Parthenocarpy is the process by which a fruit develops without fertilisation. As a result the fruit is seedless.

Chapter 6

Somatic Embryogenesis

6.1. INTRODUCTION

The act of fertilization triggers the egg cell (called the zygote after fertilization) to divide and develop into an embryo (the process of embryo development is called embryogenesis). However, fertilization is not always essential to stimulate the egg to undergo embryogenesis. As happens in parthenogenesis, the pollination stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development. Moreover, it is not the monopoly of the egg to form an embryo. Any cell of the female gametophyte (embryo sac), or even that of the sporophytic tissue around the embryo sac may give rise to an embryo. In several species of *Citrus* and *Mangifera* the development of adventive embryos from nucellar cells is a normal feature. However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development, or else they fail to mature. In nature there is no instance of ex-ovulo embryo development (Bhojwani and Bhatnagar, 1990). These in vivo observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the 'magic bath' of the embryo sac.

During the last three decades considerable information has accumulated to establish the embryogenic potential of somatic plant cells, and there has been an explosion in the number of species that form somatic embryos (SEs). Based on the recent spectacular development in cell and tissue culture of higher plants it would be fair to say that any cell, in which irreversible differentiation has not proceeded too far, will, if placed in an appropriate medium, develop in an embryo-like way and produce a complete plant. The whole complex sexual apparatus is, therefore, not an essential prerequisite for cells to acquire embryonic properties. The events occurring in the ovule after fertilization thus provide only a special case of embryogeny. For detailed recent reviews on in vitro somatic embryogenesis refer to Ammirato (1989), Carman (1990), Gray and Purohit (1991), Michaux-Ferriere and Schwendiman (1992), Zimmerman (1993), de Jong et al. (1993) and Emons (1994).

Embryos formed in cultures have been variously designated as accessory embryos, adventive embryos, embryoids, and supernumerary em-

bryos. In this chapter the embryos formed in cultures have been referred to as somatic embryos (SEs) or simply embryos.

6.2. SOME EXAMPLES OF SOMATIC EMBRYOGENESIS

The first observations of in vitro somatic embryogenesis were made in *Daucus carota* (Reinert, 1958, 1959; Steward et al., 1958). Ever since, this species has been widely used to investigate various aspects of in vitro somatic embryogenesis (Terzi et al., 1985; Molle et al., 1993; Zimmerman, 1993). Other plants in which this phenomenon has been studied in some detail are *Citrus* sp. (Rangaswamy, 1961; Sabharwal, 1963; Rangan et al., 1968; Kochba and Spiegel-Roy, 1977b; Tisserat and Murashige, 1977; Gavish et al., 1991, 1992), *Coffea* sp. (Monaco et al., 1977; Sondahl et al., 1979a,b; Sharp et al., 1980; Nakamura et al., 1992), *Macleaya cordata* (Kohlenbach, 1977), *Medicago* sp. (Redenbaugh and Walker, 1990, McKersie et al., 1993), *Ranunculus sceleratus* (Konar and Nataraja, 1969; Konar et al., 1972; Thomas et al., 1972), and *Zea mays* (Emons and Kieft, 1991; Songstad et al., 1992; Emons, 1994).

In *Ranunculus sceleratus* various floral parts (including anthers) as well as somatic tissues proliferate to form a callus on a medium containing coconut milk (10%) with or without IAA. Within 3 weeks numerous embryos appear on the callus (Fig. 6.1) (Konar and Nataraja, 1969). The embryos originate from the peripheral as well as deep-seated cells of the callus (Fig. 6.1H,I). Embryo differentiation also occurs in suspension cultures raised from these calli. The SEs germinate in situ or when they are excised and planted individually on a fresh semi-solid medium. A specially interesting feature is the development of a fresh crop of embryos from the stem surface of these plantlets (Figs. 6.2 and 6.3) (Konar and Nataraja, 1965, 1969). The number of adventive embryos formed per plantlet varied from 5 to 50. Light microscopic (Konar and Nataraja, 1965) and ultrastructural studies (Konar et al., 1972) revealed that the stem embryos originated from single epidermal cells (Fig. 6.3) through stages reminiscent of in vivo zygotic embryogeny in this species. Direct embryogenesis from intact epidermal cells also occurs in the cultures of hypocotyledonary segments and their superficial peels in carrot (Kato and Takeuchi, 1966; Kato, 1968).

