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المرحلة الرابعة

الفصل الاول

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Plant Tissue Culture lec.1

Introduction:

Plant tissue culture : is one of the most promising branch of science, which based on the inherent potential plant cell to regenerate whole plant if grown aseptically under controlled condition in nutrient medium in vitro, this technique occupies a key role in the second green revolution in which gene modification and biotechnology are being used to improve crop yield and quality.

Plant tissue culture has been employed to solve the problems in crop plants and tree species. It has played an important role in our understanding of basic and applied aspects of science and commercial applications in agriculture, horticulture, forestry and industry.

This technology has proved aboon in visualizing many areas like micrpropagation ,disease detection and elimination, production of androgenic haploids, rescue of embryos, generation and selection of genetic variability in vitro , biosynthesis and biotransformation of secondary metabolites, use of protoplasts for production of somatic hybrids, genetic transformation and production of transgenic plants .collection, storage and exchange of germplasm, on servation of endangered plant species.

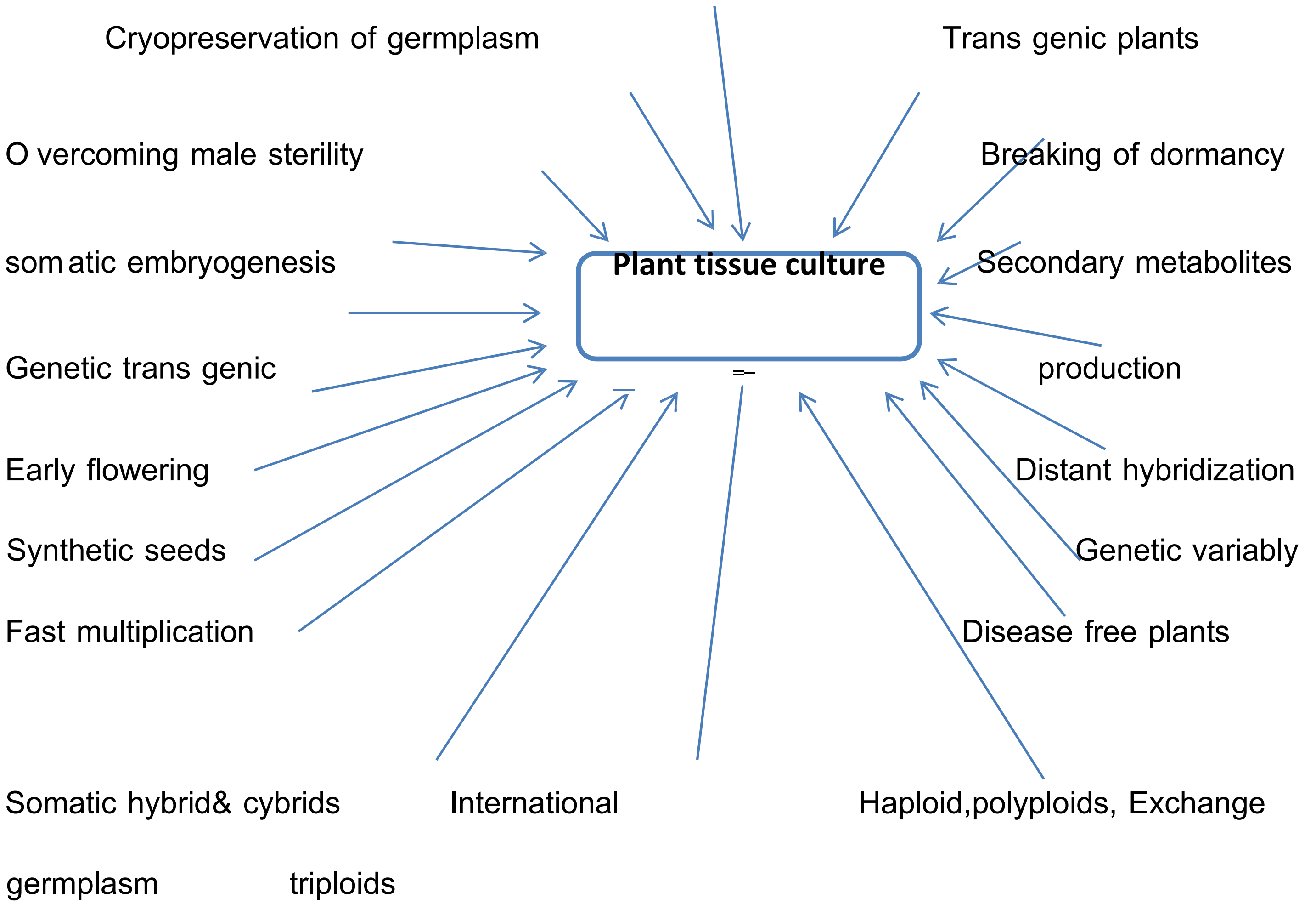

Fig. Application of plant tissue culture

History and Present Situation of Plant tissue culture and its application

The most important achievements of plant tissue culture	Scientists	The year
The first attemptin tissue culture and identify its problemess	Haberlandt	1902
The first attemptin in embryo	Hannig	1904
Tissues get rooted to tops the roots	Robbins	1922
Campium tisses get sold to some trees	Gautheret	1934
Farm get tomato root tissue	White	1934
Tissue is obtained for carrots	Nobecourt	1937
Get acellular action (callus culture)	Barun	1941
The first applied research in tissue culture	Ball	1946
Get plants free of viruses	Morel & Martin	1952
Get the plant form a single cell	Muir et al.	1954
Dis cover the hormone cytokinin (Kinten)	Miller et al.	1955
Advertise the installation of a nutritious environment (MS)	Murashige & Skoog MS	1962
Getting mono doubling plants form pollen	Bourging & Nitch	1967
The first successful attempt to isolate the protoplast out cell suspension	Eriksson & Jonassen	1969
Successful first merger protoplast fusion	Pouer et al.	1970
Get the first plant full of protoplast	Takebe et al.	1971
Successful biotransformation in cell cultures	Reinhard	1974
Discovery of plasmid	Zaenen et al. & larebke et al.	1974

Successful merger Ti– plasmid DNA separated form agrobacterium insid plant	Chilton et al.	1977
Somaclonal variations in tissue culture plant	Larkin & Scowcrot	1981
Successful genetic transformation process in plant cells using plasmid	Paszkowski et al.	1984
Published the first research on polymerase chain action PCR	Mullis	1986

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, tissues or organs isolated from the mother plant, on artificial media. It includes techniques and methods used to research into many botanical disciplines and has several practical objectives. Before beginning to propagate plants by tissue culture methods, it is necessary to have a clear understanding of the ways in which plant material can be grown and manipulated in 'test tubes'. This chapter therefore describes the techniques that have been developed for the isolation and in vitro culture of plant material, and shows where further information can be obtained. Both organised and unorganised growth are possible in vitro.

1. ORGANISED GROWTH

Organised growth contributes towards thecreation or maintenance of a defined structure. It occurs when plant organs such as the growing points of shoots or roots (apical meristems), leaf initials, young flower buds or small fruits, are transferred to culture and continue to grow with their structure preserved. Growth that is coherently organised also occurs when organs are induced. This may occur in vitro either directly upon an organ or upon a piece of tissue placed in culture (an explant), or during the culture of previously unorganised tissues. The

process of de novo organ formation is called organogenesis or morphogenesis (the development of form).

2. UNORGANISED GROWTH

The growth of higher plants depends on the organised allocation of functions to organs which in consequence become differentiated, that is to say, modified and specialised to enable them undertake their essential roles. Unorganised growth is seldom found in nature, but occurs fairly frequently when pieces of whole plants are cultured in vitro. The cell aggregates, which are then formed, typically lack any recognisable structure and contain only a limited number of the many kinds of specialised and differentiated cells found in an intact plant. A differentiated cell is one that has developed a specialised form (morphology) and/or function (physiology). A differentiated tissue (e.g. xylem or epidermis) is an aggregation of differentiated cells. So far, the formation of differentiated cell types can only be controlled to a limited extent in culture. It is not possible, for example, to maintain and multiply a culture composed entirely of epidermal cells. By contrast, unorganised tissues can be increased in volume by subculture and can be maintained on semisolid or liquid media for long periods. They can often also be used to commence cell suspension cultures. Differentiation is also used botanically to describe the formation of distinct organs through morphogenesis.

The most important kinds of organ culture are:

- **Meristem cultures**, in which are grown very small excised shoot apices, each consisting of the apical meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to give one single shoot.

- **Shoot tip, or shoot cultures**, started from excised shoot tips, or buds, larger than the shoot apices employed to establish meristem cultures, having several leaf primordia.

These shoot apices are usually cultured in such a way that each produces multiple shoots.

- **Node cultures of separate lateral buds**, each carried on a small piece of stem tissue; stem pieces carrying either single or multiple nodes may be cultured. Each bud is grown to provide a single shoot.

- **Isolated root cultures**, The growth of roots, unconnected to shoots: a branched root system may be obtained.

- **Embryo cultures**, where fertilised or unfertilized zygotic (seed) embryos are dissected out of developing seeds or fruits and cultured in vitro until they have grown into seedlings. Embryo culture is quite distinct from somatic embryogenesis (see below). These types of cultures are described in more detail later in this chapter.

In practice the following kinds of cultures are most generally recognized:

- Callus (or tissue) cultures.
- Suspension (or cell) cultures.
- Protoplast cultures.
- Anther cultures.

USING TISSUE CULTURES FOR PLANT

- from pre–existing shoot buds or primordial buds (meristems).
- following shoot morphogenesis when new shoots are induced.

- through the formation of somatic embryos which resemble the seed embryos of intact plants.

Theoretically, plant cells, organs, or plants, can all be cloned, i.e., produced in large numbers as a population where all the individuals have the same genetic constitution as the parent. Present tissue culture techniques do not permit this in every case and irregularities do sometimes occur, resulting in ‘Somaclonal variants’ (Larkin and Scowcroft, 1981). Nevertheless, as will be described in the chapters, which follow, a very large measure of success can be achieved and cultures of various kinds can be used to propagate plants.

INITIATING TISSUE CULTURES

–Explants.

–Isolation and incubation.

–The cultural environment.

– Media (Solidified media& Liquid media).

The another landmark comes in the form of somatic embryogenesis in carrot cell suspension cultures and production of complete plants (steward and Ammirato,1969).

Steward had taken suspension of callus tissue derived form wild carrot embryos and plated them out on a coca milk medium solidified with agar. Form this plated cell population thousands of embryo develop each being derived form one or a few cells of the callus tissue this has not only opened avenues of micrpropagation of plants species at an enormous rapid rate but also complete established the totipotency of the cell.

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Fig. Schematic presentation of embryogenesis in carrot

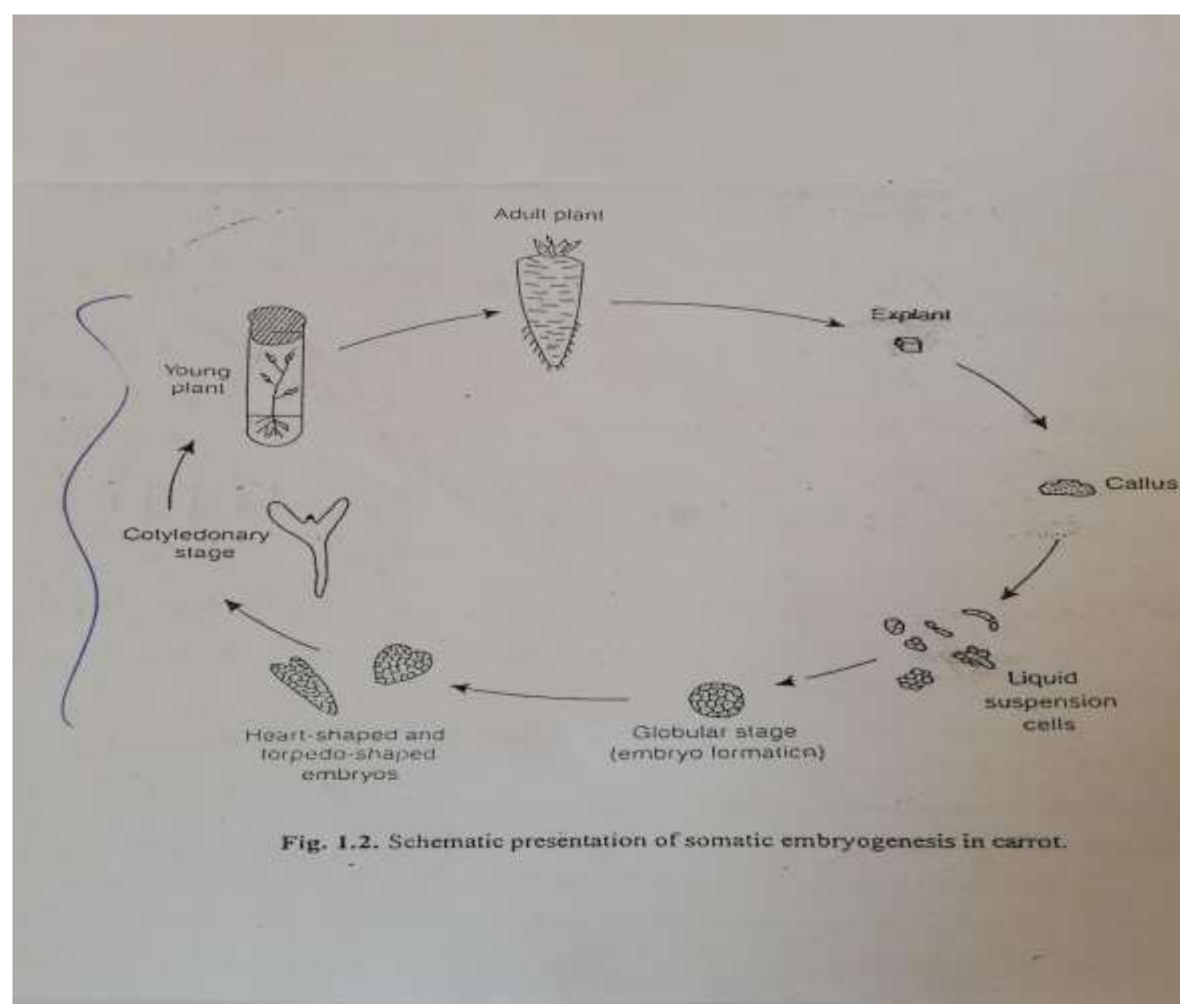

Fig. 1.2. Schematic presentation of somatic embryogenesis in carrot.

