Chapter 11

Zygotic Embryo Culture

11.1. INTRODUCTION

Embryo culture is an in vitro technique that has been practised by plant breeders for over half a century now, and is described in most plant-breeding texts. The first systematic attempt to grow the embryos of angiosperms in vitro, under aseptic conditions, was made by Hannig (1904), who cultured mature embryos of two crucifers, *Cochleria* and *Raphanus*. Subsequently, many workers raised plants by culturing embryos excised from mature seeds. Dieterich (1924) pointed out that on a semi-solid medium containing Knop's mineral salts and 2.5–5% sucrose, the mature embryos grew normally but those excised from immature seeds failed to achieve the organization of a mature embryo. Instead, they grew directly into seedlings, skipping the stages of normal embryogenesis. Dieterich described this phenomenon of precocious germination of excised immature embryos as 'Kunstliche Fruhgeburt' (see Section 11.5).

A stimulus for further progress in the field of embryo culture was provided by Laibach (1925, 1929), who demonstrated the most important practical application of this technique. In the interspecific cross *Linum perenne* × *L. austriacum*, Laibach noted that the seeds were greatly shrivelled, very light, and incapable of germination. By excising embryos from such seeds and growing them on moist filter paper or on cotton wadding containing sucrose he was able to raise the hybrid plants. This led Laibach to suggest that in all those crosses where viable seeds are not formed it may be appropriate to excise their embryo and grow them in an artificial nutrient medium. Since then the technique of embryo culture has been widely used to produce hybrids which were otherwise not possible due to embryo abortion (see Section 11.10.1).

The possibility of growing embryos outside the environment of the ovule (ex-ovulo) provides an excellent opportunity to study the nutrition of the embryos at various stages of development (see Section 11.3). Using this technique the regeneration potential of whole embryos and their segments have also been investigated.

TABLE 11.1

Progressive embryogenesis in *Capsella bursa-pastoris*[a]

Developmental stage	Length of embryo (μm)	Nutritional requirements
Early globular	20–60	Unknown for embryos smaller than 40 μm
Late globular	61–80	Basal medium (macronutrient salts[c] + vitamins[d] + 2% sucrose) + IAA (0.1 mg l^{-1}) + kinetin (0.001 mg l^{-1}) + adenine sulphate (0.001 mg l^{-1})
Heart-shaped	81–450	Basal medium alone
Torpedo-shaped	451–700	Macronutrient salts[b] + vitamins[d] + 2% sucrose
Walking-stick-shaped and mature embryos	700 and larger	Macronutrient salts[b] + 2% sucrose

[a]After Raghavan (1966).
[b]Macronutrient salts (mg l^{-1}): 480 $Ca(NO_3)_2 \cdot 4H_2O$, 63 $MgSO_4 \cdot 7H_2O$, 63 KNO_3, 42 KCl, and 60 KH_2PO_4.
[c]Micronutrient salts (mg l^{-1}): 0.56 H_3BO_3, 0.36 $MnCl_2 \cdot 4H_2O$, 0.42 $ZnCl_2$, 0.27 $CuCl_2 \cdot 2H_2O$, 1.55 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 3.08 ferric tartrate.
[d]Vitamins (mg l^{-1}): 0.1 thiamine hydrochloride, 0.1 pyridoxine hydrochloride, 0.5 niacin.

11.2. TECHNIQUES

From technical considerations the two most important aspects of embryo culture are: (a) excision of the embryo and (b) composition of the culture medium. The choice of the plant material may become important when the objective is to introduce the technique to a beginner. The composition of the medium and the preparation of the explant for aseptic culture varies with the plant and the age of the embryo to be cultured. A detailed discussion on the constituents of various media is given in Section 11.3. For compositions of some successfully used embryo culture media, refer to Tables 11.1–11.5.

11.2.1. Plant material

The selection of plant to be used for embryo culture is normally dictated by the problem in hand. However, if a choice exists, as in introductory demonstrations, it would be advisable to start with a plant material whose embryos can be easily dissected out. Mature embryos of seed leg-

TABLE 11.2

Composition of the two media used in different parts of the same petri dish to obtain uninterrupted growth of *Capsella bursa-pastoris* embryos from the globular stage (ca. 50 μm) to maturity[a]

Constituents	Amount (mg l^{-1})	
	Medium 1 (external ring)	Medium 2 (central zone)
KNO_3	1900	1900
$CaCl_2 \cdot 2H_2O$	484	1320
NH_4NO_3	990	825
$MgSO_4 \cdot 7H_2O$	407	370
KCl	420	350
KH_2PO_4	187	170
Na_2·EDTA	37.3	–
$FeSO_4 \cdot 7H_2O$	27.8	–
H_3BO_3	12.4	12.4
$MnSO_4 \cdot H_2O$	33.6	33.6
$ZnSO_4 \cdot 7H_2O$	21	21
KI	1.66	1.66
$Na_2MoO_4 \cdot 2H_2O$	0.5	0.5
$CuSO_4 \cdot 5\ H_2O$	0.05	0.05
$CoCl_2 \cdot 6\ H_2O$	0.05	0.05
Glutamine	–	600
$B_1 = B_6$	0.1	0.1
Sucrose	–	180000
Agar (Difco)	7000	7000

[a]After Monnier (1976).

umes and crucifers, possessing large seeds, are good starting materials. Consideration must also be given to the possibility of obtaining a large number of genetically uniform embryos and at the same stage of development. Plants grown under controlled conditions would normally provide uniform material for each experiment. When embryos of specific stages of development are required it is best to select plants which flower and fruit regularly in order to ensure a sufficient supply of material needed. Shepherd's purse (*Capsella bursa-pastoris*) satisfies some of these requirements (Raghavan, 1967). In this plant the inflorescence is a raceme. In each raceme ovules at various stages of development are present. Generally the younger embryos are arranged at the top and the older ones are towards the base along the inflorescence axis. Each capsule contains about 20–25 ovules which are more or less at the same stage of development (Raghavan, 1967; Torrey, 1973).

TABLE 11.3

Improved nutrient medium for *Capsella* embryo culture[a]

Constituents	Amount (mg l^{-1})
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	880
NH_4NO_3	825
$MgSO_4 \cdot 7H_2O$	370
KCl	350
KH_2PO_4	170
Na_2·EDTA	14.9[b]
$FeSO_4 \cdot 7H_2O$	11.1[b]
H_3BO_3	12.4
$MnSO_4 \cdot H_2O$	33.6
$ZnSO_4 \cdot 7H_2O$	21
KI	1.66
$Na_2MoO_4 \cdot 2H_2O$	0.5
$CuSO_4 \cdot 5H_2O$	0.05
$CoCl_2 \cdot 6H_2O$	0.05
Glutamine	400
Vitamin B_1	0.1
Vitamin B_6	0.1
Sucrose	120000
Agar	7000

[a]After Monnier (1976, 1978).
[b]2 ml of a stock solution containing 5.57 g $FeSO_4 \cdot 7H_2O$ and 7.45 g Na_2·EDTA l^{-1}.

Artificial pollination of freshly opened flowers may be necessary when the embryos are to be cultured at specific stages of development. In such studies it would be helpful to first prepare a calendar showing the relationship between the stages of embryo development and days after pollination (DAP).

When the goal is to obtain plants from otherwise abortive seeds, the embryos should be excised for culture prior to the onset of abortion.

11.2.2. Sterilization

Zygotic embryos, being enclosed within the sterile environment of the ovular and ovarian tissues, do not require surface sterilization. Entire ovules are disinfected following the standard methods described in Chapter 2, and embryos are dissected out and transferred to the culture medium under strictly aseptic conditions. In orchids, where the seeds are minute and lack a functional endosperm, and the seed-coat is highly reduced, whole ovules are cultured (in ovulo embryo culture; see Section

TABLE 11.4

Composition of the medium used for the culture of globular embryos of *Brassica juncea* (after Liu et al., 1993a)[a]

Constituents	Amount (mg l^{-1})	Constituents	Amount (mg l^{-1})
Macroelements			
NH_4NO_3	200	$KH_2PO_4 \cdot 2H_2O$	100
KNO_3	1500	$Na_2EDTA \cdot 2H_2O$	37
$CaCl_2 \cdot 5H_2O$	850	$FeSO_4 \cdot 7H_2O$	28
$MgSO_4 \cdot 7H_2O$	400		
Microelements			
KI	0.75	$Na_2MoO_4 \cdot 2H_2O$	0.25
H_3BO_3	3	$CuSO_4 \cdot 5H_2O$	0.025
$MnSO_4.H_2O$	10	$CoCl_2 \cdot 6H_2O$	0.025
$ZnSO_4.7H_2O$	2		
Sugar			
Sucrose	4000	Mannose	100
Glucose	2000	Rhamnose	100
Fructose	100	Cellobiose	100
Ribose	100	Sorbitol	100
Xylose	100	Mannitol	100
Organic acids[b]			
Sodium pyruvate	20	Malic acid	40
Citric acid	40	Fumaric acid	40
Vitamins and amino acids			
Inositol	500	Nicotinic acid	0.1
Glutamine	200	Pyridoxine·HCl	0.1
Thiamine·HCl	1	D-Biotin	0.01
Other supplements			
Coconut water[c]	300 (ml l^{-1})	Casein hydrolysate	100
Agarose (low gelling temperature Sea Plaque)		6000	

[a]Medium was filter sterilized.
[b]pH adjusted to 5.5 with NH_4OH.
[c]Obtained from green coconuts.

10.3.1). In such cases whole fruits can be surface-sterilized, and the seeds excised under aseptic conditions and spread as a monolayer on the surface of the agar medium using a sterilized needle.

