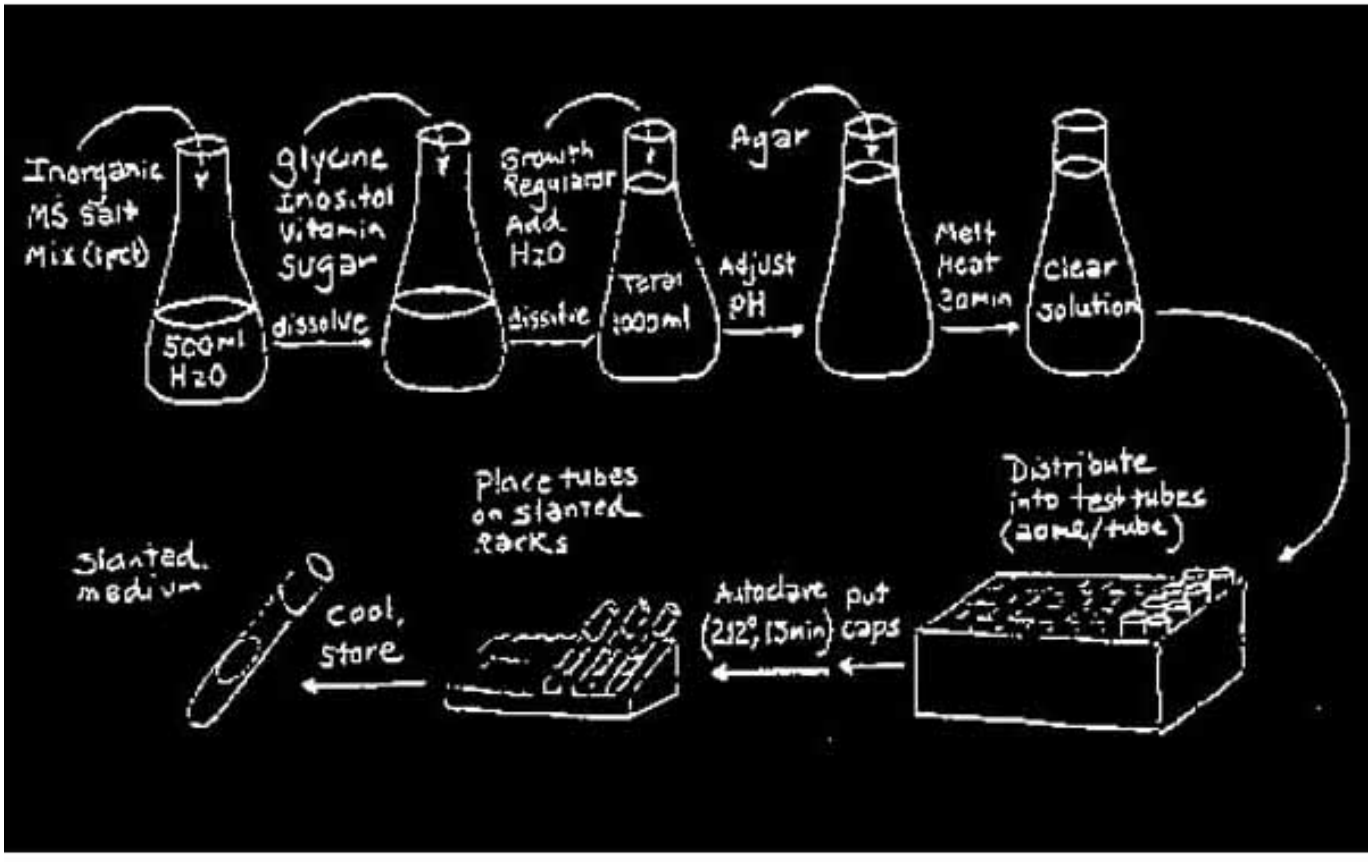
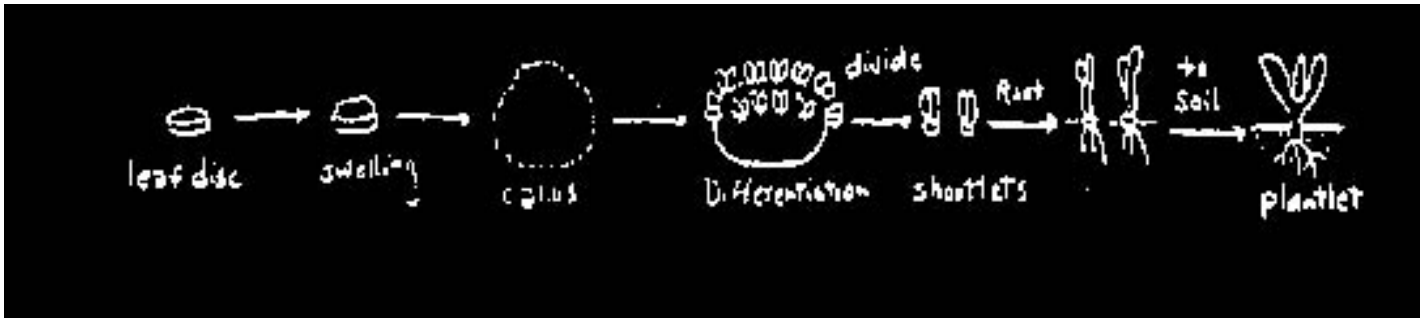
PLANT TISSUE CULTURE

Plant tissue culture may be defined as a process whereby small pieces of living tissues explants are isolated from an organism and grown aseptically on a nutrient medium under controlled conditions

Two concepts viz., plasticity and totipotency are important for understanding plant cell culture and regeneration.





TOTIPOTENCY

It is the genetic potential of a plant cell to produce the entire plant and the ability of a plant cell to perform all the functions of development which are characteristic of zygote

PLASTICITY

It is the ability of the plants to adapt to environmental conditions by altering their metabolism, growth and development to t suit their environment.

When plant cell and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity which allows one type of tissue or organ to be initiated from another type .

History of plant tissue culture

The history of plant tissue culture begins with the concept of the cell theory given independently by Schleiden 1838 and Schwann 1839 which implied.that the cell is a functional unit. The first plantlet formation *in vitro* was reported as early in 1940s Ernest Bell (1946) reported it in Tropaeolum and Lupinus. It was only during 1960s that Morel reported plantlets formation in orchids , which became commercially viable programme

1838 & 1839	Schleiden and Schwann	Cell theory, cell is the functional unit of living organisms
1839	Habertland (Father of tissue culture)	
1939	Nobecourt	Cultivating plant tissues for unlimited period
Gautheret	1955	Totipotency Establishment of habituated cultures, , tumour cells, importance of light and

		temperature for the root growth Two important functions 1. the meristem of plant tissue cultures 2. plant tissue culture
Georges Morel		First to culture monocotyledonous tissue, meristem tip culture for elimination of virus disease of orchids, discovered two unique opines of crown gall tissues, Established about protocom
1934	White	First reported for the first time successful continuous cultures of tomato root tips in liquid culture and obtained indefinite growth., in vitro cultivation of viruses on excised roots and growth of tumour tisues, Important publication 1.the cultivation of animal and plant cells
Caplin and Steward	1948	Use of coconut milk for the first time, cell cultures of single cells, various types of vessels for culture work like rotating nipple flasks,
Steward and Ammirto	1969	Somatic embryogenesis in carrot for the first time, , completely established the totipotency, Important publication
		Volumes in “plant physiology”
Reinert	1959	Bipolar embryo formation in carrot and showed embryogenesis, , cryopreservation of cells, regeneration of plantlets, Important publication First state of art in plant tissue culture “Plant cell, tissue and organ culture”
Vasil and Vasil	1965	Land mark work on single isolated cells of tobacco which

		paved way for cells cuttures in genetics, morphogenesis and vegetative propagation and proved totipotency in cells, has contributed significantly for cells culture, protoplast, somatic embryogenesis of cerals
Maheswari	1964-1967	Experimental embryology Important publication An introduction to the embryogenesis Phytomorphology
Guha and Meheswari		Development of haploids through anther and pollen culture for the first time

ORGANOGENESIS

Organogenesis means the development of adventitious organs or primordial from undifferentiated cell mass in tissue culture by the process of differentiation. This is 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 connected to the parent tissue. Morphological changes in the callus leading to the *denovo* organ formation from undifferentiated tissues are termed as organogenesis.

.This system is commonly produced in callus cultures, but can be produced directly from the *explant.* It is effected through a balance between the levels of auxins and cytokinins. A relatively a higher proportions of auxins are required for root induction and for the shoot induction a higher level of cytokinins are required. It is usual to induce shoot formation by increasing the cytokinin to auxn ratio of the culture medium. These shoots can then be rooted in auxin rich medium.

Organic connection between shoot and root primordial is essential for the regeneration of complete plantlet from the same culture. Shoot formation followed by rooting is the general characteristic of organogenesis . Organogenesis is unipolar in structure and there is vascular connection with explant and not easily separated unless cut off.

de novo It literally means “arise a new”. New plants arising from unorganized cells or tissues to form unorganized cells and tissues

Cytodifferentiation

In plant tissue culture during growth and maturation of the callus tissue or free cells in suspension culture few dedifferentiated cells undergo cytosenescence and redifferentiated to vascular tissues. The development process is termed as cytodifferentiaton.

Caulogenesis

It is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus culture

Rhizogenesis

It is a type of organogenesis by which only adventitious root formation takes place in the callus tissue

Organoids

In some cultured tissues , an error occurs in the development programming for organogenesis and an anomalous structure is formed. Such anomalous organ like structures are called organoids

Meristemoids

Meristemoid is a localized group of meristamic cells that arise in the callus tissue and may give rise to shoots and or roots

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

Precocious germination

Premature germination of the embryo radicle prior to completion of embryogenic development.

Dedifferentiation

The phenomenon of the conversion of mature cells into the meristematic state leading to the formation of callus is called dedifferentiation

Protocom

In nature, little differentiated structures developing naturally on orchid embryo which help in propagating the orchids. Through *in vitro* culture also, from cymbidium shoot tip cultures , protocoms could be produced which again could be sectioned into quarters and subculutred, each section regenerating a new protocom, within a few weeks which, in turn , could be divided. The so obtained protocom subsequently evolved in to young plantlets.

Adventitious buds:

Shoot buds originating from other than existing meristem are known as adventitious shoot buds.

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Differentiation

The process of biochemical and structural changes by which cells especially the unroganised cells becoming specialized in form and function

Redifferentiation

The component cells of callus have the ability to form a whole plant is called redifferentiation

These two phenonmenons of dedifferentiation and redifferentiation are inherent in the capacity described as cellular totipotency . This property is found only in plant cells and not in animal cells

Xylogenesis

It is the differentiation of parenchyma into cells that have localized secondary wall thickeninings as seen in the xylem and vascular plants

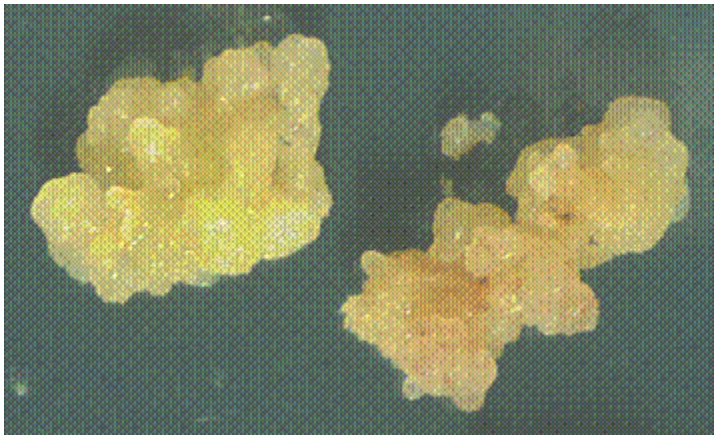
Factors affecting Regeneration

- source of explant
- nutrient media and constituents
- plant growth regulators

CALLUS INDUCTION AND MAINTANANCE

What is a callus?