Plant tissue culture

Fourth level studients

Lec 2

The period 1941 to 1970 proved to be an exciting one because of the development of suitable nutrient media to culture various plant tissues, clonal propagation and culture of ovary, embryo, endosperm, anther pollen etc. Around 1970s the protoplast culture (isolation and fusion) started, the commercial availability of cell wall degradation and fusogenic chemicals, tumor inducing principle (TIP) through *Agrobacterium* based transformation was successfully established.

During 1990 until 1993, the *in vitro* technology applications increased in number of plant species used, control of insect, weed and plant diseases, molecular, physiological and biochemical studies, cell cycle and chromosomal changes in cultured cells. Advances in molecular biology resulted in genetically engineered plants through the precise insertion of foreign genes from diverse biological systems.

The breakthroughs in P.T.C were:

1. Development of the shuttle vector for enhancing the natural gene transfer capability of Agrobacterium by direct transformation of regenerable explants obtained from plant organs.
2. The development of selectable markers with physical and mechanical means that can be used for amenable species to *Agrobacterium* mediated transformation.
3. Biolestic approach proved to be most successful in transformation for any plant species and genotype.

Over 100 species of plants including nearly all major dicotyledonous and monocotyledons crops have been genetically engineered and modified (GM) by gene transfer techniques.

Establishment of sterile cultures:

Plant tissue culture requires strict maintenance for aseptic conditions contamination may result in the loss of valuable time and efforts, the contamination of one batch culture may result in great economic loss or even loss of a plant-cultured strain. Therefore, sterile control measures are needed to be maintained in every aspect of the lab (personal and instruments). P.T.C. media like MS media are rich in nutrients so it is very suitable for microbial growth that results in the consumption of the nutrients rapidly, multiplying and finally suppressing the growth of the plant tissue by their overgrowth. Contamination by microorganisms suppresses the growth of plant tissue by releasing enzymes and toxins, selective absorption of specific nutrients leading to tissue infection and loss.

Disinfectant agents:

There many disinfectants that can used for the sterilization of the explant in P.T.C. like:

1. Sodium hypochlorite (NaOCl) for (5-30) min
2. Calcium hypochlorite ($Ca(OCl)_2$) for (10-15) min
3. Hydrogen peroxide (H_2O_2) for (1-30) min
4. Ethyl or isopropyl alcohol at 70% (v/v) for (1-5) min
5. Chlorine gas (Cl_2) for (16-18) hr.
6. Sodium dichloroisocyanurate salt (NADCC)
7. Isothiazalone biocide
8. Antibiotics (ex. Gentamicin and ampicillin) for 30 min in conc. Of 50-100 mg/L
9. Nanoparticles (NPs) (ex. Ag NPs and Fe NPs)

These disinfectants are used in different concentrations and for different period, (1-30) min. the most important thing is the washing process after the sterilization to ensure the complete removal of the disinfectant or it will be toxic to the explant.

recautions that must be taken in *in vitro* culturing:

1. Minimising air current in the working area so as to avoid spores of contaminating microorganisms to move in along with the air over the sterile area.
2. Preferably making the entire area air-conditioned.
3. Duct work and air-conditioning system can become a source of microbial contamination. Air handling ducts should be cleaned and properly maintained and prefilters venting the air into the culture room should be replaced frequently.
4. Allowing specific area for media preparation, inoculation and growth of cultures.
5. Storing properly the prepared media, nutrients and tools in the cabinets.
6. Using separate area for cleaning and washing.
7. Restricting personnel movement in the aseptic área.
8. Provision should be made for germicidal ultra-violet tube lights in inoculation room and corridors.
9. Switching on the ultra-violet tube lights of the coridors in the night.
10. Fixing ultra-violet tube lights in laminar air-flow bench/cabinet placed in the inoculation chamber and switching them on one hour before work.
11. Equipping the laboratory with laminar air-flow system so as to create positive air pressure, particularly for aseptic transfer.
12. To destroy the microorganisms, the culture vials containing the medium should be properly plugged and autoclaved.
13. There should be proper aeration of cultured tissue.
14. Washing hands, fingernails and arms with warm soapy water before entering the sterile area.
15. Using hair nets, masks and apron during working.
16. Wearing sterile latex hand gloves during aseptic transfers.
17. Working in the aseptic transfer hood not at the outer edge.
18. Minimising talking while working in the transfer hood.

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Sterilization of instruments and media

Since manipulations of plant tissue cultures are carried out under aseptic conditions, all instruments and media should be made free from M.Os through proper sterilization. There are various methods of sterilization, some of them mentioned in the table below

Table 1: various methods of sterilization in P.T.C

No.	Sterilization techniques	Material for sterilization
1	Dry heat (oven) at 160-180 °C for 3 hr. Or 140- 160 °C for 4 hr.	Empty glassware, instruments like scalp, forceps and needles
2	Flame sterilization	instruments like scalp, forceps, needles and mouths of culture vessels
3	Wet sterilization (autoclave) at 121°C at 15 psi for 15- 40 min	Media ,culture vessels, D.W and contaminated cultures
4	Filter sterilization (membrane filter 0.45 µm or smaller pore size like HEPA filter)	Heat labile compounds like plant growth regulators (PGR) for example GA, ABA and enzymes. Also sterilization of particular area
5	Wiping with 70% alcohol	Benches, platform of LFC and hands of the operator
6	Surface sterilization using sterilizing agents	Like all explants to be cultured

Use of a wetting agent such as Tween-20 or a detergent like dishwashing soap, which acts as a surfactant, helps enhance the disinfestation. In practice, usually 1-2 drops of surfactant per 100 ml of the disinfecting agent will suffice. To enhance the contact of the disinfecting agent with the explant, shaking, stirring and agitating of the explant while it is being disinfested, is required. Sonication treatment during infestation has also been found to enhance disinfestation of the explant. The sonication time can vary from 2 minutes for soft tissue to 20 minutes for seeds. Air bubbles trapped in the epidermal hairs covering the surface of the explant can be evacuated under a vacuum. The disinfecting agent should be removed from the explant by

rinsing it 3-5 times in sterile water. The concentration of disinfecting agent and the amount of the exposure time can be determined on the basis of trial and error or a literature reference. Generally, the less concentrated solution for the shortest time interval to obtain clean explants is desirable, as this will cause least amount of damage to the explant. The disinfecting agent can cause damage to explant and this damaged tissue has to be removed before culturing the explant.

Explant Source

The season of the year, the place where plant material is being grown (growth chamber, greenhouse, field) and the location of the explant on the source plant, are often singificant factors in the establishment of clean cultures. The plant material that is in active state of growth (meristematic tissues), such as spring flush shoot tip growth, is generally always cleaner as compared to dormant shoot tissue. Plant material from the fields is generally more contaminated as compared to the plant material from the greenhouse or growth chamber. It has been found that the contamination gets reduced by taking potted plant material out of the greenhouse (high humidity) and placing it in the laboratory or office (a drier environment) a few weeks prior to taking explant material. Plant material growing in the soil (roots, tubers, bulbs) or near the soil surface (stolons, rhizomes, orchid protocorms, shoots from the rosettes), is often difficult to clean than the aerial plant material. To obtain clean explants from rhizomes of aquatic plants (which are extremely challenging to clean), it is suggested to divide the rhizome into multinodal segments and to treat them in 50-100 mg/litre gibberlic acid (GA) to promote bud growth and 1 to 2-mm buds, developing from the rhizome, can be used as explant source.

Internal Microbial Contamination endophytes

It is quite likely that the explant material may harbour internal microorganisms. This poses a serious problem in establishing clean cultures. Explants from rapidly growing shoot tips, ovules of immature fruits, flower parts (both mature and immature) and runner tips are usually least likely to harbour internal contaminants. On the other hand bulbs, slow-growing shoots or dormant buds, roots, corms and underground rhizomes can have a heavy load of both external and internal contaminants. The seed can be aspetically germinated to provide clean explants from the root and seedling stem (hypcotyl, cotyledons and shoots).

The use of fungicides or antibiotics in the nutrient medium is generally not successful. Moreover, antibiotics need to be filter sterilized and added to cooled media. Both antibiotics and fungicides can suppress the growth of some microorganisms but when they are removed, microorganisms generally reappear. They can also suppress the growth of plant tissue or even kill it. In plant transformation, antibiotic as selective marker is based on the fact that antibiotic is lethal to the non-transformed tissue and only transgenic tissue containing the gene for antibiotic resistance will survive and grow. In case of known identity of the microorganisms, such as during experiments on transformation using *Agrobacterium tumefaciens*, use of clavamox, augmentin, carbanicillin and other antibiotic proves effective to eliminate or control *Agrobacterium* from overgrowing the explant.

Lec.3

Callus Culture

Cell form any plants species can be cultured aseptically on or a nutrient . the cultures are initiated by planting a sterilize tissue (an explant) on an agar medium. Within 2-4 weeks, depending upon plant species , a mass of unorganized(callus)is produced. Such a callus can be subculture indefinitely by transferring a small piece on to the fresh agar medium.

Juvenile tissues are generally most likely to produce a callus . however , callus cultures have been obtained form seedlings, young shoot or buds, root tip or developing embryos: frutis, floral parts, tubers and bulbs . any plant tissue with living cells can be used as an explant (preparation and sterilization of medium is described in earlier chapter). Under the influence of plant growth regulators, the cells of the explant may be induced to divide to form a loose mass of cells as callus. Callus tissue have been obtained form wood phloem, leaf , frutis and other tissues in which cells are highly specialized (differentiated) and not meristmatic.

Application of callus culture

callus culture are slow growing system on static medium and offers a unique system (as compared to *in vivo* grown plants) to study the following aspects of plant metabolism and differentiation:

1- Nutrition of plants

2- Cell and organ differentiation and morphogenesis.

3- Somaclonal variation and its exploitation.

4- Developing cell suspension cultures and protoplasts cultures.

5- Genetic transformation using ballistic particle gun technology.

6- In the production of secondary metabolites and their regulation.

Cell Culture

Cell suspension cultures are imitated by transferring friable callus to liquid nutrient medium. In liquid medium, plant tissues remains submerged which leads to anaerobic conditions and ultimately death of the cells. Therefore, such culture are agitated on rotary shaker at 80 – 150 rpm, with an orbital diameter of 2.5 -5.0 cms. Agitation serves

both to aerate the eultures and to disperse the cells. Cells form the inoculum are separated during this process and a suspension of cells is produced. The division rates

of suspension culture cells at the exponential phase are typically higher than callus cells, but doubling times are slower than bacterial and usually vary form 24-72 hrs. It is a common observation that if relativity small number of cells are transferred (low inoculum density) to anew medium (either static or liquid), they may fail to divide, whereas a larger quantity of tissue transferred form the same culture may proliferate rapidly on the some medium. This observation has led to the concept of critical initial cell density. This is defined as smallest inoculum per volume of medium, form which a new culture can be reproducibly grown.

There are a few conditions, which determine the critical initial density of cells. These are :

1- The culture physiological characteristics.

2- The length of time and conditions under which the culture was previously maintained.

3- The composition of the fresh medium.

The third point is of interest . as the isolated cells filed 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 of protoplasts. In cell suspension cultures, cells grow as isolated single cell and cell aggregates of a few cells to a few hundred cells. Cells aggregation vary from species to species and sometimes it is difficult to maintain fine cell suspension culture, e.g., in members of the family rutaceae.

Cell cultures are subcultured by dilution of the stock cultures, 5 to 10 times v/v (volume by volume), depending upon the growth of cells. As mentioned above, growth of cell suspension cultures is always higher than callus cultures and, there fore, requires rapid subculture(7-21days) as compared callus cultures (4-8 weeks).

Cell cultures in the liquid medium are grown as:

A. Batch cultures in shake flasks and bioreactor

B. Continuous culture system.

Batch culture

Whether cultures are grown in flasks (100ml to 2 liters capacity containing 20 to 500 ml liquid medium) or in a bioreactor, system is used as a batch. Inoculated cultures are harvested after a definitive growth period (determined by growing cultures earlier and recording the growth phases during 1 to 6 weeks growth) at the end of stationary phase and cultures are harvested for a specific objective. Such cultures are grown again and again in batches for the purpose of experiments and known as batch cultures.