TABLE 11.5

Composition of media used in embryo culture of barley[a]

Constituents	Amount (mg l^{-1})			
	B–II[b]	C–17[c]	C–21[d]	C–45[e]
Macronutrients				
KNO_3	–	300	300	900
$CaCl_2 \cdot 2H_2O$	740	250	–	400
$MgSO_4 \cdot 7H_2O$	740	325	300	300
$(NH_4)_2SO_4$	–	–	–	60
$NaH_2PO_4 \cdot H_2O$	–	100	–	75
KCl	750	150	300	–
KH_2PO_4	910	150	500	170
$Ca(NO_3)_2$	–	–	500	300
NH_4NO_3	–	200	–	500
Micronutrients				
KI	–	0.10	–	–
H_3BO_3	0.5	0.5	15.0	1.0
$MnSO_4 \cdot 4H_2O$	3.0	0.5	–	5.0
$ZnSO_4 \cdot 7H_2O$	0.5	0.25	–	5.0
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.012	–	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.012	–	0.012
$CoCl_2 \cdot 6H_2O$	0.025	0.012	–	0.012
Ferric citrate	10	3	20	20
Fe-EDTA	–	17.5	10	28
Vitamins				
Nicotinamide	–	–	–	1.0
Thiamine·HCl	0.25	0.25	10	10
Pyridoxine·HCl	0.25	0.25	–	10
Inositol	50	50	150	100
Ca-pantothenate	0.25	0.25	–	–
Glycine	–	0.75	–	–
Ascorbic acid	–	0.5	–	1.0
Amino acids				
Glutamine	400	–	–	600
Glutamic acid	–	150	300	–
Alanine	50	30	–	100
Cysteine	20	–	–	–
Arginine	10	20	50	–
Leucine	10	10	–	–
Phenylalanine	10	20	–	–
Tyrosine	10	–	–	–
Aspartic acid	–	30	100	100
Proline	–	50	50	–

TABLE 11.5 (*continued*)

Constituents	Amount (mg l^{-1})			
	B–II[b]	C–17[c]	C–21[d]	C–45[e]
Valine	–	10	–	–
Serine	–	25	25	50
Threonine	–	10	–	100
Lysine	–	10	–	–
Sucrose	34000	60000	45000	45000
Agar (Difco)	6000	–	–	–
pH	5	5.5	5.5	5.8

[a]In addition to the constituents mentioned in the table, the four media contain the following additives per litre of medium: B-II, 1 g malic acid dissolved in 50 ml of water and pH set to 5 with NH_4OH; C-17, 500 mg citric acid dissolved in 50 ml of water and pH adjusted to 5.3 with NH_4OH; 300 mg Tris-potassium citrate added to final medium and pH of medium adjusted to 5.5 with KOH (filter sterilized); C-21, 50 mg citric acid dissolved in 50 ml of water, pH adjusted to 5 with NH_4OH, added to medium and final pH brought to 5.5 with KOH (filter-sterilized); 250 mg Tris-potassium citrate added to the medium and pH of medium adjusted to 5.5; C-45, 300 mg malic acid dissolved in 50 ml of water containing 300 mg citric acid and pH brought up to 5 with NH_4OH.
[b]After Norstog (1973).
[c]Used by Jensen (1976, see Jensen, 1977) for small and non-uniform monoploid embryos.
[d]Used by Jensen (1976, see Jensen, 1977) for uniform, well-developed embryos.
[e]Used by Jensen (1976, see Yeung et al., 1981) for 1–2-week-old embryos.

11.2.3. Excision of embryo

For the in vitro culture of embryos, generally it is necessary to free them from their surrounding tissues. The mature embryos can be isolated with relative ease by splitting open the seed. Seeds with a hard seed-coat are dissected after soaking them in water. Smaller embryos require careful dissection with the aid of a stereoscopic microscope and quick transfer to the culture vial. Liu et al. (1993a) found it necessary to carry out dissections of globular embryos of *Brassica juncea* in a solution of 9% glucose to reduce osmotic shock to the proembryos. These scientists have recommended that during the dissection of very young embryos, care should be taken not to touch the embryo proper with forceps or needles. They used Gilson micropipettes to pick and transfer globular embryos from dissection plate to culture medium.

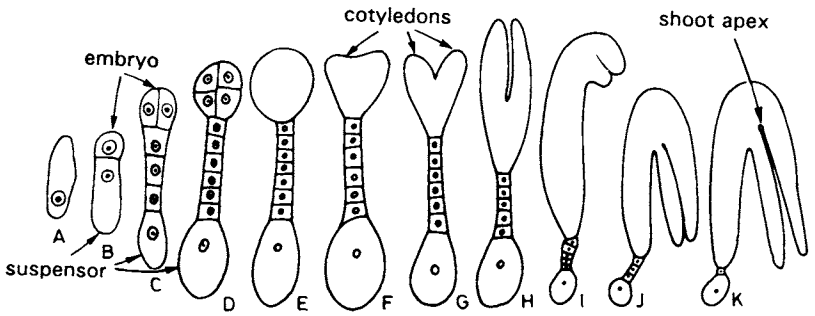

Fig. 11.1. Stages in the normal development of embryo in *Capsella bursa-pastoris*. (A) Zygote; (B) two-celled proembryo; (C–E) globular; (F) heart-shaped; (G) intermediate stage; (H) torpedo-shaped; (I) walking-stick-shaped; (J) inverted U-shaped; (K) mature (after Raghavan, 1966).

Raghavan and Torrey (1963) adopted the following procedure to isolate embryos at varying stages of development (see Fig. 11.1) from the ovules of *Capsella bursa-pastoris*. Sterile capsules (Fig. 11.2A) were kept in a few drops of the sterile culture medium. Outer walls were removed by an incision in the region of the placenta and the two halves pulled apart with forceps to expose the ovules (Fig. 11.2B). Torpedo-shaped and younger embryos are confined to one longitudinal half of the ovule (Fig. 11.2D) and are clearly visible through the chalaza either because of their green colour (intermediate and torpedo-shaped) or because of the transparent vesicle of their suspensor. To excise these immature embryos a single ovule, removed from the placenta, was placed in the depression of a new slide with a drop of medium. With the help of a sharp mounted blade the ovule was split longitudinally (Fig. 11.2D) to isolate the half containing the embryo. By carefully teasing apart the ovular tissues the entire embryo, along with the attached suspensor, could be removed. For excising older embryos a small incision was made in the ovule on the side lacking the embryo (Fig. 11.2C) and a slight pressure applied with a blunt needle. This was enough to release the intact embryo into the surrounding fluid. The whole operation, especially when dealing with immature embryos, must be performed with utmost care so that there is no apparent injury to the embryo.

In monocots, a well-studied plant for embryo culture is barley (*Hordeum vulgare*). Norstog (1965b) has described the following procedure for the isolation of immature barley embryos. The caryopsis were placed on a sterilized slide under a dissecting microscope at 20–50× magnification. Using watchmaker's forceps or fine-tipped needles embryos as small as 0.2 mm could be readily excised and transferred to

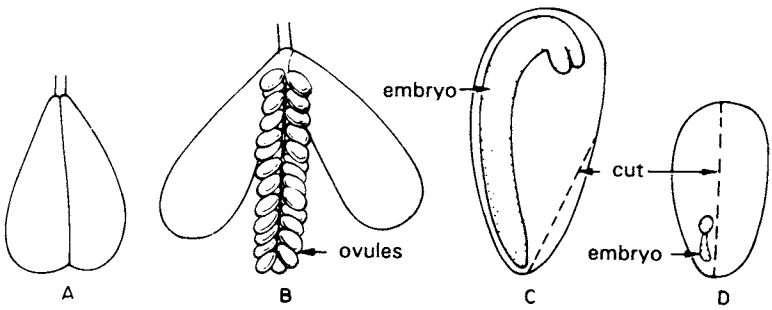

Fig. 11.2. Isolation of the embryos of *Capsella bursa-pastoris*. (A) A capsule; (B) the capsule has been opened to expose the ovules; (C) an ovule with walking-stick-shaped embryo inside; the dotted lines show the region of incision of the ovule to release the embryo; (D) an ovule with globular embryo. A cut along the dotted lines exposes the embryo (after Raghavan, 1966).

the culture medium. However, for smaller embryos a modified procedure was required because they tend to dry out during dissection and planting on the medium. In barley, the region of the ovule that contains the embryo is beak-like. The beak was excised and transferred to a drop of sterile paraffin oil. Tissues of the beak were carefully teased apart to release the embryo. The isolated embryo was lifted out in the oil film using a microspatula and transferred to the nutrient medium. The oil appeared to float free and it was possible to push the embryo to an oil-free area on the medium. The oil did not seem to interfere with the embryonic growth.

11.2.4. Embryo-nurse endosperm transplant

As a rule very young embryos are difficult to culture on artificial culture medium. Despite considerable progress in the improvement of embryo culture media, one is still often confronted with the problem of rescuing the hybrid embryos where abortion occurs at a very early stage of development (Williams and De Lautour, 1980). Ziebur and Brink (1951) had shown that in vitro growth of excised immature embryos (300–1100 μm long) of *Hordeum* was considerably promoted by surrounding it, on the culture medium, with excised endosperm from another seed of the same species. Kruse (1974) reported that implanting immature embryos on cultured barley endosperm showed significant improvement in the frequency of hybrid plant development in certain intergeneric crosses. For example, in the cross *Hordeum* × *Secale* the survival rate with the implantation technique was 30–40% compared to 1% with the traditional

method of embryo culture. Ziebur and Brink (1951) and Kruse (1974) have emphasized that endosperm older than the embryo by 5 days was more efficient as nurse tissue than the endosperm of the same age as the embryo.

A modified endosperm transplant technique for young embryo culture has been described by De Lautour et al. (1978), Williams (1978, 1980) and Williams and De Lautour (1980). They inserted an excised hybrid embryo into a cellular endosperm dissected out from a normally developing ovule of one of the parents or a third species and cultured the nurse endosperm with the transplanted embryo on an artificial medium. Using this technique these workers have produced many interspecific hybrids in the genus *Trifolium* which could not be reared by growing embryos directly on the medium. The procedure described by Williams and De Lautour (1980) is as follows. Transplant manipulation was carried out in a laminar air-flow cabinet with the aid of a stereoscopic microscope. A heat-absorbing glass filter was placed in front of the light source in order to protect the embryo from desiccation. Surface-sterilized pods carrying hybrid embryos or normally developing embryos (for nurse endosperm) were placed in sterile petri-dishes lined with moist filter paper until dissection. Two ovules, one carrying a hybrid embryo and the other with a normal embryo were removed from their pods and placed on a small (2 × 2 mm) pre-sterilized 'carrier' square of moist filter paper on the sterilized microscope stage. For the following text refer to Fig. 11.3. After making a shallow cut (c_1) across the back of each ovule, opposite the funiculus, the ovule wall was lifted back as a flap (f_1). The ovule was held steady with a needle inserted into the bar of the structural tissues (*) near the funiculus. A second shallow cut (c_2) towards the hilum allowed the ovule wall to be peeled back as two flaps (f_2) and expose the central embryo sac region. If the hybrid embryo was small a third shallow cut (c_3) was made around the top of the ovule so that the ovule wall could be peeled back further for the removal of the embryo. In the normal ovule, containing nurse endosperm, gentle left-to-right pressure with the aid of a needle along the line p-p detached the embryo suspensor from the maternal tissues and forced the embryo and the surrounding endosperm out on the filter paper through the previously cut holes (c_1 and c_2). However, to remove the hybrid embryo (without endosperm) its suspensor was gently detached from the ovule directly with the point of a needle. At this stage the normal embryo was removed from inside the endosperm (for use as a nurse tissue) and the hybrid embryo inserted in its place through the exit hole (see Fig. 11.4). The nurse endosperm enclosing the hybrid embryo was then transferred to the surface of the artificial medium.

