Plant Tissue Culture

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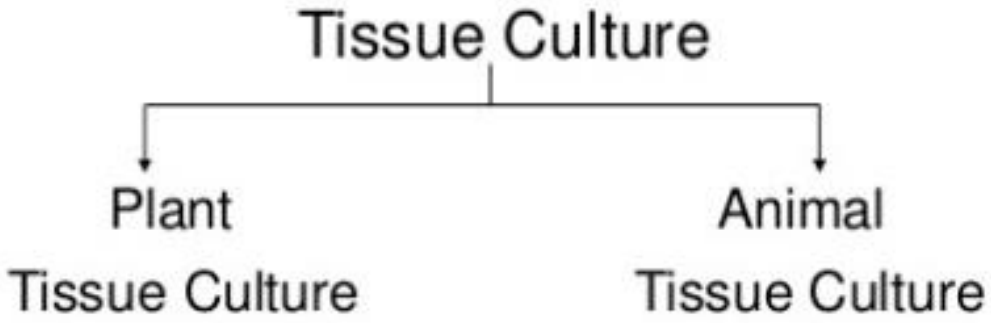
Unit 1: Plant Tissue Culture

- Historical perspective; Composition of media; Nutrient and hormone requirements (role of vitamins and hormones); Totipotency; Organogenesis; Embryogenesis (somatic and zygotic); Protoplast isolation, culture and fusion; Tissue culture applications (micropropagation, androgenesis, virus elimination, secondary metabolite production, haploids, triploids and hybrids; Cryopreservation; Germplasm Conservation).

History of Plant Tissue Culture

- A German plant physiologist Gottlieb Haberlandt (1902) cultured isolated single palisade cells from leaves in Knop's salt solution enriched with sucrose. **Haberlandt is regarded as the father of plant tissue culture.**
- Hanning(1904) **Embryo culture of selected crucifers**.
- Snow, Gautheret (1934-1939) **Importance of auxin** as growth regulator
- Morel & Martin 1952 used Meristem-tip culture for disease elimination. They recovered for the first time, **virus- free Dahlia plants**.
- Skoog and Miller (1957) **Discovery of principle of hormonal control of the organ formation in tissue culture**
- Reinert and Steward (1958-59) First report on **somatic embryogenesis.**
- Cocking (1960) Isolation of protoplasts by **enzymatic degradation method**.
- **Murashige and Skoog** (1962) Developed a universally used high salt medium containing mineral salts, vitamins, an energy source and growth hormone (**MS medium**).
- Guha and Maheshwawari (1964) Production of **First haploid plant by anther culture**.
- Power et al (1970) **Protoplast fusion**.
- Takabe et al (1971) **Regeneration of first plant from protoplast**.
- Carlson et al (1972) First report on inter-specific hybridization through protoplast fusion.

Plant Tissue Culture



- **Defination:**
- Plant-tissue culture is *in-vitro* cultivation of plant cell or tissue under aseptic and controlled environment conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant.
- In other words it is an experimental technique through which a mass of cells (callus) is produced from an explant tissue.
- The callus produced through this process can be utilized directly to regenerate palntlets or to extract or manipulate some primary and secondary metabolites.

- The plant tissue culture refers to the cultivation of a plant cell which normally forms a multicellular tissue.
- When grown on agar medium, the tisse forms a callus or a mass of undifferentiated cells. The technique of cell culture is convinient for starting and maintaining cell lines, as well as, for studies pertaining to organogensis and meristem culture.

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

1. To keep the plant cells or organs free from microbes

2. To ensure the desired development in cells and organs by providing suitable nutrient media and other environmental condition.

Advantages of tissue culture

1. Availability of raw material

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

2. Fluctuation in supplies and quality

The method of production of crude drugs is variable in quality due to changes in climate, crop diseases and seasons. All these problems can be overcome by tissue culture.

3. New methods for isolation

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

4. Biotransformation (Process through which the functional group of organic compound are modified by living cells) reactions are feasible using plant-cell cultures.

6. Disease free and desired propagule

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

7. Biosynthetic pathway

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

8. Immobilization of cells

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

9 Continuous, uniform biomass is obtained.

10. Medicinally important compound can be synthesized, which can't be synthesized chemically.

11. Useful natural compounds can be produced, independent of soil condition & change in climatic conditions.

12. Improvement of medicinal plant species.

13. Propogation of plant without seeds in defined and controlled condition.

Disadvantages of tissue culture

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

Basic requirement for tissue culture

- Plant material
- Equipments and Glasswares
- Aseptic Condition
- Washing and storage facilities
- Media preparation room
- Sterilization room
- Nutrient medium
- Transfer room
- Culture room or incubators
- Proper and optimum aeration
- Well equipped observation or recording area

Equipments and Glassware

- Incubating chamber or laminar airflow cabinet with UV light fitting for aseptic transfer
- Incubator with temperature control ± 0.5°C generally temperature recommended for most tissue culture studies is 36°C.
- Autoclave-for sterilization of glassware, media etc.
- Refrigerators and freezers-For storage of reagents, tissue culture stock solutions, chemicals etc.
- Hot air oven-for dry sterilization of glassware, media etc.
- Microscope-Simple and special microscope with a provision to take camera are required. The stage of this microscope should be large enough to accommodate large roller bottles in specific cases.
- pH meter- for adjusting the pH of the medium
- A spirit burner or gas micro burner for flame sterilization of instruments
- Washing up equipments- Washing facilities for glassware, pipette etc. in deep soaking baths or washing sinks of stainless steel or polypropylene are suitable for manual washing and rinsing of almost all types of glassware except pipettes.

Standard siphon type pipette washers are suitable for washing the pipettes soaked in detergent for overnight. The washed pipettes should be rinsed with deionised water and dried in a stainless steel pippette dryer.

- Water purifier- Pure water is required at most of the plant tissue culture study.
- Centrifuge- To increase the concentration of cell suspension culture

Equipments and Glassware

- Shakers- To maintain cell suspension culture
- Balance- To weigh various nutrients of the preparation of the medium
- Shelves- Build from rigid wire mesh to allow maximum air movement and minimum shading should be used in the culture room.
- Scissors, scalpels and forceps- For explant preparation from excies plant parts are for their transfer
- Culture vessels- Usually borosilicate glass vessels are preferred, it includes test tubes, conical flasks, bottles, special flat tubes etc.

Now, the common vessels are 100 ml conical flasks or large test tubes of 25 × 150 mm size.

- Glasswares- Like measuring cylinders, beakers, funnels, petri dishes, graduated pipette, conical flask etc. Are required for preparation of nutrient media.
- Miscellaneous-Non absorbent cotton plug, screw cap or polyurethane foam is required to close the mouth of the culture vessel. Aluminium foil is required to cover the exposed part of plug from becoming wet when autoclaved. Labels, marking pencils, hand lens, plastic disposables like syringes, plastic bottles, hot plate, stirrer etc.
- Microwave- not essential but it melts the solidified media for pouring in culture vessels like petri dishes etc.

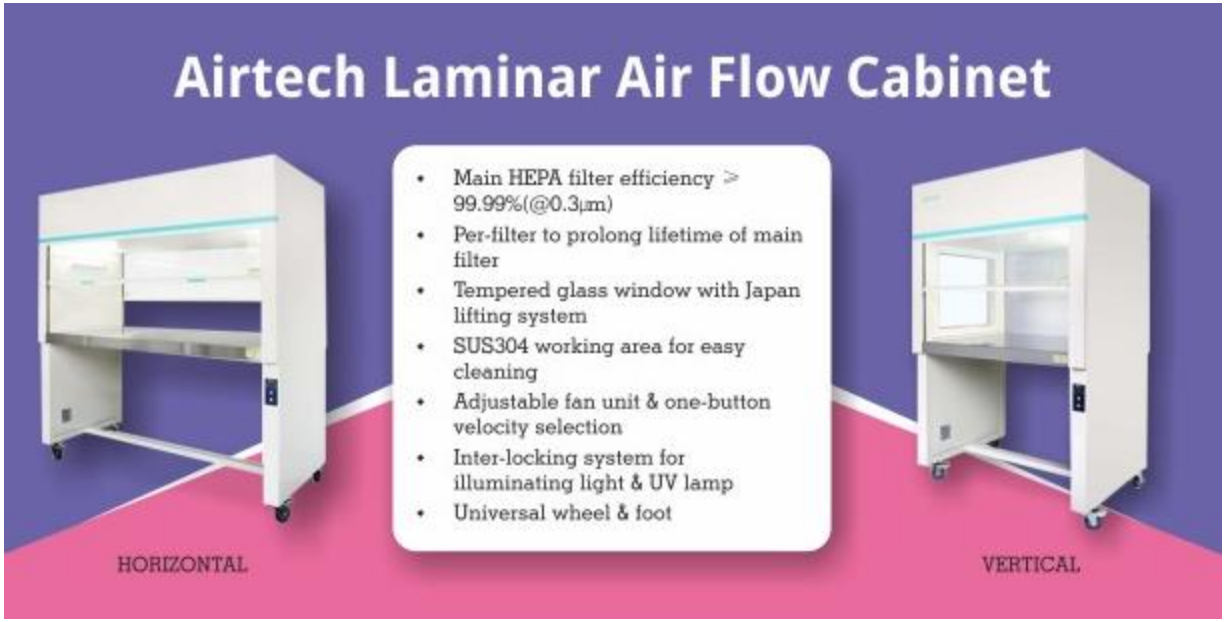
Aseptic Condition

- The plant materials (tissues), equipments, culture media and the room should be free from microorganisms.
- Usually dry heat, wet heat, ultrafiltration and chemicals are used for the sterilisation process.
- Surface sterilisation of plant materials such as seed, fruit, stem, leaf etc. by agents like
  - 9-10% calcium hypochlorite for 5-30 minutes
  - 2% sodium hypochlorite solution for 5-30 minutes. The materials need to be washed thoroughly in double-distilled water, after sterilising in these solutions.
  - 10-12% of hydrogen peroxide solution for 5-15 minutes.
  - 1-2% bromine water, for 2-10 minutes
  - 1% solution of chlorine water, mercuric chloride, silver nitrate or antibiotics etc. can also be used.
  - Absolute alcohol is used for hard tissues

- Dry heat method is used for sterilisation of equipments in hot air oven.
- Sterilisation of equipment with chromic acid-sulphuric acid mixture, hydrochloric acid, nitric acid strong detergent solution, alcohol, incubator or autoclaves etc. are use for this purpose.
- Wet heat method is used for sterilisation of glassware, culture media in autoclave at 121°C and 15 lb pressure for 15 minutes.
- Ultrafiltration is used for sterilisation of liquid media which are unstable at high temperature.
- Antibiotics are added to medium to prevent the growth of the microorganisms e.g. Potassium benzyl penicillin, strptomycin sulphate, gentamycin etc.
- Chemicals like alcohol are used for sterilisation of working area and the instruments.
- Sterilisation of the environment is done by fumigation method, the inoculation chamber is generally laminar airflow cabinet is widely used these days.