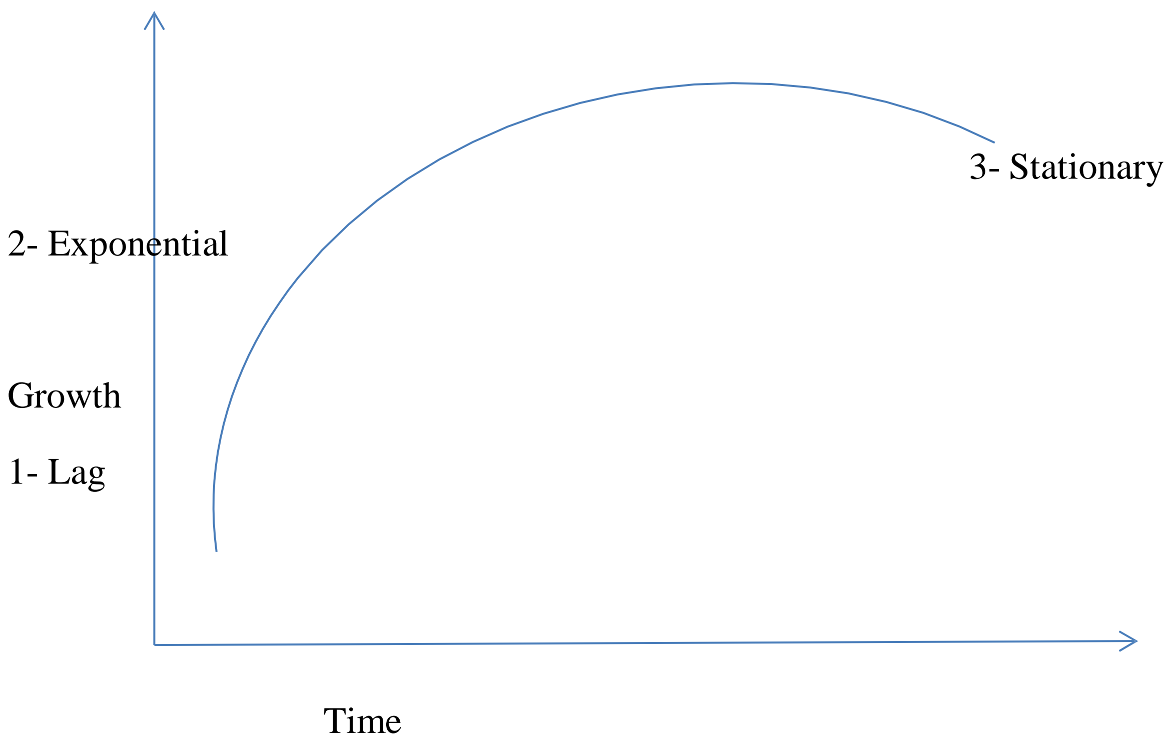

Fig. Time course of growth showing different phases of growth in a growing systime.

Continuous culture

Continuous culture are usually grown in a bioreactor, (also known as chemostate) when inputs (nutrient, oxygen) and output (spent medium, cells) are precisely controlled and cells are grown under defined conditions of nutrients, pH, oxygen, and density continuously for purpose of study. Such cultures are known as continuous cultures.

This type of work is possible only after basic study of growth and metabolism in small shake flask. In continuous culture, cells are always in an exponential phase of growth.

Time course of growth

Fresh weight of callus is determined by carefully removing the adhering agar form callus and immediately weighing the callus in a sensitive balance. Cell cultures are filtered in butchner funnel lined with filter paper, washed with distilled water (under mild vacuum) and weighed. Dry weights are determined by drying the cells to a constant weight at 06 ^{0}C in an oven and weighing in balance. Packed cell volume (PVC) of cell culture is determined by centrifuging it at 1000rpm for 5min in a graduated centrifuge tube and mastering the volume occupied by the cells out of total volume of the cell suspension. Cell are counted by a haemocytometer under compound microscope during growth period.

Comparison of callus and suspension cultures

Parameters	Callus	Cell suspension
Growth	Slow	fast
Cell to cell contact	cell in contact	dissociated all cell in
Medium	only lower layer in is	direct contact with the
Contact with the medium	medium	
Precursors	not available to all cells	available to all cells
Subculture period	long 4-8 weeks	short, 7-12 days
Accumulation of metabolites	higher than cell suspension	lower than callus culture
Scale – up in bioreactor	not possible	cell suspensions are grown in bioreator

Application of cell culture

1- cell culture offers enormous opportunities in the study of single cells and group of cells.

2-In isolation of protoplast

3- In cell cloning by the plating technique with or without specific treatment, e.g., mutagens, amino acid analogues, etc.

4- Development of cell lines for various types of resistance , e.g., salt and drought and toxin resistance lines.

5- In scale-up technology using bioreactors of various types.

6- In providing an excel system of micrpropagation on mass- scale by somatic embryogenesis in cell culture and use of bioreactor. Such system can be used for artificial seed production.

7- In the study of nutrition

8- In cellular differentiation , single cells constitute an excellent system to study cytodifferentiation in a cell leading to tracheiry element formation and plastids differentiation etc.

9- In secondary product formation , regulation and biosynthesis.

10- In the process of cell division and factors effecting cell divisions and related process.

Lec.4

Totipotency, Cytodifferentiation and Organogenesis

Totipotency is the ability of cell to form all the cell types in the adult organism. During the growth and maturation of callus tissue or free cell in suspension cultures, few dedifferentiated cells undergo cytoqurescence and cytoscence.

Totipotency

Gottlieb Haberlandt, a German botanist in 1902, stated that plant cells are totipotency (have the ability to regenerate into plants). The term totipotency, probably coined by Morgan1901, as applied to plant cell means that every cell, when provided proper conditions, is capable of developing into a whole plant. As all somatic of cells of the mature plant are derived by mitosis form the zygotic cell, each one of them must also contain all the genetic information needed for growth , development and regeneration of a whole plant .expansion of this genetic potential of a plant cell can be demonstrated by *in vitro* culture of different types of cells of the plants. In fact the totipotency of the cell in the culture is manifested through the process of differentiation . in this process , growth regulators (auxins and cytokinins) play a major role. Formation of the organs, known as organogenesis, is initiated by the balance in the ratio of auxins and cytokinins. Root formation rhigogenesis takes place at a relatively high auxin-cytokinin ratio and reverse favours shoot formation (caulogenesis). Sometimes the balance of growth regulators levels leads to the differentiation into embryoids or embryos known as embryogenesis: a process that occurs in the development of normal zygotic embryos. In some cases , the cells of some callus mass frequently differentiate into vascular elements without forming any plant organs, known as cytodifferentiation or histogenesis. Evidently , the totipotent cells may express themselves in different ways on the basis of differentiation process and manipulation.

When totipotent cells are not expressed or partially expressed, obviously the limitation on the their capacity for development must be imposed by the microenvironments. The same may be corrected by providing suitable nutrient medium favourable for the expression of totipotent cells. It is actually standardized by trial and error method. The cells of habituated callus also remain totipotent. They are capable of regenerating whole plants without any major manipulation. Crown gall tumor cells exhibit limited totipotency. As a matter of fact, the mode of expression of totipotency of plant cell in culture varies form

plant to plant. In callus culture, all the cells are not totipotent. The limited expression of totipotency is mainly affected by variation in **(#)** chromosome number in the cells of the callus. **(#)** Observation on organogenesis or embryogenesis in

callus culture may sometimes be required to provide the appropriate environment for certain individual to express their totipotency **(#)** the unavailability of a threshold level of exogenous and endogenous growth regulators in all cells of the callus tissue may be a barrier to their expression of totipotency. Apart form these factors, heterogeneity in physical structure of the cells makes a significant difference in the degree of chemo differentiation of the cells, making an asynchronous situation for which all cells are not able to express their totipotency at a time.

It is evident that form fundamental to applied aspect of plant biology cellular totipotency is highly important. The success of various aims and objectives of plant tissue culture depends upon the expression of totipotency. Expression of totipotency is of great value to agriculture, horticulture, forestry and many industries. Totipotent cells within a bit of callus tissue can be cryopreserved for germplasm storage.

CYTODIFFRENTIATION

In plant tissue culture during growth and maturation of the callus tissue or free cells in suspension culture, few dedifferentiated cells undergo cytoqurescence and cytosenescence. The twin phenomena are chiefly associated with redifferentiation of vascular elements, particularly tracheary tissues. This whole developmental process is known as **cytodifferentiation**, the preliminary steps of which are reflected by a series of histological and biochemical changes in the cell. Based on numerous observations, the proposed fate of the cultured cells during cytodifferentiation can be spread over three steps: **(*i*)** the callus tissue, at the time of its initiation, shows a mixed population of small round oval cells with dense cytoplasm **(*ii*)** with an increase in number of subculture, few cells become elongated and **(*iii*)** with further increasing subculture members, callus tissue shows maximum xylogenesis with presence of tracheary elements. In fact initiation of xylogenesis takes place form mitotical blocked elongated cells of the callus.

Ultrastructural studies have reveled some plausible clams of intracellular events leading to loss of organogenic potential for dedifferentiation. It has been observed that certain degradative changes associated with chlorophyll, endoplasmic reticulum, ribosomes, dictysomes and mitochondria and separation of bounding membranes, are the first steps in cytoqurescence leading to cytosenescence. The functions, such as transformation of living

cells into dead cells, formation of empty tracheids during cellular differentiation and biosynthesis of acid phosphatase enzyme, are functionally related to the autolysis of the cell contents and lignin synthesis. It is a matter of debate whether it is prerequisite for xylem differentiation or not. Since it has been observed that xylem differentiation is completely suppressed by ***BUdr*** an inhibitor of DNA synthesis, it becomes clear that

DNA synthesis is a crucial factor in differentiation. It has also been observed that gamma irradiated cells do not participate in xylem differentiation. Reports are there that xylem differentiation is preceded by meiotic cell division. Moreover, in many cases cell division is not a perquisite for xylem differentiation. Keeping in view variations, question of cell division prior to xylem differentiation remains unsolved.

It has been observed that a number of physical and chemical factors also have profound effect on cytodifferentiation. At low concentrations, auxins stimulate xylogenesis. Many workers have reported the influence of auxins in xylem differentiation *in vitro*. Some workers have reported that (cytokinins also play a role in cytodifferentiation in cell division and xylem differentiation). For cytodifferentiation, presence of sufficient amount of sugar (sucrose) is also very essential. Reports of effects of physical factors, such as highs, temperature, water stress etc. on vascular differentiation, are rare. There are some other factors, such as morphactins, methionine, irradiation etc. that also affect cytodifferentiation. Some lacuna still exists in our understanding of cellular and biochemical aspects of differentiation and quite a number of parameters need consideration before studding cytodifferentiation in tissue culture.

ORGANOGESIS

In vitro callus tissue can be obtained form many different parts of plants by planting single cells, cell aggregates of cell suspension cultures and form isolated protoplast. In all cases the main object is to regenerate whole plants with different plant organs. The regeneration of different organs takes place by means of organogenesis, i.e, the development of adventitious organs or primordial form undifferentiated mass of cells by the process of differentiation. Individual meristmatic cells or groups of cells within the callus tissue, the meristemoids, get transferred into cyclic nodules form which root or shoot primordial may differentiation. In most. Initiation of shoot buds (caulogensis) may precede initiation of root buds (rhizogenesis) or *vice versa* or induced shoot buds may grow as rootless shoots.

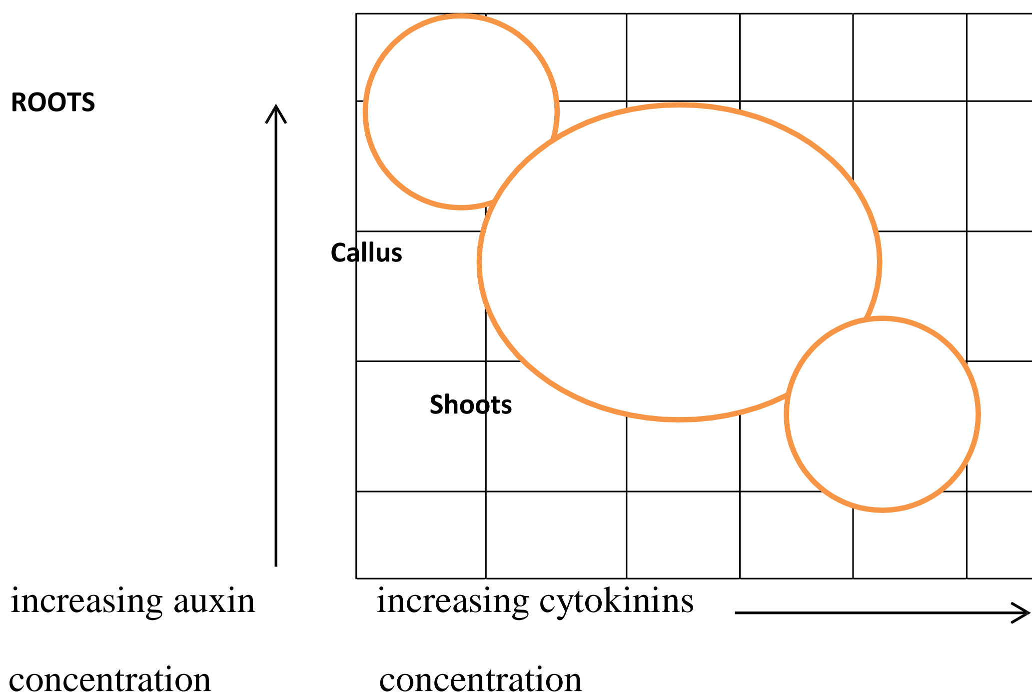

Fig. Relationship between auxin and cytokinins concentration in organ formation in the callus culture.

In many cases, the callus tissue shows a high potential for organogenesis when first initiationed but gradually a decline sets in as subculture proceeds with eventual loss of organogenic response. The loss of potential organogenesis may be due to either genetic or physiological changes induced by either prolonged cultural conditions or composition of nutrient media. The genetic effects in a callus tissue are reflected in changes in number and structure of chromosomes. Although the tissue becomes mixopolid but it is generally seen that shoot bud formation takes place form diploid cells. Long – term culture also often leads to the loss many endogenous factors at the critical stages of the growth. These endogenous factors, present at the initial stage, may not be synthesized at all or may be synthesized only in insufficient quantity at alter stages.

Biochemical analysis of organogenesis has revealed that the activity of some enzymes has direct impact on organogenesis potential of the callus tissue**. Increase in peroxidase activity** and the activates of enzymes of **carbohydrate metabolism** before differentiation of shoot and root has been demonstrated. The comparison of **malic dehydrogenase activity** under shoot and root formation stages reveled that it was more pronounced prior to shoot and root differentiation. Higher activity levels of EMP and pentose phosphate **pathways enzymes in shoot** forming sugarcane indicated generation of energy molecules, reducing power and pentose sugars vital for energy dependent reactions and synthesis of nucleic acid during shoot differentiation.

