Zygotic Embryo Culture 11

11.1 Introduction

The zygote (fertilized egg), through a series of well-defined developmental stages, forms an embryo (Fig. 11.1) the progenitor of the next generation. Several physical and chemical factors regulate the growth and development of the embryo. The surrounding tissues, especially the endosperm, also control the predetermined pattern of embryo development. Any disturbance in these factors causes abnormalities and, in extreme cases, abortion of the embryo. In vitro culture of excised zygotic embryos at different stages of development has provided useful information on the developmental and physiological aspects of embryogenesis. Embryo culture is one of the in vitro techniques, which found a practical application even before it was well established. It is now widely used to produce rare hybrids which are normally not possible due to premature abortion of the embryo. This chapter describes the technique of embryo culture, its importance in understanding the physiological and developmental aspects of embryogenesis and, finally, its applied aspects.

The first systematic attempt to grow the embryos of angiosperms in vitro under aseptic conditions was made by Hannig (1904). He cultured excised mature embryos of Brassicaceae members, *Cochlearia danica, Raphanus caudatus, R. landra* and *R. sativus* on a mineral salt medium supplemented with sucrose and obtained transplantable seedlings. Dieterich (1924) observed that on a semi-solid medium containing minerals and 2.5–5 % sucrose, mature embryos of several plants showed normal growth, but the embryos excised from immature seeds failed to achieve the organization of a mature embryo. These, instead, grew directly into seedlings, skipping the intermediary stages of embryogenesis. Dieterich described this phenomenon of precocious germination as 'kunstliche Fruhgeburt'. Stimulus for further work on embryo culture came from the work of Laibach (1925, 1929). In interspecific crosses between *Linum perenne* and *L. austriacum*, Laibach noted that the seeds were greatly shrivelled, very light and incapable of germination. By excising the embryos from such seeds and growing them on a moist filter paper or cotton wadding soaked in sucrose or glucose solution, he was able to raise full hybrid plants. This technique of hybrid embryo culture has since been widely used to raise numerous such hybrids which normally fail due to post-zygotic sexual incompatibility. The technique of growing embryos outside the ovule (ex ovulo embryo culture) provides an excellent opportunity to study the physical and chemical regulation of growth and differentiation during embryogenesis. Raghavan (2003) has published an excellent historic account of embryo culture in the centenary year of this technique. More recently, Thorpe and Yeung (2011) have reviewed the subject.

11.2 Technique

There are two important aspects of the embryo culture technique: (i) composition of the culture medium and (ii) excision of the embryo.

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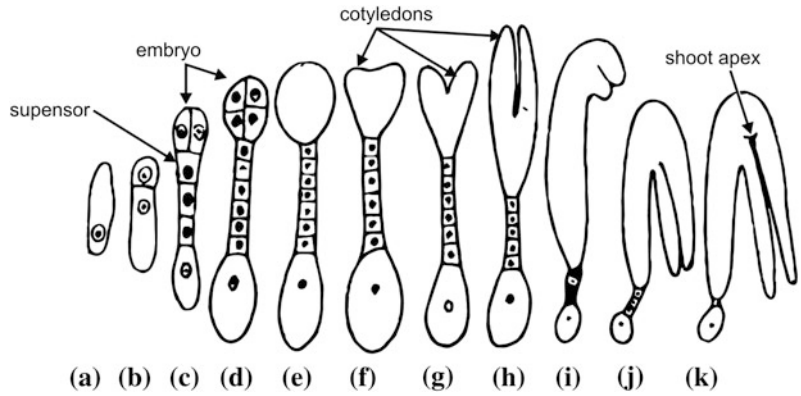

Fig. 11.1 Stages in the normal development of embryo in *Capsella bursa-pastoris*. **a** Zygote, **b** 2-celled proembryo, **c–e** Globular, **f** Early heart-shaped, **g** Late heart-shaped, **h** Torpedo-shaped, **i** Walking stick–shaped, **j** Inverted U-shaped and **k** Mature embryos (*after* Raghavan 1966)

The composition of the culture medium varies with the material and the age of the embryo to be cultured. Some of the media used for embryo culture are given in Tables 11.1, 11.2 and 11.4. For the culture of isolated zygotes and very young proembryos, the media formulations developed so far have proved inadequate. They require co-cultivation with feeder cells/nurse tissue, such as actively growing suspension cell aggregates or embryogenic microspores.

For the introduction of the technique to the students, a plant that has large seeds and whose embryos can be easily dissected out is ideal, such as some legumes and crucifers. For researchers, it would depend on the experimental objective. *Capsella bursa-pastoris* (Shepard's purse) has been a favourable material for many basic studies on embryo culture. It has racemose inflorescence, and each raceme bears flowers at various stages of development, with younger flowers at the top and older below. Each capsule bears 20–25 ovules, which are more or less at the same stage of development (Fig. 11.2a, b). When the objective is to rescue a hybrid embryo which otherwise aborts in situ, it is essential to determine the stage at which the degeneration of the embryo sets in. In such cases, the embryo is allowed to grow inside the ovule as long as possible and excised well before the abortion starts. Younger the embryo, more difficult it is to culture.