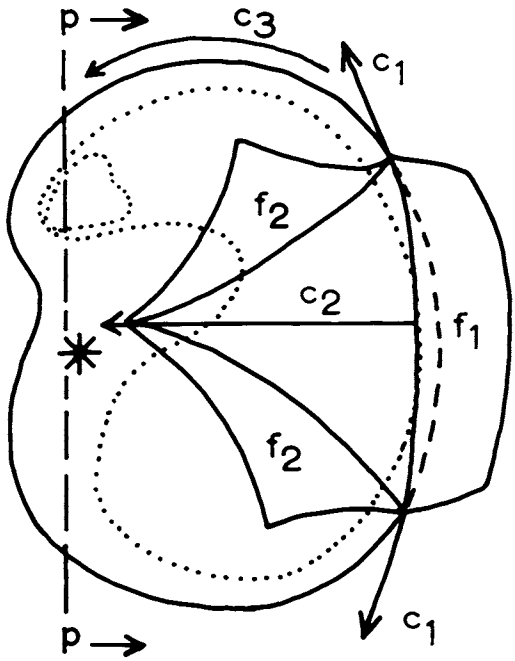

Fig. 11.3. Dissection procedure for 1.0–1.5 mm long ovules of *Trifolium, Lotus* or *Ornithopus*. c_1, c_2 and c_3 represent portions of sequential needle cuts, f_1 and f_2 are the flaps of the ovule wall peeled back after cutting to expose embryo sac contents. p– – –p is the line along which pressure (left to right) is applied with the side of a needle to expel the embryo and endosperm from the embryo sac. *Marks the position where a needle can be inserted into structural tissues to hold the ovule steady without damage to the embryo sac (after Williams and De Lautour, 1980).

11.3. CULTURE REQUIREMENTS

The most important aspect of embryo culture is the selection of the right culture medium that would support progressive and orderly development of embryos excised at different stages of development. Early reports of embryo culture were generally concerned with the development of plants from mature embryos (post-germinal development) on a simple medium. Hannig (1904) used a mineral salts–sucrose solution to culture mature embryos (2 mm long) of crucifers. Laibach (1925) reared full plants from excised hybrid embryos (1 mm long) using only 15% glucose solution. In contrast, immature embryos generally fail to grow on such a simple medium. Their nutritional requirements are more elaborate than those of the mature embryos. The persisting interest of workers to elucidate problems such as the control of embryogenesis, and the desirability of obtaining hybrids from non-viable crosses led to extensive investiga-

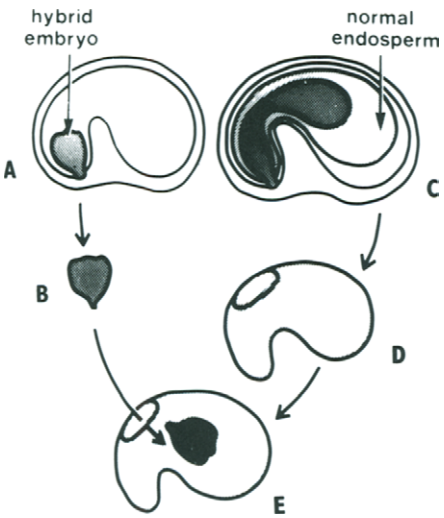

Fig. 11.4. Endosperm transplant for the culture of hybrid embryos in *Trifolium*, *Lotus* or *Ornithopus*. (A,B) The hybrid embryo is removed from the ovule in which endosperm development has failed. (C) To provide transplant endosperm a normally developing intraspecifically pollinated ovule is dissected at a stage when it contains cellular endosperm enclosing a heart-shaped to torpedo-shaped embryo. (D) The normal embryo is pressed out of the sac of endosperm leaving an exit hole. (E) The hybrid embryo is inserted into the normal endosperm through the exit hole (after Williams and De Lautour, 1980).

tions on the culture requirements of immature embryos (pre-germinal development). Continual efforts have been made to improve the culture media to rear younger embryos and duplicate, as far as possible, the in ovulo pattern of embryogenesis in the excised embryos. The two angiosperm systems studied in greatest details for embryo culture are *Capsella* and *Hordeum*. Appreciable work has also been done with *Citurs*, *Datura* and *Phaseolus*.

With respect to its nutrition, two phases of embryo development have been recognized by Raghavan (1966): (a) the heterotrophic phase: during this early phase the embryo is dependent and draws upon the endosperm and the surrounding maternal tissues; and (b) the autotrophic phase: during this the embryo is metabolically capable of synthesizing substances required for its growth from the basic mineral salts and sugar and is, thus, fairly independent for its nutrition. The critical stage at which the embryo comes out of the heterotrophic phase and enters the autotrophic phase, however, varies with the species (Raghavan, 1976a). In *Capsella bursa-pastoris* the embryos are definitely heterotrophic until the globular stage, and only in the late heart-shaped stage they turn autotrophic (Raghavan, 1976a). Even within the two phases the exoge-

nous requirements of the cultured embryos become progressively simpler with the age of the embryo. This is clearly brought out by the observations of Van Overbeek et al. (1941, 1942) on *Datura* and those of Raghavan and Torrey (1963, 1964; see also Raghavan, 1966) on *Capsella* (see Table 11.1).

Changing growth requirements of the embryos growing in ovulo is also observed in the cultures of immature embryos. This necessitates the transfer of embryos from one medium to another in order to achieve their optimal growth. Monnier (1976, 1978) described a new culture method which allows complete development of 50 μm long embryos (early globular stage) of *Capsella* up to germination in the same culture plate (Table 11.2), without moving the embryo from its original position (for details of this method see Fig. 11.5). The composition of the two media used in the culture dish is given in Table 11.2. It may be noted that the media used in this method are different from Monnier's medium for traditional embryo culture, described in Table 11.3. Similarly, a double-layer culture system, involving two semi-solid media (Table 11.4), differing only in their osmolarity, was used by Liu et al. (1993a) for successful culture of *Brassica juncea* proembryos as small as 35 μm in size (8.36 cells). The proembryos were planted on the bottom-layer medium (O.P. 0.45 mol l^{-1}) and overlaid with molten top-layer medium (O.P. 0.63 mol l^{-1}) held at 38°C. The proembryos developed into normal mature embryos with 65–95% efficiency.

Refinement of nutrient medium for the culture of heterotrophic embryos has involved, as for any other type of plant tissue cultures, considerations of mineral salts, organic nutrients, and growth regulators. In addition, osmotic pressure of the medium is a critical factor for successful culture of proembryos. Finally, there is an intimate relationship between embryo culture and the use of the plant extracts in culture media.

11.3.1. Mineral salts

Many different formulations of mineral salts have been used for embryo culture without much critical evaluation of the role of individual elements. In his studies on the effects of various standard inorganic solutions (Knop's, Heller's, Murashige and Skoog's) on cultured embryos of *Capsella*, Monnier (1976) observed that there was no correlation between the growth and survival of the immature embryos on a particular medium (see Fig. 11.6). In Murashige and Skoog's (MS) medium which supported maximum growth of embryos the survival frequency of the embryos was very low, whereas in Knop's medium, which was least toxic, the growth of the embryos was very poor. This prompted Monnier to for-

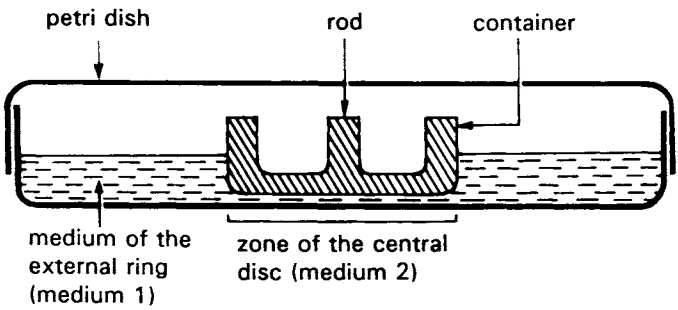

Fig. 11.5. Device allowing the juxtaposition of two media with different compositions (see Table 11.2). The first agar medium is liquified by heating and then poured around the central glass container. This medium gives the external ring. After cooling and solidification of the medium the container is 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).

mulate a new mineral solution that would favour both good growth and high survival. He altered the concentration of every salt in the MS medium to study the effect of such variations, and on the basis of these experiments developed a medium on which the growth of embryos was as good as on MS but survival was high (see Fig. 11.6). The composition of the new medium is given in Table 11.3. Compared with the inorganic composition of MS medium, Monniers medium has high concentrations of K^+ and Ca^{2+}, and a reduced level of NH_4^+ ions.

Umbeck and Norstog (1979) reported that NH_4^+ in the medium was essential for proper growth and differentiation of immature barley embryos. With NO_3^- as the sole source of inorganic nitrogen, 500 μm long barley embryos (at culture) showed very little growth, that was largely due to cell elongation. For the differentiation of scutellum, 'ligule' and 'sheath' 4.3–8.6 mM NH^+_4 was required. In *Datura stramonium* (Paris et al., 1953) and *D. tatula* (Matsubara, 1964) NH_4^+ was either essential or a preferred source of inorganic nitrogen, especially for immature embryos. Embryos of jute (*Corchorus capsularis*; Mitra and Datta, 1951a,b), on the other hand, showed an absolute requirement for nitrate nitrogen.

11.3.2. Carbohydrate and osmotic pressure of the culture medium

A suitable source of carbon energy is generally required for the cultivation of excised mature and immature embryos. Sucrose is by far the

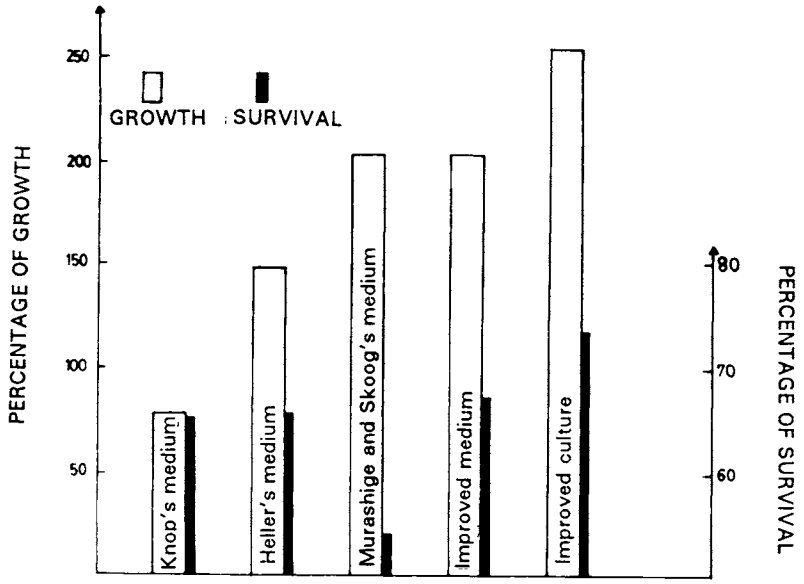

Fig. 11.6. Growth and survival of immature *Capsella* embryos in various culture media. Composition of the 'improved medium' is given in the Table 11.3. For 'improved culture' Monnier's medium (1976, 1978) (see Table 11.3) was used, and it involved agar-coating of the embryos and tyndallization of the medium (after Monnier, 1976, 1978).

best form of carbohydrate and has been most commonly used for embryo culture (Van Overbeek et al., 1944; Lofland, 1950; Rijven, 1952; Mauney, 1961; Matsubara and Nakahira, 1965; Burghardtova and Tupy, 1980).

Sucrose is added to the embryo culture media not only as a source of energy but also to maintain a suitable osmolarity which is extremely important for immature embryos (Liu et al., 1993a). For this latter function the optimum concentration of sucrose varies with the stage of embryo development. Mature embryos grow fairly well with 2% sucrose but younger embryos require higher levels of the carbohydrate. This is in harmony with the observations that in situ the proembryos are surrounded by a fluid of high osmolarity (Ryczkowski, 1960; Mauney, 1961; Smith, 1973). Eight to twelve percent sucrose is generally adequate for the culture of proembryos (*Datura*; Rietsema et al., 1953; *Hordeum*; Ziebur and Brink, 1951; Norstog, 1961; *Capsella*; Rijven, 1952; Veen, 1963; Monnier, 1978). In long-term experiments, with the age of the cultures the embryos need to be transferred to media with progressively lower levels of sucrose, or a culture system as that of Monnier (1976) or Liu et al. (1993a), as described in Section 11.3, should be used.

