Chapter

# Embryo Culture and Embryo Rescue in *Brassica*

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## Abstract

Somatic embryogenesis is the best demonstration of totipotency in higher plants in which somatic cell produce whole plant like zygotic embryo. It is also demonstrated that immature, weak, hybrid or sometimes inviable embryos can be saved through *in vitro* culture to prevents its degradation. It may help to cross the reproductive barriers when interspecific hybrids developed. *Brasssica* is an economically valuable oil yielding and vegetable crop and India is the largest producer of oil seed rape in the world. Various factors affect the embryo rescue in *Brassica* like growth stage of the embryos, types and composition of the rescue medium etc. The embryo regeneration potential can improve through the modification of culture conditions in both zygotic as well as somatic embryo. Except the embryo culture other parts like ovule, ovary culture can also be done to developed interspecific hybrids. This chapter is focused on the embryo rescue techniques in the genus *Brassica* and summarizes possible ways of improving the technique used.

**Keywords:** *Brassica*, embryo rescue, hybrids, embryo culture, somatic embryos

## 1. Introduction

*Brassica* is an important vegetable and oilseed crop of India and it is the largest producer of the oilseed rape in the world. The genus belongs to the family Brassicaceae having about 38 different species [1]. Crossing and hybridization in these species was done to developed new cultivars worldwide for improving traits and yields. The naturally occurring genetic variation is the basis of the improvements in *Brassica* to produce new morphotypes in interspecific hybridization program [2]. The interspecific hybridization is a difficult process due to pre and post-fertilization barriers and abortion of the hybrid embryo. The embryo degeneration after some hybridization experiments takes place very early [3]. This may be due to the poor endosperm development or sometimes endosperm may not be developed [4]. But in non-endospermic embryos the post fertilization barrier cannot crossed and embryos are defective, disformed and aborted early. The hybrid embryos can be regenerated through various technique that's comes under the embryo rescue. The embryo rescue by culturing on nutrient medium that would support and orderly development of embryos. If the embryos are disformed, secondary embryogenesis may be induced by the manipulation of medium and growth regulators combination [5]. New biotechnological tool like transgenic technology would be better in improving the varieties of *Brassica* crop. Large number of transgenics, both biotic and abiotic stress resistant plants were developed [6, 7]. It is desired to produce transformed plant through somatic embryogenesis. It is preferred because of the

genetic stability during culture and very low rate of genetic variations which is otherwise results into somatic variations or somaclones [8]. There are however, some exceptions as in wheat (*Triticum durum* Desf.) somaclonal variation had been reported for several agronomic and phenotypic traits, such as plant height, leaf size, pollen fertility, tolerance to aluminum toxicity, albinism and leaf malformations [9]. *Cymbopogon winterianus* Jowitt showed somaclonal variations when regenerated via somatic embryogenesis [10]. But these variations are restricted to only few plant genera and it is found that the somatic variation produced frequently during tissue culture but generally not in somatic embryo or secondary embryos culture.

The embryogenesis in higher plants is controlled by many genes and related embryos specific proteins. Loss of function mutants were used to identified the genes involved in There are about 220 *EMB* genes in *Arabidopsis* required for normal embryo development. These were identified through duplication of alleles or molecular complementation [11]. Some proteins also required for the somatic embryogenesis and in embryo rescue in Carrot (*Dacus carrota*), these are mainly glycoproteins secreted into the culture medium such as endichitinase and arabinigalactan proteins (AGPs) are required for somatic embryogenesis. These AGPs contains glucosamine and *N*-acetyl-*D*-glucosaminyl [12]. *Rhizobium leguminosarum nod* gene metabolic product contains *N*-acetylglucosamine lipooligosaccharides that promotes carrot embryo rescue [13].

