THE **EMBRYOLOGY** OF **ANGIOSPERMS**

S S Bhojwani S P Bhatnagar

A popular textbook with B.Sc. (General and Honours) and M.Sc. stude. for the last 25 years, and translated into Japanese, has been thoroughly revised and updated. This edition explains lucidly all the important topics such as development and structure of male and female gametophytes, pollination, fertilization, sexual incompatibility, development of endosperm and embryo, polyembryony, apomixis and seed development. Chapters on embryology in relation to taxonomy and experimental and applied embryology have also been included. The book is profusely illustrated with self-explanatory diagrams and appended with suggested reading.

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Prof. Bhatnagar has published extensively in both Indian and foreign journals, including a monograph on the Loranthaceae. He compiled a Bibliography on the Embryology of Angiosperms. He has also co-authored a textbook on Gymnosperms in 1996.

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THE EMBRYOLOGY OF ANGIOSPERMS

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4th Revised & Enlarged Edition

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CONTENTS

CHAPTER

1

HISTORICAL ACCOUNT

Three thousand years ago, the Arabs had some knowledge of the part played by pollen in fruit development in date palm. However, sexual cells were observed only after the invention of the microscope, and the credit for this goes to Leeuwenhoek (1677). Grew (1682), for the first time, highlighted the fact that stamens are the male organs of the flower, and the pollen grains in some way influenced the ovary to produce fruit.

Camerarius (1694) had a more scientific approach. He noticed that a female mulberry tree, near which no male plant was growing, formed only abortive seeds. He was inspired by this discovery, and experimented with some female plants of *Mercurialis annua*. These he kept isolated from the male plants. Again, fertile seeds were not produced. He made similar observations in *Ricinus* and *Zea mays*. From these findings he concluded that anthers are the male sex organs of the plant, and the ovary, with style and stigma, the female sex organ.

The significance of pollination in seed-set, and the importance of insects in pollination was recognized by Kolreuter (1761). He also made notable contribution to pollen morphology. With a primitive type of microscope, he was able to identify two distinct layers in the covering of pollen grains. However, the most significant contribution of Kolreuter concerning sexual reproduction was the artificial production of hybrids in *Dianthus, Hyoscyamus, Mathiola, Nicotiana*, etc. He observed that if a stigma was pollinated with the pollen of the same species, as well as foreign pollen at the same time, normally, only the pollen of the same species were effective in fertilization.

As happens with many important discoveries, the discovery of pollen tube was also an accident which triggered a chain of many significant events. The real credit for revealing the actual role of pollen in fertilization goes to Amici (1824), an Italian mathematician, astronomer, and a meticulous microscope maker. While examining the stigma of *Portulaca oleracea*, he saw a pollen grain adhering to a stigmatic hair. He kept it under observation and, to his surprise, the pollen grain split open followed by emergence of a tube, or 'gut', which entered the tissues of the stigma. Repeating his observation in 1830, Amici confirmed that the pollen tube, after emerging

true; some, renouncing all observations of their own, dressed up the phantom in the theoretical principles; others with microscope in hand, but led stray by their preconceptions, believed that they saw what they could not have seen, and endeavoured to prove the correctness of Schleiden's notions with the aid of countless figures, with anything but truth to recommend them; and how an academy by rewarding such work once again confirmed what has repeatedly been seen in the past, especially in our own subject, that prize essays are little suited to the solution of a doubtful question in science." (Cited from P. Maheshwari, 1963). Besides solving the controversy regarding the origin of embryo, we are indebted to Hofmeister for his other contributions to embryology. Among these are his own observations on the formation of microspore tetrads, and the organization of embryo sac.

Embryological studies gained great momentum with the advent of microtome, and refinement in staining methods leading to the discoveries of the various types of embryo sac development, endosperm and embryo.

One of the most significant discoveries was made by Strasburger in 1884. He observed the actual fusion of the male gamete with the female gamete (egg) in *Monotropa* and some other plants; this phenomenon is

Fig. 1.3 Edward Strasburger

known as syngamy. Since two male gametes are released by the pollen tube; what happened to the second male gamete? The answer was provided by Nawaschin (1898) who, working with *Fritillaria* and *Lilium*, showed that one male gamete fused with the egg (syngamy), and the other one with the polar nuclei (triple fusion). This was the discovery of double fertilization, later found to be of universal occurrence in angiosperms.

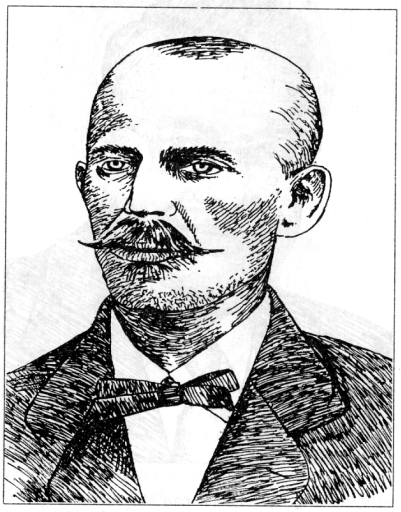

Fig. 1.4 Sergius Nawaschin

By 1900 we had enough information about the development of male and female gametophytes, and embryo. Coulter and Chamberlain (1903) presented this data in their book *Morphology of Angiosperms*. After 26 years, Karl Schnarf of Vienna published two very valuable books namely *Embryologie der Angiospermen* (1929) and *Vergleichende Embryologie der Angiospermen* (1931). In the early thirties and forties of the twentieth century, India started taking the lead in the field of embryology, and the name of P. Maheshwari stands foremost. His book *An Introduction to the Embryology of Angiosperms* (1950) is a masterly exposition of the subject, and is still unsurpassable as a text in embryology. This was followed by an edited volume by P. Maheshwari entitled *Recent Advances in the Embryology of Angiosperms* (1963). Three years later, G.L.Davis (Australia) brought out a very useful book *Systematic Embryology of Angiosperms* (1966).

out of the pollen grain, grew bit by bit and, finally, came in contact with the ovule.

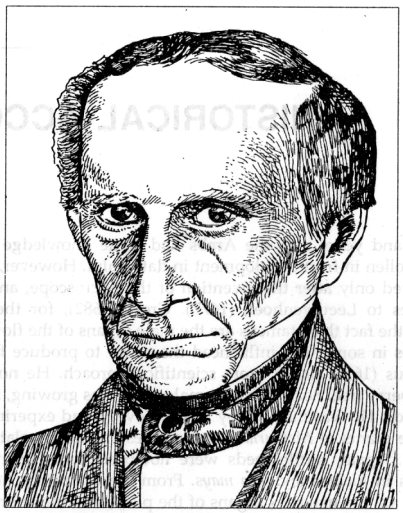

Fig. 1.1 Giovanni Battista Amici

Several other persons got interested in this problem, and mention may be made of Schleiden (who with Schwann propounded the Cell Theory in 1839), who made detailed observation on the ovule. He agreed with Amici as far as the growth of the pollen tube through the stigma and style and its entry into the ovule are concerned. A man of great imagination, Schleiden asserted that the tip of the pollen tube after entering the embryo sac developed into the embryo. According to him, the embryo sac acted only as an 'incubator' wherein the tip of the pollen tube was nourished giving rise to a new plantlet. Such a conclusion would obviously negate sexuality in plants. Nevertheless, Schleiden was able to muster enough support for his absurd idea; notable among his supporters was Schacht.

Amici (1842) opposed the observations of Schleiden and tried to show that the embryo developed from a portion already existing in the ovule, and not from the tip of the pollen tube. Schleiden (1845) ridiculed those who raised objections against his findings, and vehemently challenged the observations of Amici. Schleiden went to the extent of inviting people to come and see for themselves that what he believed was the real truth.

Amici, in the face of mounting criticism, provided conclusive evidence and demonstrated that in *Orchis* the 'germinal vesicle', which was already present inside the ovule, was stimulated by the presence of the pollen tube and developed into the embryo (Amici, 1847) . Eventually, however, it was truth that triumphed. Amici received a lot of support from many workers, prominent among them was Hofmeister. In a most convincing demonstration, Hofmeister (1849) traced the formation of embryo from the egg right up to its maturity in the seed. He confirmed this in several genera of angiosperms, leaving no doubt as to the correctness of Amici's findings.

Fig. 1.2 Wilhelm Hofmeister

Schleiden and Schacht continued to hold their views for some time. It was only when the evidence against their views began to grow that both of them withdrew their assertions. The controversy came to an end with the publication of Radlkofer (1856) in which he fully supported the detailed observations of Hofmeister (1849) that the embryo originated from a cell of the embryo sac and not from the pollen tube.

We may recall here the words of Von Mohl (1863) at the time of Amici's death: "Now that we know that Schleiden's doctrine was an illusion, it is instructive, though sad, to see how willing men were to accept the false for

With the advancement of comparative knowledge on the developmental sequence in the life history of many angiosperms it became possible to fruitfully apply the data to the study of taxonomic inter-relationships of numerous genera and families (Johri *et al.* 1992).

Fig. 1.5 Panchanan Maheshwari

While developmental and comparative embryology continued to yield interesting results, in mid 1960's the emphasis in research shifted towards the functional and applied aspects of embryological systems, involving the use of the modern methods of physics and chemistry, such as histochemistry, biochemistry, fluorescence and electron microscopy, autoradiography, computers and tissue culture.

During 1965 to 1975 considerable work was done to describe the organization of the female gametophyte and the ultrastructure of its constituent cells. In this area the contributions of W.A. Jensen and his colleagues at the University of California, Berkeley, are noteworthy. They and, subsequently, several other groups demonstrated that before fertilization the synergids are metabolically more active than the egg and play an important role in the process of fertilization. A pollen tube discharges its contents in one of the synergids from where one of the sperms migrates

to the egg and the other one to the central cell. The universal occurrence of filiform apparatus has been correlated with this function of the synergids.

Fig. 1.6 William A. Jensen

Almost the same time Heslop-Harrison and his students and associates in different parts of the world made concentrated efforts to understand the structure and development of pollen wall and the involvement of pollen wall materials in pollen-pistil interaction following pollination.

In 1973, Cass had shown that the two sperms produced by a male gametophyte remain together for considerable time. Later several workers demonstrated that the vegetative nucleus also remains in contact with the sperm cells, and the 3 cells move through the pollen tube as a "male germ unit" (*see* Chapter 4). These studies brought to light the phenomenon of sperm dimorphism, and suggested that the fate of the two sperms delivered by a pollen tube may be predetermined. Knox and his associates presented a computer-assisted, three dimensional structure of the male germ unit of *Brassica campestris* (McConchie *et al.*, 1985) and *Zea mays* (McConchie *et al.*, 1987). It is now certain that, at least, in some plants the inheritance of extranuclear genes is bi-parental.

In 1973, Cass reported separation of sperm cells from the vegetative cell cytoplasm of barley pollen. However, Russell (1986) was the first to obtain

San-Noeum. Since then gynogenetic haploids have been raised through ovary or ovule culture in at least 16 species (*see* Chapter 17).

Manipulation of embryological processes has also been attempted through genetic engineering (*see* Chapter 17). Mariani *et al.* (1990) developed a widely applicable method to induce male sterility in flowering plants. It involves inserting a chimeric gene, comprising a bacterial gene coding for ribonuclease and a tapetum-specific promoter. More recently, parthenocarpic eggplant has been produced by introducing an auxin-coding bacterial gene (Rotino *et al.*, 1997).

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CHAPTER

2

FLOWER

Flowers have served as an excellent offering to God, an invaluable aid for personal make-up, and a source of inspiration to poets. Since the beginning of civilization man has been accustomed to use flowers to make his festivals more festive. In Sanskrit literature of the ancient past flower epigrams have been used copiously to interpret romantic situations. Being so much involved with man's daily life, flowers have developed a language of their own and can speak volumes of human sentiments.

For plants, flowers are structures of sexual reproduction. The various embryological processes described in the following chapters of this book occur in the flower. Therefore, while studying embryology it is essential to acquire familiarity with the organization of flower. To deal with all aspects of flower is not possible here. However, in this introductory chapter only some general aspects of floral organization would be described. Readers interested in further details may consult Eames (1961), Sattler (1973), and Gifford and Foster (1989).

For some time, after seed germination, plant continues to grow vigorously and bears only foliar leaves. This is the vegetative phase. At certain point in the life cycle the plant switches over to the reproductive phase and starts bearing flowers. The vegetative phase may last only a few weeks, as in chickweed, or more than a year, as in many perennial trees. *Agave* is described as monocarpic because it flowers only once in its life-time. The switchover from the vegetative phase to the reproductive phase is influenced by a variety of internal and external factors, and it is now possible to shorten or prolong the vegetative phase by altering the light conditions and temperature, and by the application of certain chemical substances.