A high concentration (60 mmol l^{-1}) of NH_4NO_3 has been reported to promote the growth of immature embryos (200–300 μm) of *Datura tatula* by increasing the osmotic value of the medium (Matsubara, 1964).

Raghavan and Torrey (1964) also noted a stimulatory effect of high concentrations (12–18%) of sucrose on in vitro development of excised globular embryos (smaller than 80 μm) of *Capsella*. However, a combination of IAA (0.1 mg l^{-1}), kinetin (0.001 mg l^{-1}) and adenine sulphate (0.001 mg l^{-1}) added to the basal medium with only 2% sucrose provided the best medium for continued division and growth of the proembryos. Based on these observations, Raghavan and Torrey suggested that in vitro growth and differentiation of excised embryos is not so much dependent on the osmotic value as on the availability of specific growth factors. The effect of high osmoticum in preventing precocious germination of excised immature embryos is effectively reproduced by ABA (Crouch and Sussex, 1981). Whether the beneficial influence of the high osmotic pressure is mediated through its effect on the endogenous pool of growth regulators remains to be demonstrated.

11.3.3. Amino acids and vitamins

The addition of amino acids, singly or in combination, to the culture medium may stimulate embryo growth. Hannig (1904) reported that asparagine was very effective in enhancing embryo growth. Generally, however, glutamine has proved to be the most effective amino acid for the growth of excised embryos (Paris et al., 1953; Rijven, 1955; Matsubara, 1964; Monnier, 1978). Rijven (1955) reported that glutamine promoted the growth of embryos from nine different families of flowering plants, and the promotion was considerably more than that by asparagine. For some of these species (*Capsella, Arabidopsis, Reseda*) asparagine was even inhibitory. Matsubara (1964) tested 18 amino acids and two amides for the culture of young *Datura tatula* embryos and observed that all except glutamine were inhibitory.

Casein hydrolysate (CH), an amino acid complex, has been widely used as an additive to the embryo culture media. Sanders and Burkholder (1948) tried it for the culture of pre-heart-shaped (100–250 μm long) embryos of two species of *Datura*. When added to the basal medium (containing mineral salts, vitamins, and sucrose) with cysteine and tryptophan, CH increased embryogenic differentiation and the size of the embryo. In an attempt to identify the growth-promoting factor(s) in CH, Sanders and Burkholder tested a mixture of 20 amino acids in the proportion in which they occur in CH. In addition, 10 of these amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, lysine,

phenylalanine, proline, tyrosine), which had been earlier found to be beneficial for plant growth, were also tested individually and collectively. The mixture of 20 amino acids proved as effective as CH for embryo growth and development, suggesting that the promotive effect of CH for embryo growth was due to amino acids. The mixture of 10 amino acids promoted embryo growth similar to CH in *D. innoxia* but not in *D. stramonium*. Individually none of the amino acids matched either CH or the mixture of 20 amino acids in its effect. These observations suggest a synergistic interaction between amino acids. CH was shown by Kent and Brink (1947) to inhibit precocious germination and promote pre-germinal embryo development in *Hordeum vulgare*.

Rangaswamy (1961) reported that CH was essential for the culture of very young globular embryos (14–28 × 14–28 μm) of *Citrus microcarpa*. On a modified White's medium these proembryos collapsed within 3 days of culture. Increasing the level of sucrose up to 10% did not support normal embryogenic development. However, when White's medium was supplemented with 400 mg l^{-1} CH, the proembryos passed through all pre-germinal stages of embryo development, and in 3–4 weeks fully organized embryos were formed in 80% of the cultures.

The sensitivity of embryos to concentrations of CH may vary with the species. The optimum level of CH for *Hordeum vulgare* was 500 mg l^{-1} (Kent and Brink, 1947). The embryos of *Datura innoxia* and *D. stramonium* grew best with 400 mg l^{-1} CH (Sanders and Burkholder, 1948). The growth of embryos of *D. tatula* was promoted by CH at 50 mg l^{-1} but at 100 mg l^{-1} it proved toxic. Ziebur et al. (1950) concluded that the effect of high levels of CH on excised embryos of barley was largely due to its osmotic effect which could be reproduced by a high concentration of sucrose.

Vitamins have been used in embryo culture media but their presence is not always essential. In some cases a vitamin may even inhibit normal morphogenesis (Raghavan, 1980). It is pertinent, therefore, that vitamins are included in the media only after their desirability has been established through empirical experimentation for the system in question.

11.3.4. Natural plant extracts

From his experience of culturing embryos of several plant species LaRue (1936) remarked '...embryos 0.5 mm or slightly less in length can be grown successfully ... those of still smaller size have not yet been brought under control ... at a length of 0.5 mm we may have reached a new lower threshold which will be difficult to cross'. Obviously, the relationship between the size of the embryo and its stage of development would vary with the species. For large-seeded plants 500-μm long em-

bryos would be fairly young whereas for small-seeded plants it would represent a comparatively older stage. However, the main point of LaRue's statement is that at that time it was difficult to culture younger embryos. Van Overbeek et al. (1942) observed that post-torpedo-shaped embryos of *Datura* formed normal seedlings in a medium containing 1% dextrose, 1% agar, mineral salts, glycine, thiamine, ascorbic acid, nicotinic acid, vitamin B_6, adenine, succinic acid and pantothenic acid. However, heart-shaped and younger embryos failed to develop on this medium. Since immature embryos in the ovule are surrounded by endosperm, Van Overbeek et al. (1942) added non-autoclaved coconut milk (CM; liquid endosperm of coconut) to the above medium in an attempt to culture younger embryos. On this modified medium normal seedlings developed from embryos as small as 150–200 μm long (heart-shaped) and as young as 10 DAP. The growth-promoting factor in the coconut milk was referred to as 'embryo factor' (Van Overbeek et al., 1942), and this discovery proved a turning point in the history of embryo culture; it enabled the successful culture of young embryos of a number of species (Warmke et al., 1946; Norstog, 1956b, 1961; Matsubara, 1962). Coconut milk was indispensable for the culture of 35–80 μm proembryos of *Brassica juncea* (Liu et al., 1993a).

In order to find a suitable substitute for CM for the culture of pre-heart-shaped embryos of *D. tatula* (<150 μm long), Matsubara (1962) tested a range of natural substances including CH, dried brewer's yeast, skimmed milk, and diffusate from the endosperm of *Ginkgo* and the seeds of several angiosperms. The alcohol diffusates from young seeds of *Lupinus* and mature seeds of *Sechium* proved as effective as CM.

Kent and Brink (1947) reported promotion of the embryogenic growth and inhibition of precocious germination of immature barley embryos by extracts of dates and bananas, wheat glutein hydrolysate, milk and tomato juice (TJ). Of these TJ was most effective. On the basal medium containing 22–66% TJ, 7–9-day-old embryos underwent normal embryogenic development. Without TJ these embryos grew irregularly.

With an urge to develop purely synthetic media for the culture of immature embryos attempts have been made to substitute the 'embryo factor' of coconut milk by chemically defined substances. For the growth of barley proembryos, the requirement for CM could be replaced by phosphate-enriched White's medium fortified with glutamine and alanine as the major amino acids and five other amino acids as the minor sources of N_2, at pH 4.5. The survival rate of the embryos was considerably increased when the concentration of KCl, KNO_3 and certain organic components was increased 5–10 times (Norstog, 1967, 1973). This modified Norstog's medium was found to be the best of the various recipes tested

by Cameron-Mills and Duffus (1977) for the culture of 250-μm long embryos of five varieties of *Hordeum distichum*.

11.3.5. Growth regulators

For the callusing of embryos and embryo segments generally an auxin or cytokinin, or both, are required. However, there is not enough evidence to suggest that exogenous growth regulators are necessary for the normal development of excised mature or immature embryos apart from breaking dormancy in some plants. In fact, auxin is generally inhibitory for embryo growth (Raghavan, 1980). Monnier (1978) suggested that hormones should not be added to the embryo culture media because they bring about structural abnormalities. He felt that embryos are autonomous for most of the growth regulators. This has been clearly shown at least for GA_3 in *Phaseolus*, where the suspensor fulfils the requirement of excised embryos for this hormone (see Section 11.4).

Raghavan and Torrey (1964) reported that for cultivating globular embryos of *Capsella* on a mineral salts–vitamin medium with only 2% sucrose, it was necessary to supplement it with IAA, kinetin, and adenine sulphate. Precocious germination of cultured immature barley embryos (500 μm long), which is promoted by GA_3 and kinetin, could be suppressed, and orderly embryogenic development induced by the application of ABA, especially in combination with NH_4^+ ions (Norstog, 1972; Umbeck and Norstog, 1979). The role of ABA in supporting morphologically and biosynthetically normal embryogenesis in cultured embryos is dealt with in Section 11.5.

11.3.6. pH of medium

The pH of the ovular sap of *Capsella* is about 6.0, and its excised embryos grow equally well in the medium with pH 5.4–7.5 (Rijven, 1952). The optimal pH for early heart-shaped embryos of *Datura tatula* ranged from 5.0 to 7.5 (Matsubara, 1962). Rice embryos (8-day-old) showed best growth at two pH values, viz. 5 and 9 (Sapre, 1963). Norstog and Smith (1963) have reported that for organogenic differentiation of immature barley embryos the pH of the medium was critical. The optimum value was about 4.9. At a pH above 5.2 embryo growth occurred without any appreciable differentiation.

11.3.7. Culture storage

Embryos of most plants grow well at temperatures between 25 and

30°C (Narayanaswamy and Norstog, 1964). The optimum temperature of *Datura tatula* is reported to be 35°C (Matsubara and Nakahira, 1965). According to Narayanaswamy and Norstog light is not critical for embryo growth. Matsubara and Nakahira also concluded that the growth of young *Datura* embryos was not influenced by light. However, in barley light suppresses precocious germination of immature embryos (Norstog, 1972).

11.4. ROLE OF THE SUSPENSOR IN EMBRYO CULTURE

The suspensor is an ephemeral structure found at the radicular end of the proembryo; it usually attains its maximum development by the time the embryo reaches the globular stage (see Fig. 11.1). Detailed structural (Yeung and Clutter, 1979), cytological (Nagl, 1962; Avanzi et al., 1970), physiological (to be discussed below), and biochemical studies (Clutter et al., 1977) suggest an active involvement of the suspensor in the development of the young embryos (see also Yeung and Meinke, 1993).

