Regeneration Ability of an Explant

depends on:

1. Organ from which it is derived
2. The physiological state of explant/ Differences in the stage of the cells in the cell cycle
3. Size of the explant
4. Orientation of the explant on the medium
5. Inoculation density
6. Availability or ability to transport endogenous growth regulators
7. Metabolic capabilities of the cells

Plant Regeneration Pathways

1. Histogenesis (Micropropagation; Pre-existing Meristems)	Uses meristematic cells to regenerate whole plant (shoot culture/nodal culture)
2. Organogenesis	Relies on the production of organs either directly from an explant or callus structure
3. Somatic Embryogenesis	Embryo-like structures which can develop into whole plants in a way that is similar to zygotic embryos are formed from somatic cells

1. Histogenesis (Micropropagation)

-most commonly used tissue explants are the meristematic ends of the plants like the stem tip, auxillary bud tip & root tip

-these tissues have high rates of cell division & produce required growth regulating substances including auxins & cytokinin

Stage 0: Preparation of donor plant	-if possible, mother plant should be ex vitro cultivated under optimal conditions to minimize contamination in the in vitro culture
	-explant should be selected from young and healthy part that actively grow
	-collection prior to flowering
Stage I: Initiation stage	1. Explant isolated is surface sterilized and transferred into nutrient medium
	2. Combined application of bactericide and fungicide (generally)
	3. Cultures are incubated in growth chamber either under light or dark conditions according to the method of propagation
	*disinfectants: sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride (HgCl2)
Stage II: Multiplication stage	-aim: increase the number of propagules
	-number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained
	-repeated enhanced formation of axillary shoots from shoot tips or lateral buds
	-4-8 weeks subculturing intervals (1 cycle)
	-multiplication is very labor-intensive
	1. Higher concentration of cytokinin provided
	2. Lower concentration of auxin provided

1. Histogenesis (Micropropagation) (cont)

	3. Gibberellins (GA's) may be added to promote etiolation, especially in species that form rosettes.
Stage III: Rooting stage	1. Plants must be rooted by using media containing auxin or by dipping explant bases in auxin solutions.
	a) may use the same culture media used in multiplication stage
	b) sometimes, it is necessary to change media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth
	2. Higher concentration of auxin provided
	3. Lower concentration of cytokinin provided
Stage IV: Acclimatization Stage	-aim: in vitro plants need to be weaned and hardened by undergoing acclimatization
	1. Microshoots are moved from sucrose in jar (heterotrophic stage) to photosynthesis (photoautotrophic stage)
	2. Increasing the light intensity (to harden the plants)
	3. reducing sugar, inorganic salts and humidity (to harden the plants)

1. Histogenesis (Micropropagation) (cont)

4. The plants are then transferred to an appropriate substrate (sand, peat, compost etc.) and gradually hardened under greenhouse

*Medium must be removed prior to transplantation to prevent contamination.

Micropropagation Flow Chart

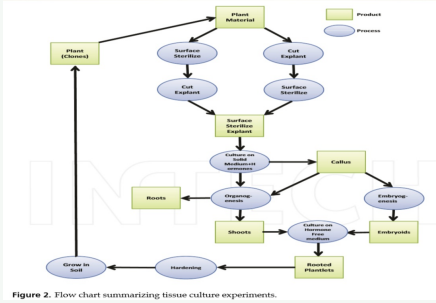


Figure 2. Flow chart summarizing tissue culture experiments.

Conventional propagation vs Micropropagation

	Conventional propagation	Micropropagation
	Cuttings, budding, grafting, layering	Tissue culture using axillary buds and meristems
Advantages	• Equipment costs minimal. • Little experience or technical expertise needed • Inexpensive • Specialized techniques for growth control (e.g. grafting onto dwarfing rootstocks)	• From one to many propagules rapidly; • Multiplication in controlled lab conditions & Reduce stock plant space **No Pressure on Land Use** • Precise crop production scheduling • Long-term germplasm storage • Production of difficult-to-propagate species **Continuous Production**

**2. Organogenesis

-refers to the production of adventitious plant organs i.e. roots, shoots and leaves that may arise directly from the meristem or indirectly from the undifferentiated cell masses (callus)