It is an unorganized or undifferentiated mass of proliferative cells produced either in culture or in nature



What is callus culture ?

Tissues and cells cultured on an agar medium form an unorganized mass of cells i.e., callus

Callus formation from explant involves the development of progressively more random planes of cell division , less frequent specialization of cells and loss of organized structures.

Callus culture is often performed in the dark and the light can encourage differentiation of the callus.

Micro callus

The initial colony of cells visible but too small to transfer by direct manipulation recovered from cultured of protoplasts single cells or very small aggregate cells.

Induction and maintenance

When freshly cut pieces of surface sterilized plant tissues are grown in agar medium with appropriate nutrients with suitable proportion of auxin and cytokinin they exhibit callusing at cut ends, which gradually extends to the entire surface of the tissue. Callus cultures need to be subcultured every 3 – 5 weeks in view of cell growth , nutrient depletion and medium drying. Repeated subculture on an agar medium improves the friability of the callus

Habituation of callus tissue

In some plant species the callus tissue is able to grow on a standard maintenance medium or basal medium which is devoid of growth hormones. This property of the callus tissue is known as habituation and the callus tissue is known as habituated callus tissue.

During the long term culture, the culture may lose the requirement for auxin and or cytokinin.

What is subculture?

Subcluture is the transfer of cultures with or without dilution from one culture vessel to another containing fresh culture medium . It is also known as passage.

The callus tissue in many cases shows a high potential for organogenesis when first initiated but gradually a decline sets in as subculture proceeds with eventual loss of organogenic response. The genetic effects in a callus tissue are reflected in changes of chromosomal structure or number such as aneuploidy , polyploidy , cryptic chromosomal rearrangements.

Callus culture are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from

which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant regeneration studies.

Applications of callus culture

1. to study nutrition requirement of plants
2. to study cell and organ differentiation and morphogenesis
3. somaclonal variations and its exploitation
4. developing cell suspension cultures and protoplasts cultures
5. genetic transformation using ballistic particle gun technology
6. in the production of secondary metabolism and regulation

SELECTION OF EXPLANTS

Appropriate selection of explants is very important for rapid *in vitro* propagation

The genotype, age and physiological state of the explant respond differently to cultures.

Selection of mother plant

It is necessary to select the mother tree/plant with the care. The explants should be taken from mother plants possessing superior phenotypes such as disease resistance , stress tolerance , high yield and product quality. Explants should proliferate readily. Even materials with strong ability to proliferate should be screened continuously

The following aspects decide the size of explant

1. the surface size
2. volume
3. cell number

. Shoot tips of 2mm size only respond well in *Manihot esculenta* whereas in *Dianthus caryophyllus* if the shoot tip size is too small i.e.., 2mm , there is lesser response or only the root are induced and not the shoots When eradication of viral infection is the main objective, very small meristem should be used

location of explants

Position of explants i.e., developmental stage is very important. In shoot tip culture of *Rosa rugosa* the success rate of terminal bud culture was higher than the using the lateral buds

season and time of explant collection

the season during which the bud is collected is another factor to be considered. In general buds about to sprout are suitable for culture. For embryo culture the dormancy state of seeds should be considered

CELL SUSPENSION CUTURE

How single cells are produced?

Callus cultures fall into two types viz., compact and friable. In compact callus the cells are compactly and densely arranged and in friable type the callus cells are loosely arranged and they are soft and breaks apart easily. Friable callus provides inoculum for cell suspension culture when friable callus is placed in liquid medim and agitated single cells are released in the medium.

What is a cell suspension culture?

It a type of culture in which single cells or small aggregates of cells multiply while suspended in agitated liquid medium . It is also referred to cell culture or cell suspension culture

What is plant cell culture?

It is an *in vitro culture* of single or relatively small groups of plant cells i.e., the callus culture

Why plant cell suspension culture?

Establishment of single cell cultures provides and excellent opportunity to investigate the properties and potentialities of plant cells and for mutation induction.

Spatial heterogeneity

The individual cells within a population of cultured cells invariably show cytogenetical and metabolic variations depending on the stage and growth cycle and culture conditions, such variables is termed as spatial heterogenity.

Critical Initial Density (CID)

The particular initial cell density that is able to grow in liquid medium is called critical initial density.

Different categories of suspension culture

Broadly speaking there are three types of suspension cultures

1. batch culture
2. continuous culture
3. immobilized cell cultures

Batch culture

It is a suspension culture where the cell cultures are maintained in a definite volume of agitaged liquid with repeated subculturing of a small aliquot of cell culture to a fresh medium at regular intervals. Generally cell suspensions are grown in flasks (100 – 250 ml) containing 20 –75 mil of the culture medium incubated on orbital platform shakers at the speed of 8- 120 rpm..

After subculture the cells divide and the biomass of the culture increases in a characteristic fashion, until nutrients in the medium are exhausted and/or toxic by - products build up. . The cell number of a batch culture exhibits a typical sigmoidal curve containing

1. a lag phase (during which period the cell or biomass remains unchanged
2. a log phase , an exponential phase which includes rapid increase in cell numberdue to active cell division
3. stationary period during which period the cell number does not changedue to the depletion of nutrients in the media and or by toxic substances

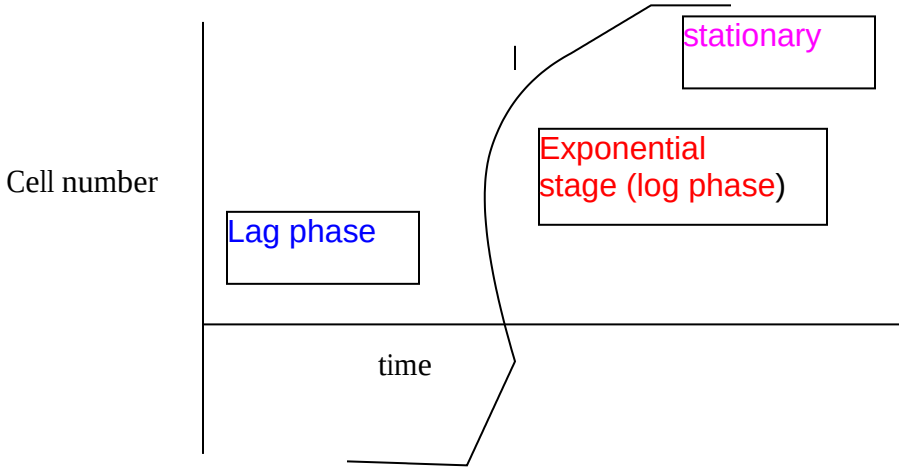


figure:. Model curve showing different growth phases in batch culture

Batch cultures are characterized by a constant change in the pattern of cell gwoth and metabolism.

Uses of batch culture

a) for cloning

b) for cell suspension

Continuous culture / mass culture

In continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the used or spent medium by fresh medium

There are two types of continuous culture types

a. closed continuous culture
b. open continous culture

closed continuous culture

cells are separated from the used medium taken out for replacement and added back to the culture so that the cell biomass keeps on increasing

open continuous cultures

both cells and the used medium are taken out from open continuous cultures are replaced by equal volume of fresh medium . Here, the steady state of suspension culture is maintained

immobilized cell cultures

plant cells and groups may be encapsulated in a suitable material or entrapped in membranes or stainless screens. To provide the nutrients to the cells, liquid medium is continuously run through the column where the immobilized cells are packed and sufficient aeration is also provided.

Assessment of cells in suspension

1. cell count

   cell count (at the beginning of culture and after certain days of incubation) , is a relatively more accurate measure adopted to determine the growth and cultures Increase in cell number depends on Mitotic Index (MI) of cells in suspension cultures

   Mitotic Index refers to the percentage of cells undergoing mitosis.

$$MI = \frac{\text{total number of divided cells}}{\text{total observed cells}} \times 100$$

2. packed cell volume (PCV) = (Biomass volume) ml / g of culture
3. cell fresh weight (gm / ml)
4. cell dry weight(gm / ml)
5. by measuring the optical density of the liquid medium the turbidity level could be estimated which is directly proportional to cell number

Test for viability of cell

using Fluoresein Di Acetate (FDA), Evans blue stains the viable cell count could be made

applications of cell culture

1. it helps in understanding of an organ formation or embryoid formation starting from single cell or small cell aggregate
2. suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloid
3. it helps in mutant selection in relation to crop improvement
4. to produce valuable products including secondary metbolites through bio transformation a technique utilized with the help of microbes

   (eg) Datura cell culture possess ability to convert hydroquinone into arbutin
5. single cell cultures are also being used for production of Single Cell Proteins (SCP)

   SCP are any microbial biomass from both uni and multicellular bacteria, yeasts, filamentous fungi or algae which can be used as food or feed additives.

(eg) Spirulina

comparison of callus and cell suspension cultures

Parameters	Callus	Cell suspension
Growth	Slow	Fast
Cell or cell content	Cells in contact	Dissociated
Medium	Only lower layer is in contact with the medium	All cells are in direct contact with the medium
Precursors	Not available to all cells	Available to cells
Subculture period	Long, 4-8 weeks	Short , 7-21 days
Accumulation of metabolites	Higher than cell suspension	Lower than callus culture
Scale up in bioreactor	Not possible	Cell suspension ar grown in bioreactor

Applications of cell culture

- cell culture offers enormous opportunities in the study of single cells and group oc cells
- in the isolation of protoplasts
- in cell cloning by the plating technique with or without specific treatment like mutagens,
- development of cell lines for various types of resistance like salt or drought tolerance, toxin resistant lines
- in scale up technology using bioreactors of various types

PRODUCTION OF SECONDARY METABOLITES

WHAT IS SECONDARY METABOLITE?

The secondary metabolites are those cell constituents which are not essential for their survival and not involved in primary metabolic activities like photosynthesis, respiration, protein synthesis etc.. These secondary metabolites include alkaloids , glycosides (steroids, phenolics), terpenoids and a variety of flavours , perfumes , agrochemicals etc.

Plants are the chief source of a wide range of biochemicals which are of medicinal importance . It has been found that the cells of the respective plants when cultured in specific medium *in vitro* , they produce the secondary metabolites which can be conveniently harvested and further processed.

Why synthesis of secondary metabolites through cell culture?

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

A list of some groups of biochemicals obtained from plants

Group	Examples
Alkaloids	Morphine, codeine, quinine, nicotine, cocaine, hyoscyamine, lysergic acid etc
Terpenoids	Menthol, camphor, carotenoid, polyterpenes
Phenylpropanoids	Anthocyanin, coumarins, flavonoids, isoflavonoids,
Quinines	Anthraquinone, nebzoquinones
Steroid	sterols

A list of some pharmaceutically valuable biochemicals obtained from plants

Compound	Plant species	Medicinal value
Shikonin	*Lithospermum erythrorhizon*	Antiseptic
Berberine	*Coptis japonica*	Antibacterial
Quinine	*cinchona*	Antimalarial
Taxol	*Taxus sp.*	Breast and ovarian cancer treatment

What is a bioreactor?