## 2. Zygotic embryos, genetic embryos and somatic embryos

In higher plants, formation and development of embryo are the two distinct phenomena that can takes place inside the ovule. An ovule has different parts like chalaza, nucellus, micropyle, integuments, and most important embryo sac. The embryo sac develops after the reduction division of the megaspore mother cell. Only one haploid megaspore cell give rise to the birth of embryo sac that's contains many cells like synergid cells, antipodal cells and one egg cell. Instead of all these cells there is a central nucleus (So called because it is not surrounded by distinct cell wall). The egg cell is polar and contain a distinct nucleus on cytoplasm-rich chalazal pole while there are vacuoles at micropylar end [14]. Egg cell when fertilized called the zygote and gives rise to the embryo. The two important process alternate in each generation, the meiosis (reduction division resulting two haploid cells called the gametophyte) and the fertilization (fusion of the two-haploid nucleus called the sporophyte). The three process are distinct with one another. Formation of zygote after fertilization, formation of the embryo and development of the embryo. Failure of any process disturbed the embryogenesis and cease the formation and development of embryo. After the formation of complete embryo successfully the mature embryo enters in desiccated and metabolically quiescent state [15] or it undergo the period of dormancy that's complete the process. The dormancy is the process of adaptation to withstand unfavorable conditions. The zygotic embryo in the angiosperm after maturation develops into seeds and it is composed of several tissues, including the embryo, the endosperm, and the testa. The mature embryo has the bipolar axis, on which root and shoot meristem are present and gives rise to the root and shoot during plant development. In contrast to the zygotic embryo, the genetic embryo is formed without fertilization through the process termed as apomixis. Apomixis refers to the formation of embryo in the ovule from the somatic cells. These somatic cells when diploid i.e. nucellus or integument directly gives rise to the embryo and termed as sporophytic apomixis. However, when embryo sac originates either from megaspore mother cells by mitosis or incomplete meiosis in diplospory is termed as gametophytic apomixis.

Totipotency is the most spectacular demonstration of potencies in the cells of higher plants. The somatic embryogenesis is the generation of bipolar structure from any somatic cell that have distinct root and shoot pole. It is very rare phenomenon present in nature and restricted to some plants like *Kalenchoe*, *Bryophyllum* etc. But it may be introduced into other plants artificially through tissue culture technique. How somatic cell triggered to gives rise to the embryo? It is somewhat unclear but it is suggested that the irregular distribution of auxin stimulates the establishment of embryonic structure [16]. However, the stages of the development of the somatic embryos resemble with the zygotic embryos.

## 3. Embryo culture

In *Brassica*, somatic embryogenesis can be induced using various auxins like 2,4-D and NAA at higher concentrations separately or in combinations with the cytokinin [17]. 2,4-D alone proved best hormone that used to induced polarity in the somatic cells as compared to the combination with the cytokinin like kinetin as it may ceased the further proliferation of somatic embryos [18]. Various culture medium was used with the auxins like SH Medium [19], B5 medium [20], MS medium [21], Kao's medium [22] etc., but rapid propagation of somatic embryos in *Brassica* proved to be the best in MS medium [17]. MS basal medium and low pH (3.5–5) was also used to induced somatic embryos in *Brassica napus* using immature seeds 14 to 28 days after pollination [23, 24]. Not only in *B. napus* but in *B. oleracea* varieties, cabbage and cauliflower immature zygotic embryos gives rise to the somatic embryos with high frequency [5], confirming that the stress condition either due to the PGR (mainly auxin) reprogrammed the zygotic immature cell to induced embryogenesis in *Brassica*. There is not only induction and establishment of polarity but also maintenance of the root and shoot meristem. It is also noticed that the zygotic embryos when immature have higher embryogenic potential than the mature embryo [23, 25]. The low pH value i.e., 3.5–5 in *Brassica napus* increase the exchange of ions and ionic nutrients that's accumulate inside the embryogenic tissue or callus. The stored food material mainly lipid bodies in the cells of *Brassica* indicates the good exchange of nutrient under auxin induced stress condition. The leaf explant is best for the induction of somatic embryos as compared to the other explants like stem and hypocotyl sections (**Figure 1A-D** and **F**) [17, 26, 27]. This is because the large number of vacuolated protoplasts that's provide the space for the storage of the food material in cotyledon and leaf explants.

