

## Techniques of *in vitro* cultures

Various terms are used to define the phenomenon in *in vitro* studies. For example differentiation, de-differentiation, re-differentiation, regeneration and morphogenesis are terms with overlapping meanings. To give a clear cut view for the usage of terms, the sharp differences among them exposed hereunder.

**Differentiation:** The term differentiation is used in many different senses in biology. In broad sense, it is defined as the process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other.

**De-differentiation:** The term is used to denote the process of formation of unorganized tissues from the highly organised tissues.

**Re-differentiation:** The process of differentiation occurring in an undifferentiated tissue.

**Regeneration:** It is defined as the structuring of any part, which has been removed or physiologically isolated from the organism. In other words, genesis of an entire plant from cultured explants directly or *via* callus indirectly is called regeneration.

### Morphogenesis

Attainment of biological organization or form is termed as morphogenesis. Under *in vitro* conditions this can be achieved by two routes: *de novo* origin of organs, either shoots or roots from the cultured tissues precisely termed as organogenesis and *de novo* origin of embryos with distinct root and shoot poles on opposite ends from the somatic cells or cells cultured *in vitro*, otherwise called as somatic embryogenesis. The historical background, achievements and the causes for the two routes are discussed below.

### Organogenesis

In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves, flowers, *etc.* The earliest report on induction of shoot organogenesis *in vitro* was by White (1939) using a tobacco hybrid; and the first observation of root formation were reported by Nobecourt (1939) using carrot callus. Till late 1950s, the basic regulatory mechanism underlying in organogenesis was not identified. 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 or de novo regeneration* is referred to the development of organized structures such as shoots, roots, flower buds, somatic embryos etc., from cultured cells or tissues. *De novo* 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.

## **Types of cultures**

**Organ cultures:** Culturing isolated organs or tissues such as roots, stem, or leaf in an artificial media under controlled conditions are known as **organ culture**. Depending on the type of organs or tissue used for establishing the culture, organ cultures are named accordingly. The following are the various types of organ culture and its specific purpose:

**Seed culture:** Increasing the efficiency of germination of seeds that are difficult to germinate *in vivo*, precocious germination by application of plant-growth regulators, and production of clean seedlings for explants or meristem culture.

**Embryo culture:** Overcoming embryo abortion due to incompatibility barriers, overcoming seed dormancy and self-sterility of seeds, and embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor, shortening of breeding cycle, etc.

**Ovary or ovule culture:** A common explant for the initiation of somatic embryogenic cultures, for the production of haploid plants, overcoming abortion of embryos of wide hybrids at very early stages of development due to incompatibility barriers, and *in vitro* fertilization for the production of distant hybrids avoiding style and stigmatic incompatibility that inhibits pollen germination and pollen tube growth.

**Anther and microspore culture:** Production of haploid plants, production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce inbred lines, and for uncovering mutations or recessive phenotypes.

## **Explant culture**

**Explant culture** is actually the tissue culture. Culturing of any excised tissue or plant parts such as leaf tissue, stem parts, cotyledon, hypocotyls, root parts, etc., is called explant culture. The primary purpose of explant culturing is to induce callus cultures or to regenerate whole plantlets directly from it without the formation of callus. Shoot apical meristem culture is an example, and its important uses are the following: Production of virus-free germplasm or plantlets, mass production of desirable genotypes, facilitation of exchange between locations (production of clean material), and cryopreservation (cold storage) or *in vitro* conservation of germplasm, etc., are the main purposes of meristem or shoot apex culture.

### **Callus culture**

**Callus** represents an unorganized or undifferentiated mass of cells. They are generally composed of parenchymatous cells and usually undergo division. When an explant is cultured in a medium supplemented with sufficient amount of auxins, it starts producing mass of cells from the surface of the explant. The concentration of auxins required for each type of explant will be different and is mainly dependent on the physiological state of the explant tissue. Callus cultures can be maintained for a very long time by intermittent sub-culturing to a fresh medium. The callus cultures can be manipulated for different purposes by changing the hormone concentrations in the media. Callus cultures can be used for regeneration of plantlets, preparation of single cells or suspension cultures, or for protoplasts preparation. Callus cultures can also be used for genetic transformation studies. In some instances, it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis. Callus cultures are suitable for the generation of useful somaclonal variants (genetic or epigenetic) and can be used for *in vitro* selection of cells and tissue variants.

