza sativa
Taxillus vestitus

B- Inflorescence culture

Brassica oleracea var botrytis
Musa species
Pennisetum americanum
Sorghum almum
Triticum aestivum
Zea mays

D-Embryo culture

Arachis hypogaea
Allium cepa
Costus speciosus
Eucalyptus citriodora
Hordeum vulgare
Podophyllum hexandrum

F-Shoot tip culture

Atropa belladonna
Acacia auriculiformis
Chrysanthemum monifolium
Gladiolus species
Morus indica
Phoenix dactilifera
Piper nigrum
Picrorhiza kurroa
Terminalia bellerica
Zinziber officinale

H-Seed and seedling callus

Acacia auriculiformis
Albizzia lebbeck
Dalbergia latifolia
Commiphora wightii
Carthamus tinctorium
Helianthus annuus
Prosopis tamarugo
Tecomella undulata
Sesbania grandiflora
Vigna mungo
Ziziphus mauritiana

Apart from the above mentioned cultures the other methods that are commonly used for culturing of plant cells/tissue are:-

Protoplast culture

Hairy root culture

Protoplast culture

Protoplasts are the naked plant cells which do not contain cell walls. The real start of plant protoplast research was made by **E.C.Cocking** in 1960 when he demonstrated that naked cells called as protoplasts can be obtained through enzymatic degradation of cell walls. In view of this the isolation and culture of protoplasts has become a very important area of research within the realm of plant biotechnology. Protoplasts are isolated by two methods namely, mechanical and enzymatic.

Mechanical method- In this method the plasmolysed cells (infact cell walls) are cut with sharp knife to release the protoplasts. This method gives poor yield of protoplasts thus it is no more practically used. It is only a historical method.

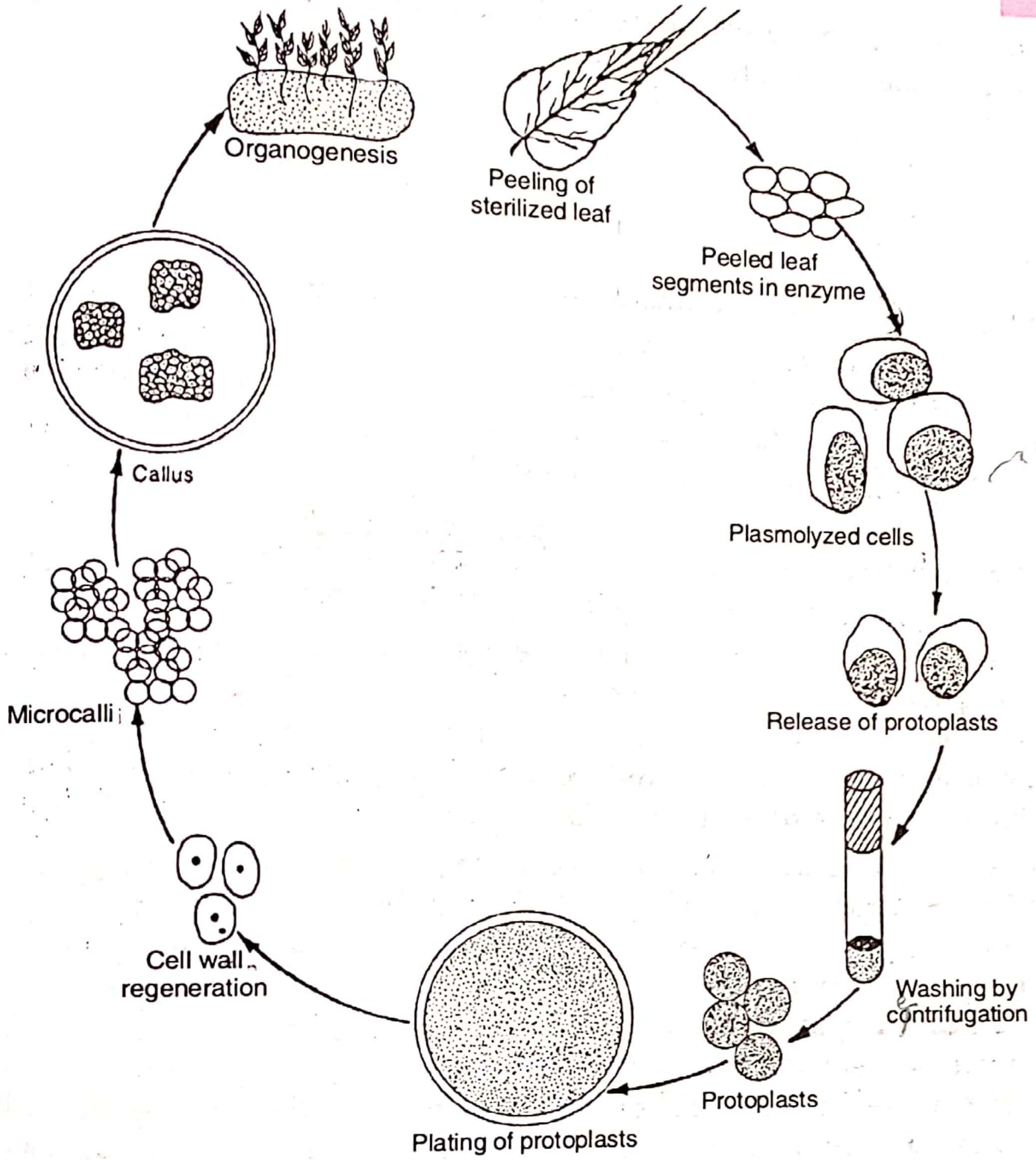
Enzymatic method- The enzymatic method is almost invariably used now for the isolation of protoplasts where cells are not broken and osmotic shrinkage is minimum. The protoplasts can be isolated from a variety of tissues including leaves, roots, *in vitro* shoot cultures, callus, cell suspension and pollen. However, the most commonly used part are leaves which can be employed for isolation of protoplasts using the following steps -