-ability of non-meristematic plant tissues to form various organs

-production of roots, shoots or leaves

-organs may arise out of pre-existing meristems or out of differentiated cells

-may involve a callus intermediate but often occurs without callus

-involves the callus production and differentiation of adventitious meristems into organs by altering the concentration of plant growth hormones in nutrient medium

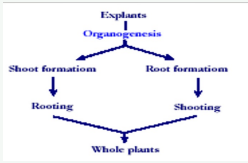
Type of Organogenesis

1. Direct Organogenesis

**2. Organogenesis (cont)

a) Directly from an explant	
b) Axillary bud formation and growth	
2. Indirect organogenesis	
a) Callus culture	1) Dedifferentiation - less committed, more plastic developmental state
	2) Induction - Cells become organogenically competent and fully determined for primordia production
	3) Differentiation
Characteristics	-relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium
	-auxin to cytokinin ratio determines which developmental pathway
	-induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium.
	-these shoots can then be rooted relatively simply
Control of Organogenesis	
1. Auxin: Stimulates Root Development	*-↑ Auxin ↓Cytokinin = Root Development*
2. Cytokinin: Stimulates Shoot Development	*-↑ Cytokinin ↓Auxin = Shoot Development*
	- Auxin = Cytokinin = Callus Development
Advantage	
1. Mass multiplication of elite germplasm.	
2. Source material for protoplast work or genetic transformation	
3. Conservation of endangered genotypes	
*Organogenesis may not produce clones!	

Organogenesis Flow Chart



Organogenesis Process

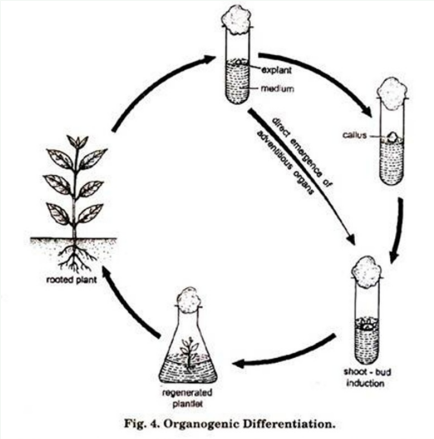


Fig. 4. Organogenic Differentiation.

3. Somatic Embryogenesis

-in vitro method of plant regeneration widely used as an important biotechnological tool for sustained clonal propagation

-process by which somatic cells or tissues develop into differentiated embryos, then develop into whole plants without undergoing the process of sexual fertilization

-Plant growth regulators play an important role in the regeneration and proliferation of somatic embryos

-usually involves a callus intermediate stage which can result in variation among seedlings

A) Plant regeneration via somatic embryogenesis occurs by the induction of embryogenic cultures from zygotic seed, leaf or stem segment and further multiplication of embryos

B) Mature embryos are then cultured for germination and plantlet development, and finally transferred to soil

1. Direct Somatic Embryogenesis	-Embryos initiate directly from explant in the absence of callus formation.

3. Somatic Embryogenesis (cont)

	-Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare
2. Indirect Somatic Embryogenesis	-Embryos initiate from callus developed from explant
	Explant → Callus induction → Callus Embryogenic development → Maturation → Germination
1) Initial stage (embryo initiation)	high concentration of 2,4-Dichlorophenoxyacetic acid (selective herbicide) is used
2) Second stage (embryo production)	embryos are produced in a medium with no or very low levels of 2,4-D
	*supplying a source of reduced nitrogen (specific amino acids/casein hydrolysate) can also improve

- also regarded as a valuable tool for genetic manipulation

-The process can also be used to develop the plants that are resistant to various kinds of stresses and to introduce the genes by genetic transformation. adventitious

3. Somatic Embryogenesis (cont)

Various terms for non-zygotic embryos

1. Adventitious embryos	Somatic embryos arising directly from other organs or embryos.
2. Parthenogenetic embryos	Somatic embryos are formed by the unfertilized egg.
3. Androgenetic embryos	Somatic embryos are formed by the male gametophyte.