Plant morphologists regard flower as a shoot of determinate growth with highly condensed internodes, and the leaves specialized variously to suit the functions of different floral organs. During floral initiation the shoot apex gets transformed into floral apex. The vegetative apex is usually conical with distinct zonation into the outer tunica and the subjacent corpus. The cells of the tunica stain lighter, possess larger nuclei, and are more vacuolate than the cells of the corpus. During floral initiation the apex forms the mantle which includes the tunica and a few outer layers of the corpus. The cells of the mantle stain uniformly. Whereas during vegetative

an enriched fraction of purified sperm cells. Since then several protocols have been described for the isolation of large numbers of live sperms by bursting of pollen or pollen tubes in a variety of angiosperms. The possibility to manipulate isolated sperms has given birth to a new field of pollen biotechnology (*see* Shivanna and Sawhney, 1997).

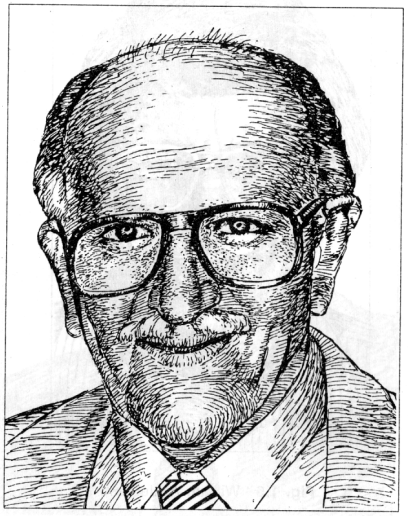

Fig. 1.7 Jack Heslop-Harrison

For long, suspensor was regarded as an ephemeral structure of not much importance in embryo development. However, a closer examination of the physiological, biochemical and ultrastructural features of this structure has established beyond doubt that, during early stages of embryogenesis, suspensor is metabolically more active than the embryo proper and plays an important role in the nutrition of the proembryo (*see* Chapter 12).

Currently, several scientists are investigating the genetics of embryogenesis in angiosperms (Goldberg *et al.*, 1994). To identify the genes involved in events such as pattern formation, and cell and organ differentiation during embryogenesis, the classical approach of screening developmental mutants defective in normal embryo development is being followed.

Experimental embryology has a long history. As early as 1904, Hannig had initiated work on embryo culture. In 1925 the potential of this technique

in increasing the success rates of hybridization programmes was demonstrated (Laibach, 1925, 1929). Since then *ex-ovulo* and *in-ovulo* hybrid embryo culture has enabled raising several rare hybrids which normally failed due to premature abortion of the hybrid embryo (*see* Chapter 17). Until recently it was not possible to culture isolated pre-globular zygotic embryos. Therefore, in the crosses where abortion occurred at an early stage embryo rescue was possible only by *in-ovulo* embryo culture. In 1993, Liu *et al.* achieved complete embryogenesis in the cultures of excised 8-celled proembryos of mustard, by using a rich nutritive medium and a modified culture technique. Almost the same time, Kranz and Lorz (1993) and Holm *et al.* (1994) succeeded in raising full fertile plants from *in vitro* and *in vivo* formed zygotes of maize and barley, respectively, by supplementing the culture medium with nurse cells (*see* Chapter 17).

In early 1960s, embryologists developed the techniques of intra-ovarian pollination (Kanta *et al.*, 1960) and test tube pollination (Kanta, 1960) to widen the scope of sexual hybridization by bypassing the barriers to crossability for which reaction occurs on the stigma or in the style. By 1990 methods were available to isolate large numbers of living male and female gametes of angiosperms, and studies had been initiated to manipulate the naked gametes. In 1993, Kranz and Lorz succeeded, for the first time, in regenerating full fertile plants of maize from zygotes produced by *in vitro* fusion of egg and sperm cells. This report of *in vitro* fertilization has opened up a new approach to crop improvement (*see* Chapter 10).

In 1958 it was reported for the first time that somatic cells can form embryos like the zygote (Reinert, 1958; Steward *et al.*, 1958). Since then somatic embryogenesis has been observed in over 100 species. Embryogenic cultures of carrot are being used to clone embryo-specific and embryo-enhanced genes (*see* Chapter 13). The possibility of producing millions of somatic embryos within a short period offers the opportunity to exploit this phenomenon in clonal multiplication of plants on commercial scale. Artificial seeds produced by encapsulating somatic embryos in a suitable jelly would allow mechanical sowing of somatic embryos in the field (Redenbaugh, 1993).

The role of haploids in breeding and genetics of higher plants has been emphasized for considerable time but the restricted availability of such individuals with gametic number of chromosomes did not allow their exploitation. In 1964 and 1966, Guha and Maheshwari demonstrated the possibility of raising a large number of haploids from pollen grains (androgenesis) of *Datura innoxia* by culturing immature anthers. To-date anther/pollen culture has enabled raising haploid plants or cell lines in over 200 species. In many plants it has been possible to raise androgenic plants by isolated microspore culture, which offers many advantages over anther culture. Androgenic haploids are being used to produce improved cultivars of crop plants (*see* Chapter 17). The limitation of anther or pollen culture in producing viable haploids of some species prompted exploitation of the alternative source of haploid cells, the female gametophyte. The first report of *in vitro* gynogenesis in *Hordeum vulgare* was published in 1979 by

phase the rate of cell division is higher in the corpus, in the floral apex the mitotic activity shifts to the mantle. The floral apex is flat and wider than the vegetative apex. Cell divisions in the mantle do not bring about elongation of the axis. Instead, it differentiates various floral whorls in acropetal succession. The floral axis bearing the floral organs is known as the receptacle. Since there is no elongation of the internodes on the receptacle, the various floral whorls differentiate close to each other.

Flowers exhibit a great variation in size, colour, shape and insertion of different floral whorls. The duckweed, *Wolffia microscopica*, has flowers about 0.1 mm across and are the smallest among the angiosperms. The largest flower known, up to a metre in diameter, occurs in certain species of *Rafflesia*, a root parasite found in the forests of Malaysia. The colour of flowers, which is usually due to the colour of petals, ranges from dead white through ivory, yellow, orange, red and, finally, violet and blue. Combinations of these can give rise to nearly the whole of the visible spectrum of colours. In spite of these and limitless other variations in the structure of flower, its basic organization is fairly uniform.

To describe the structure of a perfect flower we have chosen carnation (*Dianthus caryophyllus*; Fig. 2.1A-G), an ornamental herb of winters in Delhi. The stalk of the flower through which it is connected to the remaining part of the plant is referred to as pedicel (Fig. 2.1A, B). The leaf-like structures bearing flowers in their axil are called bracts. Similar foliar structures present at the summit of the pedicel and subtending the remaining parts of the flower, are the bracteoles (Fig. 2.1A, B). The swollen part of the floral axis above the level of bracteoles is the receptacle. Four types of organs are borne on the receptacle in distinct whorls. The outermost whorl is the calyx, comprising five green sepals fused at their base. Inner to calyx is a whorl of five brightly coloured petals, collectively called corolla. Next to corolla, towards the inside, are ten stamens arranged in two whorls of five stamens each. They are collectively called androecium and represent the male sex organs of the flower. Each stamen consists of a slender stalk (the filament) bearing at its tip purple bilobed anther (Fig. 2.1C, D). The male gametophytes (pollen grains) are formed within the anther (*see* Chapter 3). In the centre of the flower stands the female reproductive organ, the gynoecium. It comprises two units, each called a carpel. As is evident from Fig. 2.1E, the two carpels are fused at the base forming a common swollen ovary. At the top of the ovary are present two slender structures called styles. The tips and a part of the inner margin of the styles are furnished with brown papillae forming the stigma. The latter is the site for pollen landing at the time of pollination. The ovules (megasporangium with its protective coats) are borne inside the ovary attached to a central column, the placenta (Fig. 2.1F, G).

A flower is regarded as complete if it has all the four floral whorls, i.e. calyx, corolla, androecium and gynoecium, as in carnation. If any one of these parts is missing the flower is described as incomplete. An incomplete flower can either be perfect, having male as well as female sex organs, or imperfect with either of the two sexes missing. Flowers with only male sex

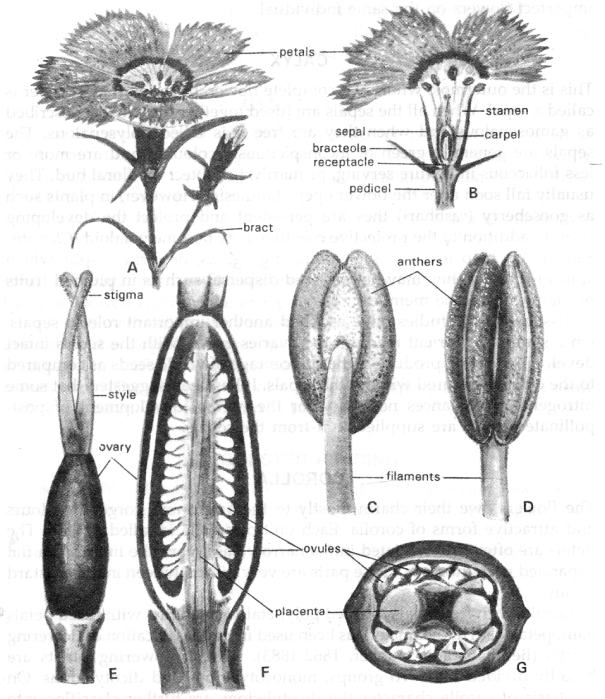

Fig. 2.1. *Dianthus caryophyllus*. **A.** Flowering twig. **B.** Longitudinal-half of a flower to show its various parts. **C, D.** Anthers with a part of the filament; **C**, dorsal view, and **D**, ventral view. **E.** Carpels; the ovaries of both the carpels are fused completely whereas the styles and stigmas are free. **F, G.** Longitudinal (**F**) and transverse (**G**) sections of the ovary. The ovules are borne on a central axis, the placenta.

CHAPTER

3

MICROSPORANGIUM

Pollen grains, which contribute the male gametes, are formed within an anther. A typical anther is tetrasporangiate (Fig. 3.1). It has a column of sterile tissue, called the connective, on either side of which is an anther lobe. Each lobe has two microsporangia separated by a strip of sterile tissue (Fig. 3.1). Externally, the partitions can often be made out by the

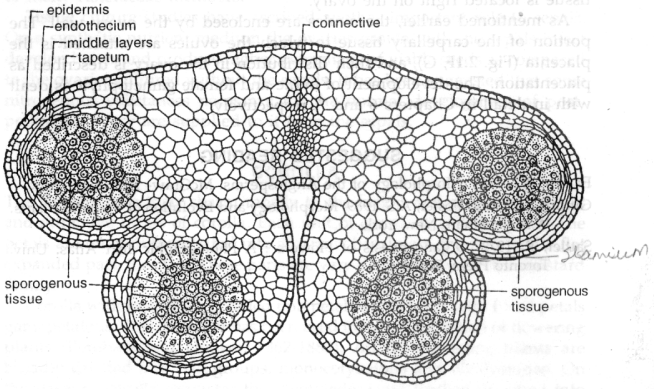

Fig. 3.1 Transverse section of a tetrasporangiate anther to show its various tissues.

deep longitudinal grooves (Figs 2.1D, 3.1). At maturity the two sporangia in a lobe become joined due to the breakdown of the partition between them. In some plants, such as *Moringa* and *Wolffia*, each anther lobe has only one microsporangium (monothecous). Rarely, there may be just one microsporangium per anther as in *Arceuthobium* (Fig. 3.2).

A very young anther comprises a homogeneous mass of cells bound by a well-defined epidermis (Fig. 3.3A, B). During its development the anther

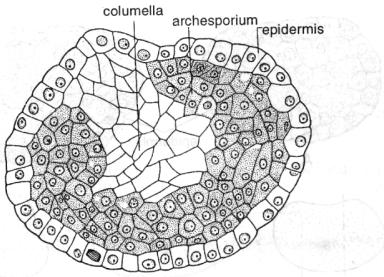

Fig. 3.2 Transverse section of an anther of *Arceuthobium minutissimum* showing a single horse-shoe-shaped archesporium and sterile central columella. (*after* Bhandari and Nanda, 1968)

assumes a four-lobed appearance (Fig. 3.3C-E), and in each lobe some hypodermal cells become more prominent than the others because of their larger size, slight radial elongation, and conspicuous nuclei (Fig. 3.3C_1, D_1). These cells constitute the archesporium. Mostly, the archesporium is composed of many vertical rows of cells, and in transverse section of the anther it appears as a plate of cells (Fig. 3.3C_1). Rarely, however, it is formed of a single vertical row of cells and, consequently, only a single archesporial cell can be seen in a cross-section of the anther.

Stages in the differentiation of sporogenous tissue are shown in Fig. 3.3. The archesporial cells divide in a plane parallel to the outer wall of the anther lobe (periclinal division), cutting off parietal cells toward the epidermis and primary sporogenous cells toward the interior of the anther (Fig. 3.3D_1). The cells of the parietal layer undergo a series of periclinal and anticlinal divisions (Fig. 3.3E_1) to form 2-5 concentric layers of anther wall. The primary sporogenous cells, either directly or after a few mitoses, function as microspore mother cells. Schematic representation of the ontogeny of various wall layers and microspore mother cells in *Alectra thomsoni* is given in Table 3.1.