Fully expanded leaves are obtained from about 10 weeks old plants and are surface sterilized by first dipping them into 70% ethyl alcohol for one minute and then treating them with 2% solution of sodium hypochlorite for 20-30 minutes. The leaves are then rinsed three times with sterile distilled water and subsequent operations are carried out under laminar air flow. The lower epidermis of the sterilized leaves is carefully peeled off and the stripped leaves are cut into small pieces. Mesophyll protoplasts can be obtained from these peeled leaf segments while those for epidermis are obtained from peeled epidermis. From the peeled leaf segments the protoplasts can be isolated using any one of the two methods:

(i)- direct (one step) method, in which treatment with macerozyme (or pectinase) and cellulase is done simultaneously, or

(ii)- sequential (two step) method, in which cells are first isolated using macerozyme and then cells are treated with cellulase to isolate protoplasts.

The isolated protoplasts are cleaned by centrifugation and decantation method. The cleaned protoplast solution of known density (1x105 protoplast/ml) is poured on sterile culture media in the petridishes and mix them gently by rotating each petridish. Allow the medium to set, seal the petridishes with paraffin film and incubate the petridishes in inverted position in BOD incubator. The protoplasts which are capable of dividing, undergo first division within 2-7 days and form callus after 2-3 weeks. The callus is then transferred to fresh medium (subculturing of callus) containing appropriate proportions of auxin and cytokinin. Embryogenesis begins and the embryo develops into plantlets. Subsequently, the plantlets may be transferred to pots.



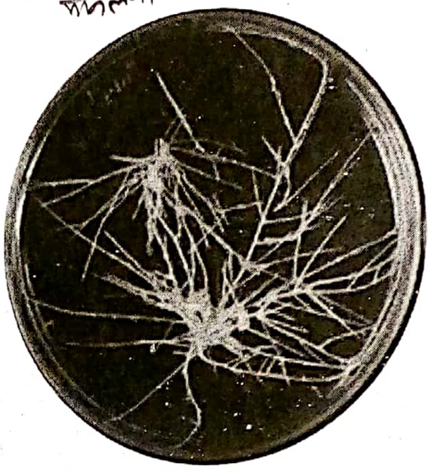
Protoplasts isolation procedure

HAIRY ROOT CULTURE

A relatively new type of plant culture which consists of highly branched roots covered with a mass of tiny root hairs originated directly from the explant in response to *Agrobacterium rhizogenes* infection. This bacteria is able to induce hairy root symptoms. These cultures can even grow on simple media of salts and sugars (devoid of hormones or vitamins). These hairy roots can be excised and cultivated indefinitely under sterile conditions. A feature of hairy root systems of paramount importance for their commercial exploitation is their stable, high level production of secondary metabolites.

In the production of hairy root cultures, the explant material is inoculated with a suspension of *Agrobacterium rhizogenes*. The bacteria contains root inducing (Ri) plasmid. This culture is generated by growing bacteria in yeast maltose broth (YMB) medium for 48 hours at 25°C with rotary shaking, pelleting by centrifugation (5×10^3 rpm, 20 min) and resuspending the bacteria in YMB medium to form a thick suspension. Transformation may be induced on aseptic plants grown from seed or on detached leaves, leaf disc, petioles or stem segments from green house plants followed by sterilization of the excised tissues. In some species a profusion of root may appear directly at the site of inoculation, but in others a callus will form initially and roots emerge subsequently from it. In either case, hairy roots appear within one to four weeks. The susceptibility of species to infection is very variable. Addition of acetylsyrigone, the compound produced during the wounding response of plants, activates the Vir(Virulence) genes of *Agrobacterium* adding plasmid T-DNA transfer.

