Chaudhary Mahadeo Prasad College

(A CONSTITUENT PG COLLEGE OF UNIVERSITY OF ALLAHABAD)

E-Learning Module

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(Study material for Post Graduate Students)

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COURSE CODE: BOT 506

Reproductive Biology, Morphogenesis and Tissue culture

Unit (V): Topic: Somatic embryogenesis

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SOMATIC EMBRYOGENESIS

In somatic embryogenesis (SE), embryo-like structures analogous to zygotic embryo are formed either directly from the tissue or via an intervening callus phase. The process is opposite of zygotic or sexual embryogenesis. The fertilization process prompts the egg cell (called zygote after fertilization) to divide and develop into an embryo (the process is called embryogenesis). However, fertilization is not always essential to stimulate the egg to undergo embryogenesis. As happens in parthenogenesis, the pollen stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development. Moreover, it is not the monopoly of the egg to form an embryo. Any cells of female gametophyte (embryo sac) or even that of the sporophytic tissue around the embryo sac may give rise to an embryo (Figure 1). The development of adventives embryos from nucellar cells is a very common feature in case of *Citrus* and *Mangifera*. However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development or else they may fail to mature. These *in vivo* observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the embryo sac. The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* and in other species like, *Citrus* species, *Medicago* species, *Ranunculus sceleratus,* etc.

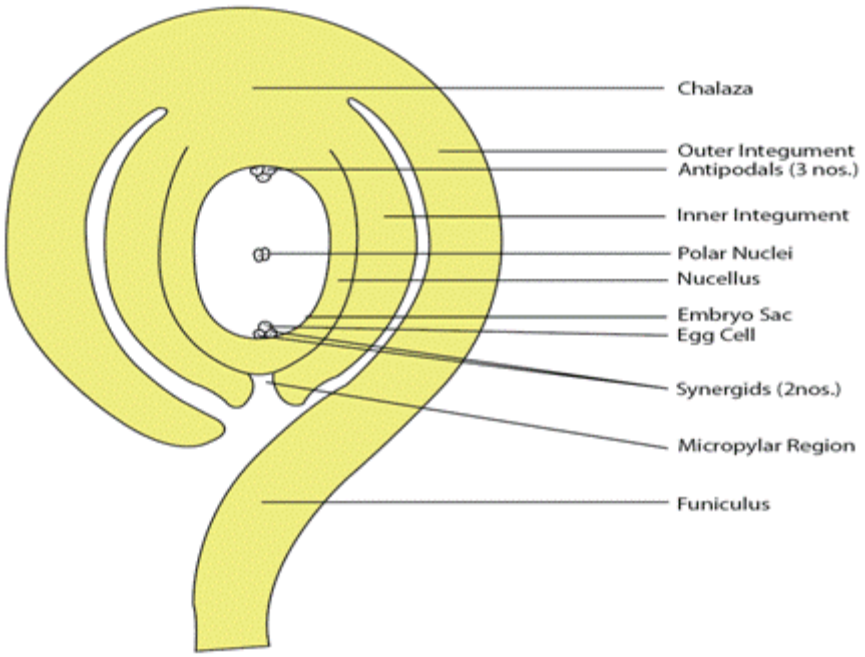


Figure 1 : Longitudinal section of an ovule

2. In vitro somatic embryogenesis

In vitro somatic embryogenesis (SE) was first demonstrated in 1958 by Reinert and Steward. There are two ways by which SE could be obtained - i) Indirect SE, where first the callusing is induced from the explant by rapid cell division and later the callus give rise to SE (Figure 2), and

ii) Direct SE, where the somatic embryos are developed directly from the explant without an intermediate callus phase (Figure 3).

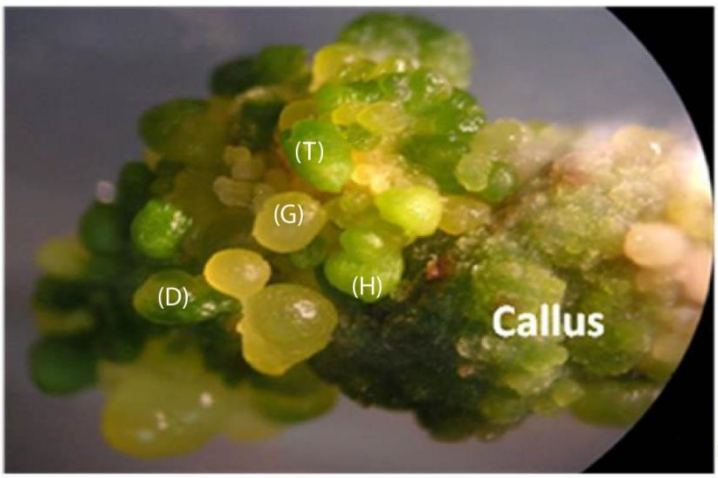


Figure 2: Somatic embryogenesis via callusing showing the development of globular (G), heart (H) torpedo (T) and dicot embryos (D) (arrow marked).