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Organogenesis is controlled by a number of factors, such as source size and age of explant , components of culture medium especially of growth regulators, pH of the medium, age of the callus, ploidy level of the cells of the callus , temperature , photoperiod , intensity and quality of light , oxygen gradient etc.

During regeneration of plants form cells or calli, organogenesis is most important because unless organs are formed the whole plant cannot develop. Applications of organogenesis are vast and varied. In fact the success of regeneration of whole plants in all in vitro culturing programmes solely depends on organogenesis.

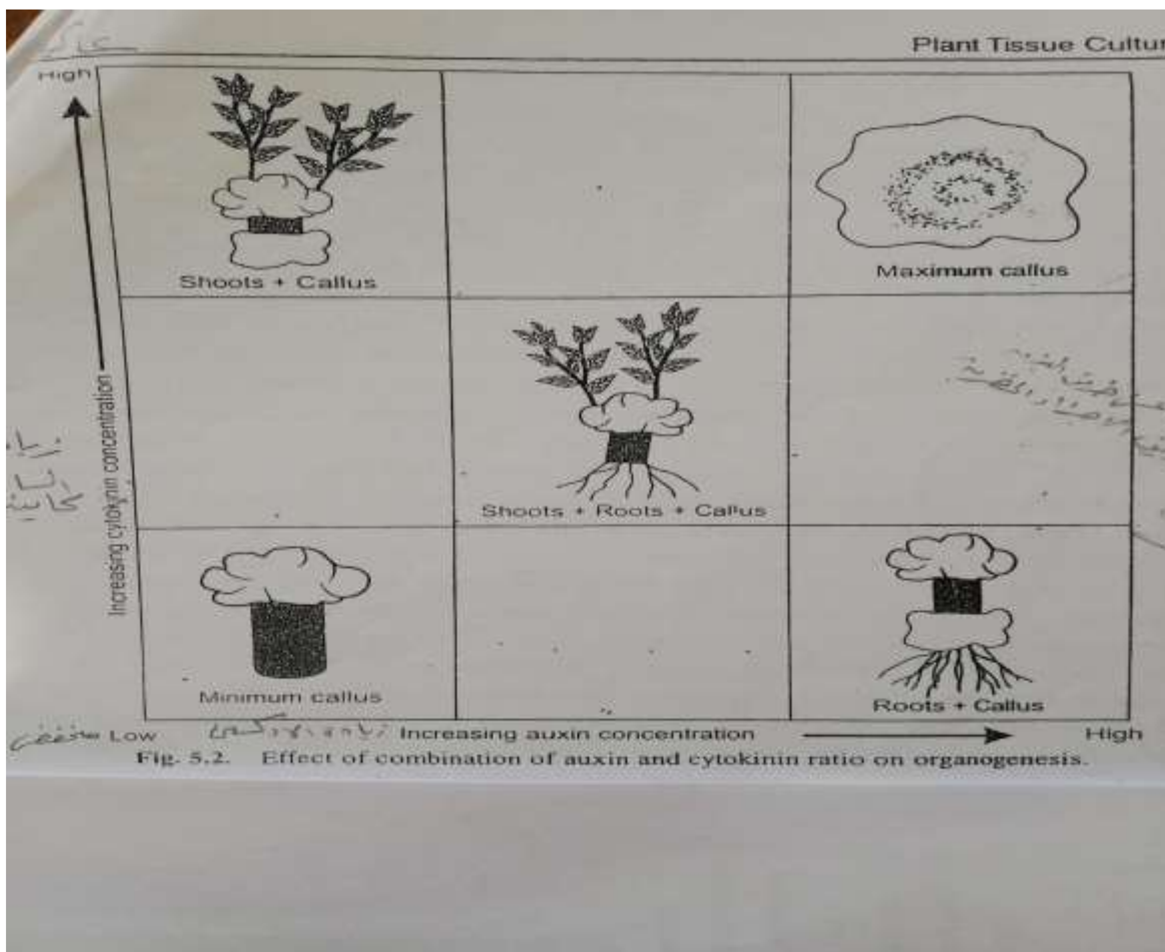

Fig. Effect of combination of auxin and cytokinins ratio on organogenesis.

Lec. 5 SOMATIC EMBRYOGENESIS

Plant cell are totipotent and can produce whole plants *in vitro* through organogenesis or somatic embryogenesis , almost simultaneously but independently, reported for the first

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time somatic embryo formation in carrot cell suspension cultures. Later rangaswamy (1961) worked out somatic embryogenesis in citrus in detail. Somatic embryogenesis is a process by which somatic (non- gametic) cells undergo differentiation to form a bipolar structure, the somatic embryogenesis containing both root and shoot axes. Somatic embryos are similar to zygotic embryos , except in their place of origin and larger size. These can mature and germinate. The tissues derived form them can again develop embryos spontaneously. Thus , it has been demonstrated that embryo development is not the monopoly of egg cell (after being fertilized) and that any cell of the embryo sac or any other part of sporophyte can be made to give rise to an embryo by *in vitro* culture.

Somatic embryogenesis can be initiated in two ways

(***i***) indirectly inducing embryogenic cells within the performed culture or (***ii***) directly form proembryogenic determined cells (without callus) which are ready to differentiate in to embryoids. In the first case , embryoids are initiated in callus form superficial cell aggregates (cutlers) where cells are highly meristmatic and contain dense cytoplasm , large granules and conspicuous nucleus. These cells have high potential for embryogenic development, it has been observed that the indirect embryogenesis occurs in most of the cases.

Somatic embryogenesis in short – term culture and this ability decreases with the increase in the duration of culture due to change in ploidy and loss of certain biochemical properties of culture cells.

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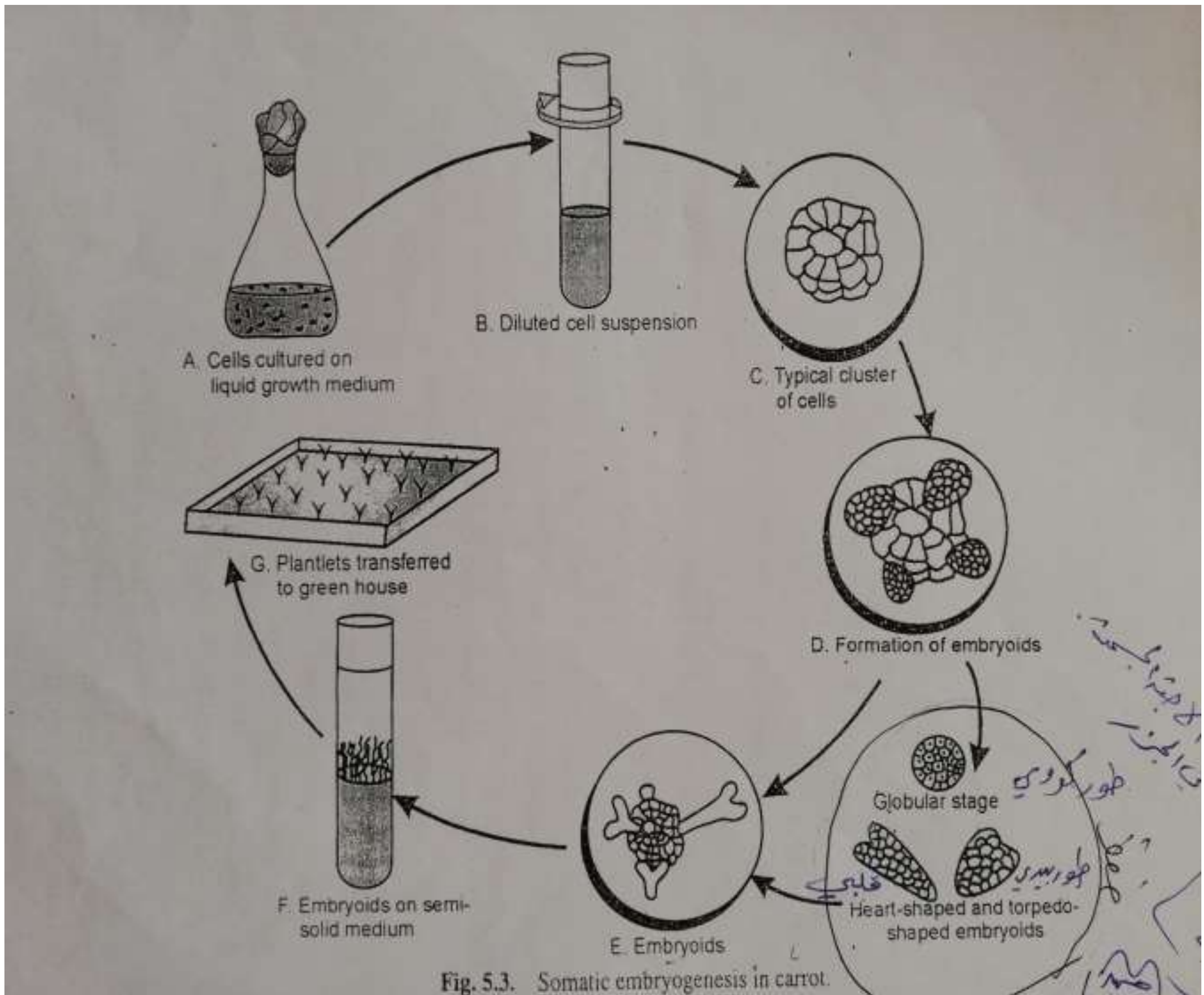

Fig. Somatic embryogenesis in carrot.

Importance of somatic Embryogenesis:

The potential applications of in vitro somatic embryogenesis are far greater than organogenesis. It is a versatile technique micro propagation of plant species. Some of the applications of somatic embryogenesis are:

1- somatic embryogenesis is used for production of adventitious embryos for plant propagation.

2- Since somatic embryogenesis multiply rapidly in cell cultures, they are of special importance for analysis of biochemical and molecular events, such as mutations that take place during induction and maturation of embryo.

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3- Somatic embryos, being bipolar structures in same unit (presence of both root and shoot), develop directly into a complete plantlets without needing rooting step as required organogenesis.

4- Somatic embryos, grow individually, making the system easy to manipulate by subcultureing.

5- Somatic embryos can be used for isolation of specific storage protein.

6- Somatic embryogenesis is an alternative approach for production of disease free plants.

7- It is possible to store culture of somatic embryos for long duration.

8- Dormancy can be induced in somatic embryos by their cryopreservation.

9- Somatic embryogenesis helps shorten breeding of deciduous trees and increases the rate of germination of hybrid embryos where delayed germination of seeds a significant handicap in rooting of plants, particularly in species of horticultural importance.

10- Somatic embryogenesis is a versatile technique for micropropagation of several herbaceous dicots and monocots.

11- Somatic embryos are devoid of food reserve. Suitable artifial endosperm can be packaged in them by encapsulation or coting them with calcium alginate which prevents them form desiccation. Such somatic embryos, called artificial seeds, are capable of producing plantlets directly into the soil/field like normal seeds.

ARTIFICIAL SEEDS

Artificial seeds , also known as **synthetic** seeds or **somatic seeds**, are the living seeds-like structure that are developed experimentally by the technique in which somatic embryoids, derived form tissue culture , are encapsulated in a suitable matrix made up of calcium alginate or sodium alginate along with substances like mycrohiza, insecticides, fungicides and herbicides. This encapsulation prevents them form desiccating. Artificial seeds can be used for the rapid mass propagation of elite plant species. They have been able to solve many problems of storage and transportation of propagules transportation against conventional plant tissue cultures for clonal propagation. Commercially viable

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artificial seeds must contain a chemical substance that can induce reversible dormancy of somatic embryos.

Kitto and Janick(1982) in their attempt were first to coat clumps of carrot embryoids with polyethylene to develop artificial seeds. Later, Redenbaugh *et al* (1988) demonstrated that hydrogels, such as sodium alginate, could be used to produce single embryoid artificial seed. It has now become possible to successfully obtain artificial seeds of some crop plant like *Zea mays* , *Daucas carota* , *Lactuca sativa* , *Medicago sativa* , *Gossypium hrisutum* , *Brassica* sp etc.

There are several different types of synthetic seeds, such as those in which: (***i***) somatic embryos are encapsulated in water gel, (***ii***) somatic embryos are dried and coated (***iii***) somatic embryos are suspended in fluid carrier and (***iv***) shoot buds are encapsulated in water gel. As yet no system for large – scale production of synthetic seeds has been developed although pilot studies for moderate quantity using somatic embryos encapsulated in water gel , have been conducted in Japan.

Production of synthetic seeds requires several steps, such as establishment of callus culture , induction of somatic embryogenesis in callus culture , maturation of somatic embryos and encapsulation of somatic embryos (Fig 5.4) . following encapsulation , artificial seeds embryoid to plant conversion , greenhouse and field planting and their yield in comparison to plants derived form true seeds. The artificial seeds (fig 5.5) produced so far require asptic storage in low temperature.

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Fig. 5.4 production of artificial seeds.

Fig. 5.5 A typical artificial seeds.

Importance of Artificial Seeds

Artificial seeds are important because the following reasons:

1- the use of artificial seeds, is an improvement over traditionally micropropagation plants in vegetatively propagated crops. In long term, it may turn out to be cost saving venture as labor intensive step of transferring plants form ***in vitro*** culture to soil / field conditions may be overcome.

2- Artificial seeds can be stored aseptically under low temperature upto a year or so without the loss of viability.

3- Artificial seeds can be made available within a short time and one has not to wait for longer time till the plant attains the reproductive phase and produces seeds.

4- Production of artificial seeds is time or season independent. They can be obtained at any time of season which is not possible with normal plants.

5- Using artificial seeds, the dormancy period may be reduced and the life cycle of plant can be shortened.

6- They can be used in the maintenance of male sterile lines and parental lines for hybrid crop production and preservation.

7- artificial seeds are easy to handle and prove useful as units of delivery.

8- They can be sown directly in the soil/field like natural seeds and do not need hardening in green house.

