LECTURE 01

HISTORY OF PLANT TISSUE CULTURE

Plant tissue culture is the technique of growing plant cells, tissues and organs in an artificially prepared nutrient medium, static or liquid under aseptic conditions

Schwann and Schleiden (1838)	Totipotency theory-Cells are autonomic, capable of regenerating to give a complete plant.
Sachs (1982)	Plants synthesize organ forming sub stances which are polarly distributed.
Haberlandt (1902)	Father of Plant tissue culture. First attempt to culture plant cells *in vitro* on an artificial medium. He has cultured stamen hair cells on a simple medium (Knops solution containing glucose and peptone) of Tradescantia . Cells enlarged but never divided.
Hannig (1904)	First attempt at embryo culture of selected crucifers.
Kuster (1909)	Fusion of plant protoplasts although the products failed survive.
Knudson (1922)	Asymbiotic germination of Orchid seeds
Robbins (1922)	*In vitro* culture of isolated root tips.
Laibach (1925)	Embryo culture applied in interspecific crosses of Linum.
Gantheret (1940)	In vitro of cambial tissues of carrot to study adventitious shoot formation.
Van overbeck (1941)	Discovered nutritional value of liquid endosperm. Coconut milk-containing a cell division factor (Diphenyl area) for the first time for culture of Datura embryos.
Skoog (1944)	First in vitro cultures of tobacco used to study adventitious shoot formation.
Skoog and Tsui (1946)	Formation of adventitious shoots and roots of tobacco determined by ratio of auxin adenin.
Skoog and Miller (1957)	Discovery of the regulation of Organ formation (roots and Shoots) by changing ratio of cytokinin/auxin.High ratio of auxin to cytokinin in the medium favored root formation, the reverse shoot formation and that intermediate ratios promoted callus proliferation in tobacco callus.

Maheswari and Rangasamy (1958)	Regeneration of somatic embryos *in vitro* from nucellus of citrus ovules.
Gautheret (1959)	Publication of first extensive hand book in Plant tissue culture.
Kanta (1960)	First successful test tube fertilization in *Papaver rhoeas*.
Cocking (1960)	Enzymatic degradation of cell walls to obtain large numbers of protoplasts.
Morel (1960, 1964)	Vegetative propagation of orchids by meristem culture, development of shoot apex culture.
Bergmann (1960)	Filtration of cell suspensions and isolation of single cells by plating.
Murashinge and Skoog (1962)	The development of famous Murashige and Skoog medium.
Guha and Maheswari (1966)	First haploid Datura plants produced from pollen grains.
Kasha and Kao (1970)	Embryo culture utilized in the production of monoploids in Barley.
Power *et al*. (1970)	First achievement of protoplast fusion.
Gengenbach and Green (1975)	Positive selection of maize callus cultures resistant to *Helminthosporium maydis*.
Hendre and Co-workers (1975)	Established technique for obtaining virus free Sugarcane Citrus, Potato and Cassava.
Seibert (1976)	Shoot induction from Cryo preserved Shoot apices of Carnation.
Power *et al*. (1976)	Inter specific plant hybridization of protoplast fusion for *Petunia hybrida* and *Petunia parodii*.
Melchers *et al*. (1979)	Somatic hybridization of potato and tomato by protoplast fusion.
Larkin and Scowcroft (1981)	Introduction of the term "Soma clonal variation".
Zimmermann (1982)	Fusion of protoplasts by electrical stimulus.
Pelletier *et al*. (1983)	Inter generic cytoplasmic hybridization in radish and rape.
Kitto and Janick (1985)	Production of artificial seeds.
Kohn and Co-workers (1985)	Somatic hybrids in tobacco mediated by electro fusion.
E. Suderburg and K. Glemelius (1986)	Somatic hybrids in Brassicaceae.

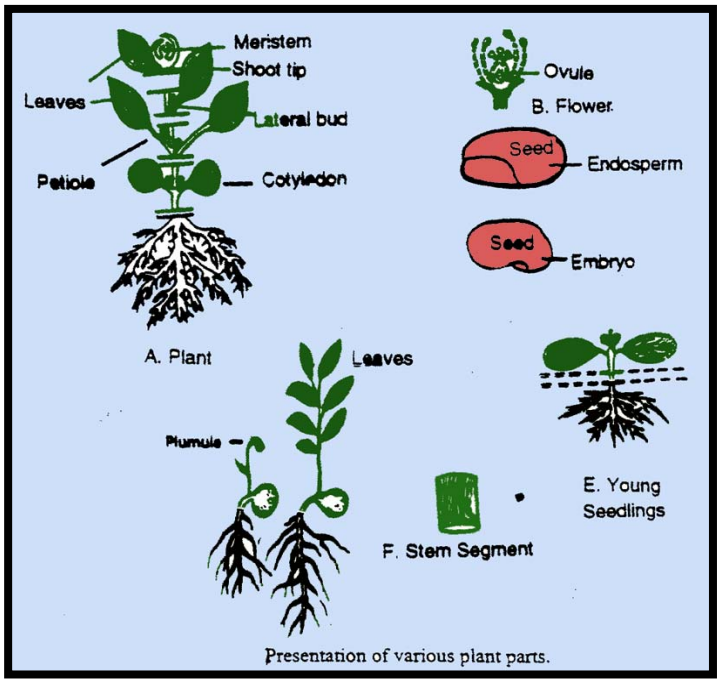
R. Nadagauda,	Isolated and planted variants of
A. Mascarenhas (1986)	sugarcane resistance to mosaic virus and turmeric plants of the variety"Tekurpeta" in the field.

Totipotency

Totipotency is the capacity and capability of a cell to grow and develop into a multi-cellular (or) multi-organ higher organism.

Explant

A piece of a plant tissue placed in an environment free from microorganisms in a balanced diet of chemicals.



Presentation of various plant parts.

Medium

Substrate for plant growth and refers to the mixture of certain chemicals to form a nutrient rich gel or liquid for growing cultures.

Sterilization methods

Dry heat sterilization	- Hot air oven (dry hot air)
Wet heat sterilization	- Autoclaves (steam under pressure)
Ultrafiltration	- Millipore filters (Using filters at room temperature)
Chemical sterilization	-LAF (ethanol, isopropanol) -Instruments(Scalpels, forceps, spatulas)-Ethanol
Surface sterilization of explants	- Surface disinfectants, antibiotics, antiseptics and wetting agents

LECTURE 02
NUTRITIONAL REQUIREMENTS

Requirements

1. Inorganic nutrients
2. Carbon / Energy source
3. Vitamins
4. Plant growth regulators (Auxins, cytokinins, gibberellins, abcicsins,ethylene, polyamines , organic acids)
5. Solidifying agent
6. Amino acids
7. Undefined supplements, and jasmonates

Physical form of medium- solid and liquid media

Solidified Medium	Liquid medium
Explants are easily seen and recovered	Recovery is difficult.
No Special aeration	Usually shaken, rotated/aerated
Shoots grow in more orderly	Growth is disoriented
Long term maintenance	
Simple containers little space	
	Advantages:
	Callus break up and shed as cells to establish a fine suspension
	Faster rate of multiplication
	A greater surface of explants is in contact with medium.
	Toxic metabolites will effectively be dispersed.
	Disadvantages:
	Seeds would not germinate; protocorms and plantlets became brown and dried. Seeds submerged will show vitrification.

Commonly used tissue culture media

MS (Murashige and Skoog, 1962) and **LS** (Linsmaier and Skoog, 1972) for plant regeneration of both monocots and dicots

B_5 (Gamborg *et al*., 1969) developed for culture of soybean cell suspensions but also has been effectively used for methods of plant regeneration. **B_5** and its various derivatives have been valuable for cell and protoplast cultures.

SH: Schenk and Hildebrandt (1972) introduced this for culture of monocots and dicots, . Widely used especially for legumes.

WPM: Lloyd and McCown (1980,1981). This is post MS media.. WPM is increasingly used for propagation of Ornamental shrubs and trees in commercial labs.

N_6 (Chu) was developed for cereal anther culture and used in the success in other cereal anther culture. In special cases , NN (Nitsch and Nitsch) was also used.

Environmental factors influencing plant tissue culture

Genotype and Explant:
Medium: (i)Nutrients, (ii)Growth regulators and (iii)Other additives
Culture Environment: (i) Temperature, (ii) Relative Humidity (RH) and (iii) Light

LECTURE 03
PLANT GROWTH REGULATORS

In plants, only five substances are classically defined as plant hormones. However, some other substances are being studied that may eventually be classified also as hormones.

The five classical hormones:

- ❒ Auxin
- ❒ Gibberellins
- ❒ Cytokinin
- ❒ Abscisic acid
- ❒ Ethylene

Additional substances gaining recognition as hormones:

- ❒ polyamines
- ❒ jasmonates
- ❒ salicylic acid
- ❒ brassinosteroid
- ❒

Auxins

Auxins stimulate new cell division, cell enlargement, the formation of shoot apices or buds, the induction of somatic embryogenesis, and may promote rooting. *e.g*. Indole-3-acetic acid (IAA), Indole-3-butyric acid, 1-Naphtalene acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichlorophenoxy acetic acid (2,4,5-T) and picloram.

Cytokinins

Cytokinins stimulate cell division, cell and/or shoot differentiation, lateral bud break, *etc*
natural : zeatin (ZEA), Isopentenyl Adenine (2iP or IPA), (adenine)
synthetic: benzylaminopurine (BAP = benzyladenine BA), kinetin (KIN)

Auxin - Cytokinin Interaction

- ❒ In case of high auxin-cytokinin ratio, root formation, embryogenesis and callus formation is initialised.
- ❒ In case of low auxin-cytokinin ratio, formation of adventitive or axillary shoots is induced.
- ❒ The auxin-cytokinin ratio is also essential for chloroplast formation (and for most of another process).

Gibberelins

Gibberelins - naturally occurring plant hormones involved in internode elongation, enhancement of flower, fruit and leaf size, germination, vernalization and others processes in plants. *e.g* gibberellic acid (GA_3)

Abscisic Acid

Abscisic acid (ABA) - A plant hormone involved in abscission, enforcing dormancy, and regulating early stages of embryo development.

Ethylene

Ethylene - A gaseous plant hormone involved in fruit maturation, abscission, and senescence. It is produced by certain tissue cultures.

Brassinosteroids

- ❒ Promote shoot elongation at low concentrations
- ❒ Strongly inhibit root growth and development
- ❒ Promote ethylene biosynthesis and epinasty

Jasmonates

- ❒ Jasmonates are represented by jasmonic acid and its methyl ester. Jasmonic acid is considered by some to be a new class of plant growth substance.
- ❒ Inhibition of many processes such as tissue culture growth, embryogenesis, seed germination, pollen germination, flower bud formation, chlorophyll formation,
- ❒ Differentiation in plant tissue culture, adventitious root formation, breaking of seed dormancy, pollen germination,

Polyamines

There is some controversy as to whether these compounds should be classified with hormones. They appear to be essential in growth and cell division.

Salicylic Acid

SA is thought by some to be a new class of plant growth substances**.** It promotes flowering, inhibits ethylene biosynthesis and reverses the effects of ABA .

LECTURE 04
PLANT REGENERATION 'DE NOVO'

The development of an adult organism from a single cell (zygote) is the result of the integration of cell division and cell differentiation. Isolated cells from differentiated tissues are generally non dividing and quiescent but to express totipotency, the differentiated cells first undergoes differentiation and then re-differentiation. The phenomenon of a mature cell reverting to a meristematic state and forming a dedifferentiated callus tissue is called dedifferentiation whereas the ability of differentiated cell to form a whole plant/plant organs is termed as re-differentiation.

Organogensis

Organogenesis = formation of individual organs, such as shoots or roots.
'*de novo'* regeneration (organogenesis) = formation of adventitious buds (roots, shoots, leaves,...) on the place (tissues) where was no meristems present before.

Organogenesis in the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often attached to the parent tissues. Organised development leading to plantlet regeneration via *de novo* organogenesis is a multistaged process consisting of atleast three distinct stages namely shoot bud formation, shoot development (and multiplication) and rooting of developed shoots. This can be controlled mostly by a balance between cytokinin and auxin. A relatively high ratio of auxin to cytokinin induces root formation in callus tissues whereas a low ratio induces shoot formation.

Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. When it is applicable for root, it is known as rhizogenis. Anomalous structures developed during organogeneiss is called organoids. The localized meristematic cells on a callus which give rise to shoots and/or roots is termed as meristemoids. Those can occur directly on an explant or indirectly via callus.

Plant tissues *in vitro* may produce many types of **primordia** (adventitious buds and organs), including those that will eventually differentiate into embryos, flowers, leaves, shoots, and roots. These primordia originate *de novo* from a cellular dedifferentiation process followed by initiation of a series of events that results in their formation.The cell or cells thought to be the direct progenitors are somehow stimulated to undergo a number of rapid cell divisions leading to the formation of **meristemoid**. Meristemoids are characterized as being an aggregation of meristem-like cells .

A developmental sequence involving an intervening callus stage is termed 'indirect' organogenesis

primary explant $\rightarrow$ *callus* $\rightarrow$ *meristemoid* $\rightarrow$ *organ primordium*

Direct organogenesis is accomplished without an intervening proliferate callus stage:

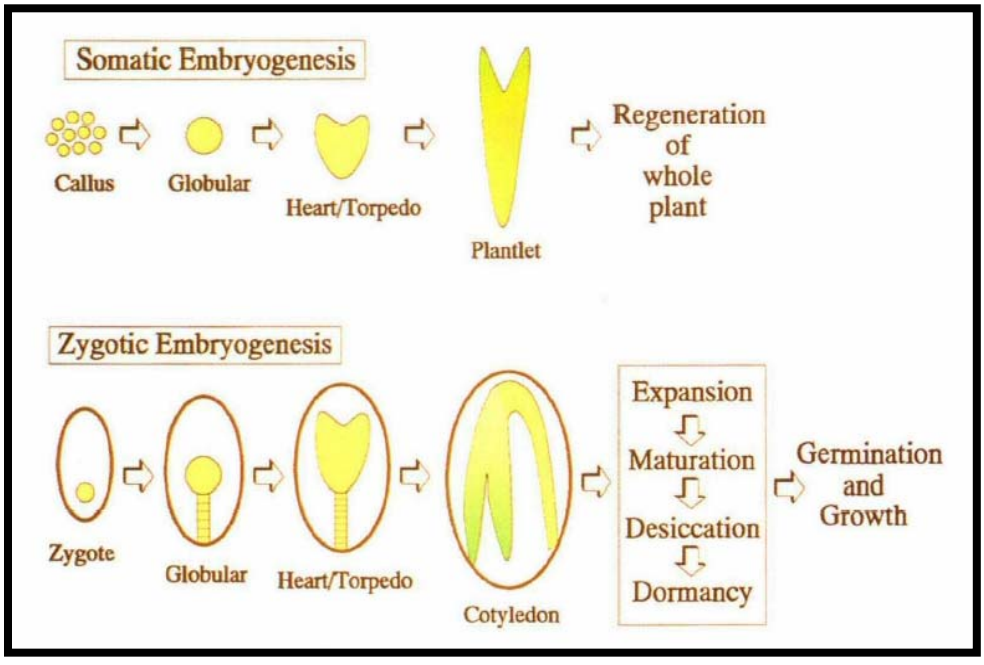
primary explant $\rightarrow$ *meristemoid* $\rightarrow$ *organ primordium*

Non-zygotic embryogenesis

Somatic embryogeneisis

Somatic embryogeneiss leads to the production of a bipolar structure containing in root/shoot axis with a closed independent vascular system. Plant regeneration via somatic embryogenesis for many species can be divided into two phases namely the selection and induction of cells with embryogenic competence and development of these cells into embryos. These embryos can again occur directly on a explant or indirectly *via* callus.