Table 11.1 Improved nutrient medium for the culture of *Capsella bursa-pastoris* embryo culture (*after* Monnier 1976, 1978)

Constituents	Amount (mg L^{-1})
KNO_3	1,900
$CaCl_2.2H_2O$	880
NH_4NO_3	825
$MgSO_4.7H_2O$	370
KCl	350
KH_2PO_4	170
Na_2.EDTA	14.9[a]
$FeSO_4.7H_2O$	11.1[a]
H_3BO_3	12.4
$MnSO_4.H_2O$	33.6
$ZnSO_4.7H_2O$	21
KI	1.66
$Na_2MoO_4.2H_2O$	0.5
$CuSO_4.5H_2O$	0.05
$CoCl_2.6H_2O$	0.05
Glutamine	400
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Sucrose	120,000
Agar	7,000

[a] 2 ml of a stock solution containing 5.57 g $FeSO_4.7H_2O$ and 7.45 g Na_2.EDTA L^{-1}

The ovules enclosing the embryo are well protected inside the ovary wall and are sterile. Therefore, the ovaries are suitably surface

Table 11.2 Composition of Kao90 medium for the culture of barley zygote (*after* Holm et al. 1994)

Constituents	Amount (mg L^{-1})	Constituents	Amount (mg L^{-1})
Inorganic nutrients			
NH_4NO_3	165	$MnSO_4.4H_2O$	22.3
KNO_3	1,900	$ZnSO_4.4H_2O$	8.6
$CaCl_2.2H_2O$	440	$Na_2MoO_4.2H_2O$	0.25
$MgSO_4.7H_2O$	370	$CuSO_4.5H_2O$	0.025
KH_2PO_4	170	$CoCl_2.6H_2O$	0.025
KI	0.83	$FeSO_4.7H_2O$	27.8
H_3BO_3	6.2	$Na_2.EDTA.2H_2O$	37.3
Organic nutrients			
Inositol	100	Biotin	0.005
Nicotinamide	1	Choline chloride	0.5
Pyridoxine HCl	1	Riboflavin	0.1
Thiamine HCl	10	Ascorbic acid	1
D-Calcium pantothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D_3	0.005
p-Aminobenzoic acid	0.01	Vitamin B_{12}	0.01
Organic acids			
Sodium pyruvate	5	Malic acid	10
Citric acid	10	Fumaric acid	10
Hormone			
2,4-D	1		
Others			
Vitamin-free casamino acid	250	Coconut water	20 ml L^{-1}
Maltose	9 %	–	–

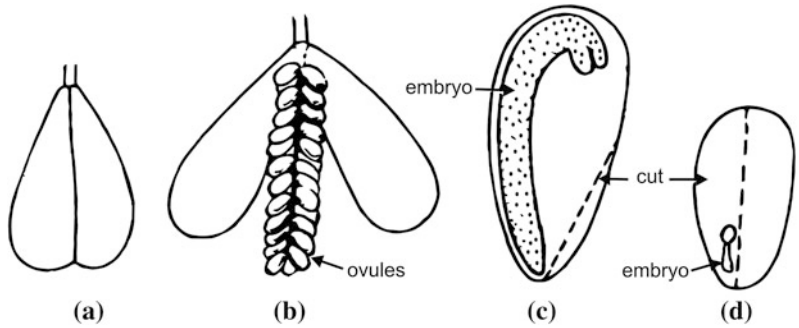

Fig. 11.2 Isolation of the embryos of *Capsella bursa-pastoris*. **a** A capsule. **b** The capsule has been opened to expose the ovules. **c** An ovule with walking stick–shaped embryo inside; the *dotted line* shows the region of incision of the ovule to release the embryo. **d** An ovule with globular embryo. A cut along the *dotted line* exposes the embryo (*after* Raghavan 1966)

sterilized, and the ovules and embryos are dissected out inside a sterile chamber. The dissections are carried out in a drop of suitable medium such as sucrose/glucose solutions or a culture medium to prevent desiccation and provide osmotic protection to the embryo.

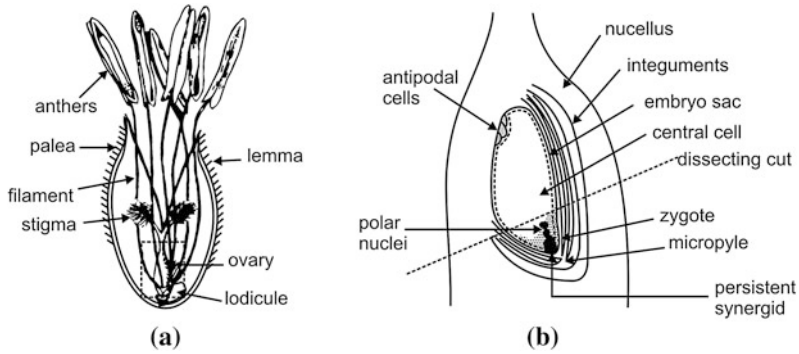

Fig. 11.3 Structure of a rice spikelet during anthesis (**a**) and dissection of zygote (**b**). The ovary emblocked in (**a**) is excised and cut open one-third its length from the micropylar end which houses the zygote (**b**). Slight pressure at the micropylar end pushes out the zygote from the cut end (*after* Zhang et al. 1999)

The procedure used for the isolation of *Capsella* embryos by Raghavan and Torrey (1963) is depicted in Fig. 11.2. The surface sterilized capsules are placed in a few drops of sterile culture medium, the outer wall is removed by an incision in the region of the placenta, and the two halves are pulled apart to expose the ovules (Fig. 11.2b) Torpedo-shaped and younger embryos are confined to longitudinal one-half of the ovule (Fig. 11.2c, d) and are clearly visible through the chalaza, either because of their green colour (older embryos) or because of transparent vesicle of their suspensor. To excise immature embryo, an ovule is placed in a drop of medium on a microscope slide and split longitudinally with the help of a sharp mounted blade (Fig. 11.2d). By carefully teasing apart the ovular tissues, the entire embryo, along with the suspensor, is carefully removed. For excising older embryos, a small incision is made in the ovule on the side lacking the embryo (Fig. 11.2c), and the embryo is pushed out by a slight pressure with a blunt needle.