It is a culture vessel generally of a large volume which has provisions for aeration, stirring to achieve medium and cell mixing , contamination control, replacement of used medium and or cells

Four types of bioreactors are

1) 1.batch bioreactors
2) continuous bioreactors
3) multistage bioreactors
4) immobilized cell bioreactors

commercial production of shikonin

Shikonin was the first commercial product from cell culture of *Lithospermum erythrorhizon* .

Steps involved in the production of shikonin

1. the high producing clone cells to be used as inoculum i.e., stock or seed clutres and maintained in jar fermentors
2. the inoculum is first added to a 200 lit fermentor containing the MG5 medium for culture growth
3. after 9 days the cells are filtered out and inoculated into a 750 lit fermentors with M 9 medium and incubate for 14 days
4. the cells are harvested by simple filteration techniques and shikonin derivatives are extracted from the cells

A 750 lit bioreactor with 600 lit medium would yield 1.2 kg Shikonin in 2 weeks . In contrast *Lithospermum erythrorhizon* roots from one hectare land would yield about 9kg Shikonin after 4 years . Shikonin is used for making cosmetics, lotion and soap in Japan

Advantages of secondary metabolite synthesis

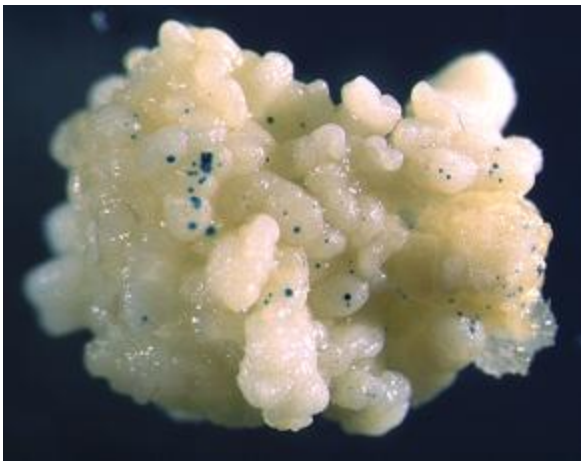
1. threat on endangered or extinct species could be eliminated (eg. *Lithospermum erythrorhizon)*
2. when the target plant is limited in supply (Trichosanthes sp.) or slow in growth (L.erythrorhizon) cell culture is highly useful for faster and continuous supply
3. cell cultures produce more secondary metabolites than the plant tissues in the field (20% more production of shikonin in cell culture)
4. cells can be stored

SOMATIC EMBRYOGENESIS

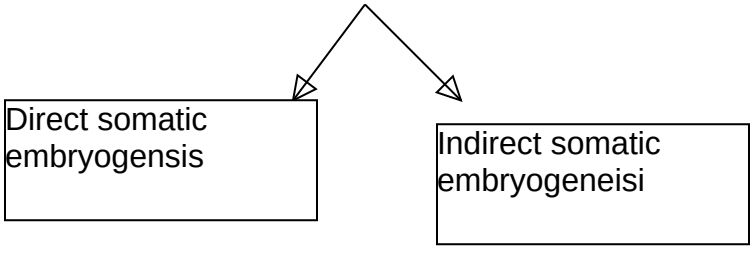
What is somatic embryogenesis?

It is the process of a single cell or a group of cells initiating the developmental pathways that lead to reproductive regeneration of non zygotic embryos capable of germinating to form complete plants.

Embryogeneic cultures are compact dry, amorphous, and white in colour as compared to non embryogenic cultures which are watery dirty white to light brown in colour and soft in nature.



SOMATIC EMBRYOGENESIS



In direct embryogenesis the embryo is formed directly from a cell or group of cells without the production of an intervening callus

In indirect somatic embryogenesis callus is first produced from the explant. Embryos are then produced from the callus

Embryoid

It is a small well organized structure comparable of the sexual embryo, which is produced in tissue culture of dividing somatic cells.which are embryogenically potential,

Embryogenic potential

The capability of the somatic plant cell of a culture to produce embryoids is known as embryogenic potential

Embryogenic callus (EC)

In somatic embryogenesis small compact cells divide asymemetrically and their daughter cells stick together to produce cell masses called proembryogenic masses or embryogenic clumps

Characters of somatic embryo

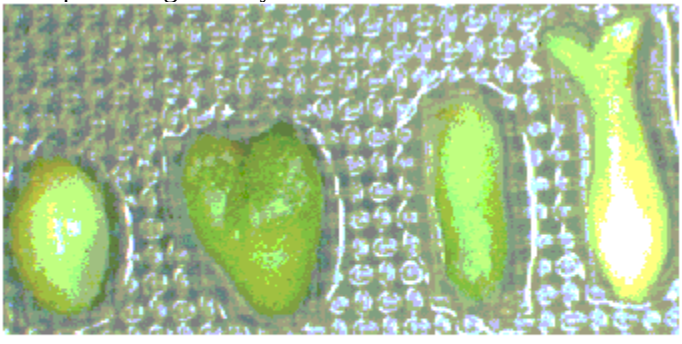
1.the origin is single cell
2.bipolar i.e., both shoot and root primordia present
3.vascular connection between the somatic embryos and the explant is absent
4. the somatic embryos are easily separated from the explant tissue

explants for initiation of SE

1.embryonic or young seedling tissues
2.excised small tissues from young inflorescence
3.scutellum
4.young roots
5.petioles
6.immature leaf
7.immature hypocotyls

Distinct stages in development of Somatic Embryogenesis

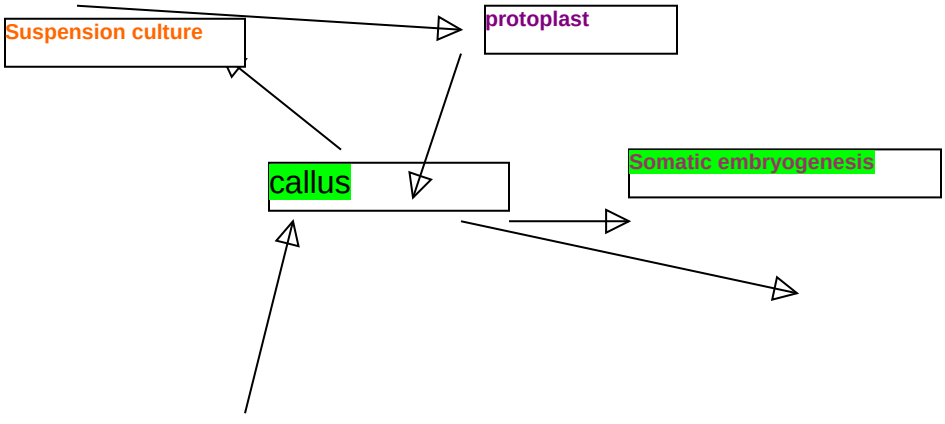
1. .single cells
2. .group of cells
3. globular stage
4. .heart shaped embryo
5. .torpedo stage embryo

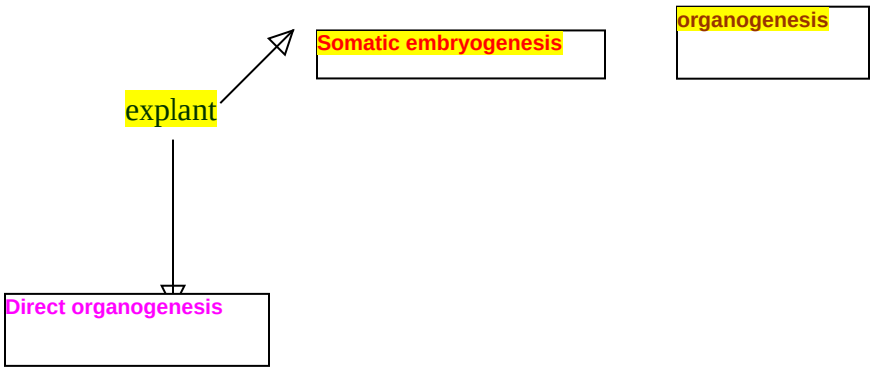


DRAW THE DIAGRAM

somatic embryogenesis may develop from single cells or from a small group of cells. Repeated cell divisions lead to the production of a group of cells that develop into an organized structure known as "globulin"stage embryo. Further development results in heart and torpedo stage embryos from which plants can be regenerated Signs of tissue differentiation become apparent at the globular stage and apical meristem are apparent in heart shaped embryo.

Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation) , a high concentration of 2,4 D is used. In the second stage (embryo production) embryos are produced in a medium with no or very low level of 2,4D In many systems it has been found that somatic embryogenesis is improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate.





Indirect somatic embryogenesis in carrot

A callus can be established from explants from a wide range of carrot tissues by placing the explants on solid medium containing 2,4D 91 mg)/lit) . This callus can be used to produce a cell suspension by placing it in agitated liquid MS medium containing 2,4D (1mg/lit). this cell suspension can be maintained by repeated subculturing into 2,4D containing medium. Removal of old 2,4D containing
medium and replacement with fresh medium containing absicic acid (0.025 mg / lit) results in the production of embryos

Direct somatic embryogenesis form alfalfa

Young trifoliate leaves are used as the *explant* . These are removed from the plant and chopped into small pieces. The pieces are washed in a plant growth regulator free medium and placed in liquid medium (B5) supplemented with 2,4D (4mg / lit) , kinetin (0.2 mg /lit) , adenine (1mg / lit) and glutarthione (10mg /lit) . The cultures are maintained in agitated liquid medium for about 10 – 15 days . Transferring the culture to maltose and PEG supplemented media results in the development of somatic embryos. These somatic embryos mature on solid medium containing absicic acid.