Cultures may be cleared of bacteria by several passage in media containing 200 mg/L cephalosporin and 500 mg/L ampicillin. The infection of plants with *Agrobacterium rhizogenes* causes one or both of two pieces of T-DNA (Tt and Tg) of Ri plasmid to be inserted into the plant genome. Integration alters the auxin metabolism of transformed tissues in such a way that the hairy root phenotype is expressed and amino acid metabolism is modified in such a way that specific metabolites such as opines are produced.



Hairy Roots

Establishment and Maintenance of various cultures-

The growth establishment and maintenance of various plant tissue cultures can be done by three main culture systems which are selected on the basis of the objective-

1- Callus culture (also called as Static culture)

2-Suspension cultures

3-Protoplast culture- The protoplast culture can be grown as-

Callus culture

Suspension culture

CALLUS CULTURE

The unorganised mass of cells which proliferates from the cells of an explant is termed as **callus**. The cultivation of callus on an agar-gelled medium under aseptic conditions is called as **callus culture**. This technique is described below-

INITIATION OF CALLUS CULTURE

(i) Selection of an explant- Callus cultures can be obtained from any organ or culture such as seedlings, young shoots or buds, root tips or developing embryos: fruits, floral parts, tubers and bulbs.

(ii) Preparation of an explant- After selection, the explant is taken and surface sterilized

It is washed with tap water and sterilized with sodium hypochlorite (2%) or mercuric chloride (0.1- 1%) solution for 15-30 minutes. Finally it is washed with sterile glass distilled water and cut into small segments of 2-5 mm. (For detail, please refer the surface sterilization).

(iii) Culture media- The culture of the medium depends upon the species of plant and objective of study. The nutrient media required should be well defined and it should contain inorganic nutrients, organic nutrients and growth hormones. The growth hormones like auxins, cytokinins and gibberelins are added to media according to the objective of culture. Auxins like IBA and NAA are widely used for rooting and in combination with cytokinins for shoot proliferation. 2,4-D and 2,4,5-T are very effective for induction and growth of callus. Cytokinins are employed for the promotion of cell division , regeneration of shoots and growth of auxillary buds.

The well defined semi solid nutrient media is prepared and pH of the medium is adjusted between 5 to 6. It is poured into culture vessels, plugged with non- absorbent cotton, covered with aluminium foil and are sterilized by autoclaving.

(iv) Transfer of an explant- Surface sterilized explant is transferred aseptically to the vessels containing semi solid nutrient media.

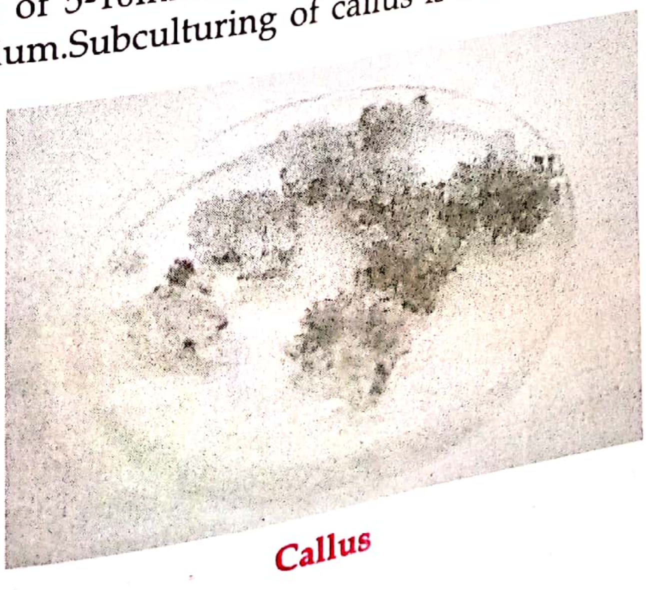
(v) Incubation- These inoculated vessels are incubated in BOD incubator at the temperature of 25 ± 2^0C using light and dark cycles of each 12 hours duration. After 3 to 8 days of incubation sufficient amount of callus is produced and after 3 to 4 weeks, callus should be 4 to 5 times, the size of an explant. Callus is formed through three stages of development viz-

(A) Induction - In this stage, metabolic activities of the cell increases therefore it accumulates the organic contents and finally divide into a number of cells.

(B) Cell division- In this stage the active cell division takes place as the explant cells revert to meristematic state.

(C) Cell differentiation- In this stage the cellular differentiation takes place i.e. the morphological and physiological differentiation occurs resulting in the formation of secondary metabolites.

Maintenance- After a period of time it becomes neccessary to transfer the callus to fresh media (subculturing of callus) chiefly due to nutrient depletion and meduum drying. In general, callus tissue of 5-10mm in diameter and 20- 100mg in weight are transferred aseptically to fresh medium.Subculturing of callus is done after every 4 to 6 weeks.



Callus

Callus cultures are slow growing systems. Cells grow as clumps or masses in callus cultures and only lower cells are in contact with the medium whereas cells in upper layers get their nutrients from cells in lower layers. The main feature of callus is its capability to develop into normal root and shoot and ultimately forming a plant. Secondary plant metabolites can also be produced from callus cultures but on the whole it is good source for establishment of suspension cultures.