9- In future, they are likely to be applicable for large – scale monocultures as well as mixed-genotype plantations.

10- their seed coating has the potential to hold and deliver beneficial adjuvants, such as growth promoting thizobacteria, plant nutrients, growth control agents and pesticides for precise placement.

11- They help in studying the role of endosperm and seed coat formation.

The only limitation of synthetic seeds is their high cost of production which in future is expected to go down. When this happens in due course , they will become popular at the commercial scale.

Lec 6

Protoplast culture

USE OF PROTOPLASTS FOR PLANT IMPROVEMENT

Plant cells from which the cell wall has been removed are termed protoplasts. Protoplasts are somewhat unique in plant cell culture in SSthat they exist as separate cells without cytoplasmic continuity among neighboring cells. Communication among protoplasts sharing the same culture environment is therefore limited to metabolites that can traverse the plasmalemma into the culture medium and influence the behavior of other cells. This limits the direct pathways of communication through cytoplasmic plasmodesmata generally present with intact plant tissues. However, in protoplast cultures, the barrier imposed by plant cell walls has also been eliminated. The plant cell wall, although critical to plant structure and function, is a major impediment in exploiting direct DNA transfer to individual cells and the production of somatic hybrids by cell fusion. Removal of the cell wall temporarily during protoplast culture can result in viable cells with properties otherwise unknown in plants. Plant protoplasts were first isolated by Klercker in 1892 by slicing onion bulb scales with a thin knife in a plasmolyzing solution, resulting in the release of protoplasts when cells were cut through the wall.

PROTOPLASTS APPLICATIONS

1-Hybrid plant production.

2- Inclusion of foreign materials and their introduction into cytoplasm, such as adding nuclei and plasmids.

3- Can be selection pure single cell, compared to suspended cells.

ISOLATION OF PLANT PROTOPLASTS

Protoplast isolation by enzymatic cell wall digestion involves the use of cellulase, hemicellulase, and/or pectinase, which are extracted from various sources, including fungi and snail and termite gut. These hydrolytic enzymes are available commercially in differing formulations of varying purity. Digestion by a combination of these three enzymes is generally conducted at a pH of 5.5 to 5.8 over a period of 3 to 18 hours. Protoplasts can then be collected and purified using centrifugation to separate broken and damaged cells from intact protoplasts by taking advantage of their differing buoyant densities.

SOURCE OF PROTOPLASTS

Protoplast form which protoplast can be obtained:

1- Leaves: the leaves is convenient source of cells form which protoplast can be separated mechanically.
2- Callus.
3- Cell suspension.
4- Roots.
5- Pollen grans.
6- Plant grown in sterile media.

METHODS OF PROTOPLASTS ISOLATION

1- Mechanical isolation.
2- Enzymatic isolation.

USES FOR PLANT PROTOPLASTS

Plant breeders have used sexual hybridization to improve cultivated crops for centuries. This process is generally limited to plants within a species or to wild species that are closely related to the cultivated crop. When possible, sexual hybrids between distantly related species have been useful for the incorporation of single gene traits such as insect and disease resistance. However, intraspecific and interspecific incompatibility barriers limit the use of sexual hybridization for accessing germplasm distantly related to crop species. Protoplast culture has been used to develop plants with improved agronomic and horticultural characteristics and improved disease resistance through recovery of culture-induced variant, parasexual hybrids, and genetically engineered plants. Regenerated plants from cultured protoplasts have included variants (somaclones) with improved characteristics. Parasexual or somatic hybrids can be obtained by fusing protoplasts of distantly related species. Through genetic engineering, foreign genes can be inserted into plant protoplasts that can be regenerated into improved plants that express the inserted genes. Improved plants obtained by protoplast manipulation can be used in breeding programs to develop new cultivars.

PURIFICATION METHODS

1-Sedimentation and washing: used solution osmosis manitol or sucrose with pH5.8
2- Flotation: using manitol, sorbitol and sucrose concentrated 0.3-0.6***M***

PROTOPLASTS VIABILITY TESTS:

1- Cytoplasmic streaming.
2- Evan blue method.
3- Measurement of photosynthesis and respiration.
4- Change in protoplasts size.

5- Staining with fluorescein dictate FAD.

CULTURE MEDIA

The best culture medium for protoplasts is often similar to, or slightly modified from, that used for organ regeneration from other explants. Adjustment of ammonium nitrate and calcium is often required to stimulate cell division. Ammonium nitrate is essential for promoting cell division, but is toxic to protoplasts at concentrations (20 mM) used in most tissue culture media. Hence, the ammonium nitrate concentration is lowered to 1/4 to 1/2 of the normal concentration. In contrast, the calcium concentration is usually increased for most protoplast procedures. The calcium concentration of most tissue culture media ranges from 0.5 to 3 mM. At this low concentration, protoplasts typically aggregate and brown rapidly. Raising the calcium concentration (14– 40 mM) promotes early cell division and cell synchronization, decrease aggregation, and reduces browning of protoplasts during the early stages of culture. Normal ammonium nitrate and calcium levels are restored after protoplasts have regenerated a new wall and divided.Organic components such as inositol, nicotinic acid, pyridoxine, thiamine, glycine, folic acid,and biotin are found in many tissue culture media. In addition, casein hydrolysate, D-Ca-pantothenate, choline chloride, cysteine, malic acid, ascorbic acid, adenine sulfate, riboflavin, and glutamine are often added to protoplast media in small amounts (0.01–10 mg/l) to hasten cell wall synthesis and promote cell division. Most of these compounds are not used after protoplasts have regenerated a new cell wall and divided. Sugars are used in protoplast culture media as osmotic stabilizers and carbon sources. Due to the elimination of wall pressure (i.e., pressure potential), a component of water potential, culture media must be osmotically adjusted to prevent rupture of protoplasts during isolation and early culture. Mannitol, sorbitol, and glucose are used as osmotica at concentrations from 0.3 to 0.7M.

Sucrose and glucose are used as carbon sources at concentrations from 0.2 to 0.6M. The sugar concentration is gradually reduced after cells synthesize a new wall and divide. Osmotic sugars are usually eliminated by the time macroscopic colonies are visible. Exogenous PGRs are necessary to promote cell division. Both auxins and cytokinins typically are used. The type and concentration differ with the species and mode of regeneration. Naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.45 to 10.7 µM are typical auxins and concentrations.

PROTOPLAST FUSION AND SOMATIC HYBRIDIZATION

Once purified protoplasts have been obtained from two different plant or tissue sources, various treatments can be applied to induce them to fuse together to form hybrid cell lines. The simplest way to do this is to use spontaneously fusogenic protoplasts. Such cell lines have been described in detail in carrot and other systems (Boss, 1987), but they are not common enough for widespread use in plant protoplast research. Generally, chemical agents

or electrical manipulation are necessary to induce membrane instability that leads to protoplast fusion.

PROTOPLASTS FUSION

The merger occurs ether natural(spontaneous fusion) or through certain influences (induced fusion). An application of fusion is the hybridization process of somatic cells it is one of the important methods in the application of genetics and plant improvement unable to form seeds.

METHODS TO INDUCE FUSION

1- Spontaneous fusion.
2- Polyetheleneclycol (PEG) fusion.
3- Fusion by calcium and high pH.
4- Fusion by sodium nitrate.
5- Immunological method.
6- Mechanical method.
7- Fusion by electrical shock.
8- Other methods for fusion.

MECHNICAL OF FUSION

It includes four main stages, which are the following:
1- Convergence: protoplasts else together by adhesion promoting factors. **2-** Agglutination.
3- Membrane fusion.
4- Dissolving of the protoplasts wall.

POLYETHYLENE GLYCOL –MEDIATED PROTOPLAST FUSION

Several aqueous solutions have been used to induce chemical fusion of plant protoplasts (Saunders and Bates, 1987). These include salt solutions (NaCl, KCl, NaNO3, KNO_3), dextran sulfate, polyvinyl alcohol, lysolethicin and polyethylene glycol (PEG). Of thes fusogenic agents, PEG has been most frequently used, in conjunction with alkaline pH and high calcium concentration.There are many steps in the fusion of plant protoplasts using PEG as a chemical facilitator Initially, the cell membranes must be brought into close physical contact (agglutination).As most cell membranes possess a net negative surface charge, adjacent cells with similar charges tend to repel each other. Chemical fusogenic facilitators, such as concanavalin A and immune antisera, promote cell fusion by overcoming the repellent effect of similar net negative surface charges on the protoplast membranes. PEG is a potent cell agglutinator and functions as a membrane modifier. Once cell membranes are in close contact, their surface proteins migrate to

create lipid-rich regions. During this period, the dehydrating effect of PEG on the cell membrane and the ability of PEG to bind to phospholipids within the membrane induce cell adhesion between adjacent cells. Subsequently, successive washing with high concentrations of calcium in a buffer with an alkaline pH effectively completes the fusion process.

REGENERATION OF PLANTS FOR PROTOPLASTS CULTURE

The process of plant emergence form cultivation of protoplasts includes the following steps:

1- The formation of cell wall.
2- Division of cells formed.
3- Organogenesis formation.
4- Embryogenesis formation.

SELECTION OF HYBRID CELL LINES

An important feature of any somatic hybridization procedure is the identification and selection of fused cells. Typically, it is desirable to separate hybrid cell lines from other cells in the mixture because cell lines that result from fusion of two or more cells from the same parent (homokaryotic fusions) or from parental cells that did fuse at all may greatly outnumber the heterokaryotic fusion products. Furthermore, there is no guarantee that just because a cell fusion occurred, nuclear fusion will follow. Fusion of the two membrane-bound nuclei within a single cell and coordination of mitosis involving the chromosomes from what were formerly two independent nuclei are required to form a stable hybrid. Unless there is some identification and selection system incorporated into each of the parental cell lines prior to fusion, collection of hybrid cell lines can be very difficult. Several selection systems have been developed based on specific requirements of the prospective parent cell lines.

SELECTION OF FUSION HYBRIDS

After the fusion process is complete the contents the protoplasts are mixed, in addition, it contains homozygous or different nuclei and cytoplasmic conjugates. There are several methods of determining franchise of selection:

1- Visual selection.
2- Fluorescent able selection.
3- Nutritional selection.
4- Selection based light sensitivity.
5- Selection based on drug sensitivity.

GENETIC TRANSFORMATION

Genetic transformation of single cells has been accomplished by co-cultivating protoplasts with Agrobacterium tumefaciens or by direct DNA transfer using polycationic chemicals, electroporation, liposomes, microinjection, or sonication.Transformation of plant protoplasts using polycationic chemicals and/or electroporation has been most often reported.

Transformed protoplasts offer the possibility of examining factors affecting transient gene expression, regenerating transformed plants where other methods have been difficult or unsuccessful, as in many monocots, or for studies of gene function or protein targeting.

UPTAKE OF CELLULAR COMPONENTS BY PROTOPLASTS

The inclusion of protoplasts in cludes several applications they are following:

1- Uptake of chromosomes.
2- Uptake of mitochondria.
3- Uptake of bacteria.
4- Uptake of chromoplasteds.
5- Uptake of plasmids.
6- Uptake of viruses.
7- Uptake of micro organisms.
8- Uptake of cellular components by protoplast.

Lec 7

Meristem Culture for Virus-free Plants

I. INTRODUCTION

Development of virus free plants is one of the most significant applications of plant biotechnology. The technique has applications in diverse areas such as rapid clonal multiplication of vegetatively propagated crop plants, virus elimination and germplasm preservation. From a plant pathologist's point of view, control of viral diseases can be classified under the following headings:

a. Eliminating the sources of virus infection.

b. Avoiding the disease carrying vectors.

c. Directly attacking the vectors.

d. Breeding resistant varieties.

e. Curing virus infected plants.

f. Adopting special methods of propagation like meristem culture.

Virus is a intracellular, obligate parasite and its multiplication is so intimately associated with normal metabolic process in plants that selectively interfering with it is not possible at all. Moreover,such methods can not eliminate virus from a whole plant. Above mentioned all the methods except meristem culture are conventional. When plant pathological approaches and the control pressure is removed, virus population is increased to its early concentration. Fortunately, the majority of known viruses are not transmitted by seeds and-seeds of infected plant usually develop into healthyplant. However, seedlings are useless for maintenance of clones, hybrids and varieties because they do not produce plants identical to parents. Seeds are produced by gametic recombination (sexual reproduction) and thus they produce offspring different from parents. This is not required in plantation and horticultural crops. Once systemically infected, the disease is transmitted from one vegetative generation to the next in vegetatively propagated crops. By this process of multiplication and perpetuation of virus, all the varieties of various crops become completely infected. It is presumed that most of the clonally propagated crops are infected by one or more viruses. Some of them may be latent and not detectable by their symptoms.

Virus-free plants are free from virus for which they are tested or produced experimentally but they may contain undetectable (latent) viruses for which they are not tested. Therefore,

another term 'virus tested' has been suggested in place of virus free plants to give a clear idea about the virus for which the plant is tested.

There are four stages in therapeutic method for virus elimination. These are: a. Identification of virus (es) present in the clone.

b. Therapy.

c. Testing of treated plants.

J. propagation and continued testing of cure plants under conditions that avoid reinfection.

The most common and widely used method of virus elimination for planting material is heat treatment. Keeping the sugarcane cutting at 5O-52°C in water for 30 minutes can eliminate several viruses. This technique has been used in several plant species like potato, tomato, apple, strawberry and raspberries.

MERISTEM CULTURE

Limasset & Comnet (1949) observed that in systematically infected plants virus concentration decreased as they approached the apical meristem. In apical meristem, no virus was detectable in 50% of the materials tested. On the basis of th is observation, Morel and Martin (1952) proposed that it might be possible to isolate the apical meristem of a systematically infected plant in vitro to obtain a virus-free plant. They were successful in confirming this hypothesis and obtain virus-free Dahlia plants through meristem culture of the infected plants. Since then this technique has been used for obtaining virus-free plants in a number of genera. the meristem is a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm long. As rule meristem of both main shoot and axillary buds are used for obtaining virus-free plants.