ANTHER WALL

The mature anther wall comprises an epidermis followed (on the inside) by a layer of endothecium, 2 or 3 middle layers, and a single-layered tapetum (Fig. 3.1).

Epidermis

During anther development the epidermal cells undergo repeated anticlinal divisions in order to cope up with the rapidly enlarging internal tissue. In

organs are described as staminate, and those with only female sex organs are called pistillate. In corn, the staminate and pistillate flowers are present on the same plant, whereas in hemp they occur on different individuals. The plants of corn-type are called monoecious and those of hemp-type dioecious. Polygamous plants, as for example mango, bear perfect and imperfect flowers on the same individual.

CALYX

This is the outermost whorl of a complete flower. Its individual member is called a sepal. When all the sepals are fused together the calyx is described as gamosepalous and when they are free it is called polysepalous. The sepals are generally green or inconspicuously coloured and are more or less foliaceous in texture serving, primarily, to protect the floral bud. They usually fall soon after the flower opens (anthesis). However, in plants such as gooseberry (rasbhari) they are persistent and protect the developing fruit. In addition to the protective role they may become petaloid (*Clematis, Salvia*) and help in attracting pollinating agents, or form a spur which stores nectar, or they may help in seed dispersal such as in plumed fruits of many Asteraceae members.

Tissue culture studies have ascribed another important role to sepals. On a synthetic nutrient medium the ovaries grown with the sepals intact develop better and produce higher percentage of viable seeds as compared to the ovaries cultured without the sepals. It has been suggested that some nitrogenous substances necessary for the normal development of post-pollinated ovary are supplied to it from the sepals.

COROLLA

The flowers owe their charm mostly to the bright and gorgeous colours and attractive forms of corolla. Each unit of corolla is called a petal. The petals are often differentiated into a narrow basal part, the limb and a flat expanded part, the claw. These parts are very distinctly seen in the mustard family.

Corolla with free petals is called polypetalous, and that with fused petals gamopetalous. This character has been used in the classification of flowering plants (Bentham and Hooker, 1862-1883). All the flowering plants are broadly divided into two groups, monocotyledons and dicotyledons. On the basis of corolla character the dicotyledons are further classified into three groups: (i) *Gamopetalae* - plants with gamopetalous corolla, (ii) *Polypetalae* - plants with polypetalous corolla, and (iii) *Monochlamydeae* - plants lacking a distinct corolla.

When the sterile appendages in a flower are not distinguishable into calyx and corolla, as in Monochlamydeae, they are collectively called perianth and their individual member a tepal. The corolla and perianth protect the young reproductive organs and help in pollination through their attractive colours and curious forms.

ANDROECIUM

This is the collective name for the male reproductive elements, the stamens. In the so-called primitive flowers a stamen is a broad leafy structure bearing a pair of elongated pollen sacs embedded on its inner side well removed from the apex. A typical stamen, however, has an elongated sterile filament bearing at its distal end a fertile bilobed anther (Fig. 2.1C). The structure and development of anther are described in Chapter 3.

GYNOECIUM

The female reproductive apparatus in a flower is gynoecium, and its functional units are called carpels. As a rule, the carpels are borne laterally on the receptacle. Even the most apparent terminal position of the carpel in pea family is actually lateral.

A typical carpel comprises a basal swollen ovary with a terminal style surmounted with the stigma. Whereas the ovary and stigma seem to be essential for the normal functioning of carpel, it can do away with the style. In plants, such as *Drimys*, carpels lack a style, and the stigmatic tissue is located right on the ovary.

As mentioned earlier, the ovules are enclosed by the ovary wall. The portion of the carpellary tissue to which the ovules are attached is the placenta (Fig. 2.1F, G), and their distribution in the ovary is described as placentation. The development of ovule and female gametophyte is dealt with in detail in Chapters 6 and 7, respectively.

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Sattler, R. 1973. Organogenesis of Flowers : A Photographic Text Atlas. Univ. Toronto Press, Toronto.

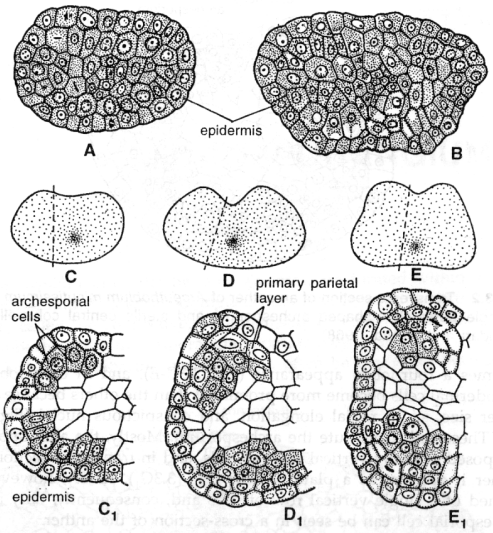


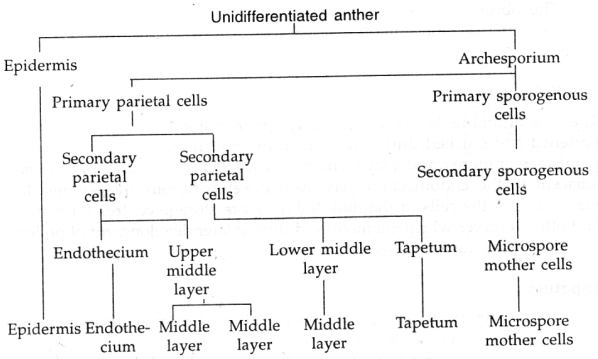
Fig. 3.3 Microsporogenesis in *Centaurium ramosissimum* as seen in transverse sections of the anther. C_1, D_1 and E_1 are cellular details of the portions marked in **C**, **D** and **E**, respectively. **A**, **B**. Undifferentiated anthers. In **B** the anther has become slightly lobed. C_1. Some hypodermal cells have differentiated into archesporial tissue. D_1. Primary parietal layer (toward the outer side) and primary sporogenous tissue (toward the inner side) have differentiated. E_1. The cells of the primary parietal layers are in the process of cutting wall layers. (*after* Vijayaraghavan and Padmanabhan, 1969)

a mature anther they are greatly stretched and flattened. The epidermis performs its usual protective function. In *Zeuxine longilabris* the epidermal cells simulate tapetum (Karnath *et al.*, 1979). They may even become binucleate. An unusual feature is the development of fibrous thickenings in the epidermal cells of *Arceuthobium* (Bhandari and Nanda, 1968) forming exothecium, which is characteristic of the microsporangia of gymnosperms.

Endothecium

Normally the endothecium is single-layered, but in some plants, it may become multilayered. The endothecium originates from the parietal layer,

TABLE 3.1



and is present in the protuberant part of the anther. A similar tissue, when on the inner side, is formed by the cells of the connective. In *Triticale*, however, the entire ring of endothecium is formed by the parietal layer (Bhandari and Khosla, 1981).

The cells of the endothecium are radially elongated. This layer attains its maximum development when anther is ready to dehisce for the discharge of pollen. Cells of this layer develop fibrous bands (Fig. 3.4) which arise from the inner tangential walls (rarely from the radial walls) and run outward and upward, ending near the outer wall of each cell. The outer tangential walls remain thin. The endothecial thickenings, according to de Fossard (1969), contain a high proportion of α-cellulose. Lignin was found to be absent from the endothecial bars. These findings were reported in *Chenopodium rubrum*. According to Vasil (*see* de Fossard, 1969) the endothecial thickenings are always composed of cellulose and are slightly lignified at maturity. Additionally, small amounts of pectin and lignin are present in *Pisum* and *Lens*, respectively (Biddle, 1979). In the anthers which open by longitudinal slits the endothecial cells around the junction of the two sporangia lack these thickenings (*see* Fig. 8.1A, B). The presence of fibrous bands, differential expansion of the outer and inner tangential walls, and the hygroscopic

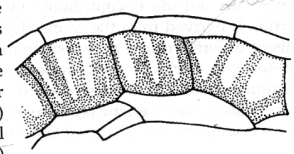


Fig. 3.4 Endothecial cells of *Mimusops elengi*, showing fibrous bands. (*after* Bhatnagar and Gupta, 1970)

nature of the endothecial cells help in the dehiscence of anthers at maturity. The fibrous bands are altogether absent in certain members of the Hydrocharitaceae and some cleistogamous forms, whose flowers never open.

Middle Layers

The cells of middle layers are generally ephemeral and, as a rule, become flattened and crushed during meiosis in the pollen mother cells. In some plants one or more middle layers may persist (*Lilium, Ranunculus*), and one adjacent to the endothecium may even develop fibrous thickenings. In many species the cells of the middle layers are storage centres for starch and other reserves which get mobilized during later development of pollen (*see* also Reznickova and Willemse, 1980).

Tapetum

This is the innermost layer of anther wall and attains its maximum development at the tetrad stage of microsporogenesis. It completely surrounds the sporogenous tissue and is of considerable physiological importance because all the food material to the sporogenous tissue must pass through it. Typically, the tapetum is composed of a single layer of cells characterized by the presence of dense cytoplasm and prominent nuclei (Fig. 3.1).

The tapetum is of dual origin in most angiosperms. The tapetal portion toward the outside is contributed by the parietal layer, while the inner portion is derived from the connective tissue. In a few species, the tapetal cells of different origin may also be different morphologically (dimorphic tapetum). In *Alectra thomsoni*, for example, the tapetal cells toward outside are much smaller than those toward inside (Fig. 3.5).

In *Triticale*, however, the tapetum originates solely from the parietal layer and is homogeneous (Bhandari and Khosla, 1982). Sometimes, the tapetum

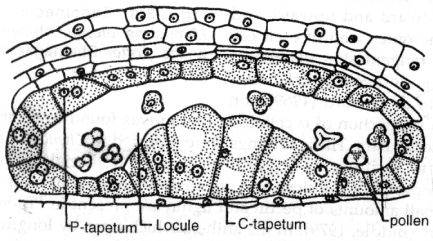


Fig. 3.5. Dimorphic tapetum in *Alectra thomsoni*. The portion of the tapetum with large cells (C-tapetum) is derived from the connective and the remaining portion (P-tapetum) from the parietal layer. (*after* Vijayaraghavan and Ratnaparkhi, 1973)

may also arise from the sporogenous tissue, as in *Antirrhinum majus* and *Impatiens globulifera*.

Based on its behaviour, the tapetum is of two types :

1. AMOEBOID (also called *INVASIVE* or *PERIPLASMODIAL*). This type of tapetum is characterized by an early breakdown of the inner and radial walls of its cells. The protoplast masses move into the anther cavity. This is followed by the fusion of protoplasts to form a tapetal periplasmodium closely investing the pollen mother cells or the microspores. The movement of protoplasts into the locule may take place during meiotic prophase or may be delayed until the tetrad stage. This type of tapetum is found in *Alisma, Arum, Butomus, Tradescantia, Typha, Helianthus, Mahonia,* etc.

In a detailed ultrastructural study of amoeboid tapetum in *Tradescantia*, Mepham and Lane (1969) have shown that the tapetum possesses an organized and functional structure. As development proceeds the cell organelles in the periplasmodium undergo reorganization rather than degeneration. The nuclei in the periplasmodium also show divisions. The breakdown of the tapetal cell walls, which occurs at the premeiotic stage of sporogenous tissue, is probably due to the hydrolytic enzymes released by dictyosomes in the tapetal cells themselves. Often these enzymes reach the anther locule and digest the walls of some sporogenous cells also. During meiosis the periplasmodium surrounds each microspore mother cell. After meiosis the callose wall around the spores is degraded, and the individual spores are bathed in the tapetal cytoplasm just before anthesis. The enzymes which are responsible for the degradation of the callose wall are also considered to be derived from the tapetal periplasmodium.