Generally, embryos have been cultured without an intact suspensor because the small size and delicate structure of the suspensor make it difficult to excise it along with the embryo proper. Some painstaking studies have, however, shown that, in cultures, the presence of a suspensor is critical for the survival of young embryos (Corsi, 1972; Cionini et al., 1976; Yeung and Sussex, 1979). Cionini et al. (1976) observed that the older embryos (500 μm or over in length) of *Phaseolus coccineus* grew equally well whether cultured with the intact suspensor or without it. However, in the culture of young embryos removal of the suspensor significantly reduced the frequency of plantlet formation. Confirming the importance of the suspensor for the growth of young embryos of *P. coccineus*, Yeung and Sussex (1979) stated that the suspensor, when intact with the embryo proper or when detached from it but placed in its close proximity on the culture medium, strongly stimulated the further development of the embryo in comparison to the embryo cultured in its absence (see Table 11.6). According to these authors, the growth-promoting activity of the suspensor is maximal at the early heart-shaped stage of the embryo (see Table 11.6). Of the various growth regulators tested, gibberellin at a concentration of 5 mg l^{-1} most effectively substituted for the requirement of the suspensor (Yeung and Sussex, 1979; also see Cionini et al., 1976). This is in accordance with the observations of Alpi et al. (1975) that in *P. coccineus*, at the heart-shaped stage gibberellin activity in the suspensor is about 30 times higher than that in the embryo proper. After the formation of cotyledons, when the suspensor is in its initial stages of degeneration, it shows a sharp decline in the level of GA_3 with a

TABLE 11.6

The effect of suspensor on in vitro growth and development of *Phaseolus coccineus* embryos[a]

Initial[b] stage (fresh weight)	Treatment	Fresh weight[c] ± SE (*N*)	% Embryos[e] forming plants (no. of embryos cultured)
Early heart-shaped (0.87–0.02 mg)	Embryo proper only	3.19 ± 0.52 (10)	41.5 (89)
	Embryo proper with suspensor attached	8.91 ± 1.16[d] (10)	88.4 (95)
	Embryo proper with detached suspensor in direct contact	6.22 ± 0.78[d] (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 mg)	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 mg)	Embryo proper only	20.3 ± 2.5 (7)	100 (18)
	Embryo proper with suspensor attached	24.4 ± 2.75 (11)	100 (19)

[a]After Yeung and Sussex (1979).
[b]Seed sizes: early heart-shaped stage - 4.5 mm; late heart-shaped stage - 6.5 mm; early cotyledon stage - 7 mm.
[c]Fresh weight was taken 10 days after culture; (*N*) represents the sample size.
[d]Significant at the 1% level.
[e]Assessed 8 weeks after culture.

concomitant increase in its level in the embryo proper. Kinetin also promoted the growth of young embryos in the absence of the suspensor and was effective over a wide range of concentrations (0.001–1 mg l^{-1}) but at no level did its effect match that of gibberellin.

Recently, Liu et al. (1993a) have reported that removal of suspensor had only a small effect on in vitro development of 55 μm or smaller embryos of *Brassica juncea* and no effect on the embryos larger than 55 μm.

However, these authors used a very complex culture medium rich in organic nutrients and was supplemented with 30% coconut water, which may contain sufficient plant hormones to substitute for the suspensor.

11.5. PRECOCIOUS GERMINATION

To an embryologist, embryo development is almost synonymous with embryogenesis which embraces developmental stages from the zygote to the fully formed embryo characteristic of the species. However, plant physiologists and biochemists conceive embryo development as a linear progression from zygote formation to germination (Dure, 1975; Sussex, 1978; Walbot, 1978). Walbot (1978) has classified embryo development into five stages (see Table 11.7). Initially the growth of the embryo is predominantly by cell division resulting in the formation of a proembryo which comprises small meristematic cells. This is followed by tissue differentiation during which the meristematic activities become localized. After its full-term development the embryo becomes dehydrated and enters a phase of metabolic quiescence and developmental arrest. During this phase (dormancy) which, depending on the genotype and the environmental conditions, may last from a few days to several months or even years, the embryo is normally incapable of germination. Thus, embryo development is a highly regulated process.

Embryos of mangroves and some viviparous varieties of cultivated plants germinate while still attached to the parent without any diminution of its rate of growth. Excised immature embryos of even other plants when cultured on nutrient medium not only bypass the stage of dormancy but also cease to undergo further embryogenic mode of development. They do not exhibit normal biosynthetic activities characteristic of late embryogeny (Choinski and Trelease, 1978; Crouch and Sussex, 1981; Dure and Galau, 1981). Instead, the embryos develop into weak seedlings displaying only those structures already present at the time of embryo excision (see Fig. 11.7). This phenomenon of seedling formation without completing normal embryogenic development is called precocious germination. One of the aims of culturing immature embryos has been to stimulate normal embryogenic development in order to understand the factor(s) that regulate the orderly in ovulo development of embryos in nature.

The extension of embryogenic development and delayed germination by CH in the cultures of excised immature embryos of barley was reported by Kent and Brink (1947). Later, Ziebur et al. (1950) found that, although not as effective as CH, a high sucrose level (12.8%) in the medium also inhibited precocious germination of cultured immature em-

TABLE 11.7

Major stages in the development of *Phaseolus* embryos[a]

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 materials
4. Dormancy	Developmental arrest
5. Germination	Renewed cell expansion and division; embryo growth

[a]After Walbot (1978).

bryos of barley. Precocious germination of *Capsella* embryos was similarly inhibited by the presence of a high concentration of sucrose (12–18%) in the medium (Rijven, 1952). These observations are in agreement with the findings that both in monocots and dicots the values of osmotic pressure and sugar concentration of the ovular sap are high during the early stages of embryo development and they progressively decrease with the age of the ovule (Ryczkowski, 1974). Norstog (1972) and Norstog and Klein (1972) have shown that exogenous factors, besides high osmolarity, which suppress precocious germination of excised immature barley embryos are reduced O_2 tension, elevated temperature and high light intensity.

Andrews and Simpson (1969) observed that naked embryos from freshly harvested mature seeds of a highly dormant strain of *Avena fatua* failed to germinate on a medium that supported the germination of naked embryos from non-dormant (after-ripened) seeds of the same strain. However, the dormant embryos germinated in the same medium if they were supplied with exogenous GA_3 or cultured after they had been leached on an agar medium. Seeds of *Taxus baccata* require an after-ripening period to complete embryogeny. During this period the embryo is normally incapable of germination. Culturing the dormant embryos in liquid medium for 8 days rendered them germinable. Apparently some leachable inhibitor(s) residing in the embryo is(are) responsible for preventing precocious germination (Le Page-Degivry and Garello, 1973). One of the inhibitors of germination seems to be abscisic acid (ABA) which is known to accumulate in high concentrations during the later stages of seed development in some plants (Dure, 1975; King, 1976; Hsu, 1979). The hypothesis that ABA like substance(s) is(are) involved in preventing

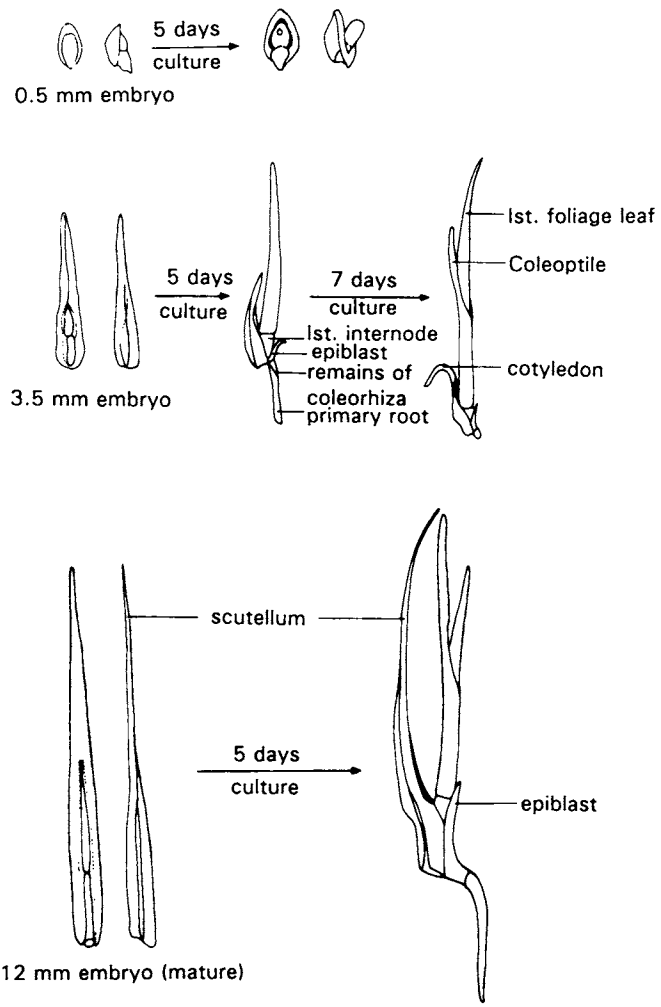

Fig. 11.7. In vitro growth of *Zizania aquatica* embryos excised from ovules at different stages of development. Embryos 0.05 mm long showed very little in vitro growth. Older, immature embryos (3.5 mm long) germinated precociously and formed seedlings which were not as well developed as those formed by the mature embryos in the same culture period (after LaRue and Avery, 1938).

precocious germination in nature gains support from the work of Norstog (1972), who noted that precocious germination of immature barley embryos may be induced under culture conditions which normally inhibit it (high osmolarity of the medium, high light intensity and elevated temperature) by GA_3 application. This GA_3-induced precocious germination was suppressed by ABA treatment.

In cotton, mRNA for the synthesis of enzymes carboxypeptidase C, isocitrase and protease, required to hydrolyze the reserve material during first few hours of germination, are transcribed much before the seed attains maturity and the embryo enters dormancy (Ihle and Dure, 1969, 1972a,b). However, translation of the mRNA remains inhibited during the dormancy period due to the presence of high levels of ABA in the tissues surrounding the embryo (Dure, 1975). Walbot (1978) has proposed that ABA may be preventing precocious germination by suppressing water uptake by the embryo which is essential for germination. Temporal separation of transcription and translation of the germination-specific mRNA in cotton is prevented during precocious germination of the young embryos excised at a stage before the mRNA is normally transcribed (Ihle and Dure, 1972a,b). The germination of the excised embryos and the appearance of the enzymes are inhibited by the aqueous extract of the ovules as well as exogenously applied ABA, suggesting that in cotton the factors suppressing the precocious germination of the embryo reside in extra-embryonal tissues of the seed.

11.6. MORPHOGENESIS IN THE CULTURES OF SEEDS WITH PARTIALLY DIFFERENTIATED EMBRYOS

In many flowering plants (belonging to 19 families) fully developed seeds on the plant contain embryos that lack differentiation into radicle, plumule and cotyledons (Rangaswamy, 1967). In *Eranthis* (Ranunculaceae) seeds at shedding enclose a pear-shaped embryo (comprising only a few hundred cells) with a long suspensor. After the seed has fallen on the ground the embryo undergoes intraseminal growth and achieves the usual morphology of a mature dicotyledonous embryo (Maheshwari and Rangaswamy, 1965). In orchids and root parasitic members of the Orobanchaceae also the seed harbours an unorganized embryo but in these plants the intraseminal growth is absent and the seedling is formed by the globose embryo without undergoing further embryogenic differentiation. In such plants the embryonal end proximal to the micropyle is regarded as the 'radicular pole' and that distal to the micropyle is termed the 'radicular pole'. Interestingly, both in orchids and the Orobanchaceae members, only one of the poles of the embryo is involved in the development of the seedling.