TECUNIQUE AND MEDIA

Shoots of all angiosperms and gymnosperms grow by virtue of their apical meristems. The apical meristem is usually a dome of tissue located at (he tip of shoot and measures approximately 0.1mm in diameter and O.2-O.3mm in length. From plant anatomy point of view, this mass is separated as tunica, corpus, central mother cells, flank meristem and rib meristem.

The apical meristems are first formed during embryo development and they remain (except in dormant buds) in an active state of division throughout the vegetative phase of the plant. The totipotency of the plants cells form the basis of meristem culture technique.

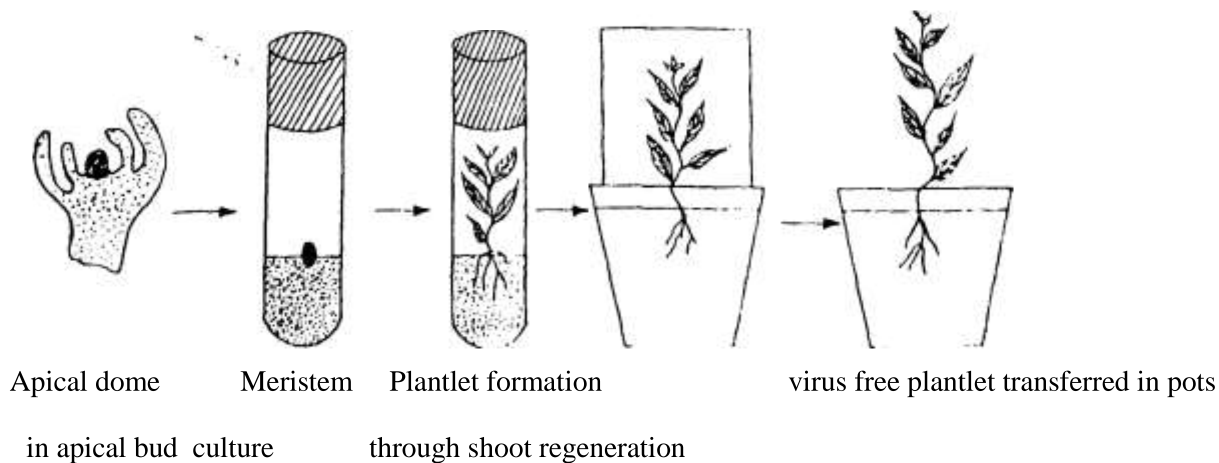

Fig. 1. Schematic presentation of isolation and meristem culture and regeneration of virus-tree plantlets.

After proper sterilization of shoot buds, (apical, or axillary) apical meristem 0.3 to 0.5mm is removed with the help of fine forceps and scalpel tinder d stereo binocular microscope. Before removing the meristem, apical dome is cleared from leaves. Isolated meristem is immediately transferred on to the surface of solid medium and incubated in the culture room. The medium composition and culture conditions are usually the same as used for the material under investigation. Usually low salt and high vitamins are considered favorable for the growth of isolated meristem. There is no simple rule for the success of meristem tip culture. The in vitro culture of meristem and shoot tip involves several phases such as initiation of culture and establishment of explant, growth and differentiation, proliferation of shoots and finally plantlet formation by rooting of shoots. Therefore, any technique involving micropropagation through explant culture has all the factors of micropropagation influencing the growth and multiplication. All the conditions of nutrients, plant growth regulators and meristem have to be decided empirically to obtain success. Virus-free plants obtained in different species through this technique are presented in the Table below. It may be concluded from the results obtained with these species that all the factors which influence the shoot growth and proliferation, also influence the meristem culture, apical or lateral position of the meristem as in *Chrysanthemum cinerariaefolium*, concentrations and composition of plant growth regulators, pre-condition of the plants from which meristems are obtained,

pH of the medium, and addition of gibberellic acid. In some cases, use of filter paper with liquid medium was found better for growing meristem as compared to static medium.

Table: Effect of use of meristem culture for virus elimination.

Plant species	virus eliminated
Allium sativum (Garlic)	Mosaic virus
Ananas sativus (Pineapple)	Mosaic virus
Brassica oleracea (Cauliflower)	Tumip mosaic virus, cauliflower mosaic virus
B. oleracea var. gemmifera	Tumip mosaic virus, Cauliflower mosaic virus
Caladium hortulanum	Dasheen mosaic virus
Colocasia esculenta (Taro)	Dasheen mosaic virus
Chrysanthemum spp.	Virus B.

MERISTEM CULTURE ANI) HEATTREATMENT

It has been observed that without heat therapy, percentage of virus-free plants produced through meristem culture is low. Therefore, Pre-treatment of plantlets with heat therapy, increases the chance of obtaining more virus-free plants. In this technique, the temperature and its duration for the treatment of apical buds has to be determined experimentally. Neither the treatment should kill the plant material (excessive treatment) nor it should be ineffective (less treatment). This will depend upon the precondition of plant, type of virus infection and its population, and treatment itself. Heat treatment is also helpful in eliminating certain viruses which are not eliminated by normal meristem culture, e.g., in potato infected with PVX, PVS. In such crops, heat treatment improves the efficiency of the technique. Usually heat treatment is given to the plant before excision of meristem. However, in case of Nicotiana rustica, excised meristems grown in static or liquid medium were heated at 32°C and elimination of cucumber mosaic virus and alfalfa mosaic virus was achieved as compared to cultures grown at 22° C, which remained infected. TMV was not eradicated by this method. Thus, heat treatment to the mother plant or excised meristem is beneficial in improving the efficiency of the method to obtain virus-free plants.

MECHANISM OF VIRUS ELIMINATION IN MERISTEMS

Several reasons have been provided or the absence of virus particles in the meristems by different workers based on experimental evidences obtained by them. No reason is conclusive and applicable to all the plant materials or tested for different crop plants. Therefore, none of these is considered as general rule or the exact mechanism operative by which virus particles are eliminated from the growing meristem. The various mechanism proposed for virus elimination from the apical meristems are as follows.

a. During 1950s, it was presumed that virus inhibiting effect of plant growth regulators might be responsible for virus-free apical meristem as apical meristem has always high concentration of plant growth regulators. this hypothesis has never been proved.

b. Wu and Co-workers (1960) suggested that rapid cell division is the cause of vims free cells. Norma) nucleo-proteins are synthesized first and viral nucleoprotein is synthesized during cell elongation. Therefore, there is a competition between cell division and vital multiplication as evidenced by experiments with tobacco callus

c. (t has been demonstrated on the basis of experiments with carnation that carnation mottle virus containing meristems on transfer to nutrient medium become free of virus after 30-40hrs. It was concluded that contact with nutrient was responsible for virus elimination from the meristems grown in vitro.

EFFECT OF VIRUS ELIMINATION

Several workers have used meristem culture for the production of virus-free plants in many plant species. However, this process does not impart resistance in the species. Therefore, virus-free plants will become diseased when exposed to the virus. The duration in which virus reinfect the plant will depend upon the agricultural practices and epidemiology of virus. The following effects can be noticed in virus-free plants raised through meristem cultures:

a. It will be not surprising that new pathological (virus) symptoms are produced by virus free plants. This may be because the removal of major virus provides the host free for latent or less infectious virus to produce symptoms.
b. Increased growth of plants, e.g., as observed in *Narcissus tazetta*, rhubarb, cauliflower. *Pelargonium*, *Chrysanthemum*.

c. Improvement in rooting of stem cutting, e.g., in Pelargonium.

d. Increased flower size, e.g., *Chrysanthemum*, *Narcissus*.

e. In potato, healthy tubers produce higher yield of potato tuber than infected tubers.

VIRUS INDEXING

The availability of a rapid, effective and sensitive procedure for virus indexing is of significant importance for success of a program designed at production of virus-free plants. All the plant materials should be certified for virus free status for international exchange. Usually biological assays were used for virus indexing. These methods were slow and not useful for large number of plants
produced through biotechnological methods. Later on, transmission electron microscopy, serology or a combination of both were developed for virus detection in plant tissues. Among various serological techniques, the enzyme linked immunosorbent assay (ELISA) has been extensively used for virus indexing. With the availability of recombinant DNA technology, nueleic acid hybridization techniques were applied for detection of viruses. Meristem culture is also used extensively in germplasm preservation of endangered and crop species.

Lec.8
Transgenic plants

Genetic engineering of crop plants represents a major milestone in modern agricultural science. The advent of recombinant DNA technology in the early 1970 and the subsequent development of DNA transfer techniques provided exciting opportunities for plant scientists to insert foreign genes from both prokaryotic and eukaryotic organisms into the genome of crop plants and achieve transgene expression. Technological advancements in plant tissue culture techniques facilitated introduction of foreign genes into the plant genome to produce transgenic plants. Transgenic plants expressing novel traits now are being widely cultivated for their improved yield, quality, and other value-added characteristics. It should be noted, however, that in most instances genetic engineering techniques provide only an alternative approach to conventional breeding programs. In crop improvement, conventional breeding and hybrid seed production are the mainstay in ongoing efforts

directed toward varietal development. Nonetheless, modern genetic engineering technologies offer several unique advantages over conventional hybridization approaches.

DNA transfer techniques permit introduction of genes and other genetic elements among sexually unrelated organisms, thereby bypassing biological barriers. Such genetic manipulation can be accomplished using a large quantity of plant materials in a relatively small space with a year-round artificially controlled growth environment. Hence, use of genetic engineering techniques complements and expedites conventional breeding programs by increasing diversity of genetic resources, enhancing efficiency and reducing length of time needed to introgress desirable traits into existing elite crop varieties. Genetic engineering also allows utilization of exotic genes for development of transgenic plants to produce proteins with novel nutritive, pharmaceutical, agrichemical, and industrial characteristics.

GENETIC TRANSFORMATION

Is the acquisition of good genes by plants using genetic engineering technology. The transformation processes in the plant include the coloration of the desired genes, transferring them in different methods and introducing them to the genome to obtain transgenic plants (Genetic Modified Plants GMPs) and the production of plants with high productivity.

The process of transferring a gene to plant has many advantages:

1- transfer of genes to produce new plants that can't be achievedn with fertilization and natural cross breeding (hybridization).

2- Its plant gene can be introduced into plant.

3- the addition of good trait to the plant , or the expression of genes present in the plant can be changed.

4- the addition of certain genes without affecting other traits.

the main steps for genetic transformation in plant:

1- Transfer of desired DNA to a single plant cell.

2- Embedding desired DNA into the plant cell genome.

3- Converting transgenic cell into complete plant.

Methods of genetic transfer:

1- Direct transformation.

2- Indirect transformation.

Indirect transformation:

The genes are transferred to plant using vectors as a suitable colon and in corporate it the plant change its qualities.

There are two types of vectors:

1- Transformation ***Agrobacterium tumfaciens***.

2- Transformation by plant virus.

AGROBACTERIUM -MEDIATED TRANSFORMATION

A. tumefaciens is a Gram-negative, soil-borne, phytopathogenic bacterium responsible for inciting crown gall disease in a large number of gymnosperms and angiosperms. The development of crown gall disease occurs through an intricate interplay between bacterial genetic elements and plant host responses. Early molecular studies revealed that this bacterium was capable of transferring a short piece of DNA (T-DNA) from its tumor-inducing (Ti) plasmid into the genome of susceptible host plant cells. The T-DNA contains genes encoding proteins that are involved in the biosynthesis of phytohormones (oncogenes) and novel conjugates of organic acids and amino acids or sugars called opines (the opine synthesis genes). These phytohormones and bacterial metabolites are necessary for survival and proliferation of bacterial cells in

the modified host cell environment. They also stimulate tumor formation (i.e., the "crown gall"). In addition, genes located in the virulence (vir) region of the Ti plasmid encode a number of virulence proteins (Vir proteins) that mediate the TDNA transfer process. Activation of these genes is controlled by plant regulatory factors that are produced by infected host plant cells. For producing transgenic crop plants, there are several advantages associated with Agrobacterium -mediated transformation. Agrobacterium -mediated transformation results in transfer of DNA with defined ends and with minimal rearrangement. Relatively large segments of DNA also can be accommodated within the T-DNA region and subsequently transferred.

In addition, compared to other gene transfer techniques, only one or a few copies of the transferred genes are generally integrated into plant chromosomes with Agrobacterium -mediated transformation. With a minimum degree of gene disruption resulting from genomic integration of transgenes, transgenic plants with normal agronomic performance and fecundity can be obtained.

TRANS FORMATION BY USING BACTERIA (***Agrobacterium tumfaciens***)

Transformation is done with the following steps:

1- Design the appropriate cloning vector of the plasmid Ti

2- Transfer of the desired gene to the bacteria cells.

3- Joint cultivation with plant parts.

4- Screening of plants generated depending on the expression of a gene called a registered gene and transmitted in piece T-DNA.

5- After the metamorphic plants are detected the branches are rooted to form complete plants.

6- A plant tested to detect the ability of a transferred gene to be expressed in mutant plants.

7- Production of the progeny transformed plants and the study of the inheritance stability of the transferred trait.

THE T-DNA TRANSFER PROCESS

The T-DNA transfer process can be separated into the following major steps:

1. Activation of virulence genes.

2. T-strand processing and transfer.

3. T-DNA integration into the plant cell genome.

For successful DNA transfer, Agrobacterium detects the presence of low molecular mass phenolic and sugar compounds (e.g., acetosyringone) produced by wounded plant cells. This molecular sensing process is mediated by an inner membrane protein, VirA, which subsequently transduces information to a transcriptional activator, VirG, by a mechanism involving protein phosphorylation.

Activated VirG then triggers transcription and expression of the virregion contained in the Ti plasmid, leading to production of up to 20 Vir proteins from six operons: virA ,B - , - C, - D , - E , and –G.