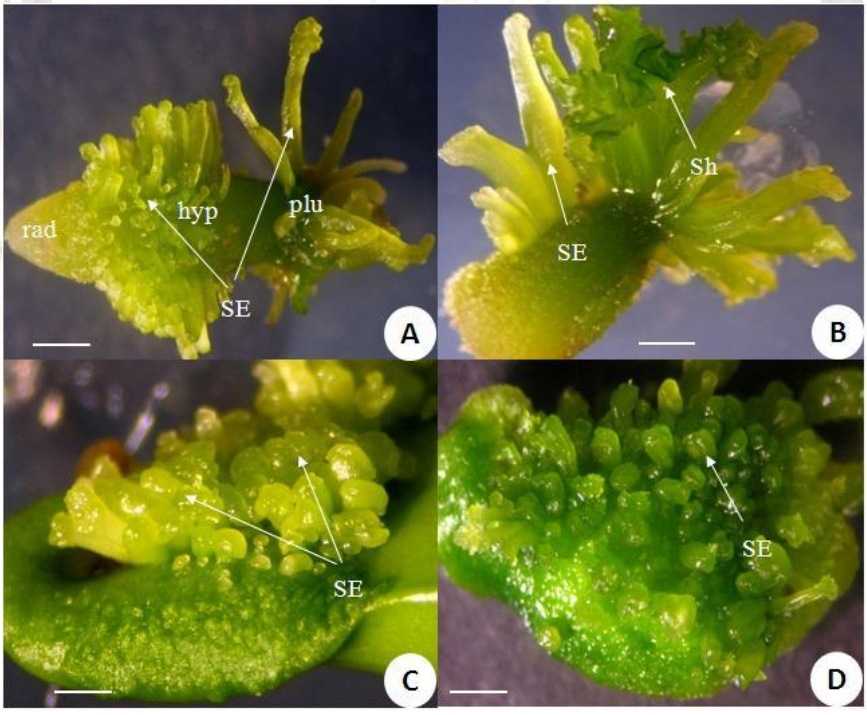


Figure 3: Direct somatic embryogenesis from cotyledon explant showing embryos at various stages of development

In either of the cases, the somatic embryos resemble the zygotic embryos. In dicotyledonous plants, the somatic embryos passes through the globular, heart, torpedo and cotyledonary stages, as happens in zygotic embryos (Figure 4). The embryos germinate and develop into complete plantlets. The only major difference between somatic and zygotic embryogenesis is that somatic embryos do not pass through the desiccation and dormancy phases as happens in zygotic embryos, but rather continue to participate in the germination process.

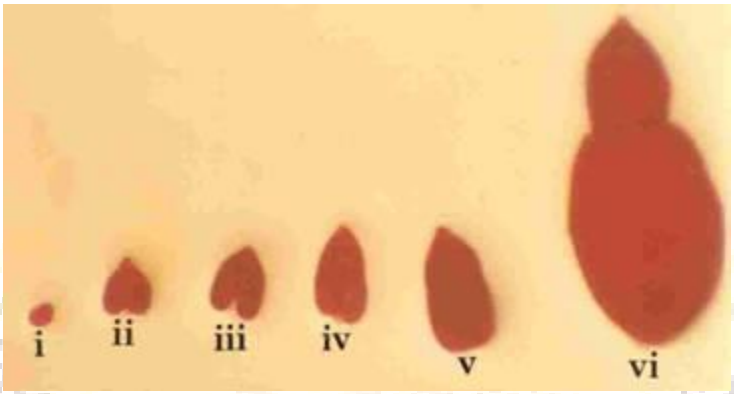


Figure 4: Different stages of development of zygotic embryos: (i) globular, (ii) early heart shape, (iii) late heart shape, (iv) torpedo shape, (v) early dicot, and (vi) fully developed dicot embryo

Whether originating directly or indirectly via callusing, somatic embryos arise from single special cells located either within clusters of meristematic cells in callus mass or in the explant tissue. Somatic embryogenesis is regarded as a three step process:

- i. Induction of embryo

  ii. Embryo development

  iii. Embryo maturation

3. Organogenesis versus embryogenesis

In tissue cultures, plant regeneration via somatic embryogenesis may offer many advantages over organogenesis, such as

1. i. Embryo is a bipolar structure rather than a monopolar one.

   ii. The embryo arises from a single cell and has no vascular connection with maternal callus tissue or the cultured explant. On the other hand during organogenesis shoots or roots develop from a group of cells resulting into chimera formation which later establish a strong connection with the maternal tissue.

iii. Further, induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant, while in organogenesis, it requires two different hormonal signals to induce shoot first and then root organ.

4. Factors affecting somatic embryogenesis

4.1. Genotype and type of explant

Like organogenesis, SE is also genotype dependent for a given species and significant variations in response between cultivars have been observed in several plants like, wheat, barley, soyabean, rice, alfalfa etc. Genotypic variations could be due to endogenous levels of hormones, therefore, if the species has not shown SE previously, then it is required to test number of different cultivars of that species.

The next problem comes up is what tissue should be used as an explant in a particular species to induce SE? It can be decided very easily by closely examining of what explants were used in related species, genus or family. The explant selection is much more important than the media selection for SE process. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures as somatic embryos will form more readily from cells that are already in embryonic state. In *Azadirachta indica* (neem), the immature zygotic embryo at different stages of development, viz. globular, early to late heart shape, torpedo shape and early dicotyledonous stage, when cultured showed varied potential for SE. The globular embryos did not show any response. The older embryos germinated, formed calli or differentiated three types of organized structures, viz. shoots, somatic embryos and neomorphs (abnormal or embryo-like structures with varied morphology). Often the same explant differentiated more than one kind of regenerants. The most responsive stage of embryos was early dicotyledonous, followed by torpedo shaped embryos.

4.2. Growth regulators

Auxin : Auxin plays a major role in the development of somatic embryos. All the well-studied somatic embryogenic systems, such as carrot, coffee and most of the cereals require a synthetic auxin for the induction of SE followed by transfer to an auxin-free medium for embryo differentiation. The synthetic auxin 2,4-D is the most commonly used auxin for the induction of SE. Besides, other auxins, NAA, IBA, picloram (4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid) and IAA, have also been used. A naturally occurring auxin IAA is a weak auxin and more readily broken down compare to 2,4-D and NAA. The auxins, particularly 2,4-D, in the concentration range of 0.5 – 1.0 mgl^{-1} (**proliferation or induction medium**), stimulates the formation of localized group of meristematic cells in the callus called 'proembryogenic masses' (PEMs), which are cell clusters within cell population competent to form somatic embryos (Figure 5)

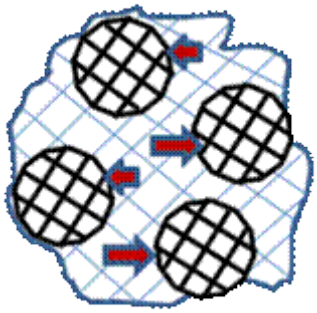


Figure 5: Embryogenic callus with PEMs (indicated by arrows) in the induction medium

In repeated subcultures on the proliferation medium, the embryogenic cells continue to multiply without the appearance of embryos. However, if the PEMs are transferred to a medium with a very low level of auxin (0.01-0.1 mgl^{-1}) or no auxin in the medium (**embryo development medium** ; ED medium), they develop into embryos. The presence of an auxin in the proliferation medium seems essential for the tissue to develop embryos in the ED medium. The tissues maintained continuously in auxin-free medium would not form embryos. Therefore, the proliferation medium is called the 'induction medium' for SE and each PEMs as an unorganized embryo.

Cytokinin : There are reports of somatic embryo induction and development in cytokinin containing medium, but these reports are very few compared to those reporting induction by auxin alone or auxin plus cytokinin. Cytokinin, in general, induced SE directly without the callusing of explant. In most cases, TDZ is used as cytokinin, a herbicide, which mimics both auxin and cytokinin effects on growth and differentiation. The other cytokinins are also used when zygotic embryos are used as the explant source. The most commonly used cytokinins are BAP and Zeatin.