- ❒ Non-zygotic embryos have been shown to be functionally equivalent to zygotic embryos.
- ❒ The bipolar structure of the somatic embryo contains both shoot and root meristems.
- ❒ Generally iinduction of somatic embryogenesis in most species requires a high concentration of auxin, usually 2,4-D, in the culture medium (for induction of 'proembryogenically determined cells' - PEDC).
- ❒ The pro-embryogenic cell then either continues to divide irregularly to forma pro-embryonal complex, or divides in a highly organized manner to form a somatic embryo
- ❒ As the embryos (both zygotic or non-zygotic) develop, they progress through the distinct structural steps of the globular, heart, torpedo, cotyledonary, and mature stages



Induction Factors

- ❒ Effects of gene expression
- ❒ Effect on intercellular interactions
- ❒ Role of Cytokinins

- ❒ Miscellaneous factors: a) Genotype and explant characteristics, b) Medium Components, c) Culture environment

d) Bacterial compounds

Synthetic seeds:

Synthetic seeds /Artificial seeds are the living seed like structure derived from Somatic embryo *in vitro* after encapsulation by a hydro gel. The preserved embryoids are termed as synthetic seeds. Such seeds are contaminated with microbes and desiccate quickly when subjected to field conditions. So, encapsulated in Calcium alginate.

Steps

Induction of SE
↓
Maturation of SE
↓
Encapsulation of SE
↓
Evaluation of Embryoid and plant conversion
↓
Planting

Procedure

Somatic embryos are mixed with 3 % sodium alginate solution and the mixture is added drop by drop into $CaCl_2$ solution with a pipette. After 30 to 60 min, the drops are gelled completely. Those with a single embryo are collected. Nutrients and/or pesticides may be added to the alginate solution.

High costs of production and very low field germination are the main reasons why the artificial seeds are not widely used until now.

LECTURE 5
ESTABLISHMENT AND MAINTENANCE OF CALLUSES

Isolated cells from differentiated tissues are generally non dividing and quiescent but to express totipotency,the differentiated cells first undergoes differentiation and then re-differentiation.

Dedifferentiation: The phenomenon of a mature cell reverting to a meristematic state and forming a dedifferentiated callus tissue

Redifferentiation is the ability of differentiated cell to form whole plant/plant organs is termed as re-differentiation.

Callus induction, subculture and maintenance

Callus

A callus consists of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. For callus initiation, aseptically transfers explants to the required semi solid medium and gently press them into the agar so that good contact is made. Radical tips will callus well if laid horizontally on the agar whereas stem section may produce more callus if placed vertically with one cut end in the agar.

Within 2-3 weeks, explants should show new growth as pustules or protuberances or as a fine mat across the surface depending on the distribution and mitotic activity of the parenchyma residing in the excised tissues. Some callus growths are heavily lignified and hard in texture, whereas others break easily into small fragments. Assuming a suitable medium has been selected, most explants should have produced sufficient callus to allow for subculture within 3-7 weeks. When cultured for several weeks, any callus will show signs of ageing, noted as deceleration of growth, necrotic or browning and finally desiccation. The newly formed callus will be removed from the initial explant at this stage by cutting with the sterile scalpel. Once well established, most callus cultures will require regular subculture at approximately 4 weekly intervals.

Cell suspension culture

The term "suspension culture" has simply aggregates of cells suspended in liquid medium. Most suspension cultures are obtained by transfer of friable callus lumps to agitated liquid medium of the same composition as that used for callus growth. Agitation rates on orbital shakers should be in the range of 30-150 rpm. The degree of cell separation of established cultures of high friability can be modified by changing the composition of the nutrient medium particularly the concentration of growth regulators.

When the plant material is first placed in the medium, there is an initial lag period prior to any sign of cell division. (Figure). This is followed by an exponential rise in cell number and a linear increase in

cell population. There is a gradual deceleration in the division rate. Finally the cells enter a stationery or non dividing stage. In order to maintain the viability of the culture, the cells should be sub cultured early during this stationery phase.

Subculture and Measurement of Growth

Cell suspension usually requires regular subculture at more frequent intervals than callus growth from which they are derived. Subculture involves the aseptic transfer of suitable size inoculums to fresh medium using either pipettes (or) autoclavable metal syringes, and transfer by simply tipping culture into the new vessel up to a graduation mark to ensure approximately constant inoculum's size. The latter method is only one practicable with cultures containing large cell aggregates.

The growth of cell suspension cultures may be monitored by measurement of one or more of the following parameters.

Medium conductivity

A Conductivity change of the culture medium is inversely proportional to cell fresh weight.

Cell viability

n addition to microscopic examination for protoplast streaming and the presence of an intact nucleus, cell viability may also be assessed by use of vital stains may of type which are excluded by intact living cells eg. Evan's blue (0.025% w/v) or colored (Tetrazolium salts) or those which are metabolized in living cells to give either fluorescent products (Fluorescein diacetate).

Mitotic Index =

$$\frac{\text{No. of Nuclei in mitosis}}{\text{Total no. of nuclei examined in the sample}}$$

In Batch cultures, cells are nurtured in a fixed volume of medium until growth ceases whereas in Continuous cultures, cell growth is maintained by continuous replenishment of sterile nutrient medium.

Secondary metabolites production in cell lines

Higher plants are valuable sources of industrially important natural products which include flavors, fragrances, essential oils, pigments, sweeteners, feed stocks, anti microbial and pharmaceuticals which belong to metabolic groups collectively referred to as secondary metabolites. These substances do not participate in vital metabolic functions of the host plant tissues in the same manner as amino acids, nucleic acids or other primary metabolites but appear to serve as a chemical interface between the producing plant and its surrounding environment. These are used to ward off predators and to attract pollinators. They may also help in combating infectious diseases.

Extraction of certain secondary metabolites from cell lines has definite advantages.

- ❒ Plant cells are relatively easy to grow.
- ❒ The rate of cell growth and biosynthesis in cultures initiated from a very small amount of plant material is quite high and the final product may be produced in a considerably short period of time.
- ❒ Plant cell cultures are maintained under controlled environmental and nutritional conditions which ensure continuous yields of metabolites.
- ❒ Suspension cultures offer a very effective way of incorporating precursors into cells
- ❒ New routes of synthesis can be recovered from deviant and mutant cell lines which can lead to production of novel compounds not previously found in whole plants.
- ❒ Some cell cultures have the capacity for bio transformation of specific substrates to more valuable products by means of single or multiple step enzyme activity.
- ❒ Culture of cells may be more economical for those plants which take long period to achieve maturity.

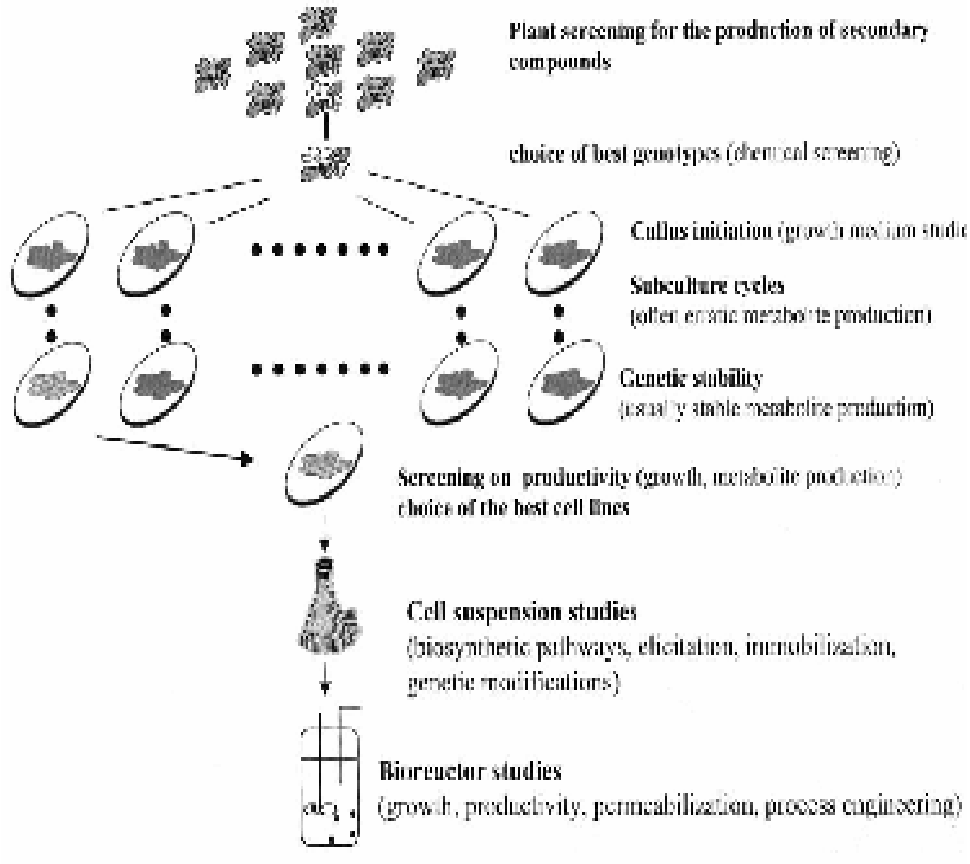


Fig. 1. Guidelines for the production of secondary metabolites from plant cell.

LECTURE 06
PROPAGATION FROM PREEXISTING MERISTEMS

Many crops are vegetatively propagated. Often the rate of multiplication under field conditions is fairly low. Many important crop and ornamental species are difficult or impossible to manipulate by conventional propagation. Therefore, it takes a lot of time **to build up a large population of disease free material or of a new desirable genotype**. Micropropagation (rapid multiplication) using plant tissue culture techniques offers an elegant solution for this problem as long as these techniques do not affect the genetic stability in the regenerated plantlets.

Micropropagation = *in vitro* propagation – is defined as true-to-type propagation of selected genotypes using *in vitro* culture techniques.

Four basic methods are used to propagate plants *in vitro*. Depending on the species and cultural conditions, *in vitro* propagation can be achieved by:

- ❒ enhanced axillary shoot proliferation (shoot culture)
- ❒ node culture
- ❒ *de novo* formation of adventitious shoots through shoot organogenesis
- ❒ non-zygotic embryogenesis.

Currently, the most frequently used micropropagation method for commercial production utilizes enhanced axillary shoot proliferation from cultured meristems. This method provides genetic stability and is easily attainable for many plant species.Besides propagation, shoot meristems are cultured *in vitro* for two other purposes:

- ❒ production of pathogen eradicated plants; and
- ❒ preservation of pathogen eradicated germplasm.

Shoot and Node Culture

Both methods rely on the stimulation of the axillary shoot growth from lateral buds following disruption of apical dominance of the shoot apex.

Shoot culture

Shoot culture refers to the in vitro propagation by repeated enhanced formation of axillary shoots from shoot tips or lateral buds cultured on medium supplemented with PGRs, usually a cytokinin. The axillary shoots produced are either subdivided into shoot tips and nodal segments that serve as a secondary explants for further proliferation or are treated as microcuttings for rooting.

Compared to other micropropagation methods, shoot cultures:

- ❒ provide reliable rates and consistency of multiplication following culture stabilization;
- ❒ are less susceptible to genetic variation;
- ❒ may provide for clonal propagation of periclinal chimeras.

Node culture, a simplified method of shoot culture, for plants that do not respond well to the cytokinin stimulation of axillary shoot proliferation. Axillary shoot growth is promoted by the culture of either intact shoots positioned horizontally on the medium (in vitro layering) or single or multiple node segments. Shoots (microcuttings) are either rooted or acclimatized to ex vitro conditions or repeatedly subdivided into nodal cuttings to initiate additional cultures. Although node culture is the simplest method , it is associated with the least genetic variation.

Summary of stages in micropropagation

A typical sequence of events in the micropropagation of a plant is:

selection and culture of the source plants
↓
establishment of aseptic cultures (culture initiation)
↓
multiplication
↓
preparation for transfer - rooting of shoots
↓
plant establishment - transfer to non-sterile conditions

0 - Selection and culture of source plants

Choosing of mother plant

- ❒ It is necessary to know origin (variety, cultivar)
- ❒ Should be healthy plant cultivated in optimal conditions (greenhouse
- ❒ Very important factor is the season when explants are taken (stage of active growth is preferable).

Explant - the tissue taken from a plant or seed and transferred to a culture medium to establish a tissue culture system or regenerate a plant.

1 - Establishment of aseptic cultures (culture initiation)

a) In semi-aseptic laboratory - initiation of aseptic culture (surface sterilisation)
washing of explant (stem, leaf, seed , etc.) with running warm water (30'-2 hours)
Sterilisation in one or combination of disinfection agents
b) Under aseptic conditions - in laminar flow
after disinfection the disinfect agent have to be fairly wash down from explant
repetitional washing in sterilised distilled water (at least 3 times)

then it is necessary cut out (with scalpel) destroyed parts of the explant and fit the explant into required form

finally the explant is placed into cultivation vessel on the media surface (or into liquid media)

2 - Shoot multiplication

- ❒ Main aim of this step is multiplication of cultured plant material
- ❒ plant material is continuously sub cultured: clusters of plants or shoots are removed out of vessels (in laminar flow), cut into small pieces and transferred to vessels with fresh media
- ❒ the process can be leaded by stimulation of **axillary branching** (high cytokinin concentration inhibit apical dominance) or by formation of **adventitious shoots** (can rise from stems, leaves, tubers, bulbs, etc.)
- ❒ this process is running until required number of shoots (explants or plantlets) is obtained

3 - Preparation for transfer - rooting of shoots

- ❒ obtained shoots can form the roots either *in vitro* (3) or *in vivo* (4)
- ❒ rooting can also be influenced by light and temperature

4 - Plant establishment - transfer to non-sterile conditions

After rooting of plantlets (either *in vitro* or *in vivo*), acclimatization to outside conditions shall start:

- ❒ much lower humidity than in the vessels
- ❒ pass onto autotrofic nutrition (photosynthesis in *in vitro* is very low or no one)
- ❒ eaves formed *in vitro* serve mostly as 'storage organ' after transfer *in vivo* and new leaves must be formed quickly

Plants in *in vitro* culture sometimes have an abnormal, water soaked, or glassy appearance - this phenomenon is called **'hyperhydricity'** (previously **'**vitrification**'**). It is caused by high humidity in the vessels and insufficient light intensity in cultivation rooms.

<u>Advantages of micropropagation:</u>

Clonal mass propagation

The important point here is that extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting, one can obtain upwards of 1,000,000 plants per year from one initial explant.

- culture is initialised from small parts of plants and small plants are propagated (no need of much space: $1m^2$ in culture room = 20000 - 100000 plants per year)

Pathology -Eliminate viruses,bacteria,fungi etc .

Use heat treatment and meristem culture.Used routinely for potatoes,carnation,mum,geranium, garlic,gypsophila

- it is propagation in aseptic conditions, so once culture is established, there is no loss of plants because of diseases and pests
- virus free plant production = simplification of international exchange of plants

Difficult or slow to propagate plants

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

- conditions of cultivation are exactly defined can be regulated (increases the coefficient of propagation)

Introduction of new cultivars

Dutch iris. gets 5 daughter bulbs annually and takes 10 years for commercial quantities of new cultivars to be built up. One can get 100-1000 bulbs per stem section through micropropagation

Vegetative propagation of sterile hybrids

used as parent plants for seed production. Eg.cabbage.

- it can be used for plants which cannot be propagated by common methods (it is decreasing price of such plants)
- can be done during the whole year
- no space (or not big) for mother plants
- no special demands between subculturing of plants (weeding, irrigation, aeration, etc.)
- rejuvenilization can be reached - one of the basic presumptions for cloning of trees

Storage of germplasm

Generally the only successful method to date is keeping them in refrigerator. Slows down,but does not eliminate, alterations in genotype.

- *in vitro* cultures can be stored for long time - cryopreservation
- breeding cycle can be shortened .

Disadvantages of micropropagation

- ❒ expensive laboratory equipment and service
- ❒ no possibility of using mechanization
- ❒ obtained plants are not autotrophic in culture
- ❒ acclimatization (hyperhydricity)
- ❒ risk of genetic changes if 'de novo' regeneration is used

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

- -Regeneration is often not possible. Especially with adult woody plant material. More problems with roots cf.to shoots.
- -May not get uniform growth of original plant from tissue culture. Have different growth rates and maturation *in vitro*. Thus cannot be used thus for floriculture crop production where uniformity is critical.

LECTURE 07
MERISTEM CULTURE FOR VIRUS ELIMINATION

Meristem:
- A localized group of actively dividing cells, from which permanent tissue system, i.e., root, shoot, leaf, and flower, are derived.