Arum italicum has a two-layered, amoeboid tapetum. A semi-diagrammatic summary of the events in the tapetum in relation to the stage of meiocyte development is presented in Fig. 3.6. In the pre-meiotic phase tapetal cells are inter-connected by plasmodesmata (Fig. 3.6A). During the meiotic prophase (Fig. 3.6A-C) the innermost middle layer flattens and certain walls of the tapetal cells start to dissolve. At first the plasmodesmatal connections widen and are transformed into cytomictic channels (Fig. 3.6B). During pachytene the tangential walls, common to both rows of tapetal cells, disappear and many microtubules are seen lying parallel to the radial walls (Fig. 3.6C). By anaphase most of the radial walls between the tapetal cells also disappear, and microtubules are seen toward the inner tangential wall (Fig. 3.6D). The protoplasts of the tapetal cells, now fused into a single mass, start to penetrate between the microspore mother cells at the telophase I (Fig. 3.6E). By the tetrad stage the inner tangential wall of tapetal cells disappears and the amoeboid tapetum begins to envelop the tetrads (Fig. 3.6F). The plasma membrane of tapetal cells, which now surrounds the tetrads, makes close contact with their callosic wall, and some microtubules in the tapetal cytoplasm are seen running as arcs more or less parallel to the tetrad wall (Fig. 3.6F). By this stage the inner row of the middle layers shows degeneration of their nuclei. With the release of microspores from the tetrads (early uninucleate stage) the outer wall of the tapetal cells also disappears. At this stage the tapetal contents can be separated into two

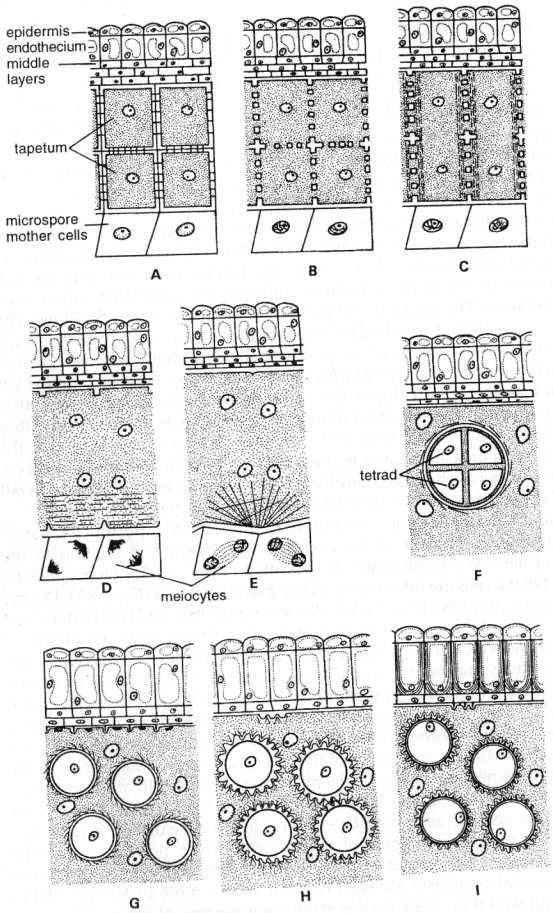


Fig. 3.6A-I. Diagrammatic summary of events in the plasmodial tapetum in relation to microspore development in *Arum italicum*. For details see text. (*after* Pacini and Juniper, 1983)

distinct zones. The inner zone surrounding the microspores contains polyribosomes, microtubules arcing parallel to the surface of the microspores, vesicles and a few dilated ER cisternae. The outer zone, on the other hand, contains a few small vacuoles, nuclei, ribosomes, polyribosomes, mitochondria and plastids. The cytoplasm of the inner zone adheres to the microspore surface (Fig. 3.6G). At the mid-uninucleate stage the tapetal plasmalemma surrounding the microspores retracts in a convoluted fashion from the exine surface leaving roughly cone-shaped spaces (Fig. 3.6H). This phenomenon also occurs at the periphery of tapetum but discontinuously and with less precision. The spaces left between the microspore surface and tapetal periphery by localized retraction of the plasmalemma are filled with the material forming spines on the exine (Fig. 3.6I). At this late uninucleate stage of the microspores the tapetal cytoplasm is no longer distinguishable into the two zones. The polyribosomes and the individual ribosomes reduce in number and the microtubules are no longer seen.

The close association between the developing microspores and the tapetal cytoplasm until just prior to anthesis suggests that the latter must be exercising a selective influence on the transport of material into the pollen.

2. SECRETORY (also called *PARIETAL* or *GLANDULAR*). In this type of tapetum its constituent cells remain in their original position throughout the microspore development. Substances are contributed to the anther sac by secretion from the inner faces of the cells until their total breakdown at the time of pollen maturity. The secretory type of tapetum is more common among angiosperms and has been studied in far more detail than the amoeboid tapetum.

Echlin and Godwin (1968) have described in detail the events which take place in the tapetal cells of *Helleborus foetidus* (an example of secretory tapetum) from the premeiotic stage to the stage of pollen maturation. At the stage of sporogenous tissue the tapetal cells possess mitochondria, plastids, a number of spherical bodies (pro-Ubisch bodies), and dictyosomes with only a few peripherally associated vesicles. The cell walls are relatively thin at this stage and appear to be composed of middle lamella with a small amount of cellulosic primary wall. Before the onset of meiosis the tapetal cell walls become thick, and the cytoplasm appears more dense due to the increased number of ribosomes and pro-Ubisch bodies. The thickenings of the inner tangential walls of the tapetum (the wall facing the locule) are irregular. During meiosis there is further increase in the number of pro-Ubisch bodies and the size of the nuclei. The pro-Ubisch bodies, although distributed throughout the cytoplasm, appear to be more in the region of the cells nearest the anther cavity.

At the tetrad stage the number of ribosomes further increases and that of microtubules and plastids decreases. At this stage two features of the tapetal cells deserve special mention: (a) The pro-Ubisch bodies, whose number has further increased, now appear to be surrounded by a zone of ribosomes which radiate from them like the spokes of a wheel. The limiting membrane of the pro-Ubisch bodies is discontinuous at those places which

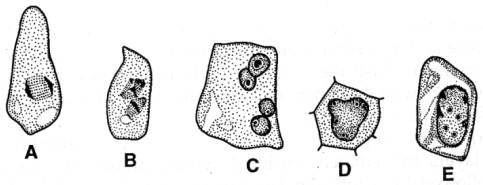


Fig. 3.7 Tapetal cells: **A-C, E.** *Mimusops elengi.* **D.** *Iodina rhombifolia.* **A.** A uninucleate cell in division. **B.** A binucleate cell showing synchronous mitoses. **C.** A tetranucleate cell. **D.** Probably a fusion product of four nuclei is seen in the cell. **E.** Probably an octaploid cell. (A-C, E. *after* Bhatnagar and Gupta, 1970; D. *after* Bhatnagar and Sabharwal, 1969)

a large polyploid nucleus. In the uninucleate tapetum of *Cucurbita pepo* nuclei of four sizes corresponding to their degree of ploidy (2n, 4n, 8n and 16n) are formed as a result of endomitosis.

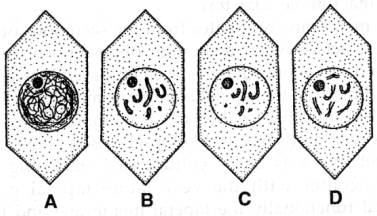


Fig. 3.8 Diagrams to show the process of endomitosis. **A.** Endoprophase. **B.** Endometaphase. **C.** Endoanaphase. D. Endotelephase.

3. *Formation of restitution nuclei.* This also results in polyploid nuclei. Mitosis goes on normally up to the early anaphase stage but the two sets of chromosomes finally get incorporated into a common nuclear membrane to form a restitution nucleus (Fig. 3.9).

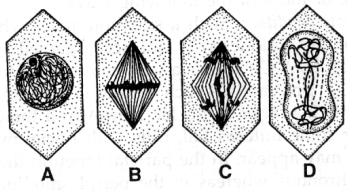


Fig. 3.9 Diagrams to show stages in the formation of a restitution nucleus. **A.** Prophase. **B.** Metaphase. **C.** Anaphase; note the chromosome bridges. **D.** Both the sets of chromosomes are enclosed in a common nuclear membrane to form a restitution nucleus.

4. *Polyteny.* This refers to an increase in the number of chromonemata per chromosome. This mode of DNA increase does not alter the number of chromosomes per nucleus (Fig. 3.10).

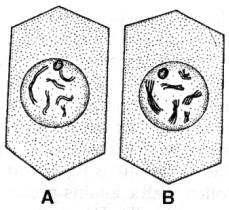


Fig. 3.10 Diagrams to illustrate the phenomenon of polyteny. **A.** A diploid cell with 6 chromosomes. Each one of the chromosomes has only two chromatids. **B.** A tetraploid cell. The number of chromosomes is maintained at six but now each chromosome possesses four chromatids.

FUNCTIONS OF TAPETUM. The tapetum appears to play a significant role in the development of pollen. Its precocious degeneration during premeiotic and meiotic stages or its cellular persistence for unusually long period results in pollen sterility. Some of the roles assigned to the tapetum are:

1. During meiosis it is presumed to be simply transporting the nutrients to the inside of the anther locule since this is the only channel through which material can reach the meiocytes. At this stage the nutrients are not derived from the tapetum.

2. Mepham and Lane (1969) have demonstrated that in *Tradescantia bracteata* the plasmodial cytoplasm derived from the tapetum has callase activity. Shortly before callose degradation starts, the cytoplasm of tapetal cells shows certain vesicles which are probably associated with callase activity. Stieglitz and Stern (1973) measured callase activity separately in anther wall and the microsporocytes from early meiosis to tetrad dissociation. The activity was found to be altogether absent from developing microsporocyte but it was present in wall tissue, reaching its peak at the time of release of microspores from tetrads. This suggests that sporophytic tissue, presumably tapetum, is involved in the synthesis of callase enzyme for the release of microspores in a tetrad by degrading the callose wall.

3. As mentioned earlier, morphological and cytological studies on anther development in male fertile and male sterile lines have shown that formation of non-viable pollen in sterile lines is associated with non-functional or faulty development of tapetum. Frankel *et al.* (1969) and Izhar and Frankel (1971) reported that in cytoplasmic male sterile (CMS) *Petunia,* the microspore mother cells do not show callose wall after prophase-I. Estimation of callase activity in fertile and sterile lines revealed that this abnormality is due to faulty timings of callase appearance in the CMS plants. Whereas in fertile plants callase appears only at late meiosis and tetrad stage, in sterile plants strong callase activity is detected during prophase-I. Based on point 2, it may be concluded that precocious release of callase by tapetum is probably responsible for cytoplasmic male sterility in *Petunia.*

4. During post-meiotic period tapetum plays an important role in pollen wall formation. Its contribution of sporopollenin through Ubisch bodies has been described in Chapter 4.

correspond to the insertion of ribosome rays. (b) The dictyosomes now appear to be associated with a large number of vesicles at the periphery. The dictyosomes and vesicles are never seen associated with the pro-Ubisch bodies. The tapetal cell membrane at this stage appears highly convoluted.

Soon after the microspores have separated from one another the ribosomes surrounding the pro-Ubisch bodies become more prominent, and the inner region of the cytoplasm contains abundant dictyosomes. The pro-Ubisch bodies eventually pass through the tapetal cell membranes (facing the locule) without creating discontinuities in the latter. Once the pro-Ubisch bodies have been extruded from the tapetal cytoplasm into the space between the membrane and the cell wall they rapidly become coated with sporopollenin, and are now called Ubisch bodies. The inner wall of the tapetal cells now appears considerably thinner, and a space appears between the wall and the cell membrane. Pro-Ubisch bodies continue to be formed within the tapetal cytoplasm and extruded from the cells, and sporopollenin is deposited on them. The deposition is initially on the side nearest the locule. All the tapetal cell walls have by this time disappeared, and the cytoplasm has completely disorganized. The limiting membrane is ruptured and only a few recognizable organelles may be found in the residue.

The Ubisch bodies are spheroidal structures only a few microns in diameter. However, frequently they fuse into larger compound aggregates. It seems the Ubisch bodies are produced only by the glandular tapetum and not by the amoeboid tapetum. The Ubisch bodies are involved in the external thickening of the exine, whose pattern is laid down by the spore cytoplasm in the tetrad stage. Often the Ubisch bodies are capable of organizing exine pattern without any contribution from the spore itself. This is suggested by the fact that in spite of the degeneration of the protoplasm in the pollen grains of *Mirabilis jalapa* the exine patterning develops normally. Also, in the male sterile *Silene pendula* normal exine sculpturing develops.

TAPETAL MEMBRANE. In secretory type of tapetum (*Nigella damascena*) at the tetrad stage the inner tangential walls of the tapetum disintegrate and a new membrane, called tapetal membrane, is formed around the tapetal protoplasts along the thecal face (Bhandari and Ram Kishori, 1971, 1973). On this membrane are studded the Ubisch granules. In the Asteraceae, where the tapetum is of the plasmodial type, an extra-tapetal membrane is organized adjacent to the middle layer (Heslop-Harrison, 1969). Unlike other cell membranes, the tapetal and extra-tapetal membranes are acetolysis-resistant. These membranes enclose the developing pollen grains, in the anther locule, like a sac. The intact pollen sacs can be easily isolated in class rooms by macerating the anthers, preferably at the uninucleate pollen stage. Take a few anthers in a test tube, add to it a mixture of acetic anhydride (9 parts) and concentrated sulphuric acid (1 part), and heat the tube. During this treatment the tissue outside the tapetal membrane will disintegrate releasing the pollen sacs. Anthers of *Cryptostegia*, *Hibiscus* and *Ocimum* require heating for only a fraction of a minute to release intact

pollen sacs but the optimum duration of heating may vary with the plant. Overheating would rupture the membrane. After adequate heating, cool the mixture and add some water. Centrifuge it at slow speed and discard the supernatant. Wash the sediment with distilled water twice and stain the pollen sacs with 0.005 per cent aqueous aniline blue for about 24 hours and mount them in glycerine.

