Organogenesis and Embryogenesis

Medium and growth conditions are manipulated to obtain a complete plant from explant through either organogenesis or embryogenesis; both of them may be direct or following callus (a mass of unorganized cells) phase. Organogenesis is the ability of plant tissue to form various organs de novo. It describes many processes including in vitro multiplication of pre-existing shoot meristems, regenerating shoots or roots on explant, callus or even in vivo root formation on stem cuttings. However, the focus in tissue culture is on organogenesis in vitro on non-meristematic plant tissues. Shoots are regenerated first then transferred to rooting medium. If root is formed first, shooting is usually not attained. Organogenesis is divided into three phases: dedifferentiation, induction and differentiation.

- Dedifferentiation involves the reversion to a less committed, more flexible developmental state that may or may not give rise to callus tissue. At the end of this phase, cells acquire a state of competence which is defined by the ability to respond to organogenic stimuli.

- The induction phase is characterized with a cell or group of cells become fully committed to the production of shoot or root.

- At differentiation phase, morphological differentiation and development of the nascent organ begins. It is worth to mention that cytological studies showed that regenerated organs are multicellular in origin.
Direct Organogenesis

Shoot formation

Root formation

Shoot

Explant

Root

Callus
Embryo is a morphologically distinct entity that functions as an intermediate stage in the transition between gametophytic to sporophytic life cycle. In higher plants, embryos develop within seeds as a result of gametic fusion and are called zygotic embryos. In tissue cultures, somatic embryos (non-zygotic embryos) resulted without gametic fusion. In contrast to organogenesis, any somatic embryo is unicellular in origin. Somatic embryogenesis involves initiation of embryogenic cell, embryo development and embryo maturation phases.

Initiation of embryogenic cell is a dramatic change in the fate of non-embryogenic cell (in explant or callus) including dedifferentiation followed by redetermination toward an embryogenic cell type. Some genetic mechanisms for somatic embryogenesis can be related to mechanisms of zygotic embryo formation. Immature zygotic embryo can be used as explant; in such case, we need only to propagate these embryogenic cells in vitro.

Embryo development is the observable transition from non-embryogenic to embryogenic cell; it occurs when the cell undergoes an unequal division, resulting in a larger vacuolated cell and a smaller, densely cytoplasmic (embryogenic) cell. Embryogenic cell divides to form somatic embryo. Zygotic and somatic embryos share the same gross pattern of development (globular, heart, torpedo and cotyledonary stages).
Embryo maturation is the terminal event of embryogenesis; it is characterized, in zygotic embryo, by attainment of mature embryo morphology, accumulation of storage food materials, reduction in water content and often gradual cessation of metabolism. Complete maturation is not absolutely necessary to obtain plants from somatic embryos. It is required to achieve high rate of plant recovery. High sucrose concentration, ABA and polyethylene glycol are shown to enhance maturation of somatic embryos. Generally, somatic embryos tend either to grow and germinate without normal maturation, become disorganized into embryogenic tissue or die. In some rare cases, somatic embryo enters resting stage like that observed for zygotic embryos.
Callus culture

Explant tissues generally show distinct planes of cell division, various specializations of cells, and organization into specialized structures such as the vascular system. Callus formation from explant tissue involves the development of progressively more random planes of cell division, less frequent specialization of cells and loss of organized structures. Consequently, callus is defined as a mass of undifferentiated cells. It is naturally formed on plants in response to wounding. Auxins like 2,4-D have strong effect on initiation of cell division in tissue culture. Callus growth passes five phases:

- Lag phase: Explant cells prepare to divide.
- Exponential phase: The rate of cell division reaches its maximum.
- Linear phase: Cell division slows but rate of cell expansion increases.
- Deceleration phase: Rate of cell division and elongation decrease.
- Stationary phase: Number and size of cells remain constant.

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

Synthetic seed

The seed (or zygotic seed) is the vehicle that connects one generation to another in much of the plant kingdom. By means of seed, plants are able to transmit their genetic constitution in generations and therefore seeds are the most appropriate means of propagation, storage and dispersal. Synthetic seeds have great potential for large scale production of plants at low cost as an alternative to true seeds. A synthetic seed is often described as a novel analogue to true seed consisting of a somatic embryo surrounded (or not according to type) by an artificial coat (like media used in tissue culture solidified with alginate instead of agar) which is at most equivalent to an immature zygotic embryo, possibly at post-heart stage or early cotyledonary stage. There are various advantages of synthetic seeds such as; better and clonal
plants could be propagated similar to seeds; preservation of rare plant species extending biodiversity could be realized; and more consistent and synchronized harvesting of important agricultural crops would become a reality. In addition; ease of handling, potential long-term storage and low cost of production and subsequent propagation are other benefits.