Transfer room

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



Culture room or incubator

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

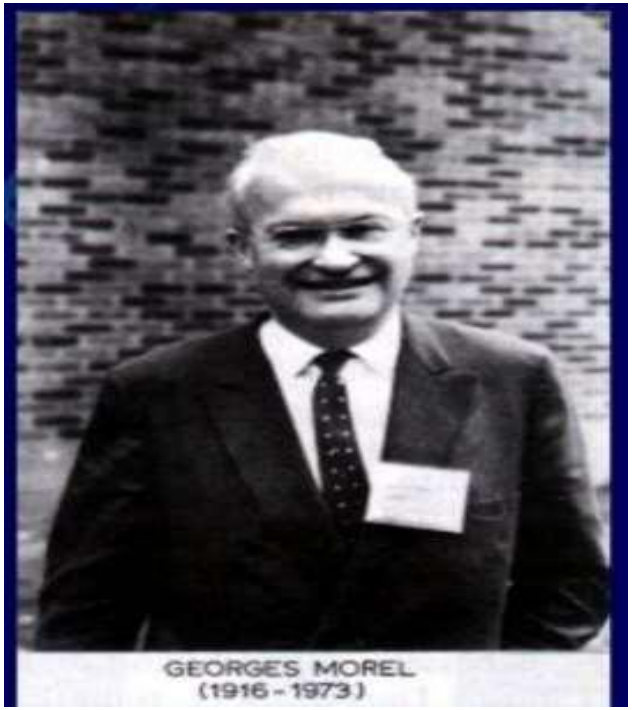
Techniques for plant tissue culture

The general technique used in the isolation and growth of culture is described as follows:

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

HISTORY 0F VIRUS FREE PLANT CULTURE

1952 Morel & Martin:
First virus-free plant through shoot tips culture (*Dahlia*)
1960 Shoot tip culture of *Cymbidium*



- Morel and Martin (1952) developed meristem culture technique and recovered Dahlia shoots, free from viruses, by meristem tip culture. In 1955, they recovered virus free potato. This attained wide application of plant tissue culture to raise virus free plants in agriculture.

Callus culture

Callus is **defined** as an unorganized **tissue** mass growing on solid substrate. **Callus** forms naturally on plants in response to wounding, infestations, or at graft unions (Bottino, 1981). **Callus** formation is central to many investigative and applied **tissue culture** procedures.

- It may initiate from explants of any multi-cellular plant.
- The organs such as root, stem tips, leaves, flowers and fruit are grown on solid media.
- The cell groups are initiated from:
  - Explant/Segments of root, stem or leaf either from the mature or embryogenic plant
  - Explant/Excised fragments of parenchyma or mixed tissue containing cambium or endosperm
- The longer the tissue explant the more complex the range of cell types & greater the possibilities of initiating a culture of mixed cells.
- Callus can be induced to undergo organogenesis and/or embryogenesis and eventually whole plant by providing suitable nutrient medium.
- To study the biosynthetic pathway of various metabolic processes by using tracer elements in callus culture.

Plant Tissue Culture Terminology

- **Adventitious**---Developing from unusual points of origin, such as shoot or root tissues, from callus or embryos, from sources other than zygotes.
- **Agar**---a polysaccharide powder derived from algae used to gel a medium. Agar is generally used at a concentration of 6-12 g/liter.
- **Aseptic**---Free of microorganisms.
- **Aseptic Technique**---Procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cultures.
- **Autoclave**---A machine capable of sterilizing wet or dry items with steam under pressure. Pressure cookers are a type of autoclaves.
- **Chemically Defined Medium**---A nutritive solution for culturing cells in which each component is specifiable and ideally of known chemical structure.
- **Clone**---Plants produced asexually from a single source plant.
- **Clonal Propagation**---Asexual reproduction of plants that are considered to be genetically uniform and originated from a single individual or explant.
- **Coconut milk**---The liquid endosperm of coconut contain the cytokinin *zeatin* and will support the continued cell division of mature cells, leading to the formation of callus.
- **Contamination**---Being infested with unwanted microorganisms such as bacteria or fungi.
- **Culture**--- plant growing in vitro.
- **Detergent**---Increasing the efficiency of sterilization.

Callus

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Plant Tissue Culture Terminology

- **Differentiated**---Cells that maintain, in culture, all or much of the specialized structure and function typical of the cell type *in vivo*. Modifications of new cells to form tissues or organs with a specific function.
- **Explant**---Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.
- **Horizontal laminar flow unit**---An enclosed work area that has sterile air moving across it. The air moves with uniform velocity along parallel flow lines. Room air is pulled into the unit and forced through a HEPA (High Energy Particulate Air) filter, which removes particles 0.3 µm and larger.
- **Hormones**---Growth regulators, generally synthetic in occurrence, that strongly affects growth (i.e. cytokinins, auxins, and gibberellins).
- **Internode**---The space between two nodes on a stem
- **Media**---Plural of medium
- **Medium**---A nutritive solution, solid or liquid, for culturing cells.
- **Micropropagation**---*In vitro* Clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.
- **Node**—A part of the plant stem from which a leaf, shoot or flower originates.
- **Pathogen**---A disease-causing organism.
- **Pathogenic**---Capable of causing a disease.
- **Petiole**---A leaf stalk; the portion of the plant that attaches the leaf blade to the node of the stem.

Plant Tissue Culture Terminology

- **Plant Tissue Culture**---The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.
- **Regeneration**---In plant cultures, a morphogenetic response to a stimulus that results in the products of organs, embryos, or whole plants.
- **Somaclonal Variation**---Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.
- **Somaclones**---Plants derived from any form of cell culture involving the use of somatic plant cells.
- **Sterile**--- (A) Without life. (B) Inability of an organism to produce functional gametes. (C) A culture that is free of viable microorganisms.
- **Sterile Techniques**---The practice of working with cultures in an environment free from microorganisms.
- **Subculture**---See "Passage". With plant cultures, this is the process by which the tissue or explant is first subdivide, then transferred into fresh culture medium.
- **Tissue Culture**---The maintenance or growth of tissue, *in vitro*, in a way that may allow differentiation and preservation of their function.

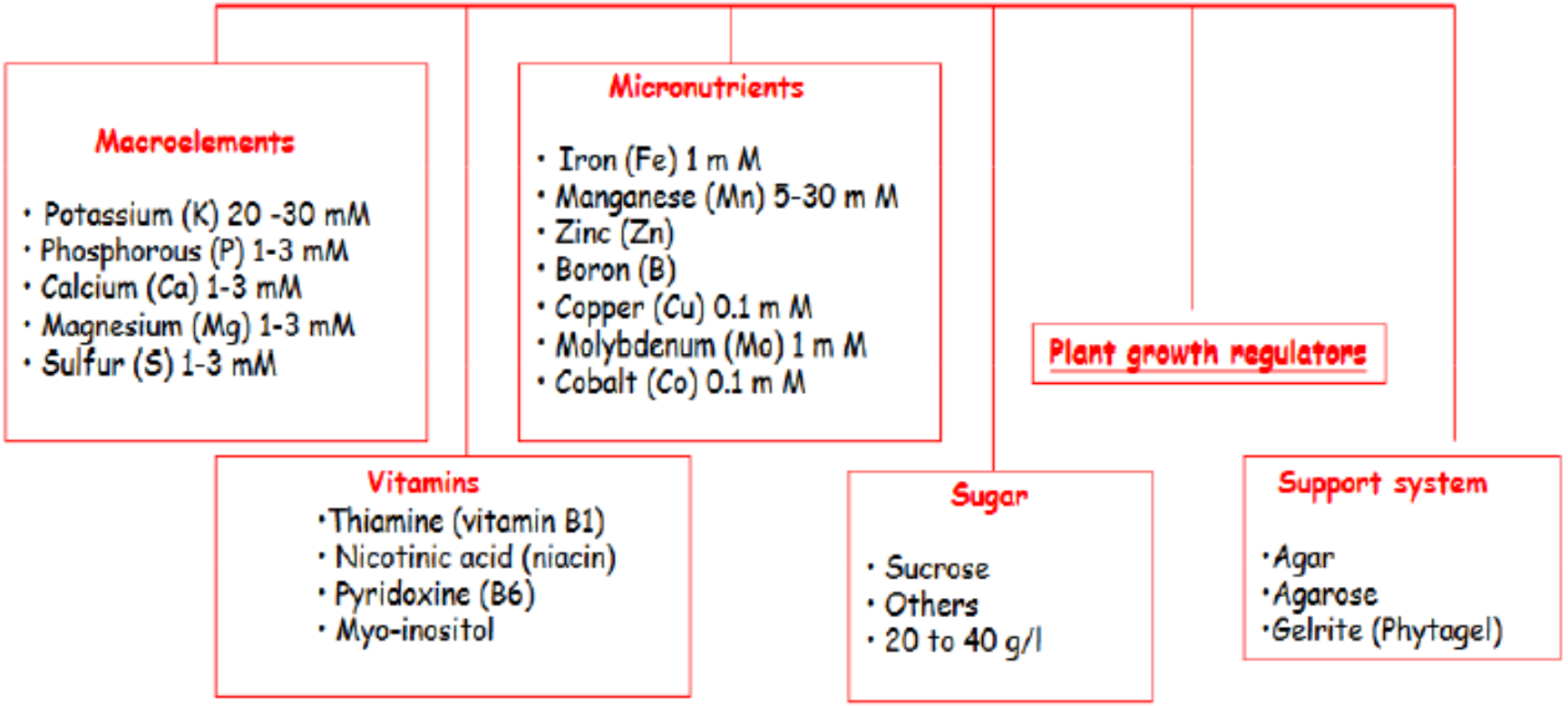
1. **Totipotency**---A cell characteristic in which the potential for forming all the cell types in the adult organism are retained.

- **Undifferentiated**---With plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo

Nutrient medium

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

Culture Medium



Essential elements for plant growth

Element	Function
Nitrogen (N)	Component of proteins, nucleic acids and some coenzymes Element required in greatest amount
Potassium (P)	Regulates osmotic potential, principal inorganic cation
Calcium (Ca)	Cell wall synthesis, membrane function, cell signalling
Magnesium (Mg)	Enzyme cofactor, component of chlorophyll
Phosphorus (P)	Component of nucleic acids, energy transfer, component of intermediates in respiration and photosynthesis
Sulphur (S)	Component of some amino acids (methionine, cysteine) and some cofactors
Chlorine (Cl)	Required for photosynthesis
Iron (Fe)	Electron transfer as a component of cytochromes
Manganese (Mn)	Enzyme cofactor
Cobalt (Co)	Component of some vitamins
Copper (Cu)	Enzyme cofactor, electron-transfer reactions
Zinc (Zn)	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum (Mo)	Enzyme cofactor, component of nitrate reductase

Plant growth regulators used in plant tissue culture media
Normal concentration range is 10^{-7} ~ 10^{-5}M

Class	Name	Abbreviation	MW	Stock solution
Auxin	p-chlorophenoxyacetic acid	pCPA	186.6	All auxins dissolved in dilute NaOH or aqueous ethanol
	2,4-Dichlorophenoxyacetic acid	2,4-D	221.0	
	Indole-3-acetic acid	IAA	175.2	
	Indole-3-butyric acid	IBA	203.2	
	1-Naphthaleneacetic acid	NAA	186.2	
	2-Napthoxyacetic acid	NOA	202.2	
Cytokinin	6-Benzylaminopurine	BAP	225.2	All cytokinins dissolved in dilute NaOH or aqueous ethanol
	N-Isopenteylaminopurine	2iP	203.3	
	6-Furfurylaminopurine (Kinetin)	K	215.2	
	Zeatin***	Zea	219.2	
Gibberellin	Gibberellic acid***	GA_3	346.4	Dissolved in water
Abscisic acid	Abscisic acid	ABA	264	Dissolved in aqueous ethanol

Murashige and Skoog (MS) Medium (1962)

Macroelement (10x)		Microelement (1000x)		Organics	
NH_4NO_3	1.65 g/L	H_3BO	6.2 mg/L	Nicotinic acid	0.5mg/L
$MgSO_4$	370 mg/L	$CoCl_2$	0.025 mg/L	Pyridoxin-HCl	0.5 mg/L
$CaCl_2$	440 mg/L	$CuSO_4$	0.025 mg/L	Thiamine-HCl	0.1 mg/L
KPH_2O_4	170 mg/L	$ZnSO_4$ ($7H_2O$)	8.6 mg/L	*myo*-Inositol	100 mg/L
KNO_3	1.9 g/L	$NaMoO_4$ ($2H_2O$)	0.25 mg/L	Glycine	2 g/L
		$MnSO_4$ ($4H_2O$)	22.3 mg/L	Sucrose	30 g/L
		KI	0.83 mg/L		
		*$FeSO_4$ ($7H_2O$)	27.8 mg/L		
		*Na_2EDTA	37.3 mg/L		

Composition of nutrient medium

Table 1. Inorganic salt composition of Murashige and Skoog (13), Hoagland and Arnon (7) and White's (20) media.