During seed germination in orchids, the plumular pole of the embryo enlarges to form a spherule-like structure, called the protocorm. Initially the protocorm is non-chlorophyllous but gradually it turns green. After attaining a certain size the protocorm differentiates roots and shoot. In this way in orchids the entire seedling develops exclusively by the divi-

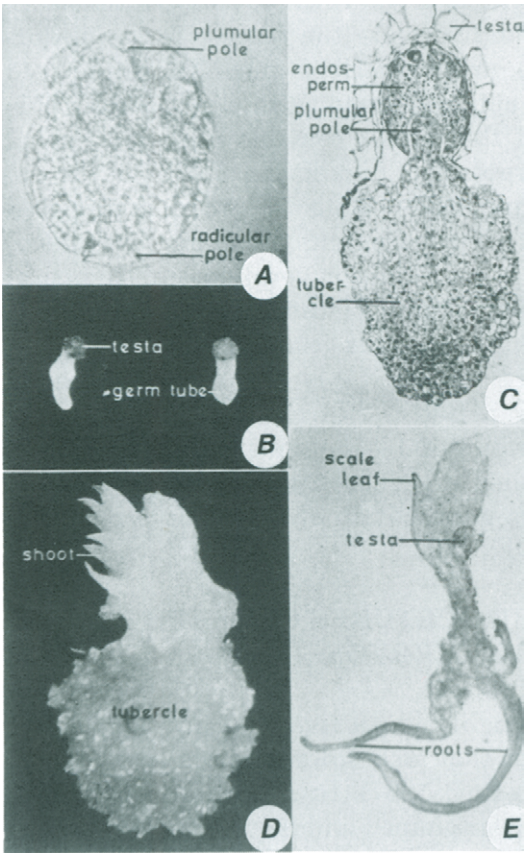

Fig. 11.8. Embryo morphogenesis in seed cultures of two members of the Orobanchaceae, with reduced embryos. (A,E) *Orobanche aegyptiaca* and (B–D) *Cistanche tubulosa*. (A) Whole mount of excised, mature embryo; it lacks a radicle and a plumule. (B) Three-week-old culture on White's medium containing CM and CH, showing the formation of the germ tube. (C) Longisection of a seed at the stage shown in (B); the plumular pole of the embryo has remained quiescent inside the seed whereas the radicular pole has formed a massive holdfast. (D) Differentiation of a shoot from the proliferated embryo after transfer to Tepfer's H-medium and storage in the dark. (E) Five-week-old culture on medium containing strigol. The seedling is formed by bipolar activity of the embryo. (A) After Rangaswamy, 1963; (B–D) after Rangan and Rangaswamy, 1968; (E) after Kumar and Rangaswamy, 1977.

sion of the plumular pole of the embryo (Carlson, 1943; Rao and Avadhani, 1963). Almost the same pattern of seedling development occurs in the seed cultures of orchids (Rao and Avadhani, 1963).

Unlike orchids, in the root parasitic members of the Orobanchaceae normally a seedling is derived by the sole activity of the radicular pole of the embryo (see Fig. 11.8A). According to Kadry and Tewfic (1956) during

seed germination in *Orobanche crenata* the radicular pole of the embryo forms a 'radicular cylinder' (see Kumar and Rangaswamy, 1977) whose tip penetrates the host root. The portion of radicular cylinder that remains outside the host root proliferates into an irregular mass of tissue, called the tubercle, from which the shoot differentiates. Rangan and Rangaswamy (1968) germinated the seeds of *Cistanche tubulosa*, another member of the Orobanchaceae, in vitro and recorded a similar pattern of germination (see Fig. 11.8B–D) as described for *O. crenata* by Kadry and Tewfic (1956). Rangaswamy (1963) also reported monopolar seedling development in seed cultures of *O. aegyptiaca*. However, Kumar and Rangaswamy (1977) have shown that the pattern of seedling development in this parasite can be modified from monopolar to bipolar by modifying the composition of the culture medium. On TB medium (salts after Tepfer et al., 1963, glycine, niacin, thiamine·HCl, calcium pantothenate and sucrose) supplemented with CM or yeast extract the germination was monopolar (formation of seedling by the sole activity of the radicular pole). The addition of IAA (0.1 mg l^{-1}), kinetin (0.5–10 mg l^{-1}), GA_3 (5–30 mg l^{-1}) or strigol (0.01 μg l^{-1}) to the TB medium induced a bipolar pattern of seedling development (see Fig. 11.8E); the plumular pole differentiated into a shoot bud and the radicular pole produced roots. Substitution of sucrose in the TB medium by glucose, mannose or raffinose also favoured bipolar germination (Usha, 1968). The highest frequency of bipolar seedling development occurred in TB medium + GA_3 (7.5 mg l^{-1}).

The mature embryos of *Cuscuta* have a distinct plumule but lack a radicle (Haccius and Troll, 1961; Truscott, 1966). According to Truscott (1966) there is no indication of the existence of a protoderm, root meristem or hypocotyl either during embryogenesis or in the mature embryos of *C. gronovii*. During in vivo seed germination the plumule forms a shoot but the radicular end of the embryo starts to die off 4–5 days after the initiation of germination. Thus, in this plant a root system is absent altogether. Truscott (1966) cultured mature and immature embryos of *C. gronovii* to test if roots can be artificially induced by applying growth substances, such as adenine, kinetin and GA_3, or complex nutrient mixtures, such as CM, and CH. However, none of the treatments induced rooting. This led Truscott to conclude that *C. gronovii* lacks a root growth potential. Similarly, root differentiation never occurred in the cultures of embryos or shoot apices of *C. reflexa* (Maheshwari and Baldev, 1962).

11.7. MICROSURGICAL EXPERIMENTS

A typical plant embryo is a bipolar structure, possessing the basic organization of the adult plant. During seed germination different parts of

the embryo perform their specialized functions and follow pre-determined mode of development. In vitro microsurgical experiments with embryos have thrown light on, at least, two aspects of growth and development: (a) interaction between different parts of the embryo during seed germination, and (b) the morphogenetic potentials of the various constituent parts of the embryo.

Studies to-date suggest that the cotyledon(s) plays an important role in the normal development of the embryonal axis into a seedling. This is true for dicots, monocots and, at least, one gymnosperm (Raghavan, 1980). Kruyt (1952) and Kester (1953) demonstrated the necessity of intact cotyledons for normal growth of seedlings in pea and peach, respectively. In *Zea mays* (Andronescu, 1919), *Avena sativa*, *Hordeum vulgare* and *Pennisetum typhoideum* (Narayanaswamy, 1963) removing the scutellum (equal to the single cotyledon of other monocots) inhibited the development of root and shoot. Tilton (1981) has reported that in *Zea mays*, hypocotyl-scutellum explants were more rhizogenetic than were epicotyl explants. Since the hypocotyl segments, in culture, showed complete lack of root growth and total deformity of the explant, it is suggested that in maize the scutellum provides the growth factor(s) which promotes root growth and normal root morphology during germination. For epicotyl growth, however, the presence of the scutellum was slightly inhibitory (Tilton, 1981). A quantitative relationship between the amount of cotyledon removed and the extent of growth of root, hypocotyl and shoot in embryo cultures of *Vigna sesquipedalis* has been noted by Hotta (1957); the larger the portion of cotyledons severed the greater was the suppression of seedling development.

Rangaswamy and Rangan (1971) made similar observations with *Cassytha filiformis* (a stem parasite); these are summarized in Table 11.8 (see also Fig. 11.9). It is evident from the data that in *C. filiformis* the growth factor(s) for shoot morphogenesis resides in the 'radicular-halves' of the cotyledons. If the 'plumular-halves' of both the cotyledons are severed (treatment D) the seedling is well developed but if 'radicular-halves' are removed (treatment E) the plumule does not grow. Further, the 'radicular-half' of only one cotyledon alone (treatment G) or along with the 'plumular-half' of one of the cotyledons (treatment C,F) is not sufficient to support the growth of the plumule. The minimum fraction of the cotyledons necessary to permit normal morphogenesis of the plumule is the 'radicular-half' of one cotyledon together with 'plumular-half' of both the cotyledons (treatment A) or the 'radicular-halves' of both the cotyledons (treatment D).

In the embryo cultures of *Phaseolus vulgaris* an increase in the weight of embryonal axis was directly proportional to the amount of cotyledonary tissue left on it (Monnier, 1978).

TABLE 11.8

Effect of cotyledons on seedling formation in *Cassytha filiformis*[a,b]

Portion of the cotyledons severed	Response
A. Radicular-half of one cotyledon	Seedling well developed
B. Plumular-half of one cotyledon	Seedling well developed
C. One entire cotyledon	Hypocotyl and radicle inhibited
D. Plumular-halves of both cotyledons	Seedling well developed
E. Radicular-halves of both cotyledons	Plumule quiescent
F. Radicular-half of one cotyledon and plumular-half of the other	Plumule quiescent
G. One entire cotyledon and plumular-half of the other	Plumule quiescent
H. One entire cotyledon and radicular-half of the other	Plumule quiescent
I. Both cotyledons; leaving behind the embryonal axis	Plumule quiescent

[a]After Rangaswamy and Rangan (1971).
[b]Decotylated embryos were cultured on medium after Rangaswamy (1961).

Thevenot and Come (1971, 1973) observed that the growth of partially after-ripened apple (*Pyrus malus*) embryos was much better when they were planted with the cotyledons in contact with the medium than in any other orientation, suggesting that the cotyledons are the chief structures involved in the absorption of nutrients from the medium and transferring them to the other parts of the embryo. In gymnosperms, Bulard (1952) showed that cultured mature embryos of *Ginkgo* displayed normal shoot growth only when the cotyledons were in contact with the medium. Paradoxically, completely decotylated embryos of *Cajanus cajan* (Kanta and Padmanabhan, 1964) and *Dendrophthoe falcata* (Bajaj, 1966a) have been reported to grow into normal seedlings. In *Pinus* normal root development occurred irrespective of whether the cotyledons were in contact with the medium or away from it (Sacher, 1956) or when completely removed before culturing the embryos (Berlyn and Miksche, 1965). However, an appreciable shoot growth never occurred in these cultures.

In some genotypes of maize (Harms et al., 1976) and barley (Dale and Deambrogio, 1979) embryo callusing was much better when the embryo was planted with the scutellum in contact with the medium than when the scutellum was away from it.