Banerjee (1967) reported that in grasses the tapetal membrane consists of three layers. Immediately outside the tapetal protoplast is the fenestrated layer, followed by a reticular layer interconnecting the Ubisch granules. The Ubisch granules themselves constitute the third layer. However, in *Sorghum* (Christensen *et al.*, 1972) and *Nigella* (Bhandari and Ram Kishori, 1973) the membrane does not show the reticulate layer, and comprises only two layers. According to Bhandari and Ram Kishori, in *Nigella* the layer comparable to the fenestrated layer does not stain with spirit blue whereas the layer of Ubisch bodies takes up the stain. On the basis of this selective staining of one of the layers the authors suggested that the fenestrated layer, like the Ubisch granules, consists of sporopollenin but, presumably, in a different macromolecular form.

The tapetal membrane originates from the secretions of the tapetal cells which are most active when maximum deposition of exine takes place. Occasionally, a direct connection has been observed between sporopollenin strands protruding from the tapetal membrane and tips of spinules of exine. The functional significance of the tapetal membrane ("orbicular wall") is not clear. However, according to Heslop-Harrison (1969) the extra-tapetal membrane probably acts as a "culture sac" enclosing the developing microspores together with the very labile tapetal periplasmodium. Chemically and functionally the tapetal membrane and the extra-tapetal membrane appear to be similar. Christensen *et al.* (1972) have suggested that "... orbicular wall could be regarded as a vestigial capacity of the tapetum in that, phylogenetically, this layer was once active sporogenous tissue" (*see* also Bhandari and Ram Kishori, 1973).

NUCLEAR BEHAVIOUR IN TAPETAL CELLS. A common feature of tapetum, amoeboid as well as secretory, is that the total DNA content of the tissue increases enormously through the meiotic prophase. This is achieved in one or more of the following ways:

1. *Multinucleate condition.* Such a situation arises when during mitosis nuclear division (karyokinesis) is not accompanied by wall formation (cytokinesis) (Fig. 3.7A-C). This is a very common feature of tapetal cells. There may be 1, 2, 3 or, rarely, 4 such mitoses in a tapetal cell. Accordingly, the cell will have 2, 4, 8, or 16 nuclei. However, if there is nuclear fusion (Fig. 3.7D,E) or some mitoses fail, cells with nuclei number outside the expected series may appear. In the parietal tapetum the nuclear divisions are rarely synchronous whereas in the periplasmodium they are highly synchronous.

2. *Endomitosis.* This is a type of mitosis in which the chromosome duplication and chromatid separation take place within the intact nuclear membrane and without the formation of a spindle (Fig. 3.8). The result is

5. Most conspicuous example of transfer of material from tapetum to pollen is of pollenkitt substances (*see* page 52) and tryphine. Whereas the former appears to be complex mixture of hydrophobic liquids and carotenoids, the latter seems to be a complex mixture of hydrophilic substances including proteins. Ultrastructural studies by Dickinson (1973) have revealed that both these substances are synthesized in a special population of plastids in the tapetal cells. Biological significance of the pollenkitt materials is described in Chapter 4 (page 53).

6. Pollen wall contains proteins derived from the gametophyte as well as the tapetal cells. The proteins of latter origin are present in the spaces and cavities of the exine. These proteins are rapidly released when the pollen grains are moistened and are responsible for the common hayfever and pollen caused allergy. Another biological significance of these proteins is in the recognition of compatible pistils. If a pollen lands on an incompatible stigma these proteins induce the formation of callose plugs in the stigma or style and block the growth of pollen tube. Similar callose plugs are formed if the tapetum excised at the onset of its dissolution is applied to the incompatible stigma, confirming the tapetal origin of the substances responsible for the "rejection reaction" (Heslop- Harrison *et al.*, 1974).

SPOROGENOUS TISSUE

The sporogenous cells may directly function as microspore mother cells (also called pollen mother cells or PMCs) or they may undergo a few mitoses to add up to their number before entering meiosis. Each PMC, by a meiotic division, gives rise to a group of four haploid microspores. Aggregates of four microspores are referred to as microspore tetrads.

Meiosis

The meiotic division is of great significance in the sexual life cycle of an organism. It is by this division that the diploid sporophytic cells are able to give rise to haploid gametophytes. It is also responsible for the variations due to recombinations. Here we shall not concern ourselves with the chromosomal changes that take place during the division. For this some standard text-book on cytology may be consulted.

Meiosis is a continuous process but for the sake of convenience in describing, cytologists have distinguished the following sequential stages in this division:

Prophase-I	Meiosis-I
Preleptotene	
Leptotene	
Zygotene	
Pachytene	
Diplotene	
Diakinesis	
Metaphase-I	
Anaphase-I	
Telophase-I	
Prophase-II	Meiosis-II
Metaphase-II	
Anaphase-II	
Telophase-II	

Meiosis-I is a reduction division resulting in two haploid cells or nuclei, and Meiosis-II is more or less a normal mitosis.

What precisely is the nature of the stimulus for inducing meiosis in the sporogenous cells is not clear as yet. However, there are indications that the stimulus originates in some other part of the plant and is transmitted to the anther. Clutter and Sussex (1965) suggested, from their work on the culture of isolated fern leaves, that the meiotic stimulus arises in the vegetative shoots of the plant. The stimulus seems highly specific in its action. It is effective only on the sporogenous cells, not even on the tapetal cells through which it must pass in order to reach the sporogenous tissue.

In the preleptotene stage the PMCs are much like the meristematic somatic cells possessing normal cellulosic walls. All the PMCs in an anther locule are interconnected by plasmodesmata (Fig. 3.11A). At this stage plasmodesmatal connections also exist between the tapetal cells and PMCs. With the entry of PMCs into meiosis the connections between the tapetal cells and the PMCs are broken, and the walls of the PMCs become thicker by the deposition of callose (β-1,3-glucan; Fig. 3.11B), contributed by the dictyosomes which appear in abundance in the periphery of the cytoplasm of microspore mother cells. Rarely, as in *Pergularia daemia*, which shows compound pollen grains or pollinia, the callose wall does not develop during or after meiosis (Vijayaraghavan and Shukla, 1977).

Fig. 3.11 Diagrams to show cellular interconnections in developing anther; only single-layered tapetum and the pollen mother cells (PMCs) are drawn. **A**. Preleptotene stage; plasmodesmatal connections exist between PMCs, and between the tapetal cells and the PMCs. PMCs are thin walled. **B**. Meiotic prophase-I. The PMCs are now enclosed in thick callose walls (dark areas) and are interconnected by wider cytoplasmic channels; no cytoplasmic continuities persist between the tapetal cells and PMCs. **C**. Tetrad stage. All the spores are independent cells, no cellular communications are seen.

The deposition of callose usually starts at the corners of the cells between the plasma membrane and the original wall. The latter is finally degraded. Bhandari *et al.* (1981) have, however, noticed the persistence of the original mother wall until the late tetrad stage in *Allium tuberosum* and *Cyclamen persicum*. Although the reason for the persistence of this wall is not clear, the authors opine that this, together with callose, probably acts as a barrier to the entry of macromolecules ensuring autonomous development of microspores. Concurrently with the deposition of callose, the plasmodesmatal connections between the PMCs are replaced by massive cytoplasmic channels (Fig. 3.11B; for a summarized representation of intercellular connections in tapetum, meiocytes and microspores during microsporogenesis *see* Table 3.2). These channels are 1-2 µm in diameter. They attain their maximum development in the zygotene-pachytene stage. Thus, at this stage the whole mass of PMCs in an anther locule forms a large meiocytic syncytium. In some families, such as Mimosaceae and Orchidaceae, the sporogenous tissue in an anther lobe is partitioned by plates of somatic cells. In such plants the syncytium is formed in small groups.

The massive cytoplasmic channels provide passage for the movement of cytoplasmic contents from one cell to the other. Gates (1911) had noted the movement of nuclear material from one PMC to another and termed the phenomenon as cytomixis. It is now believed that cytomixis is a pathological phenomenon rather than a normal event. Movement of chromatin material from one PMC to another occurs through the cytoplasmic channels only upon mechanical injury or squeezing of the anther.

The cytoplasmic continuities of the PMCs impose a mutual influence of one cell over the other. This helps in maintaining a close synchrony during meiosis in the large number of PMCs in an anther locule. The positive role of these channels in maintaining the synchrony is suggested by the following facts:

(a) with the blockage of the cytoplasmic channels at late prophase or, occasionally, during the second meiotic division the synchrony is also gradually lost, and (b) in various orchids where the cytoplasmic channels persist between the sister spores in a tetrad, even pollen mitoses are synchronous.

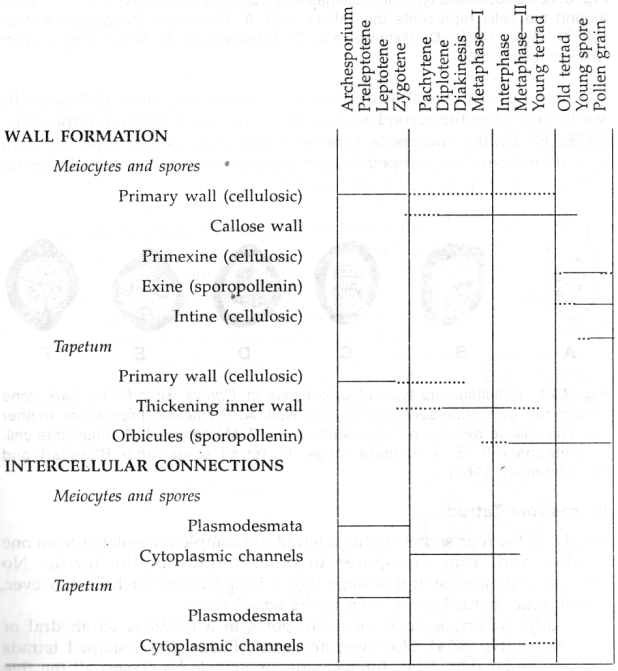
At the end of the meiotic prophase (occasionally at the end of the first meiotic division) the callose walls of the PMCs close up, and the cytoplasmic channels are cut off. With this all the PMCs become independent and go through the rest of the meiosis as isolated cells. Therefore, additional callose walls are formed between the daughter cells (microspores) of the PMCs (Fig. 3.11C).

Cytokinesis. Wall formation, after the meiotic divisions in PMCs, is of two types:

1. *Successive*. After the first meiotic division a wall is formed separating the two nuclei (Fig. 3.12A, B). This leads to the formation of a dyad. The wall formation in this case is centrifugal. A cell plate is formed in the centre and extends laterally. This is followed by the deposition of callose on either side of the plate. The two cells of the dyad undergo the second meiotic division (Fig. 3.12C, D), which may not be synchronous. After the second division callose walls are again formed the same way, resulting in a tetrad (Fig. 3.12E). This type of cytokinesis is common in monocots.

2. *Simultaneous*. In this type of cytokinesis the first meiotic division is not followed by wall formation (Fig. 3.13A- C). Consequently, a binucleate cell is formed after meiosis-I; there is no dyad stage. The two haploid nuclei synchronously undergo the second meiotic division (Fig. 3.13D). The

TABLE 3.2 Formation of walls and intercelluar connections in tapetum, meiocytes and microspores during microsporogenesis. The solid bars represent the period in which the feature is predominant and the interrupted bars indicate the period of early development (when it is present before the solid bar) or dissolution (when it is present after the solid bar). The tapetal sequences refer to the secretory type. (*after* Heslop-Harrison, 1972).



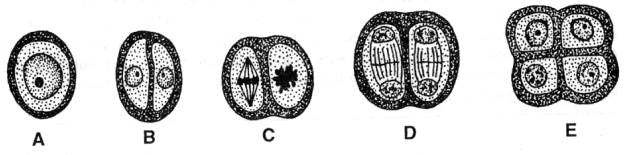


Fig. 3.12 Successive type of cytokinesis in *Commelina subulata*. The dark zone around the cells represents the callose wall. **A.** Premeiotic microspore mother cell. **B.** Dyad stage. **C.** Metaphase-II. **D.** Telophase-II. **E.** Tetrad stage. (*after* Chikkannaiah, 1960)

synchrony exists, probably, due to the common cytoplasm. Callose walls are formed after the second meiotic division, giving rise to a tetrad (Fig. 3.13E, F). Unlike successive type of cytokinesis, in this type the wall formation occurs by centripetally growing furrows which meet in the centre of the cell and divide it into four parts.

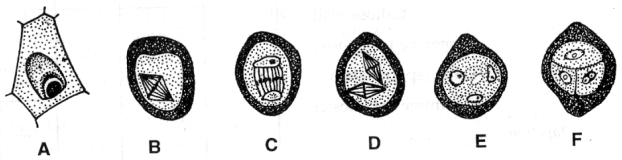


Fig. 3.13 Simultaneous type of cytokinesis in *Drimys winteri*. The dark zone around the cells represents the callose wall. **A.** Premeiotic microspore mother cell. The callose wall has not appeared as yet. **B.** Metaphase-I. **C.** Binucleate cell. **D.** Metaphase-II. E. 4-nucleate stage. **F.** Tetrad stage.(*after* Bhandari and Venkataraman, 1968)

Microspore Tetrad

Mostly all the four spores within a tetrad are completely isolated from one another and from the spores in other tetrads of the locule. No interconnections exist at this stage (Fig. 3.11C). In some orchids, however, cytoplasmic channels exist even in the tetrad stage.