### **Cell-suspension cultures**

Single-cell cultures and suspension cultures can be established from callus cultures by transferring a piece of callus tissue into liquid medium and subjecting it to continuous shaking. The growth rate of the suspension-cultured cells is generally higher than that of the solid culture. The former is more desirable, particularly for the production of useful metabolites on a large scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmeyer flask and the vessel placed on a rotary or reciprocal shaker. The culture conditions depend on plant species and other factors, but in general, the cells are cultivated at 100 rpm on a rotary shaker at 25°C. By subculturing for several generations, a fine cell suspension culture containing small-cell aggregates and single cells is established.

The time required to establish the cell-suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The cells in suspension are also used for a large-scale culture with jar-fermentors and tanks. The suspension cultures can be grown either as batch cultures or as continuous cultures for producing phytochemicals. Enzymatic methods can also be adopted for establishing a fine cell-suspension culture. This is based on the use of certain pectin digesting enzymes in the culture medium, such as pectinase or macerozyme. These enzymes act on the pectin, which joins two adjacent cells in plant tissues, so that the cells become independent and grow freely as single cells. The cell-suspension

cultures can be used for inducing somatic embryogenesis and the preparation of artificial seeds, induction of somatic mutation, and selection of mutants by screening the cells just like microbial cultures. The main application of plant cell-suspension cultures is that it can be used for the bioproduction of certain important phytochemicals or secondary metabolites by applying the principle of biochemical engineering. The suspension cultures can be cultivated in specially designed bioreactors known as **airlift bioreactors** for the **mass-cell cultures** for the production of plant secondary metabolites on industrial scale. Normal bioreactors with mechanical stirrer cannot be used in plant-cell cultures because it can result in the breaking of cells and thereby the cell viability can be drastically reduced. Whereas the airlift bioreactor can provide both stirring and air inflow to meet the high demand of oxygen. The cells can also be used for genetic transformation experiments to produce transgenic plants.

### **Protoplast cultures**

Protoplasts are plant cells without cell walls. The cell wall can be removed with an enzymatic method. The cells may be from the leaf tissue or from any other part of the plant or may be the cells from the suspension cultures. These cells are incubated in an enzyme mixture consisting of cellulase, hemicellulase, and pectinase for a specific period of time. The enzyme mixture acts on the cell wall and is completely digested, so that the underlying cell membrane is exposed. This protoplast on culturing in a proper medium will regenerate its cell wall and becomes a normal cell and then can regenerate into a whole plant. The plant protoplasts can be used for various biochemical and metabolic studies and it can be used for the somatic cell fusion to create somatic hybrids. Fusion of aenucleated and nucleated protoplasts can result in a special type of somatic hybrids known as cybrids, in which there is no fusion of nucleus, but fusion in between the cytoplasm. Protoplasts can also be used for genetic transformation studies with biolistic methods, electroporation techniques, by PEG-mediated DNA transfer or by direct injection of DNA into the nucleus of the protoplast using micro-syringes.

### **Plant regeneration pathways**

There are two methods of whole plant regeneration: **organogenesis** and **somatic embryogenesis**.

**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 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, you 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).