To isolate zygote, time taken from pollination to fertilization or from anthesis to syngamy is determined, preferably under controlled light and temperature regimes. In cereals, the spikelets at the appropriate stage are removed, surface sterilized and washed thoroughly before mechanically isolating the zygote in a suitable medium. Whereas Holm et al. (1994), working with barley, used a modified MS medium supplemented with 9 % maltose, Zhang et al. (1999) used Kao and Michayluk (1975) medium with 9 % maltose for rice. For the isolation of rice zygote, the ovaries excised from surface-sterilized spikes were placed in a drop of the isolation medium and decapitated at approximately one-third of their length from the micropyle to open the embryo sac (Fig. 11.3a, b). The sudden pressure change in the embryo sac releases the zygote. The section of the ovary containing the zygote is then transferred to 35 mm plastic Petri dish containing 3 ml of the isolation medium. The zygote is gently pushed out of the embryo sac through the cut end of the ovary by carefully pressing from the micropylar end towards the cut end. The isolated zygotes are picked up with micropipette and transferred to culture dish. In maize, softening of nucellar tissue by a brief treatment with a mixture of cell wall–degrading enzymes prior to microdissection of zygote proved useful (Kranz and Kumlehn 1999).

11.3 Culture Requirements

Brown (1906) applied the technique of embryo culture to study the relative efficiency of various organic nitrogenous compounds on the growth of excised barley embryos and demonstrated that the amino acids, aspartate, glutamate, amide and asparagine, are superior nitrogen sources, causing increased dry weight and nitrogen content of the cultured embryos. Further studies have contributed significantly to our knowledge about

the importance of mineral nutrients, source and concentration of carbohydrate, nitrogenous compounds and growth regulators for the development of isolated zygotic embryos.

11.3.1 Mineral Nutrients

Many different mineral formulations have been used for embryo culture without much critical evaluation of the role of individual elements. Monnier (1976) studied the effect of various mineral solutions for the culture of zygotic embryos of *Capsella bursa-pastoris* and concluded that there was no correlation between the growth and survival of embryos on a particular medium. MS medium supported maximum growth, but the survival frequency of the embryos was very low. On the other hand, the Knop's medium was least toxic, but growth of the embryo was very poor. After a detailed study, Monnier developed a medium (for composition see Table 11.1) that supported as good growth of the embryo as MS and the survival was high. As compared to the MS medium, this medium has higher concentrations of K^+ and Ca^{2+} and lower level of NH_4^+ ions.

For immature embryos of many plants, such as barley (Umbeck and Norstog 1979), *Datura stramonium* (Paris et al. 1953) and *D. tatula* (Matsubara 1964), NH_4^+ was either essential or a preferred source of inorganic nitrogen. This has been ascribed to the lack of necessary enzyme to reduce NO_3^- to NH_4^+ at the early stage of embryo development.

11.3.2 Amino Acids and Vitamins

Hannig (1904) reported that asparagine was very effective in enhancing the embryo growth. Generally, glutamine has proved to be the most effective amino acid for the growth of excised embryos (Rijven 1955; Matsubara 1964). Casein hydrolysate, a complex mixture of amino acids, has been widely used as an additive to embryo culture media, especially for young embryos (Sanders and Burkholder 1948; Rangaswamy 1961).

Vitamins have been used in embryo culture media, but their presence is not always necessary.

11.3.3 Carbohydrates

Sucrose is by far the best form of carbohydrate and has been most commonly used for embryo culture. Sucrose is added to the medium not only as the source of energy but also to maintain a suitable osmolarity which is extremely important for immature embryos (Liu et al. 1993). The optimum concentration of sucrose, therefore, varies with the stage of embryo development. Mature embryos grow well with 2 % sucrose, but younger embryos require higher levels of the carbohydrate. This is in harmony with the observation that in situ, the proembryos are surrounded by a fluid of high osmolarity that gradually drops as the embryo grows (Ryczkowski 1960). Sucrose at 8–12 % is generally adequate for the culture of proembryos of *Datura* (Rietsema et al. 1953), *Hordeum* (Ziebur and Brink 1951) and *Capsella* (Monnier 1978). Artificially increasing the osmotic pressure of the culture medium by the addition of enough mannitol, in the presence of a moderate level (2 %) of sucrose, has enabled the culture of proembryos of wheat (Fischer and Neuhaus 1995) and *Capsella bursa-pastoris* (Rijven 1952).

11.3.4 Growth Regulators

Growth regulators are not always necessary for the normal development of embryo. Addition of hormones may cause structural abnormalities (Monnier 1978).

The requirement for high osmolarity of the culture medium for globular embryos (55 μm long) of *Capsella bursa-pastoris* could be substituted by a balanced combination of IAA, kinetin and adenine sulphate (Raghavan and Torrey 1963, 1964). Some other examples of growth promotion of proembryos by growth regulators are as follows: *Linum usitatissimum* by kinetin (Pretová 1986), heart-shaped embryos

of *Medicago scutellata* and *M. sativa* by IAA and BAP (Bauchan 1987) and proembryos of maize by zeatin and BAP (Matthys-Rochon 1998). Substitution of suspensor by GA_3 (see Sect. 11.5) is another example of the involvement of a growth regulator in embryo development. ABA is a well-known hormone to support morphologically and biosynthetically normal embryogenesis in cultured embryos (Fischer-Iglesias and Neuhaus 2001).