Kohlenbach (1965) and Lang and Kohlenbach (1975, 1978) demonstrated the ability of mechanically isolated, fully differentiated mesophyll cells of *Macleaya cordata* to yield an embryogenic callus (Fig. 6.4). On a medium containing 2,4-D and kinetin at an equimolar concentration of 5 μM the individual mesophyll cells divided to form tissues which could be continuously multiplied on this medium. A reduction in kinetin con-

The auxin/cytokinin ratio concept does not seem to apply equally well to other species. (1) In some species, *e.g.*, *Convolvulus, Bacopa, Citrus*, shoot regeneration occurs on GR-free media, but is promoted by a cytokinin. (2) In some other cases, *e.g.*, *chickpea* immature cotyledons, shoot regeneration occurs only when cytokinin is provided; presence of a low concentration of auxin may have a promotory effect. (3) In plants like *Cyclamen*, auxin concentration alone determined whether a shoot or root would regenerate; in contrast, adenine (a cytokinin) affects only the frequency of their regeneration. (4) Alfalfa presents a peculiar case: callus is first initiated on a 2, 4-D and kinetin containing medium; shoot or root regeneration occurs when it is subcultured on a GR-free medium. The ratio of auxin to kinetin in the first medium determines the type of organ formed: a high 2, 4-D concentration results in shoot regeneration, while a high kinetin level supports root regeneration. In general, GA_3 inhibits shoot bud regeneration. But in some species, *e.g.*, *Chrysanthemum, Arabidopsis*, GA_3 promotes shoot regeneration. In contrast, a large number of species show enhanced shoot regeneration due to ABA (abscisic acid), which counteracts many effects of GA_3.

The variable responses of different plant species to the exogenous GRs may be explained as follows. Shoot and root regeneration require specific levels of the different growth hormones, *viz.*, auxin, cytokinin, and gibberellin. However, the endogenous levels of these hormones may vary considerably among different plant species so that a hormone may be either suboptimal, optimal or supraoptimal for shoot (or root) regeneration. The response of a plant species to an exogenous GR would, therefore, depend mainly on the endogenous level of that GR (and of other GRs as well) in that species. Thus it has been postulated that in species like *Chrysanthemum*, GA_3 occurs in suboptimal concentrations so that exogenous GA_3 has a promotory effect. But in those species where its endogenous concentration is supraoptimal, ABA promotes shoot regeneration. It may be added that in some species auxin inhibitors enhance shoot bud differentiation.

6.6.1.2. *Other Factors.* Shoot regeneration is markedly affected by the genotype of explant in that different varieties of a given species show quite different frequencies of shoot regeneration. In alfalfa, breeding and selection drastically increased regeneration ability. In wheat, callus growth and regeneration ability are governed by genes called, tissue culture response (TCR) genes, which have been mapped on specific chromosomes. In addition, physical condition of medium (liquid or agar) has a marked influence on shoot regeneration; in some cases liquid medium was superior, while in others it was drastically inferior to agar medium. Light seems to have an

inhibitory effect, and even the quality of light may be important. The optimum temperature for shoot regeneration may vary with the plant species.

6.6.2. Somatic Embryogenesis

A ***somatic embryo*** (SE) is an embryo derived from a somatic cell, other than zygote, usually on culture *in vitro*, and the process is known as ***somatic embryogenesis.*** In contrast, embryos developing from zygotes are called ***zygotic embryos*** or often simply ***embryos***, while those derived from pollen are known as ***pollen embryos*** or ***androgenetic embryos***. By 1993, somatic embryogenesis was reported from over 100 species.