SUSPENSION CULTURE

Tissue and cells cultured in a liquid medium (without agar) produce a suspension of single cells and cells clumps of few to many cells; these are called as suspension cultures.

Initiation of suspension culture

Cell suspension cultures are initiated by transferring the friable callus to liquid nutrient medium (without agar). In liquid nutrient medium plant tissue remains submerged which leads to anaerobic conditions and ultimately there is death of cells. Therefore such cultures are agitated by a rotary shaker at 50-150 rpm. Agitation serves both to aerate the cultures and to disperse the cells. After the production of sufficient number of cells, subculturing can be done in fresh liquid medium.

It is common observation that if relatively small number of cells are transferred (low inoculum density) to a new medium (either static or liquid), they may fail to divide whereas a larger quantity of tissue transferred from the same culture may proliferate rapidly on the same medium. This observation has led to the concept of 'critical initial cell density'. This is defined as the smallest inoculum per volume of medium, from which a new culture can be reproducibly grown. There are few conditions which determine the critical initial density of cells. They are :

(i)-The cultures physiological characteristics.

(ii)-The length of time and conditions under which the culture was previously maintained.

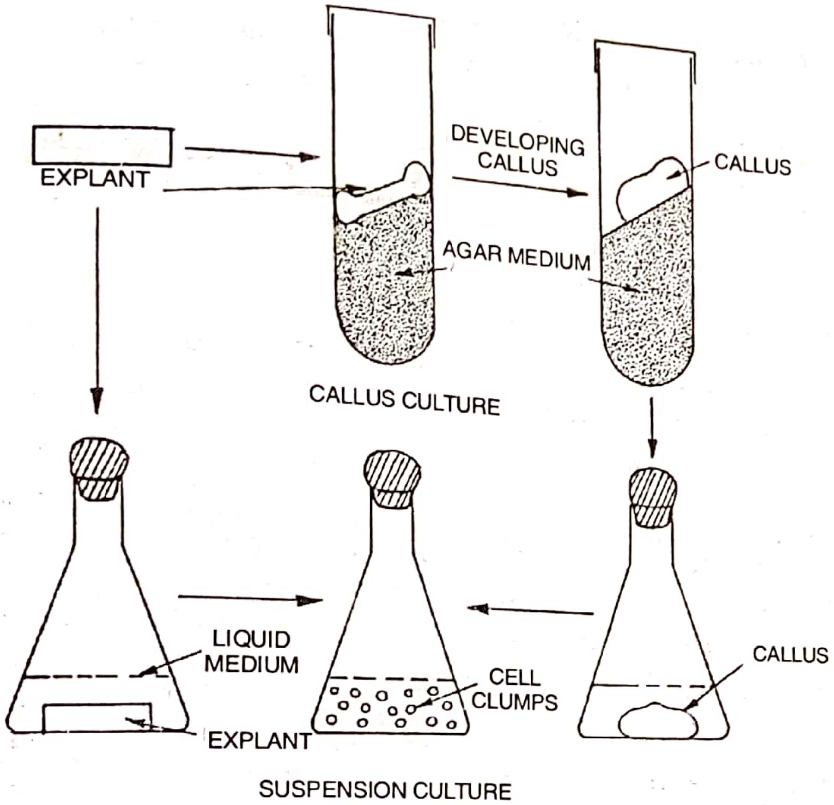
(iii)-The composition of fresh medium.

The third point is of interest. As the isolated cells failed to grow on fresh medium, 'conditioned medium' or 'nurse tissue' conditions are used to grow isolated cells or protoplasts. A 'conditioned medium' is the medium on which some tissues were previously grown. Conditioning makes the minor adjustment in the nutrients and chemical substances released in the medium by the callus, promotes the growth of isolated cells of protoplasts. In suspension cultures, cells grow as isolated single cells and cell aggregates of a few hundred cells. Cell aggregation vary from species to species.

MAINTENANCE OF SUSPENSION CULTURE

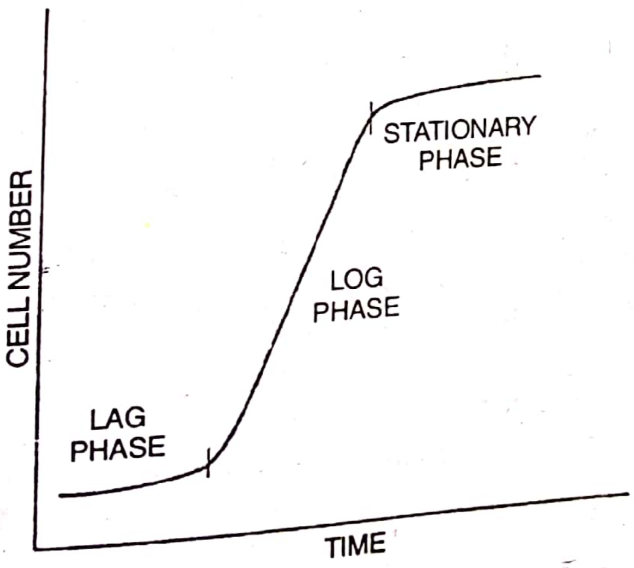
The suspension culture can be maintained by the following ways:

(a) Batch culture- In the batch culture technique the cells are allowed to multiply in liquid medium which is continously agitated. Except for circulation of air, the system is 'closed' with respect to addition or substraction from the culture. To get the growth again on the stationary phase either the cells are transferred to fresh medium or more amount of liquid medium is added to the original culture. Each fresh medium containing culture (suspension) constitutes a batch. Such cultures are grown again and again in batches for the purpose of experiment.



Initiation of callus and suspension cultures

In batch culture there is no steady state of growth. The cell number or biomass of a batch culture exhibits a typical **sigmoidal** curve having a lag phase during which the cell number of biomass remains unchanged, followed by a logarithmic (log) phase (Exponential phase) when there is rapid increase in cell number and finally ending in a stationary phase during which cell number gradually declines.



A model curve for cell number in a batch culture.

The lag phase duration depends mainly on inoculum size and growth phase of culture from which inoculum is taken. The log phase lasts about 3-4 cell generations (a cell generation is the time taken for doubling of cell number) and duration of a cell generation may vary from 22-48 hours, depending mainly on the plant species. The stationary phase is forced on the culture by a depletion of the nutrients and possibly due to an accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period, the cells may die. Therefore subculturing should be done.

(b) Continous culture- In this tecnique the cell population is maintained in a steady state for a long period by draining out the used medium and adding fresh medium. Such culture systems are of two types-

(i) Closed type- In closed continous culture, cells are separated from the used medium taken out for replacement and added back to the culture so that cell biomass keeps on increasing.

(ii) Open type- In open continous culture, both cells and the used medium are taken out and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at submaximal growth indefinitely. Further open continous culture are of two types viz. turbidostat and chemostat types.

Turbidostat type- In turbidostat, cells are allowed to grow up to a preselected turbidity (usually measured as OD) when a predetermined volume of the culture is replaced by fresh normal culture medium.

Chemostat type- In this a chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting while other nutrients are still in concentration higher then required. In such a situation any addition of the growth- limiting nutrient is reflected in cell growth.

SUBCULTURE

The growth of cell suspension culture is always higher than callus culture therefore they should be subcultured every 3-14 days. The inoculum volume should be 20-25% of the fresh medium volume; in any case the initial cell density of the fresh culture (just after inoculation) should be around $5x10^4$ cells ml^{-1} or higher otherwise the cells may fail to divide.

Estimation of growth

The various parameters used for estimating the growth of cultured cells are like fresh weight, dry weight, cell number and packed cell volume.

Fresh weight- This parameter is employed to measure the growth of both suspension and callus cultures. In case of callus cultures, the cell mass is placed on a pre- weighed dry filter paper or nylon filter and weighed to determine fresh weight.

In case of suspension cultures, the cells from suspension cultures are filtered on to a filter paper or nylon filter and washed with ditilled water. The excess of water is removed under vacuum and weighed along with the filter (filter is pre weighed in wet conditions).

Dry weight- This parameter is also employed to measure the growth of both suspension and callus cultures. Dry weights are determined by drying the cells and filter in an oven at 60°C for 12 hrs and weighed ; the filter is pre- weighed in dry conditions.

Cell number- Cell number is the most informative measure of cell growth and is applicable to only suspension cultures. Cell aggregates are treated with pectinase or 5-15% chromic acid. To the 1 volume of cell suspension culture, 2 volumes of 8% chromic acid and trioxide solution is added and it is heated at 70°C for 5-15 minutes . The mixture is cooled and agitated for 10 minutes. The suspension so obtained is certrifuged, chromic acid is removed and the pellet is suspended in 8% saline solution. After few minutes, free cells are counted by haemocytometer.

Packed cell volume- This is determined by pipetting a known volume of suspension culture (4-7ml) into a 15 ml graduated centrifuge tube, spinning at 200 x g for 5 min and reading the volume of cell pellet which is expressed as ml cells/ L of culture.

APPLICATIONS OF PLANT TISSUE CULTURE IN PHARMACOGNOSY

Now a days the plant tissue culture technique is widely used in all the fields of biosciences including pharmacognosy also. It's applications are-

1- Production of secondary metabolites
2- Biotransformation
3- Clonal propagation or Micropropagation
4- Somaclonal variation
5- Cell Immobilization

1-Production of secondary metabolites-

It is well known that plants are an important source for a variety of chemicals used in pharmacy, medicine and industry.