Ingredients	Media		
	Murashige and Skoog	Hoagland and Arnon	White
	Macronutrients (µmoles/liter)		
Nitrogen	60.0	15.0	2.0
NH^+_4	20.6	-	-
NO^-_3	39.4	15.0	2.0
Phosphorus	20.0	1.0	0.1
Potassium	1.3	6.0	1.7
Calcium	3.0	5.0	1.2
Magnesium	3.0	2.0	3.0
Sulfur	3.2	2.0	4.5
	Micronutrients (µmoles/liter)		
Boron	100.0	46.3	-
Chlorine	2,993.0	10.9	870.0
Cobalt	0.1	-	-
Cooper	0.2	0.3	-
Iodine	5.0	-	4.5
Iron	10.0	9.0	10.0
Manganese	103.0	10.9	30.0
Molybdenum	1.1	0.1	-
Sodium	3.2	-	-
Zinc	3.0	0.8	9.0

Composition of nutrient medium

- **Salts** are supplied in the form of macronutrients viz. N, Mg, K, Ca, P
- **Micronutrients** Cu, Ni, Mn, Co, etc.
- **Iron** is supplied in the chelated, Fe-EDTA (Ferric-Sodium Ethylene-Amine Tetra Acetate) form.
- **Vitamins** viz. meso-inositol, thiamin (B1), nicotinic acid (B3), pyridoxine (B6), etc.
- **Aminoacids**, mostly glycine is used.
- **Carbohydrate** is supplied usually in the form of sucrose.
- **Phytohormones** (auxins and cytokinins), their chemical form, concentration and ratio may vary from plant to plant.
- In general Auxins, such as IAA (Indole Acetic Acid) NAA (Naphthalene Acetic Acid), IBA (Indole Butyric acid); Cytokinins viz. Kinetin (6-furfuryl amino purine) 6-BAP (6, Benzyl Amino Purine) and Zeatin are used in nutrient medium.

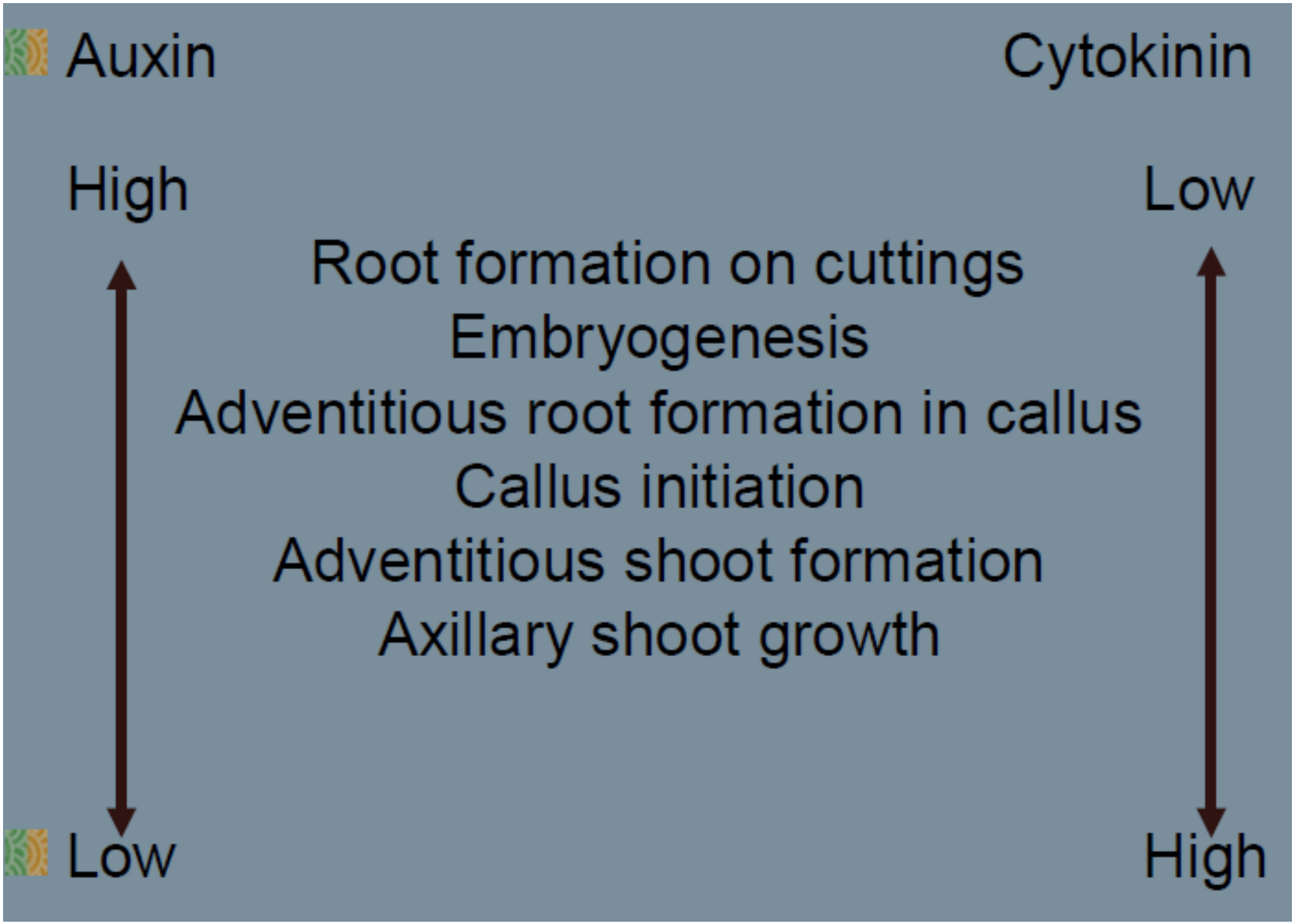
Plant growth regulators

Two major hormones affect Plant Differentiation:

- **Auxins:** Stimulates Root Development &
- **Cytokinin:** Stimulates Shoot Development

- Generally, the ratio of these two hormones can determine plant development:
  - ↑ Auxin ↓Cytokinin = Root Development.
  - ↑ Cytokinin ↓Auxin = Shoot Development.
  - Auxin = Cytokinin = Callus.

Hormonal balance



Types of medium

- **Chemically defined nutrient medium**
  - Chemical composition and structure is known
- **Chemically undefined nutrient medium:**
  - Complex additives viz. coconut milk, Casein hydrolysate, yeast extract, water melon juice, etc. are added in the medium.

1. Solid medium: 6-8% agar-agar

2. Semi solid medium: Less amount of agar

3. Liquid medium: Agar is not added. It is used for cell suspension culture.

Tissue culture

"... A method of biological research in which fragments of tissues from an animal or plant are grown in vitro in artificial medium under aseptic conditions and continue to survive and function."

"... the aseptic culture of plant protoplasts, cells, tissues or organs under aseptic conditions which lead to cell multiplication or regeneration of organs or whole plants."

Basic concepts of plant tissue culture(PTC)

Two concepts, are central to understanding plant cell, tissue, organ culture and regeneration.

Plasticity:

- -ability to initiate cell division from almost any tissue of the plant.
- -ability to regenerate lost organs or undergo developmental pathways in response to particular stimuli.

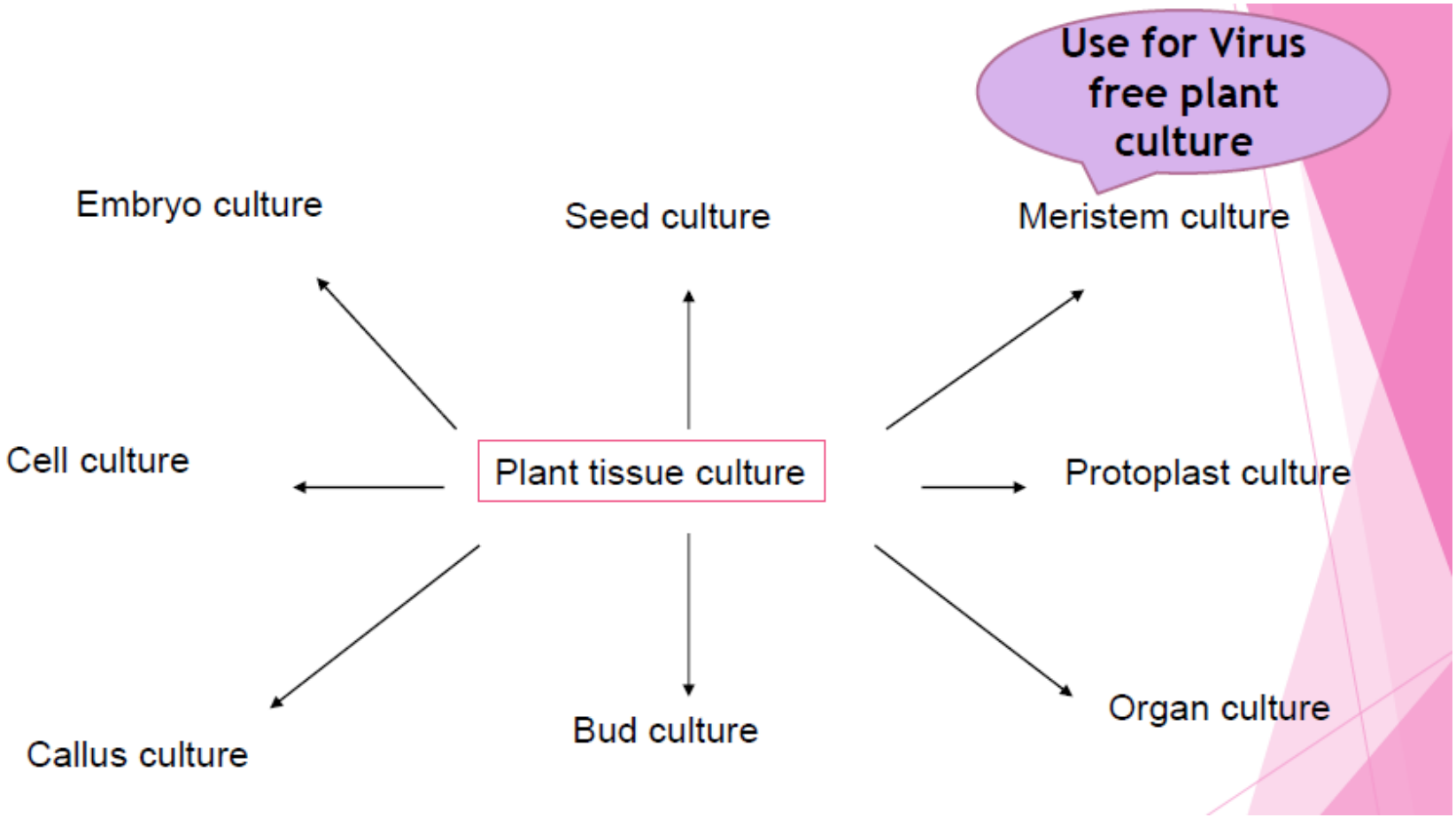
Totipotency:

- -each cell has the capacity to regenerate the entire plant.