Involvement of polar auxin transport has been implied in modulating cell divisions in a localized region of the globular embryo where the cotyledons arise. Treatment of cultured globular embryos with auxin transport inhibitors (TIBA, N-1-naphthylphthalamic acid) leads to the formation of a ring-like structure around the shoot apex akin to fused cotyledons, instead of two separate cotyledons in *Brassica juncea* (Liu et al. 1993) and differentiation of additional shoots in wheat (Fischer et al. 1997).

11.3.5 Natural Plant Extracts

The use of liquid endosperm of coconut (coconut water or coconut milk) enabled van Overbeek to culture 200–500-μm embryos of *Datura stramonium,* which otherwise could not be cultured on a defined medium. This led to the suggestion that CM contains an 'Embryo Factor'. This was followed by several reports on in vitro culture of proembryos of other plants by the use of plant extracts of endospermic or non-endospermic origin or by using endosperm as the nurse tissue. Matsubara (1962) found alcohol diffusates from young seeds of *Lupine* to be as effective as CM for the culture of heart-shaped embryos (150 μm) of *Datura tatula*.

11.4 Culture of Proembryos and Zygote

The nutritional requirements of very young embryos and zygote remain undefined. Liu et al. (1993) successfully cultured 35 μm long early globular embryos (8-celled) of *Brassica juncea* using double-layer culture system of Monnier (1976, 1978), involving two complex semi-solid culture media differing only in their osmolarity. In vitro culture of excised zygotes has been possible so far only by co-cultivation with actively growing feeder suspension cells or embryogenic microspores. Holm et al. (1994) cultured zygotes of barley on a complex Kao90 medium (Table 11.2), which is a modified MS medium with NH_4NO_3 reduced to 165 mg L^{-1} and supplemented with vitamins and organic acids of the Kao and Michayluk (1975) medium, 20 ml L^{-1} liquid endosperm of coconut (CM), 250 mg L^{-1} vitamin-free casamino acids, 1 mg L^{-1} 2,4-D and 9 % maltose as the source of carbohydrate. On this medium, the isolated zygotes of barley, excised 6 days after pollination, divided a few times and formed spherical microcalli, after which the cells degenerated. However, co-cultivation with embryogenic microspores of barley (younger than 19 days old) in the liquid Kao90 medium for 23–30 days resulted in 75 % of the zygotes developing beyond the microcalli stage, and most of the cultures formed plantlets. Kumlehn et al. (1998) reported regeneration of fertile plants from isolated wheat zygotes through direct embryo differentiation with 90 % success. In contrast, only 36 % of the zygotes of a japonica cultivar (Taipei 309) of rice grew into fertile plants, which was similar to the frequency of zygote regeneration in maize (Leduc et al. 1996). In rice, the zygotes divided 24–48 h after culture and formed calli, which differentiated fertile plants only after transfer to MS medium (with 3 % maltose) supplemented with 0.4 mg L^{-1} BAP (Zhang et al. 1999).

Kumlehn et al. (1999) developed a method in which the wheat zygotes were immobilized in 100 μl droplets of 0.75 % agarose placed on a microscope slide and co-cultured with an appropriate population of embryogenic microspores in liquid medium (Fig. 11.4). The system facilitated direct observation and photographic documentation of the developmental changes during 5–40 days after pollination. The first division of

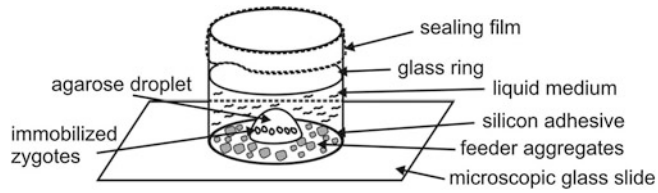

Fig. 11.4 Representation of the culture technique employed to monitor individual development of isolated wheat zygote (*after* Kumlehn et al. 1999)

the zygote was symmetrical, and it developed into a germinable normal embryo which formed fertile plants.

So far, the success with excised zygote culture is restricted to monocots. In *Arabidopsis thaliana*, as in many other dicots, in ovulo culture of zygote has been successful, but excised embryos could be cultured only after the heart-shaped stage (Sauer and Friml 2008). For the culture of in vitro formed zygotes, see Chap. 13.

11.5 Changing Growth Requirements of the Embryos

With respect to its nutrition, Raghavan (1966) recognized two phases of embryo development: (i) *Heterotrophic Phase*—the early phase of development that may last up to the globular or even a slightly later stage, during which the embryo draws upon the endosperm and suspensor for its nutrition and (b) *Autotrophic Phase*—this phase starts at the late heart-shaped stage when the embryo becomes metabolically capable of synthesizing substances necessary for its growth and morphogenesis from basic mineral salts and sugar and draws upon its own metabolites. The critical stage at which the embryo enters the autotrophic phase varies with the plant species (Rhagavan 1976). Even within the two phases, the exogenous requirements of the cultured embryo become simpler as it ages. This can be illustrated by two well-known examples, viz. *Datura* (van Overbeek et al. 1941, 1942) and *Capsella bursa-pastoris* (Raghavan 1966).

In *Datura*, excised mature embryos develop into normal seedlings on mineral salts and dextrose. Young torpedo-shaped embryos (up to 0.5 mm) require a medium containing mineral nutrients, dextrose, glycine, thiamine, ascorbic acid, nicotinic acid, vitamin B_{12}, adenine sulphate, succinic acid and pantothenic acid (basal medium). Embryos smaller than 0.5 mm in length could be cultured only with the addition of coconut milk to the basal medium. A similar increasing autonomy of *Capsella* embryos with age is evident from the data presented in Table 11.3.