6.6.2.1. *Developmental Pattern of SEs*. SEs generally originate from single cells, which divide to form a group of meristematic cells. Usually, this multicellular group becomes isolated by breaking cytoplasmic connections with the other cells around it and subsequently by cutinization of the outer walls of this differentiating cell mass. The cells of meristematic mass continue to divide to give rise to globular (round ball-shaped), heart-shaped, torpedo and cotyeledonary stages (Fig. 6.5). In general, the essential features of SE development, especially after the globular stage, are comparable to those of zygotic embryos.

Somatic embryos are bipolar structures in that they have a radicle and a plumule. The radicular end is always oriented toward the centre of callus or cell mass, while the plumular end always sticks out from the cell mass (Fig. 6.5). In contrast, a shoot bud is monopolar as it does not have a radicular end (Table 6.3). In many SEs, radicle is suppressed so that they often do not produce roots; in such cases, roots have to be regenerated from the shoots produced by germinating SEs. SEs often show abnormal developmental features, *e.g.*, 3 or more cotyledons, bell-shaped cotyledon, larger size etc.; these problems are often overcome by the presence of ABA or a suitable concentration of mannitol. In some species, normal looking somatic embryos are formed, but they fail to germinate. Somatic embryogenesis is influenced by several factors, *e.g.* (1) GRs (2) nitrogen source, (3) explant, (4) genotype and (5) others.

6.6.2.2. *Growth Regulators*. In most species an auxin (generally 2,4-D at 0.5-5 mg/l) is essential for somatic embryogenesis. The auxin causes dedifferentiation of a proportion of cells of the explant, which begin to divide. In carrot, these small, compact cells divide asymmetrically, and their daughter cells stick together to produce cell masses called ***proembryogenic masses*** or ***embryogenic clumps*** (ECs). In the presence of auxin, the ECs grow and break up into smaller cell masses, which again produce ECs. But when the

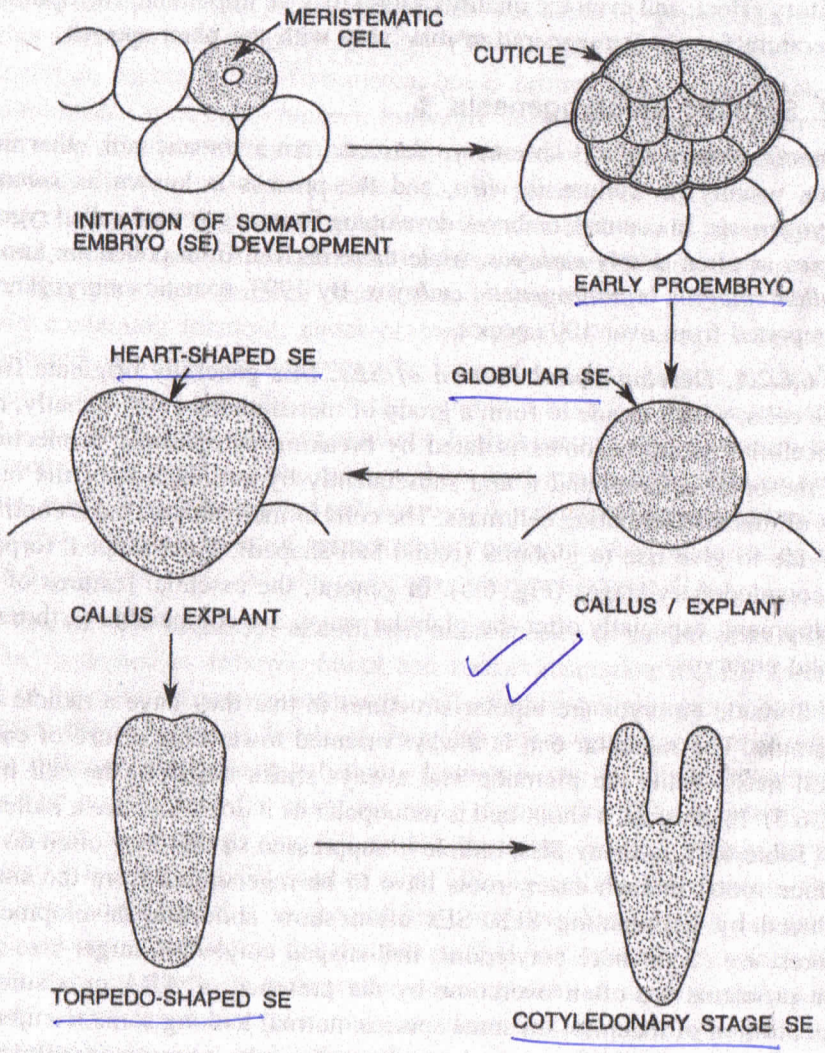

Fig. 6.5. Development of a somatic embryo from a single superficial cell of the explant.