In recent years, plant cell suspension cultures, callus cultures and immobilized cells are being utilized for the production of these chemicals on commercial scale due to following advantages over extraction from plants-

1. The yield and quality of the product is more consistent in cell cultures because it is not influenced by the environment.
2. The production schedule can be predicted and controlled in the laboratory or industry.

The most important chemicals produced using cell cultures are secondary metabolites which are defined as 'those cell constituents which are not essential for survival'. These secondary metabolites include alkaloids, glycosides, terpenoids, steroids and a variety of flavours, perfumes,colours etc. The yield of these chemicals in cell culture is though generally lower than in whole plants, it can be substantially increased by manipulating physiological and biochemical conditions. In some cases cell cultures accumulate these secondary metabolites at levels higher (2-10 times) than those found in whole mother plants, from which cell culture has been prepared. Automation in cell cultures can be used for industrial production of secondary metabolites. However, sometime immobilized plant cells are used instead of suspension cultures to increase the efficiency of production system. Some of the important secondary metabolites obtained from plants are listed in following tables.

TABLE NO.3

Alkaloids produced in culture and their pharmacological activity

Plant species	Product	Culture type	Activity
Atropa belladonna	Atropine	S	Anticholinergic
Catharanthus roseus	Vincristine	Shoot culture	Anticancer
	Vinblastine	Shoot culture	Anticancer
	Ajmalicine	S	Hypotensive
Cinchona officinalis	Quinine	S	Antimalarial
Colchicum autumnale	Colchicine	C	Antimitotic
Coffea Arabica	Caffiene	C	Stimulant
Cephaelis ipecacuanha	Emetine	Root culture	Emetic
Datura stramonium	Scopolamine	Hairy root culture	Antihypertension
Ephedra gerardiana	Ephedrine	S	Spasmolytic
Nicotiana tabacum	Nicotine	S	Stimulant
Ochrosia elliptica	Ellipticine	S	Antitumour
Papaver somniferum	Morphine	S	Analgesic
	Papaverine	S	Spasmolytic
	Codeine	S	Sedative, Analgesic
Rauwolfia serpentine	Reserpine	S	Antihypertensive

S- Suspension culture

C- Callus culture

FACTORS AFFECTING THE PRODUCTION OF SECONDARY METABOLITES

The factors that affects the production of secondary metabolites are :-

(1) Physical factors

(2) Effect of nutrients

(3) Selection of cells

(1) Physical factors- The effect of **light** on growth and metabolite production has been extensively studied. Light is involved in light mediated enzyme metabolism and photomorphogenesis which indirectly affects the secondary metabolites. Phytochemical responses are affected by both irradiance and light quality. Blue light induced maximum anthocyanin formation in *Haplopappus gracilis* cell suspension. White light induced the anthocyanin synthesis in *Catharanthus roseus* and *Populus* species. In contrast to these, white or blue light completely inhibited naphthoquinone biosynthesis in callus culture of *Lithospermum erythrorhizon*. The production of chlorogenic acid in *Haplopappus gracilis* was stimulated by white, blue and red light; of which blue light was the most effective. Anthocyanin synthesis in cultures of *Daucus carota*, *Linum usitatissimum*, *Vitis vinifera* and *Helianthus tuberosus* required white light. Callus cultures of *Ephedra gerardiana*, *Scopolia acutangula* and *Peganum harmala* produce more alkaloid in light than in dark.

Effect of **temperature** on secondary metabolites production is little studied. Work on *Catharanthus roseus* cell culture is widely cited for demonstrating effect of temperature. Indole alkaloid production increased two fold when cells of *C.roseus* were incubated at 16°C instead of 27°C. However at lower temperature (16°C) growth was three fold slower. Thus produc-

TABLE NO. 4

Saponine& steroids produced through tissue culture

Plant species	Product formation
Saponins	
Aesculus hippocastamum	Aescin
Agave insalna	Hecogenin
Dioscorea deltoidea	Diosgenin
Glycyrrhiza glabra	Glycyrrihizin
Ponax ginseng	Ginseng saponins
Cardiac glycosides	
Digitalis lanta, D.purpurea	Digoxin, Digitoxin
Strophantus species	Quabain
Urginea maritime	Proscilariddin
Other steroids	
Holarrhenna antidysenterica	Sitosterol, stigma sterol, cholesterol
Solanum xanthocarpum	Solasodine
Withania somnifera	Withanolides

TABLE NO. 5

Food additives produced by tissue culture

Plant species	Product
Colour	
Daucus carota	Anthocyanin
Euphorbia milli	Anthocyanin
Vitis vinifera	Anthocyanin
Beta vulgaris	Betalaines
Crocus sativus	Crocin, crocetin
Flavours	
Allium cepa	Onion flavor
Capsicum annuum	Capsicum, capsaicin
Capsicum frutesceus	Capsicum, capsaicin
Crocus sativus	Safranal
Vanilla planifolia	Vanilla,vanillin
Sweetner	
Stevia rebaudiana	Stevioside
Thaumatococcus danielli	Thaumatin

tivity of cultures remained same. Change in incubation temperature of *C.sinensis* or *N. tobacum* resulted in decreased synthesis of caffeine and nictotine respectively.