As mentioned before, the osmotic value of the nutrient medium seems to be an important factor for the proper growth of the embryo. Ryczkowski (1960–1972) had shown that in

Table 11.3 Progressive nutritional autonomy during embryogenesis in *Capsella bursa-pastoris* (*after* Raghavan 1966)

Developmental stage	Length of embryo (μm)	Nutritional requirement
Early globular	21–60	Could not be cultured
Late globular	61–80	Basal medium (macronutrients + trace elements + vitamins + 2 % sucrose) + kinetin (0.002 mg L^{-1}) + IAA (0.1 mg L^{-1}) + adenine sulphate (0.001 mg L^{-1})
Heart-shaped	81–450	Basal medium alone
Torpedo-shaped	451–700	Macronutrients + vitamins + 2 % sucrose
Walking stick–shaped and mature	>700	Macronutrients + 2 % sucrose

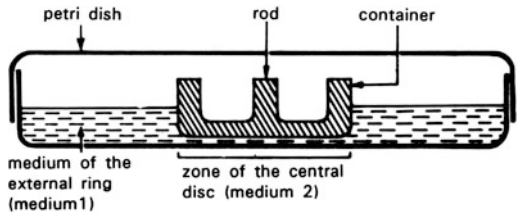

Fig. 11.5 Device allowing the juxtaposition of two media with different compositions (see Table 11.4). The first agar medium is liquified by heating and then poured around the central glass container. The medium gives the external ring. After cooling and solidification of the medium, the container is removed. In the central ring, thus formed, a second medium of a different composition is poured. The embryos are cultivated on the second medium in the central part of the Petri dish. As a result of diffusion, the embryos are subjected to the action of variable medium with time (*after* Monnier 1976, 1978)

monocots as well as in dicots, the values of osmotic pressure, viscosity, specific gravity and concentration of sugar and amino acids of the ovular sap that surrounds the developing embryo decrease with the age of the ovule. Accordingly, isolated mature embryos grow fairly well with 2 % sucrose in the culture medium, but younger embryos require higher levels of the carbohydrate for normal embryogenic development. With the lower level of sucrose, proembryos directly develop into weak seedlings displaying only those structures already present at the time of embryo excision (see Sect. 11.7).

Changing growth requirements of developing embryos necessitates their transfer from one medium to another in order to achieve their optimal in vitro growth. Monnier (1976, 1978, 1990) described a culture method which allowed complete development of 50 µm long embryos (early globular stage) of *Capsella* into embryos that germinated in the same culture plate without moving the embryos from its original position (for details see Fig. 11.5). The compositions of the two media used in the culture dish are given in Table 11.4. Following a similar procedure, Fischer and Neuhause (1995) obtained normal direct embryogenesis in the cultures of globular embryos (100–160 µm) of wheat. Liu et al. (1993) used a similar technique for ex ovulo culture of 8-celled proembryos of *Brassica juncea*.

Table 11.4 Composition of the two media used in different parts of the same Petri dish to obtain uninterrupted growth of *Capsella* embryos from the globular stage (ca. 50 µm) to maturity (*after* Monnier 1976)

Constituents	Amounts (mg L^{-1})	
	Medium 1 (external ring)	Medium 2 (central ring)
KNO_3	1,900	1,900
$CaCl_2.2H_2O$	484	1,320
NH_4NO_3	990	825
$MgSO_4.7H_2O$	407	370
KCl	420	350
KH_2PO_4	187	170
Na_2.EDTA	37.3	–
$FeSO_4.7H_2O$	27.8	–
H_3BO_3	12.4	12.4
$MnSO_4.H_2O$	33.6	33.6
$ZnSO_4.7H_2O$	21	21
KI	1.66	1.66
$Na_2MoO_4.2H_2O$	0.5	0.5
$CuSO_4.5H_2O$	0.05	0.05
$CoCl_2.6H_2O$	0.05	0.05
Glutamine	–	600
Thiamine HCl	0.1	0.1
Pyridoxine HCl	0.1	0.1
Sucrose	–	18 %
Agar (%)	0.7	0.7

11.6 Role of Suspensor in Embryo Development

Suspensor is an ephemeral structure found at the radicular end of the embryo. It attains maximum size by the globular or early heart-shaped stage. Thereafter, it starts degenerating, and in mature embryos, only remnants of suspensor may be seen. Based on detailed structural, cytological, physiological and biochemical studies, an active involvement of suspensor in early embryo development has been suggested (Bhojwani and Bhatnagar 2008). Embryo culture provides direct experimental evidence to support the nutritive role of suspensor in embryo development. Continued in vitro growth of proembryos of *Eruca sativa* (Corsi 1972), *Phaseolus coccineus* (Yeung and Sussex 1979) and *Capsella bursa-pastoris*

(Monnier 1984, 1990) is enhanced by the presence of suspensor.

Cionini et al. (1976) observed that while the older embryos (5 mm or larger) of *Phaseolus coccineus* grew equally well when cultured with or without the suspensor, in the cultures of heart-shaped or early cotyledonary stage embryos the removal of suspensor drastically reduced the frequency of plant formation. Confirming the importance of suspensor for the growth of young embryos of *P. coccineus*, Yeung and Sussex (1979) showed that intact suspensor when detached but placed in the proximity of the embryo on the culture medium greatly stimulated further development of the embryo as compared to the embryo cultured in the absence of the suspensor (Table 11.5). The growth promoting activity of the suspensor was maximal at the early heart-shaped stage of the embryo. Of the various growth regulators tested, gibberellin at a concentration of 5 mg L^{-1} most effectively substituted the suspensor. This is in accordance with the observation of Alpi et al. (1975) that gibberellin activity in the suspensor of *P. coccineus* is maximal at the heart-shaped stage.