### **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.

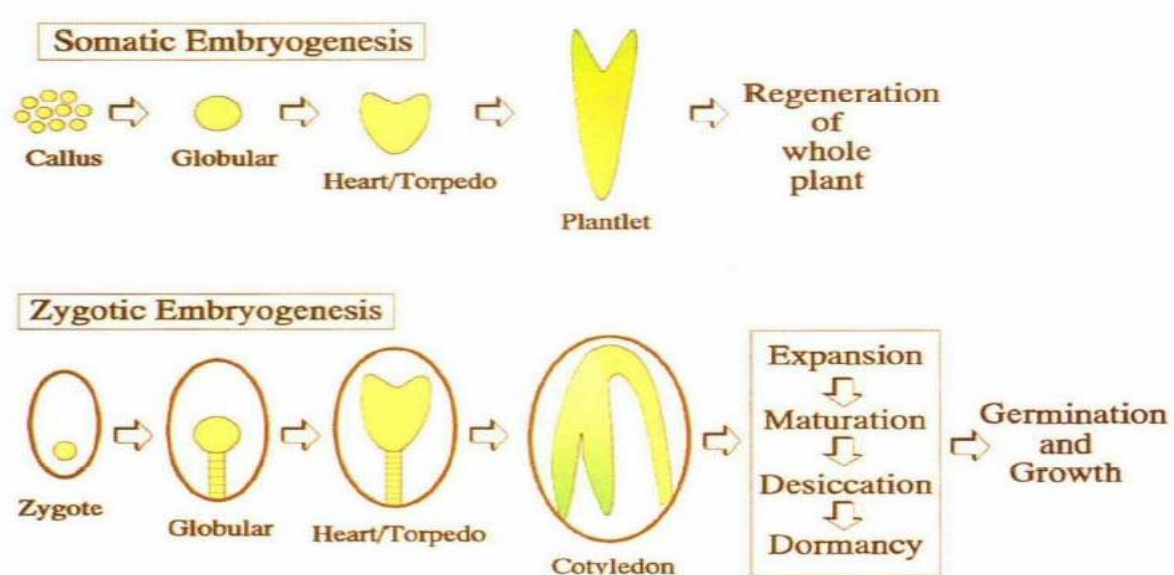
#### **Zygotic embryogenesis**

Embryos developing from zygotes (resulting from regular fusion of egg) are called as *zygotic embryos* or often simply *embryos*.

## Non-zygotic embryogenesis

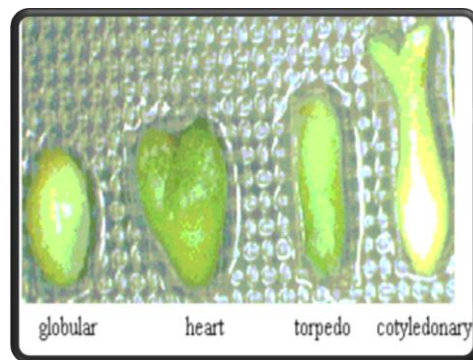
Usually non-zygotic embryos are formed by cells other than the zygote. E.g. *Parthenogenetic embryos* - formed from unfertilized eggs or a fertilized egg without karyogamy. *Androgenetic embryos* – formed from microspores, micro-gametophytes or sperm. *Somatic embryos* (also called as embryoids, accessory 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*.

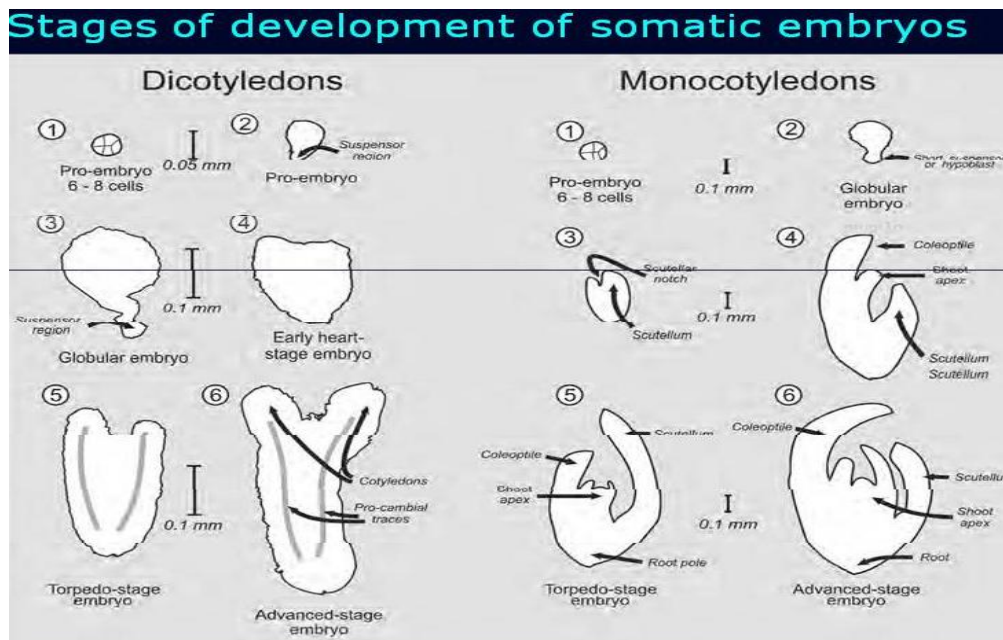


## Stages in development of somatic embryoids

Plant regeneration via somatic embryogenesis for many species can be divided into two phases: 1. Selection and induction of cells with embryogenic competence, 2. Development of these cells into 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 (Figures). 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.