Existing studies related to the interaction between different constituent tissues of the embryo cannot be critically evaluated especially when the media used by different authors are not identical. For example,

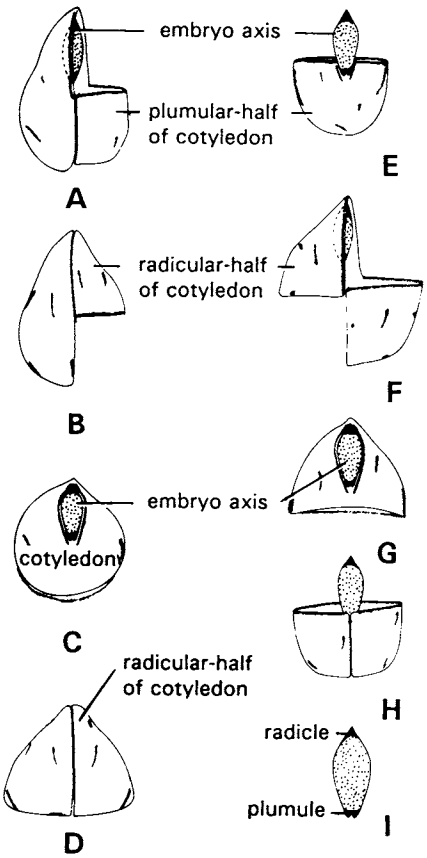

Fig. 11.9. Decotylation patterns of *Cassytha* embryos in microsurgical experiments. For the responses of various treatments refer to Table 11.8 (after Rangaswamy and Rangan, 1971).

Cassytha embryo segments were cultured on a simple medium comprising mineral salts, vitamins, amino acids, and sucrose whereas for *Dendrophthoe* embryos the medium was enriched with casein hydrolysate.

11.8. EMBRYO AND SEED CULTURE OF PARASITIC ANGIOSPERMS

From the viewpoint of morphogenesis, parasitic angiosperms constitute an interesting group of plants. Depending on the mode of their attachment to the host plant these parasites fall into two broad categories: root parasites (establishing contact through host roots) and stem parasites (establishing contact through aerial parts of the host plant).

Root parasites are dependent on the host stimulus to various degrees. In nature most of them require a host contact for normal seedling morphogenesis, especially shoot development (Kuijt, 1969). Some root parasites, including *Cistanche, Orobanche* and *Striga*, are also dependent on the host plant for seed germination (Shivanna and Rangaswamy, 1976). In cultures the seeds of parasites belonging to the second category will not germinate in a salts–sugar–vitamins–agar medium. However, the addition of extract or exudate of the host roots to the medium may stimulate seed germination independent of the host (Saunders, 1933; Brown and Edwards, 1944; Cezard, 1965; Okonkwo, 1966). Many different chemically defined substances have been shown to substitute the stimulus provided by the host for seed germination in obligate root parasites: cytokinins for *Striga euphrasioides* (Rangaswamy and Rangan, 1966), gibberellins for *Orobanche ludoviciana*, *O. ramosa* (Nash and Wilhelm, 1960) and *O. aegyptiaca* (Kumar and Rangaswamy, 1977), scopotelin for *Striga asiatica* (Worsham et al., 1962) and strigol for *S. lutea* (Cook et al., 1966) and *O. aegyptiaca* (Kumar and Rangaswamy, 1977).

Seeds of *Alectra vogelii* germinate without a host stimulus but seedling formation does not proceed beyond the stage of radicle emergence unless the radicle establishes contact with the roots of a suitable host (Okonkwo, 1975). Similarly, in *Sopubia delphinifolia* a germinating seed normally fails to develop a shoot without a host contact. In seed cultures of these two obligate root parasites full seedling development occurred independent of a host stimulus when an exogenous carbohydrate, such as sucrose and glucose, was supplied through the medium (Okonkwo, 1975; Shivanna and Rangaswamy, 1976). In seed cultures of *S. delphinifolia* shoot development without a host stimulus was also favoured by high light intensity (3000 lx) (Sahai, 1978).

In vitro seed germination and shoot development independent of host stimulus has also been observed in the root parasites, *Osyris wightiana* (Bhojwani, 1968), *Exocarpus cupressiformis* (Johri and Bhojwani, 1965; Bhojwani, 1969a) and *Santalum album* (Rao and Rangaswamy, 1971). However, most of the root parasites did not form haustoria in cultures without a host. Aseptic seedlings of *Striga senegalensis* formed haustoria only if grown with seedlings of *Sorghum bicolor* (Okonkwo, 1966). Cultured seedlings of some scrophulariaceous root parasites have been reported to develop haustorial structures without a host stimulus when treated with chemically undefined substances, such as aqueous extracts of gum tragacantha (*Agalinis purpurea,* Riopel and Musselman, 1979; *S. delphinifolia,* Sahai and Shivanna, 1981), cotton strings and soya beans (*Orthocarpus purpurascens,* Atsatt et al., 1978). None of the common growth hormones or sugars was effective in haustorial induction.

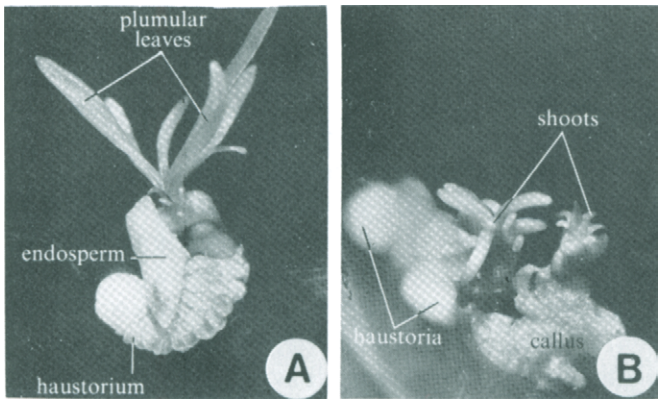

Fig. 11.10. *Scurrula pulverulenta.* (A) Seedling formed in vitro independent of a host, showing a worm-like haustorium and several plumular leaves. (B) Differentiation of shoot-buds and haustoria from the embryo callus (after Johri and Bhojwani, 1970).

Unlike root parasites, loranthaceous stem parasites frequently form distinct haustorial structures, comparable to those formed in nature, without a host or a plant extract (Johri and Bajaj, 1963; Bhojwani, 1969b; Johri and Bhojwani, 1970; Nag and Johri, 1976; see Fig. 11.10A). Even callused embryos (Johri and Bhojwani, 1970; Nag and Johri, 1976; see Fig. 11.10B), and endosperm (Bhojwani and Johri, 1970) of leafy mistletoes differentiated haustorial structures. These mistletoes are also distinct from root parasites and other stem parasites in the ability of their seeds to germinate on simple nutrient media lacking growth hormones or a complex nutrient mixture like casein hydrolysate and coconut milk (Johri and Bhojwani, 1971).

11.9. MORPHOGENIC POTENTIAL OF THE EMBRYO CALLUS

One of the most critical needs of applying methods of somatic cell genetics to crop improvement has been to establish cell and callus cultures from which complete plants can be regenerated. Embryo calluses have been shown to possess a high regeneration capacity in relation to those derived from mature organs like leaf, stem and root (Merkle et al., 1990).

Dale and Deambrogio (1979) cultured meristem tips, mesocotyl, leaf sheath, immature embryos and mature embryos of barley. Best growing calluses originated from root and embryo explants but shoot differentiation occurred only from the immature embryo callus. Green and Phillips (1975) reported that in four different genotypes of maize the embryos excised 18 DAP gave the highest frequency of differentiating calluses. No differentiation occurred in calluses of mature embryo origin (see Table

TABLE 11.9

Influence of embryo age and genotype on percent cultures showing plant regeneration from the scutellar callus in maize[a,b]

Genotype	Embryo age (DAP)					
	14	16	18	20	22	24
A188	18	15	21	14	9	0
A619	0	5	6	5	0	0
A632	0	0	0	0	0	0
B9A[C]	0	5	8	0	0	0
W64A	0	0	11	0	0	0

[a]After Green and Phillips (1975).
[b]Each value represents the % of 60 embryos.
[c]These cultures died during the fourth month of growth (fourth subculture).

11.9). That the age of the embryo in maize considerably influences the regeneration ability of their calluses was confirmed by Freeling et al. (1976). Immature embryos of *Avena* (Cummings et al., 1976) and *Sorghum* (Gamborg et al., 1977; Thomas et al., 1977) have also been found to be good explants for initiating calluses capable of plant regeneration. Several other crop plants, such as most of the cereals, grain legumes, cotton and several tree species, which were once regarded as recalcitrant for plant regeneration in tissue cultures have become amenable to improvement by the biotechnological methods simply because highly embryogenic cultures could be established from immature zygotic embryos.

11.10. PRACTICAL APPLICATIONS

11.10.1. Obtaining rare hybrids

The most useful and popular application of zygotic embryo culture has been in raising rare hybrids. In many interspecific and intergeneric crosses fertilization occurs normally and the embryo shows early development, but the poor or abnormal development of the endosperm causes premature death of the hybrid embryo, and germinable seeds are not formed. In 1925 Laibach made a very significant contribution when he demonstrated that in such unsuccessful crosses the embryo is often potentially capable of normal growth, and full hybrid plants can be raised by excising the embryos before the onset of abortion and culturing them on nutrient medium. In this context, Raghavan (1976a) has remarked

that 'The triumph of the embryo culture method is nowhere better seen than in the dramatic demonstration of the ability of the embryos removed from the seeds of inviable crosses to grow in cultures.' Since the time of Laibach (1925) the technique of embryo culture has been extensively used to produce agriculturally and horticulturally useful hybrids from crosses which normally fail due to the post-fertilization barriers to crossability. In this section only some selected examples are mentioned. Raghavan (1976a, 1977) has dealt with many more examples.

All attempts to cross *Lilium henryi* and *L. regale* were unsuccessful until the embryo culture technique was adopted (Skirm, 1942). Emsweller and Uhring (1962) crossed *L. speciosum-album* with *L. auratum*. The seeds obtained in this cross were larger in size due to a large amount of endosperm. The embryo was also very long. During the storage of seeds or soaking them in water the embryo underwent degeneration and no seedlings could be obtained. This has been ascribed to some sort of embryo-endosperm incompatibility. By growing excised immature embryos on nutrient medium Emsweller and Uhring (1962) could raise full hybrid plants from this cross.

Embryo culture has been successfully applied to produce many interspecific hybrids in the family Fabaceae. *Melilotus officinalis* is an agronomically important species but the plants have high levels of coumarin which is harmful to cattle. Attempts have been made to introduce into this species the genes for low coumarin content by crossing it with another species (*M. alba*) which possesses the trait. In the reciprocal cross between *M. officinalis* and *M. alba* the hybrid embryo grows for some time but fails to mature because the ovules abort 8–10 DAP. Only through embryo culture could Webster (1955) and Schlosser-Szigat (1962) obtain some hybrid plants from this cross. Interspecific hybridization in the genus *Trifolium* has often proved difficult due to the occurrence of both pre- and post-fertilization barriers. Embryo culture was successfully applied to many such crosses where hybridization was normally impossible due to endosperm abortion and consequent abnormal differentiation and starvation of the hybrid embryo (Williams, 1978, 1980; Williams and De Lautour, 1980).

The interspecific cross *Phaseolus vulgaris* × *P. lunatus* is normally unsuccessful due to failure of the hybrid embryo to grow beyond the heart-shaped stage. Kuboyama et al. (1991) obtained a full hybrid plant from this cross with the aid of embryo culture but it was fully sterile.