Usually the arrangement of microspores in a tetrad is tetrahedral or isobilateral (Fig. 3.14). However, decussate, linear, and T-shaped tetrads are also found (Fig. 3.14). Interestingly, in *Aristolochia elegans* all the five types of tetrads have been recorded.

Eventually, the microspores are set free in the anther locule by the breakdown of the common callose wall which is supposed to be tapetum mediated. While still within the callose wall the microspores start synthesizing their individual walls. Callose seems to play an effective role in the laying down of the very first pattern of exine. For convenience and continuity of the description up to maturation, pollen wall development has been described in Chapter 4.

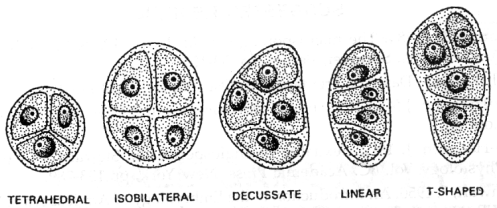


Fig. 3.14 Types of microspore tetrads in *Aristolochia elegans*. (*after* Johri and Bhatnagar, 1955)

RARE FEATURES

Usually the microspores separate from one another shortly after meiosis. However, in some plants the spores tend to remain together in tetrads for longer period and develop into compound pollen grains (*Drimys, Drosera*). In many members of Orchidaceae and Asclepiadaceae all the microspores in a sporangium remain together to form what is called a pollinium. An intermediate situation is met with in Mimosaceae. Here a number of groups each with more than four spores are formed in a sporangium.

The envelope around the pollinium of *Pergularia daemia* is sporopollenin in nature (Vijayaraghavan and Shukla, 1976). It has been found homologous to the tapetal membrane which invests the secretory tapetum.

Occurrence of more than four spores in a tetrad is called polyspory. A number of plants have been reported to show this phenomenon as an abnormality. Mahabale and Chennaveeraiah (1957) reported polyspory in *Hyphaene*, which is often due to divisions of the members of the tetrads (Fig. 3.15A, B). Tetrads with as many as eleven microspores have been observed in *Cuscuta reflexa*.

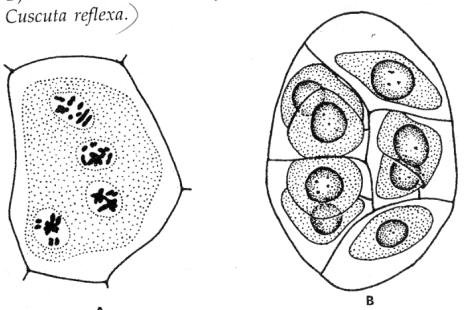


Fig. 3.15 Polyspory in *Hyphaene indica*. **A.** Microspore mother cell after meiosis. The four daughter nuclei are seen in division. **B.** Octad; very likely derived from the microspore mother cell shown in **A**. (*after* Mahabale and Chennaveeraiah, 1957)

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CHAPTER

4

MALE GAMETOPHYTE-DEVELOPMENT

Microspores represent the beginning of the male gametophytic generation. Older microspores, particularly after their release from the tetrads, are referred to as pollen grains. During gametogenesis, the pollen nucleus divides to form a generative cell and a vegetative cell. The former undergoes another mitosis to form two sperms (the male gametes). The generative cell may divide either inside the pollen grain or in the pollen tube, after the pollen has germinated. At the same time when these changes are going on the microspore is also synthesizing its wall. The surface of mature pollen grains shows curious sculpturing patterns. At one or more loci the pollen wall is very thin. These regions are called germ-pores. Palynologists refer to these pores as apertures.

A freshly formed microspore is richly cytoplasmic with a prominent, centrally located nucleus (Fig. 4.1A). Soon after its release from the tetrad the microspore (hereafter called pollen grain) undergoes a rapid expansion by the uptake of locular fluid, and its volume increases many fold in a short period. The initial expansion of the pollen grain is without an apparent vacuolation but later vacuoles appear and the cytoplasm comes to form a thin film lining the wall (Fig. 4.1C).

In many plants the DNA synthesis prior to first pollen mitosis takes place immediately before the division. In *Tulbaghia violacea*, however, it begins soon after meiosis.

FORMATION OF VEGETATIVE AND GENERATIVE CELLS

The first division in a pollen grain results into two unequal cells. The larger one is the vegetative cell, which eventually forms the pollen tube. The smaller one is the generative cell, which produces the sperms by another mitosis. The generative cell is initially attached to the wall of the pollen grain but, later, comes to lie freely in the cytoplasm of the vegetative cell.

In most tropical angiosperms the period between the end of meiosis and the first pollen mitosis is very short. However, in some woody species of the colder regions this interval may be several months. Often the pollen

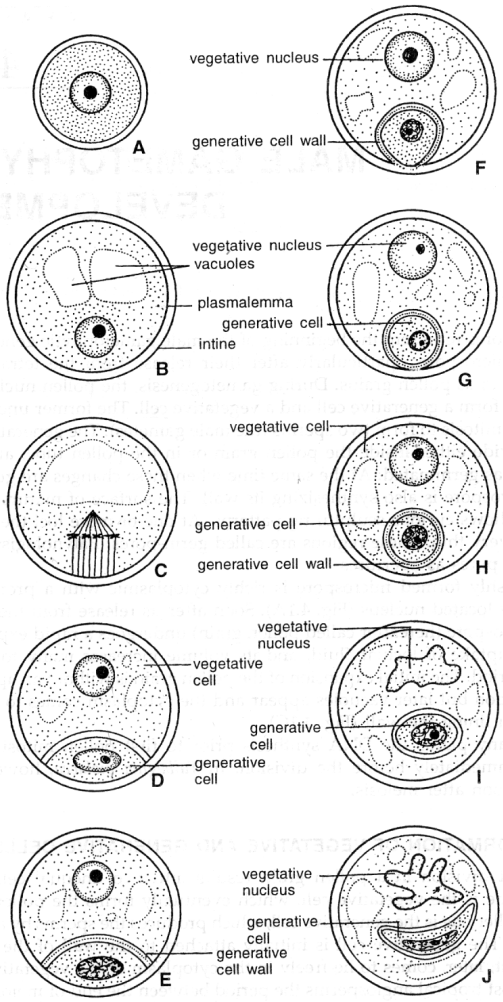


Fig. 4.1 →

mitosis in these plants is postponed until the spring of the year following meiosis. Pollen mitosis is not synchronous unlike meiosis where a high degree of synchrony is often maintained. This is, probably, due to the lack of cytoplasmic interconnections between the pollen grains in an anther locule. As described in Chapter 3, during meiosis, the meiocytes in an anther locule are interconnected by massive cytoplasmic channels, which allow a mutual influence of one cell over the other. Where the spores remain in tetrads during pollen mitosis (*Spiranthes australis*), all the four spores in a tetrad are in the same stage of division (Fig. 4.2A), but not all tetrads in the locule. A complete synchrony may be expected where the microspores are united into pollinia or massulae (Fig. 4.3; Orchidaceae, Asclepiadaceae, Mimosaceae).

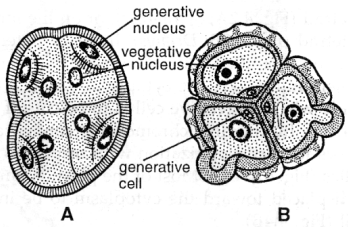


Fig. 4.2 Pollen mitosis in intact tetrads. All the spores are in the same stage of development. **A**. *Spiranthes australis*; the generative cells are cut-off on the outer side of the spores. **B**. *Drimys winteri*, the generative cells are cut-off on the inner side of the spores. (**A**. *after* Maheshwari and Narayanswami, 1952; **B**. *after* Bhandari and Venkataraman, 1968)

Before pollen mitosis starts, two distinct changes in the pollen protoplast are noticed: (1) The nucleus is displaced from the centre toward one side of the cell (Fig. 4.1B). The displacement is always in a particular direction and marks the position of the generative cell. This position of the generative cell is genetically controlled and is constant for a species. Thus, the generative cell may be cut-off either on the outer side, the side away from

Fig. 4.1 Diagrammatized stages in the formation of vegetative and generative cells, generative cell wall formation and pinching-off of the generative cell into the cytoplasm of the vegetative cell. **A**. Microspore soon after release from the tetrad. **B**. The cytoplasm of the pollen grain has become highly vacuolated, and the nucleus has been displaced to one side. **C**. Pollen mitosis; note the asymmetric spindle. **D**. Two-celled pollen soon after pollen mitosis. **E**. Generative cell wall has appeared in between the plasma membranes of the vegetative cell and the generative cell. **F-H**. Stages in the detachment of the generative cell from the pollen wall. Immediately after detachment the generative cell appears spherical. **I**. The vegetative nucleus has become lobed and the generative cell has lost its spherical shape. **J**. The generative cell has become ellipsoidal.

Vegetative Cell

After pollen mitosis the vegetative cell continues to grow. The cell organelles increase in number as well as in size. The vacuole gradually disappears, and RNA and protein contents of the cell increase dramatically. The nuclear envelope becomes highly convoluted (Fig. 4.1J). Normally, the vegetative nucleus remains arrested in G_1 phase of cell cycle and does not synthesize DNA. At mature stage the nucleus often lacks a nucleolus (Fig. 4.1I, J). In *Haemanthus* the large nucleolus, initially present, is replaced by a small nucleolus. In certain orchids also the vegetative nucleus possesses a nucleolus.

According to Sanger and Jackson (1971), in the development of the vegetative cell in *Haemanthus* four distinct stages can be identified:

1. *Immature stage*, during which the nucleus begins to lose its spherical shape.
2. *Mature stage*, which is characterized by an increase in the number of dictyosomes and change in the staining density of lipid bodies from dark to light. Starch accumulates in the plastids.
3. *Stage, shortly before anther dehiscence*, during which the nucleus is highly lobed, lipid bodies are absent, and the plastids no more contain starch.
4. *Pollen tube stage* (changes at this stage are described in Chapter 9).

Generative Cell

Immediately after becoming detached from the pollen wall the generative cell is spherical (Fig. 4.1H). It undergoes a lot of change in shape during pollen development. Usually, the cell elongates (Fig. 4.1J), sometimes to such an extent, that in mature pollen grain it appears vermiform. The elongated shape of the generative cell, probably, facilitates its movement into the pollen tube. The elongation of the generative cell has been attributed to the microtubules which are oriented longitudinally, parallel to the long axis of the cell. Sanger and Jackson (1971) have shown that if a pollen grain containing ellipsoidal generative cell is treated with a substance, such as colchicine or Isopropyl N-phenylcarbamate, which selectively destroys the microtubules, the generative cell tends to regain its original spheroidal shape (Fig. 4.5).

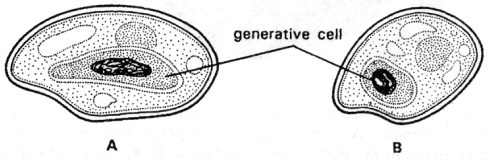


Fig. 4.5 Effect of treating mature binucleate pollen grain of *Haemanthus* with colchicine or Isopropyl N-phenylcarbamate. **A.** Untreated pollen. **B.** Treated pollen; note the change in the shape of the generative cell. (*after* Sanger and Jackson, 1971)

The generative cell cytoplasm is highly reduced but it contains the usual cell organelles - mitochondria, ribosomes, endoplasmic reticulum, microtubules, and dictyosomes. While most of the species investigated (*e.g., Beta, Haemanthus, Petunia*) lack plastids in the generative cell there is evidence of their being present in some other species (*e.g., Oenothera hookeri, Medicago sativa*). When present, the plastids do not contain starch. The mitochondria in the generative cell are poorly developed except in barley where they show well developed internal membrane system.

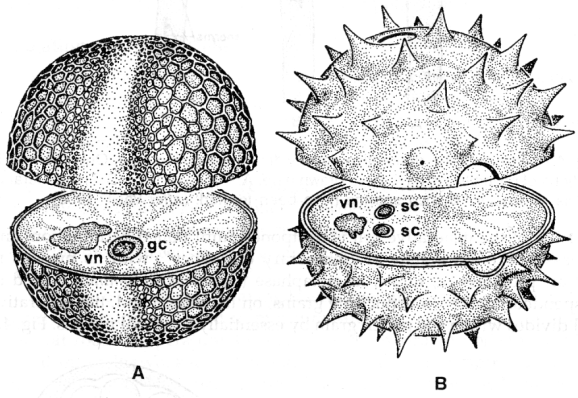


Fig. 4.6 Diagrams of two-celled (**A**) and three-celled (**B**) pollen grains, showing a vegetative nucleus (*vn*), and a generative cell (*gc*) or two sperm cells (*sc*). (*after* Iwanami *et al.*, 1988)

Formation of Sperms

Sperms are formed by a mitotic division in the generative cell. This may take place after the release of pollen from the anther or while the pollen are still confined to the anther. Thus, in the former condition the pollen are shed at the 2-celled stage (Fig. 4.6A) and in the latter at the 3-celled stage (Fig.4.6B). In those plants where pollen grains are shed at the 2-celled stage the generative cell may divide inside the pollen grain after it has alighted on the stigma (*Holoptelea integrifolia*), or in the pollen tube before it reaches the embryo sac (this is the most common condition; Fig. 4.7) or, rarely, after the pollen tube has reached the embryo sac (*Euphorbia terracina*).