Somatic embryogenesis leads to the production of a bipolar structure containing in root/shoot axis (radicle/plumule) with a closed independent vascular system. The radicular end is always sticks out from the cell mass. In contrast, a shoot bud is monopolar as it does not have a radicular end.



Somatic embryos show abnormal developmental features, e.g. 3 or more cotyledons, bell-shaped cotyledon, larger size etc.; these problems are often overcome by the presence of ABA or a suitable concentration of mannitol. In some species normal looking somatic embryos are formed but they fail to germinate; at least some somatic embryos do not germinate in most of the cases.

As mentioned before, these embryos can occur directly on an explant or indirectly *via* callus. The somatic embryos regenerating from explant or callus are termed as *primary somatic embryos*. In many cases, somatic embryos regenerate from the tissues of other somatic embryo or the parts of germinating somatic embryo. Such somatic embryos are called as *secondary somatic embryos* (and the process is called as *secondary embryogenesis* or *recurrent embryogenesis*).

#### **Additional readings.....**

#### **Factors affecting somatic embryogenesis**

1. *Growth regulators*: In most species, an auxin is essential for somatic embryogenesis. The auxin causes dedifferentiation of the explant which begins to divide. In carrot, small compact cells divide asymmetrically and their daughter cells stick together to produce cell masses called *proembryogenic masses* or *embryogenic clumps* or '*proembryogenically determined cells*' (*PEDC*). In the presence of auxins, the embryogenic clumps grow and break up into smaller cell masses which again produce embryogenic clumps. But when the auxin is either removed or reduced and cell density is lowered, each embryogenic clump gives rise to few to several somatic embryos. Some glycoproteins produced by totipotent cells are secreted into the medium; when these proteins are added into the culture medium they speed up the process of acquisition of totipotency. A class of proteins, called arabinogalactan proteins (90% carbohydrates with a protein backbone) induces somatic embryo regeneration in undifferentiated carrot cells, indicating their role in this process. Auxins promote hypermethylation of DNA which may have a role in totipotency acquisition. In alfalfa, recurrent cycles of somatic embryogenesis- *secondary embryogenesis* or *recurrent embryogenesis* - occur in growth regulator free medium and each somatic embryo can give rise to about 30 somatic embryos.
2. *Sucrose*: When embryogenic clumps transferred to an appropriate medium, somatic embryo proceeds from globular, heart-shaped, torpedo to cotyledonary stages. This is called as somatic

embryo development phase. In most species, somatic embryos begin to germinate immediately after the cotyledonary stage, and this is termed as somatic embryo conversion. But the plantlets will be very weak. Therefore, the somatic embryos are subjected to maturation phase. In this phase, somatic embryos do not grow but undergo biochemical changes to become more sturdy and hardy. This is achieved by culturing them on a high sucrose medium or in presence of a suitable concentration of ABA or by subjecting them to desiccation (usually this is achieved by enclosing somatic embryos in a sterile, sealed, empty Petri dishes). This improves the somatic embryo conversion by several folds.

3. *Nitrogen source*: The form of nitrogen has marked effect on somatic embryogenesis. In carrot,  $\text{NH}_4^+$  is essential during somatic embryo induction, while somatic embryo development occurs on a medium containing  $\text{NO}_3^-$  as the sole nitrogen source. The yield of alfalfa somatic embryo has considerably increased when amino acids such as proline, alanine, arginine and glutamine were added to the medium.
4. *Genotype of explant*: Explant genotype may determine the regeneration of somatic embryo. Of the 500 varieties of rice screened, 19 showed 65-100% embryogenesis, 41 showed 35-64% embryogenesis and the remaining 440 cultivars were less efficient in regeneration. These genotypic variations could be due to endogenous levels of hormones. In wheat, major and regulatory genes affecting regeneration were mapped on 2A, 2B and 2D. Variation for regeneration ability is highly additive and heritable in maize, rice and wheat. But in barley and alfalfa, dominance seems to be more important. Mitochondrial genome has also had an influence on regeneration (e.g. wheat). It has been shown that a loss of an 8 kb mitochondrial DNA segment in the non-embryogenic cells played a special role in the ability of dedifferentiated cells to regenerate. The success in obtaining regenerating cultures of several recalcitrant species (cereals, grain legumes and forest tree species) has been possible largely due to explant selection rather than media manipulation. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures of recalcitrant plants. However, cotyledons from somatic embryos of soybean gave considerably higher embryogenic response than those from zygotic embryos.