Meristem culture:
- *In vitro* culture of a generally shiny, dome-like structure measuring less than 0.1 mm in length when excised, most often excised from the shoot apex.

Meristem tip culture

Only the meristematic dome and 1 pair of subtending leaves should be excised.

If larger pieces are taken, it is likely that the virus will be transmitted. The size of a meristem plus the subtending leaves ranges from 0.1-0.5 mm. The apical dome itself measures from 0.1-0.25 mm depending on the species. There is a balance in size. The meristem tip must be small enough to eradicate viruses and other pathogens, yet large enough to develop into a shoot. Although roots may form on the shoot directly in the same medium, often the shoot has to be transferred to another medium in order for roots to develop.

Meristem tip culture is used successfully to remove viruses, bacteria, and fungi from plants. In a majority of cases heat therapy is combined with meristem tip culture in order to produce the greatest number of plants that are "virus free".

The term *meriste*m, *shoot ti*p, *meristem tip* are often interchanged. Here we will use the term *shoot tip* to refer to an apical tip ranging from 1-3 cm. The *meristem* is strictly the meristematic dome without any primordial leaves. The term *meristem tip* will be used to denote the meristem together with 1-2 primordial leaves and measuring between 0.1 and 0.5 cm in height. The term *virus-free* is a misnomer. After successful heat therapy and meristem tip culture **one can only say that a plant is free from the actual viruses for which one teste**d. *Pathogen-free* is another term that is misleading. To be more exact one should use the term "specific virus-free" or 'specific pathogen-free'. Both heat treatmentand meristem culture are used in many crops.

In case of meristematic propagation, elimination of virus particles in explant cells is reached within a short time. In many cases meristematic cells do not contain virus particles because of non-existing vascular connection with other plant parts. For plant treatment proper meristematic apex without adjacent leaf primordia (size - 0.2-1.0 mm) is used as starting explant.

Thermotherapy /Heat treatment: Heat treatment is used in those plants in which viruses cannot be eradicated just by meristem tip culture alone.

In plant tissue cultures viruses can also be eliminated with incidence of higher temperatures (heat treatment.).In such case explants are exposed to the incidence of higher temperatures, which are not lethal for plant cells, but they are lethal for viruses Mostly used temperature range is 50-52^{o}C with exposition about 10-30 minutes. In case this method is applied on whole plants, lower temperatures have to be used (32-40^{o}C) with exposition about 4 - 30 days(depends on plant species and virus type).

Chemotherapy : Virus-free plants can be also obtained when antivirus matters are added into nutrient solution (Ribavirin or 2-thiouracil).

Mostly combination of thermo-therapy and meristem culture is used for virus-free plants production (e.g., cassava, bananas, citruses, strawberry, Irish potato, apples, chrysanthemas, garlic). Heat therapy combined with meristem tip culture is able to eradicate viruses, bacteria, and fungi but does not remove viroids. Unlike viruses, viroids are RNA without a protein coat – thus they are known as 'naked' RNA and are very difficult to eradicate. Usually the infected plant must be destroyed.

Shoot tip grafting (STG) /Micrografting

In a number of species including those of citrus, attempts at meristem culture remained unsuccessful. As an alternative, shoot tips of 0.14-0.18 mm in length isolated aseptically from a diseased plant were grafted on to young etiolated root stock seedlings grown *in vitro*. STG had been used to produce virus free plants in other crops including peach, and apple.

Why virus eradication works

Several hypotheses exist to explain why heat therapy and meristem tip culture when used together are effective in eradicating viruses.

- ❒ Virus distribution is uneven in a plant and is much less in a meristem.
- ❒ Viruses cannot travel quickly enough through plasmodesmata to keep up with actively growing tip.

Plants in which virus eradication is commonly used

Garlic, pineapple, dahlia, cymbidium, orchid, carnation ,strawberry,hyacinth sweet potato, iris, lily, apple, cassava,banana, narcissus , perlagonium (geranium),gooseberry, raspberry, sugarcane,potato, grape and ginger.

One of meristem propagation advantages is that one of facilitating of plant material international exchange.

Virus Indexing

The biological assays are reasonably accurate, but too slow and difficult but to use for screening a large number of plants. Alternatively, techniques involving electron microscope, use of indicator plants, serology or a combination of both were developed for virus detection in plant tissues. Among serological techniques, enzyme linked immunosorbent assay (ELISA) and nucleic acid hybridization techniques are popular.

LECTURE 08
ANTHER CULTURE & POLLEN (MICROSPORE) CULTURE

Anther and microspore cultures provides a method for the production of homozygous lines over the course of a few months, rather than the several generations (years) required using conventional whole plant techniques.

The doubled haploid plants resulting from anther and/or microspore culture are homozygous and breed true.

Because they harbour no hidden traits, the use of doubled haploids for breeding also improves the efficiency with which superior genotypes can be identified..

Haploid Plants

In this technique, immature anthers containing pollen at a particular stage of development are excised and placed on a culture medium. This process inhibits typical gametophytic differentiation and instead allows cell division and regeneration to occur In culture, although androgenesis can be induced in anthers at the tetrad stage/at bi nucleate pollen stage, microspore just before or at the time of first mitosis are however most suitable for the induction of androgenesis. Other method for the production of haploids includes the culture of unfertilised ovules or ovaries, resulting in gynogenesis (it is not used so often as microspore culture).

Induction of androgenesis (Pathways)

In vitro as a result of meiosis in pollen mother cells (PMC), pollen tetrads are formed which are eventually released in the form of microspores. The newly formed microspore is highly cytoplasmic with a central nucleus. With increase in volume of microspore and vacuolation, the nucleus is pushed towards periphery. By first mitosis, a large and diffuse vegetative cell and a small dense generative cell are formed. The former remains quiescent while later divides to form sperms.

Microspores in cultured anthers exhibit various modes of development leading to androgenesis.

- ❒ Division is unequal. After first mitosis, Generative nucleus does not divide or does so once or twice and degenerates. The vegetative cell divides repeatedly to form a haploid embryo as in case of Nicotiana, Datura , triticale,capsicumand Hordeum.
- ❒ Division is unequal.The generative nucleus usually remains quiescent and aborts after a few divisions but occasionally it does take part in androgenesis. E.g. *Hyoscyamus niger*
- ❒ The microspore nucleus instead of dividing to form a generative and a vegetative nucleus give rise directly two similar nucleus or there is a direct segmentation where both the daughter cells are involved in androgenesis. E.g. *Datura innoxia*
- ❒ Division is asymmetrical as in pathway II. Both the vegetative and generative nuclei contribute for the development of haploid embryo

- ❒ In some cases, as in Datura two similar nuclei are formed as a result of direct division of the microspore nucleus or of the vegetative one, fuse with one another and this results in formation of homozygous diploids.

Spontaneous duplication of chromosomes often occurs within anther culture-derived callus cells, resulting in the production of fertile doubled haploid plants. Because the two copies of genetic information within such plant are identical such plants are fully homozygous. COLCHICINE can be used to induce polyploidization and offers the possibility to increase the number of dihaploid plants produced, especially when direct androgenesis is involved.

With experience, haploid plants can be distinguished from diploid plants with about 90% certainty at the point at which plants are transferred to the greenhouse. (Subtle difference in stature, colour, leaf shape, tillering and root development, stomata number and size can be used to eliminate most haploids at this stage if greenhouse resources are limited.

Main factors influencing the success of anther culture

- ❒ Genotype of the plant from which anthers are obtained;
- ❒ Condition of the donor plant;
- ❒ Thermal shock pre-treatment of the anthers;
- ❒ Composition of the nutrient media;
- ❒ Developmental stage of the pollen.

Microspore culture

The ideal culture system for production of haploids is isolation and culture of microspores after separation from anther wall tissue.

Reasons:

The influence of anther tissue can be detrimental.
Diploid tissue - Connective tissue is growing activity which is competitive with growth of haploid microspore which is soon submerged by profuse diploid callus. So, variable and numerous chromosomal alterations are noticed during culture.

Methods

Spontaneous

A combination of pretreatment and incubation is given. -Anthers will dehisce in liquid medium and produce callus/embryo which will float from somatic tissue. eg. Brassica, cereals, solanaceae.

Homogenisation and filtration

Pretreated anthers are cultured form 3-4 days gently crushed with a glass rod/syringe piston in liquid medium to allow the microspores squeezed out. The suspension with anthers and microspores are filtered through a nylon sieve which allows microspore to pass through. The filtrate is centrifuged for 5 minutes at 100g. After discarding the supernatant, wash pollen at least once and re suspend in liquid medium at initial density in petridish and incubate.(e.g. Solanaceae, rape, sugarcane

Slit Technique

Cutting the anther wall to release the microspore calluses/embryos rather than relying on natural dehiscence but this is a time consuming process(e.g.) Tobacco, pennistem.

Uses of haploids

- ❒ Production of homozygous varieties in self pollinated crops.
- ❒ In cross-pollinated crops, the derivation from heterozygous material of pure lines for use as parents of the intended single cross or double cross hybrids.
- ❒ The obvious advantage of haploids is that they display mutations with successive effects in single dose.
- ❒ Effective fixation by chromosome doubling on transformation.
- ❒ Double haploid plants are also used in mutagenesis, biochemical, and physiological studies.
- ❒ Development of pure lines and disease resistant lines for mildew and yellow mosaic- Barley
- ❒ Parthenogenetic haploids in Maize
- ❒ Recovery of sexual inter specific hybrids between wild and domestic species **-** Tomato
- ❒ Development of pure lines and 100% male plants in Asparagus
- ❒ Complex hybrids for disease resistance in Coffee

LECTURE 09
OVARY AND EMBRYO CULTURE

Embryo rescue and other applications

Gynogenesis

Flowers are collected in a stage at which there is maximum elongation and enlargement of the flower where ovules contain a mature sac. Flowers are sterilized inoculated on pre culture medium. After 10-14 days, ovules are removed from flowers and plated on medium. Ovule culture requires extensive labour, therefore a more efficient method would be to culture ovaries or flowers.

Advantages

- ❒ Gynogenetic haploids may be a valuable substitute for the production of homozygous lines in cases where cytoplasmic male sterility precludes the use of micropsores.
- ❒ Another advantage is the reduction in the frequency of albino plants in some species especially cereals.

Techniques: Culture of ovule is advantageous as they can be excised even at the zygote stage and are thought to provide a "maternal environment" for the developing embryo.

Technique

Pollination $\rightarrow$ Ovary collected and surface sterilized
(1-12d) post pollination
Ovary cut open, ovules scooped out placed on medium.

For unfertilized ovules $\rightarrow$ Ovary is collected 24-48h prior to anthesis.

- ❒ In many inter specific and inter generic crosses, the hybrid embryo frequently aborts in the developing seed. By resorting to ovule culture or a combination of ovule embryo culture, it might be feasible to obtain the hybrid progeny.
- ❒ Unfertilized ovule culture may prove to be a promising approach to obtain gynogenic haploids.

Ovary culture

Following pollination, whole flower buds are excised (2-15 days post pollination) Calyx, corolla and stamen are removed. Ovaries are then surface sterilized and inoculated. To obtain un pollinated ovaries, flower buds are removed 24-48 hours prior to anthesis.

Advantages

- ❒ Utilized to obtain hybrids of normally incompatible species.
- ❒ In umbelliferae, poly embryony has been observed in ovary culture. The embryo which usually follows normal development some undergoes cleavage and budding resulting in poly embryonal mass with embryoids emerges by rupturing the pericarp and eventually form plantlets.

Bulbosum technique

Principle

The fertilization proceeds readily between *H. vulgare* and *H. bulbosum* Zygote induction is high and chromosomes of *H. bulbosum* are rapidly eliminated from the cells of developing embryo. The develops for two to five days and then aborts. In the developing monoploid embryo cells, the division and increment in slower than in diploid cells. This comparatively slow growth of the monoploid condition, together with the disintegration of the endosperm leads to the formation of small embryos which have to be dissected out of the fruits and provided with nutrients *in vitro* in order to complete their development. Following *in vitro* embryo culture, the developing plantlets are raised under normal green house conditions and chromosome doubling is induced on established plants.

Advantages

- ❒ The method of hybridization followed by chromosome elimination proves to be of general interest for haploid production in other species of Hordeum and also of hexaploid wheat.
- ❒ It is possible to produce mono ploids of barely in a cytoplasm of *H. bulbosum* by using *H. vulgare* as male and *H. bulbosum* as female. Using embryo culture as vehicle, high frequency foreign cytoplasm monoploids can be obtained.
- ❒ Hordeum species is not the only one where chromosome elimination is found in higher plants. In Haplopoppus, mono ploids has been examined with only two chromosome.*H. bulbosum* need not be the ideal partner for *H. vulgare* to induce mono ploids of barley via somatic chromosome elimination . There can well be a range of Hordeum that might be tried as a more efficient pattern than *H. bulbosum*.

Embryo culture

Embryo culture is the sterile isolation of an immature or mature embryo *in vitro* with the goal of obtaining a viable plant.

Two types

- ❒ Culture of immature embryos originating from unripe seeds which is mainly to avoid embryo abortion with the purpose to produce a viable plant.
- ❒ Culture of mature embryos derived from ripe seeds.

Factors affecting the success of embryo culture

1. Genotypes
2. Developmental stage of the embryo at isolation
3. Growth conditions of the mother plant
4. Composition of the nutrient media
5. Light
6. Temperature

Practical applications

1. Elimination of (absolute) inhibition of seed germination
    a. Endogenous Inhibitors: E.g. Iris
    b. Dry storage: wild oat (*Avena fatua*)
    c. Immaturity of embryo: Orchid seeds
2. Germination of seeds of obligatory parasites
3. Shortening breeding cycle: Cultivated varieties of rose
4. Overcoming self sterility of seeds
5. *Musa bulbisiana* and tubers crops *Colocasia esculenta & C. antiquorum* .
6. Seed Testing: Rapid means of determining viability of particular lot of seeds eg. seeds of conifers, shrubs, vines and fruit trees.
7. Prevention of embryo abortion in early ripe fruits e.g. "Peach, cherry, apricot, plum.
8. Prevention of embryo abortion as a result of incompatibility (embryo rescue) In interspecific(*Phseolus*, lily flex, cotton, tomato, rice and barely), intergenic (Hordeum x Seale and Triticum x Seale.)and with crosses between diploids and tetraploids (barley and Rye).

9. Production of Haploids .*H. vulgare x H. bulbosum*, Following the bulbosum technique haploids were also obtained with Agropyron after crossing *H. vulgare* and in some cereals.
10. Vegetative propagation In Gramineae and coniferae embryos are often used as a starting material.

Other applications

- ❒ To study some fundamental problems in experimental embryogenesis.
- ❒ Host pathogen interaction. *e.g*. formation of ergot by infection of rye embryos by *claviceps purpurea* and fusarium wilt of seedlings. In latter case, incorporation of fungal toxin fusaric acid into culture medium interfere with water uptake by germinated embryos of *Phaseolus vulgaris* and induce characteristics wilting of embryonic leaves.
- ❒ Cultured embryos have been used as test objects to evaluate the mutagenic ability of irradiated substrates on living tissues. For this embryos of certain cereals were planted on X-irradiated nutrient medium for evaluation.

LECTURE 10
PROTOPLASTS- ISOLATION, CULTURE AND REGENERATION

Protoplasts are naked cells that can be obtained through mechanical/enzymatic degradation of cell walls. They are plant cells with a plasma membrane but without cell wall.

Sources: Young leaves, roots stems, petals, reproductive organs, friable callus, tissues/cells, fast growing cell suspensions. Coleoptiles, aleuronic layers, Plant cell tumors/galls.

Commercial enzyme preparations

- ❒ Onozuka R10 Cellulase (Japan)
- ❒ Cellulase R 10 (Japan)
- ❒ Macerozyme R 10, Pectinase (USA)
- ❒ Hemicellulase (USA)
- ❒ Driselase, Cellulase (Japan)
- ❒ Celulysin, Cellulase (USA)
- ❒ Pectinase (USA, Sigma)
- ❒ Macerase (USA)
- ❒ Rhozyme HP 150, Hemicellulase (USA).