These proteins perform a variety of functions to ensure the success of TDNA transfer. Of particular interest is the site-specific endonuclease VirD2. This protein, assisted by VirD1 protein, recognizes the T-DNA border sequences (25 bp direct repeats at its ends), creates a nick site and initiates the formation of a single strand (ss) copy of the T-DNA (T-strand). VirE2 is a sequence-nonspecific ssDNA binding protein. It functions to bind ssDNA regardless of sequence, forming a fully coated VirE2:T- strand complex (T-complex) that effectively protects the T-DNA from nucleolytic degradation during the transfer process. In addition, nuclear localization signals (NLSs) present in VirE2 and VirD2 facilitate the import of T-complex into the plant cell nucleus.

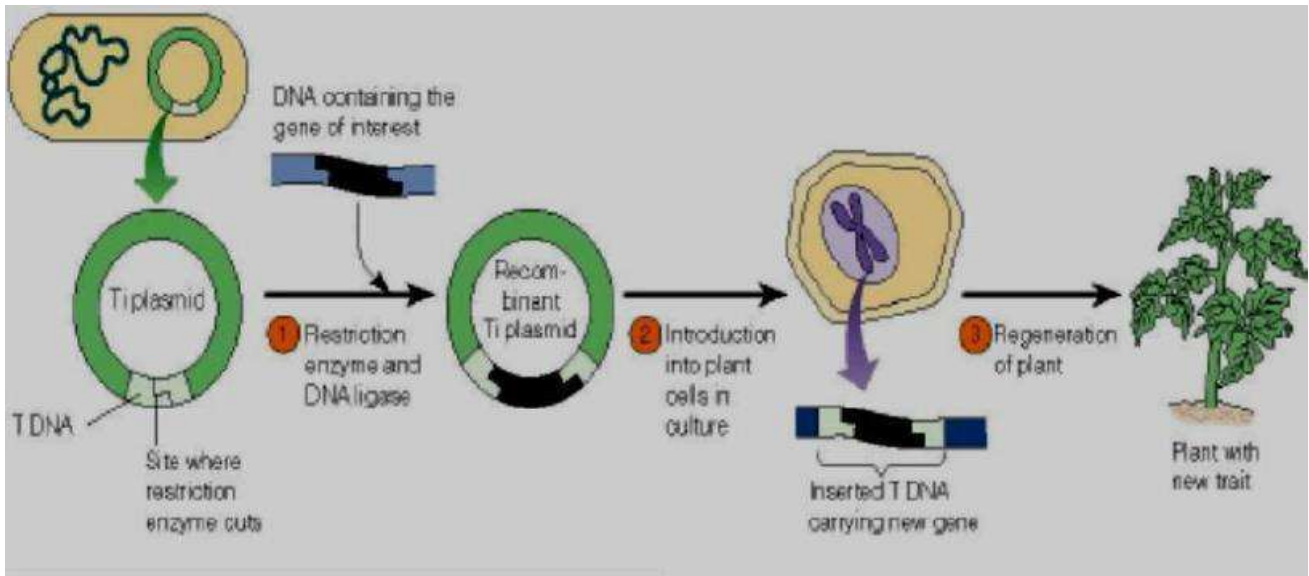

Fig. the stages of transformation using by *Agrobacterium tumfaciens* and the production of genetically modified plants .

FACTORS AFFECTING AGROBACTERIUM –MEDIATED TRANSFORMATION

T-DNA transfer is a complex biological process necessitating activation of a large number of Ti plasmid and related bacterial-encoded genes that involve active molecular interactions between Agrobacterium and host plant cells. Accordingly, development of efficient Agrobacterium –mediated transformation systems for different crop species can only be accomplished if the essential requirements for improving vir gene expression and plant cell responses to Agrobacterium

infection are met. Among factors that affect the efficacy of DNA transfer into plan cells and the obtainment of transgenic plants are virulence of the Agrobacterium **Methods of direct gene transfer in plant.**

A- Gene transfer to protoplast.

The methods used to transfer a gene directly to protoplasts are:

1- Electroporation mediated gene transfer.

2- Chemically stimulated DNA transfer.

3- Liposomes mediated gene transfer.

4- Microinjection mediated.

5- Sonication mediated gene transfer.

PROTOPLAST-MEDIATED TRANSFORMATION

Protoplasts are cells without cell walls . In a protoplast system, the only barrier between living protoplasm and the external environment is the plasma membrane. Thus, the semi-fluid nature of the plasma membrane allows direct movement of macromolecules, such as DNA, into protoplasts by using relatively simple physical or chemical treatments. When cultured under suitable conditions, protoplasts are capable of cell wall regeneration and subsequent growth into whole plants.

These unique characteristics of protoplasts permit development of efficient protoplast-mediated DNA transfer and transformation systems.

PROTOPLAST –MEDIATED DNA T RANSFER

Electroporation and polyethylene glycol (PEG) treatments are the two most widely used methods for delivering DNA into protoplasts. Cationic liposome- mediated transformation of protoplasts is less frequently used. Electroporation treatment provides a technically simple way to introduce DNA into protoplasts through electrically induced membrane pores. Electroporated protoplasts can be immediately subjected to culture treatments facilitating the recovery of transformed cells.

B- Transfer of the gene to plant tissue

1- Gun acceleration of DNA loaded micro particles.

2- Laser micro beam mediated gene transfer.

3- Silicon carbide mediated gene transfer.

Applications of genetic engineering in plant:

1- Applications of genetic engineering in insect resistance.

2- Applications of genetic engineering in virus resistance.

3- Applications of genetic engineering in bacterial and fungal resistance diseases.

4- Plant genetic engineering for salinity and drought tolerance.

5- Plant genetic engineering for herbicide resistance.

6- Engineering the plant to genetically improvement is nutritional value.

Secondary Products from *In Vitro* Culture

INTRODUCTION

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same Valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's market place for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, commercial significance alone does not drive the research Initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes.

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, biobased fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds. A series of distinct advantages exist to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include the following:

- Production can be more reliable, simpler, and more predictable
- Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants
- Compounds produced *in vitro* can directly parallel compounds in the whole plant
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures
- Cell cultures can yield a source of defined standard phytochemicals in large volumes
- Cell cultures are a superb model to test elicitation
- Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically

Plant cell cultures have great potential for the production of secondary metabolites. In recent years, considerable success has been achieved in increasing the secondary metabolites using cell suspension cultures in several plant species. Plant cells grown in culture have potential to produce and accumulate chemicals similar to the parent plant from which they were derived. There are nu- merous reports describing the production of diverse secondary metabolites, viz., anthocyanins, al- kaloids, carotenoids, flavones, coumarins, naphthaquinones, saponins, sesquiterpenes, steroidal al- kaloids, sterols, tannins, terpenoids and several others.

ALKALOIDS

Alkaloids have been known to man for several centuries and have been arc used for human welfare since long. Among various groups of secondary metabolites, alkaloids are the most exten- sively investigated compounds. Of all known natural products, about 20% (i.e., about 16,000) arc classified as alkaloids. The biological activity of several of these has been investigated but only 30 alkaloids are produced at the commercial level. Most are medicines but some arc used as flavouring, poison, and model compounds for pharmacological activity. These alkaloids can be classified as novel chemicals, as their world production is limited, e.g., quinine and quinidine have a yearly production of 300-500 kg, while compounds such as vincristine and vinblastine are produced in a **few** kilogram range. -If we compare this system to cane sugar production from sugar cane (plant based primary product), biomass utilization for extraction of alkaloids is very small. For previously mentioned quantities of alkaloids, 5,000-10,000 tons *of Cinchona* bark, 200-300 tons of *Catharanthus roseus* roots arc required for quinine and vincristine, respectively. The price of these compounds is exorbitant, which is why secondary metabolites arc termed low-volume- high-value products.

Why Tissue Culture ?

The pharmacologically active novel compounds are extracted from plants. In plant systems, they accumulate iif leaves (nicotine in *Nicotiana),* roots (ajmalicine in *Catharanthus roseus),* bark (quinine in *Cinchona)* or in the whole plant (ephedrine in *Ephedra).* Sometimes these products are produced in specialized differentiated tissues such as resin in resin ducts aryd-4atex in laticifers. " Except the herbaceous cultivated plants (e.g., *Papaver somniferum),* most of the secondary metabolites are accumulated after a certain age or maturity of the plant. In the case of tree or shrub species, e.g., *Cinchona, Rauwolfia, Camptotheca, Ochrosia,* etc., plants attain maturity in a few years be- fore they accumulate the

active principle in high amounts. It is difficult to increase the area under plantation for a particular species and growth of plants takes it own time. To meet the ever-increasing demand (e.g., vincristine) the natural resources are not sufficient. The world political scenario may also affect the supply of a particular raw material. To overcome all these hurdles, the industry requires alternative methods of assured supply of uniform material throughout the year.

Harvesting of plants (except cultivated species) from natural forest resources is not only difficult, but also makes them endangered species; e.g.. *Ephedra gerardiana* and several other Himalayan plants. When plant material is not available throughout the year in a quantity sufficient for industrial production and chemical synthesis is not possible, particularly for large complex molecules, biotechnological methods offer an excellent alternative. But before implementing this approach, cost of the product and its demand should justify production by biotechnological means.

Approaches

Production of alkaloid from *in vitro* culture requires basic information about botany, phytochemistry and importance of alkaloids (in pharmaceutics or otherwise) before commencing work. After collection of proper information about the material, cultures are raised as static or cell suspension cultures. Callus cultures are slow growing systems and accumulate more as compared to fast- growing cell suspension cultures. But cell suspension cultures have obvious advantages of growing at a large-scale in a bioreactor essentially required for ultimate industrial production of the compound. By manipulation of medium components, growth is optimized. It is well established that cultures produce very low amounts of secondary metabolites (alkaloids) as compared to the intact plant. Therefore, production has to be increased to make the system productive and ultimately commercially viable.

Optimization of alkaloid production is done using physical factors (light, temperature, vessel type), nutrients [carbon source, nitrogen source - nitrate versus ammonium nitrogen, organic (reduced) nitrogen, precursor molecules, phosphate, etc.], plant growth regulators (auxin type and concentration, cytokinin) and perhaps complex organic supplements (casein hydrolysate; coconut milk, yeast extract, etc.). IT has been established from a large number of reports that fast-growing cultures accumulate alkaloids in low amounts during exponential phase of growth and in high amounts during the stationary phase. During this phase nutrients are exhausted and primary metabolism comes, to a halt and the stored pool of primary metabolites is diverted to the synthesis of secondary products.

Zenk *et al.* (1977) used a two step culture system: medium for optimal growth (growth or maintenance medium) and medium for production of alkaloids (production medium). In the latter medium, growth is practically arrested by manipulating the nutrients, viz., high sucrose (4-10%) and low phosphate, 2,4-D and nitrogen. On transfer of growing cells to the production medium, product yield is increased several-fold.

The other strategy is to manipulate the plant component. Cultures are mixture of producer and non-producer cells and selection of high alkaloid producing cells/aggregates enhance the product yield. But these high-yielding cultures are not stable and repetitive clonal selection is required to maintain high yields of alkaloids. Other strategies include shoot culture, root culture, transformed root culture and single cell (protoplasts) selection on the basis of fluorescence of the alkaloid. When all the conditions of product yield are standardized in batch cultures, large-scale cultivation and product yield can be attempted using bioreactor. More recent approaches seek to know the mechanism of transport and accumulation, enzymes involved in the synthesis of alkaloids, identification of gene/s and transfer of such genes (recombinant technology) for expression in other eukaryotes or prokaryotes. These are strategies to improve the production of alkaloids in particular and secondary metabolites in general.

a. Catharanthus alkaloid

Catharanthus roseus, *Ochrosia elliptica* and *Rauwolfia serpentina* are the most extensively investigated phytochemically and biotechnologically, plants among the large number of alkaloid-containing taxa of Apocynaceae. Several plants of this family contain large number of indole alkaloids and other classes of alkaloids. *C. roseus* can be considered as one of the most extensively investigated plants as far as the production of secondary metabolites (alkaloids) is concerned.The herb is native to Madagascar, and cultivated in tropical regions. *C. roseus* has attained importance on account of the presence of anti-cancerous drugs, vicristine and vinblastine (0.0005% dry weight basis obtained from the roots). Medicinal properties of *Catharanthus roseus* have been described in traditional and folk medicine of several countries. The antineoplastic activity of the alkaloidal constituents of the plant was independently discovered by Canadian and American scientists. Beneficial effect of its extract in diabetes mellitus was known but later on active principles suppressing neoplasm were isolated. The extracts yielded four active dimeric .alkaloids- vinblastine, vincristine, vinleurorine and vinrosidine. Vinblastine and vincristine arc used climically. Vinblastine and vincristine, two indole dihydroindole alkaloids, are typical representatives of *Catharanthus oncolytic* alkaloids, are typical representatives of Catharanthus oncolytic alkaloids and have been developed alkodeial drugs. Subsequent results have shown that C. roreca is a store house of more than 75 alkaloids vizajmalcine, serpenane, tabersonine, catharanthinèe, etc) and several of them possess antineoplastic activity but none of the other compounds are as active as vinblastine or vincristine.

Vincristine and vinblastine are cell-cycle specific agents, and similar to colchicine and podophyllotoxin, block mitosis by dissolution of cell mitotic spindles and causes metaphase arrest. These alkaloids bind with tubulin protein and prevent tubulin formation from the protein. Though vincristine and vinblastine have anti-proliferative properties, both have different patterns of cytotoxic effect and are used in combination.