Basic concepts of plant tissue culture(PTC)

- Cells lines differentiate to form specialized tissues and organs.
- Unlike animal cells, living plant cells re-differentiate.
- Therefore, tissue can be regenerated from explants such as cotyledons, hypocotyls, leaf, ovary, protoplast, petiole, root, anthers, etc.

Types of culture



Callus

- It is an unspecialized , unorganized, growing and dividing mass of cells.
- It produced when explants are cultured on the appropriate solid medium, with both an auxin and a cytokinin in a correct conditions. 2,4-D are commonly used.
- During callus formation there is some degree of dedifferentiation both in morphology and metabolism, resulting in the lose the ability to photosynthesis.
- Callus cultures may be compact or friable.
  - Compact callus shows densely aggregated cells .
  - Friable callus shows loosely associated cells and the callus becomes soft and breaks apart easily.
- **Habituation**: it reduce the requirement of auxin and/or cytokinin by the culture during long-term culture.

Principle / procedure of callus culture

There are Three criteria for callus culture are

1. Aseptic preparation of plant material
2. Selection of suitable nutrient medium
3. Incubation of culture under controlled physical condition

Aseptic preparation of plant material

- Surface sterilization :-

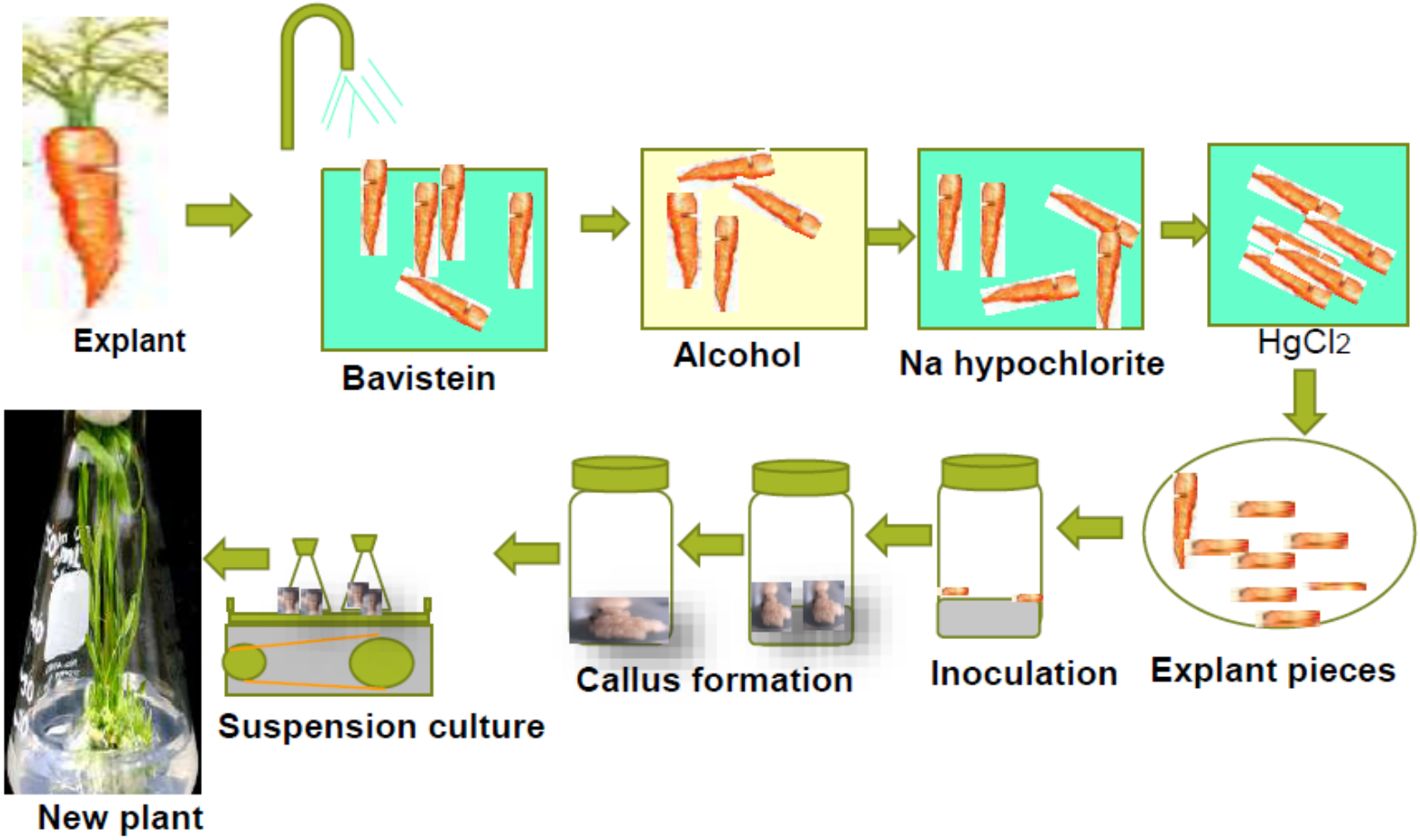
Selection of suitable nutrient medium

- Auxin/cytokinin –
  - 10:1-100:1 induces roots.
  - 1:10-1:100 induces shoots
  - Intermediate ratios around 1:1 favour callus growth .
- Agar solidified or semi – solid nutrient medium are used.
- That media are autoclaved at 15 psi pressure for 15 – 20 min at 121 0c.

Incubation of culture under controlled physical condition

- Temperature : 25 ± 2 oC
- Photoperiod : 16 hr Light , 8 hr Dark
- Light intensity : 2000 – 3000 lux
- Relative Humidity : 55 % - 60 %

STEPS INVOLVED IN CALLUS CULTURE



Three stages of callus culture

1. **Induction:** Cells in explant dedifferentiate and begin to divide

2. **Proliferative Stage:** Rapid cell division

3. **Morphogenesis stage:**

   - Organogenesis
   - Somatic embryogenesis

Organogenesis

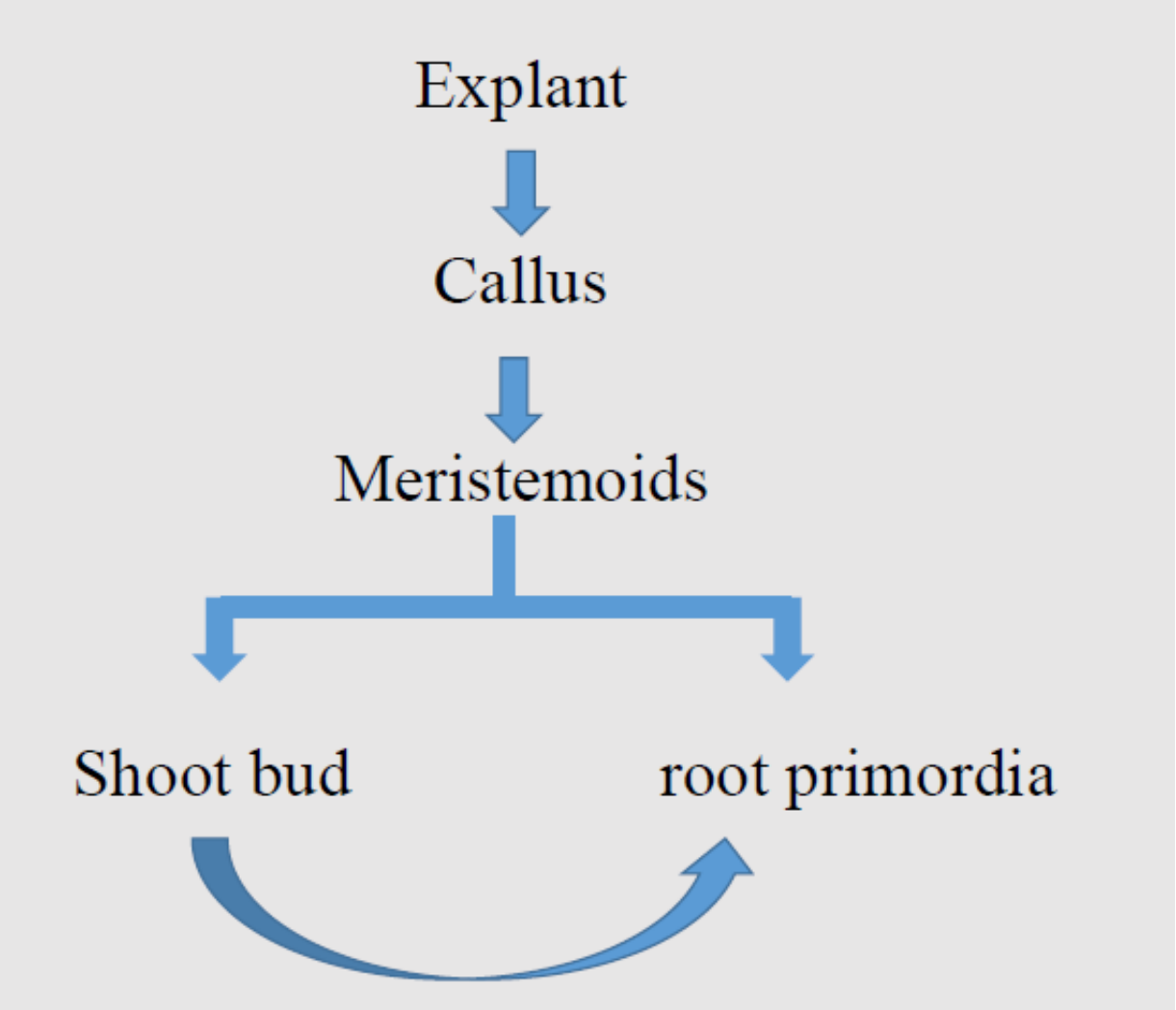
- The process of initiation and development of an organ is called organogenesis.
- In plant tissue culture, Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation.
- Organogenesis in plant tissue culture involves two distinct phases: dedifferentiation and redifferentiation.
- Dedifferentiation begins shortly after the isolation of the explant tissues with an acceleration of cell division and a consequent formation of a mass of undifferentiated cells (called callus).
- Redifferentiation, also called budding in plant tissue culture, may begin any time after the first callus cell forms. In this process of tissue called organ primordia is differentiated from a single or a group of callus cells. The organ primordia give rise to small meristems with cells densely filled with protoplasm and strikingly large nuclei.