*Other factors*: Polyamines (putrescine, spermidine and spermine) are required for embryo development *in vivo or in vitro*. High  $\text{K}^+$  levels and low dissolved  $\text{O}_2$  levels promote somatic embryo regeneration in some species. The need for reduced dissolved oxygen could be

substituted by the addition of ATP to the medium, suggesting that, probably, oxygen tension enhanced the level of cellular ATP. In *Citrus*, some volatile compounds like ethanol inhibit somatic embryo regeneration.

Miscellaneous factors:

- a) Culture environment
- b) Bacterial compounds or contamination during tissue culture process.
- c) Electric stimulation: Stimulation of shoot bud differentiation in wheat, tobacco and alfalfa callus culture was achieved by exposure to mild electric field (0.02 V).

**Large scale production of somatic embryos:**

As the multiplication of embryogenic cells and the subsequent development of somatic embryos can occur in liquid medium, somatic embryogenesis offers a potential system for large scale plant propagation in automated bioreactors, with low labour inputs. For mass production of somatic embryos in bioreactors, callus is initiated on a semi-solid medium. Pieces of undifferentiated or embryogenic callus are transferred to liquid medium in small flasks and agitated in shaker. After a few cycles of multiplication in flasks, the embryogenic suspension may be filtered through a sieve of suitable pore size and proembryogenic masses (PEM) or globular embryos transferred to the bioreactor flask. Since the somatic embryos are individually propagules, a 2-5 litre bioreactor with a production capacity of 10-100 X 10<sup>3</sup> embryos should be sufficient for commercial micropropagation (Figure).

**Difference between shoot buds and somatic embryos**

Characteristics	Shoot bud	Somatic embryo
Origin	Many cells; usually	Single cell, superficial
Polarity	superficial Unipolar; only the shoot	Bipolar; both shoot and root
Vascular connection with callus/explant	pole present Present; vascular strands	Absent; there is no vascular
Separation from callus/explant	connected with those present in callus/explant Not easily separated unless	connection with callus/explant Easily separated since the
	cut off.	radicular end is cutinized.

### ***Events during organogenesis***

It is a general rule that the organ formation would be through a process of differentiation in the undifferentiated mass of parenchyma. Most of the parenchymatous cells are highly vacuolated and with inconspicuous nuclei and cytoplasm, sometimes with lignification. In this group of cells, regions showing random cell division would occur, leading to radial files of differentiated tissues. These scattered cell division regions would form regions of high mitotic activity resulting in the formation of meristematic centres, otherwise termed as meristemoids. These meristemoids may be either on the surface of the calli or embedded in the tissue. Continued cell division in these meristemoids would produce small protruberences on the surface of the calli, giving nodular appearance to the tissues. From the meristemoids, the primordia of organs by repeated mitotic activity form either shoot or root. This was discovered by Torrey in 1966.

The meristemoids consist of a spherical mass of small isodiametric meristematic cells with dense cytoplasm and a high nucleo-cytoplasmic ratio. Normally, callus tissues accumulate starch and other crystals before organogenesis, but the substances disappear during meristemoid formation. During the initial stages of meristemoid formation, the cytoplasmic protrusions enter the vacuoles thus distributing the vacuoles around the periphery of each cell or dispersed throughout the cytoplasm. The nucleus is in the centre with maximum possible size. Thus cells in the meristemoids resemble the cells of highly active meristems in an intact plant.

### **Embryogenesis**

An embryo is defined as a plant in its initial stage of development. Each embryo possesses two distinct poles, one to form root and the other shoot, and is the product of fusion of gametes. In some plant species, embryos are produced without the fusion of gametes and termed as asexual embryogenesis or adventitious embryony.