Importance of somatic embryogenesis

1.clonal propagation:- the mass production of adventitious embryos in cell culture is one of the ways of clonal propagation
2.for genetic transformation
3.raising somaclonal variation in tree species with the help of mutation
4synthesis of artificial seeds
5.synthesis of metabolites

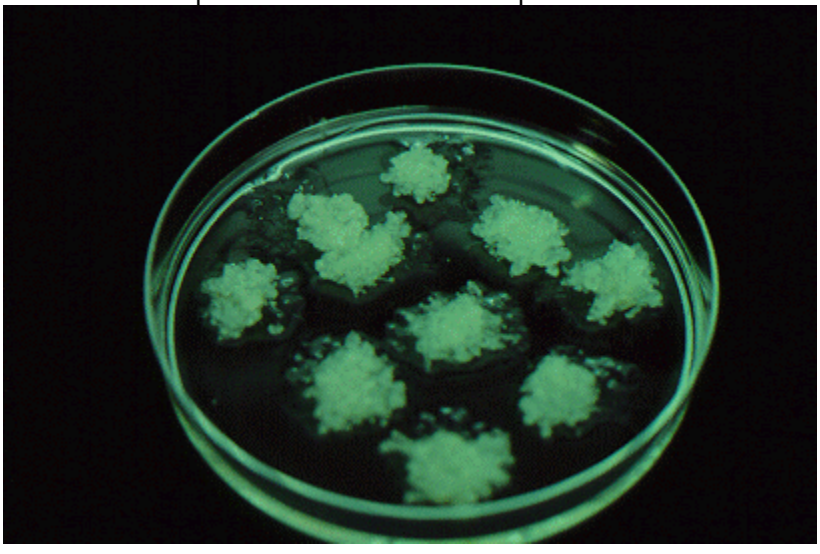
Differences / comparison between organogenesis and embryogenesis

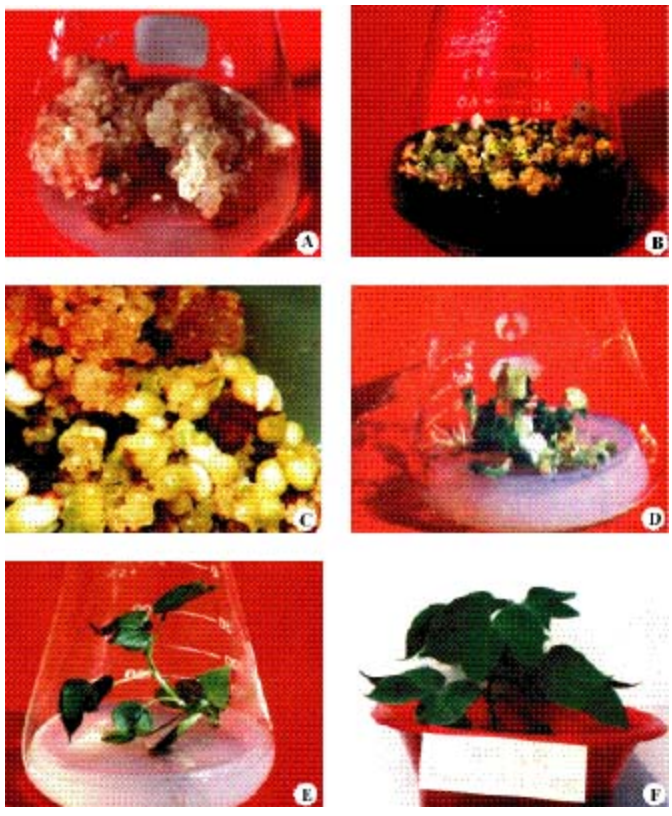
Charactersistc	Organogenesis	embryogenesis
Origin	Many cells , usually superficial	Single cell, usually superficial
Polarity	Unipolar	Bipolar
	Present; vascular strands	Absent; there is no vascular

Vascular connection with callus / explant	connected with those present in callus / explant	connection with callus/ explant
Separation from callus/ explant	Not easity separated unless cut off	Easily separated since the radicular end is cutinized

Applications of somatic embryogenesis

1. somatic embryogenesis provides potential plantlets in the form of somatic seeds. These are available in enormous number once the culture is established. The somatic embryos can be used for the production of synthetic seeds for direct sowing in the field.
2. somatic embryo provides organized culture system comparable to intact plant in sterile liquid cultures. Such cultures produce organ specific or differentiation related compounds in higher amount compared to cell culture of the species

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ARTIFICIAL SEEDS OR SYNTHETIC SEEDS

When somatic embryoids derived from plant tissue culture are encapsulated by a hydrogel and such encapsulated embryoids behave like true seeds if grown in soil and can be used as substitutes of natural seeds

Methods of artificial seeds production

1.gel complexation
2.molding

Gel complexation: in this method isolated somatic embryoids are mixed with 0.5 to 5% (W/V) Sodium alginate and dropped into 30 – 100 μM Calcium nitrate solution. Surface comlexation begins immediately and the drops are gelled completely within 30 seconds

molding method:- In this method isolated somatic embryos are mixed in a temperature dependent – gel such as gelrite and placed in the well of a microtiter plate and it formsgel when the temperature is cooled down.

Four types of synthetic seeds have been proposed on the basis of embryos and its encapsulation

- uncoated desiccated somatic embryos eg orchard grass
- coated desiccated somatic embryos eg. Carrot
- encapsulated coated hydrated somatic embryos eg. Alfalfa
- uncoated hydrated embryos (in a fluid drilling gel) eg. Carrot

plants produced from synthetic seeds sown in vitro and in soil

- in vitro – Apium graveiolens, Brassica sp. Carrot, cotton, alfalfa, rice, maize
- in soil – Apium graveolens, carrot, alfalfa
-

Importance of artificial seeds

1. they canbe stored upto a year without loss in viability
2. they are easy to handle and useful as units of delivery
3. they can be directly sown in the field without hardening
4. artificial seeds are available within a short time
5. season / time independent
6. no dormancy
7. artificial seed coating also has the potential to hold and deliver beneficial adjuvants such as growth promoting thiozobacteria, plant nutrients and growth control agents and pesticidies for precise placement
8. artificial seeds help to study the role of endosperm and seed coat formation

limitation

1. when the artificial seeds are stored at low temperature the embryos show a characteristic drop in conversion

2. cost of production is high

Common hydrogel used for encapsulation

Sl.no	Gel	Complexing agent
1	Sodium alginate	Calcium salts
2	Sodium alginate with gelatin	Calcium chloride
3	Carnagenan with locust beam gun	Potassium or ammonium chloride
4	Gelrite	Temperature lowered

SOMACLONAL VARIATION

Somaclonal variation is the genetic variability which is regenerated during tissue culture . Such variations manifests themselves as heritable mutation and present in the plant population even after transplantation to the field. Larkin and Scowcroft (1981) proposed the term somaclone to describe the plants originating from any type of tissue culture. The somaclonal variation may be attributed to either I) pre existing variation in the somatic cells of the explants) or ii) variation generated during tissue culture)

Mechanisms causing somaclonal variation

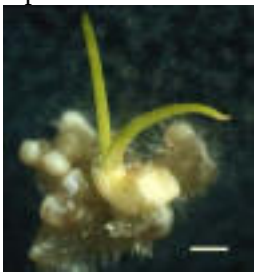
1.endomitosis

2.chromosomal abberation

3.DNA amplification

4.transposable elements

5.point mutation



why or how these chromosomal changes occur?

1. alterations in DNA methylation

a decrease in methylation causes the genes more active

2.lack of nucleic acid precursors due to very rapid cell division

3.role of growth regulators like 2,4D and sometimes kinetin in inducing variation

4.nutrient composition

KNO3 influences the albino plants from wheat cultures , level of organic N_2 chelating agents and other micro nutrients

5.Culture conditions: temperature , method of culture

6. duration of culture (longer the duration of culture more the variation)

SELECTION OF SOMACLONAL VARIATION

1.analysis of phenotypic characters

the morphological characters of somaclones are compared with the parental types for three tof four generations and finally the genetic variability is assessed

2.cytological study of variants

3.mesurement of DNA content of the variants

4.gel electrophoresis of proteins and enzymes

5. by exerting selection pressure using particular chemicals in the media
    a) selection for disease resistance - the toxin produced by the pathogen can be used in the media (phytophthora resistance in potato)
    b) herbicide resistance - by adding interested herbicide in the media (tobacco)
    c) salt tolerance - by adding NaCl (tobacco)
    d) drought/water logging tolerance - by adding PEG (tomato)
    e) mineral toxicity/deficiency tolerance - adding and reducing the quantity of interested nutrient in the media (tomato, sorghum)
    f) temperature stress / chilling response - exposing the culture to very high or low temperature (tobacco for chilling response, pear for temperature stress)

Somaclonal variation among regenerated plants from callus and protoplast culture has been presented to a lot of significant contributions to plant science. Somaclonal variation among regenerated plants from

Callus and protoplasts culture has been suggested as a useful source of potentially valuable germplasm for plant breeding and improvement. The major benefit of somaclonal variation is to create variation in adapted genotype.

List of crop species where desirable and heritable somaclonal variation has been reported

Species	Characters which were modified
Allium sativa	Bulb size, shape, clove no. aerial bulbil
Avena sativa	Plant height, heading date, awns
Hordeum spp	Plant ht, tillering
Oryza sativa	Plant ht, heading date , seed fertility, grain no., wt
Saccharam officinarum	Disease (eye spot, fiji disease, downey mildew)
Triticum aestivum	Plant and ear morphology, awns, gliadins, amylase, grain wt., yield
Zea mays	T toxin resistance, male fertility , Mt DNA
Lycopersicon esculentum	Leaf morphology, branching habit, fruit colour, pedicel, male fertility, growth
Medicago sativa	Multifoliate leaves, elongated petioles, growth branch number plant height, dry matter yield
Solanum tuberosum	Tuber shape, maturity date, plant morphology, resistance for early andlate blight,

	photoperiod, leaf color, vigour, height, skin colour

.problems with somoclonal variation

- variation is cultivar dependent
- frequencies of change vary
- many changes are undesirable
- some changes are unstable
- many changes are not novel
- characters of interest may not change

methods of assessment of somaclonal variation

- phenotypic parameters(quantitative (leaf size, plant height, qualitative, branching pattern, flowercolour)
- physiological parameters(protein patterns by electrophoresis, secondary products formation)
- genetic parameters (chromosome number and structure, banding pattern)

MICROPROPAGATION / CLONAL PROPAGATION

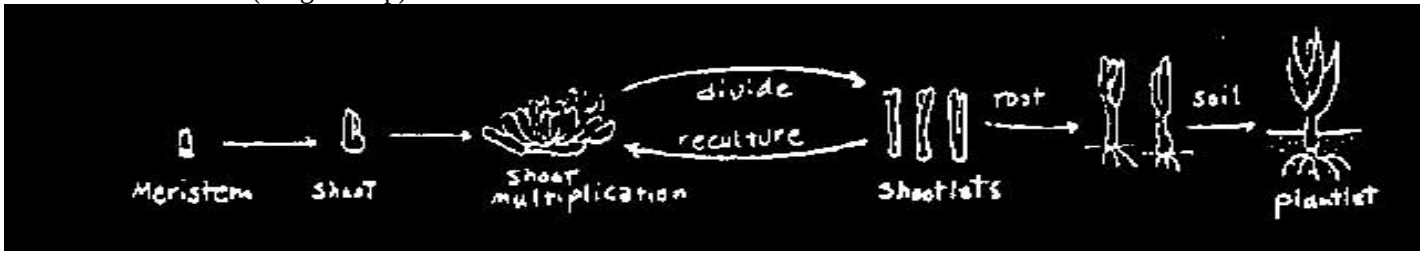
What is a micropropagation?

