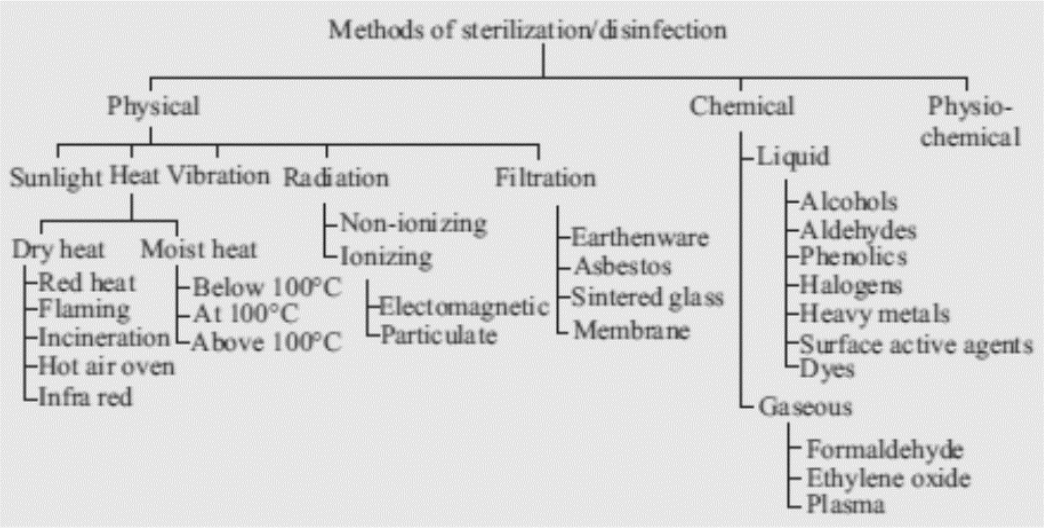
METHODS OF STERILIZATION

The various methods of sterilization are:

1. **Physical Method**
   (a) Thermal (Heat) methods
   (b) Radiation method
   (c) Filtration method
2. **Chemical Method**
3. **Gaseous method**



Nutrient medium

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media. Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that plants in nature can synthesize their own food material. However, plants growing in vitro are mainly heterotrophic i.e. they cannot synthesize their own food.

Composition of Media:

The composition of the culture media is primarily dependent on two parameters:

1. The particular species of the plant.
2. The type of material used for culture i.e. cells, tissues, organs, protoplasts.

Thus, the composition of a medium is formulated considering the specific requirements of a given culture system. The media used may be solid (solid medium) or liquid (liquid medium) in nature. The selection of solid or liquid medium is dependent on the better response of a culture.

Major Types of Media:

The composition of the most commonly used tissue culture media is given in the following Table, and briefly described below.

White's medium:
This is one of the earliest plant tissue culture media developed for root culture.

MS medium:
Murashige and Skoog (MS) originally formulated a medium to induce organogenesis, and regeneration of plants in cultured tissues. These days, MS medium is widely used for many types

of culture systems.

B5 medium:
Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.

N6 medium:
Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.

Nitsch's medium:
This medium was developed by Nitsch and Nitsch and frequently used for anther cultures. Among the media referred above, MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.

Composition of common Tissue culture media

Components	Amount (mg l^{-1})				
	White's	Murashige and Skoog (MS)	Gamborg (B5)	Chu(N6)	Nitsch's
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thlamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

Synthetic and natural media:

When a medium is composed of chemically defined components, it is referred to as a synthetic medium. On the other hand, if a medium contains chemically undefined compounds (e.g., vegetable extract, fruit juice, plant extract), it is regarded as a natural medium. Synthetic media have almost replaced the natural media for tissue culture.

Expression of concentrations in media:

The concentrations of inorganic and organic constituents in culture media are usually expressed as mass values (mg/l or ppm or mg I^{-1}). However, as per the recommendations of the InternationalAssociation of Plant Physiology, the concentrations of macronutrients should be expressed as mmol/$l^{-}$and micronutrients as µmol/l^{-}.

Constituents of Media:

Many elements are needed for plant nutrition and their physiological functions. Thus, these elements have to be supplied in the culture medium to support adequate growth of cultures in vitro. A selected list of the elements and their functions in plants is given in the Table below.

Selected list of elements and their functions in plants

Element	*Function(s)*
Nitrogen	Essential component of proteins, nucleic acids and some coenzymes. (Required in most abundant quantity)
Calcium	Synthesis of cell wall, membrane function, cell signalling.
Magnesium	Component of chlorophyll, cofactor for some enzymes.
Potassium	Major inorganic cation, regulates osmotic potential.
Phosphorus	Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, cysteine and cystine, and some cofactors).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes, involved in electron transfer.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}.
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Required for chlorophyll biosynthesis, cofactor for certain enzymes.

The culture media usually contain the following constituents:

1. Inorganic nutrients
2. Carbon and energy sources
3. Organic supplements
4. Growth regulators
5. Solidifying agents

Culture initiation and regeneration through different pathways.