Plant cells are usually cultured on media having a pH range of 5 to 6. There are several reports which clearly demonstrate that the pH of the growth medium can drastically influence the production of phytochemicals by cultured cells, e.g. anthocyanins, anthraquinones and alkaloids etc. Cultures of *Daucus carota* produced less anthocyanin when grown at pH 5.5 than when grown at pH 4.5. It was suggested that it was because of increased degradation of anthocyanin at higher pH. Anthocyanin contents decreased by 90% at pH 5.5 compared to tissues grown at pH 4.5.

pH must be low

(2) Effect of nutrients- Cultured plant cells are usually grown on medium containing all the elements required for their sustained growth. Plant cell cultures are totipotent and possess all the capabilities of the intact plant to synthesize primary and secondary metabolites. Therefore it is imperative that medium ingredients such as carbohydrate, nitrogen, phosphorous and plant growth regulators affect the growth and metabolism of cultured cells and the production of secondary metabolites.

(a) Effect of carbon source- Carbohydrates are incorporated at 2-5% concentration in the medium and are known to influence the production of phytochemicals. In *Catharanthus roseus* cultures alkaloid content fluctuated with sucrose concentration in the medium; it increased as the sucrose concentration was increased (4-10%). Similarly the nature and concentration of the carbohydrate source had a significant effect on diosgenin production by *Dioscorea deltoidea* cell suspension cultures. It was recorded that on 1.5% sucrose supplemented medium, tissues yielded a higher amount of diosgenin in *D.deltoidea* compared to tissues grown on media with same amount of fructose, galactose lactose or starch. Cells of *D.deltoidea* with the greatest diosgenin productivity were those grown on medium containing 3% sucrose.

(b) Effect of nitrogen source- A mixture of nitrate and ammonium compounds is used in all the standard media as a source of nitrogen. The nitrogen source also affects the production of secondary metabolites. However, different types of results in relation to secondary metabolites by varying the nitrogen in the medium are obtained. It is reported that synthesis of 1,4- naphthaquinones in callus cultures of *Lithospermum erythrorhizon* increased with increase in total nitrogen from 67mM to 104 mM, while further increase in nitrogen in the medium suppressed yield. Zenk and co-workers reported that anthraquinone production by *Morindra citrifolia* cells decreased when KNO_3 levels were varied either above or below the range 2 to 4.5 g/L. Changes in total ubiquinone production in *Nicotiana tobacum* suspension cultures were recorded with changed ammonium to nitrate ratio in the medium from 3:1 to 1:3 but keeping the total nitrogen level constant. The biosynthesis of indole alkaloids in *Peganum harmala* decreased when ammonia or glutamine were substitued for nitrate.

(c) Effect of plant growth regulators- Effect of growth regulators on cultured plant cell is manifested in growth, metabolism and differentiation. The production of all secondary metabolites is affected by growth regulators. There are several reports in literature stating that by reducing the concentration of 2,4-D in the medium or replacing it with another auxin, the accumulation of secondary metabolites can be enhanced e.g. alkaloids in the cultures of tobacco, ephedra and pigment (shikonin) in the cultures of *Lithospermum erythrorhizon*. But the inhibitory effect of 2,4-D is not universal since there are many instances of an increase in metabolite content e.g. 2,4-D stimulates the production of ubiquinone and scopolatin in tobacco cultures and solasodine content in *Solanum eleagnifolium*. There are also examples available where in other auxins inhibited the production of secondary metabolites e.g. NAA and IAA inhibited, similar to 2,4-D the synthesis of anthocyanin in cell suspension cultures of carrot. It may be generalised that to a certain extent increase in concentration of an auxin, the medium has adverse effects on alkaloid content of the tissues.

The effect of cytokinins is similar to that of auxins as far as secondary metabolites are concerned, e.g. (i) activation of production of metabolites : DOPA in the tissues of Stizolabium, scopolin and scopoletin in the tissues of tobacco and carotenes in the cells of *Ricinus*, ajmalicine in *Catharanthus roseus* or (ii) inhibition of metabolites : anthraquinones in the tissues of *Morinda citrifolia*, shikonin of cells of *Lithospermum erythrorhizon* and nicotine of cells of tobacco etc. It is worth mentioning that the concentration and combination of plant growth regulators modulate growth of the tissues and production of secondary metabolites.

(d) Precursors- Precursors are molecules which are directly incorporated into secondary metabolites but perhaps with some structural changes. When such precursors are fed to culture medium they affect the growth and concentration of secondary metabolites. For e.g. addition of phenylalanine to the cultures of *Ephedra gerardiana* increases the ephedrine production. Vanillylanine and isocarpic acid precursors increases the production of capsaicine in the cultures of *Capsicum frutescens*. Addition of phenyl propane to the cultures of *Podophyllum hexandrum* increases the production of podophyllotoxin by 128 folds. Similarly addition of tryptamine and secologanin to the cultures of *Catharanthus roseus* improves the production of ajmalicine.