## 4. Embryo rescue in *Brassica*

Embryo rescue is an *in vitro*-culture technique that is used to save weak, immature and hybrid or sometimes inviable embryos to prevents its degradation. The procedure involves excising weak, immature plant embryos and culture them on specially devised culture medium. It plays an important role in plant breeding of important crop plants. In *Brassica*, the interspecific and intergeneric hybridization was done from very earlier because the crop yield losses due to disease, biotic and abiotic stresses is very high. Interspecific, intergeneric and intervarietal hybrids have been generated in mango, banana, seedless grape, papaya and seedless citrus using embryo rescue [28]. It is not only an important oilseed crop but used as vegetable, and fodder for the farm animals. The earlier attempts were made in Chinese cabbage varieties i.e. *B. oleracea* and *B. campestris* [3]. The hybrid embryos were carefully removed and cultured on the nutrient medium *in vitro*.

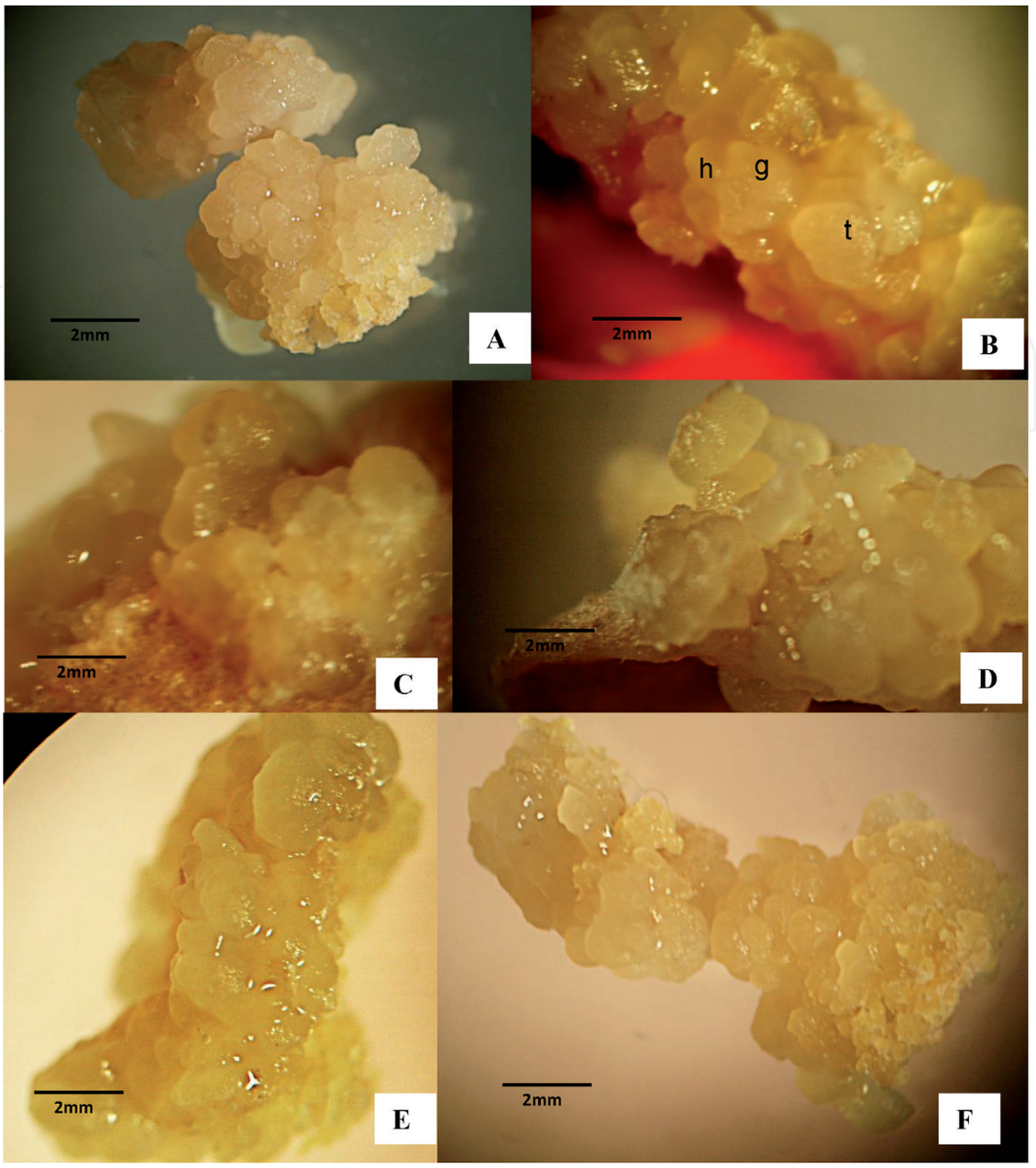


**Figure 1.**
*Somatic embryos of* Brassica juncea *L. A, B, C, and D, The cotyledon derived somatic Embryos, E and F, hypocotyl derived somatic embryos.*

The survival rate of the embryos can be increased when rescued because wide hybridization crosses fail to complete normal sexual reproduction cycle. In *Brassica* the best adopted methods for the embryo rescue are the direct ovule culture, siliqua culture and immature embryos culture. Sometimes embryo-nurse endosperm for embryo transplant was also adopted [29]. Very young embryo is difficult to culture on artificial culture medium. Due to this young fertilized pistil were cultured. Therefore, the chances of embryo abortion can be minimized. For siliqua culture young fertilized pistils excised 4 to 6 days after pollination and cultured on the MS medium. It absorbed the medium start to grow gradually but regular subculturing is required. The swollen pistil again excised to dissect out developing ovule. Ovules can also be culture in a similar manner and selection day after pollination may varies from plant to plant and to identify the fertilized pistil, pollinated pistil fixed in 70% ethanol 24 to 48 hour after pollination and stained in aniline blue, the pollen germination and pollen tube growth may be observed under the microscope [30]. Ovary culture in *Brassica* can be done 4 to 14 days after pollination. The stalk