Today synthetic seeds represent capsules with a gel envelope, which contain not only somatic embryos but also axillary and apical buds. These plant materials are encapsulated in protecting material (eg: hydrogel or alginate gel) and can be developed into a plant. The coating protects the explants from mechanical damage during handling and allows germination and conversion to occur without inducing undesirable variations. They behave like true seeds and sprout into seedlings under suitable conditions.

**Procedure for the Production of Synthetic Seeds**

The general procedure of synthetic seed production varies according to the type of artificial seed produced, need of artificial seeds and the economic feasibility. The development of the ideal viable, quiescent, low-cost artificial seed can be summarized as follow:
• The optimization of the clonal production system (optimizing protocols to synchronize and maximize the development of normal mature embryos capable of conversion to normal plants).
• Post-treatment of mature embryos to induce quiescence,
• Development of an encapsulation and coating system.
• Optimization of the encapsulation system.
• Optimization of requirements for greenhouse and field growth (watering, fertilizer, transplantation, etc.).
• Identification and control of any pest and disease problems that may be unique to artificial seeds and determination of the economic feasibility of using the artificial seed delivery system for a specific crop compared with other propagation methods (cost–benefit analysis of encapsulation versus other options).

**The need for synthetic seed**

Zygotic embryo seeds carry traits from both parents. Production of seeds carrying certain traits requires homozygous parents for such traits which is not easy and time consuming. After the discovery of somatic embryogenesis in 1950 it was possible to have an alternative of conventional zygotic seeds. Somatic embryo arises from the somatic cells of a single parent. They differ from zygotic embryos since somatic embryos are produced through *in vitro* culture, without nutritive and protective seed coats and do not typically become quiescent. Somatic embryos are structurally equivalent to zygotic embryos, but are true clones, since they arise from the somatic cells of a single parent. The structural complexity of artificial seeds depends on requirements of the specific crop application. Therefore, a functional artificial seed may or may not require a synthetic seed coat, be hydrated or dehydrated, quiescent or non quiescent, depending on its usage. The field that seeks to use somatic embryos as functional seed is termed “artificial or synthetic seed technology”.

**Types of synthetic seeds**

There are various types of artificial seeds; first two are essentially uncoated somatic embryos; i. uncoated non quiescent somatic embryos, which could be used to produce those crops
micro propagated by tissue culture; ii. uncoated, quiescent somatic embryos would be useful for germplasm storage. The other categories are; iii. Non quiescent somatic embryos in a hydrated encapsulation constitute a type of artificial seed that may be cost effective for certain field crops that pass through a greenhouse transplant stage such as carrot, celery, seedless watermelon, and other vegetables and iv. dehydrated, quiescent somatic embryos encapsulated in artificial coatings are the form of artificial seed that most resembles conventional seed in storage and handling qualities. These consist of somatic embryos encased in artificial seed coat material, which then is dehydrated. Under these conditions, the somatic embryos become quiescent and the coating hardens. Theoretically, such artificial seeds are durable under common seed storage and handling conditions. Upon rehydration, the seed coat softens, allowing the somatic embryo to resume growth, enlarging and emerging from the encapsulation.
Artificial seeds and their germination
Breeding by somatic cell genetics

A) Protoplast (naked cell without cell wall) fusion

Cells of primary plant tissues possess cellulosic walls with a pectin-rich matrix, the middle lamella, joining adjacent cells. The living cytoplasm of each cell, bounded by the plasma membrane, constitutes the protoplast. Normally, intimate contact is maintained between the plasma membrane and the wall, since this membrane is involved in wall synthesis. However, in hypertonic solutions, the plasma membranes of cells contract from their walls. Subsequent removal of the latter structures releases large populations of spherical, osmotically fragile protoplasts (naked cells), where the plasma membrane is the only barrier between the cytoplasm and its immediate external environment.
Protoplast isolation is now routine from a wide range of species; viable protoplasts are potentially totipotent. Therefore, when given the correct chemical and physical stimuli, each protoplast is capable, theoretically, of regenerating a new wall and undergoing repeated mitotic division to produce daughter cells from which fertile plants may be regenerated via the tissue culture process. A remarkable progress has been made in the number of species for which protoplast-to-plant systems exist.

First attempts for releasing protoplasts involved incubation of tissues in hypertonic solution (eg: concentrated sucrose solution) to shrink protoplast away from cell wall, then plasmolysed tissues are cut at such a thickness that only the cell walls are cut without damaging protoplast. The protoplasts are released by osmotic swelling when strips of tissues are placed in a hypotonic solution.