Enzyme mixture is normally dissolved in culture media together with an osmotic stabilizer. The osmotic agents may be sugar, alcohols, sorbitol or mannitol (13% w/v). Sucrose can be used for this purpose.

Isolation Methods

Mechanical

e.g. Onion bulbs, scales, radish roots. This method gives poor yield of protoplasts and is not suitable for isolating protoplasts from meristematic and less vacuolated cells. This method is tedious and not often in use.

Enzymatic method

Preplasmolysis –Enzyme digestion-Washing- Purification-Culture

Advantages

- ❒ Protoplasts can be obtained in large quantities.
- ❒ The cells are not broken like in mechanical isolation.
- ❒ Osmotic shrinkage is much less.

Direct (one step) method

Treatment with Macrozyme or Pectinase and Cellulase is done simultaneously. The enzyme mixture in direct method consists of 0.5% Macerozyme + 2% cellulase in 13% sorbitol or mannitol at pH 5.4.

Sequential (two step) method: In two step method, leaf segments with mixture A (0.5% macerozyme+ 0.3% potassium dextran sulphate in 13% Mannitol at pH 5.8) are vacuum infiltrated (in a desiccators) for about 5 min and washed cells are then, incubated with enzyme mixture B (2% Cellulase in a 13% solution of Mannitol at pH 5.4) for above 90 min at 30˚C. After incubation, the mixture is centrifuged at 100g for 1 minute, so that protoplasts form a pellet, which is cleaned three times as in one step method.

Protoplast culture and Regeneration of plants

Like the totipotency of plant cells, isolated protoplasts regenerate a cell wall around themselves to reconstitute a cell and undergo repeated division to form callus. Protoplasts can be cultured following 'Bergman's cell plating technique', hanging drops or in micro chambers or following multiple drop array method Isolated protoplasts is mixed with 1.0% agar culture medium and maintained at 40-45°C. Small amount of agar (liquid) protoplasts mixture is then poured into sterile plates. The parameters to be calculated before culture are:

1. Viability and Plating density of protoplasts
2. Minimum Plating density (MPd) :In general, protoplast density within a range of 5 x 10^4 to 10^5/ml seems to be suitable. Normally, for the induction of division in the protoplasts, they have to be plated at final densities higher than 10^4/ml of the medium.

Protoplasts in culture start to regenerate a wall within a few hours and may take two to several days to complete. After three weeks, colonies are visible. Once small colonies are formed, their further growth is slowed down/inhibited altogether if they are allowed to remain on original high osmotic medium. The colonies therefore should be transferred to Mannitol free medium.

Applications

Virus uptake : Studies on the mechanism of infection and host parasite relationships
Bacterial uptake: Symbiotic nitrogen fixing bacterium (Rhizobium, Azotobacter) can be introduced into legume. Direct DNA transfer and expression of a bacterial gene in protoplasts of exogenous DNA by cells or protoplasts of *T. Monococcum* and *N. tabucum* are reported.
Incorporation of Cyanobacterial *(e.g.):* Cyanobacteria or BGA Co incubate algal preparation with isolated protoplasts with 25% PEG and high planting density. Protoplasts begin engulf algal cells.
Incorporation of exogenous DNA
Exogenous DNA can be taken up by higher plant cells/protoplasts and this is known as Trasngenosis.
Transplantation of nuclei: Organelles such a large nuclei can be introduced through plasma lemma into protoplasts. Both intra and inter specific nuclear transplantations have been observed in *Petunia hybrida, Nicotiana tabacum* and *zea mays*.

Chloroplast implantation: This has been done in Petunia and carrot.

Mutation and Genetic variability: Like callus cells, isolated protoplasts especially haploid ones would make an ideal system for studying the effect of irradiation and for the induction of mutations by planting them in media supplemented with various chemical mutagens.

LECTURE 11
PROTOPLAST FUSION AND SOMATIC HYBRIDIZATION

Protoplast fusion is especially important in sexually incompatible plants and in cases where conventional methods of breeding fails to operated.

Protoplast fusion can be classified into three categories.1. Spontaneous fusion, 2. Mechanical Fusion 3.Induced fusion.

Spontaneous fusion

Young leaves are more likely to produce such spontaneously induced multi nucelate protoplasts. It is strictly inter specific.

Mechanical fusion

This is a mechanical method to bring isolated protoplasts into intimate contact through micro manipulators and perfusion micropipette. By this method, occasional fusions of protoplasts from soybean. *Arachis hypogea* and *Vinca rosea* were observed. In this procedure, protoplasts are likely to get injury.

Induced fusion

Induced fusion of protoplasts does not necessarily involve fusion of the same plant species which requires an inducing agent.

Use of fusogens

1. Treatment with $NaNO_3$: Isolated protoplasts are suspended in an aggregation mixture of 5.5% Sodium nitrate in 10% sucrose solution
2. Effect of proteins: Gelatin and early products of its degradation at a concentration of 2.5% induced aggregation at high frequency within one hour E.g. Vicia, Glycine and Allium.
3. Immunological Method: Soybean and brome grass antibody cross reacted with and agglutinated *Vicia* protoplasts..
4. Calcium ions at High pH: This involves spinning the protoplasts in a fusion inducing solution (0.05M $CaCl_2$ $2H_2O$ in 0.4 M mannitol at pH 10.5)

Polyethylene Glycol (PEG)

- When protoplasts are available in sufficient quantities, 1 ml of the protoplasts suspended in a culture medium is added to 1 ml of 56% solution of PEG and the tube shaken for 5 Seconds.

- ❒ When micro quantities of protoplasts are available drop cultures can be used. Two types of protoplasts are mixed in equal quantities, 4-6 micro drops (100μl each) are placed in small plastic petridishes and allowed to settle for 5-10 minutes at Room temperature.

5. Miscellaneous: Additives such as Poly L-ornithine, poly D lysine, cocanavalin DM so, lysozyme, cytocholasin B and protamine sulphate have been employed.

6.Electric fusion: There are two steps:

- ❒ Protoplasts are exposed to high frequency alternating electric field (0.5-1.5 NH_2) that generates dipoles through dielectricphores. So, protoplasts find together to form, pearl chains.
- ❒ Application of one/more short (10-100μs) direct current (DC) (1.3 k/cm_{-1}). Cause reversible membrane breakdown resulting in pores in the aligned membranes. The contact membranes can be fused and fusion opens the way for hybrid cell formation. To insure maintenance of close membrane contact during fusion, AC field is reapplied.
- ❒

Selection of fused protoplasts

After fusion, the protoplast population consists of a mixture of parental types, homokaryons and heterokaryons, of which heterokaryons (potential source of future hybrids) often make only 0.5%-10%.

1. Visual selection: This utilizes protoplasts for fusion studies that are visually distinct at the light microscope level. This is laborious. Only limited fusion products can only be selected.
2. Biochemical basis of selection/Nutrional selection: This is based on nutritional requirements of parents and hybrids
3.Complementation Selection is based upon the ability of the two genomes present in the heterokayon/hybrid to complement E.g. *N. tabaccum Petunia* Datura.
4. Drug resistance/sensitivity: Parental types are sensitive to actinomycin. (*Petunia parodic, P. hybrida*). Somatic hybrids (*P. hybrida* + *P. parodri*) are resistant to actinomycin.
5. Labelling: Protoplasts of two parents may be labeled by different fluorescent agents, which will then enable the selection of hybrids
(1) Octadeconyl amino fluorscein.
(2) Octadecyl palamine.
6.Fluorescent Assorted cell sorter (FACS):

Uses

Somatic hybrids for gene transfer

- **Interspecific** hybrids in genus-Daucus and its relatives.
  Rice and *Echinochola oryzicola* (Barnyard grass) hybrids have been obtained E.g. Nicotinana, Brassica, Petunia, Solanum, Lycopersicon and Datura interspecific hybrids.
  -Intergeneic

Raphanus sativus + *B. oleracea* - Rhanobrassica
Moricandia arvensis + *B. oleracea* - Moricandio brassica
N. tabacum + *L. esculentum* - Nictoipersicon
Solanum tuberosum + *L. esculentum* - Solanopersican
Datura innoxia + *Atropa belladona* - Daturotropa.
O.sative + *Echinochloa oryzicola*-Oryzochloa.
Intertribal (bet. family, Brassicaceae)
A. thaliana + *B. campestris* - Arabidobrassica
Barbarea vulgaris + *B. napus* - Barbareobrassica

Symmetric hybrids between potato and tomato have been shown to exhibit intermediate cold tolerance.

Asymmetry in somatic hybrids for gene transfer

An important example of use of asymmetric hybrids in gene transfer is transfer of blackleaf resistance to rape seed.

Somatic hybrids for CMS (Melchers, 1992).

Substitute nucleus of one species into the cytoplasm of another species, whose mictochondria are inactivated.which led to generation of CMS.
E.g. Mesophyll protoplasts of *Solanum acaule* (potato) and tomato.
In five tomato cultivars male sterility induced in this manner was inherited maternally over several generations.

Advantages

- ❒ Only one step is required.
- ❒ The nuclear genotype of cultivar remains unaffected.
- ❒ There are prospects that 100% of the progenies of somatic hybrids will be CMS.

Cybrids for CMS and Herbicide tolerance

LECTURE 12
SOMACLONAL VARIATION AND CROP IMPROVEMENT

Larkin and Scowcroft (1981) proposed the term somaclone to describe the plants originating from any type of tissue culture. Genetic variation (Genotypic and Phenotypic Variability) found to occur between somaclones in plant tissue cultures was then called somaclonal variation.This variation includes aneuploids, sterile plants and morphological variants, sometimes involving traits of economic importance in case of crop plants. The usefulness of variation was first demonsrated through the recovery of disease resistant plants in potatao (resistance against late blight and early blight) and sugarcane (resistance against eye-spot disease, Fiji disease and downy mildew)

Genetic variation - mutations or other changes in the DNA of the tissue those are heritable .This is only transmitted to the next generation and is thus important for crop improvement. Therfore it is necessary to study the transmission of variation to sexual progeny to facilitate the estimation of its utility for improvement of a sexually propagated crop. In several crops R0,R1 and R2 progeneies were analysed for genetic analyses and 3:1 segregation leading to the isolation of true breeding variants was observed.

Epigenetic variation- non-heritable phenotypic variation. Epigenetic changes can be temporary and are ultimately reversible. However, they may also persist through the life of the regenerated plant.
Physiological variation- emporary in response to stimulus and disappear when it is removed.

Causes for variation

Changes of mother plant origin

Chimeral rearrangement of tissue layers Many horticultural plants are periclinal chimeras, that is, the genetic composition of each concentric cell layer (LI, LII, LIII) in the tunica of the meristematic tissues is different. These layers can be rearranged during rapid cellular proliferation. Therefore, regenerated plants may contain a different chimeral composition or may no longer be chimera at all. Cell variation also occurs if callus is initiated from explants containing differentiated and matured tissue s that have specialized function.

Explant derived variation

The most stable cultures are obtained from meristematic tissue of a mature plant or tissues of a very young organ of meristematic nature.Polyploid cells can give more variability than diploids

Genetic changes arising in culture

Ploidy Changes Three phenomena that occur during mitosis lead to most changes in ploidy:

endomitosis (sister chromatids separate within the nuclear membrane, but there is no spindle formation nor cytoplasmic division)
endoreduplication (chromosomes at interphase undergo extra duplications)
spindle fusion (giving binucleate or multinucleate cells).

Gross structural rearrangements appear to be a major cause of somaclonal variation. These involve large segments of chromosomes and so may affect several genes at a time.

Deletions (genes missing, for example 1,2,3,4 now 1,2,4)
Inversions (gene order altered, for example 1,2,3,4 now 1,3,2,4)
Duplications (1,2,3,4 now 1,2,2,3,4)
Translocations (whole chromosomal segments moved to a new location, for example 1,2,3,4 now 1,2,3,4,A,B,C).

Transposable elements are segments of DNA that are mobile and can insert into coding regions of genes, typically resulting in a lack of expression of the gene. The culture environment may make the transposable elements more likely to excise and move.

Point mutations (the change of a single DNA base), if they take place within a coding region of a gene and result in the alteration of an amino acid, can lead to somaclonal variation. Point mutations are often spontaneous and are more difficult to detect. Note that they result in single gene changes

Structural changes in the DNA sequence
Chromosomal rearrangements, point mutations, or transposition of transposable elements can occur during culture. These changes can occur spontaneously or can be induced with chemicals or radiation

DNA methylation: Most of the mutational events occasioned by tissue culture are directly or indirectly related to alterations in the state of DNA methylation. A decrease in methylation correlates with increased gene activity
Lack of nucleic acid precursors: Shortage of the precursor necessary for rapid nucleic acid biosynthesis, which occurs in many tissue cultures
Growth regulators: One of the triggers of polyploidy *in vitro* is growth regulators; both kinetin and 2,4-D have been implicated.
Composition of culture medium: The level of KNO_3 influence the albino plants from wheat cultures. Level of organic N_2, chelating agents and other micro nutrients are other factors.
Culture conditions: Temperature, Method of culture

Effect of the genotype

Effects of the culture process itself (lengthy culture periods, growth and other aspects of the culture medium may also affect the ploidy of the cultured cells. Medium that places cells under

nutrient limitation will favor the development of "abnormal" cells. Chromosomal alterations, like ploidy changes, increase with increased lengths of culture.

In mixed populations of cells with different ploidys, diploid cells retain their organogenic potential better than polyploid and aneuploid cells (probably due to an enhanced ability to form meristems).

One common alteration seen in plants produced through tissue culture is *rejuvenation*, especially in woody species. Rejuvenation may lead to changes in morphology, earlier flowering, improved adventitious root formation, and/or increased vigor.

Selection of somaclonal variants on subjecting the cells to selection pressure

Selection	**Selection of cells in the presence of**
Resistance to herbicide	- Herbicide
Resistance to salt	- Sodium chloride / Aluminium
Resistance to drought	- PEG / Mannitol
Resistance to frost	- Hydroxy proline resistant lines
Resistance to pathogens	- Pathotoxin / Culture filtrate

Crop improvement through somaclonal variation for desirable characters

Crop	**Characters modified**
Sugarcane	Diseases (eye spot, fiji virus,downy miledew, leaf scald)
Potato	Tuber shape, maturity date, plant morphology, photperiod, leaf colour, vigour, height, skin colour,Resistance to early and late blight
Rice	Plant height, heading date, seed fertility, grain number and weight
Wheat	Plant and ear morphology, awns,, grain weight and yield, gliadin proteins,amylase
Maize	T toxin resistance, male fertility, mt DNA
Medicago sativa	Multifoliate leaves, elongated petioles, growth, branch, no.of plants, dry matter yield.
Tomato	Leaf morphology, branching habit, fruit colour, pedicel, male fertility, growth
Avena sativa	Plant height, heading date,awns
Hordeum spp	Plant height and tillering
Lolium hybrids	Leaf size, flower,vigour, survival

LECTURE 13
CRYOPRESERVATION AND GERMPLASM STORAGE

In situ hybridization is defined as conservation of genetic resources with their eco system and natural habitat. *Ex situ* conservation means conservation of components of genetic material of biological diversity outside their natural habitat. This refers to man made gene bank conservation that includes *ex situ* seed conservation in seed gene bank, *ex situ* plant conservation in field gene bank and *ex situ in vitro* conservation of explants/organs in *in vitro* bank and cryo bank, DNA library and DNA bank.

In vitro conservation: Conservation of genetic diversity under aseptic condition using culture methods is called in vitro conservation

Strategies

1.Normal growth: short to medium storage

2. Slow growth: Using different enclosures for culture vessels, reduction in temperature, inclusion of osmoticum, growth retardants, modification of gaseous environment, induction of storage organs, minimal growth media

3.Suspended growth: Cryo stroage./ Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra low temperature preferably at that of liquid nitrogen..

Cryo preservation-Steps

1. Rising sterile tissue cultures and cell suspensions.
2. Addition of cryoprotective agent.
3. Subjecting cell cultures to super low temperature by regulated slow rate of cooling or after pre freezing.
4. Storage of frozen cells in liquid nitrogen.
5. Thawing or rapid re warming of cells.
6. Removal of cryoprotectant by repeated washing.
7. Determination of viability.
8. Re culture of the retrieved cells.
9. Induction of growth and regeneration of plants.