It is the *in vitro* regeneration of plants from organs, tissues , cells or protoplasts and the true to type propagation of a selected genotype using *in vitro* culture technique

A variety of plants species can be conveniently propagated through the techniques of cell , tissue or organ culture. This is particularly described as clonal propagation or micropropagation

Explants for micropropagation

- apical shoots
- axillary buds
- adventitious shoots
- bulbs
- leaf size (Begonia sp)



stages in micropropagation

in vitro clonal propagation is a complicated process requiring many steps or stages . Murashige proposed four distinct stages that can be adopted for overall production technology of clones commercially. Stages I to III are followed under in vitro conditions, whereas stage IV is accomplished in a green house environment

stage 0 – Preparative stage

This step involves selection of mother plant to provide a suitable *explant* of good quality for initiation of aseptic cultures. Selection of suitable *explant* is essential to the success of in vitro propagation Wherever possible mother plants should be grown under protected environment like

glasshouse, greenhouse etc. while taking *explant* from the field age of the mother plant, yield potential and healthiness should be considered

Stage 1 initiation of aseptic culture

This step is meant for obtaining an aseptic culture of the plant which is under investigation. The culture may be initiated from shoot tips , buds, stem root explants, flowers, sterile plantlets, callus etc. At this stage it is absolutely essential that the explant to be cultured should be totally free from microbial infection and that a high percentage of the explants survive and show rapid growth. This depends on the correct development of medium

Stage II shoot proliferation stage

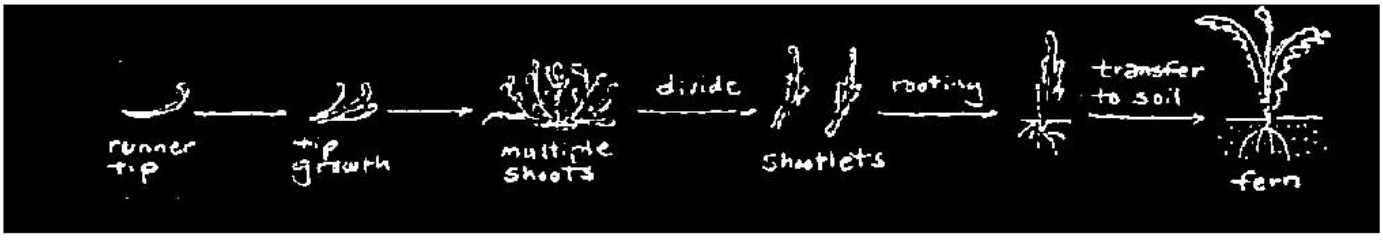
6. This stage aims at obtaining a rapid increase in the number of shoots or asexual embryos which can utilimately be used to provide the large number of plants. This cycle generally lasts for 4 – 10 weeks The cytokinin in the media stimulates pre existing shoot buds present in the explants (apical meristems in shoot tips, and axillary buds in nodal explants) to develop into shoots. After sometime (4-6 weeks) the axillary branching in a culture reaches the maximum. The individual shoots are then excised and subcultured onot fresh medium to initiate a new cycle of multiplication by axillary branching with a final yield of $5^{10} - 6^{12}$ plants in one year.

Stage III root development

This stage involves preparation of shoots for rooting. The proliferated shoots are transferred to a rooting medium. Sometimes shoots are directly established in soil as micro cuttings to develop roots.

Stage IV hardening

An important and critical stage in this process is when the plantlets under aseptic conditions of laboratories have to be shifted to galssshouse or greenhouse . The sterile plants are hardened or acclimatized to their new surroundings .



Advantages of micropropagation

1) rapid multiplication of superior clones and maintenance of uniformity
2) multiplication of disease free plants
3) multiplication of sexually derived sterile hybrids
4) supplies planting material irrespective of season
5) facilitates storage of a large scale
6) eases the transportation of planting material
7) stocks of germplasm can be maintained for many years
8) multiplication of cloning dioecious species
9) requires minimum growing space in commercial nurseries
10) in case of forest trees mature elite trees can be identified and rapidly cloned by this technique
11) in ornamentals tissue culture plants give better growth , more flowers,
12) very valuable when limited tissue is available as explant

limitations

- high investment cost
- requirement of more skill
- high electricity consumption
- presence of traces of somaclonal variation
- possibility of loss of rare genetic material when there is a severe contamination
- suitable techniques of micropropagation are not available for many valuable speices
- vitrification may be a problem in some species

Explants used in micropropagation of some of the economically important ornamental and horticultural platns

Group	Plant	Explant
Bulbs and corms	Allium, lilium, tulips, narcissus,	Scale leaves, leaf blades, stems
	Gladiolus	Corms, stem
Houseplants	Petunia	Small leaf pieces
Orchard crops	Musa	Shoot tip
Conifers	Picea abies	Needles
Field crops	Sugarcane,	Meristem tip culture and shoot tip culture

PRODUCTION OF VIRUS FREE PLANTS

Mersitem culture is often useful in recovering virus free plants from virus infected plants or clones

Eg. (potato, sugarcane)

What is a meristem?

A localized group of actively dividing cells from which permenant tissue system, i.e., root, shoot, leaf , and flower are derived. Thye contain only one pair of subtending leaves

Apical meristem?

The apical meristem is a group of cells , situated at the extreme tip of the shoot or root in the shape of dome. This shoot meristem measures approximately 0.1 mm in diameter and 2.5 to 3 mm in length covered by developing leaf primordial. The apical meristem remain in an active state during the vegetative growth phase and they are in a permanent embryonic stage.

How meristem tip culture useful for micropropagation ?

The cells constituting the meristems are highly conservative and hence genetically stable so the plants regenerated from the plants are genetically identical.

Why plants derived from meristem tip culture are free from virus?

Since, the rate of cell division is higher the virus is not able to cope up with the same and get eliminated. Further the absence of plasmodesmata and vascular elements in the meristem greatly hinders the transport of virus particles leading to very low concentration in meristem.

Meristem culture

In vitro culture of a generally shiny, domelike structure measuring less than 0.1 mm in length when excised , most often excised from the shoot apex

First application of meristem was to obtain virus free plants of Dhalias. Considerable expertise is required to dissect out the shoot apical meristem with only one or two leaf primordia.

Meristem tips are carefully dissected away from the apical or lateral shoot buds under a steremicroscope under aseptic condition. After this though most mersitems can be directly on suitable culture medium without surface sterilization . Meristem can be directly inoculated on a suitable culture medium without surface sterilization . Meristems are usually cultured on solid medium occasionally on liquid media supplemented with a low concentration of cytokinins and a moderate level of auxins. Further, transferred to root development media for improving root growth. After a period of 2-6 months the plants are transferred to green house and sample plants are tested for the presence of virus by ELISA and PAGE techniques

Sometimes mersitem tip cuture have failed to eliminate virus infection because the explant contains shoot apices with vascular tissue instead of true meristem. Meristem tip culture combined with heat treatment (thermo therapy) or chemical treatment (chemotherapy) has proved to be very effective in virus eradication.

Thermo therapy

Explants are exposed to the incidence of higher temperature which are not lethal for plant cells, but they are lethal for viruses. Mostly used temperature range is 50 – 52 ° C with exposition about 10 30 minutes. In case of whole plant, lower temperature has to be used (32 – 40 °with exposure for 4 to 30 days

Chemotherapy

By using some of the chemicals viz., malchite green, ribavirin or 2 thiouracil when added to the medium it kills the virus in the explants

Shoot tip grafting *in vitro* and micrografting

Shoot tip grafting (STG) in vitro, also known as micrografting is extremely beneficial with woody species . This method involves micrografting of aseptically isolated, very small shoot tips consisting of meristems with 2 – 3 leaf primordia to in vitro grown virus free seedling (root stock) . This method is employed in citurs, apple, prunus plants.

Virus indexing

It may be emphasized that all the plants obtained through meristem culture with or without chemo or thermotherapy are not virus free. Therefore, such plants have to be tested for the presence of the concerned virus. This is called virus indexing.

- The simplest method for virus indexing is to score the plants for the presence of specific symptoms produced by the relevant virus
- The saps from test plant may be used to inoculate highly sensitive and healthy indicator plants
- A highly sensitive and precise technique for virus indexing embryos is ELISA test (Enzyme Linked Immuno Sorbant Assay) It is more convenient, rapid and efficient when a large number of plants are handled
- DNA and RNA probes can also be used for virus detection

  Virus have eliminated form a number of economically important plant species including the following:
  - Ø Mottle virus free from cassava
  - Ø Cauliflower mosaic virus from cauliflower
  - Ø Other crop spp include garlic, pineapple, dahlia, cymbidium, orchid, carnation, straw berry, iris, lily, apple, cassava, banana, raspberry, sugarcane, grape, potato, ginger

ANTHER CUTLURE AND MICROSPORE CULTURE

By carefully selection of developing anthers at a precise and critical stage it is possible to establish the anther culture that will give rise to haploid plants.

Guha and Maheswari (1964) first reported the direct development of embryos form pollen grains of Datura by anther culture

What is androgenesis?

Androgenesis is the *in vitro* development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation.

Direct androgenesis

Microspores behave like a zygote and undergoes change to form embryoid which ultimately gives rise to a plantlet.

Indirect androgenesis

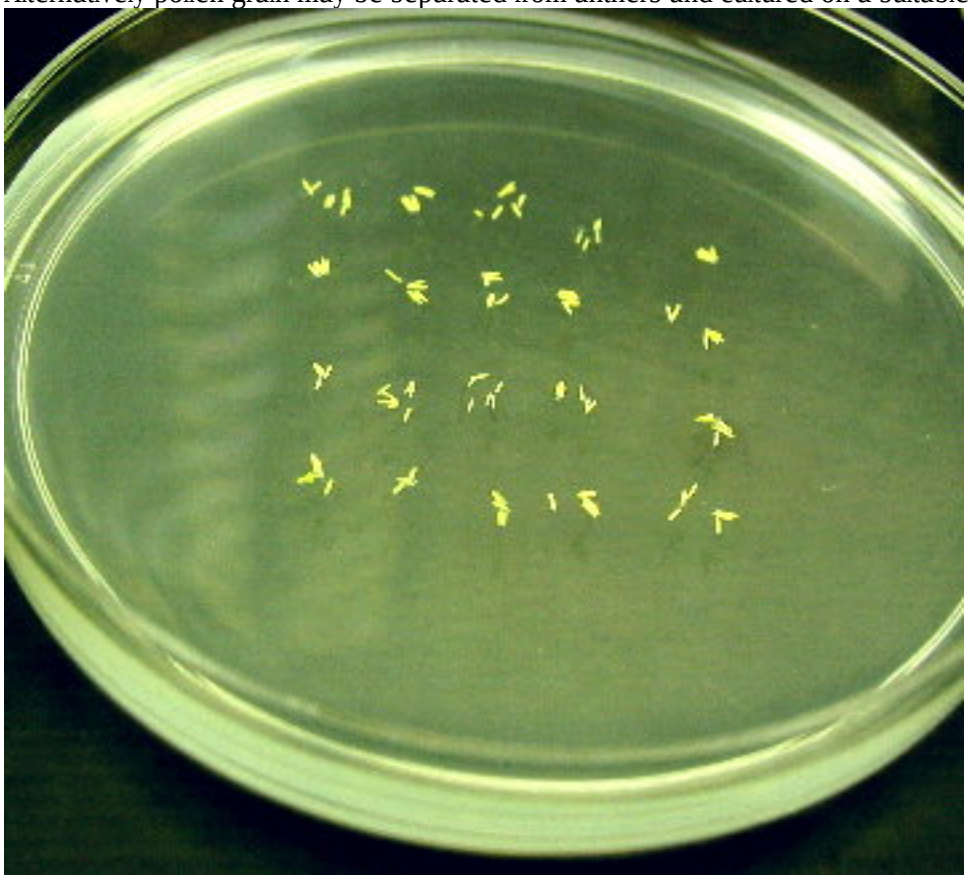
In contrast to the direct androgenesis the microspore instead of undergoing embryogenesis divide repeatedly to form a callus tissue which differentiates into haploid plantlet.