Mechanical procedures are now rarely employed for protoplast isolation, but are useful with large cells and when limited (small) numbers of protoplasts are required. When large populations of protoplasts are required, enzymatic digestion of source tissues is essential. Cellulase and pectinase enzymes became available commercially for routine use. Some salts and nutrients (eg: sucrose or the sugar alcohol sorbitol) are used as osmoticum to prevent plasma membrane from rupturing. Pretreatment of donor tissues (plasmolysis or cold treatment) is used to reduce cytoplasmic damage and spontaneous fusion of protoplasts from adjacent cells.

The physiological status of the source tissue influences the release of viable protoplasts. Furthermore, seasonal variation, which affects the reproducibility of protoplast isolation from glasshouse-grown plants, can be effectively eliminated using in vitro grown (axenic) plant material. Some authors recorded persistence of a seasonal clock even in vitro. Several factors influence protoplast release, including the extent of thickening of cell walls, temperature, duration of enzyme incubation, pH optima of the enzyme solution, gentle agitation, and nature of the osmoticum.
Isolated protoplasts were observed to fuse spontaneously that is now used to produce hybrids from sexually incompatible species. Protoplast fusion is achieved through high Ca\(^{++}\), high pH, PEG or electric field.
B) Haploid Cultures

Sporophyte: is an independent plant with diploid chromosome number. In higher plants, the sporophyte is dominant and performs vegetative and sexual reproduction. Gametophyte: is an independent plant with haploid chromosome number. In higher plants, the gametophytes are very much reduced and represents the gametes only which fuse to form sporophyte. Life cycle alternates between sporophyte and gametophyte.

Plants with gametophytic chromosome number in their sporophyte are referred to as haploids. Haploids can result through the culture of haploid explants like ovules or pollens. The process of haploid regeneration though unpollinated female gametophytes is usually described as gynogenesis, while androgenesis is used when starting with pollen. The regenerated haploid plants are generally sterile, requiring chromosome doubling for use in breeding programmes. Chromosomes can be doubled (to produce homozygous individual at all loci) either spontaneously or artificially, and haploid plantlets are usually treated with colchicine as a means of inducing chromosome doubling.

Stress treatments are the most common factor affecting embryogenesis, with cold/heat shock and starvation treatment being commonly used. Without stress, the change from gametophytic to sporophytic phase is very difficult. Certain physical treatments (e.g., low/high temperature, dark period or starvation medium) applied to donor plants may have a strong influence on embryo induction, and are also important to switch from gametophytic pathway to saprophytic development. Pretreatments differing in type, level, and duration can be applied at different levels of explants.

In addition to stress treatments, several studies have focused on culture media constituents. In general, the concentration of salts in the culture media is lower compared to micropropagation media, but there is no general rule. Choices and concentrations of carbohydrates are often essential. The most commonly used carbohydrate is sucrose and is added in high concentrations (i.e., 13%), while substitution of sucrose by maltose has been an important innovation, first discovered for barley anther culture. Although not always
required, plant growth regulators might be essential. The influence of all groups of growth regulators has been tested, with positive effects of polyamines being among the latest studied.

During the production of homozygous lines, various undesired heterozygous plantlets can be obtained. In anther culture and in vitro gynogenesis, such plants can be regenerated from the somatic tissue of inoculated plant organs such as anther wall cells, somatic cells of flower buds or ovaries. Reliable and fast selection of regenerants is therefore necessary before further employment of putative haploids and doubled haploids. Several direct and indirect approaches are available for determining the ploidy level of regenerated plants. Indirect approaches are based on comparisons between regenerated and donor plants in terms of plant morphology (plant height, leaf dimensions and flower morphology), plant vigor and fertility, number of chloroplasts and their size in stomatal guard cells. They are fairly unreliable and subject to environmental effects but do not require costly equipment. Direct methods for ploidy determination are more robust and reliable and include conventional cytological techniques, such as counting the chromosome number in root tip cells and measurement of DNA content using flow cytometry.

The induction and regeneration of haploids followed by spontaneous or induced doubling of chromosomes are widely used techniques in advanced breeding programs of several agricultural species. Completely homozygous plants can be established in one generation thus saving several generations of selfing comparing to conventional methods, by which also only partial homozygosity is obtained. Homozygosity of regenerants and true breeding propagation enables the fixation of mutations in the first generation after mutagenic treatment. The mutagenic agent is usually applied soon after microspore isolation, before the first nuclear division in order to avoid heterozygosity and chimerism caused by spontaneous diploidization through nuclear fusion. In vitro mutagenic treatment can be followed by in vitro selection of desired traits, such as disease and herbicide resistance.