Organogenesis

- Key factor of Organogenesis is the ratio of Endogenous Auxin – Cytokinin (T. A. Thorpe, 1980). The Explant develops into callus tissue in a medium containing either a particular concentration of Auxin or definite Auxin - Cytokinin ratio.

Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation.

Process



Factors effecting organogenesis

Genetic or a physiological change

- In a callus tissue the changes of chromosome structure or number such as aneuploidy, polyploidy, etc. Such chromosomal changes may lead to loss of totipotency of the cells.
- At the early stage of culture, the callus tissue exhibits maximum number of diploid cells. Eventually at the later stage of culture, the cells of callus tissue become mixaploid due to alteration of chromosome number and organogenesis could not be induced in such callus tissue, Occasionally, rooting occurs but shoot bud does not develop.

Factors effecting organogenesis

Phytoharmones

- For organogenesis the required balance of phytohormones by an exogenous supply of auxin, cytokinin or gibberellin either separately or in combination is essential .
- Generally high concentration of cytokinin brings about shoot bud initiation, whereas high levels of auxin favours rooting.
- Therefore, to obtain organogenesis, different permutation and combination of hormones as well as various concentrations of hormones are supplemented in the culture medium.

Factors effecting organogenesis

Other Chemicals

- Certain **phenolic compounds also induce shoot initiation in tobacco** callus- Phenolic compounds actually inactivate the auxins and consequently rise in the physiologically effective level of cytokinins which ultimately bring about the initiation of shoot buds.
- The use of **auxin inhibitor or auxin antagonist via culture medium also** causes the induction of shoot bud.
- Additions of **adenine in the culture medium also induce shoot bud in the** callus tissue.
- **Chelating agent like 1, 3 diamino-2- hydoxypropane-N.N.N'.N' tetraacetic acid initiates Shoot bud in haploid tobacco cultures.**
- **Abscisic acid in place of cytokinin also induces shoot bud formation in** root tuber tissue of sweet potato and stem tuber tissue of potato.

Factors effecting organogenesis

Enzymes

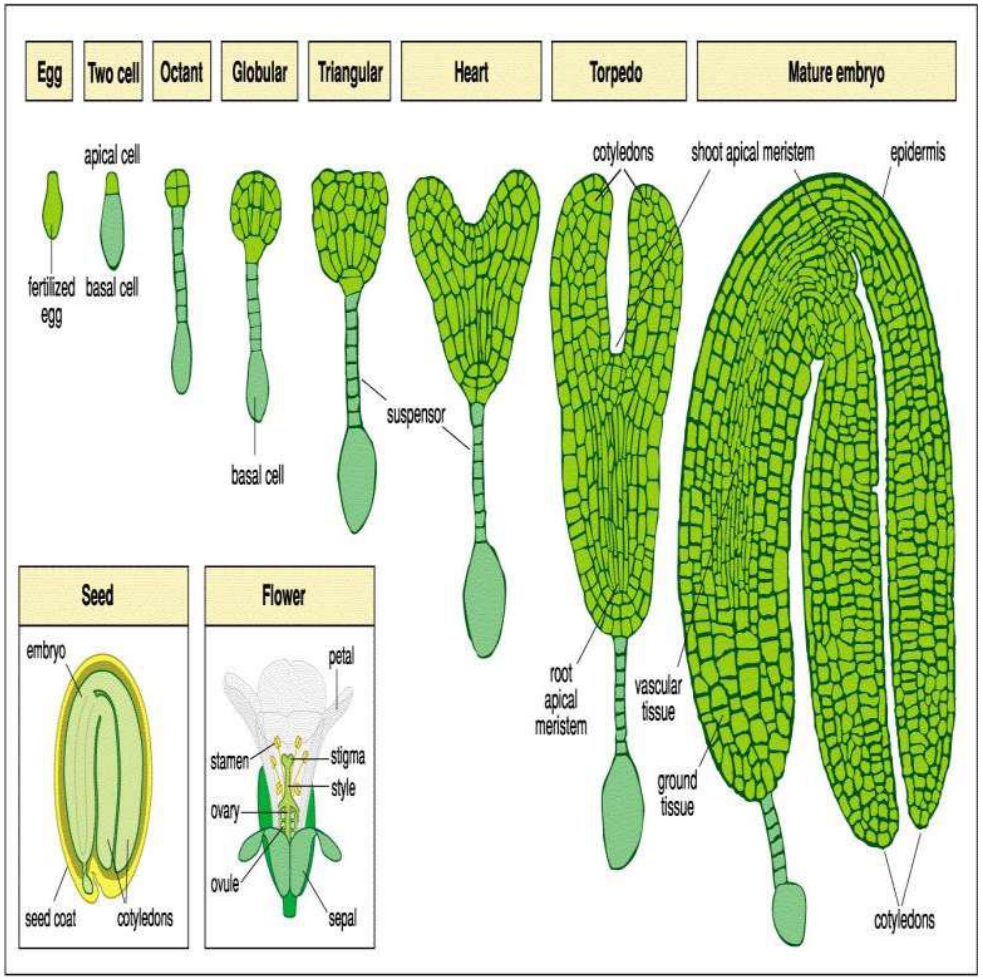
- **Peroxidase- One of the most important functions of peroxidase is** involvement in the metabolism of auxin.
- **Enzymes involving in carbohydrate metabolism- Gibberellic acid,** which represses starch accumulation by mobilising high amylase synthesis/activity, also inhibits shoot formation.
- **Embden Meyerhof-Parnas (EMP) and Pentose Phosphate (PP)** Pathway enzymes namely phosphoglucose isomerase, aldolase, pyruvate kinase, glucose-6- phosphate dehydrogenase, 6-phosphogluconate dehydrogenase etc. also involving in the shoot formation.

Somatic embryogenesis

- The process of formation of an embryo is called embryogenesis.
- Embryogenesis starts from a single embryogenic cell, that can be a zygote or an undifferentiated callus cell.
- Embryos developing from zygotes are called **zygotic embryos**, while those derived from somatic cells are called **somatic embryos**.
- In plant tissue culture, the developmental pathway of numerous well organised, small embryoids resembling the zygotic embryos from the embryogenic potential of somatic plant cell of the callus tissue or cells of suspension culture is known as **somatic embryogenesis**.
- Embryoid is a small, well-organised structure comparable to the sexual embryo, which is produced in tissue culture of dividing embryogenic potential of somatic cells.

Somatic embryogenesis

- Zygotic and somatic embryos share the same gross pattern of development.
- Both types of embryos develop as passing through typical developmental stages, such as globular, scutellar and coleoptilar stages for monocots, or globular, heart, torpedo and cotyledonary stages for dicots and conifers.
- Embryo development is bipolar, having a shoot and a radicular pole at opposite end.



Importance of Somatic Embryogenesis

- The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system.
- The adventitious embryo is a bipolar structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture.
- Somatic embryo has no food reserves, but suitable nutrients could be packaged by coating or encapsulation to form some kind of artificial seeds. Such artificial seeds produce the plantlets directly into the field.
- Unlike organogenesis, somatic embryos may arise from single cells and so it is of special significance in mutagenic studies.
- Plants derived from asexual embryos may in some cases be free of viral and other pathogens. So it is an alternative approach for the production of disease-free plants.

Comparison of zygotic and somatic embryogenesis

Differences between Zygotic and Somatic embryo:-

Zygotic embryo	Somatic embryo
➢ Fertilized egg or zygote.	➢ Sporophytic cells.
➢ Contain seed coat.	➢ Did not contain seed coat.
➢ Produce seed.	➢ Only form embryo.
➢ Plantlets are healthy.	➢ Plantlets are weaker
➢ Not like to mother plant.	➢ Like to mother plant.
➢ Propagation is low.	➢ Propagation is high.

Significances of callus culture

Callus culture as such has no major importance unless and until it is used for other experimental objectives . Still ,callus culture got some importance

1. The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium . This phenomenon is known as plant regeneration or organogenesis or morphogenesis .
2. Callus tissue is good source of genetic variability.
3. Cell suspension culture in moving liquid medium can be initiated from callus culture.
4. Callus culture is very useful to obtain commercially important secondary metabolites.
5. Several biochemical assay can be performed from callus culture.

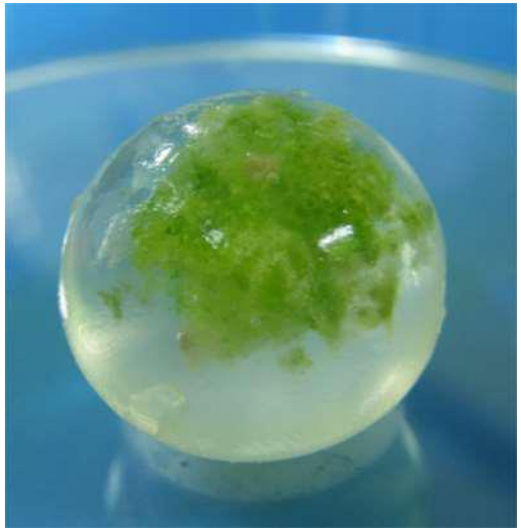
Synthetic Seeds

- Synthetic seeds are encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing as a seed or that possesses the ability to convert into a plant under *in vitro or ex vitro conditions and that can retain this* potential also after storage.
- Synthetic seeds are produced by encapsulating a plant propagule in a matrix which will allow it to grow into a plant. Plant propagules consist of shoot buds or somatic embryos that have been grown aseptically in tissue culture.
- Encapsulation is necessary to produce and to protect synthetic seeds. The encapsulation is done by various types of hydrogels which are water soluble. the gel has a complexing agent which is used in varied concentrations.

Gelling agent (% w/v)	**Complexing agent (µM)**
Sodium alginate (0.5 – 5.0)* Sodium alginate (2.0) with Gelatin (5.0)* Carragenan (0.2 – 0.8) Locust beam gum (0.4-1.0) Gelrite (0.25)	Calcium salts (30 –100) Calcium chloride (30 –100) Potassium chloride Ammonium chloride (500) temperature lowered

Principle and Conditions for Encapsulation with Alginate Matrix

- The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the somatic embryos when dropped into the CaCl2.2H2O solution form round and firm beads due to ion exchange between the Na+ in sodium alginate with Ca2+ in the CaCl2 . 2H2O solution.



Application of Synthetic Seed

1. Artificial seed provides low price production.
2. It's going to act as distinctive delivery system.
3. It plays a task of reproductive structure in embryo development.
4. Artificial seed technology has evolved as another and probably economical technique for mass propagation of various plant varieties.
5. By the employment of artificial seed technology species may be propagated.
6. Cereals, fruits and healthful plants may be studied with the assistance of artificial seed development at any place in the world.
7. Artificial seeds area unit terribly little therefore, they're straightforward to handle.
8. Artificial seed may be transported from one country to a different while not any obligations from the quarantine department.
9. Direct inexperienced house and field delivery of elite(seeds) chosen genotypes, genetically built plants area unit doable.
10. Artificial seed crop area unit sometimes straightforward to handle attributable to uniform genetic constituent.

Micropropagation

"… the *art and science of multiplying* plants *in vitro*

Stages of Micropropagation

Stage I - Selection & preparation of the mother plant – sterilization of the plant tissue takes place Initiation of culture – explant placed into growth media

Stage II – Multiplication – explant transferred to shoot media; shoots can be constantly divided.

Stage III - Rooting– explant transferred to root media.