auxin is either removed or reduced (0.01-0.1 mg/l) and cell density is lowered, each EC gives rise to few to several SEs; each SE is believed to develop from a single superficial cell. The ability to regenerate SEs, *i.e.*, totipotency, is acquired by cells during dedifferentiation in response to high auxin treatment but the mechanism is not well known.

Some glycoproteins produced by totipotent cell masses are secreted into the medium; when these proteins are added into the culture medium they

TABLE 6.3
A comparison between shoot buds and somatic embryos

Characteristic	*Shoot bud*	*Somatic embryo*
Origin	Many cells, usually superficial	Single cell, usually superficial
Polarity	Unipolar; only the shoot pole present	Bipolar; both shoot and root poles present
Vascular connection with callus/explant	Present; vascular strands connected with those present in callus/explant	Absent; there is no vascular connection with callus/explant
Separation from callus/explant	Not easily separated unless cut off	Easily separated since the radicular end is cutinized.

speed up the process of acquisition of totipotency. A class of proteins, called ***arabinogalactan proteins***, induces SE regeneration in undifferentiated carrot cells, indicating their role in the process. Auxins promote hypermethylation of DNA which may have a role in totipotency acquisition.

In many species like carrot, coffee; alfalfa etc., somatic embryogenesis is a two step process: (*i*) SE induction on high auxin (up to 40-60 mg/l 2, 4-D) and (*ii*) SE development on a low auxin or GR-free medium. In the ***SE induction*** phase, explant cells dedifferentiate, became totipotent and, in many species, form embryogenic clumps (ECs). Cells can be maintained in embryogenic stage on the induction medium for prolonged periods (over 10 years in carrot).

When ECs are transferred from induction medium to an appropriate medium, SE differentiation proceeds from globular, heart-shaped, torpedo to cotyledonary stages; this is called ***SE development phase***. Clearly in species like carrot, etc., GR requirements for the two phases are drastically different. In most cases, SEs begin to germinate immediately after the cotyledonary stage; this is called ***SE conversion.*** But often the plantlets so obtained are rather weak. It is, therefore, desirable to subject SEs to a ***maturation phase,*** following their development; in this phase the SEs usually do not grow but undergo biochemical changes to become more sturdy and hardy. SE maturation is achieved by culturing than on a high sucrose (up to 6% or even 40%) medium or in presence of a suitable concentration (0.2-0.4 mg/l) of ABA, or by subjecting them to partial desiccation. In most species, SE maturation improves their conversion, often by several-fold.

In some species, *e.g.*, *Cicer arietinum,* wheat etc., SE induction and development may take place on the same high auxin medium, although the frequency of mature embryos is rather low. In some species, SEs are produced

in response to a cytokinin, *e.g.*, BAP induces SEs in hypocotyls of young zygotic embryos of *Trifolium* sp., pea, etc. But SEs are produced on immature cotyledons of these explants when 2,4-D is used in the medium. It seems that cytokinins are effective in SE regeneration from embryogenic cells of young zygotic embryos, while auxins are effective on differentiated cells of both embryos and somatic tissues. Many workers have used combinations of auxins and cytokinins for SE regeneration in different species, but the role of cytokinin in these systems is not known.