In an intact plant this type of embryogenesis may occur in sporophytic tissues like integuments and nucellar tissues or from unfertilized gametic cells. Apart from the normal course of embryo formations *viz.*, zygotic embryogenesis and adventitious embryony, instances of embryo formations from the tissues cultures *in vitro* were reported. This phenomenon termed as somatic embryogenesis was first observed by Steward and his co-workers (1958) in suspension cultures of carrot followed by Reinert (1959). Since then, a number of reports of embryo formation have been published.

Somatic embryogenesis or embryogenesis *in vitro* produces embryo like structures resembling the zygotic embryos in structure and morphogenetic potential. Despite this resemblance, the ontogeny of an embryo like structure from somatic cell differs from that of zygotic embryo, where the origin is from a single cell.

Embryoid is generally used to denote the embryo like structure from cultured tissues. These embryoids possess bipolarity, no vascular connection with the mother tissue and origin from a single cell or a group of cells.

### ***Events during embryogenesis***

In 1959, Reinert made the remarkable claim that following a succession of changes of the nutrient media, root derived callus tissue of *Daucas carota* produced normal bipolar embryos. The changes made or observed in the nutrient medium were as follows: maintenance of callus in White's medium with high level of auxin (IAA at 10 mg/litre) and subculturing of callus for several months on White's basal medium with additives like vitamins, amino acids, amides and purines. As a result of these manipulations, the calli showed small protruberences on the surface. Histological sections of these calli showed centres of organised development. These tissues with organised centres on transfer to auxin lacking but coconut milk containing medium produced embryoids and from embryoids, whole plants.

### **Theories on embryogenesis:**

Several theories have been proposed to explain the phenomenon of somatic embryogenesis, of which the following are considered important.

***Cell isolation theory:*** Steward and his co-workers proposed this theory in 1964. According to them, the embryo producing cells are isolated from the neighbouring cells in a cell mass. The isolation of cells, favours the embryogenesis. The isolation of cell may be induced by the constraints in the surrounding cells, due to physical and physiological separation of cells. In most cases, the connection of plasmodesmata was severed. But this generally appears to be secondary to the induction process.

***Differentiation theory:*** This theory states that the embryos would not be produced from the differentiated cells of the explants. The cells of explants have to undergo de-differentiation to form callus. Then the cells of callus will produce embryos. In other words, de-differentiation in cells is a prerequisite for the production of somatic embryos *in vitro*. That the embryos can be formed directly from the epidermal cells of the stem or hypocotyl indicate the possibility of

embryo formation without de-differentiation. The need for differentiation depends on the explant material used during primary culture. Epidermal cells of the stem, hypocotyl and young embryos may begin embryo development without going through a callus stage, while cortical cells and cells of xylem and phloem explants require de-differentiation. This theory was proposed by Halperin in 1970.

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**Intercellular communication and cytodifferentiation theory:** According to this theory, cytodifferentiation in cells due to intercellular communication induces embryo formation. The cytodifferentiation is regulated by diffusion gradients of nutrients, endogenous plant growth regulators and gaseous factors like O<sub>2</sub>, CO<sub>2</sub> and ethylene. The changing microclimate in the culture environment affects intercellular communication and in turn cytodifferentiation. This concept was proposed by Street (1973).

**Explant physiology and culture environment theory:** This concept was developed by Street in 1976. He is of the view that the embryogenesis is a dependent phenomenon on the explant and the culture environment. Explants like flower buds, young embryos and parts of young seedling are most responsive to produce somatic embryos, but not from those of mature plants. Apart from the explant physiology, culture environment is also a factor influencing the embryogenesis. For example, highly embryogenic callus culture can be maintained non-embryogenic if the medium is supplemented with high level of auxin and the same may be induced to produce embryos when transferred to auxin free medium.

**Pre-determination theory:** This was proposed by Tisserat *et al.* (1979). It states that the embryo production potential is pre-determined phenomenon in the cells and the *in vitro* culture provides the opportunity for embryogenesis. In other words, embryogenesis from a cell is an inherent one which is facilitated to produce embryos by optimal culture environment.