In *Azadirachta indica* , somatic embryo differentiation was influenced by the culture medium as well as the stage of embryo at culture. Maximum somatic embryogenesis occurred directly from the explant on BAP containing medium when early dicotyledonous stage of embryos were cultured. Medium with 2,4-D induced only neomorph differentiation directly from the explant. While torpedo shaped embryos showed both neomorph formation as well as somatic embryogenesis on BAP containing medium (Figure 6).

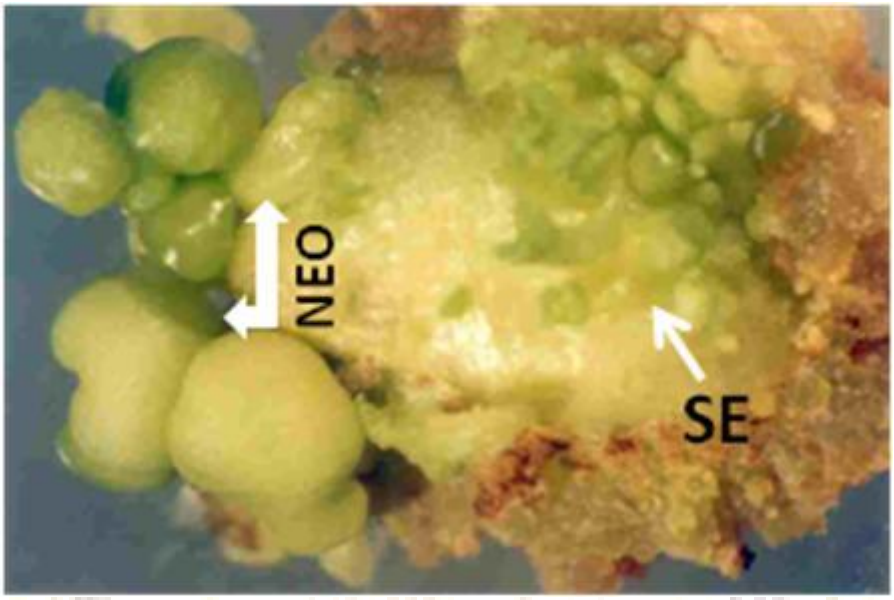


Figure 6: An explant showing differentiation of neomorphs (NEO) and somatic embryos (SE) on the same explant

Neomorphs were suppressed embryos with green, smooth, shiny surface and solid interior (Figure 7A). Although they were epidermal in origin like somatic embryos with heart shape notch but showed monopolar germination and no clear cut radicular region (Figure 7B).

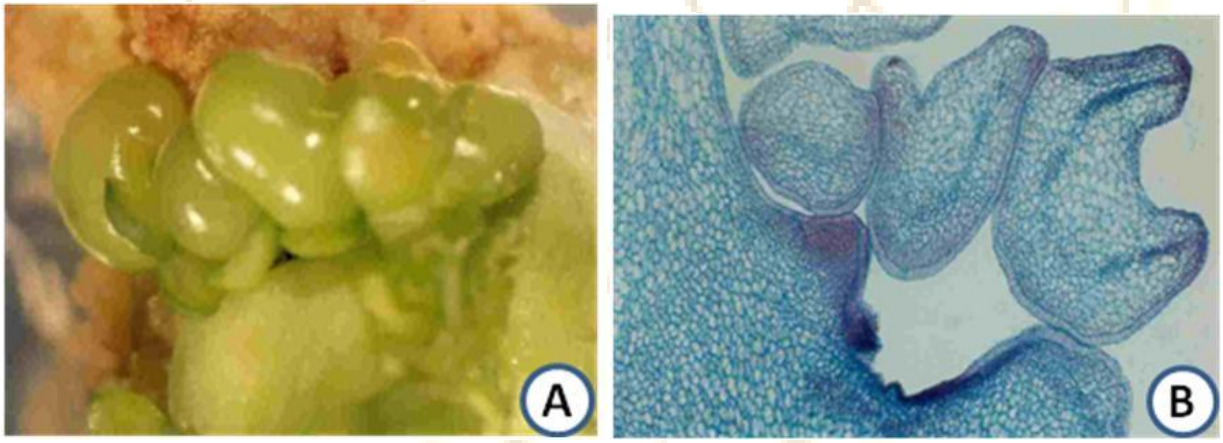


Figure 7: A. An explant showing direct differentiation of neomorphs. Some of these structures also show cotyledon-like flaps. The portion of the explant in contact with the medium has proliferated into a brownish green callus

B. A histological section of **A,** showing epidermal origin of a neomorph of various shapes. It has a well differentiated epidermis and compactly arranged internal cells. These structures are loosely attached to the explant and show provascular strands.