of the ovary cut from the base and cultured on the nutrient medium [31]. For embryos culture, the siliqua was collected after 10, 15, 20, 25, and 30 days after pollination and after the sterilization the ovules are dissect out from siliquae and the young embryos cultured on the MS medium [21]. All these methods of embryo rescue depend on the type and the condition of the hybrid embryos and the type of hybridization experiments [32]. Considerable progress in embryo rescue has been takes place but the rescue of hybrid embryos seems to be difficult when abortion occurs at very early. Embryo implantation technique was adopted to overcome this problem which is known as embryo-nurse endosperm transplant where excised hybrid embryo inserted into a cellular endosperm dissected from third species or one of the parents. The nurse endosperm with the transplanted embryo cultured on an artificial medium [33]. Recently double haploids were developed through embryoids derived from isolated microspore culture [34].

## 5. Factors affecting embryo rescue

Various factors affect the embryo rescue in *Brassica* and other plants. It depends on the age of the embryo, intactness of the suspensor [35], excision procedure, sterilization, culture medium supplementation, temperature and light requirements etc. Highly immature embryo rescue is very difficult and requires special medium requirements. Excision procedure is designed in that way, no or minimum injury should be there in embryo proper specially when embryo excised at very early stage of development. The aim should be rescue for primary embryos secondary embryogenesis should be avoided.

### 5.1 Nurse tissue

The early stage embryo culture during rescue is very difficult to culture on artificial medium. The nurse tissue provides a natural condition and nutrition so that the chances of embryo abortion reduces. The best nurse tissue for the embryo culture is endosperm and if it is some days old it efficiently increases the chances of survival of the young embryo. The somatic embryos can be used as nurse tissue for culturing the hybrid zygotic embryos as done in *Pinus* [36]. The ovule is another nurse tissue which is used to generate very young stage embryos. It is not necessary to excise young embryo from the ovule but excision of ovule is easy. Sometime ovary culture can also be used and this was done when the embryos are very small and inconspicuous.