Exploration of the effects of structural modifications on the anticancer activity and toxicity of dimeric Catharanthus alkaloids resulted in the synthesis of several analogues. One of the examples is 5"-norunhydrovinblastine, which possesses better activity and lower toxicity. The compound has recently been introduced in France as an anticancer drug. The second important drug is vindesine, which resembles vincristine in its spectnum of activity but its neurotoxic potential appears to be less than that of vincristine. The most remarkable characteristic of vindensine is related to the absence of cross resistance with vincristine as demonstrated in the treatment of acute lymphoid leukaemia. Catharanthus roseus is cultivated in the tarai region (plains of Ganges) of India and roots are exported to USA, where the antitumour alkaloids are isolated. More than 2 dozen laboratories are working all over the world to produce catharanthus alkaolids by altenantive biotechnological methods, including vincristine and vinblastine. Diverse technological methods like repeated cloning for high yield, cloning for a particular alkaloid, novel compound production, short culture for differentiation related alkaloids, and cultures of cells and organs in bioreactor have been attempted to obtain the high yields of the alkaloids and to understand the mechanism. To the dismal of most of the scientists working on C. roseus, unorganised cultures did not produce antitumour alkaloids. Recently, Dutta and Srivastava (1997) recorded presence of these alkaloids using HPLC in callus cultures. They reported that vinblastine content increased as the seedlings matured and the content becomes table when the plants become 3-month old. As the callus differentiated into multiple shoots, the vinblastine production increased rapidly, comparable to that of in vivo seedlings of similar age. They concluded that increased vinblastine content was correlative to the increased cellular differentiation and maturity both in vivo and in vitro.

Earlier Miura et al. (1988) reported vinblatine production in multiple shoot culture obtained from seedling callus. They obtained 15 ug/g dry weight alkaloid level which was higher than that recorded in unorganized cultures. They also concluded that the production of vinblastine is closely associated with morphological differentiation. In a slightly different approach, a cell tree extract of

C. roseus was used to obtain bioconversion and formation of dimeric alkalod. **b. Tobacco alkaloids**

In the class of simple alkaloids (Pyrrolidine, piperdine and pyridine), nicotine and nicotinic acid derivatives are one of the most extensively studied secondary metabolites in plant tissue culture.(Though commercial production of nicotine through plant tissue culture is not a viable project, tobacco cultures are used as model system to develop understanding and technology for the production of secondary metabolites. Nicotine is synthesized in roots and accumulated that nicotine synthesis is an energy expensive process and about 1/3 of CO_2 fixed in photosynthesis is used for the nicotine synthesis (Robinson, 1974). The practice of tobacco smoking was made known to Europeans about the year 1492 when they visited West Indies, after the discovery of New World. Since then tobacco was introduced in several countries and extensively cultivated. Tobacco is mainly used for smoking and plant tissue culture is used to create variation and to develop varieties with low nicotine but containing aroma which were less harmful to man.

Nicotine was first isolated from cell cultures of *Nicotiana tabacum* by Speake *et al.* in 1964 but the most of the cell lines were not stable. As mentioned above, tobacco cultures have been used k J as a model system to understand secondary metabolism, which involves manipulation of tissue and medium components. In cultures raised from isogenic lines differing only in two loci for alkaloid production, Kinnersley and Dougall (1980) showed variation in alkaloid production as influenced j by genotype. Similarly, variation in growth and alkaloid level was evident in cell lines raised from parental and hybrid (gametic and somatic) plants.

Variability : Variation in alkaloid level was also observed when cultures were raised from different explants, viz., embryo, stem pith, and leaf explant. High nicotine content was recorded in cultures raised from embryo followed by stem pith and leaf callus. In contrary to this, other workers observed no change in nicotine level when cultures were raised from different organs.

Optimization and metabolism : Tobacco cultures have been extensively used for the investigations related to effect of nutritional factors, plant growth regulators, precursors, and physical factors on alkaloid synthesis in the cultures, including biosynthesis of nicotine. Ravishankar and Mehta (1982) observed increased ornithine decarboxylase activity and decreased activity of ornithine carbomoyltransferase in floral bud callus of N. *tabacum* cv Anand-2 grown on MS medium with lO mM urea as sole source of nitrogen. Different auxins had different effects on nicotine accumulation and higher-concentrations were inhibitory, particularly synthetic auxin 2, 4-D. **c. Cinchona alkaloids**

Cinchona alkaloids (quinine, quinidine) arc still obtained from bark of the *Cinchona* (C. *ledgeriana, C. succirubra, C. officinalis)* tree. The plant is native to Andes of South America but planted in other countries including India. The first report on tissue culture and micropropagation of high-yielding clones of *Cinchona* tree was published in 1974 by Chatterjee, while Staba and Chung were the first to report callus and cell suspension cultures of *Cinchona.* They obtained 4mg alkaloid per g dry weight of the cultures, which remained the highest production, is culture for this plant. Accumulation of alkaloids takes place in the bark of mature tree (7-12 years old). Fast growing cultures produce very low amount of alkaloids and production of alkaloids by biotechnological methods using cell cultures is not a viable project. However, levels of alkaloid produced in shoot cultures of C. ledgeriana is an example where the accumulation of secondary product is greater than in intact plant of similar age and plant tissue culture can be used for the propagation of high-yielding clones.

Applications of secondary plant products

1. Antitumor agents

   For example taxol from *taxus baccata* plant and vinblastine and vincristine from *Catharanthus roseus* plant.

2. Production of steroids and saponins

   An example of steroids is solasodine from *Solanum xanthocarpum* while glycyrrhizin from *Glycyrrhiza glbara* is an example of saponins.

3. Food additives

   Food additives produced by plant tissue culture were classified to: a.

   Colours: like anthocyanine from *Daucus carota*

   b. Flavours like onion flavor from *Allium cepa* and vanilla from *Vanilla planifolia*

   c. Sweetner like stevioside from *Stevia rebaudiana*

4. Insecticides

   Like nicotine from *Nicotiana tabacum* and pyrethrins from *Tagetes erecta*

Lec. 10

Nanoparticles and plant tissue cultures

Employing nanotechnology in plant tissue culture have resulted in positive outcome. NPs have been employed to boost crops production and plant growth, also improvement seeds germination, enable plant genetic modification, attain plant protection and increase the production of bioactive compounds. When tomato seeds were treated with silicon dioxide (SiO_2), the SiO_2NPs boosted seed germination and seedling growth. The usage of iron (Fe) and magnesium (Mg) Nano-fertilizers significantly increased the seed protein content as well as the number of seeds per pod in black-eyed peas. The content of flavonoids, glycyrrhizins, anthocyanins, tannins and phenolic compounds was increased after treating licorice seedlings with zinc oxide (ZnO) and copper oxide (CuO) NPs. Silica– silver NPs were reported to have an antimicrobial effect against number of plant pathogens. Treating infected green squash plants with silica–silver NPs has been found to be helpful in fighting powdery mildew disease. Studies have demonstrated that surface disinfection of explants with NPs noticeably decreases microbial contamination in different plants. Adding NPs to the tissue culture media to remove bacterial contamination and improve explants' morphogenetic potential. The inclusion of nanoparticles in the medium also induced soma-clonal variation. bioactive compounds content in plant cell, tissue or organ cultures has been increased after treatment with nanoparticles. Engineered nanoparticles have been utilized to carry proteins or DNA into plants.

Nano-technological achievements in plant tissue culture

1. Input of Nanomaterials towards Rooting, Shoot Growth, Organogenesis and Callus Induction

Multiple researches have demonstrated the positive effects of nanoparticles on callus induction and shoot growth and regeneration. when stem explants of *Tecomella undulata* were cultured on MS medium with Ag NPs, shoots and callus formation and shoot induction percentage were elevated. The impact of silver nanoparticles (Ag NPs) on organogenesis could be attributed to inhibition of ethylene production. Percentage of produced shoots, Shoot number and length of induced shoots were considerably enhanced upon culturing the nodal explants of *T. undulata* on MS medium with Ag NPs. In addition, the application of Ag NPs increased the explant survival and slowed there senescence via the down regulation of the TuACS gene. Nevertheless, a detrimental effect on shoot regeneration has started to appear at higher concentrations. Sharma *et al.*, 2012 reported that growth characteristics of *Brassica juncea* seedlings were improved after incorporating Ag NPs into MS basal medium, due to the decrease of proline and hydrogen peroxide content which was caused by the activation of antioxidant enzymes. still, seedling growth was deteriorated at higher concentrations of Ag NPs. In *Brassica nigra*, seed germination was noticeably inhibited after the addition ZnO NPs to the MS medium. The shoot and root lengths were significantly affected after the treatment with ZnO NPs .

Kumar and his team's in 2013 reported that in *Arabidopsis thaliana,* the percentage of seedling growth and seed germination was enhanced after incorporation of Au NPs into MS basal medium. Plants treated with gold nanoparticles (Au NPs) had a longer pod and a higher number of seeds. Treating *A. thaliana* with Au NPs enhanced the antioxidant enzyme activity and caused a decrease in the expression of microRNAs in the plant. The molecular and

physiological variation might be accountable for the advantageous impact of Au NPs. The rooting, number of shoots and shoot length in *Mentha longifolia* were increased after the addition of Cu NPs to modified MS medium. The highest frequency of shoot formation was acquired when nodal explants of *Stevia rebaudiana* were cultured on MS medium incorporated with ZnO NPs. MS medium supplemented with Ag NPs had given The highest number of shoots for vanilla plants. The incorporation of CuO NPs increased organogenesis in rice cultivars. from all the previous informations it can be deduced that adding NPs to a plant tissue culture medium impacts shoot multiplication, callus proliferation, rooting and somatic embryogenesis through changing antioxidant enzymes activity, gene expression, inhibiting production of ethylene.

2. Nanomaterial enhancement of secondary metabolites

Bioactive secondary metabolites can be found in abundance in Plants, these compounds are essential in the survival of plants in their environments. The production of secondary metabolites is highly dependent on the *in vitro* plant cell and organ cultures. providing suitable culture conditions, optimizing the composition of the culture medium and incorporating precursors and elicitors have significantly enriched the content of secondary compounds in cell and organ cultures. The Nanoparticles supplemented to the plant *in vitro* culture medium could serve as an elicitor and a nutrient source.

Poborilova *et al.* 2013 reported that the phenolic content in tobacco cell suspension cultures was significantly raised after adding of Al_2O_3 NPs. dose and exposure time determine quantity of phenols accumulating in the cells. Al-Oubaidi and Ameen in 2014 reported that incorporating MS medium with Ag NPs has significantly increased the content of essential oil in *Calendula officinalis* calli. TiO2
NPs have significantly enhanced chlorogenic acid, cinnamic acid Gallic acid, tannic acid and Coumaric acid content in embryonic calli of *Cicer arietinum*. In callus cultures of *Prunella*

vulgaris, the addition of Au–Ag NPs in (1:3) caused maximum accumulation of total flavonoids and phenolic compounds.

Chamani and others in 2015 indicated that the accumulation of particular bioactive compounds in *Lilium ledebourii* dependency on the concentration of ZnO NPs in the MS medium. The highest content of anthocyanins, flavonoids and phenols was acquired on MS medium supplemented with (100, 75 and 25) mg/l ZnO NPs respectively. In shoot cultures of *Stevia rebaudiana,* the fortification of MS medium with ZnO NPs has significantly boosted the accumulation of steviol glycosides.

Desai and others in 2015 reported that In shoot cultures of *S. rebaudiana,* incorporation of Zn NPs lowered the stevioside production. When *Verbena bipinnatifida* seedlings grown on MS medium containing 5 µM $CuSO_4$ NPs, The total phenolic content was doubled. The essential oil content in *Mentha longifoliaby* was increased after the Application of Co and Cu NPs respectively.

3. Nanomaterials as antimicrobial in P.T.C

Plant tissue culture is a basic and fundamental component of plant biotechnology and progress in various fields of biotechnology greatly depends on the improvement of this technique. Nowadays, nanomaterials especially Nano silver (NS) are frequently being used as an antimicrobial agent in different fields of sciences including in vitro propagation of plants. Microbial contamination is one of the most serious problems in plant tissue culture and various techniques are being employed to reduce it. Antimicrobial activity of nanoparticles is importance to control conventional antimicrobial agents. Bacterial contamination is a serious problem in plant tissue culture procedures. Nano dioxide titanium (TiO 2) is used to remove bacterial contaminants. Experiment involved NS and TiO 2 in MS medium which resulted in microbial free growth of the explant in the culture media. The addition of nonmaterial to the culture for contamination removal is achieved either by adding the NPs directly to the culture medium or by

sterilizing the explant with it. There are many NPs used as antimicrobials in plant tissue culture like iron, silica, gold, zinc and magnesium.

The toxicity concerns and safety issues surrounding exposure of plant tissue cultures to nanomaterials

Nano toxicology is a flourishing branch which has developed to document the adverse aspects of nanomaterials. Due to their large surface area to volume ratio and quantum size effects, nanomaterials possess unique characteristic in compression with their larger counterparts; one of these unique characteristics is an additional toxicological attribute that the nanomaterials may have in contrary to their equivalent bulk materials. Nanomaterials are extremely active at nanometer dimensions, even when they are produced of inert elements such as gold (Au). Nano toxicological studies decides whether and to what degree these characteristics may present a threat to living beings and environment. The toxicity evaluation of nanomaterials on plants has been mainly estimated throughout *in vitro* seed germination and the following growth of seedlings. Mishra *et al.*, 2017 suggest that the nanomaterials incorporated in the culture medium can bring about a significant and negative effects on explant survival, shoot growth, organogenesis, seed germination, seedling development and cell viability.

Nanomaterials phytotoxicity relies on their chemical composition, size, dose, type and stability, the explant type, plant species, the application method and the composition of the culture medium. *In vitro* seed germination and seedling growth of wheat, maize, barley, tomato, rice and alfalfa were described to be negatively affected by large doses of metal NPs. The addtion of NPs to the cell suspension decrease the viability of the cells by causing DNA damage, changing nucleic acid expression, disturbing chlorophyll synthesis, raising the production of ROS, causing electrolyte leakage and causing cell membrane damage. To this day, the uptake of nanoparticles into plants has not been evaluated or recorded. The intake of NPs by plant cell, tissue and organ cultures is described to be widely associated with the absorption of nutrients and moisture from the medium.