11.7 Precocious Germination

From the viewpoint of plant physiologists and biochemists, embryo development is a continuous process starting from zygote to germination. Walbot (1978) identified five stages in embryo development (Table 11.6). Initially, the growth of the embryo is essentially by cell divisions, resulting in the formation of a proembryo with small meristematic cells. This is followed by tissue differentiation, during which the meristematic activities become localized. After its full development, the embryo accumulates reserve food and gets dehydrated, leading to a phase of metabolic quiescence and developmental arrest (dormancy) during which the embryo is normally incapable of germination.

Embryos of mangroves and some other plants germinate while still attached to the parent plant (vivipary). Excised immature embryos of several other plants when cultured on nutrient medium not only bypass the stage of dormancy but also cease to undergo further embryogenic mode of development and develop into weak seedlings displaying only those structures already present at the time of embryo excision. This phenomenon of seedling formation without completing normal embryogenic development is called precocious germination. It can be prevented and normal embryogenic development promoted in the cultures of immature embryos by increasing the osmotic value of the culture medium having moderate level of sucrose (2 %) by raising the concentration of sucrose or adding a suitable concentration of mannitol.

It has been suggested that the beneficial influence of the high osmotic pressure may be mediated through its effect on endogenous pool of growth regulators. Raghavan and Torrey (1969) noted that for continued cell divisions and growth of the globular embryos (80 μm) of *Capsella bursa-pastoris* in vitro, a combination of IAA, kinetin and adenine sulphate added to the medium with only 2 % sucrose was better than high concentration of sucrose (12–18 %). Similarly, abscisic acid has been shown to replace high level of osmoticum in preventing precocious germination of excised immature embryos of cotton (Dure 1975).

11.8 Applications

Embryo culture is one of the few techniques that found an important application, while it was still in its infancy. Over the years, it has found many other applications in basic and applied areas of plant sciences.

11.8.1 Basic Studies

Embryo culture offers a useful system to study very fundamental problems of embryogenesis, which are normally difficult to address due to difficult approach to embryo under the in situ conditions. Embryo culture has unravelled many

Table 11.5 The effect of suspensor on in vitro growth and development of *Phaseolus coccineus* embryos (*after* Yeung and Sussex 1979)

Initial stage (fresh weight in mg) ±SE	Treatment	Fresh weight[a] 10 days after culture (mg) ±SE (N)	% embryos[c] forming plants (No. of embryos cultured)
Early heart-shaped (0.87 ± 0.02)	Embryo proper only	3.9 ± 0.52 (10)	41.5 (89)
	Embryo proper with suspensor attached	8.91 ± 1.16[b] (10)	88.4 (95)
	Embryo proper with detached suspensor in direct contact	6.22 ± 0.78[b] (10)	72.5 (51)
	Embryo proper with heat-killed detached suspensor in direct contact	4.10 ± 0.43 (5)	37.0 (43)
	Embryo proper with suspensor 1 cm away	–	33.3 (30)
Late heart-shaped (1.07 ± 0.07)	Embryo proper only	17.2 ± 2.84 (5)	94.4 (18)
	Embryo proper with suspensor attached	15.4 ± 1.41 (6)	94.4 (18)
Early cotyledon (3.92 ± 0.19)	Embryo proper only	20.3 ± 2.5 (7)	100 (18)
	Embryo proper with suspensor attached	24.4 ± 2.75 (11)	100 (19)

[a] Figures in parenthesis represent the sample size
[b] significant at 1 % level
[c] Assessed 8 weeks after culture

Table 11.6 Major stages in the development of *Phaseolus* embryos (*after* Walbot 1978)

Stage	Characteristics
1. Cleavage and differentiation	Cell division with little growth; differentiation of all major tissues
2. Growth	Rapid cell expansion and division
3. Maturation	Little or no cell division or expansion; synthesis and storage of reserve material
4. Dormancy	Developmental arrest
5. Germination	Renewed cell expansion and division; embryo growth

intricacies related to embryogenesis, such as the nutrition of embryo, the physical and chemical control of orderly development of embryo, role of various parts of the embryo in its development, maturation and induction of dormancy. Embryo culture has provided experimental support to the view that suspensor plays an important role in the nutrition of proembryos. Nutritional requirements of zygote and very young proembryo still remain a challenge.

In *Arabidopsis thaliana*, ex ovulo embryo culture has been possible only after the heart-shaped stage. However, embryos as young as the 4-celled stage could be cultured in ovulo (Sauer and Friml 2004, 2008). Embryo culture is also being used to study the physiology and genetics of embryogenesis (Fischer-Iglesias and Neuhaus 2001; Schrick and Laux 2001).

Embryo culture has been applied to study morphogenesis in the cultured seeds with partially differentiated embryos and those of parasitic plants. For several crop plants, including legumes, cereals, cotton and a number of tree species, highly regenerable callus and cell cultures could be established only from immature embryos. This enabled extending the application of biotechnological methods of crop improvement to these economically important species.