### 5.2 Culture medium

There are various nutrients formulations used to culture the embryos during rescue. Murashige and Skoog formulation [21] and Gamborg's B-5 [20] are most frequently used in *Brassica* [37]. Sometimes both hormones were used as in *B. oleracea* [5]. However, there are examples in which B5 vitamins are used with MS salts in embryo rescue of *R. sativus* [38]. Other mediums used for the embryo rescue in other plants are Knop's medium; Heller's medium [39], Monnier's medium [40] etc. The early heterotrophic immature embryos take its nutrition form the endosperms and surrounding tissue but when embryos mature it is partly autotrophic and it requires only basic mineral salts and sucrose. In *Brassica*, the embryos become autotrophic very late after globular stages [41]. The early globular stage of proembryo culture was achieved earlier using double-layer culture system and in embryo culture medium which contains mineral salts, sugars, amino acids, organic acids and

coconut water [42]. The coconut water induces cell division in plant and necessary for the development of very young embryo [43]. The other additive components that used during embryo culture are tomato juice, banana pulp, different fruit juices, fish emulsion, leaf extract, potato extract etc. [44]. Sucrose is a very important constituent of the culture medium as an energy source and it maintains the osmotic potential of culture medium. High sucrose contents trigger the formation of embryos because it mimics the high osmotic potential of the embryo sac [29], and it help for the induction of embryos from embryogenic calli [17].

### 5.3 Silver nitrate

The silver nitrate is another very important ethylene antagonistic component that's play important role in *Brassica* embryo culture. It is added into the medium at a concentration that varies between (1–10 $mgl^{-1}$). It significantly increases the regeneration potential in various *Brassica* species through both embryogenesis and organogenesis. During somatic embryogenesis the presence of $AgNO_3$ increase the no of embryos significantly specially in *B. oleracea* and *B. rapa* [45].

### 5.4 Temperature and light

Embryo culture during embryo rescue influenced with temperature and light and their requirements are also varies in different plants [46]. The low temperature regime is best for the embryo rescue but in some cases at high temperature regime (26.4 °C/10.4 °C embryo rescue through ovule culture method gives better results than low temperature regime period (19.4 °C/4.3 °C) (1.75%) in *B. oleracea* [47]. According to Mei et al., [48] there is a significant quadric relation between the effective accumulated temperature (EAT) and the efficacy of ovule culture. The hybrid *B. napus* x *B. oleracea* siliqua can be collect for the excision of ovule on the basis of EAT instead of siliqua age.

## 6. Cytology of cultured embryos

Several biochemical and molecular changes occurs at cellular level as the embryo formed, grow in length and approaches to the maturity. The developmental studies of the somatic embryos showed that the lipid, protein and polysaccharides produced during at varying degrees when embryos were cultured on ABA containing medium. At first one or two weeks polysaccharides were produced and after that polysaccharides lipid and protein accumulated [49]. The analysis of the cultured embryos cells of oil yielding crop like *Brassica*, under electron microscopy revealed the presence of lipid bodies (spherosomes) associated with the endoplasmic reticulum [50]. The embryos cell contains several other components like some dense granules (ribonucleoprotein), endoplasmic reticulum, mitochondria, amyloplast and some irregular bodies etc. It was noted earlier that when a cell start to convert into an embryo, it start to contains smaller vacuoles in dance cytoplasm, large nucleus (Nu) with numerous organelles and stored bodies [17]. These are the steps towards the development of seed and the stored food material is the reserve for germination. The stored food material in the small somatic embryos is similar to as in the endosperms of zygotic embryos as in *Acromia aculeata*. The endosperm of zygotic embryos showed the accumulation of lipid and proteins which may consumed in the initial stages of germination and plantlet establishment (**Figure 2A-E**). However, their somatic embryos does not showed such types of deposition and this results in low conversion of these

embryos into plants [51]. In other plants like orchids some early stage protocorm behave like somatic embryos and contains protein bodies and starch granules and it is supposed to be similar with the zygotic embryo development [52]. The deposition of the polysaccharides starts toward the basal end of the suspensor