Stage IV - Transfer to soil– explant returned to soil; hardened off.

Steps involved in the *in vitro micropropagation*

Cleaning of glassware

↓

Preparation of nutrient medium

↓

Selection and sterilization of *explant*.

↓

Inoculation of aseptic *explant* in to nutrient medium.

↓

Proliferation of shoots on a multiplication medium.

↓

Transfer of shoots for sub-culturing.

↓

Rooting and hardening of plantlets

↓

Field trials.

Procedure for cleaning of glassware

Soak glassware in 10% soap water (teepol) for 1 hour.

Transfer glassware to conc. HCl and keep for 2 hours.

Rinse glassware in tap water.

Wash the glassware at least twice with distilled water.

Keep glassware for drying in oven at 100 °C for 1 hour.

Autoclave/ keep glassware in oven at 140-160 °C for 2 hours.

SOMATIC HYBRIDIZATION
&
CYBRIDS

Importance of Protoplast Isolation And Culture

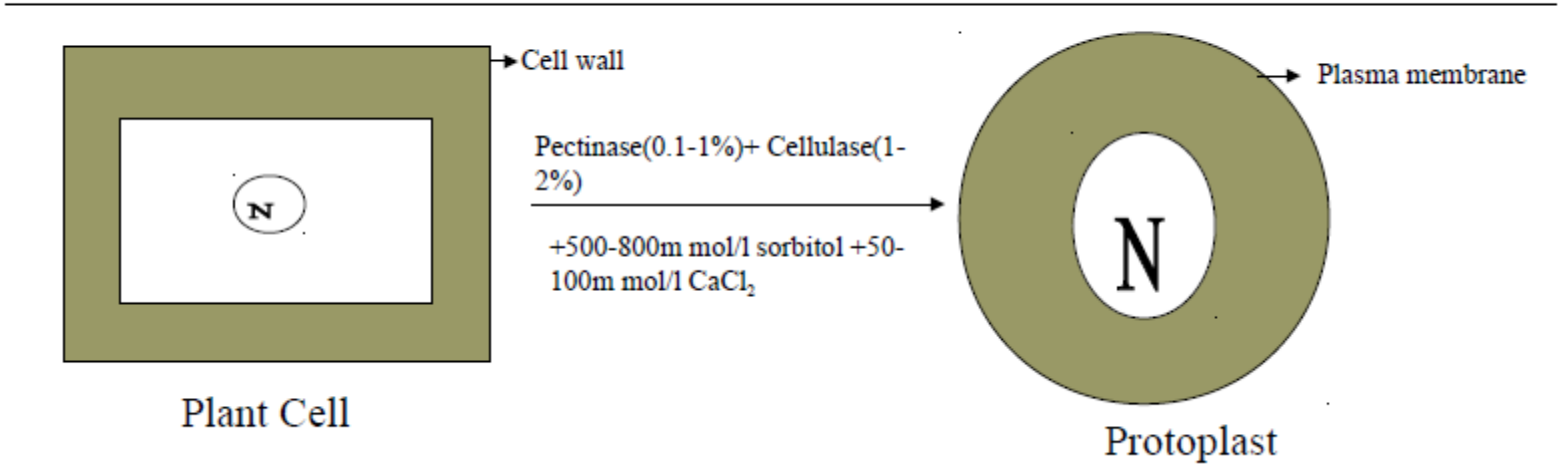
The isolation, culture and fusion of protoplast are one of the most fascinating fields of research. The techniques are important for the following reasons:-

- To develop novel hybrid plant through protoplast fusion, genetic engineering would continued to be an exciting area of research in modern plant biotechnology. This technology holds great promises to synthesise a plant of desired characteristics.
- This helps in crop improvement by somatic hybridisation and cell modification.
- The protoplast in culture can be regenerated into an entire plant.
- It provides a tool for isolating protoplasts and exploring the possibilities of genetic engineering.
- The technique in future will be one of the most frequently used research tools for tissue culturists, physiologists, pathologists molecular biologists, cytogenetics and biotechnologists

SOMATIC HYBRIDIZATION

- Development of hybrid plants through the fusion of somatic protoplasts of two different plant species/varieties is called somatic hybridization.
- This is a non conventional genetic procedure involving fusion between isolated protoplast under in vitro condition and subsequent development of their product (heterokaryon) to a hybrid plant.
- The production of hybrid plants through fusion of two different plant protoplasts (wall less naked cells) is known as SOMATIC HYBRIDISATION and such hybrids are called SOMATIC HYBRIDS.
- Somatic hybridisation involves the following 5 steps:-
  - Isolation of protoplasts.
  - Fusion of the protoplasts of desired species.
  - Identification and Selection of somatic hybrid cells.
  - Culture of the hybrid cells
  - Regeneration of hybrid plants from them.

Production of protoplasts

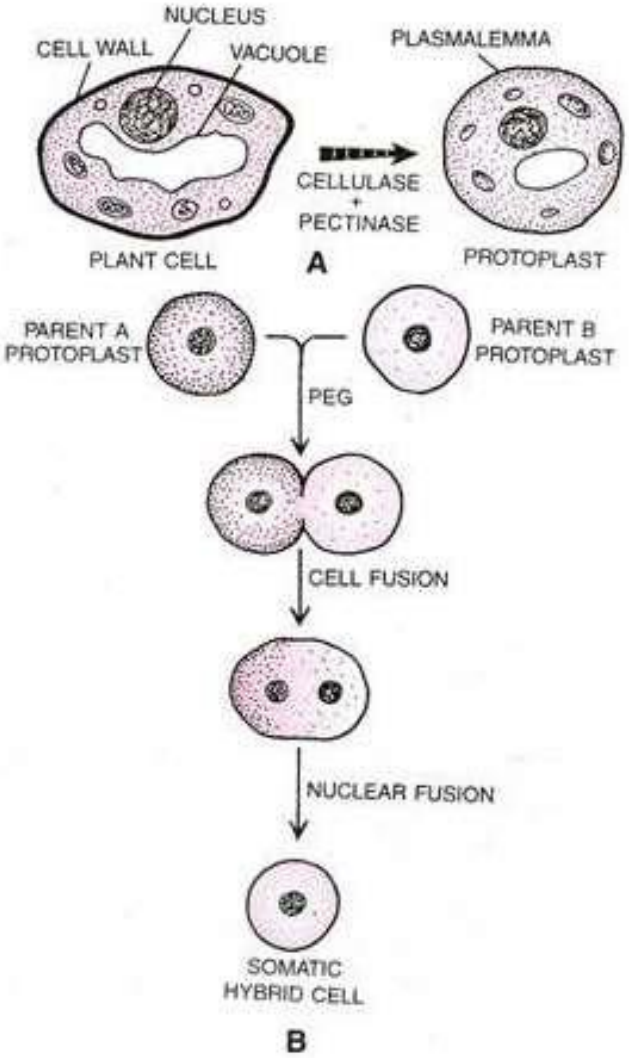


- Production of protoplasts by enzyme treatment. Osmoticum is added to stabilise the protoplasts and prevent them from bursting.

Isolation of Protoplasts

- The word “PROTOPLAST” was coined by “Hanstein” in 1880 for the living matter surrounded by the cell membrane.
- The isolated protoplast is highly fragile and outer plasma membrane is fully exposed. The plasma membrane is the only barrier between the interior of the living plant cell and the external environment.
- Isolation of protoplast can be done by three methods:-
  - (i) Mechanical (non-enzymatic)
  - (ii) Sequential enzymatic (two-step)
  - (iii) Mixed enzymatic (simultaneous)

Procedure



Somatic hybridisation. A, Production of protoplasts using a combination of pectinase and cellulase. B, Protoplast fusion induced by PEG ultimately yields somatic hybrid cells.

Mechanical Method

- Mechanical method of protoplast isolation was first done by Klercher (1982).
- Cut the tissue which are first plasmolysed with a sharp knife into small pieces. Then these pieces are deplasmolysed by using dilute solution to release the protoplasts. Generally protoplasts were isolated from highly vacuolated cells of storage tissues (onion bulbs, scales, radish root, beet root).

Sequential Enzymatic Method

- This method was first used Takebe and others in 1968 in two steps.
- The macerated tissue was first incubated in pectinase (degrade pectin cell wall) and then treated with cellulase (degrade cellulosic cell wall) for liberation of protoplasts

Mixed Enzymatic Method

- This is one step procedure in which both enzymes are used together to reduce time. Power and Cocking (1968) used this method for isolation of protoplasts.
- Protolplasts can be isolated by treating cells, with a suitable mixture of cell wall degrading enzymes. The mixture of Pectinase or Macerozyme (0.1-1.9%) and Cellulase (1-2%) is suitable for majority of plant parts.
- The commercially available enzyme has enabled the isolation of protoplasts from practically every plant tissue. There pH value is adjusted between 4.7 to 6 and is kept at temperature 25-30 Degree C.

Testing The Viability of Isolated Protoplasts

- The isolated protoplasts should be healthy and viable in order to undergo proper division and regeneration. This can be done by microscopic observation of untreated cells or after staining the cells with suitable chemicals to indicate active metabolism in the protoplasts. It is done by several methods-

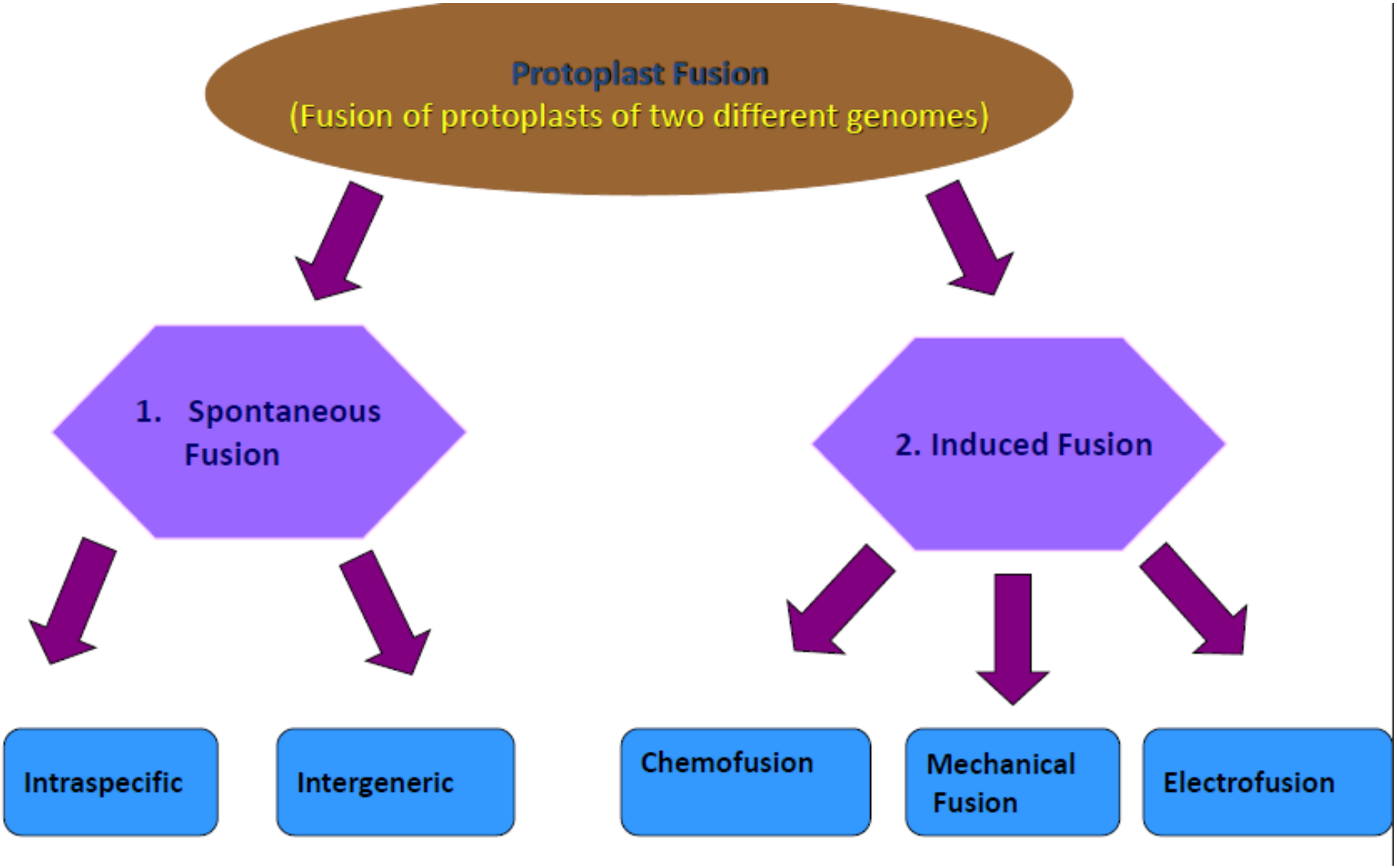
Methods for detection of viable and healthy protoplasts