Somatic Embryo Development

-Auxin must be removed for embryo development	Continued use of auxin inhibits embryogenesis

-Polarity is established early in embryo development.

-Signs of tissue differentiation become apparent at the globular stage and apical meristems are apparent in heart-stage embryos.

Development Stages

1. Zygote	4. Torpedo
2. Globular	5. Cotyledonary
3. Heart	6. Germination

Characteristics

1. Bipolar structure – shoot and root pole
2. Source of protoplasts and suspension cultures.
3. Clonal propagation

Somatic Embryogenesis Process

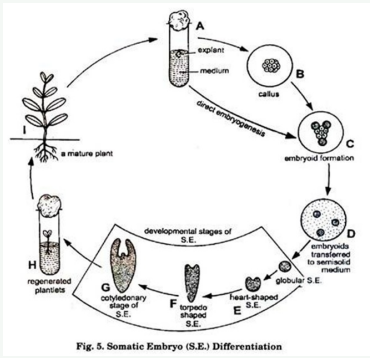
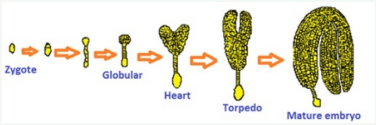


Fig. 5. Somatic Embryo (S.E.) Differentiation

Somatic Embryo Development Stages



Embryogenesis, Organogenesis, Micropropagation

-Both of these technologies can be used as methods of micropropagation.

-It is not always desirable because both of them may not always result in populations of identical plants which is needed for micropropagtion.

-The most beneficial use of somatic embryogenesis and organogenesis is in the production of whole plants from a single cell (or a few cells).

a) High probability of mutations

b) The method is usually rather difficult.

c) Losing regenerative capacity become greater with repeated subculture

d) Induction of embryogenesis is very difficult with many plant species

e) A deep dormancy often occurs with somatic embryogenesis

Difference of S. Embryogenesis and Organogenesis

Organogenesis	Somatic Embryogenesis
-monopolar structure	-bipolar structure with a closed radicular end
-has vascular connection with the mother tissue	-has no vascular connection with the mother tissue

Compare Organogenesis and Embryogenesis

Organogenesis	Embryogenesis
-Explant or callus is subcultured on shooting medium to induce shoot formation	-Explant or callus is subcultured on embryogenesis medium to induce formation of pro-embryogenic cell masses (PEMs)

Compare Organogenesis and Embryogenesis (cont)

-Group of cells differentiate to form shoots (5,000-10,000)	-PEMs are form from single cells and subcultured into the same medium for PEM proliferation (hundred thousands to million)
-Each shoot of appropriate size is identified and excised individually and subculture on rooting medium to induce rooting (labour intensive)	-PEMs are split and subcultured onto medium with less auxin in batches (less labour) to grow and differentiate further
	-Somatic embryos are subcultured on medium without hormone for germination

Different problems in Plant tissue culture

Problem	Description	Way to Overcome
1. Recalcitrance	-inability of plant tissue culture to respond to culture manipulation	-Antioxidant Protection: Antioxidants are special compounds that have the capability of neutralizing reactive molecules and particles - so called free radicals
	-loss of morphogenetic competence and totipotency capacity	-Juvenile tissue can be selected as explant
	-Free radical-mediated stress has a role in tissue culture recalcitrance.	-Parts of the desired plant rejuvenated by treatments like cytokinin spray on selected branches

Different problems in Plant tissue culture (cont)		
	-Free radicals and their reaction products react with macromolecules such as DNA, proteins and enzymes, causing cellular dysfunction and, as a result, the cultures become recalcitrant	-
	-All aerobic organisms are totally dependent upon redox reactions and the transfer of single electrons and many life processes involve free radical intermediates.	-
2. Contamination	-source: a) carry over of microorganisms on the surface or in the tissues of explants; b) faulty procedures in the laboratory	-Wear gloves and a lab coat and keep long hair tied back.
	-Bacteria, fungi, mould and yeasts are common contaminating microorganisms in tissue culture.	-Work in a laminar flow hood when passaging cells.