Cryo protectants

Most of the experimental systems (meristem, shoot tips, cultured cells etc) contain high amount of cellular water and hence are extremely sensitive to freezing, injury protection from freezing and thawing injury has to be imposed artificially. A number of compounds such as glycerol, DMSO, ethylene glycol, polyethylene glycol, sugars and sugar alcohols either alone or in combination protect cells against damage during freezing and thawing. There are two types of cryo protectants.

Permeating: DMSO and Glycerol. DMSO permeates into cells more rapidly than glycerol and requires shorter treatment duration. Glycerol is less toxic to cells than DMSO while DMSO is superior in cryoprotection of plant cells and organs.

Non-permeating: Sugars, sugar alcohols and high molecular additnes, polyvinyl pyrrolidone, PEG, dextran hyroxy ethyl starch etc.

Freezing Methods

1. Slow freezing: This is the most common method of freezing meristems, somatic embryos and cell cultures which is by regulated slow cooling at a rate of 0.5-1.0° C/min to either -30, -35 (or) -40°C followed by storage in liquid nitrogen.

2. Rapid Freezing: Meristems and somatic embryos for few plant species have been successfully cryo preserved by rapid freezing. Here, the temperature is rapidly lowered.

3. Droplet freezing: Mostly suitable for meristems E.g. Cassava. Here, the cryo protectant solution, (15% DMSO and 3% sucrose) is dispensed into droplets of 2-3μl in an aluminum foil contained in a petri dish. The advantage of droplet freezing on aluminum foil is homogeneous cooling due to efficient thermal conductivity.

4. Vitrification: This is based on the ability of highly concentrated solutions of cryoprotectants to super cool to very low temperature upon imposition of rapid cooling rates to become viscous at sufficiently low temperatures and solidity without the formation of ice. The advantage is being simple and doesn't require regulated cooling. The disadvantage is toxicity owing to high concentration of cryoprotectants.

e.g. Cell cultures of *Brassica*, naval orange, Somatic embryos of *Asparagus* and Daucus, mesophyll protoplasts of Secale and shoot tips of Mint, Potato, Papaya and Carnation.

Cryostorage and Thawing Long term preservation is accomplished at ultra low temperatures such as that of liquid nitrogen (-196°C). Thawing is generally carried out by immersing specimens rapidly for 1-2 minutes in 35-40°C water bath.

Viability assays: Viability can be assayed by fluorescent di acetate staining (FDA), Triphenyl tetrazolium chloride(TTC) reduction assay, and using other parameters like mitotic index, Cell number Cell volume, dry and fresh weight and plating deficiency.

Applications

1. In vegetatively propagated crops, to avoid high level of heterozygosity, they are clonally propagated through tubers or cuttings which have a limited life span, labor intensive and expensive and risks are associated with field maintenance. In seed crops problems on non-viability recalcitrance to storage, deterioration and heterozygosity exists. To circumvent the problems, Cryo preservation technology can be used.
2. Preservation of rare genomes.
3. Freeze storage of cell cultures.
4. Conservation of genetic uniformity.

5. Maintenance of Disease Free Material which is ideal for the international exchange.
6. Cold Acclimation and Frost Resistance.
7. Retention of Morphogenetic Potential.
8. Slow metabolism, which would prevent or virtually 'stop' the ageing process.

LECTURE 14
ACHIEVEMENTS MADE THROUGH TISSUE CULTURE IN AGRICULTURE, HORTICULTURE AND FORESTRY

Introduction

Tissue culture consists of growing plants cells as relatively on organized masses of cells on an agar medium (callus culture) or as a suspension of free cells and small cell masses in a liquid medium (suspension culture). Tissue culture is used for vegetative multiplication of many species and in some cases for recovery of virus-free plants. It has potential application in production of somatic hybrids, organelle and cytoplasm transfer, genetic transformation and germplasm storage through freeze-preservation. Having the right plant material the right media and the right working environment crop improvement through tissue culture becomes less difficult. Crops which have gone the process of tissue culture have several advantages.

The various applications of plant tissue and cells culture techniques are as below:

Production of disease free materials

They take less time to mature and the yields are higher since they are free from diseases caused by fungi bacteria, nematodes or other pathogens. Materials infected with viruses can also be made virus free through thermotherapy and meristem tissue culture.

Rejuvenation plant materials

Plant tissues from an old plant can be rejuvenated through tissue culture and able to grow again as new. e.g old cassava material have been rejuvenated to produce young plantlets through tissue culture.

Anther Culture

Plants produced through anther culture are haploid meaning they contain half the chromosomes Homozygous plants can be produced by doubling the chromosomes without going into series of backcrossing . This technique can have profound application to plant breeder and could shorten the time of breeding by half.

Hybridization

This is usually applied when all other techniques of breeding have failed. Plant cells are isolated and cell walls removed. The naked protoplast is then made to produce hybrid. In vitro hybridization is rarely applied, for crop improvement.

Gene Transformation

Important crops can be greatly improved by genetic engineering by isolating a specific gene and

then transfering it to selected crops e.g A gene resistant to sweetpotato feathery mottle virus have been identified isolated and transfered to sweetpotato in which one of the major diseases are viruses. A gene from a drought resistant weed can be identified and isolated to be inserted in a crop e.g maize so that it can grow in drought prone areas.

Embryo Rescue

Many important plants are difficult to propagate through seeds. They take a long time for seeds to germinate or the seeds do not germinate at all. This can be overcomed through embryo culture. The seeds are surface sterilized and split open in aseptic condition and the tiny embryo is excised and planted in a nutrient medium and then grows to a complete plant.

Clonal Propagation

Tissue culture is well suited for quick vegetative propagation of plant species. It is used for asexual propagation in many species of fruit and timber trees and also used for obtaining disease free and virus-free plants. The major difficulty in the use of this technique in clonal multiplication is the occurrence of genetic variation among the regenerated plants. This problem can be reduced to a large extent by using young tissue cultures, preferably during the first few subcultures.

Mutant Isolation

Biochemical mutants are far more easily isolated from cell cultures than from whole plant populations. This is because a large number of cells, 10^6-10^9, can be easily and effectively screened for biochemical mutant cells. Biochemical mutants could be selected for disease resistance, improvement of nutritional quality, adaptation of plants to stress conditions, e.g. saline soils, and to increase the biosynthesis of plant products used for medicinal or industrial purposes.

Somaclonal Variation

Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits; such a variation is known as somaclonal variation. Somalconal variation has been described in sugarcane, potato, tomato etc. Some variants are obtained in homozygous condition in the plants regenerated from the cells in vitro (R_0 generation), but most variants are recovered in the selfed progeny of the tissue culture-regenerated plants (R_1 generation). Somaclonal variation most likely arises as a result of chromosome structural changes, e.g., small deletions and duplications, gene mutations, plasma gene mutations, mitotic crossing over and possibly, transposons. Somaclonal variation may be profitably utilized in crop improvement since it reduces the time required for releasing the new variety by at least two years as compared to mutation breeding and by three years in comparison to back cross method of gene transfer.

Amino Acid Analogue Resistant Mutants

Cereal grains are deficient in lysine; maize (*Zea maize*) is also deficient in trytophan, while wheat (*T.aestivum*) and rice (*O.sativa*) are deficient in threonine. Pulses are deficient in methionine and trytophan. Amino acid analogue-resistant cells may be expected to show a relatively higher concentration of that particular amino acid. For e.g., carrot (*D.carota*) and tobacco (*N.tabacum*) cell lines resistant to trytophan analogue 5-methyl trytophan show a 10-27-fold increase in the level of trytophan. Similarly, rice cells resistant to lysine analogue 5-(*B-aminoethyl*)-cysteine, show much higher levels of lysine. This technique may prove useful in the development of crop varieties with a better-balanced amino acid content.

Disease Resistant Mutants

Many pathogenic bacteria produce toxins that ae toxic to plant cells. Plant cell cultures may be exposed to lethal concentrations of these toxins and resistant clones isolated. Plants regenerated from these resistant clones would be resistant to the disease producing pathogen. This technique should be applicable to all the pathogens, which produce the disease through the action of toxin. An e.g., an application is in the case of wildfire disease of tobacco (*N.tabacum*) produced by *Pseudomonos tabaci*. Tobacco cells resistant to methionine sulfoximine, which is similar to the toxin produced by the pathogen, were isolated. Plants regenerated from these clones were resistant to wildfire disease, although to a somewhat lesser degree. The technique can be applied to those cases only where the disease is the result of a toxin produced by the pathogen. But many of the pathogens do not seem to produce a toxin, or the toxin does not appear to be the primary cause of the disease.

Stress Resistant and other Mutants

Plant cells resistant to 4-5 times the normally toxic salt (NaCl) concentration have been isolated. Attempts to insolate such cells are being made. Similarly, attempts are being made to isolate clones that would produce more substances of medicinal or industrial value.

Somatic Hybridization

Protoplasts can be isolated from almost every plant species and cultured to produce callus. Protoplasts of two different species may be fused with the help of polyethylene glycol.

Genetic Transformation

There is some evidence that gene transfer may be achieved by feeding cells with DNA in case of eukaryotes, such as, Drosophila, Neurospora, cultured mammalian cells and in some plants. Genetic changes may be brought about by DNA or by radiation-killed pollen grains. This raises the possibility of genetic modification of plant cells with the help of both homologous (from the same species) and heterologous (from a different species) DNA. It

is also proposed that DNA plant viruses, such as cauliflower (*B.oleracea*) mosic virus and potato leaf roll virus, plasmids (e.g., *Ti plasmid of Agrobacterium*) and transposons, may be used as the carriers of genes for genetic modification of plant cells.

Organelle Transfer

In some cases, it may be desirable to transfer only organelles or the cytoplasm into a new genetic background. This may be achieved through the use of plant protoplasts. Chloroplasts have been transferred, and other organelles including nucleus may be transferred.

Germplasm Conservation

Tissue cultures may be frozen and stored in liquid nitrogen at -196^0C for long-term storage of germplasm. This would be of great value in the conservation of germplasm of those crops which normally do not produce seeds, e.g. root and tuber crops, or where it may not be desirable to store seeds. For freeze-preservation, the cells are cooled at a slow rate and are then transferred to liquid nitrogen for storage. Thawing of the cells must be very rapid for increased survival. A cryoprotectant, such as dimethylsufoxide (DMSO), is used to protect the cells from injury due to freezing and thawing. The technique of freeze-preservation i.e., *crybiology*, of plant cells is still in the developing stages.

Achievements and Future Prospects

Tissue culture techniques are being exploited to enhance crop production and to aid crop improvement efforts. Faster clonal multiplication is being exploited on commercial scale for many horticultural species e.g. oil palm, mentha, roses, carnation etc. Tissue cultured somatic tissues are now routinely being used for conservation of those species whose seeds are recalcitrant or ones which do not produce seed at all.

Embryo culture has helped in rescuing hybrid embryos enabling the recovery of many interspecific hybrids and haploid plants. Shoot tip (meristem) culture plays a vital which is of great importance in germplasm exchange, and the development of serological techniques for the detection of viruses in plant materials is a great help to the efforts in this direction.

GLOSSARY ON PLANT TISSUE CULTURE

Abiotic elicitors: Elicitors of non-organic nature eg. UV light, metal ions etc.

Absolute plating efficiency: Ratio of individual cells inoculated producing colonies to the total cells inoculated in a culture.

Acclimatization: A process by which micropropagated shoots are made to adjust to the outside culture condition after they are planted out.

Activated charcoal: Charcoal which has been treated to remove hydrocarbons and to increase its adsorptive properties is considered activated charcoal. The adverse factors such as phenolic compounds may be adsorbed to activated charcoal included in the medium.

Active polymerization: Entrapment of plant cells in some sort of gel or combination of gels which are allowed to polymerize around them.

Acute contamination: Contamination in cultures occuring at the time of culture initiation due to incomplete surface sterilization.

Adsorption filtration: A process by which organic contaminants and free chlorine are removed from water by activated carbon. Dissolved impurities are not removed by this method.

Adventitious budding. Formation of buds from places other than nodes.

Adventive embryogenesis: Development of embryos from cells that are not the product of gametic fusion.

Agar (Agar-Agar): A gelatinous polysaccharide obtained from red algae. It is used as a solidifying agent in the tissue culture media and is added at the rate of 0.6 to l.0%.

Agarified medium: Nutrient medium solidified with agar or medium in which agar is used as gelling agent.

Agarose bead type culture: Type of culture in which the protoplasts are first embedded in agrose beads, and then once the divisions have started, they are transferred to liquid medium.

Agarose disc type culture: Type of culture in which the protoplasts are embedded in agarose and plated in small droplets (disc) surrounded by liquid medium.

Agarose embedding: Embedding protoplasts in media solidified with low melting point agarose. The method increases plating efficiency of protoplasts. The aggregate formation of protoplasts as in liquid culture does not occur since the embedded protoplasts remain in a fixed position in the gel matrix throughout the culture period.

Agrobacterium mediated gene transfer: Transfer of foreign genes to new hosts using *Agrobacterium tumifaciens* and *A. rhizogenes* as vector systems.

Agroinfection: Transfer of viral DNA integrated into the T-DNA of the Ti plasmid to the host cell using Ti plasmid as the vector. The viral DNA entering the plant cells as part of the T-DNA will integrate into the plant genome.

Albino: An organism lacking normal pigmentation. In plants, the lacking pigments are chlorophylls.

Algenate gel: A block of co-polymer of D-manouronic and L-guloronic acids, in which the carbohydrate moisties are held together by ionic bridges formed between the carboxyl groups of the sugars and multivalent cations which are added as gelling agents.

Alien addition line: Line with an extrachromosome or chromosome pair from another species.

Alien gene introgression: Introgression of a gene from one species to another species.

Alien substitution line: Line in which chromosome or chromosome pair from a donor species replaces a chromosome or chromosome pair of recipient species.

Androgenesis: Formation of embryoids and plantlets from the pollen grains with the haploid set of chromosomes passing through typical stages simulating zygotic embryogenesis.

Aminoacids: Organic acids containing a basic amino group (NH_2) and an acidic carboxyl group (COOH). Some of the amino acids are added to plant tissue culture media, especially glycine and glutamine.

Amitosis: Division of nucleus into two parts without the formation of chromosomes, usually without cell division, so that the cell contians two or more nuclei.

Analogue resistance: Resistance to compounds related to, but slightly different structurally from, a biologically significant molecule.

Anergy: A process in which the exogenous application of hormones to culture medium will not alter the growth *i.e.,* the tissues grow without the exogenous application of hormones. Lack of an expected immune response.

Aneuploidy: A condition in which the cells will have chromosome number other than euploid number.

Aneusomaty: The occurrence of aneuploid cells in euploid tissues, organ or organism.

Anther: The pollen bearing part of a stamen.

Anther culture: Culture of anthers under *in vitro* conditions with an objective to produce monoploid plants.

Antiauxins: Compounds inhibiting the action of auxins. These compounds promote somatic embryo maturation by counteracting the effect of growth promoters. (*e.g.* 5-hydroxynitrobenzylbromide 7, azaindole).

Antibiotic: Complex chemical substance produced by microorganisms like fungi which are bactericidal or bacteriostatic.

Antibiotics: Substances that are toxic to microorganisms. They have the property to retard or arrest the growth of organisms in culture medium of the tissue to be cultured. A range of antibiotics are available for use to prevent microbial contamination in *in vitro* culture.

Antioxidants: Substances added to the medium to inhibit or prevent oxidative browning of the culture medium due to exhudation of phenolic compounds.

Antioxidant mixture: A mixture containing citric acid and ascorbic acid which prevents browning in plant tissue culture.

Anucleate protoplast: See cytoplast

Artificial seeds: Artificial seeds are novel analogues to botanic seed consisting of a somatic embryo surrounded by artificial seed coat.

Asexual embryogenesis: See adventive embryogenesis

Asexual reproduction: Reproduction which does not involve fertilization or fusion of dissimilar gametes.