Types of *in vitro* culture:

1. culture of intact plants e.g. seed culture in orchids
2. embryo culture e.g. immature embryo culture
3. organ culture e.g. meristem culture, shoot tip culture root culture anther culture
4. callus culture
5. single cell culture
6. Protoplast culture.

Culture initiation: selection of explants, sterilization, media optimization and establishment of the plants from *in vivo* to *in vitro*

Organogenesis

This is a major path of regeneration that involves the differentiation of culture cells or callus tissue into organs such as shoot and roots. Plant regeneration through the formation of shoots and roots is known as plant regeneration through organogenesis. Organogenesis can occur directly or indirectly from the explants depending on the hormonal combination of the medium and the physiological state of the explants. Miller and Skoog demonstrated that the initial formation of roots or shoots on the cultured callus or explant tissue depends on the relative concentration of auxins and cytokinins in the culture media. Medium supplemented with relatively high auxin concentration will promote root formation on the explants and high cytokinin concentration will promote shoot differentiation. In tissue culture practices there may be three types of medium in relative combinations of auxins and cytokinins, which promote either the shoot formation or root formation or both simultaneously. In the latter case, we can get the complete plantlets, having both shoot and roots, which can be directly transferred to the pots in the greenhouse. Whereas in other cases, after the formation of shoots, individual shoots are transferred to the rooting medium, which promote root formation. The rooted plantlets can be transferred to a greenhouse for acclimatization. Plant regeneration through organogenesis is commonly used for mass multiplication, for micropropagation, and for conservation of germplasm at either normal or subzero temperatures (cryopreservation)

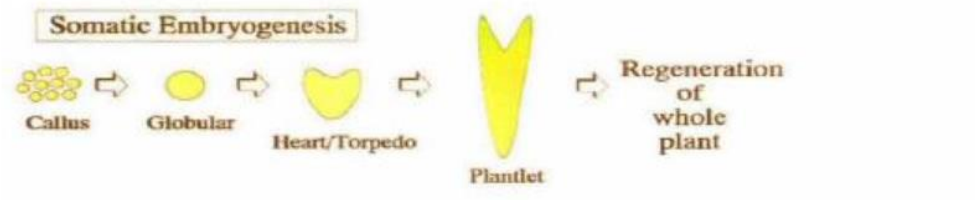
Skoog and Miller (1957) were responsible to recognize the regulatory mechanism as a balance between auxin and cytokinin. As per their finding, a relatively high level of auxin to cytokinin favoured root formation and the reverse favoured shoot formation. Using this concept, it has now become possible to achieve organogenesis in a large number of plant species by culturing explants, calli and cell suspension in a defined medium. In organogenesis, the shoot or root may form first depending upon the nature of growth hormones in the basal medium. The genesis of shoot and root from the explants or calli is termed as caulogenesis (caulm = stem) and rhizogenesis (rhizo = root) respectively.

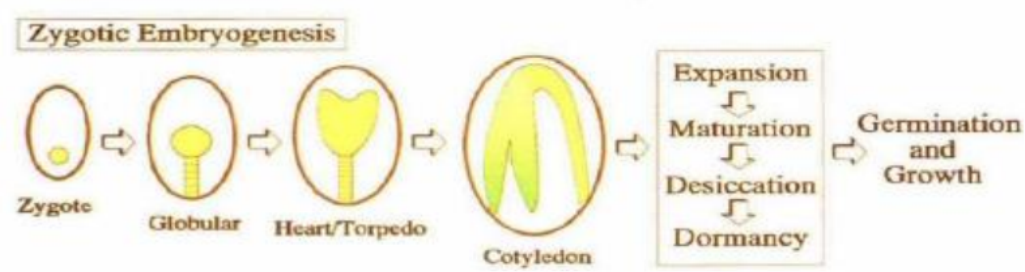
Organogenesis leading to complete plantlet regeneration is a multistage process consisting of at least three distinct stages. 1. shoot bud formation, 2. shoot development and multiplication 3. rooting of developed shoots. Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. When organogenesis leads to root development, then it is known as rhizogenesis. Abnormal structures developed during organogenesis are called organoids. The localized meristematic cells on a callus which give rise

to shoots and/or roots are termed as meristemoids. Meristemoids are characterized as an aggregation of meristem-like cells. These can occur directly on an explant or indirectly via callus. Thus, there are two kinds of organogenesis. A developmental sequence involving an intervening callus stage is termed 'indirect' organogenesis: Primary explant → callus → meristemoid → organ primordium Direct organogenesis is accomplished without an intervening proliferate callus stage: Primary explant → meristemoid → organ primordium In vitro plant tissues may produce many types of primordia (adventitious buds and organs) including those that will eventually differentiate into embryos, flowers, leaves, shoots, and roots. These primordia originate de novo from a cellular dedifferentiation process, followed by initiation of a series of events that results in to an organ.