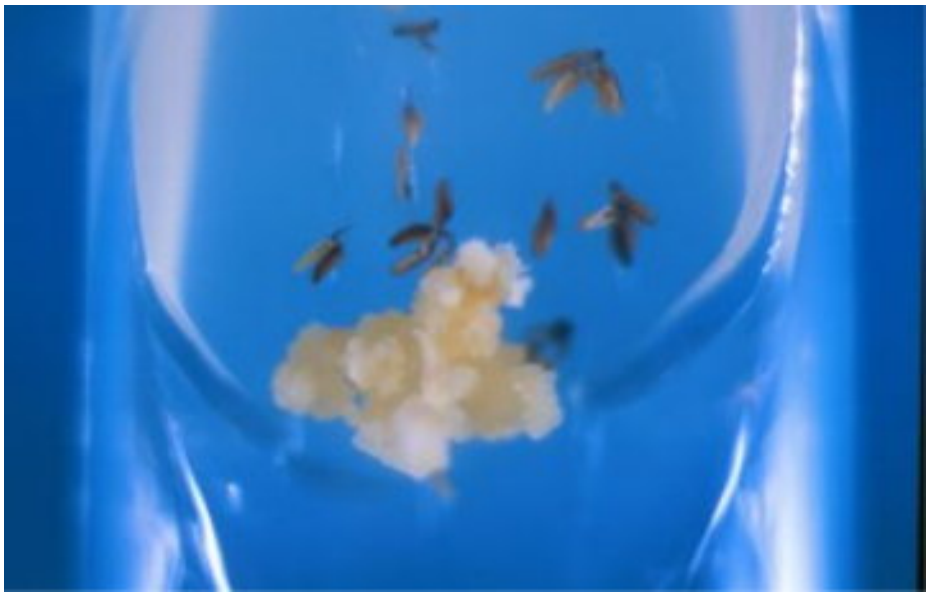
Principle of anther culture

The basic principle of anther and pollen culture is the production of haploid plants exploiting the totipotency of microspore and the occurrence of single set of chromosome (n) in microspore. The principle behind the anther culture is that without disturbing the natural habitat and environment of the enclosed anther, pollen can be grown by culturing the intact anther. In culture condition, the diploid tissue of anther will remain living without proliferation at the selective medium and at the same

time it will encourage the development of pollen by nursing and providing nutrient. In anther culture the gametophytic pathway of pollen is broken and it undergoes sporophytic pathway.

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





Pathways of development of pollen

The early divisions in responding pollen grains may occur in one of the following four ways.

Pathway I

The uninucleate pollen grain may divide symmetrically to yield two equal darughter cells called vegetative and generative nuclei both of which undergo further divisions

Eg. Datura innoxia

Pathway II

The uninucleate pollen divides unequally. The generative cell degenerates immediately or after undergoing one or two divisions. The callus / embryo originates due to successive division of vegetative cells

Eg. Barley, wheat, triticale, chillies, tobacco

Pathway III

Here the the vegetative cell degenerates and the generative cell divide repeatedly to form multinucleate pollen

Eg. Hyoscyamus niger

Pathway IV

In some species, the uninucleate pollen grain divide unequally producing generative and vegetative cells, but both these cells divide repeatedly to contribute to the developing embryo callus

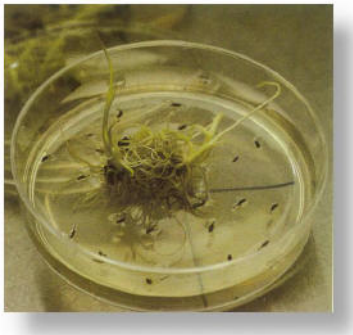
Eg. Datura innoxia

Pretreatment

Low temperature 3 – 5 °C for 2 days ----tobacco

High temperature 35°C for 24 hours ----- brassica

Prolonged low temperature 4 – 10 °C for 3 – 28 days -- cereals



MICROSPORE CULTURE OR POLLEN CULTURE

Isolated pollen grains when cultured in vitro gives rise to haploid embryos or callus and this approach is called pollen culture. Pollen may be isolated either by squeezing or float culture of anthers.

Squeeze culture

About 50 anthers may be placed in 20 ml of medium and squeezed with a glass rod; the solution is filtered through a nylon mesh of suitable pore size and centrifuged at 500 – 800 RPM for 5 minutes. The pollen pellet is collected washed twice and suspended at a final density of $10^3 – 10^4$ pollen /ml

Float culture:

Excised anthers are floated on a shallow liquid medium in petridishes; the anthers dehisce in a few days releasing their pollen grains into the medium

Initially isolated pollen grains were cultured either in hanging drops or on a filter paper raft placed on cultured anthers. Subsequently Nitsch and Co Workers first replaced the nurse tissue by an extract of cultured anthers and finally devised a completely synthetic medium for pollen culture the crucial ingredients of which were glutamine, L-Serine, and inositol

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 eg. Tobacco, pennisetum

Advantages of haploids / anther culture / pollen culture

1) Haploid are useful in cytogenetic studies
2) Production of homozygous inbreds / isogenic diploids within a year as compared to the long inbreeding method which takes 4-6 years
3) To trace the parents of the hybrids
4) Haploids are valuable for mutation studies
5) By comparing the heterozygous diploid with haploid or homozygous diploid population recessive phenotypic characters can be identified
6) Development of pure lines and 100% male plants in Asparagus
7) Recovery of sexual inter specific hybrids between wild and domestic species (tomato)
8) For understanding the phylogenetic relationship between species the study of meiotic behaviour of haploid provide clues for chromosome duplication within a species

9) To study the inheritance pattern
10) Use of haploids in the production of monosomics, nullisomics, and other anueploids
11) Double haploid, that are homozygous and fertile are readily obtained enabling the selection of desirable gene combination

How to double the chromosomes in haploid?

Spontaneous duplication

Homozygous diploid callus or embryoids may be formed by the spontaneous fusion of two similar nuclei of cultured pollen after first division. Haploid cells are unstable in culture and have a tendency to undergo endomitosis.

Induced duplication

Colchicines treatment:- the young plantlets regenerated through anther culture are treated with 0.5% colchicines solution for 24 – 48 hours. Treated plantlets are replanted in the medium after through washing. In case of mature haploid plantlets , 4% colchicines paste may be applied to the axil of the leaves

OVARY CULTURE

GYNOGENESIS

Culture of unfertilized ovaries to obtain haploid plants from egg cell or other haploid cells of embryo sac is called ovary culture and the process is known as Gynogeneis

- Ø The first report on gynogenesis was by San Noem in 1976 in case of barley

During induction of ovaries are floated on a liquid medium having low auxin and kept in dark while for regeneration they are transferred to an agar medium with higher auxin concentration and incubated in light.

In vitro parthenogenesis

Haploid plants generally originate from egg cell in most of species (rice)

In vitro apogamy

When haploid plants originate from other than egg cell called synergid (rice) or antipodal(Allium tuberosum)

Limitation

- Ø The frequency of responding ovaries (1 – 5%) and the number of plants / ovary (1-2) is low
- Ø It has been successful only in lesser number of plant species

Advantages of ovary culture

1) Ovary/ ovule culture may be useful when there is male sterility in crop species
2) For in vitro pollination and fertilization or for embryo rescue
3) To produce parthenogenetic haploids (wheat, barley,)
4) To understand the physiology of fruit development
5) Reduction in the frequency of albino plants

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

Ovule culture

Flowers are sterilized inoculated on pre culture medium. After 10 to 14 days ovules are removed from flowers and plated on medium. Ovule requires exclusive skill . Ovule culture is

mainly tried only in those cases where embryo aborts very early, and embryo culture is not possible due to difficulty of its excision at a very early stage.

EMBRYO CULTURE

Embryo culture is the aseptic isolation and growth of sexually produced embryos *in vitro* with the objective of obtaining viable plants.

In 1904, Hanning published the first report on embryo culture in cruciferae.

Types of embryo culture

1) Culture of immature embryos (hybrid seed0
2) Culture of mature embryos (to avoid inhibition in germination)

Isolation of embryo

The embryo excision operation is performed aseptically in a laminar flow hood. A stereomicroscope equipped with a coolary fluorescent lamp is required for excision of small embryos . Soaking a hard coat seed in water facilitates easy excision. The excision of smaller or immature embryos require careful dissection

Culture strategies

Sometimes young embryos may prove difficult to culture directly on the medium . In such cases, embryos may be placed onto or implanted into developing endosperm that are cultured in vitro. This technique is known as embryo-nurse endosperm technique

Precocious germination

When excised immature plant embryos are grown in vitro , they do not proceed further for its maturation and start germinate . This phenomenon without completing normal embryogenic development is known as precocious germination

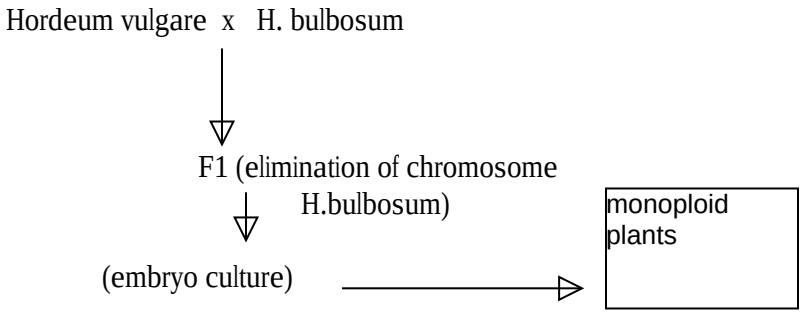
Practical applications of embryo culture

1. rescuing embryos from incompatible crosses
   in interspecific and intergeneric hybridization programmes, incompatability barriers often prevent normal seed development and production of hybrids due to post zygotic barriers and causes embryo abortion. . In such cases isolating the embryo and culturing in vitro may give useful results
2. overcoming dormancy and shortening breeding cycle
   a) the life cycle of Iris was reduced from 2 – 3 years to less than one year. It is possible to obtain two generations of flowering against one in Rosa spp.
   b)germination of the immature embryos of orchid seeds

c) dry storage wild oat (Avena fatua)

3. overcoming seed sterility
   in early ripening fruit cultivars seeds do not germinate because their embryos are still immature
   eg. Peach, apricot, plum , coconut
4. production of monoploids
   bulbosum technique
   In interspecific hybridization between Hordeum vulgare and Hordeum bulbosum the zygote is formed but during the development of embryo subsequently the chromosomes of H.bulbosum are eliminated and monoploid embryos of H.vulgare are formed . However the monoploid embryos are weak and show slow growth and on the other hand when these

monoploids are dissected and cultured in vitro they develop into monoploid plants are produced.



5. clonal micropropagation
   eg. Pinus elliotti, graminae, coniferae
6. seed testing : rapid means of determining viability of particular lot of seeds eg. Seeds of conifers, shrubs, vines and fruit trees

other application

a) to study some fundamental problems in experimental embryogenesis
b) host pathogen interaction, eg.formation of ergot by infection of rye embryos by *Claviceps purpurea* and fusarium wilt of seedlings. In later cases , 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
c) cultured embryos have been used as test objects to evaluate the mutagenic ability of irradiated substrates on living tissue. For this embryos of certain cereals were plated on X irradiated nutrient medium for evaluation

PROTOPLAST ISOLATION,PROTOPLAST FUSION AND SOMATIC HYBRDISATION

WHAT IS A PROTOPLAST?

The entire plant cell without its cellulosic cell wall and protoplasts are functional individual cell with plasma membrane as outermost layer .