6.6.2.3. *Nitrogen Source.* The form of nitrogen has a marked effect on somatic embryogenesis. In carrot, NH^+_4 has a promotive effect on SE regeneration. In fact, induction of SEs in carrot occurs only when about 5 m mol/kg of cell fresh weight NH^+_4 is present in the cells. This level of endogenous NH^+_4 is reached with only 2.5 m mol/l of exogenous level of NH^+_4, while 60 m mol/l NO^-_3 is needed for the same. Therefore, the presence of a low level of NH^+_4 (in carrot 10 m mol/l is optimal) in combination with NO^-_3 is required for SE regeneration. In carrot, NH^+_4 is essential during SE induction, while SE development occurs on a medium containing NO^-_3 as the sole nitrogen source.

6.6.2.4. *Genotype of Explant.* Explant genotype has a marked influence on SE regeneration, and in many cases it may determine whether or not SE regeneration will occur. Strong genotypic effects have been shown in many species, *e.g.*, alfalfa, wheat, maize, rice, chickpea, etc. In case of alfalfa, individual genes affecting SE regeneration have been identified. In case of wheat, chromosome 4B is important in regeneration, a major gene affecting regeneration is located on the long arm of chromosome 2D, minor genes on the long arm of chromosome 2A and short arm of 2B, and a regulatory gene on the long arm of chromosome 2B. Variation for regeneration ability is mainly additive and highly heritable in maize, rice and wheat, but in barley dominance seems to be more important. In case of wheat, rice and maize cytoplasm has a strong influence on regeneration.

6.6.2.5. *Other Factors.* Certain other factors are reported to affect SE regeneration. For example, high K^+ levels and low dissolved O_2 levels promote SE regeneration in some species. In some other species, *e.g. Citrus medica,* some volatile compounds like ethanol inhibit SE regeneration. In soybean, low sucrose concentrations (5 and 10 g/l) promote SE regeneration as compared to high concentrations (20 and 30 g/l). In alfalfa, use of maltose as carbon source improves both SE induction and maturation (including germination) as compared to those on sucrose.

6.7. ANTHER CULTURE

Haploid plants may be obtained from pollen grains by placing anthers or isolated pollen grains on a suitable culture medium; this constitutes ***anther*** and ***pollen culture,*** respectively. The anthers may be taken from plants grown in the field or in pots, but ideally these plants should be grown under controlled temperature, light and humidity; the optimum conditions may differ from species to species. Often, the capacity for haploid production declines with the age of donor plants.

Flower buds of the appropriate developmental stage are collected, surface sterilized, and their anthers are excised and placed horizontally on culture medium. Some workers prefer to partially embed the anthers in the culture medium. Flower buds with small anthers may themselves be cultured and, in some cases, the entire inflorescence has been cultured. Care should be taken to avoid injury to anthers since it may induce callus formation from anther walls. Alternatively, pollen grains may be separated from anthers and cultured on a suitable medium.

In many plant species, SEs from the pollen grains of cultured anthers are directly produced, *e.g.*, in *Datura, Atropa, Brassica campestris, B. napus,* several *Nicotiana* sp. (including *N. tabacum* and *N. rustica*), *Petunia axillaris,* etc. In such cases, the plants obtained from germination of embryos are generally haploid, but some polyploids are also produced. But in many other species like rice (*O. vulgare*), barley (*H. vulgare*), wheat, tomato, triticale, etc. pollen grains produce callus from which plantlets may be regenerated under suitable culture conditions (Fig. 6.6). In these cases, the ploidy level of plants varies considerably more than in those where embryos are produced.

Haploid plantlets have been regenerated from pollen grains of about 200 species of over 50 genera and 25 families. Of these, the following are examples of important crop species: potato (*S. tuberosum*), barley, wheat (*Triticum* sp.), rice, *Brassica campestris, Triticale,* many members of Solanaceae and some vegetables.

6.7.1. Pathways of Development

The early divisions in responding pollen grains may occur in one of the following four ways. (*i*) The unicleate pollen grain may divide symmetrically to yield two equal daughter cells both of which undergo further divisions, *e.g., Datura innoxia* (***Pathway* I**). (*ii*) In some other cases, *e.g., N. tabacum, Datura metel,* barley, wheat, triticale, chillies, etc., the unicleate pollen divides unequally (as it does in nature). The generative cell degenerates callus/embryo