Crossing cultivated tomato (*Lycopersicon esculentum*) with wild tomato (*L. peruvianum*) has been considered desirable from the point of view of transferring pest and disease resistance from the latter to the former. The cross *L. peruvianum* × *L. esculentum* does not succeed due to pre-

fertilization barriers. In the reciprocal cross, however, fertilization occurs but the cross fails because of embryo abortion, and no viable seeds are formed (Cooper and Brink, 1945; Hogenboom, 1972; Thomas and Pratt, 1981). Smith (1944), Chowdhury (1955) and Alexander (1956) reported that they were able to produce hybrid plants in the cross with *L. esculentum* as the female parent with the aid of embryo culture. However, Thomas and Pratt (1981) did not obtain even a single culturable hybrid embryo out of 401 under-developed seeds formed in the cross *L. esculentum* var. VENT × *L. peruvianum* var. LA 1283-4. These workers followed an alternative approach of 'embryo-callus culture' to obtain full hybrid plants. The poorly developed hybrid embryos were dissected out from ovules 35 DAP and cultured to form a callus followed by plant differentiation. The hybrid plants thus obtained were diploid or tetraploid. The embryo-callus culture approach has also yielded hybrids from the crosses *Lycopersicon 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).

Numerous laboratories have attempted interspecific crosses in the genus *Oryza* with the objective of developing high yielding rice varieties resistant to diseases and pests and capable of withstanding adverse environmental conditions. Embryo abortion, however, has been a serious stumbling block in achieving this goal, and embryo culture has enabled this hurdle to be bypassed. By culturing 10–25-day-old hybrid embryos from several interspecific crosses of rice, which normally failed, Iyer and Govilla (1964) were able to raise full hybrids. On an agar medium containing 10% CM and 0.1% malt extract, 88% of the excised hybrid embryos germinated. After the seedlings had grown fully in the test tubes, they were transplanted to the soil.

In the intergeneric cross *Hordeum jubatum* × *Secale cereale* the hybrid seeds collapse 13–16 days after fertilization, leading to the failure of the cross. Histological studies revealed that in this cross although the hybrid embryos showed considerable growth they ceased to grow prematurely due to incompatible endosperm. Brink et al. (1944) excised embryos from 9 to 12-day-old seeds and cultured them in White's medium. Of the 81 embryos cultured only one developed into seedling; most of the other surviving embryos showed undifferentiated growth. Regeneration of plants from the hybrid embryo callus was not attempted.

A significant improvement in the frequencies of hybrid production from the intergeneric crosses *Hordeum* × *Secale*, *Hordeum* × *Triticum* and *Hordeum* × *Agropyrum* was achieved with the aid of embryo culture (Kruse, 1974). Embryo culture methods have also been used successfully for raising intergeneric hybrids from the crosses *Triticum* × *Aegilops*

(Chueca et al., 1977) and *Triticum* × *Secale* (Taira and Larter, 1977ab, 1978).

In certain intergeneric crosses it is essential to give special treatment to the female parents, following pollination, to obtain embryos suitable for in vitro culture. When tetraploid *Triticum turgidum* (female parent) is crossed with diploid *Secale cereale* fertilization occurs with a high frequency but embryos abort 14–20 DAP. Depending on the strains of the parent used the number of culturable embryos formed may be very low. Taira and Larter (1977a) observed that treatment of the ovules with ε-amino-*n*-caproic acid or its analogue L-lysine daily for 10 days, beginning 3 DAP, significantly promoted the yield of normally differentiated embryos capable of forming normal seedling when cultured in vitro. Optimum day/night temperatures during the treatment was 17°C (Taira and Larter, 1977b). Cooper et al. (1978) reported that spraying the spikes, following pollination, with a mixture of GA_3, NAA and 2-ip was required to obtain culturable embryos in the cross barley × rye. In the non-treated spikes fruit development ceased before the embryo attained a size suitable for their culture.

11.10.2. Haploid production

A novel application of embryo culture technique has been in the production of haploids through directional elimination of chromosomes following distant hybridization (see Section 7.7). Briefly, in the cross *Hordeum vulgare* × *H. bulbosum* fertilization proceeds readily but the chromosomes of *H. bulbosum* are preferentially lost during the first few divisions of embryogenesis. As a result, the haploid embryos show slow growth. This, coupled with the disintegration of the endosperm 2–5 days after fertilization, necessitates culture of the excised embryo to raise haploid *H. vulgare* plants.

11.10.3. Shortening the breeding cycle

Occasionally the breeding work on horticultural plants is delayed due to long dormancy periods of their seeds. By growing excised embryos in nutrient medium this period may be reduced. For example, using embryo culture Randolph and Cox (1943) could shorten the life cycle of *Iris* from 2 or 3 years to less than 1 year. *Rosa* normally takes a whole year to come into flowering; through embryo culture it has been possible to produce two generations in a year and, thus, shortening its breeding cycle (Lammerts, 1946; Asen, 1948). Nickell (1951) reported that in cultures the excised embryos of weeping crabapple (*Malus* sp.) start germi-

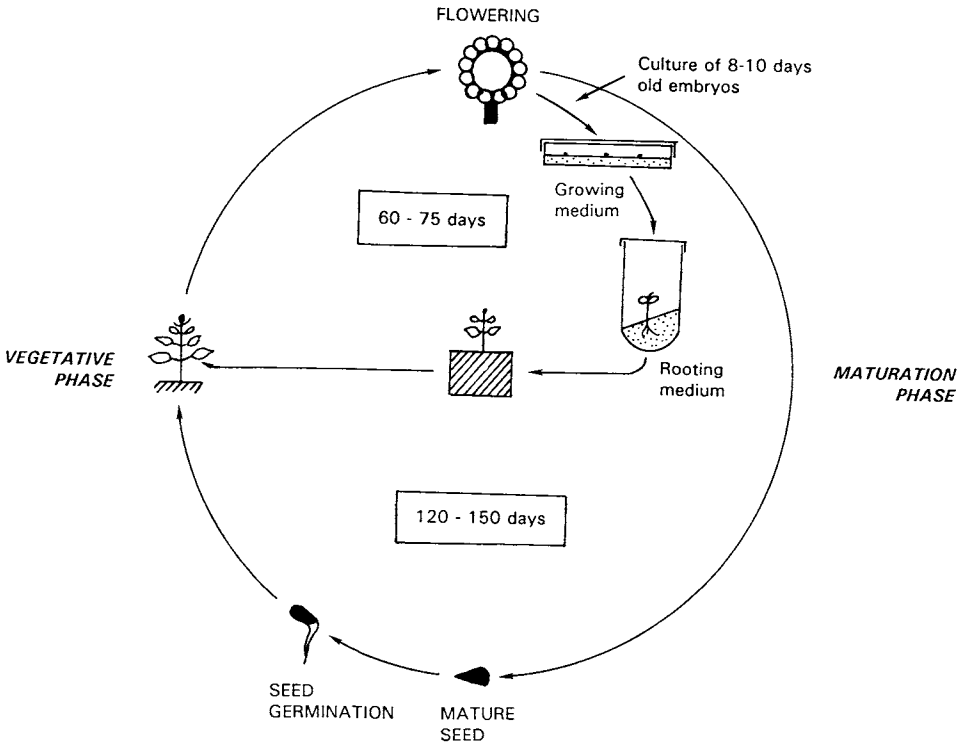

Fig. 11.11. Diagrammatized summary of immature embryo (8–10 DAP) culture to shorten the life cycle duration of sunflower from 120–150 days to 60–75 days (after Serieys, 1992)

nation within 48 h, and within 4 weeks transplantable seedlings are formed. By the end of 5 months the seedlings attain a height of about 1 m. On the other hand, seeds planted in the soil take about 9 months to germinate.

Seed maturation in soybean and sunflower takes 50–60% of the life cycle duration (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 and 10–18-day-old embryos, respectively (Fig. 11.11).

11.10.4. Rapid seed viability test

The possibility of breaking seed dormancy by embryo culture also allows the use of this technique for rapid testing of the viability of a par-

ticular batch of seeds. Germination of excised embryos is regarded as a more exact and reliable test than the commonly used staining methods for seed viability (Barton, 1961). A good correlation has been shown between the growth of excised embryos of non-after-ripened seeds and the germination of after-ripened seeds of peach (Tukey, 1944).

11.10.5. Propagation of rare plants

Seeds of *Musa balbisiana*, a wild relative of the commercial banana, do not germinate in nature, However, seedlings can be readily obtained by culturing their excised embryos (Cox et al., 1960).

As an abnormality, some coconuts develop soft, fatty tissue in place of the liquid endosperm (Mohan Ram, 1976). Such nuts are called 'makapuno'. Being rare, makapunos are very expensive and served only at special banquets in the Philippines. It had been felt that plants propagated from makapuno nuts would be truly makapuno-bearing (Zuniga, 1953). However, under normal conditions the coconut seeds fail to germinate. Using the technique of in vitro culture of excised embryos, De Guzman (1969) and De Guzman and Del Rosario (1974) succeeded in raising plantlets from makapuno nuts. Eighty-five percent of the field-grown plants obtained with the aid of embryo culture bore makapuno nuts (De Guzman et al., 1976).

11.10.6. Others

Embryo culture is a useful technique to study basic aspects of embryogenesis, such as the effect of nutrients, phytohormones and other chemical and physical factors on embryonal growth and differentiation (Monnier, 1990; Liu et al., 1993b).

In desiccated zygotic embryos the plasma membranes are perforated with large pores, making them permeable to molecules of certain size. However, during inbibition the membrane rapidly becomes intact and uniform. Taking advantage of this unique feature of the embryos, Topfer et al. (1989) transformed some cereals and legumes by imbibing their mechanically isolated embryos in a solution containing plasmid DNA with a chimeric NPT-II gene, and chemicals which enhance the permeability of the membrane.

Establishment of highly regenerable cultures from immature embryos of a large number of agronomic, horticultural and forest species has considerably expanded the scope of application of biotechnological methods to crop improvement.

11.11. CONCLUDING REMARKS

Embryo culture technique has already established its credibility as an invaluable tool for obtaining hybrid plants in some difficult crosses. Considerable progress has also been made in understanding the various aspects of embryo morphogenesis. However, factors regulating embryo development inside the ovules (in ovulo) are still not fully understood. The ovules excised at the zygote stage have been reported to show normal embryogenesis in cultures (see Chapter 10) but embryos excised prior to the differentiation of organ primordia either fail to survive in culture or exhibit callusing. In this context the recent success of Liu et al. (1993a) to culture 35 μm early globular embryos of *Brassica juncea*, using a double layer culture system and a complex nutrient medium is noteworthy. Even more remarkable is the recovery of full fertile plants from in vitro fertilized egg (in vitro formed zygote) of maize (see Chapter 10). In zygotic and non-zygotic embryogenesis an attachment of the proembryo to the parent tissue seems to be essential for normal embryogenic development. This attachment may be necessary to maintain the gradient of nutrients and other growth factors supplied by the parent tissue for proper polarized differentiation of the embryos. Liu et al. (1993b) have demonstrated that the transition from radial symmetry of globular embryos to bi-lateral symmetry of heart-shaped embryos of dicotyledonous plants is brought about by the polar transport of auxin. Interference with the polar transport of auxin resulted in the formation of embryos with fused cotyledons. More such studies are likely to enhance our understanding of the process of embryogenesis.