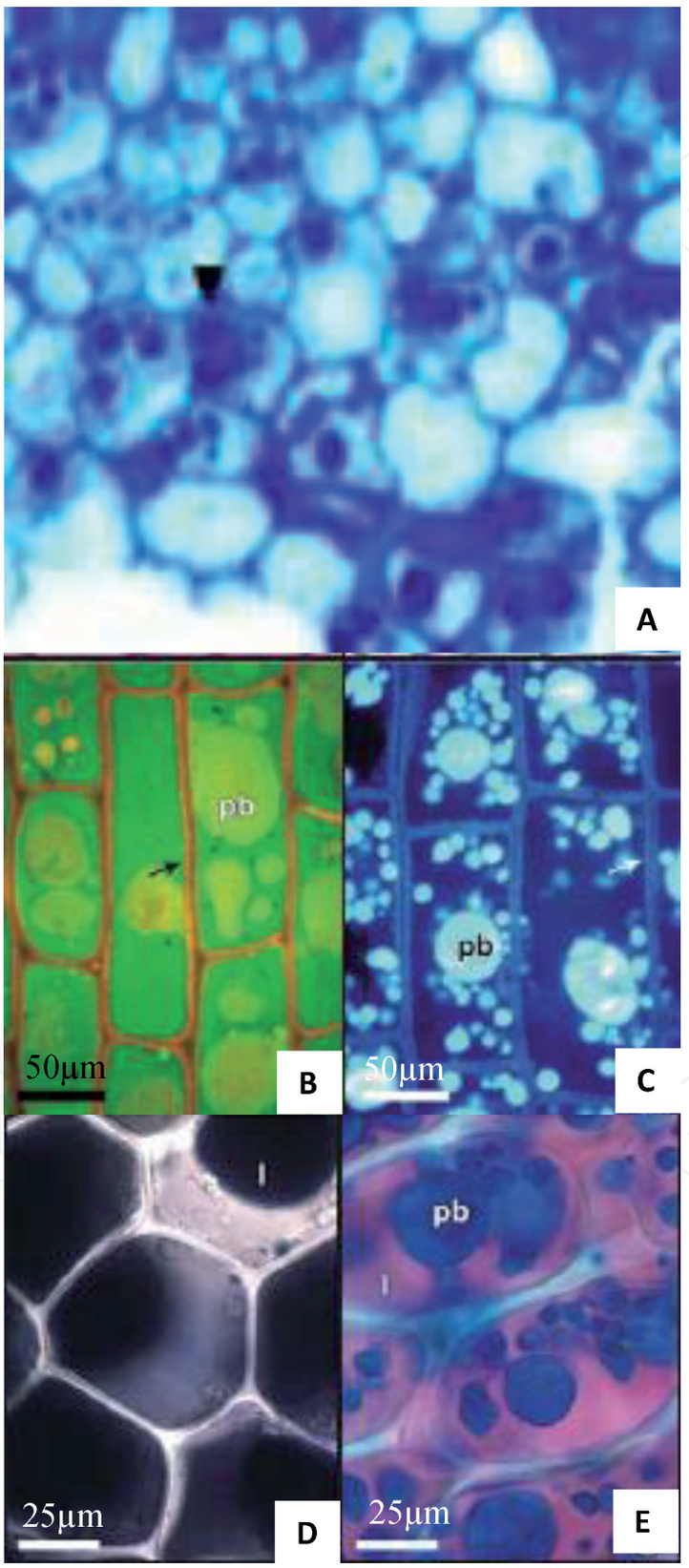


**Figure 2.**
*A Light micrographs of* Brassica juncea *somatic embryo sections, cells showed the stored food material in the form of lipid B, C, D and E Light micrographs of different histochemical tests of* Acromia aculeata *endosperm of zygotic embryos showed lipid (l) and protein bodies (pb). Source: Akmal et al [17], Moura et al. [51].*

and gradually it increases and move inwards in the cortex of developing embryos. The stored lipid bodies in embryo at its peak during the cotyledon development and after that decreases. The most abundant fatty acids in somatic embryos of *Picea abies* are linolic, oleic, palmitic and 5,9-octadecanioc acids [53]. There is a quantitative difference in the composition of the fatty acids in *in vitro* and *in vivo* cultured somatic embryos [54].