Generally, DNA synthesis in the generative nucleus starts immediately after pollen mitosis. This is followed by a more or less prolonged mitosis. During this period the generative cell undergoes elongation to acquire its characteristic vermiform appearance. In species with binucleate grains the

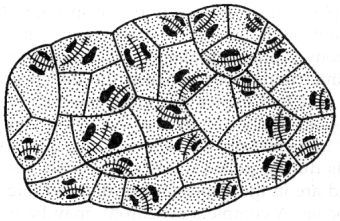


Fig. 4.3 A massula of microspores of *Peristylis spiralis*. Note the synchrony of first pollen mitosis in spores of the massula. (*after* Swamy, 1949)

the centre of the tetrad (Fig. 4.2A; *Spiranthes*), or on the inner side, toward the centre of the tetrad (Fig. 4.2B; *Drimys*). Inner or outer position of the generative cell can be identified only where the microspores remain in tetrads during pollen mitosis. (2) The cytoplasm between the nucleus and the wall, on the side where vegetative cell is to be cut-off becomes highly vacuolated (Fig. 4.1C). By the time chromosome condensation has begun the cytoplasm shows marked polarization with respect to the distribution of the cell organelles (Fig. 4.4A-C). Most of the plastids, mitochondria, and lipid bodies are displaced toward the cytoplasm to be incorporated into the vegetative cell (Fig. 4.4B).

The spindle of pollen mitosis is short and asymmetric (Fig. 4.1C). The wallward pole of the spindle is blunt and the free pole acute. Following the division of the nucleus, on the wallward side is the generative nucleus and toward the inside of the pollen is the vegetative nucleus. At the end of the nuclear division the cell plate is formed curving around the generative nucleus and is hemispherical (Fig. 4.1D).

Initially, the cytoplasms of the vegetative cell and that of the generative cell are separated by two plasma membranes (Fig. 4.1D). The wall of the generative cell is soon formed in between the two cell membranes and adjoins the intine (inner layer of pollen wall) on either side of the generative cell (Fig. 4.1E). The wall material in many taxa (*Chlorophytum, Hyacinthus, Tradescantia*) has been identified as callose. In *Haemanthus*, however, the wall seems to be made up of unidentified fibrillar material. A unique event now follows. The wall of the generative cell grows inward between the plasmalemma of the generative cell and the intine (Fig. 4.1F) until the two ends of the wall meet and fuse (Fig. 4.1G), and the cell is finally pinched off (Fig. 4.1H). At this stage the generative cell is spherical (Fig. 4.1H) and moves to the centre of the vegetative cell. Soon afterwards the wall of the generative cell disappears, and the cytoplasm of the generative cell remains enclosed in two plasma membranes, its own and the detached invagination of the plasmalemma of the vegetative cell (Fig. 4.1I). In *Chlorophytum, Hyacinthus* and *Tradescantia* the generative cell wall lasts only for 10-20 hours.

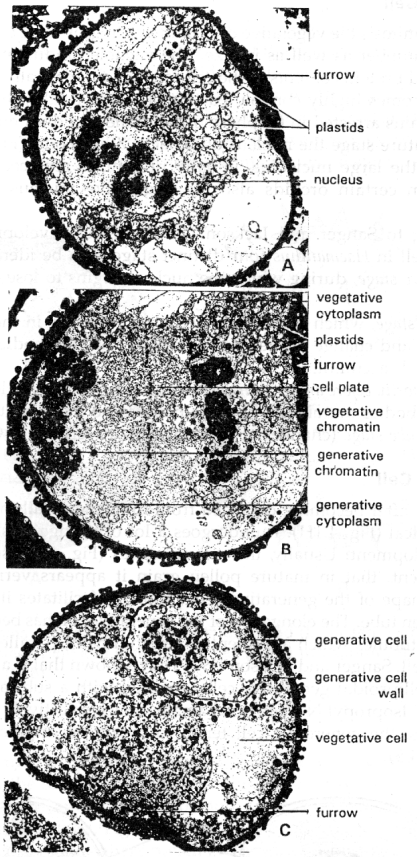


Fig. 4.4 Pollen showing the formation of vegetative and generative cells in *Haemanthus katherinae*. **A.** At the prophase of pollen mitosis; most of the cell organelles lie between the nucleus and the furrow. **B.** At the telophase of pollen mitosis, almost all the plastids and most of the other organelles are present between the furrow and the cell plate where the vegetative cell is going to be formed. **C.** A binucleate poilen before the generative cell is detached from the intine. (*after* Sanger and Jackson, 1971)

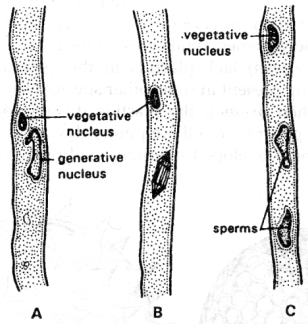


Fig. 4.7 In cotton the generative cell divides in the pollen tube. Only the middle portions of pollen tubes have been drawn here. **A.** Before division. **B.** The generative cell is in division. **C.** Two sperms have been formed. (*after* Vasil, 1958)

division of the generative cell is postponed until pollen germination, and it passes through a state of "temporary dormancy", with its nucleus in mitotic prophase or, rarely, in metaphase (*Impatiens balsamina*), a kind of suspended mitosis. In trinucleate grains, on the other hand, the generative cell divides within the pollen grain by essentially a normal mitosis. Fig. 4.8

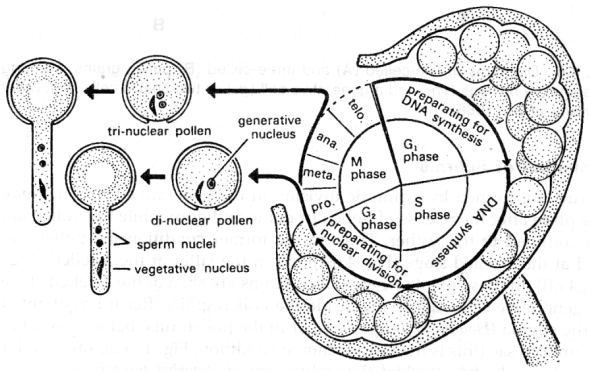


Fig. 4.8 Diagram to show division of the generative nucleus in 2-celled and 3-celled pollen grains. In the former, the grains are released during early M-phase, while in the latter, the grains are released during late M-phase of the cell division cycle. (*after* Iwanami *et al.*, 1988)

gives a diagrammatic representation of the division of the generative nucleus in 2- and 3-celled pollen grains. Pollen grains are released from the anthers during M-phase of cell division cycle (mitotic nuclear division stage) after going through the G_1-phase (preparatory stage for DNA synthesis), S-phase (DNA synthesis stage) and G_2-phase (preparatory stage for nuclear division). The 3-celled pollen grains are released during late M-phase, having two sperm nuclei. In the 2-celled pollen, the generative nucleus remains undivided and contains double the amount of DNA of sperm nucleus. Here the generative nucleus divides in the pollen tube.

Isolation of Sperm Cells

Routine isolation of large numbers of live sperm cells of both monocot and dicot flowering plants has opened up a new field of research in plant biology. It provides the opportunity to investigate the structure, physiology and biochemistry of these unique plant cells (Cass, 1997).

The procedure for sperm isolation depends on the condition of pollen at dehiscence. In plants with 3-celled pollen, sperm isolation involves osmotic bursting of freshly collected pollen. However, in plants with 2-celled pollen, it is possible only after pollen germination. The procedure followed for the isolation of sperm cells of maize, an example of 3-celled pollen, is as follows (Wang *et al.*, 1992; Cass, 1997).

Pollen are collected by shaking tassels over a tray, sieved through 80 µm nylon mesh and hydrated in moisture-saturated atmosphere for about 20 min, at room temperature. One gram of the hydrated pollen is incubated in 10 ml of Brewbaker and Kwack's medium (for composition see page 134), containing 15% sucrose and pH set at 5 (Bursting medium), at room temperature. Kranz *et al.* (1990) used 540 mosmol Kg^{-1} H_2O-mannitol solution as the bursting medium to isolate maize sperm cells for *in vitro* fertilization (see page 170). Within 20 min the pollen grains burst, releasing their contents, including the sperms, through the germ pores. Gentle shaking (130 rpm) during incubation in the bursting solution favours the release of the sperms. The bursting medium is then filtered through 50 µm nylon mesh to remove the exine fragments. Further cleaning, to remove starch grains and other cytoplasmic contents of the vegetative cell, is done by discontinuous percoll gradient centrifugation. The percoll gradient is prepared by layering 30%, 15% and 10% percoll solution (prepared in the bursting medium) in 15 ml centrifuge tubes, and 5-10 ml of the filtrate is loaded on the top of 10% percoll. After centrifugation at 3000g, for 60 min, at 4°C, sperm cells are collected at the interface between 15% and 30% percoll. The sperm cells (ca 7 µm) are pipetted out, washed with the bursting medium and centrifuged at 3000g for 7 min. The supernatant is discarded and the sperms, settled at the base, are collected in 2 ml of the bursting solution and used as desired.

Viability of the isolated sperms can be determined by NMR (correlates with water content of the sperms), phase contrast microscopy (viable sperms appear quite dark, relatively uniform and spherical), or Evan's blue

exclusion test (viable sperms with intact membrane retain the dye whereas the non-viable sperms with damaged membrane exclude it). However, fluorescence microscopy after FDA staining is the most commonly used viability test (see page 124).

Male Germ Unit

At maturity the pollen grains of about 70% species are bicellular and in the remaining 30% species tricellular (Brewbaker, 1957). Some of the major families showing bicellular pollen are Betulaceae, Rosaceae and Solanaceae, and those with tricellular pollen are Asteraceae, Brassicaceae, Caryophyllaceae and Poaceae. Tricellular grains show higher metabolic activity than bicellular grains (Hoekstra, 1983). Both types of pollen grains contain a vegetative nucleus (also called tube nucleus) which is involved with the metabolism of the pollen grain and later pollen tube. The generative cell may produce sperms before germination (3-celled pollen) or after germination (2-celled pollen). The generative cell and sperm cells lie in the cytoplasm of the vegetative cell, surrounded by their own cell membranes and an internal membrane of the vegetative cell.

Russell and Cass (1981) made a significant observation that in *Plumbago zeylanica*, an example of 3-celled pollen, the two sperms and the vegetative nucleus occur in intimate association (Fig.4.9). The two sperms are directly linked to each other by a common transverse cell wall perforated with plasmodesmata. One of the sperms is consistently associated with the vegetative nucleus through a sperm extension that embraces the vegetative

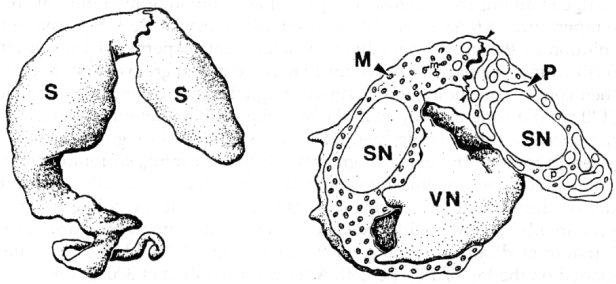


Fig. 4.9 Male germ unit of *Plumbago zeylanica*. **A**. Computer-assisted reconstruction of the two sperms (*S*). Note differences in the shape and size of the two sperms, and that they are interconnected to each other by a common wall. **B**. Internal view of the two sperms in **A**, showing predominance of plastids (*P*) in the smaller sperm cell and most of the mitochondria (*M*) in the larger sperm cell. The latter is associated with the vegetative nucleus (*VN*) by an extension. Unlabelled arrowheads indicate the common wall between the two sperms. *SN*, sperm nucleus. (*after* Russell, 1984)

nucleus over a large surface area and is partially surrounded by lobes of the vegetative nucleus. These associations, established within mature pollen grains are maintained throughout pollen tube growth. The sperms are highly cytoplasmic, containing plastids, mitochondria, ER, ribosomes, vesicles and microtubules.

Application of the techniques of serial ultrathin sections, isolation of live sperms and computer-assisted 3-D reconstructions revealed that in *P. zeylanica* the two sperms of a pair differ not only in cell size and shape but also in the size of their nucleus and the number of cytoplasmic organelles (Russell, 1984; Fig. 4.9B). The smaller sperm, which is not associated with the vegetative nucleus, contains on an average 24 plastids and 40 mitochondria. The larger sperm, which is closely associated with the vegetative nucleus through a narrow extension, usually lacks plastids and has on an average 256 mitochondria.