4.3. Nitrogen source

The most important nutrient of the culture medium is nitrogen which affects SE significantly. The form of nitrogen have a strong influence on the induction of SE. Often the presence of ammonium or some other source of reduced nitrogen is required, such as glycine, glutamate or casein hydrolysate. The ratio of ammonium to nitrate has also been shown to affect SE. In few cases, the calli initiated on a medium with KNO_3 as the sole source of nitrogen failed to form embryos upon removal of auxin. However, the addition of a small amount (5mM) of nitrogen in the form of NH_4Cl in the presence of 55mM KNO_3 allowed embryo development.

5. Embryo maturation and germination

Germination of somatic embryos can occur only when it is mature enough to have functional shoot and root apices capable of meristematic growth. Somatic embryos show poor germinable quality with respect to their convertibility into complete plantlets. This is because these embryos do not go through 'embryo maturation' phase which is the characteristic of seed or zygotic embryos. During this phase, accumulation of embryo-specific reserve food materials and proteins imparts desiccation tolerance to seed embryos and thereby promote their normal development for germination. Abscisic acid (ABA), which prevents precocious germination and promotes normal development of embryos by suppression of secondary embryogenesis and pluricotyledony, is reported to promote embryo maturation in several species. A number of other factors, such as temperature, shock, osmotic stress, nutrient deprivation and high density inoculums, can substitute for ABA, presumably by inducing the embryos to synthesize the hormone. ABA is known to trigger the expression of genes which normally express during the drying down phase of seeds. Probably the products of these genes impart desiccation tolerance to the embryos.

6. Secondary somatic embryogenesis

Secondary SE is a process in which new somatic embryos are proliferated from originally formed primary somatic embryos. Secondary SE is reported to have some advantage over primary somatic embryogenesis, such as high multiplication rate, long term repeatability and independency of an explant source. By repeated secondary SE selected embryogenic lines can be maintained for long period, in large quantities until the lines have been tested in field conditions particularly in perennial plants. Secondary SE also overcomes post fertilization barriers of the embryo, immature embryos of interspecific plants from incompatible crosses may be rescued by culturing them for secondary SE. It can also be used for the production of somatic embryos of species in which the embryos are the reservoir of important secondary metabolites. In *Azadirachta indica*, for secondary SE, primary embryos were used as the explant and when cultured on medium with TDZ and GA_3, secondary embryos were differentiated directly from hypocotyls region without any intervening callus (Figure 8A). Whereas, a combination of BAP and IAA resulted into secondary SE preceded by callusing of the primary somatic embryo (Figure 8B).

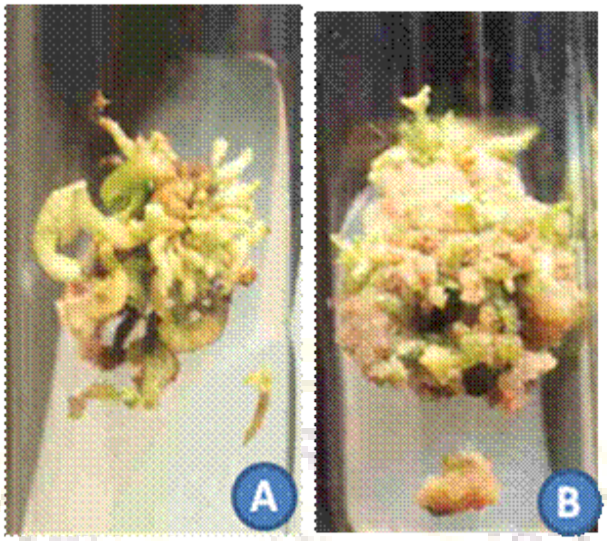


Figure 8: Primary somatic embryo showing: **A**. direct secondary somatic embryogenesis **B**. indirect secondary somatic embryogenesis

7. Synchronization of embryo development

Generally, the differentiation of somatic embryos in semi-solid medium or liquid medium is highly asynchronous which adversely affect the germination rate. Synchronization of embryo development is very important for artificial seed technology. Of the several approached tried to achieve this, the most effective method are the physical separation of embryogenic stages and use of growth regulators to physiologically synchronize the development. The other alternative methods are the fractionation of embryos of different stages by filtration of suspension through meshes of different sizes or by gradient centrifugation. Besides, the most effective method to achieve synchronous development of somatic embryos is the use of substances that would induce reversible cessation of embryo development at a particular stage. ABA at low concentration is the most satisfactory substance for the purpose. For example, in carrot it inhibits the growth of roots and enhances suspension with torpedo shaped embryos.