## 7. Genes involved in embryogenesis

When a somatic cell enters into embryogenic state it starts to modify the gene expression level. There are number of genes involved in somatic embryogenesis, showed increase expression. These can be categorized in various groups [55]. These are hormone responsive genes, house-keeping genes, genes expressed during maturation, genes coding for extracellular proteins, homeotic genes, HSPs,(Heat-Shock Proteins) germins, zygotic mutants and genes for signal transductions, except these there are the genes of transcription factors. The various transcription factors regulate several genes that showed expression in the somatic embryo induction [15]. In *Arabidopsis thaliana* the basic pattern of embryo formation is the polarity of apical-basal axis and other perpendicular to the axis. It consists of shoot meristem, cotyledons, hypocotyl, root, and root meristem along the apical–basal axis and a concentric arrangement of epidermis, subepidermal ground tissue, and central vascular cylinder along the radial axis For the apical-basal axis formation asymmetric *PIN7* and *WOX2* expression is important to establish apical cell identity at the apical and basal cell of the zygote while another *gnom* mutant argues against the possibility that the different sizes of the daughter cells per se are important for apical–basal development [56]. There are some genes that interact with one another and control the pattern formation. The mutant analysis showed that the resulting mutant disturbed the pattern formation related to the primary shoot and root meristems [57]. Some embryogenesis-related genes similar expression pattern in both in vitro and in vivo embryogenesis and these were *LEA* (Late Embryogenesis Abundance) genes, *SERK*, (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE*), *AGL15 (AGAMOUS-LIKE15*), *BBM* (BABY BOOM gene*), LEC1*(LEAFY COTYLEDON*)*, *FUS3* (FUSCA3, B3-domin transcription factor) and *AB13 (ABSCICSIC ACID INSENSETIVE3)* [58]. There are about 250 EMB genes required for the normal development of the embryo and for complete seed development additional 219 genes product required [11, 59]. A dataset of about 510 *EMBRYO-DEFECTIVE* (*EMB*) genes were identified in Arabidopsis [60]. The TARGET OF RAPAMYCIN (TOR) kinase has been recognize as a key developmental regulator in both plants and animals which integrates environmental and nutrient signals to direct growth and development [61]. The rapamycin kinase receptor gene in *Arabidopsis.*

(AtRaptor1) is responsible for the early development of embryo [62]. The genes also essential for the post-embryonic plant growth because the AtRaptor1A, AtRaptor1B double mutants are defective in meristem driven growth during post embryonic stage [63].

In near future, the techniques of somatic embryos induction, and embryo rescue in *Brassica* can be further improved so that there should be about 99% chances of hybrid embryos survival. This will not only increase their survival rate but number of plantlets regenerated also improved, especially in those intergeneric crosses where the hybrid embryos survival rate is very low. This will give an opportunity to increase the productivity of the *Brassica* crop through various crop improvement programs.

## 8. Conclusion

In this chapter, the somatic embryos and zygotic embryos with embryo rescue techniques are discussed. The hybrid embryo rescue technique in *Brassica* and other plants provide a useful tool to developed intergeneric and interspecific hybrids. The modification of the culture conditions, use of plant growth regulators and other complex medium components, the immature hybrid embryo can be successfully rescued at an early stage. The hybrid embryo in *Brassica* directly gives rise to new plants or some time it is desired to generate secondary embryos. The cytology of the somatic and zygotic embryos and the genes involved are also briefly discussed.

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## Acronyms and abbreviations

| | |
|---|---|
| *LEA* | Late Embryogenesis Abundance |
| *BBM* | BABY BOOM gene |
| *SERK, LEC* | Leafy cotyledon |
| *AB13* | Abscicsic acid insensetive3 |
| *AGL15* | Agamous-like15 |
| *WUS* | WUSCHEL |
| *SERC* | Somatic embryogenesis receptor-like kinase |

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