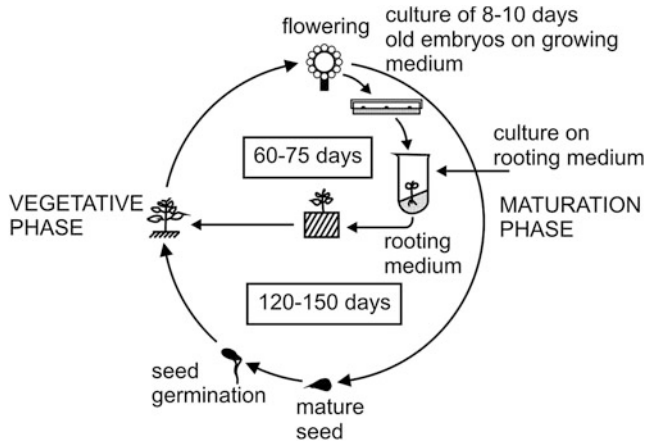

Fig. 11.6 Diagrammatized summary of immature embryo (8–10 DAP) culture to shorten the life cycle duration of sunflower from 120–150 days to 60–65 days (*after* Serieys 1992)

11.8.2 Shortening of Breeding Cycle

Breeding work on horticultural plants can be delayed due to long dormancy periods of their seeds. Excised embryo culture can circumvent the various causes of dormancy and thus reduce the period from seed to flowering. Randolph and Cox (1943) could reduce the life cycle of *Iris* from 2–3 years to less than a year by embryo culture. In cultures, the excised embryos of weeping crab apple *(Malus* sp.) start germination within 48 h, and in 4 weeks, transplantable seedlings are formed (Nickell 1951).

Seed maturation in soya bean and sunflower takes 50–60 % of the life cycle duration, which is 120–150 days (Serieys 1992). By in vitro culture of 10-day-old immature embryos of sunflower, Plotnikov (1983) could reduce the duration of the life cycle by half. Similarly, Alissa et al. (1986) and Aspiroz et al. (1988) could raise four generations of sunflower in a year by culturing 7-day-old and 10- to 18-day-old embryos, respectively (Fig. 11.6).

11.8.3 Rapid Seed Viability

The possibility of breaking seed dormancy by embryo culture also allows the use of this technique for rapid testing of the viability of a particular batch of seeds. A good correlation occurs between the growth of excised embryos of non-after-ripened seeds (freshly harvested dormant seeds) and germination of after-ripened seeds (which have completed the dormancy period) of peach (Tukey 1944). Germination of excised embryo is considered as more exact and reliable test than the commonly used staining methods for seed viability (Barton 1961).

11.8.4 Propagation of Rare Plants

As an abnormality, some coconuts develop soft, fatty tissue in place of liquid endosperm. Such nuts are called macapuno. Being rare, macapunos are expensive. Plants propagated from macapuno nuts are largely macapuno. Using the technique of excised embryo culture, De Guzman (1969) and De Guzman and Del Rosario (1974) raised plants from macapuno nuts, 85 % of which bore macapuno nuts (De Guzman et al. 1976)

11.8.5 Haploid Production

A unique application of embryo culture has been in the production of haploids through selective elimination of chromosomes, following distant

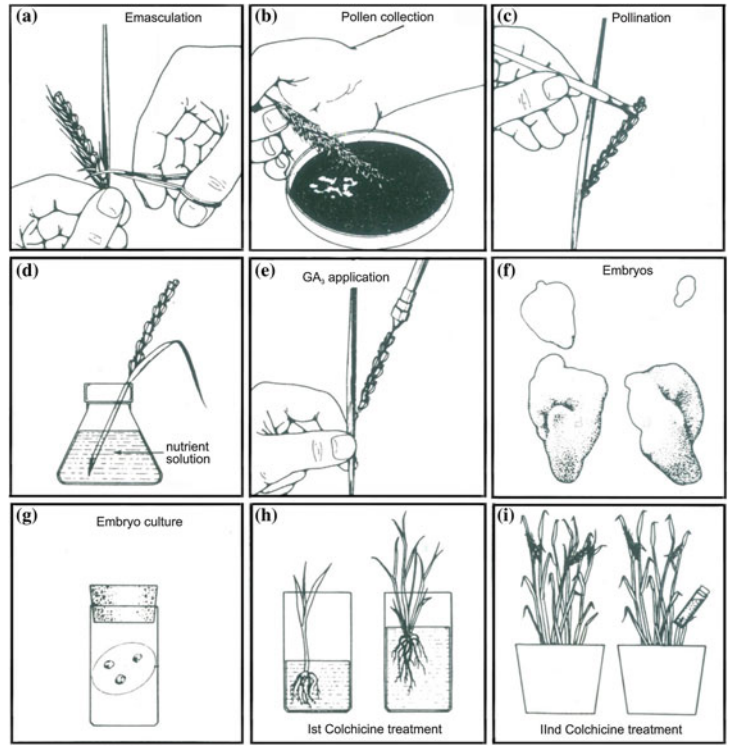

Fig. 11.7 General procedure for haploid production in barley following interspecific hybridization (*courtesy* N.C. Subrahmanyam, India)

hybridization. In the cross *Hordeum vulgare* × *H. bulbosum*, double fertilization proceeds readily, but the chromosomes of *H. bulbosum* are preferentially lost during the first few divisions of embryogenesis. As a result, haploid embryos, with single set of chromosomes of *H. vulgare*, are formed. However, the haploid embryos abort due to disintegration of endosperm 2–5 days after fertilization. In this cross, full haploid plants of barley can be raised by in vitro culture of excised immature embryo (Fig. 11.7). Wheat haploids are routinely produced by crossing it with maize, followed by immature embryo culture (Laurie and Reymondie 1991). Lei et al. (2004) reported development of haploid plants of *Cucumis sativus* by pollination with irradiated pollen, followed by embryo culture.