Asymmetric hybrid: Somatic hybrid possessing asymmetric nuclear constitution due to the partial elimination of chromosomes from one species. In other words not possessing the exact haploid genomes of both the partners.

Autotrophy: Potentiality of organisms to manufacture their own food. The organisms may be photoautotrophs or chemoautotrophs.

Auxin habituation: The attainment of auxin autotrophy in prolonged cell cultures.

Auxotroph: An organism defective in synthesis of an essential metabolite and hence requiring a supply of that metabolite for growth, or an organism not capable of building up metabolites from the medium on which it grows.

Auxillary budding: Formation of buds from the axils of the leaves.

Auxillary bud breaking: Inducing the axillary buds in grow under *in vitro* conditions.

Auxins: A group of plant hormones inducing apical dominance, promote cell elongation, rather than cell division and increase cell wall plasticity. Auxins may be natural or synthetic.

Auxin synergists: The phenolic compounds specifically acting on the induction of roots in cultures synergetic to certain auxins.

Backcross: Cross between a hybrid and one of its parents.

Basal medium: Formulation of empirically constituted macronutrients, micronutrients, hormones, vitamins and other additives to culture plant tissues. Several basal media are available for culturing plant tissues of different plant species.

Batch culture: The cells are cultured in a finite volume of medium in which the growth of cells ceases when essential nutrients are exhausted.

Binary vectors: Vectors have a host Agrobacterium strain containing Ti plasmiddeleted of its T-DNA and a cloning vehicle containing the T-DNA repeats, a selectable marker active in plant cells, and a sequence containing multiple restriction sites. Generally these vectors have a wide host range.

Biochemical markers: Markers such as proteins, allozymes, isozymes and DNA level variation are used in inheritance and phylogenetic studies because of the lesser environmental influence on them.

Bioconversions: see Biotransformation

Biolistic method: See microprojectile bombardment.

Bioreactors: Facilities for the mass cultivation of plant cells as cell suspensions in volumes larger than those possible in shake flasks.

Bioregulants: See growth hormones.

Biosynthesis: Biological synthesis, the production of compunds by a living organism.

Biotic elicitors: Elicitors of organic nature obtained from biological organisms.

Biotransformation: Conversion of one compound into another in a biolgoical material by one or two actions (one or two step biotransofmration) or series of reactions (multistep biotranformation).

Broad host range: See host range.

Bud culture: *In vitro* culture of undeveloped or unemerged stem, leaf, or flower often enclosed by reduced or specialized leaves called leaf scales.

Calcium hypochlorite: Surface sterilant of explants used at 7% concentration (70 g of calcium hypochlorite in l litre of double distilled water).

Calcoflour white (CFW): A stain used to test viability of protoplasts 0.l% V/V solution is used. CFW stains newly formed cellwalls giving a ring of fluoscence around the plasmamembrane.

Callus: Undifferentiated mass of parenchymatous tissues from fresh wounds or tissues or cells cultured *in vitro.*

Callus culture: The cultivation of callus formed from explants on solidified or liquid medium.

Callus induction: Initiation of callus from explants under *in vitro* conditions using specific auxins.

Carrier material: Compound used as substrate or solidifying agent in culture media.

Caulimoviruses: Double stranded DNA viruses used as potential vectors for genetransfer. These viruses can be directly introduced into plants.

Caulogenesis: Formation of shoots from the explants or calli.

Cell: The fundamental unit of life with an outer boundary enclosing protoplasm and nucleus. In animals the outer boundary is plasma membrane. In plants it is cell wall followed by cell membrane.

Cell cooperativity: A phenomenon by which the autotrophic cells crossfeed (generally hormones) the non-autotrophic cells in the same culture.

Cell division: Division of a mother cell into daughter cells after karyokinesis and cytokinesis. The daughter cells will be two in mitosis and resemble the mother cells and four in meiosis and have half of the chromosomes of mother cells.

Cell generation time: Time interval between consecutive divisions of a cell.

Cell immobilization: Process of separation of growth phase of cells from production phase to prolong the use of cells in a stationary or very slow growing state so that the production of secondary metabolites is encouraged.

Cell isolation theory: Theory proposing that the embryogenesis is due to isolation of cells forming embryos from other surrounding cells.

Cell line : A heterogeneous group of cells derived from a primary culture. Any culture maintained in isolation constitutes an individual cell line.

Cell line divergence: Variation between independently maintained cell lines.

Cell line diversity: Variation between individually maintained cell lines.

Cell line selection: Selection of mutant cells *in vitro* from the callus or cell suspension cultures. Selection of potentially useful cell lines following culture-induced variation or mutagenization *in vitro*

Cell sorter: see flow cytometer cell sorter.

Cell sorting: Sorting out of cells, especially somatic hybrids from non hybrids by flow cytometry.

Cell strain: a cell line cloned from a primary culture or other cell line with specific properties differing from its source.

Cell typing: Identification specific cell types using probes such as lectins and antibodies.

Cellular cohesiveness: The degree of attachment of cells together to form clusters.

Chemical fusion: A method of inducing cell fusion by use of high concentration of various chemical fusogens such as PEG (polyethylene glycol) dextran, PVA (polyvinyl alcohol).

Chemical sterilization: Sterilization by using chemicals. It is adopted to sterilize working area, needles, and explants.

Chemostat culture system: Achieving steady state in culture by fixing the volume of biomass when biomass increases beyond a fixed limit, a control device operates and the new medium is added to the culture to dilute the biomass to the required value.

Chilling injury: Response of a tissue to reduction in temperature.

Chimeral callus: Callus composed of more than one kind genetically altered tissues. The alteration in genetic material may be at chromosome level or genome level.

Chlorinated lime: Calcium oychloride an unstable chlorine carrier; on exposure to air it becomes moist and rapidly decomposes.

Chromosome addition: Addition of chromosome(s) to the chromosome complement.

Chromosome rearrangement: Any structural change in chromosomes involving the gain or loss or relocation of chromosome segments.

Chromosome recombination: A condition resulting from the exchange of chromosome segments during the process of meiosis between homologous chromosomes.

Chromosome substitution: Replacement of one or a group of chromosomes by homologous or hemoeologous chromosomes from another strain of same species or related species.

Chromosomal aberrations: Irregularities in chromosome distribution structure or arrangement during cell division.

Chromosomal elimination: Elimination of chromosomes of any of the partners during the course of cell division, generally in the hybrid cells of unrelated species.

Chronic contamination: Contaminations in cultures with latent or symptom less types of viruses or mycoplasmas.

Clones: A population of cells derived from a single cell by mitoses or a group of plants propagated only by vegetative and asexual means, all members of which have been derived by repeated propagation from a single individual.

Coconut milk: The liquid endosperm of coconut used as an organic additive in tissue culture media. It is collected from several nuts, heated to 80°C with stirring filtered and stored frozen.

Coculture: Culture of protoplasts (2 to 3 days old) and *Agrobacterium* together for 36 to 48 hours. Then the cultures are washed with antibiotics to kill the bacteria followed by selection for transformants.

Co-immobilization: Immobilizing two different cell lines of which one could be a cell line with its ability to produce in quantity a precursor to a more valuable product synthesized by a second co-immobilized cell line lacking that synthetic capacity.

Colchicine: An alkaloid obtained from a plant species *Colchicum antumnale*. It inhibits the formation of spindles there by favouring chromosome doubling.

Cold shock: see chilling injury.

Cold storage: see low temperature storage.

Column bioreactor: An improved version of bioreactors in which instead of horizontal types, vertical type vessels are used to have better control over the supply of nutrients, a saving of space and greater suitability for scaling up to an industrial scale.

Column culture: Culture of cells using column bioreactors.

Competence: State of reactivity or capacity of cells to respond to specific stimuli. Competence refers to the transient state in which cells can be induced to be determined.

Competence loss: Loss in capacity of a cell to respond to specific stimuli.

Concanavalin A: A lectin derived from *Canavalia enoiformis*, chemical used to strengthen the attachment of protoplasts induced by polyethylene glycol. Abbreiviated as Con A.

Conditioned medium: Medium after supporting normal-high density growth for a period and harvested harvested medium is incorporated into fresh medium to increase the plating efficiency.

Continuous culture: Cells are cultured in medium throughout where the inflow of fresh medium is balanced by an outflow of the medium (closed continuous) or with the efflux of cells and spent medium (open continuous).

Cotransformation: The independent integration of two markers in one protoplast originally present in different DNA molecules added together to protoplast transformation medium. In other words,simultaneous transfer of two or more genes in a gene transfer system.

Cosmid: Plasmid into which lambda *cos* sites have been inserted as a result, the plasmid DNA can be packaged *in vitro* in the phage coat.

Counter selection: A method of selection wherein the variant cell lines are selected by using a counter selective agent. The counter selective agent kills the wild type cells and not the variant cell lines. For example,

CPW solutions: Composition of washing solutions.

Critical minimum inoculation density: A typical density (10^4 cells ml^{-1}) of cells below which cells in culture will not divide.

Cryobiology: Field of science dealing with ultra low temperature storage of cells and tissues.

Cryopreservation: Ultra low temperature storage of cells, tissues, embryos or organs. This storage is usually carried out using temperatures below -l96°C.

Cryoprotectant: A substance used in cryopreservation of cells and tissues. It prevents freezing and thawing damages to cells and tissues.

Cryoprotection: Stage of culture in ultra low temperature storage where cryoprotectants are added for the survival of high plant tissues.

Culture initiation: The initiation of proliferating cultures from tissue explants by providing suitable stimuli.

Cybrids: Hybrids having two different cytoplasms (cytoplasmic hybrids).

Cytodifferentiation: Differentiation of a cell as to its function and morphology from the parent or sister cells.

Cytokinins: A group of phytohormones having the properties of causing cell division, cell differentiation and suppression of apical dominance.

Cytokinin habituation: The attainment of cytokinin autotrophy in prolonged cell cultures. In other words, loss in requirement for the exogenous cytokinin in a prolonged culture.

Cytoplast: The cytoplasm surrounded by cell membrane but without nucleus.

Cytoplast-karyoplast fusion: Alteration in the nuclear or cytoplasmic genomes.

Cytovariant: Variant originated due to chromosomal alterations either in number or structure.

Deceleration phase: Phase in which the rate of cell division slows down.

Dedifferentiation: A process of formation of unorganized tissues from the highly organized tissue. The resumption of meristematic activity by mature cells.

Deionization: A method of water purification by which ionized impurities are removed from water by passing water through synthetic resins that exchange H^+ (cations) and OH^- (anions) for the ionized impurities. No sterilization or organic removal is accomplished.

Demineralization: See Deionization.

Determinate meristem: Meristems that are incapable of continuous proliferation and form organs of fixed size.

Determined tissue: A tissue destined to form a particular structure when placed in a new environment.

Differentiation: Development of physiologically and morphologically specialized cells from unspecialized cells. Refers to the formation of cells, tissues and organs or a process of conversion of simple tissues such as meristems into complex metabolically different tissues.

Differentiation medium: Medium used to regenerate plants from cells or callus. Usually has higher level of cytokinin.

Differentiation theory: Theory proposing that the embryogenesis cannot occur from the differentiated tissues. De-differentiation in cells is a prerequisite for the production of embryos *in vitro*.

Dihaploidy: A condition in which a cell, tissue or an organism will have diploid state derived from a haploid condition by chromosome doubling.

Dimethyl Sulfoxide (DMSO)): A cryoprotectant used as 1M DMSO in IM glecyrol and 2M sucrose (final concentrations are half of these values). DMSO gives an unpleasant penetrating odour and is best dispensed in a fume hood. Filter sterilization has to be adopted. Repeated freezing and thawing of DMSO is not recommended.

Diploid: A condition wherein the cells will have two sets of chromosomes.

Diplontic selection: Selection of cells or cell lines in diplophase.

Direct embryogenesis: Origin of embryos directly from the tissues cultured *in vitro* without any callus stage.

Direct gene transfer: Transforming cells with foreign DNA without any specialized vector.

Direct somatic embryogenesis: Embryogenesis without the intervention of a callus stage from explants.

Donor recipient method: A method adapted in cytoplasmic hybridization between two partners differing in respect to both nuclear and cytoplasmic characters.

Donor-recipient protoplast fusion: Fusion of protoplasts in which nuclear division has been arrested by X-or gamma irradiation in one and other remains normal. The fused heteroprotoplasts are regenerated into plants. In this system, the irradiated protoplasts serve as organelle donors, while the non-irradiated protoplasts act as recipients of the organelles.

Double fertilization: Fusion of male gametes one with egg to form embryo and other with polar nuclei to form endosperm.

Double layer technique: A technique developed to identify calli resistant to fungal toxins. In this technique respective fungus is grown in a layer of medium. After the growth, a second medium with

fungicide is overlaid on the fungus. The fungicide kills the fungus and the toxin diffuses to second layer medium on which callus culture is done.

Dry freezing: Storage of dehydrated tissues at low temperature.

Dry heat sterilization: Sterilization of media, solutions and water by high latent heat of steam. It is done under a steam pressure of 20 pounds per square inch at a temperature of 121°C for 20 minute.

Dye exclusion method: A staining method to estimate the viability of the protoplasts. The test depends on the ability of the plasmamembrane to block the entry of the dye. Live protoplasts will not be stained since the plasmamembrane does not allow the stain.

Electrofusion: Fusion of protoplasts by employing high DC electricfield pulses. Protoplast pairs are produced by an AC field induced dielectrophoresis. Also known as Zimmermann cell fusion.

Electrofusion mediated cell reconstitution: Fusion of a karyoplast and a cytoplast to reconstitute a cell by using microfusion.

Electroporation: A method of gene transfer in which solution containing protoplasts and foreign DNA is subject to electric pulses to induce gentle rupture of the membrane at places to form small reversible poes sufficient to take DNA molecules.

Elicitation: A process by which the production of secondary metabolites in culture is increased, because of the addition of bioproducts (fungal homogenates) or inorganic salts.

Embryo: An organism in the initial stages of development. In plants, the embryo is a miniature sporophyte derived from the zygote.

Embryo abortion: Prevention of further growth of formed embryo due to various postzygotic fertility barriers.

Embryo conversion: Successful germination of encapsulated somatic embryos into seedlings.

Embryo culture: The *in vitro* cultivation of embryos excised from ovules and seeds under aseptic conditions in a medium of known chemical composition.

Embryogenesis: The process of embryo initiation and development.

Embryo implantation: Implanting young hybrid embryos of one species into normal endosperms of another seed of the same or related species, to produce a plantlet *in vitro* later. The normal endosperm acts as nurse tissue. Also known as embryo-nurse endosperm transplant technique.

Embryo-nurse endosperm transplant technique: See embryo implantation.

Embryo rescue: A technique adopted to overcome the problem of post zygotic fertility barriers. In this technique, the embryos isolated immediately after fertilization are cultured *in vitro* to produce plants.

Embryo transplantation: See embryo implantation.

Embryogenic callus: Callus capable of forming embryos under cultural conditions.

Embryogenic cells: Cells capable of producing embryoids and inturn plants.

Embryoids: Small embryolike structures derived from tissue culture capable of producing individual plants.

Encapsulation: Wrapping an *in vitro* derived embryo by a suitable compound with an analogy to natural seed coat.

Endomitosis: Reduplication of chromosomes without the division of nucleus, thus the chromosome number is increased in a cell or chromosome duplication within intact nuclear membrane.

Endoreduplication: Duplication of chromatids of each chromosome during metaphase.

Endopolyploidy: Condition of polyploidy due to endomitosis.

Endopolyploidization: See endomitosis

Endosperm: A triploid nutritive tissue for the embryos in plants formed by the fusion of secondary nuclei and sperm nucleus.

End product inhibition: See Feedback Inhibition

Endosperm culture: *In vitro* culture of isolated endospersms.

Entrapment: A technique in cell immobilization see foam entrapment and gel entrapment.

Enucleated Microplast: See cytoplast

Enucleated Protoplast: See cytoplast

Enucleation: Removal of nucleus from a cell.

Enzymatic isolation: Isolation of protoplasts by digesting the cell walls with cellwall degrading enzymes.

Epigenesis: Developmental process whereby each successive stage of normal development is built upon the foundations created by the preceeding stages of development.