Embryogeneis/ Somatic embryogenesis

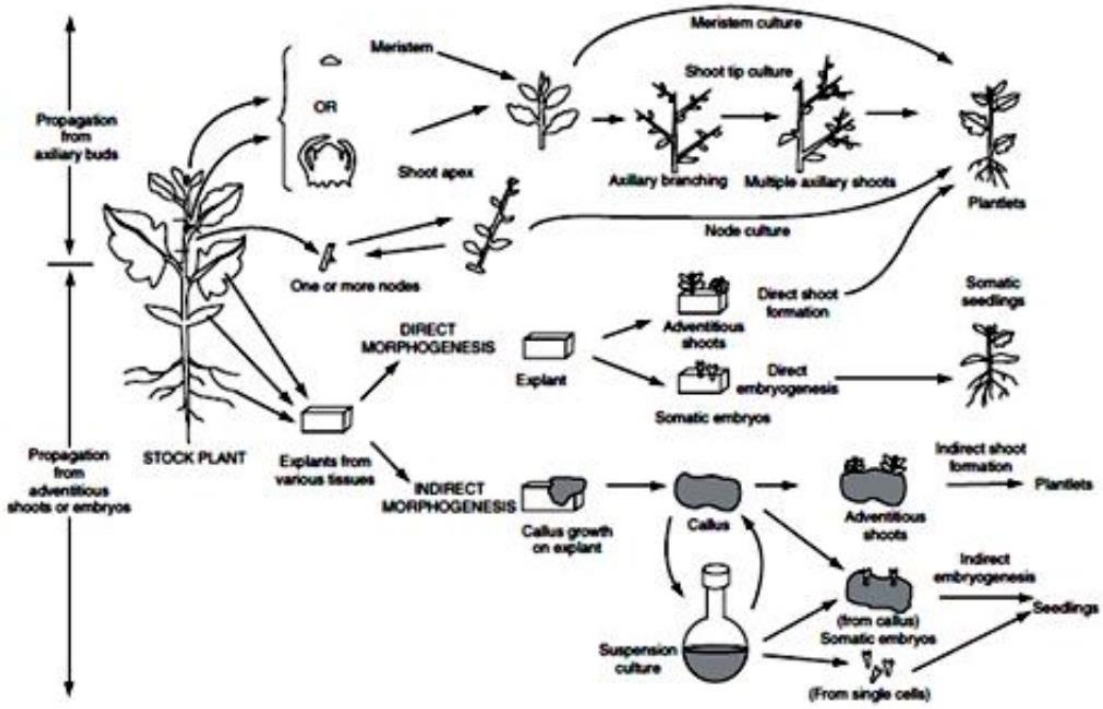
This is another major path of regeneration and development of plantlets for micropropagation or mass multiplication of specific plants. The cells, under a particular hormonal combination, change into the physiological state similar to zygotes (somatic zygotes) and follow an embryonic path of development to form somatic embryos. These somatic embryos are similar to normal embryos (seed embryos) developed from zygotes formed by sexual fertilization. The somatic embryos can develop into a complete plant. Since somatic embryos can germinate into a complete plant, these can be used for the production of artificial seeds. Somatic embryos developed by tissue or cell cultures can be entrapped in certain inert polymers such as calcium alginate and used as artificial seeds. Since the production of artificial seed is amenable to mechanization and for bioreactors, it can be produced in large numbers. Embryogenesis Embryos have been classified into two categories: zygotic embryos and non-zygotic embryos. Zygotic embryogenesis Embryos developing from zygotes (resulting from regular fusion of egg) are called as zygotic embryos or often simply embryos. Non-zygotic embryogenesis Usually non-zygotic embryos are formed by cells other than the zygote. E.g. Parthenogenetic embryos - formed from unfertilized eggs or a fertilized egg without karyogamy. Androgenetic embryos – formed from microspores, micro-gametophytes or sperm. Somatic embryos (also called as embryoids, accessory embryos, adventitious embryos and supernumerary embryos) – formed by somatic cells either in vivo or in vitro. A somatic embryo is an embryo derived from a somatic cell, other than zygote, usually on in vitro culture. The process of somatic embryo development is called as somatic embryogenesis.





Stages in development of somatic embryos

Somatic embryos generally originate from single cells which divide to form a group of meristematic cells. Usually, this multi-cellular group becomes isolated by breaking cytoplasmic connections with the other cells around it and subsequently by cutinization of the outer walls of this differentiating cell mass. The cells of meristematic mass continue to divide to give rise to globular (round ball shaped), heart-shaped, torpedo and cotyledonary stages. Somatic embryo genesis begins with active division of cells which leads to increase in size but retains the spherical shape. At this stage the primary meristem (protoderm, ground meristem and procambium) becomes visible. Following this stage, the callus continues to divide and differentiate into a heart-shaped embryo, with initiation of cotyledon primordia. As the cotyledon develops the embryo passes into the torpedo-shaped stage. The cells inside the cotyledonary ring divide to form shoot and root apical meristem and procambium differentiation takes place. In general, the essential features of somatic embryo development, especially after the globular stage, are comparable to those of zygotic embryo. The somatic embryogenesis can also be either direct or indirect depending up on the hormonal composition.



Diagrammatic representation on direct and indirect regeneration

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