- ***Phase Contrast Microscopy:*** Cytoplasmic streaming movement (cyclosis) and the presence of clear, healthy nucleus indicate that the cells are in viable state.
- ***Tetrazolium Reduction:*** In this test respiratory efficiency of cells is measured by reduction of 2,3,5- triphenyl tetrazolium chloride (TTC) to the red dye formazon. The formazon formed can be extracted and measured spectrophotometrically.
- ***Fluorescein Diacetate Method:*** The 0.5% fluorescein diacetate (FDA) in acetone is preparednand stored at 00C. This was added at 0.01% of finalbconcentration to protoplasts suspension with osmotic stabilizer. After 5min incubation the cells are observed under microscope with suitable filter.
- ***Evan's Blue Staining:*** The 0.025% of Evan's Blue stain solution was used for staining the protoplasts. The stain gives colour to the dead protoplasts by becoming permeable to dead ones. Whereas viable protoplasts remains colourless due to impermeability of plasma membrane to the stain.

Protoplast Fusion

- Plant protoplasts represent the finest single cell system that could offer exciting possibilities in the fields of somatic cell genetics and crop improvement.
- Protoplast fusion can be used to make crosses within species (intraspecific), between species (interspecific), within genera (intrageneric) and between genera (intergeneric).
- Number of methods have been used to induce fusion between protoplasts of different strains and successful result are obtained.
- The protoplasts fusion may be of 3 kinds:
  - 1. Spontaneous fusion
  - 2. Mechanical fusion
  - 3. Induced fusion

Protoplast Fusion



Protoplast Fusion

- ***Spontaneous Fusion:*** In spontaneous fusion, the adjacent protoplasts in enzyme mixture have tendency to fuse together to form homokaryons (having same type of nucleus).
- ***Mechanical Fusion:*** Gentle tapping of protoplasts suspension in a depression slide results in protoplasts fusion. The giant protoplasts of Acetabularia have been fused mechanically by pushing together two protoplasts. This fusion doesnot depend upon the presence of fusion inducing agents.
- ***Induced Fusion:*** Freshly isolated protoplasts can be induced to undergo fusion, with the help of a range of fusogens .e.g., NaNO3 , artificial sea water, lysozyme, high pH/ Ca++, PEG, polyvinyl alcohol, electrofusion.
- The following treatment have yielded success in producing somatic hybrid plants

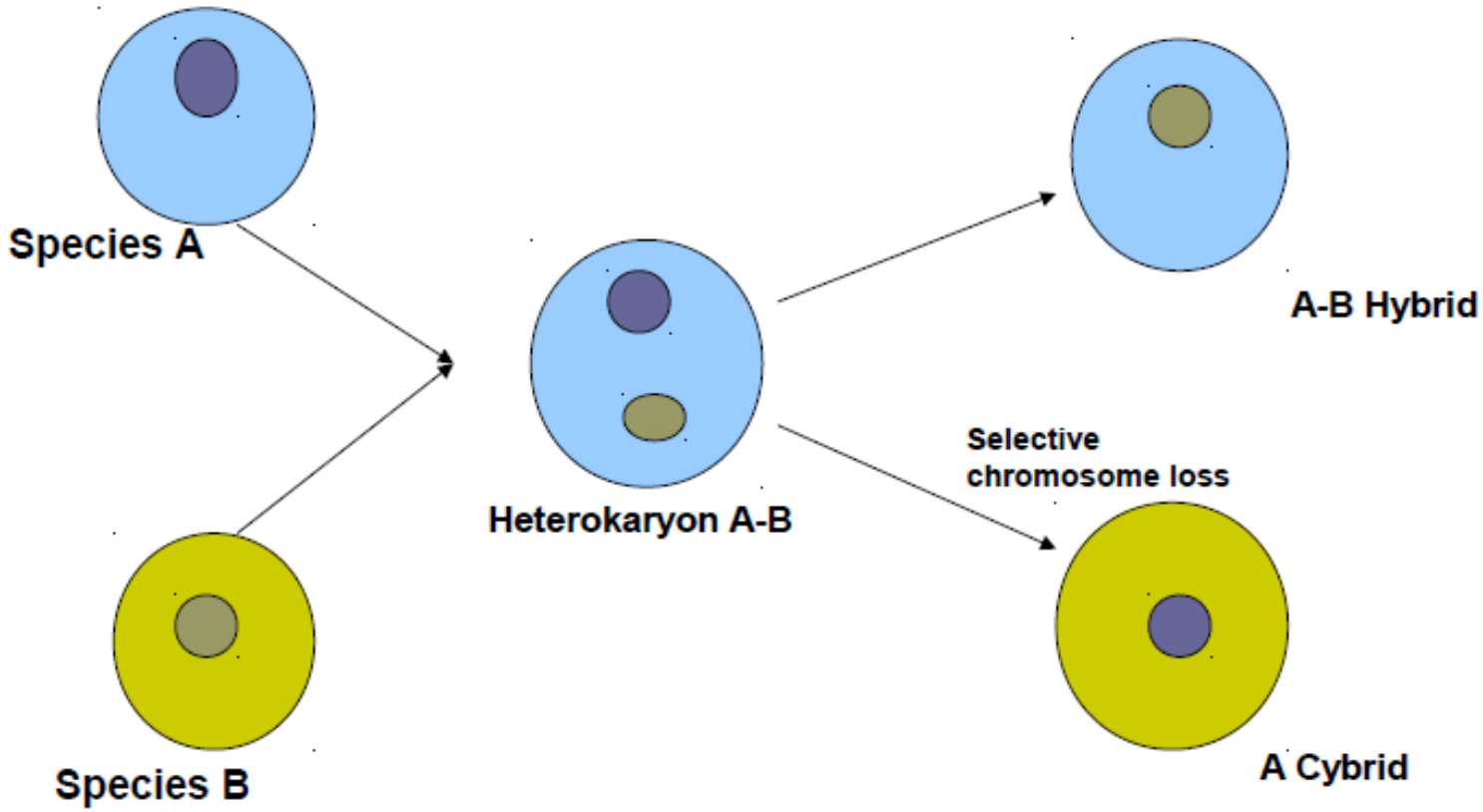
Methods for detection of viable and healthy protoplasts

- ***Auxotrophic Mutants:*** The orgininal protoplasts have the capacity to grow in minimal medium is known as Prototroph. The mutants of the prototroph which is not having the capacity to grow in the minimal medium is known as Auxotroph. The hybrid protoplasts are known to grow in the minimal medium and parental protoplasts are not able to grow in the minimal medium. It helps in the selection procedures.
- ***Visual Selection****:* In this selection method the fused protoplasts are identified by fusing the chlorophyll rich parent with chlorophyll deficient perent. The products of fusion are identified by using microscope because heterokaryons are bigger and green in colour, whereas parental protoplasts are either small and colourless. This is further differentiated by using suitable selective medium which supports good growth of only hybrid cells.
- ***Fluorescent Labels:*** In this method fluorescent labelled dyes are used to detect fusion products. If the two original protoplast cultures are pre-incubated for 12-15hours, one in octadeconyl aminoflurescein and the other in octadecyl palamine B each group of protoplasts takes on a specific fluorescence colour. The dyes are non- toxic and do not affect viability, wall regeneration or growth. After fusion of the protoplasts fusion products may be identified by their fluorescence characteristics under a fluorescence microscope.

Cybrids (Cytoplasmic Hybrid)

- Cybrids are cells or plants containing nucleus of species and cytoplasm of both the parental species. These are generally produced during protoplast fusion in variable frequencies.
- Cybrid formation may result by fusion of normal protoplasts of one species with enucleated protoplasts, elimination of the nucleus of one species from a normal heterokaryon, gradual elimination of the chromosomes of one species from a hybrid cell during the further mitotic divisions. The cybrids can be produced in high frequencies by irradiation of one parental protoplast before fusion in order to inactivate the nuclei or by preparing enucliate protoplast of one species and fusing them with normal protoplast of other species

Some fusion products resulting from protoplast culture



Culture of Protoplasts

- The first step in the protoplast culture is the development of a cell wall around the membrane of isolated protoplasts or their hybrid cells
- Isolated protoplasts or their hybrid cells are cultured either in a liquid or agar medium. The common practice of using a liquid culture medium includes either incubating protoplasts/heterokaryons in a thin layer or as small drops of nutrient medium inside a petri dish which, in turn is covered by another petri plate and finally sealed with parafilm. The culture dish is then maintained at low light or dark conditionsat 25-28 Degree C.
- For culturing protoplasts in the nutrient medium containing agar. About 2ml aliquots of isolated protoplasts of suitable density are mixed with an equal volume of agar nutrient medium, the temperature of which should not exceed 45 Degree C. On solidification of agar, the culture plates are sealed and maintained in an inverted position at 25-28 degree C. With this method, individual protoplasts or heterokaryons can be conveniently observed under a microscope and plating efficiency readily determined.

Regeneration Of Cell Wall

- In culture, protoplasts start developing a wall around itself within few hours and it takes only few days to complete the process.
- Wall materials are progressively deposited at the surface of the plasmalemma. The cellulose is deposited either between the plasmalemma and the multilamellar wall material or directly on the plasmalemma. The nature of biosynthesis of the cell wall depends on the plant material and the system of protoplast culture.nThe newly built cell wall can be observed either by plasmolyzing the protoplast by transferring it in a hypertonic solution, or by staining the cell wall with **calcofluor white fluorescent stain.**

Regeneration Of Cell Wall

However, electron microscopic studies and freeze etching studies have revealed much about the structure and progressive development of cell wall around the protoplast in culture medium.