Epigenetic changes: Changes occurring regularly in response to specific inducers; potentially reversible; not transmitted meiotically.

Epigenetic variation: Phenotypic variability which has a non-genetic base.

Established cell lines: Cells that have been adapted to indefinite growth in culture.

Evacuolated protoplasts: See miniprotoplast

Evan's blue: 1% solution of Evan's blue is used for 5 minutes to stain the dead protoplasts (See dye exclusion method).

Exponential phase: Phase in which cells divide rapidly.

Expression vector: Cloning vector designed so that a coding sequence inserted at a particular site will be transcribed and translated into protein.

Fed-batch culture: The culture is established on a nutrient medium, and the nutrients are being added according to the cell growth during the period.

Feed back control: The switching off of any biosynthetic patway by its end product.

Feeder layer: A layer of mixture of protoplasts of different species inactivated but not killed by irradiation on which protoplasts to be cultured are plated.

Field gene banks. Long-term genetic conservation of plant species in the form of *in situ* or *ex situ* plantations and orchards, since the seeds from these species are recalcitrant.

Flatbed reactor: A cell immobilization system available wherein callus cultures are seated on a horizontal substratum of polypropylene fabric matting contained within a glass vessel. Liquid nutrient medium is supplied from a reservoir above the vessel.

Fluid drilling: A process of dispersing mature somatic embryos to suitable liquid carrier (eg. hydrogels) for germination.

Fluorescein diacetate (FDA): Chemical used to assess the cell or protoplast viability. The chemical actually measures the membrane bound esterase activity. FDA is prepared as a 5 mg/ml of acetone and stored at 4°C. To prepare a working solution 0.l ml of stock solution is diluted to 5 ml with distilled water.

Fluorescence activated cell sorting (FACS): In this process two parent protoplasts are separately labeled with vital stains: Fluorescin isothiocyanate (FITC) showing green fluorescence) and rhodamine isothiocyannate (RITC) showing red fluorescence). The hybrids are identified on the basis of showing two types of fluorescence.

Foam entrapment: Passive entrapment of cells in liquid suspensions in intrices of preformed polymers of meshes added to the cell suspension.

Formula weight: See gram molecular weight

Freeze preservation: See Cryopreservtion.

Freezing: Period of culture wherein cryoprotected specimens are usually transferred to a suitable container and maintained at sub zero temperature.

Friable callus: Callus of easily separable or fragmenting; preferred callus type to initiate cell suspension cultures.

Gametoclonal variation: Variation in clones regenerated from the cultures of haploid gametophytes.

Gametoclones: Plants regenerated from cell cultures derived from gametophytes.

Gameto-somatic hybrid: Hybrids between haploid protoplasts (from microspores microspecies) and a somatic cell protoplast.

Gel entrapment: See Active polymerization.

Gelling agents: Compounds used to solidify the culture media and in cell immobilization. (agarose, gelatin, carrageenan, copolymers of alginate or agarose and gelatin, Hypol 3000 (polyurethane) Polyphenyleneoixide.

Geminiviruses: Single stranded DNA viruses.

Gene banks: Long term storage facilities wherein representative stocks of seeds of crop species are stored. Periodic testing to detect any viability loss and replenishment by regrowth and harvesting are routine activities in these banks.

Gene expression: Manifestation of the genetic material of an organism as a collection of specific traits.

Gene transfer: Transfer of specific genes to a recipient cell lacking that gene by adopting *in vitro* culture and DNA technique.

Gene silencing: Expression of one gene is silenced or suppressed by the presence of other gene.

Gene targeting: Integration of a stretch of foreign DNA at a precise, pre determined site in the genome.

Genetic mutations: Mutations that are essentially irreversible.

Genetic variability: Phenotypic variability in individuals due to changes in genetic make up.

Generative cell: The smaller cell formed due to the division of pollen grain responsible for the production of male gametes.

Genome plastome incompatibility: Incompatibility between nuclear and cytoplasmic genes occurring in a somatic hybrid.

Genome segregation: Segregation of chromosomes according to the genomes, in a mitosis of polyploid.

Genomic shock: The shock exerted on the genomes of cultured cells during the culture period inducing mutation *in vitro.*

Genovariant: Variant originated due to mutation.

Germ plasm stores-active collections: Short term germplasm stores wherein the mateirals are multiplied, evaluated, indexed and then distributed.

Germplasm stores-base collections: Longterm germplasm stores wherein the materials are maintained and not distributed.

Gibbrellins (GAs): Plant growth hormones causing shoot elongation in intact plants.

Glassiness: See vitrification.

Globular embryo: Sixteen celled globular mass of cells from the zygote.

Gram molecular weight: Sum of the weights of the atoms in a substance expressed in grams.

Growth hormones: Organic substances in small amounts induce changes in plant growth and development eg. auxins and cytolicinins.

Gynogenesis: The development of haploid individual from female gametophyte.

Habituation: (See anergy)

Hairy root culture: Infection of plant roots with *Agrobacterium rhizogenes* produces abundant production of roots and is called hairy root disease. The abundant root production due *A. rhizogenes* is exploited in increased secondary metabolite synthesis *in vitro* and is called hairy root culture.

Hanging drop culture: The droplets of liquid culture are placed on the lid of petridish. The solution of culture medium is placed on the lower bottom of petridish. Then the upper dish with culture is oriented in such a way to touch the medium inverting the lid.

Haploid: A condition wherein the cells will have a single set of chromosomes.

Haploidy: A condition wherein cells possess only one set of chromosomes in their nucleus.

Haploid embryogenesis: see androgenesis

Hardening: See acclimatization

Heat sterilization: Destruction of microorganisms by using heat.

Heterokaryon: a cell having two genetically different nuclei.

Heteroploid: A condition wherein, the group of diplophase cells will have cells with varying chromosomes numbers of diplophase and the group of haplophase cells will have cells with varying chromosome number of haplophase.

Heterozygosity: A condition in higher organisms where the alleles at one or more loci will differ from each other.

Homokaryon: a cell having genetically identical nuclei.

Homogenetic induction: The process by which a cell induces a similar or identical phenotype in adjacent cells.

Homozygosity: A condition in higher organisms where alleles at one or more loci will be similar to their counterparts.

Hormone carry over: Carry over of hormones from one medium to another simply by adherance to the cells or as cellular pools.

Hormone habituation: Loss in requirement for the exogenous hormones in a aprolonged culture. In other words the capacity of cultured plant cells to continue their growth without the addition of exogenous hormones to the culture medium.

Hormone processing: A process by which the availability of hormones in plant cells is controlled. In this, conjugation of hormones to aminoacids and sugars occur controlling the availability.

Host range: The ability of the vectors to infect the hosts for an efficient gene transfer. Some vectors doo not have specific species barrier (Broad host range) while some are efficient vectors in certain species (narrow host range).

Hybridization: Crossing of two genetically different individuals to generate hybrid progenies.

Hybrid sorting: Selection of specific hybrid combinations for further doubling based on the array of variations available in the gametoclones.

Hybrid sterility: The inability of some hybrids to produce viable gametes or suppression of the reproductive capacity of F_1 between genetically different parents.

Iodoacetate: A chemical used for the inactivation of protoplasts. The pretreatment of protoplasts of with iodoacetate will cause the degeneration of non-fused and autofused protoplasts. This favours the selection of viable protoplasts hybrids.

Immobilization: A process by which the cells are made adhering to the matrices.

Indeterminate meristem: Meristems that are capable of indeterminate growth.

Indirect embryogenesis: Origin of embryos via callus stage.

Induced embryogeny determined cells (IEDC): The cells that need sosme induction to produce embryos.

Induced embryogeny: Potential of certain cells forming embryos *in vitro* only by the addition of suitable mitogeneic substances.

Induced fusion: Fusion of protoplasts induced by a variety of treatments.

Induction medium: Medium used to induce callus from explant.

Inoculam: Material introduced into a host or a medium.

Intra genome recombination: See mitotic crossing over.

Intraovarian pollination: See *In vitro* pollination.

Intergeneric hybridization: Crossing between two different genera.

Inter organelle competition: Competition between organelles of two

***In vitro* clonal propagation**: See *In vitro* micropropagation

***In vitro* culture**: Culture of cells or tissues under artificial conditions.

***In vitro* fertilization**: Effecting fertilization by introducing pollen grains directly to the ovary.

***In vitro* gene bank**: A facility for the storage of genetic materials where slow growth is adopted for short and medium term storage of shoot cultures and ultra low temperatures storage is followed for long term storage.

***In vitro* manipulation**: Manipulation of cells or tissues under artificial cultural conditions.

***In vitro* tuberization**: Production of mini tubers from explants to have better distribution of germplasm and seed tuber production.

***In vitro* ovular pollination**: Application of pollens to excised ovules.

***In vitro* placental pollination**: Application of pollens to ovules attached to the placenta.

***In vitro* pollination**: Pollinating *in vitro* cultured ovaries or ovules by introducing pollengrains.

***In vitro* propagation**: See micropropagation.

***In vitro* stigmatic pollination**: Application of pollen to the stigma *in vitro* cultured ovaries.

***In vitro* storage**: Storage of cells, tissues or organs under artificial storage conditions.

Isozymes: Enyzmes with the same function and some tissue with same activity but with different structure.

Karyoplasts: Sub-protoplasts containing nucleus and small volume of cytoplasm.

Lagphase: Phase in which cells regain the ability to divide in a fresh medium.

Leaf disc transformation: A method of transformation wherein leaf mesophyll cells that are still within the tissue of a leaf slice or disc are cocultivated with bacteria containing a transfer DNA.

Linear phase: Phase in which the rate of cell division is comparatively less than the rate in exponential phase.

Liposome: Microscopic lipid vesicle produced when phospholipids are dispersed in an aqueous phase.

Liposome coated DNA: The DNA coated with phospolipid vesicles. The possible advantage of liposome mediated delivery of DNA are low toxicity and protection of phospholipid coated DNA from degradation by nucleases present in the culture medium and cell.

Liposome encapsulation: Entrapment of DNA into phospholipids. The phospholipids from multilamellar vesicles (MLV) or unilamellar vesicles (ULV) depending upon the method of encapsulation.

Liposome fusion: Fusion of DNA containing liposomes with plasmamembrane of a protoplast to deliver the contents to the cytoplasm and nucleus.

Liposome injection: Delivery of liposome coated DNA into the vacuole. The vacuole delivered liposome fuses with the tonoplast and then passes to the cytoplasm.

Liquid shaken culture: Culture of stem cuttings with three to four nodes in liquid shaken culture to rapidly produce large numbers of nodes for single node cutting culture.

Low density growth media: Nutrient formulation which favours culture of protoplasts at low density.

Low oxygen storage: Growing plant tissues on a medium and covering the tissues with a thin layer of mineral oil. The tissues immersed in mineral oil continue to grow at a slow rate due to greater stability of oxygen.

Low pressure storage: Storage of plant tissues at reduced atmospheric pressure which results in increased gaseous exchange.

Low temperature storage: A method of *in vitro* germplasm storage at 9°C where the cultures are maintained at minimal growth conditions because of low temperature and minimal medium.

Macroinjection: Delivery of foreign DNA in the plant parts using injection needles.

Macronutrient: essential element normally required in concentrations more than 0.5 mmol.

Mass embryogenesis: The mass production of adventitious embryos in cell suspension cultures.

Mechanical isolation: Isolation of protoplasts by inducing plasmolysis and deplasmolysis to squeeze out the protoplasts through cut ends.

Membrane filtration: A method of sterilization by which most of the particulate materials and bacteria are removed. The membrane filters have pore size to the range of 0.20 pm .

Meristem: Cluster of small near isodiametric cells characterized by thin walls, high metabolic activity and the ability to divide sustainedly.

Meristemoids: Regions of high mitotic activity forming meristematic centres in a growing callus, later to form shoots or roots.

Meristerm culture: *In vitro* culture of meristematic dome tissue without adjacant leaf primordia or stem tissue.

Microcalli: Calli developed from individual protoplasts.

Microculture: The culture of fusion products obtained by microfusion. The fusion products are selected with the aid of the selection microcapillary connected to a microculture chamber.

Microfusants: Fusion between two subprotoplasts, a single cytoplast and a protoplast, a karyoplast and a protoplast, or a karyoplast and cytoplast.

Microinjection: Delivery of foreign DNA into defined plant cells using microcapillaries and microscopic devices.

Micromole (µmol or µM): One millionth of a mole or one thousandth of mmol.

Micronutrient: essential element required in concentrations less than 0.5 mmol.

Micronucleated protoplast: A micronucleus surrounded by some cytoplasm and a plasmamembrane.

Micronuclei: The treatment of cells with Amiprophos-methyl (APM) results in accumulation of metaphase chromosomes. The individual metaphase chromosome or groups of chromosomes, decondense and develop nuclear membranes. These are called micronuclei.

Microprojectible bombardment: Delivery of high density particles with DNA into cells by accelerating the particles to high velocity by a particle gun apparatus.

Microprotoplasts: Sub protoplasts containing minor fraction of cytoplasm and few chromosomes.

Micropropagation: Plant propagation by *in vitro* techniques by the application of nutritional and hormonal regimes under aseptic conditions.

Microspore: A haploid uninucleate male gametophyte formed from microspore mother cell.

Microspore culture: *In vitro* culture of microspore.

Millimole (mmol or mM): One thousandth of a mole.

Minimal cell density: Minimum number of cells needed to have favourable growth on a medium, since growth of cells is a function of an equilibrium between an inflow of substances from the medium to cell and an outflow of substances from the cells to the medium.

Minimum plating density: Inoculation of protoplasts to a certain level to achieve sustained growth of protoplasts in culture.

Miniprotoplast: Nucleus surrounded by some cytoplasm and a plasmamembrane.

Minoploidy: Occurence of more than one chromosome number in a group of cells.

Mitotic arrest: Preventing cell division.

Mitotic crossing over: A process occuring in mototic cells favouring recombination within a genome.

Mneomovariant: Variant originated due to Non-Mendelion but heritable change asymmetrically transmited in reciprocal crosses.

Mobile DNA elements: DNA elements such as plasmids, episome, insertion sequences or transposons. These can insert into mtDNA, cpDNA or nuclear DNA causing epigenic variation.

Molarity (M): Number of moles of a substance contained in one litre of a solution.

Molecular weight: See gram molecular weight

Monoploid: The basic number of a polyploid series.

Monohaploids: Progenies possessing half the numer of chromosomes from a diploid species.

Morphogenesis; Origin of form associated with differentiation of all internal cells and tissues.

Morphogenetic competence: Refers to the capacity of the cell to form an organised structure.

Morphogenetic potential: The ability of the cells to undergo differentiation to attain an organised from.

Multipolar spindle: Formation of spindles from different poles of a dividing cell, causing irregular segregation of chromosomes.

Multistep biotransformation: See biotransformation

Mutant: An individual resulting from a mutation.

Mutant cell lines: Cell lines differing genotypically from parental plant or cell culture.

Mutation: Any sudden detectable and heritable change in an organism.

Natural hormones: Hormones isolated from living organisms.

Narrow host range: See host range.

Net entrapment: See foam entrapment

Non-disfunction: Failure of chromatids to move to opposite poles during mitosis or meiosis.

Non-oncogenic vectors: Vectors that do not produce tumours in transformants which allow the regeneration of transformed phenotypically normal plants.

Non embryogenic callus: Callus which is not having the competence to form plantlets.

Novel variations: Variations either do not exist or rare in the natural gene pools.

Nuclear cytoplasmic incompatibility: See Genome plastome incompatibility.

Nucleoplast: See miniprotoplast.

Nucleate cytoplast: See miniprotoplast.

Nucleate microplasts: See miniprotoplast

Nucleated miniprotoplasts: See miniprotoplast

Nuclear fusion: Fusion of two or more nuclei to form polyploids.

Nuclear fragmentation: Nuclear division wherein the chromosome distribution was not equal resulting odd polyploid series (3n, 5n, 7n etc.).

Nurse callus: Callus of one species or variety used as a feederlayer to culture other species or variety.

Nurse culture: Culture of plant cells or tissues on already established callus layer of different species or variety with a filter paper in between them.

Nutritionmedium: Culture medium with all the macro and micro nutrients including all the other compounds including phytohormones. Used for in vtiro culture.