But sometimes the precursor may cause toxicity in the medium for the cells or may be degraded by extra-cellular enzymes. Positive influence of ornithine, phenylalanine, tyrosine and Na-Phenylpyruvate or alkaloid biosynthesis in *Datura* cell cultures was recorded with growth inhibition by these precursor amino acids. Once entered in the cell, the pecursor is stored in the cellular compartments and thus may not be available for incorporation. Therefore, the incorporation of precursors in the medium may not be encouraging.

(e) Production medium- It has been concluded from the results obtained from the various studies on optimization of secondary product formation in cultured cells that-

(i) Higher concentration of auxin in the medium particularly 2,4-D suppresses secondary metabolites.

(ii) Lower carbohydrate level (sucrose) favours cell proliferation while higher concentration arrests cell growth and increases secondary product formation.

(iii) Higher concentration of phosphate in the medium causes cell growth and lower concentration enhances secondary metabolite levels.

(iv) In certain cases higher nitrogen level in the medium enhances cell proliferation while low concentration increases secondary product formation.

(v) Increased synthesis of secondary products occurs during the stationary phase of cultures when primary metabolism and cell proliferation comes to halt.

On the basis of above conclusions, a secondary metabolite production or induction medium was devised by Zenk et al in 1977 in which the above conditions were combined. Cells grown on maintenance medium proliferate rapidly and such cultures are then transferred to induction or production medium (optimal for secondary metabolites) in which growth is arrested or cells enter in a stationary phase of growth. Such induction medium contains the same constituents but with low levels of phosphate, nitrogen (not always) and auxin (2,4-D) and very high sucrose concentration (6-10%).

Therefore, if during exponential phase of growth, cells in maintenance medium are transferred into production medium, growth comes to halt and a carbohydrate and other nutrients are available. So primary metabolites are rapidly diverted to synthesis of secondary metabolites instead of cell growth, thereby enhancing the secondary product synthesis.

(3)-Selection of cells- In this topic we will discuss how selection procedures are helpful in increasing the yield of cultures. Before producing secondary metabolites at the industrial

TABLE NO. 8

Production of secondary metabolites in immobilized cells

Species	Product	Increase (X folds)
Capsicum frutescens	Capsaicin	>100
Capsicum annuum	Capsaicin	>100
Coffea Arabica	Methyl xanthenes	13
Catharanthus roseus	Ajmalicine	35

(ii)Biotransformation- Hydroxylation of cardiac glycosides has proved to be an interesting application of immobilized plant cells. Bioconversions of b-methyl digitoxin into b-methyl digoxin has been achieved using *Digitalis lanata* immobilized cell cultures up to 70 days.

TABLE NO. 9

Selected one-step bioconversion by immobilized cells

Cell-culture species	Reaction type	Precursor	Product	Matrix
Digitalis lanata	Hydroxylation	β-methyl digitoxin	β-methyl digoxin	Alginate
Daucus carota	Hydroxylation	Digitoxigeni	Periplogenin	Alginate
Mentha species	Reduction	(-)-menthone	(+)-necomenthol	PAAH
Papaver somniferum	Reduction	Codinone	Codeine	Alginate & PUR

EDIBLE VACCINES

Edible vaccines are transgenic plant and animal based production of or those that contain agents that trigger an animals immune response. In simple terms edible vaccines are plant or animal made pharmaceuticals.

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.

The concept of edible vaccines was developed by Arntzen in 1990s (Head of department of plant biology at Arizona State University). Although the idea seemed quite simple in the beginning but 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 *Steptococcus mutans* in tobacco.

There are several advantages of edible vaccines :

i) They are cheap so they can be produced in large.

ii) They can be ingested by eating the plant/part of the plant. So the need to process & purify does not arise.

iii) Extensive storage facilities like cold storage are not required.

v) 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.

Despite the advantages there are various disadvantages of edible vaccines.

i) There is a question mark in the survival of antigen in the acidic conditions of the stomach & if they did will they be able to trigger the immune system in right way. Although initial trials have shown promising results in human subjects but it is not clear what will happen when the person comes in contactwith actual virus.

ii) To control the dose of vaccine is the most difficult task. There seems to be danger that too high dose could provoke oral tolerance of an invading bacteria or virus instead of an immune response. Also the dosage requirements for children & adults will be different.

iii) Plants are living organism that change, so the continuity of the vaccine production might not be guaranteed.

iv) People may develop an allergy to the fruit or vegetable expressing the foreign antigen.

v) Glycosylation patterns in plants differ from those in humans & could affect the functionality of vaccines.

So the research is on its way to find the solution of above problems.

SUGGESTED READINGS

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