What is protoplast culture?

In vitro culture of protoplast

Sources of protoplast

a) mesophyll cells of leaves
b) cultured suspension cells
c) callus culture
d) preconditioned plant material

Isolation of protoplast

methods of protoplast isolation can be classifed into two main groups

1) mechanical method (non enzymatic)

2) enzymatic → sequential method (two step method)

→ mixed method (single step method)

Mechanical method:

It is done by cutting plasmolysed cells with a sharp edged micro scalpel or knife after keeping the material under microscope. The protoplasts are released and and the cells are deplamolysed. This method is useful for isolation of protoplasts from vacuolated cells (eg. 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. The mechanical method though was used as early as 1982, in now only rarely used for isolation of protoplasts

Enzymatic method

Commercially available enzymes

a) pectolyase Y 23
b) cellulase =onozyka R10
c) meicelase
d) rhozyme
e) macerozyme R 10
f) hemicellulase
g) pectinase
h) drieselase

the role of enzymes is to dissolve middle lamella and dissolving cell wall. A combination of these enzymes in a concentration of 0.5 to 20% is used . In many cases the macerozyme and cellulase are sufficient to obtain protoplast in significant umber. The enzyme solution is prepared in 10-15% sorbitol or mannitol containing small amount of $CaCl_2$ (7mM) for membrance stability.

Sequential method:

This involves initial incubation of macerated plant tissue with pectinase (macroenzyme) which inturn are then converted into protoplasts by cellulase treatment.

Mixed enzymatic

Plant tissue are plasmolysed in the presence of a mixture of pectinase and cellulases thus inducing simultaneous separation of cells and degradation of cell wall to release protoplast directly.

After enzyme treatment protoplast suspensions are collected by centrifugation (60 –100 rpm) for 2 – 5 minutes. Then washed in medium without enzyme. Cell debries are removed and the protoplasts are placed in a medium with appropriate concentration of sucrose or mannitol..

This method is widely used since

1) large quantities of protoplasts can be obtained
2) the cells are not broken as in case of mechanical isolation
3) Osmotic shrinkage is less
4) Takes reduced time
5) Lesser contamination

Osmoticum

Osmoticum is a solution causing changes in osmatic pressure. During isolation and culture , protoplasts require osmotic protection until they regenerate a strong wall. Inclusion of an osmoticum in both isolation and culture media prevents rupture of protoplasts. The most widely used osmotica are sorbitol, mannitol, glucose, or sucrose.

Subprotoplasts

Subprotoplast do not contain the entire contents of plant cells and include the following

Cytoplast

The protoplasts lacking a nucleus and each contains entire cytoplasm of a cell

Miniprotoplast / karyoplast

Isolated protoplasts contain a nucleus surrounded by some cytoplasm and the original outer plasma membrane

Microplast

The protoplast contain only a fraction of cytoplasm and outer membrane

Microprotoplast

The protoplats containing only a few of all chromosomes and a fraction of the cytoplasm

Viability of protoplast

The viability of protoplast is tested by

1) Observing the presence of cytoplasmic streaming
2) Exclusion of Evans Blue dye
3) Change in protoplast size due to change in the level of osmoticum
4) Presence of photosynthetic and respiratory activity
5) FDA (Fluorescein DiAcetate) or CFW Calcofluor white (CFW) test.

Minimum plating density

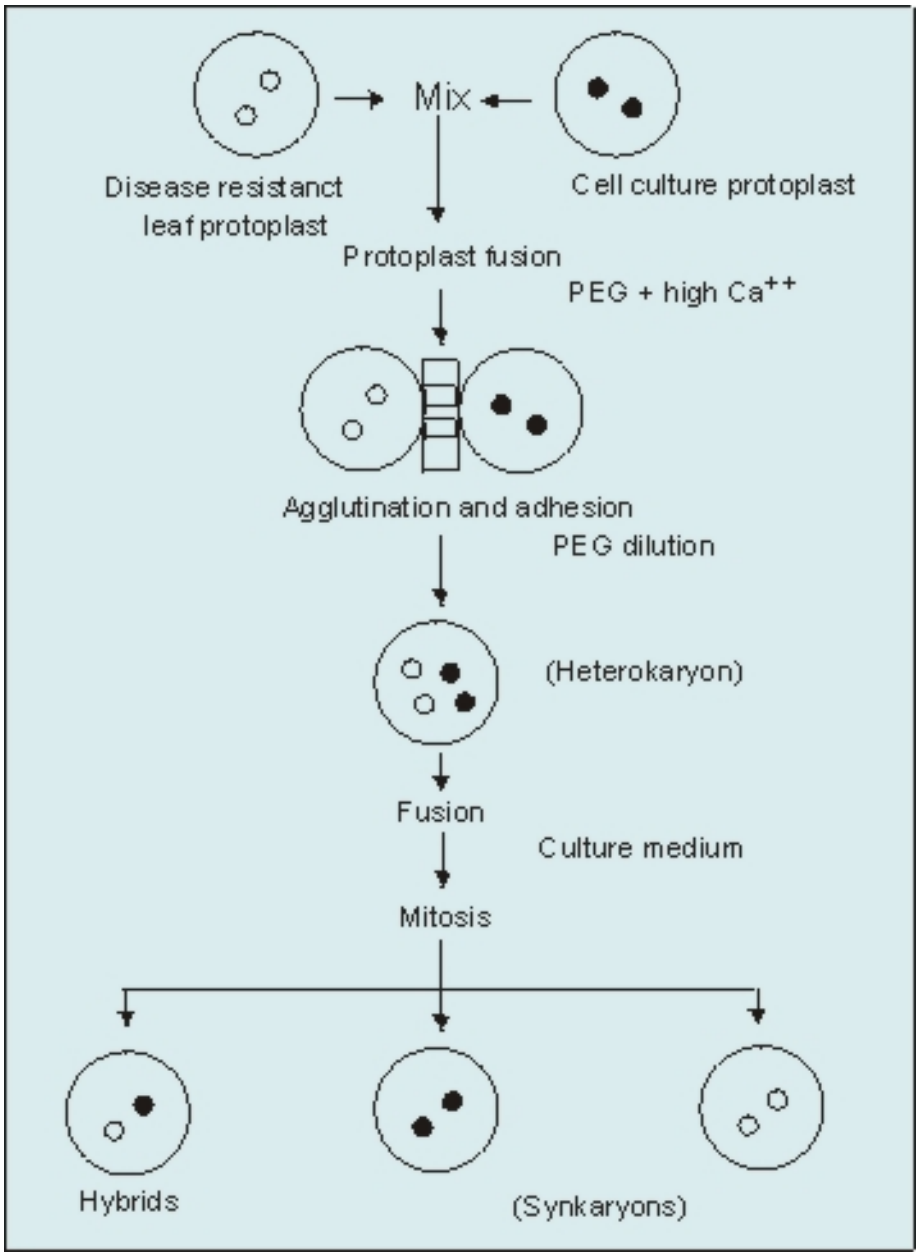
A minimum plating density of protoplasts is requied for growth to begin eg. 5×10^3.to 1×10^5protoplast / cm^3 for tobacco

Culture of protoplast

The first step in the protoplast culture is the development of a cell wall around the membrane of isolated protoplast. This is followed by induction of division in the protoplast derived new cell giving rise to a small cell colony. By manipulation of the nutritional and physiological conditions in the nutrient media, cell colonies may be induced to grow callus continuously or to regenerate whole plants

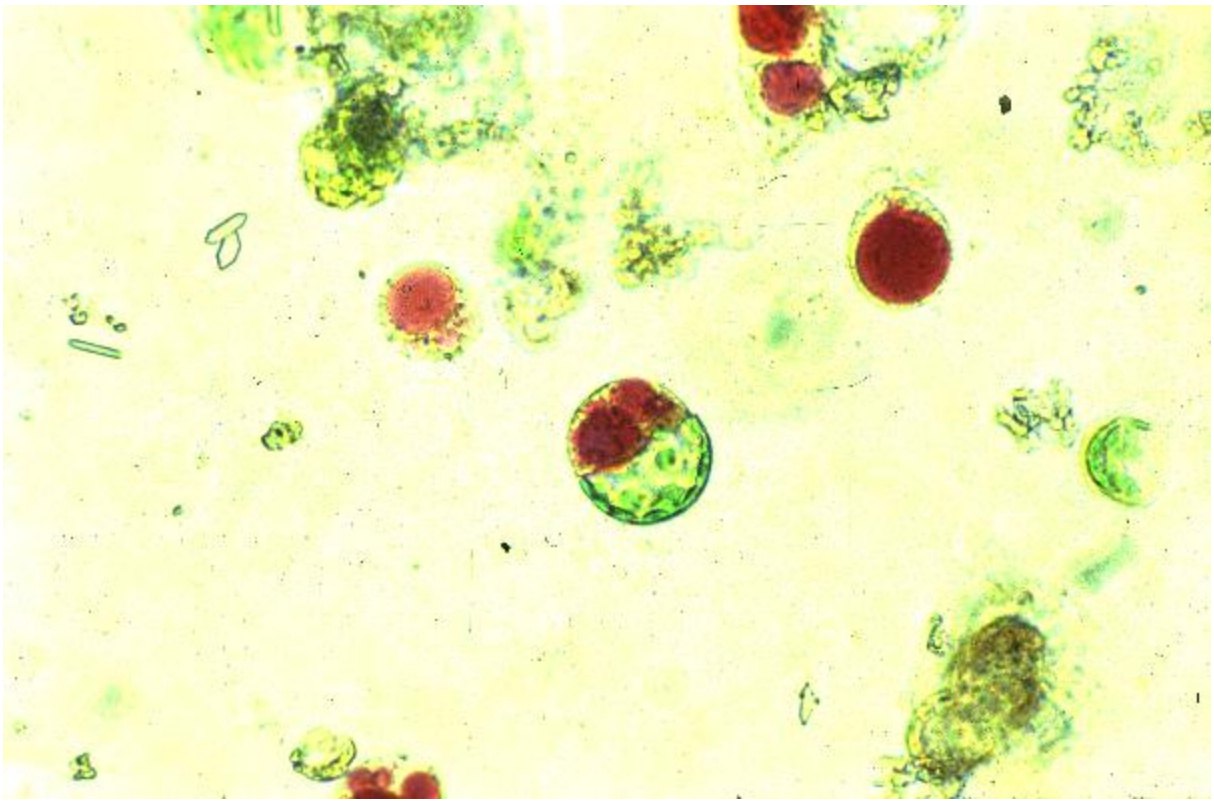
Protoplast fusion

Protoplasts fusion or somatic hybridization is one of the most important uses of protoplast culture. This is particularly significant for hybridization between species or genera, which can not be made to cross by conventional method of sexual hybridization.



Somatic hybridization

In vitro fusion of plant protoplasts derived either from somatic cell of somatic plant or from two genetically different plant is called somatic hybridization



Methods of protoplasts fusion

1) Spontaneous fusion
2) Induced fusion

Spontaneous fusion

Protoplasts during isolation often fuse spontaneously and this phenomenon is called spontaneous fusion. Simply physical contact is sufficient to bring about the spontaneous fusion among similar parental protoplasts. The occurrence of multinucleate fusion bodies is common when cells are prepared from actively dividing cells. Spontaneous fusion usually gives rise to homokaryon usually intraspecific. Protoplasts of young leaves undergo spontaneous fusion frequently. This type of fusion can be done with the help of micromanipulators or micropipettes.