8. Production of synthetic seeds or artificial seed

Although it is possible to use naked embryos for large scale planting, it would be beneficial to convert them into 'synthetic seeds' or 'synseeds' for large scale clonal propagation at commercial level. This can be achieved by encapsulating the viable somatic embryos in a protective covering. The coating material should have several qualities:

- i. It must be non-damaging to the embryos.

ii. The coating should be mild enough to protect the embryos and allow germination but it must be sufficiently durable for rough handling during manufacture, storage, transportation and planting.

iii. The coat must contain nutrients, growth regulators and other components necessary for germination.

iv. The quality of somatic embryo should be good enough, they all are of uniform stage with reversible arrested growth and with high rate of conversion to plantlets.

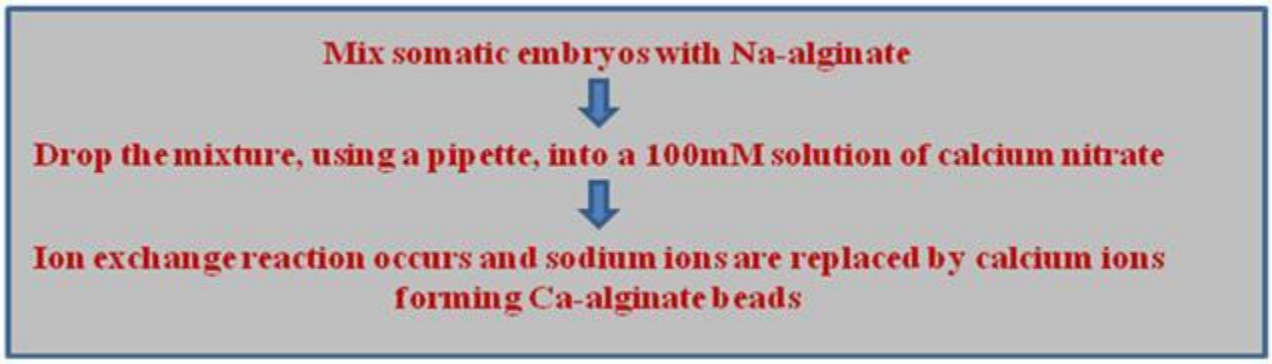
Two types of synthetic seeds are produced:

I. Desiccated synthetic seeds II. Hydrated synthetic seeds

I. Desiccated synthetic seeds : It involves encapsulation of somatic embryos followed by their desiccation and can be prepared by following methodology:

The polyox is readily soluble in water and dries to thin film. It does not support the growth of microorganism and is non toxic to the embryos. Embryo survival and conversion of seeds are determined by redissolving the wafers in embryogenic medium and culturing the rehydrated embryos.

II. Hydrated synthetic seed: Several methods have been examined to produce hydrated artificial seeds of which Ca-alginate encapsulation has been the most widely used. It can be prepared by following steps:



9. Applications of somatic embryogenesis

Following features of somatic embryos prompted many scientists to achieve regeneration via somatic embryogenesis using various explants, most popular ones being zygotic embryos, or excised cotyledons or hypocotyls

- i. Somatic embryogenesis offers immense potential to speed up the clonal propagation of plants being bipolar in nature.

  ii. Being single cell in origin, there is a possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds.

  iii. The bipolar nature of embryos allows their direct development into complete plantlet without the need of a rooting stage as required for plant regeneration via organogenesis.

  iv. Epidermal single cell origins of embryos favor the use of this process for plant transformation.

  v. It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds.

  vi. The production of artificial seeds using somatic embryos is an obvious choice for efficient transport and storage.

  vii. The embryo culture technique is applied to overcome embryo abortion, seed dormancy and self-sterility in plants.

10. Limitations of somatic embryogenesis

1. i. Complete conversion into plantlets or poor germination of embryos is a major limitation of somatic embryogenesis in many plants. Therefore, the process of germination needs to be studied in detail for successful plantlet conversion.

   ii. Compared to other plant species active research on somatic embryogenesis involving forest trees has been very slow.

   iii. The paucity of knowledge controlling somatic embryogenesis, the synchrony of somatic embryo development and low frequency of true to type embryonic efficiency are responsible for its reduced commercial application

   iv. To obtain a complete conversion into plantlets it is necessary to provide optimum nutritive and environmental conditions.