Different problems in Plant tissue culture (cont)	
-Many of the microorganisms that are likely to be present intercellular, in plant tissues will be capable of growth on the plant tissue culture medium, although some may be inhibited by the high salt or sucrose concentration and the pH	-Wipe down working surfaces with ethanol.
	-Use sterile equipment.
	-Inspect all equipment and media for visible contamination before use.
	-NO cross over - Do not pass your hands/arms over any open bottle, plate or tube.
	-Use proper antibiotics in your culture media.
	-When finished, dispose of materials properly, wipe down working surfaces with ethanol, and turn on UV lamp within laminar flow hood for 10 minutes to sterilize the area.

Different problems in Plant tissue culture (cont)		
3. Phenolic browning	-Many plants are naturally rich in polyphenolic compounds that are commonly regarded as inhibitory agents.	-Culture bottles are kept in dark condition
	-In most of the cases, when these plants are cultured in vitro, the culture medium turns brown.	-Addition of antioxidants (Polyvinylpyrrolidone, PVP-40) to medium was more effective to reduce the browning.
	-Phenolic browning caused by the accumulation and oxidation of phenolic compounds.	-inhibiting the activity of the phenylalanine ammonia lyase enzyme (PAL), thereby reducing the biosynthesis of phenolic compounds
4. Seasonal variation	-relative humidity, dry season affects the medium and nutrient medium evaporates rapidly when too dry	-Choose explant in its most responsive season
	-extreme moist climate such as poor tropical region, fungi is effected on media	-Use in vitro plantlets as explant
	-dust in air is also a major source of bacterial contaminants	-Controlled environment

Different problems in Plant tissue culture (cont)		
	-germination of shoots and roots also delayed due to the seasonal variation	-
5. Vitrification(hyperhydricity)	Hyperhydricity is the physiological malformation due to excessive hydration, low lignification and reduced mechanical strength of tissue culture generated plants.	-Culture are sub-cultured frequently to overcome this vitrification
	Hyperhydricity in plant tissue cultures are those factors triggering oxidative stresses such as high salt concentration, low calcium content in culture medium, gas built up within the container, high relative humidity, low light intensity, gas accumulation in the atmosphere of the jar, length of time intervals between subcultures.High ammonium concentration, culture bottles kept in same container.	-Vitrification can be lessen by raising the agar and/or sugar concentration, addition of ethylene-inhibitors, amino acid, phenolic glycosides phloridzin, naringin or esculin hydate, using two-phase media, bottom cooling of the culture vessels,ventilation of the vessels, adding silver nitrate

Different problems in Plant tissue culture (cont)

6. Somaclonal Variation	-genetic variations along with phenotypic changes found in the in vitro cultured cells	-Avoiding long term cultures
	-Somaclonal variations occur as a result of genetic heterogeneity (change in chromosome number and/or structure) in plant tissue cultures.	-Axillary shoot induction systems
	-cause: a) Expression of chromosomal mosaicism or genetic disorders; b) ii. Spontaneous mutations due to culture conditions	-Regularly reinitiating clones from new explants.
	-factors: a) Genotype and explant source; b)Duration of cell culture; c) Growth hormone effects	-Prevent usage of 2,4-D IN media

Limitations of Somaclonal Variations

i. Most of the somaclonal variations may not be useful.

ii. The variations occur in an unpredictable and uncontrolled manner.

iii. Many a times the genetic traits obtained by somaclonal variations are not stable and heritable.

iv. Somaclonal variations are cultivar-dependent which is frequently a time consuming process.

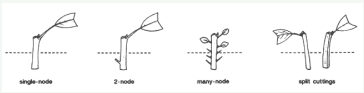
v. Somaclones can be produced in only those species which regenerate to complete plants.

vi. Many cell lines (calli) may not exhibit regeneration potential.

Nodal Cutting

Function: Removes the inhibitory effect of the shoot apex on bud outgrowth (Apical dominance)

Nodal Cutting Image



Gibberellins

Growth hormones that stimulate cell elongation and cause plants to grow taller.

Rosette

Circular arrangement of leaves or of structures resembling leaves

Etiolation

Etiolation is a process in flowering plants grown in partial or complete absence of light.

It is characterized by long, weak stems; smaller leaves due to longer internodes; and a pale yellow color.