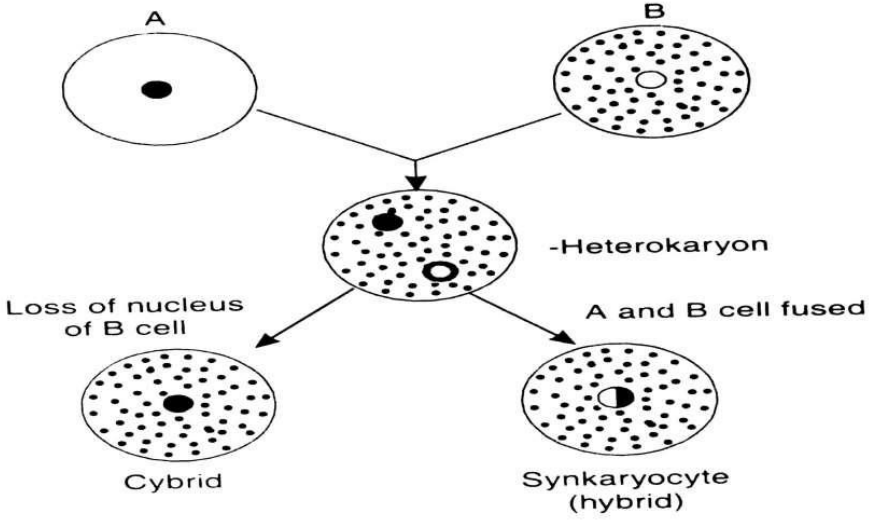
- Observe regularly the regeneration of cell wall, cell division and small callus formation under inverted microscope.
- Examine cell wall formation in protoplasts with a droplet of 0.1% calcofluor white R, American Cyanamid, Bound Brook, USA, in 0.4M sorbitol solution on a slide. The cell wall regenerated protoplasts fluoresce.
- Small cluster of calli are observed after 2-3weeks of culturing protoplasts.
- Subculture the cell clusters on a freshly prepared protoplast culture medium with or without ½ the mannitol and 0.8-1.6% agar.

Development of Callus/ Whole Plant

- Soon after the formation of wall around the protoplasts, the reconstituted cells show considerable increase in size and first divisions usually occur within 7 days. Subsequent divisions give rise to small cell colonies. After 2-3 weeks macroscopic colonies are formed which can be transferred to an osmotic free medium to develop a callus. The callus may be induced to undergo organogenic differentiation, or whole plant regeneration.

CYBRID

- Plants or cells witch containing nucleus of one species but cytoplasm from both the parental species .
- Cytoplasmic hybrids.
- Involves fusion of two protoplasts (donor and recipient) in which donor nuclear genome is eliminated while its plastome and/or chondriome are merged with that of the recipient.
- For production of a cybrid cell uses two parental cells but should be one complete cell (cytoplasm with nucleus) and other should be without nucleus or in active nucleus



Cryopreservation

- The process of storing or preserving the biological samples in extremely cold or subzero temperatures in a deep freeze, commonly at –196°C is termed as cryopreservation. The word "Cryopreservation" is derived from the Greek word "krúos", meaning icy cold or frost.
- At such low temperatures, all the biological activities of the cells stop and the cell dies. Cryopreservation helps the cells to survive cooling to extreme temperatures and again thawing them to physiological conditions.
- The ice formation inside the cells breaks the cell membrane and causes cell death. The freezing rate and the composition of the freezing medium are the two factors that can prevent this intercellular freezing.
- ***"Cryopreservation is the use of very low temperatures to preserve the cells and tissues that are structurally intact."***

Cryopreservation Process

- In this process, biological materials including cells, tissues, organs, oocytes, spermatozoa, ovarian tissues, pre-implantation embryos, and other prepared culture media are preserved in extremely cold temperatures for extended periods without affecting the cell's viability.
- Dry Ice and liquid nitrogen are generally used in this method of preservation.
- At these subzero temperatures, all the biological activities of cells, tissues and other biological materials cease or effectively stopped and are presumed to provide indefinite longevity to cells.

Cryopreservation Steps

- The complete procedure steps involved in preserving the obtained biological samples are as follows:
- **Harvesting or Selection of material**– Few important criteria should be followed while selecting the biological materials such as – volume, density, pH, morphology, and damaged free.
- **Addition of cry-protectant** – Cryoprotective agents such as glycerol, FBS, salts, sugars, glycols are added to the samples as it reduces the freezing point of the medium and also allow slower cooling rate, which reduces the risk of crystallization.
- **Freezing** – Different methods of freezing are applied in this method of cryopreservation to protect cells from damage and cell death by their exposure to the warm solutions of cryoprotective agents.
- **Storage in liquid nitrogen**– The cryopreserved samples are stored in extreme cold or -80°C in a freezer for at least 5 to 24 hours before transferring it to the storage vessels.
- **Thawing-** The process of warming the biological samples in order to control the rate of cooling and prevent the cell damage caused by the crystallization.

Applications of Cryopreservation

Cryopreservation is a long-term storage technique, which is mainly used for preserving the biological material without decline or decaying the biological samples for an extended period of time at least for several thousands of years.

This method of preservation is widely used in different sectors including cryosurgery, molecular biology, ecology, food science, plant physiology, and in different medical applications. Other applications of cryopreservation process are:

1. Seed Bank.
2. Gene Bank.
3. Blood transfusion.
4. In vitro fertilization.
5. Organ transplantation.
6. Artificial insemination.
7. Freezing of cell cultures.
8. Storage of rare germplasm.
9. Conservation of biodiversity.
10. Conservation of endangered and disease free plant species.

GERMPLASM AND ITS CONSERVATION

- A germ is a collection of genetic resources for an organism. For plants,the germplasm may be stored as a seed collection(even a large seed bank) or for trees in a nursery.
- Germplasm is a living tissues from which new plants can be grown.It can be a seed or another plant part-a leaf,a piece of stem,pollen or even just a few cells that can be turned into the whole plant.
- It contains the information for a species genetic make up,a valuable natural resources of plant diversity.

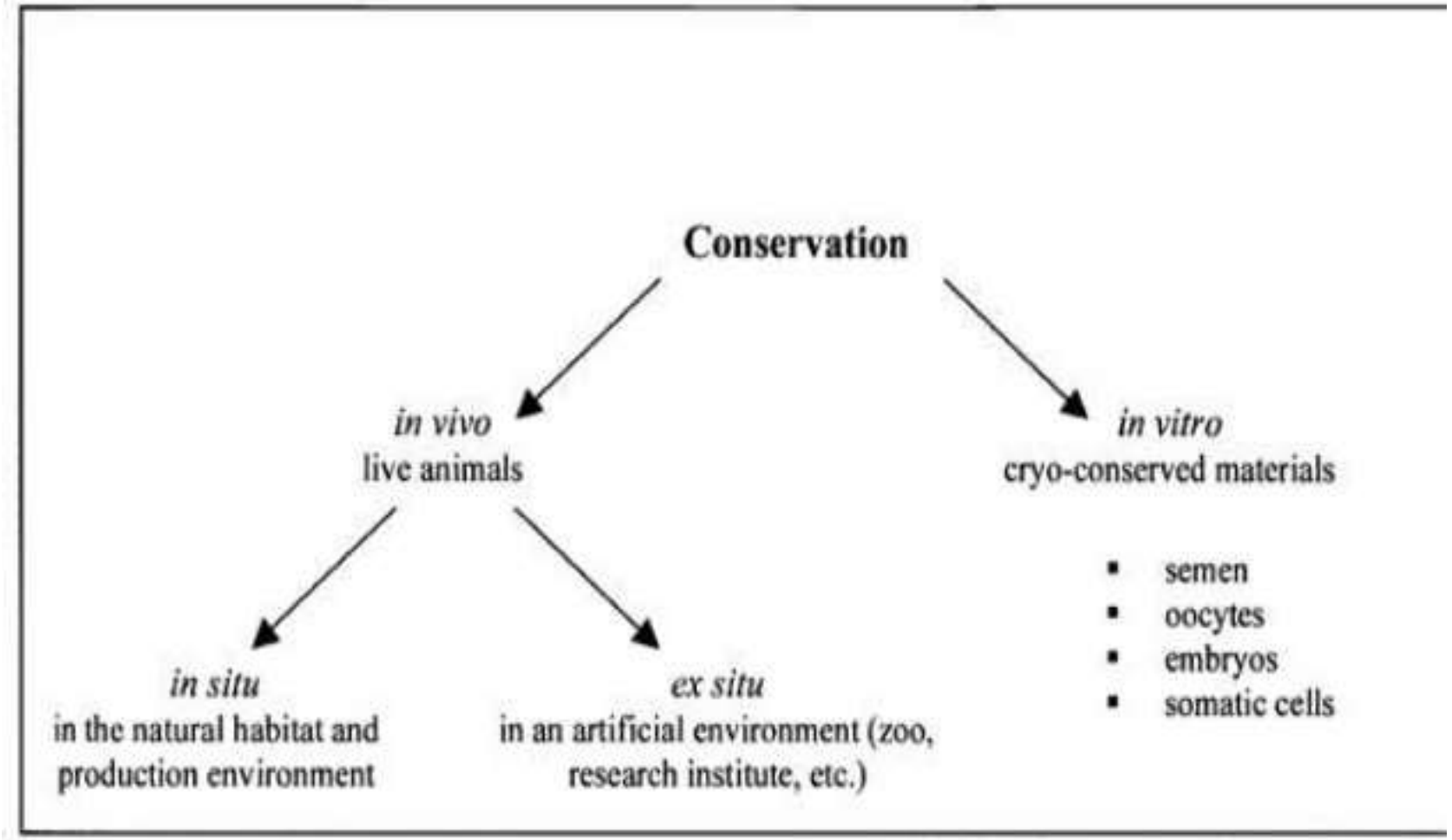
WHAT IS GERMPLASM CONSERVATION?

- Plant germplasm is the genetic sources material used by the plant breeders to develop new cultivars. They may include:
  - Seeds
  - Leaf
  - Stem
  - Pollen
  - Cultured cells
- Germplasm provide the raw material (genes) which the breeder used to develop commercial crop varieties.

NEED FOR CONSERVATION OF GERMPLASM

- Loss of genetic diversity among plant species.
- Human dependence on plant species for food and many different uses.E.g. basic food crops,building materials,oils,lubricants,rubber and other latexes,resins,waxes,perfumes,dyes fibres and medicines.
- Species extinction aand many other are threatened and endangered-deforestation.
- Great diversity of plants is needed to keep the various natural ecosystems functioning stablyinteractions between species.
- Aesthetic value of natural ecosystems and diversity of plant species.

MODE OF CONSERVATION



IN SITU CONSERVATION

- In situ conservation is on- site conservation or conservation of natural resources in a natural population of plants such as forests genetic resources in natural population of tree species.
- It is the process of protecting an endangered plant in its natural habitat either by protecting or cleaning up the habitat itself or by defending the species from predators.
- It is applied to conservation of agriculture biodiversity in agro ecosystem by farmers, especially those using unconventional farming practice.

EX SITU CONSERVATION

- Ex situ conservation means literally,"off-site conservation ".It is the process of protecting an endangered species of plants or animal outside of its natural habitat;for example, by removing part of the population from a threatened habitat and placing it in a new location ,which may be a wide area or within the care of humans.

EX SITU CONSERVATION CAN BE CARRIED OUTBY SEVERAL METHODS

- Seed gene bank
- In vitro storage
- Dna storage
- Pollen storage
- Field gene bank
- Botanical gardens