Pre and induced embryogenic determined cell theory: Though the embryogenesis is pre-determined one there are instances of non-formation of embryos directly from the explants. In these cases, an intervening callus stage comes between the primary explant and the embryos. The cells in the calli are induced to produce embryos by the manipulation of medium with relevant growth regulator. Based on this, the above theory was proposed by Sharp and his co-workers. According to this theory, there are two types of embryogenic cells: pre-embryogenic determined cells (PEDC) and induced embryogenic determined cells (IEDC).

In pre-embryogenic determined embryogenic cells, embryogeny is determined prior to mitosis while induced embryogenic determined cells the embryogeny is induced by providing suitable mitogenic substance i.e., the embryogeny is induced in the cells of callus by the application of plant growth regulators. Thus in the callus, embryogenic precursor cells or embryogenic mother cells are formed which then develop into embryogenic cells. Later these cells undergo polarised cell divisions typical of normal embryogenesis by forming globular, heart and torpedo shaped embryos.

***Patterns of embryogenesis***

Two general patterns of embryogenesis *in vitro* are identified:: Origin of embryos directly from the tissue cultured *in vitro* (direct embryogenesis) and origin of embryos *via* callus stage (indirect embryogenesis) .

**Table. Differences between direct and indirect embryogenesis**

<b>Direct embryogenesis</b>	<b>Indirect embryogenesis</b>
Embryos form from the explants directly	Embryos arise from the callus induced from the explants
A promoting substance to induce the embryo formation is needed	Auxin is need to induce callus, and cytokinin is needed to induce differentiation
The embryogenic nature of a cell is predetermined	The embryogenic nature of a cell is induced in the culture
The origin of embryos mostly from individual cells; sometimes from a group of cells	The origin may be either from single cells or from a group of cells called pro-embryonal complex

## Questions

1. The process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other is .....  
**a). Differentiation**    b). De-differentiation    c). Re-differentiation    d). Regeneration
2. Term is used to denote the process of formation of unorganized tissues from the highly organised tissues is .....  
a). Differentiation    **b). De-differentiation**    c). Re-differentiation    d). Regeneration
3. The process of differentiation occurring in an undifferentiated tissue  
a). Differentiation    b). De-differentiation    **c). Re-differentiation**    d). Regeneration
4. Genesis of an entire plant from cultured explants directly or *via* callus indirectly is called regeneration  
a). Differentiation    b). De-differentiation    c). Re-differentiation    **d). Regeneration**
5. Attainment of biological organization or form is termed as .....  
a). Differentiation    b). De-differentiation    c). Re-differentiation    **d). Morphogenesis**
6. Morphogenesis can be achieved by .....  
a). *de novo* origin of organs    b). *de novo* origin of embryos  
**c). Both a and b**    d). None of the above
7. The earliest report on induction of shoot organogenesis *in vitro* was .....  
**a). White**    b). Nobecourt    c). Skoog    d). Miller
8. The earliest report on induction of root organogenesis *in vitro* was .....  
a). White    **b). Nobecourt**    c). Skoog    d). Miller
9. The induction of shoot organogenesis *in vitro* was achieved primarily in .....  
**a). tobacco**    b). carrot    c). Both a and b    d). None of the above
10. The induction of root organogenesis *in vitro* was achieved primarily in .....  
a). tobacco    **b). carrot**    c). Both a and b    d). None of the above
11. A developmental sequence involving an intervening callus stage is termed



- a). **'indirect' organogenesis**                      b). *'direct' organogenesis*  
c). Morphogenesis                                      d). None of the above

12. Abnormal structures developed during organogenesis are called .....

- a). **Organoids**    b). Meristemoids    c). Both a and b    d). None of the above

13. The localized meristematic cells on a callus which give rise to shoots and/or roots are termed as .....

- a). Organoids    **b). Meristemoids**    c). Both a and b    d). None of the above

14. Seed culture is used for .....

- a). To increase the efficiency of germination of seeds    b). Precocious germination by application of PGRs  
c). Production of clean seedlings for explants or meristem culture    **d). All the above**

15. Embryo culture is used for .....

- a). To overcome embryo abortion due to incompatibility barriers    b). To overcome seed dormancy and self-sterility of seeds  
c). Embryo rescue in distant hybridisation    **d). All the above**

16. Ovary or ovule culture is used for .....

- a). Initiation of somatic embryogenic cultures    b). Production of haploid plants  
c). To overcome abortion of embryos of wide hybrids    **d). All the above**