Induced fusion

Induced fusion

For achieving interspecific and intergeneric fusion the following methods are followed

1) Chemical method
2) Electric method
3) Mechanical method

The induced fusion requires a suitable agent called fusogen. The inducing agents first brings the protoplasts together and then causes them to adhere to one another for bringing about fusion.

Chemofusion

The following chemicals are used as fusogens

1) $NaNO_3$ (isolated protoplasts are suspended in an aggregation mixture of 5.5% $NaNO_3$ in 10% sucrose solution)

2) Poly Ethylene Glycol (PEG)(1 ml of protoplasts suspended in a culture medium with 1ml of 56% of PEG and tube shaken for 5 seconds. The protoplasts are allowed to sediment for 10 minutes washed with growth medium and examined for successful agglutination and fusion)

When the quantity of protoplast is less drop cultures can be used. Protoplasts are placed as microdrops in petriplates to which PEG (50µl each)are added to each drop and kept for 5-10 minutes.

3) Calcium ions (Ca^{++})(spinning the protoplasts in a fusion inducing solution (0.05M $CaCl_2 2H_2O$ in 0.4M Mannitol at high pH of 10.5)
4) Proteins: (Gelatin and early products of its degradation at a concentration of 2.5% induced aggregation at high frequency within one hour (eg. Vicia, glycine, allium).

Electric fusion

If protoplasts are placed into a small vessel containing electrodes and a potential difference is applied then the protoplasts will line up between the electrodes. Afterwards an extremely short square wave electric shock is applied protoplasts can be induced to fuse.

Mechanical fusion

The isolated protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulatorand perfusion micropipette.

Selection of somatic hybrids

The protoplasts suspension recovered after a treatment with fusogens consists of following types

1. unfused prototplasts of two species/strains
2. products of fusion between two or more protoplasts of the same (homokaryon)
3. hybrid protoplast produced by fusion between protoplasts of two species(heterokaryon)

effective strategy has to be employed for the identification somatic hybrids of heterokaryon in nature and they should be isolated. This step is called selection of hybrid cells.

Methods of selection of somatic hybrids

1. Somatic visual markers

   Hybrids of Petunia parodii (green) with P.hybrida (white) could be isolated in the form of green callus which represented only hybrid cells.
2. biochemical basis for complementation

   This selection strategy exploits thse natural properties of the two parental species which show complementation in the hybrid cells and at same time permit their selection.
   a. auxin autotrophy: the parental protoplasts of Nicotina glauca and Nicotiana longsdorffi requires an auxin compound in order to proliferate, whereas hybrid callus tissue needs no such requirement since the hybrid cells are auxin autotrophic
   b. protoplast of Petunia hybrida form calli on the MS medium while those of P.parodii produce only small cell colonies. Further actinomycin (1µg/ml) inhibits cell division of P.hybrida protoplasts, but it has no effect on those of P.parodii, Thus protoplasts of both these Petunia species fail to produce macroscopic colonies on MS medium supplemented with 1µg/ml actinomycin D whereas hybrid cells divide normally and produce macroscopic calli.
3. to culture the entire protoplast population
4. labeLling: protoplasts of two parents may be labeled by different fluorescent agents, which will then enable the selection of hybrids
   i. octadeconyl amino fluorescent
   ii. octadecyl palamine

5. Fluorescent Assorted Cell Sorter (FACS)

Symmetric hybrids:

Some somatic hybrid plants retain the full or nearly full somatic complements of the two parental species ; they are called symmetric hybrids.

Asymmetric hybrids

Many somatic hybrids exhibit the full somatic complement of one parental species are lost during the preceding mitotic divisions; such hybrids are referred to as asymmetric hybrids.

CYBRIDS

Cybrids or cytoplasmic hybrids are cells or plants containing nucleus of one species but cytoplasm from both the parental species.

Cybrids can be produced in the following methods

1. fusion of normal protoplasts from one parent with enucleated protoplasts from the other parent. Enucleated protoplasts can be obtained by high speed centrifugation (20,000 – 40,000g for 45 – 90 minutes) of protoplasts or by irradiation treatment
2. fusion of normal protoplasts form one parent and protoplasts containing non viable nuclei from the other
3. selective elimination of one of the nuclei from the heterokaryon
4. selective elimination of chromosomes of one parent at a later stage after fusion of the nuclei

Major application of cybrids

- to transfer the cytoplasmic male sterility (tobacco, tomato)
- to transfer antibiotic resistance character(tobacco)
- to transfer herbicide resistance (brassica)

Production of cytoplsmically male sterile lines in tomato

i. Mesophyll protoplasts of tomato (Lycopersicon esculentum) were treated with iodoacetamide (IOA) to inactivate mitochondria and

ii. Mesophyll protoplast of Solanum acaule (S.tuberosum) wre irradiated with ϒ or X rays to inactivate nuclei. The protoplast were mixed in 1:1 ratio and induced to fuse using $Ca^{++}$and PEG, leading to the production of heterologous hybrids. Among the fusion from the original cultivars with respect to morphology, physiology and chromosome number (2n = 24), but exhibited various degrees of male sterility

iii. The nuclear genotype of cultivar remains unaffected

iv. 100% probability of getting somatic hybrids

Applications of somatic hybridisation and cybridisation

i. For effecting wide hybridization

Interspecific hybrids

in genus Daucus and its relatives

in genus Nicotiana and Brasscia

Intergeneric

Rice + Echnichloa = oryzochloa

Raphanus sativus + B.oleracea = raphanobrassica

N.tabacum + Lycopersicon esculentum = solanopersicon

Solanum tuberosum + L.esculentum = solanopersicon

ii. To create recombinants in asexually propagated crops or sterile plants
Eg. Potato

iii. Overcoming the barriers of self incompatability
Eg. Nicotiana tabacum x N.nesophila (disease resistant)

iv. Production of cms lines

v. Production of herbicide tolerant plants (brassica)

CRYOPRESERVATION / IN VITRO GERMPLASM PRESERVATION

Modes of conservation of germplasm

i. ln situ conservation

The method of conserving land races and other genotypes in their own habitat. This mode of conservation has some limitations . Due to environmental hazards some of the material may be lost. The cost of maintaining a large proportion of available genotypes in nurseries or fields is very high and tedious

ii. Ex situ conservation

This is a modern method of preserving germplasm.. Seeds or *in vitro* maintained plant cells, tissues and organs are preserved under appropriate conditions for long term storage as gene banks

Advantages of this type of conservation

- large amount of material can be preserved in small area
- it overcomes the destruction due to environmental hazards
- it provides large amounts of plant material for culturing

What is cryopreservation?

Preservation of seeds, cells , tissues and organs in liquid nitrogen is called cryopreservation It is based on the reduction and subsequent arrest of metabolic functions of biological material by imposition of ultra low temperature. At the temperature of liquid nitrogen (-196C) almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods. However, only few biological materials in their natural state, can be frozen to sub freezing temperatures without adversely affecting the cell viability . However, it requires efficient regeneration protocols through tissue culture of the species.

What materials can be used for cryopreservation?

Embryos, embryoids(conifers, pollen embryos in tobacco), shoot tips (carnation, solanum sp.), callus cultures (chrysanthemum sp) , cell suspension (capsicum), protoplast (carrot)

Strategies

- Normal growth (short to medium storage0
- Slow growth;

the growth rate can be smothered by 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

- Suspension growth:

Cryo storage / cryopreservation of cells, protoplasts etc.

what is a cryoprotection?

It is a process that prevents the formation of ice crystals in cells and protect them from toxic solution effect due to water loss from the cytoplasm

Cryoprotectants

Several chemicals such as dimethyl sulphoxide (DMSO) , glycerol, various sugars and sugar alcohol protect living cells against damage during freezing and thawing i.e. they make the storage material not to become frozen . These compounds lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates. The colligative properties of the cryoprotectants minimizes the harmful action of electrolytes concentration resulting from conversion of water into ice. High solubility in aqueous phase and low toxicity to the cells are the two essential characteristics for cryoprotectants.

Two types of cryoprotectants are permeating and non permeating

What is permeating cryoprotectant

cryoprotectant that can permeate are called permeating cryoprotectant eg. DMSO, methanol, glycerol

what is non permeating cryoprotectant?

Not able to permeate eg. Sucrose, mannitol, sugar alcohol, dextran, polyvinyl pyrrolidone, hydroxy ethyl starch

Steps in cryopreservation

1) Freezing
2) Storage
3) Thawing
4) Reculture

Freezing and storage:

a. slow freezing method

initially the temperature is frozen at the rate of 0.5 to 4°C per minute from the temperature of 0° C till it reaches -100°C and finally transferred to liquid medium

b.rapid freezing method

the material is frozen rapidly by or by pouring plunginig into liquid nitrogen

c. droplet freezing: In this method the cryoprotectant treated meristems are dispensed in droplets of 2-3 microlit on an aluminium foil in a petriplate. The specements are frozen by slow cooling (0.5 C /min) to a subzero temperature between –20C to 40C prior to immersion in liquid nitrogen.

stepwise method

slow freezing rate initially (1 to 5 °C per minute) for 30 minutes and afterwards rapidly cooled by pluning into liquie N_2

dry freezing method

materials dehydrated by drying in an oven or under vacuum

a cryoprotectant like DMSO (5-8%), Glycerol (10%) must be added to the culture medium to protect the cells from ice injury. The frozen cells and tissues are stored in a liquid nitrogen refrigerators.

Thawing

Thawing of the frozen materials is achieved by plunging vials into warm water (37 – 40 °C for 90 seconds , washed several times to remove the cryoprotectant and preserved in ice bath

Reculturing

The cells/tissue/shoot tips may be transferred to the required fresh medium after thawing for regeneration

Slow growth culture

Slow growth of cultures may be achieved by maintaining the plantlets either at low temperature (4-9°C) or on a suitable medium having high osmotic concentration. In addition the nutritional status may be lowered down to restrict the growth.

Advantages of in vitro conservation

- Ø within a little space , a large number of plants can be maintained
- Ø pathogen free condition is maintained
- Ø protection against natural disorders
- Ø easy movement of planting material across the countries beacuase it ensures appropriate quarantine measures
- Ø storage of pollen for enhancing longevity
- Ø establishment of germplasm banks
- Ø conservation of cell lines producing medicines
- Ø conservation of somaclonal and gametoclonal variation in culture

Vitrification: It involves a freeze induced cell dehydration step prior to liquid nitrogen storage. The efficiency of this method depends on the ability of highly concentrated solutions of cryoprotectants to supercool to very low temperature with rapid coolingto become viscous at sufficiently low temperatures, and solidify without the formation of ice.

Encapsualtion:

This technique is used in synthetic seed technology by coating somatic embryos in alginate beads.

Applications of cryopreservation

1. conservation of genetic uniformity
2. preservation of rare genomes
3. freeze storage of cell cultures and cell lines
4. maintenance of disease free material
5. cold acclimation and frost resistance
6. retention of morphogenetic potential in long term cultures.

Organizations involved in cryopreservation

IBPGR – International Board for Plant Genetic Resources
CIAT – Centro International de Agricultura Tropical
IRRI – International Rice Research Institute
NBPGR – National Bureau of Plant genetic Resources