11.8.6 Transformation

Protoplasts of zygotes, capable of regenerating whole plants, are ideal targets for marker-free transformation by microinjection. This is particularly attractive for the agronomic crops because it avoids the toxic marker genes that have raised concern about the safety of the edible transgenic crops (Wang et al. 2006).

11.8.7 Production of Rare Hybrids

Embryo abortion is a common problem in breeding programmes. Distant crosses are often unsuccessful even after normal fertilization because the hybrid embryo aborts on the mother plant due to the failure of normal endosperm

development or embryo–ovular tissue incompatibility. In several such crosses, in vitro culture of excised embryo (ex ovulo) has been very successful in rescuing the embryo and raising full hybrid plants.

The first successful interspecific hybrid produced with the aid of embryo culture was in the genus *Linum* by Laibach (1925). In these crosses, the embryo was unable to grow to maturity in situ, but when it was removed from the seed and cultured on nutrient medium, it developed into a full plant. This work of Laibach laid the foundation of a method to surmount post-zygotic barriers to crossability where fertilization occurs normally. The technique has become a routine tool in the hands of plant breeders to enhance the scope of hybridization to raise new genotypes.

For instance, hybrids between *Triticum* and *Aegilops* were attempted to introduce resistance to leaf rust in wheat. However, pollination of wheat with *Aegilops* pollen yielded only very few and mostly abortive seeds. A large number of hybrid plants could, however, be raised by means of embryo culture (Chueca et al. 1977). Utilizing the same technique, Ben Rajeb and Bendalis (1989) could make a cross between *Phaseolus coccineus* and *P. acutifolium*, providing useful drought-resistant hybrids. Similarly, the interspecific cross *P. vulgaris* x *P. lunatus*, which normally fails due to abortion of embryo after the heart-shaped stage, could be successfully made with the aid of embryo culture (Kuboyama et al. 1991).

All attempts to cross *Lilium henryi* and *L. regale* were unsuccessful until the embryo culture technique was adopted (Skirm 1942). The seeds obtained on crossing *L. speciosum album* and *L. auratum* enclosed a large embryo and endosperm. However, during the storage of the seeds or soaking them in water, the embryo degenerated due to embryo–endosperm incompatibility. By growing excised immature embryos, Emsweller and Uhring (1962) could raise full hybrid plants from this cross.

The cross *Lycopersicon esculentum* × *L. peruvianum* is highly desirable with the view to transfer pest- and disease-resistant traits from *L. peruvianum* to *L. esculentum*. However, in this cross, fertilization occurs normally, but the embryo aborts, and no viable seeds are formed. Smith (1944), Chowdhury (1955) and Alexander (1956) obtained hybrid plants from this cross through embryo culture. Thomas and Pratt (1981) also raised hybrids from this cross but found that the poorly developed embryo excised 35 days after cross-pollination callused, followed by differentiation of plants. This embryo-callus approach has also yielded hybrids from the crosses *L. esculentum* × *L. chilense* and *L. esculentum* × *Solanum lycopersicoides*, in which embryos capable of direct plant formation do not develop (Scott and Stevens; cited in Thomas and Pratt 1981).

Where embryo abortion occurs at an early stage when it is either difficult to excise and/or culture the embryo in isolation, in ovulo embryo culture (ovule culture) has been used to rescue the hybrid embryo. In the cross *Gossypium arboreum* × *G. hirsutum*, the hybrid embryo develops only up to 8–10 days after pollination. Efforts to raise full plants by culturing excised embryo were unsuccessful (Beasley 1940; Weaver 1958). Pundir (1967) excised the ovules 3 days after cross-pollination, when they contained zygote or a 3-celled proembryo and cultured them on MS medium containing 50 mg L^{-1} inositol. By the 5th week, in culture, fully differentiated embryos were formed, and by the 7th week, 70–80 % ovules developed hybrid seedlings. Following a similar technique, Steward and Hsu (1978) raised four different interspecifc hybrids in the genus *Gossypium*, which were otherwise unknown.

The cross *Trifolium repens* × *T. hybridum* has not been successful by the conventional or by embryo rescue method. However, excising the ovules 12–14 days after pollination and culturing them on a medium containing 15 % cucumber juice for 5–6 days provide culturable embryos

(Przywara et al. 1989). Similarly, Espinasse et al. (1991) succeeded in obtaining some hybrid plants from the crosses *Helianthus annuus* × *H. maximiliani* and *H. annuus* × *H. tuberosum* with a combination of in ovulo embryo culture for 1 week, followed by excised embryo culture.

11.9 Concluding Remarks

Zygotic embryo culture is a well-established in vitro technique being routinely used for the production of rare hybrids that could not be raised by the conventional method of hybridization due to post-zygotic sexual incompatibility between the parents. This application of embryo culture was demonstrated as early as in 1925 when the technique of tissue culture in general and embryo culture in particular was not well developed. During 1960s and 1970s, considerable work on embryo culture was done to understand the nutritional and hormonal requirements of zygotic embryos of different developmental stages. It is now possible to rescue hybrid embryos which abort even at the globular stage of development, and the technique of hybrid embryo culture has become an integral part of the techniques of plant breeding.

It has been possible to successfully culture isolated in vivo and in vitro formed zygotes, but not without supplementing the medium with a suitable nurse tissue. The role of nurse tissue, however, remains to be understood.

Over the years, zygotic embryo culture has found many other applications, such as haploid production by distant hybridization, shortening of breeding cycle, propagation of rare plants, in vitro fertilization (Chap. 13) and understanding the physiology and developmental genetics of embryogenesis (Schrick and Laux 2001; Bhojwani and Bhatnagar 2008).

Suggested Further Reading

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