Oncogenic vectors: Vectors causing tumour growth in the transformants and phenotypically normal plants cannot be regenerated from such transformants.

One or two step biotransformation: See biotransformation

One to one microfusion: Predictable transfer of partial genomes by using subprotoplasts (cytoplasts and karyoplasts). See microfusion.

Organ cultures: The culture of isolated organs.

Organelle transfer: Transfer of organelles from one protoplast to another by protoplast fusion (see donor-recipient protoplast fusion).

Organic additives: Organic substances added to culture media such as aminoacids, nitrogenbases, organic acids, vitamins, sugars and sugar alcohols.

Organogenesis: De novo origin of organs, either shoots or roots from the cultured tissues.

Osmoticum: Compounds used in plant tissue culture to maintain a balanced osmotic pressure between cell interior and exterior to avoid stress on cells. The osmotic pressure is manipulated by adding various sugars or sugar alcohols.

Packed cell volume: Millilitre cell pellet per millilitre of culture. This can be determined by transfer of known aliquot of cells to a graduated tube, centrifuging the contents at 2000 g for 5 minutes.

Parafilm: Parafinned paper which comes in a sterile roll used to seal the culture vessels.

Parasexual cycle: Systems by which genetic recombinations can be achieved, i.e. without regular alternation of generations. In other words, life cycle enabling non-meiotic recombination.

Parasexual hybridization: Hybridization by means other than through fertilization of gametes, hybridization by non-sexual methods.

Particle gun method: See microprojectile bombondant.

Partitioned petridish technique: A petridish is made into 4 quarters. Fungus is grown in two opposite quarters. Medium supplemented with fungicide is added in other two opposite quarters. This prevents fungal growth. The fungal toxin diffuses from other two quarters and callus culture is done for screening.

Passage: see subculture

Passive entrapment: See foam entrapment

PEDC: The cells that are already committed to embryonic development.

PEG method: The DNA transformation procedure wherein protoplasts are incubated with polyethylene glycol followed by incubation at high calcium ion concentration.

Perfusion culture: A method of continuous culture in which, the volume of the medium is kept constant, with part being continuously replaced by fresh medium.

Periodic imersion culture: Suspension culture wherein the cells are alternatively submerged in culture solutions.

pH - Hydrogen ion concentration; (one litre ofpure water contains l/l0000000 moles of hydrogen ions or $l0^{-7}$ moles of hydrogen ions (=pH7).)

Phenosafranine: A stain used to test viability of protoplasts. A concentration of 0.00l% solution is used. The protoplasts preparation treated with 0.0l% phenosfranine red staining in dead ones and viable protoplasts will remain unstained.

Phenovariant: Variant originated due to epigenetic variation.

Phytohormones: Chemical substances produced by plants, which in low concentrations regulate plant physiological process.

Plasmids: Circular extra chroimosomal DNA molecules present in prokaryootes and some yeasts. Used as potential vectors in genetransfer.

Plasmone: Extrachromosomal genomes.

Plastome: Chloroplast genome.

Plating efficiency: Number of cell colonies formed to the total number of cells plated on a medium, expressed in percentage.

Phages: Cloning vehicles for genomic eukaryotic DNA.

Physiological variability: Phenotypic variations that are produced due to an altered environment and are ephemeral and will persist only in that environment, disappearing when normal conditions are restored.

Pollen embryogenesis: See androgenesis

Pollen embryogenic pathway: The developmental pathway of pollen embryos.

Pollen grain: Transformed microspore into uninucleate vacuolate structure with an spindy or sculptured exine and smooth intine.

Polyaccrylamide: A gelling agent for cell immobilization.

Polyethylene glycol: A chemical used to induce fusion between two protoplasts. The protoplasts are brought in physical contact by agglutination induced by polyethylene glycol.

Polyhaploids: progenies possessing half the number of chromosomes from a polyloid species.

Polyphenol oxidation: Oxidation of polyphenolic compounds in the explants by polyphenol oxidases making the explants brown or black. Addition of antioxidants will reduce this problem.

Polyploidy: The condition in which the somatic cells will have more than two sets of chromosomes.

Polysomaty: The condition in which the chromosomes in some of the somatic cells of a tissue are present in multiples of the somatic chromosome number.

Polyvinyl alcohol: A chemical used to increase the fusion frequency among protoplasts in combination with 0.05 M calcium chloride and 0.3M mannitol.

Population doubling time: Time taken to double a population of cells.

Post-fertilization barriers: See post zygotic barriers.

Post zygotic barriers: The processes preventing the zygote to form a viable embryo.

Precocious germination: Growth of cultured immature embryos into rudimentary weak seedlings in the medium.

Precursor feeding: Providing an initial or intermediate precursor of a secondary biosynthetic pathway to increase the final product *in vitro*.

Predetermination theory: Theory proposing that the embryogenesis is a predetermined phenomenon in specific cells. In other words, embryogenesis is an inherent potential of certain cells.

Predetermined embryogeny: Potential of certain cells forming embryos *in vitro* is determined before mitosis.

Prefertilization barriers: See prezygotic barriers.

Pregrowth: A period of culture after dissection of explant and before freezing.

Pregrowth medium: Medium used to culture the cells or tissues to be cryopreserved.

Prezygotic barriers: The processes preventing fertilization.

Primary culture: culture of cells or tissues directly from organisms in other words culture initiated first time.

Primary metabolism: The metabolic processes which are vital for the life of an organism and form the base for the other pathways in the body (e.g. Photosyntehsis and Respiration).

Proembryonic cluster: Cluster of cells determined to form an embryo.

Protoclones: Plantlets obtained from protoplasts.

Protoplast: A plant cell without cell wall.

Protoplast culture: The isolation and culture of plant protoplasts, *in vitro*.

Protoplast isolation: Isolation of protoplasts, i.e. cells without their cell walls by mechanical or enzymatic methods.

Protoplast plating efficiency: Ratio ofdividing protoplasts to initial number of plated protoplasts expressed in percentage or Proportion of initially plated protoplasts that proliferated to microcallus stage expressed in percentage.

Protoplast purification: Recovery of debris free protoplasts after the enzymatic treatment of cells by filtration, centrifugation and washing.

Prototroph: An organism capable of building up its metabolites from the medium on which it grows.

Rapid freezing method: The material in storage vials are directly plunged into liquid nitrogen.

Recovery medium: Medium used to regenerate cryoprotected cells or tissues.

Redifferentiation: The process of differentiation occuring in an undifferentiated tissues.

Regeneration: Genesis of an entire plant from cultured explants directly or via indirectly from a callus.

Relative plating efficiency: ratio of individual cells inoculated producing colonies to the cells producing colonies in a control culture.

Relative transformation frequency: Ratio of transformed clones surviving after selection to the number of clones in an unselected aliquot. (Gene Transfer).

Reporter genes: Genes those which encode easily detectable enzymatic activity and can supply information concerning the regulation or action of sequences from a different gene.

Resistance selection: The selection method where the variant phenotypes are selected based on the resistance to either an anti metabolite in the culture medium or adverse culture conditions as low temperature.

Restitution nucleus: Nucleus formed due to endomitosis wherein chromosome duplication occurs within intact membrane due to failure of spindle formation.

Reversibility: Reactivity of new phenotypes to attain original status.

Rhizogenesis: Formation of roots from the explants or calli.

RIM: Root inducing medium.

Ri plasmid: Circular DNA present in Agrobacterium rhizogenes responsible for causing abundant root growth (hairy root disease).

Rooting: Inducing root formation by subculturing regenerated shoots in medium without cytokinin or by treating the shoots as conventional cuttings after removal from sterile culture.

Rotary culture: Culture of cell suspensions involving slow rotation. The culture vessels are mounted on a circular tumble wheel-type platform. The culture vessels are either tuber (for a small volumes) or nipple flasks (for larger volumes).

Screenable markers: Transformants are screened based on the expression of the market gene to the specific compounds constructs. Both transformants and non-transformants survive in the media. Non-transformants do not show any expression of the gene construct e.g. gluco\uronidase and luciferase (GVS). In other words, markers which facilitate their detection by the presence of their gene products.

Scorable markers: see screenable markers.

Secondary culture: Culture established from cells of primary culture.

Secondary metabolism: The metabolic processes that are subpathways of primary pathways.

Secondary metabolites: The products resulted from the secondary metabolism.

Selectable marker: Markers (DNA level) expressed at a particular cellular stage t allow selection of transformed cells with a selectable change in the phenotype of the transformed organism.

Semi continuous culture: The cell culture system wherein, the medium is periodically drained and replaced with fresh medium.

Semisolid medium: Medium solidified with agar or other solidifying agents.

Sequential method: A method in enzymatic isolation of plant protoplasts wherein the cells are treated with pectinase first followed by cellulase.

Sexual sterility: Sterility that occur in the progenies of two parents (plants) due to preygotic and postzygotic fertilization barriers.

Shake culture: Culturing cell suspension on an orbital shaker at the speed of 60-70 rpm with an orbital movement of 2 to 5 cm range.

Simultaneous method: A method in enzymatic isolation of plant protoplasts wherein the cells are treated both pectinase and cellulase are mixed and used.

Single node cutting culture: Culture of single nodes with leaves excised from small *in vitro* plantlets. A method of micropropagation.

Slow growth: A phenomenon exhibiting slow growth in cultured cells when they are maintained at low temperature.

Slow freezing method: The tissues are brought to - l00^{O}C, with a cooling rate of 0.l to - l0^{O}C/minute and transferred to liquid nitrogen.

Sodium hypochlorite (0.5% to l0.0%) Surface sterilant of explants used at 0.5% to 20.0% concentration.

Solid phase cultures: Growth plant cells as callus.

Solidifying agent: The compound used to solidify the tissue culture media.

Somaclonal variation: Variation originating in cell and tissue cultures.

Somatic embryogenesis: De novo origin of embryos from the somatic tissues in culture by a developmental pathway resembling the zygotic embryo formation.

Somatic incompatibility: Incompatibility existing in somatic hybrids, leading to alteration in genetic constitution of a somatic hybrid.

Sonication method: Transfer of DNA is effected by subjecting the sonication buffer containing tissues and foreign DNA to ultrasonic pulse generator at 0.5 c/cm^2 acoustic intensity for 30 minutes.

Spheroplasts: The bacterial protoplasts with remains of cell wall after the lysosyme treatment. Te spheroplasts with recombinant plasmids are introduced into the plant protoplasts.

Spin culture: Culturing cell suspension in an arrangement inclined at 45^{o}C one or two culture vessels (bottles of l00 litre capacity) are rotated mechanically at the rate of 80-l20 rpm.

Spontaneous fusion: Fusion of protoplasts without any fusants or fusogens.

Stable gene expression: Consistent expression of the gene in the transformants.

Stable regenerating callus: Callus having sustained regeneration potential even after many subcultures.

Stability: Maintenance of new phenotypes obtained after determination in competent cells.

Stage 1: Selection of suitable starting materials and their sterilization and transfer to culture media in a tissue culture process.

Stage 2: Stage representing callus formation and proliferation.

Stage 3: Stage representing regeneration from cultured callus.

Stage 4: Stage representing transfer of regenerated plantlets for

Stationary phase: Phase in which the cells cease dividing.

Stepwise freezing method: Method involves slow cooling -20^{o}C-40^{o}C followed by rapid cooling to - l96^{o}C or rapid cooling to subzero/temperature followed by another rapid cooling to-196^{o}C with a gap between. (See slow cooling and rapid cooling).

Sterilization: Removal of microorganisms already present in explants, culture vessels, media, stock solutions etc.

Stir culture: Culture of large volume of suspension with stirring by magnetic stirners rotated at 200-300 rpm or supplying compressed air (5 to l0 lb/square inch) from the top through a sterile inlet.

Storage: A step in cryopreservation in which tissues are stored in liquid nitrogen cooled refrigerators.

Strain: Chemical or physical change in organisms due to a stress. The strain may be elastic (reversible) or plastic (permanent).

Stress: any environmental factor capable of eliciting a harmful chemical or physical change in organisms.

Stress avoidance: The capacity of the organisms to exclude the stress and thus avoiding its potential strain.

Stress tolerance: Increased ability of an organism to overcome the strain produced by the stress.

Subculture: Transfer of culture from one vessel to other.

Subculture interval: Interval between two subsequent subcultures.

Surface disinfectants: Compounds used to remove the organisms contaminating the explants.

Surface sterilant: A compound used to surface sterilize the explants.

Surface sterilization: Sterilization of explants to remove wide range of microbial contaminants from this surface.

Surfactants: see surface disinfectants.

Suspension cultures: The culture of isolated cells or very small cell aggregates remaining dispersed in liquid culture.

Synthetic hormones: Hormones synthesized from various synthetic compounds.

T-DNA: The DNA of Ti-plasmid transferred to plants resulting in tumour formation and opine synthesis (Homologous to T-region of Ti plasmid.

Ti plasmid: Circular DNA present in Agrobacterium turnifaciens responsible for causing tumurous growth (crown gall disease).

Tissue culture: Techniques involving aseptic culture of plant organs, tissue cells and protoplasts. Each type of culture requires slightly different methods but the principle of culture is the same.

Tissue grafting: Establishing cultures from non-responding tissues by grafting them to responding tissues such as cambium.

Torpedo shaped embryo: Embryo with the clearcut root apex with considerable cell differentiation and elongation in cotyledons and hypocotyl.

Total selection: Screening of large number of individual colonies, applying appropriate tests to establish their phenotype. It is generally a tedious approach.

Transient gene expression: Gene expression with an early maximum followed by a subsequent decline.

Transdetermination: Change in determination of tissues from one state of competence to another.

Transduction: Process of gene transfer that is mediated by a bacteriophage.

Transformation: Process by which the foreign naked DNA is introduced into cells to get an altered phenotype.

Transformation frequency: Ratio of transformed clones to the total number of clones in a culture.

Transgenosis: Phenotypic changes of plant cells brought about by transducing phages (Syn. Transduction of bacterial cells).

Triphenyultetrazoluim chloride (TTC): TTC is used to assess the viability of cell or protoplast. The viability is detected based on the colour development in line cells. The colourless TTC solution is reduced to water insoluble red formazan by dehydrogenase activity or mitochondual activity. The dead cell cannot reduce the TTC to red colour formazan since the above activities.

Tubular hollow fibre membrane bioreactors: Use of tubular hollow fibres (eg. cellulose acetate or silicon polycarbonate, organised in parallel bundles within the bioreactors.

Turbidostat culture system: Achieving steady state in culture by adjusting the rate of dilution or concentration of a limiting nutrient medium provided other factors are not limiting.

Two-stage culture: Culture method developed for the production of specific compounds *in vitro*. In the first stage maximum cell proliferation is achieved and then the cells are allowed to produce the compound at the maximum rate in the second stage tank.

Ultra-low temperature storage: Storage of seeds in liquid nitrogen (-l96°C). This eliminates the need for testing and replenishment.

Unidirectional sorting of organelles: Segregation of organelles belonging to a particular cytoplasm as a group during the further divisions of a somatic hybrid.

Variant: A variant cell line is one which differs in some observable respect, phenotypically from the normal population.

Variant cell lines: Cell lines differing phenotypically from the parent plant or cell culture.

Vectors: DNA molecules used as cloning vehicles to transfer passanger DNA. These DNA molecules in conjunction with the passanger DNA forms the recombinant DNA and are transferred to host cells. These vehicles possess autonomous replication, unique-cleavage sites to integrate foreign DNA and markers to select recombinants.

Vegetative cells: The larger cell formed due to the division of pollen grain. In normal cases, it forms pollen tube.

Vegetative propagation: Propagation of organisms without involving any sexual phenomenon.

Virus indexing: Process of assessing the presence or absence of viruses in the progenies of virus eradicated materials.

Visual selection: Selection of cell lines based on difference in phenotype.

Vitrification: A condition in which the regenerated plants in culture become irreversibly translucent with varying degrees of distortion and swelling, sometimes followed by necrosis and death otherwise called glassiness or water soaking.

Water soaking: See vitrification.

Zygotic embryo: The embryo formed from the zygote, a fusion product of egg cell with sperm nuclei.