Interconnection of sperm cells and their association with the vegetative cell nucleus were soon described in *Spinacia* (Wilms and van Aelst, 1983) and *Brassica* (Dumas *et al.*, 1985), both with 3-celled pollen (Fig. 4.10). These plants, which lack plastids in the sperm cells, also exhibit "sperm dimorphism" with respect to size, shape and the number of mitochondria (Wilms, 1986; McConchie *et al.*, 1987). The occurrence of a "polarized fertilization unit" (Heslop-Harrison and Heslop-Harrison, 1984), more commonly called "Male Germ Unit" (MGU; Dumas *et al.*, 1984), has also been observed in several species with 2-celled pollen grains, where sperms are not formed until pollen germination, such as *Hippeastrum vitatum* (Mogensen, 1986) and *Petunia hybrida* (Wagner and Mogensen, 1987). Of these, *Petunia*, which has been investigated using 3-D reconstructions and morphometric analysis, does not show the sperms in a MGU to be significantly different morphologically (Wagner and Mogensen, 1987). Accordingly, a more generalized feature of a MGU is "connected sperms in

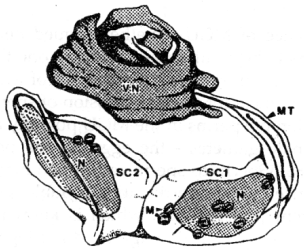


Fig. 4.10 Computer-assisted three-dimensional schematic diagram of the male germ unit from a pollen grain of *Brassica campestris*, showing placement of vegetative nucleus (*VN*) and sperm cells (SC_1, SC_2) with nuclei (*N*), mitochondria (*M*) and arrays of microtubules (*MT*). (*after* McConchie *et al.*, 1985)

close association with the vegetative nucleus". On the basis of this concept the list of species showing MGU could be very large, including dicots with 2-celled pollen (*Rhododendron* spp), *Acacia retinoides, Cyphomandra betacea, Medicago sativa, Nicotiana tabacum*, monocots with 2-celled pollen (*Aloe ciliaris, Galanthus nivalis, Gladiolus gandavensis*), and dicots with 3-celled pollen (*Cichorium intybus, Catananche caerulea, Gerbera jamesonii, Sambucus nigra*).

In the grasses, which are monocots with 3-celled pollen, neither the sperms are in close association with vegetative nucleus nor are they dimorphic (barley - Cass, 1973; Cass and Karas, 1975; rye - Karas and Cass, 1976; wheat - Zhu *et al.*, 1980, Mogensen, 1986; *Triticale* - Schroeder, 1983). A quantitative 3-D study confirmed that in barley MGU does not exist at the mature pollen stage, and the two sperms are essentially isomorphic (Mogensen and Rusche, 1985). However, within 5 min after *in vitro* pollination a close association begins to form between the vegetative nucleus and the sperm cells and within 15 min, while still within the pollen, the two sperms form cellular extensions and closely align with each other with a minimum distance of 60 nm and no intervening cytoplasm in various regions (Mogensen and Wagner, 1987) and the vegetative nucleus surrounds one sperm cell extension over a distance of 3 µm. As the pollen tube grows through the stigma and style the sperms remain connected and move as a unit. Thus, in this species, and possibly in other grasses, the MGU is established after pollination but before their exit from the pollen grain into the pollen tube. However, the association of sperms with the vegetative nucleus is brief, and in no case was the vegetative nucleus seen to accompany the sperm cells within the pollen tube (Mogensen and Wagner, 1987). Similar post-pollination events leading to establishment of an intimate association between vegetative nucleus and the sperm cells have been reported in *Hippeastrum* (Mogensen, 1986), *Rhododendron* (Kaul *et al.*, 1987) and maize (Rusche, 1988; Rusche and Mogensen, 1988). In maize the vegetative nucleus becomes closely associated with sperms only after pollen activation, and this association is maintained during their movement in the pollen tube.

Thus, the occurrence of MGU, whether formed before or after pollen germination, appears to be a universal feature among the flowering plants. Dumas *et al.* (1984), who introduced the concept of MGU, postulated that MGU functions as a vehicle for the transmission of sperms, and the quality and spatial disposition of sperms in the MGU possibly allow their targeted fusion with the female gametes - the egg and the polar nuclei (*see also* Mathys-Rochon, 1988). Sperm-to-sperm connection may also have a role in reducing the incidence of heterofertilization (double fertilization involving sperms from different pollen tubes) which is known to occur in maize (Sprague, 1932) and barley (R.T. Ramagi; cited in Mogensen, 1992). On the practical side, if individual sperms of a pair are recognizable and if it can be determined which one will combine with a given female target cell, it may be possible to genetically engineer a given sperm cell and utilize it in *in vitro* fertilization to improve crop plants.

INHERITANCE OF CYTOPLASMIC TRAITS

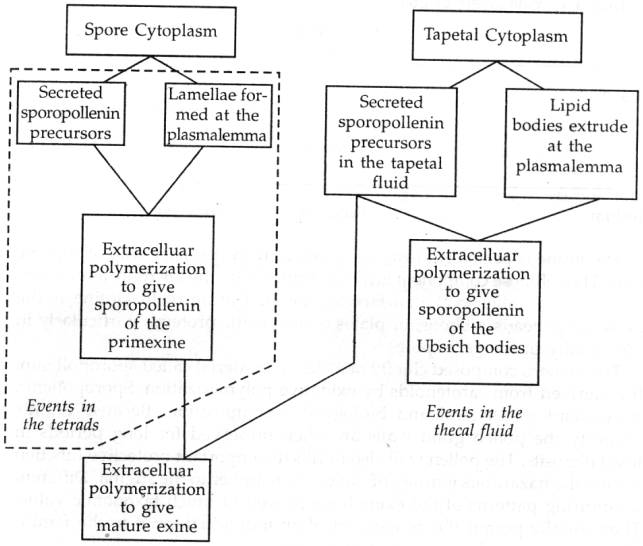
The vast majority of flowering plants transmit their extra nuclear DNA to sexual progeny exclusively or predominantly from the female parent. Strictly maternal inheritance of mitochondria is more common among flowering plants than uniparental maternal inheritance of plastids (Mogensen, 1996).

Usually the generative cell receives no plastids and, therefore, the sperms formed by its division are devoid of plastids but contain mitochondria. In such cases plastid inheritance is exclusively maternal (Hagemann, 1992). In some orchids the generative cell totally lacks both plastids and mitochondria (Yu and Russell, 1992). Some species do contain plastids in their generative cell but this does not ensure paternal inheritance of the plastids because the plastids may get eliminated before or after sperm formation by a process such as the activity of autophagosomes, exclusion of male cytoplasm at gametic fusion, or degradation of organelle DNA during pollen maturation, as indicated by the appearance of albinos in pollen derived plants. The albinos lack plastid genome products (16S and 23S ribosomes and large sub-units of Rubisco).

Based on the distribution of plastids during pollen development and their final fate, Hagemann (1983) made 4 categories of flowering plants: (1) *Lycopersicon* type- During the first pollen mitosis all the plastids of the microspores are included in the vegetative cell cytoplasm, and the generative cell is devoid of them (*e.g., Antirrhinum, Hordeum, Lycopersicon*). (2) *Solanum* type- The apportionment of the plastids during the first pollen mitosis is asymmetric; the majority of plastids are included in the cytoplasm of the vegetative cell. The generative cell receives only a small number of plastids, and these also are lost during further pollen development (*e.g., Hosta, Hyoscyamus, Solanum*). (3) *Triticum* type- This category is similar to category 2 except that the loss of plastids occurs as late as during the process of syngamy. (4) *Pelargonium* type- Plastids are distributed equally between the vegetative and the generative cells. In plants belonging to this category the sperms also carry the plastids, which are regularly transmitted to the zygote (*e.g., Oenothera, Pelargonium, Secale*). Thus, the genetic effect of the behaviour of plastids in categories 1 to 3 is the same: no transmission of paternal plastids to the zygote. Biparental inheritance of plastids occurs only in category 4 plants.

Alfalfa exhibits biparental inheritance of plastids and chloroplast DNA, but the degree of transmission of paternal and maternal plastids is genotype specific (Mogensen, 1992, 1996). The brother sperms are essentially alike with respect to plastid numbers. Quantitative 3-D reconstructions of the female gametophyte revealed that the distribution, size and number of plastids in the mature egg may influence the pattern of plastid inheritance among genotypes of alfalfa (Rusche *et al.*, 1995). The genotypes with strong female bias with respect to plastid transmission show significantly larger and more plastids in the egg cell than those with weak female bias. Moreover, in weak females plastids are located below the equatorial plane of the nucleus (Fig. 4.11A), and the distribution of plastids in strong females

TABLE 4.2 Schematic representation of the formation of Ubisch bodies and exine development in *Lilium*. (*after* Heslop-Harrison, 1972)



The first layer of the pollen wall to be formed is cellulosic and is known as primexine. The cellulose microfibrils of this layer are deposited in between the convoluted plasmalemma of the spore and the callose wall (Figs 4.12B, 4.13B). At certain regions in the cytoplasm of the spore, just below the plasmalemma, are positioned plates of endoplasmic reticulum (Fig. 4.12A, B). The cellulosic primexine is discontinuous in these regions (Fig. 4.12B, C), and the gaps thus created mark the position of germ-pores. When the cellulosic primexine has reached a certain thickness additional gaps appear in it, and columns of convoluted lamellae are deposited in these gaps at the surface of the plasmalemma (Figs 4.12C, 4.13C, D). These columns are called probacula. Now the cytoplasm of the spore starts synthesizing the precursors of sporopollenin which are polymerized and deposited on the surface of the lamellae. The columns are now called bacula. The distribution and orientation of the bacular columns vary a great deal, and are according to the pattern of the mature exine. Later, the lower ends of the bacula spread sideways into the cellulosic layer of the primexine and coalesce to form the foot-layer (Figs 4.12D, 4.13G). The foot-layer forms a sort of floor

on which are raised the columns or bacula. The tops of the bacula-columns may also spread sideways in all directions to form the tectum (Figs 4.12D, 4.13E-H). The intricate exine patterns are often due to the tectum. The tips of the bacula may also simply enlarge to form knobs. To this extent the exine is formed inside the callose wall of the tetrads. A point to appreciate at this stage is that the pattern of mature exine has already been laid down before the spores are set free.

With the age of tetrads, the callose is gradually digested (Figs 4.12D, 4.13F-H), and the individual spore (correctly pollen grain) lies free within the anther locule. In this free state pollen grains synthesize the intine and the innermost layer of the exine (Figs 4.12E, 4.13H). The formation of the

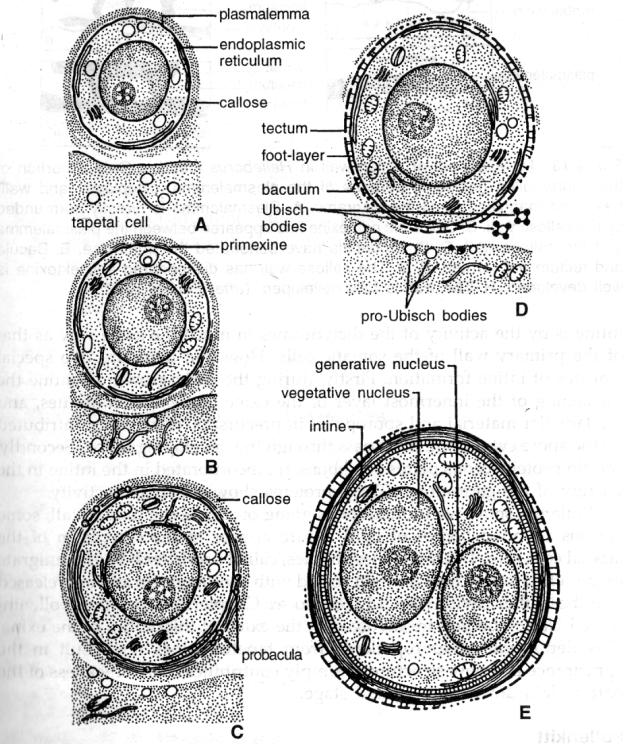


Fig. 4.12 Formation of Ubisch bodies and development of pollen wall. For details *see* text. (*after* Echlin, 1968)

is perinuclear (uniformly around the nucleus; Fig. 4.11B) (Zhu *et al.*, 1993). The distribution pattern of plastids in the egg cell is maintained in the zygote and 2-celled proembryo. Thus, in strong maternal genotypes, after the first unequal division of the zygote both apical and basal cells receive equal number of maternal plastids. On the other hand, in weak maternal genotypes, where the plastids are mostly below the middle transverse line of the egg nucleus, after first division of the zygote most of the maternal plastids are included in the basal cell which forms the ephemeral suspensor. The smaller apical cell, which forms the embryo proper, receives very few maternal plastids. Similar, unequal distribution also occurs for paternal plastids but, comparatively, the apical cell receives more male than female plastids. In the progeny of a cross between a weak female and a strong male genotype of alfalfa 90% plants showed paternal plastids.

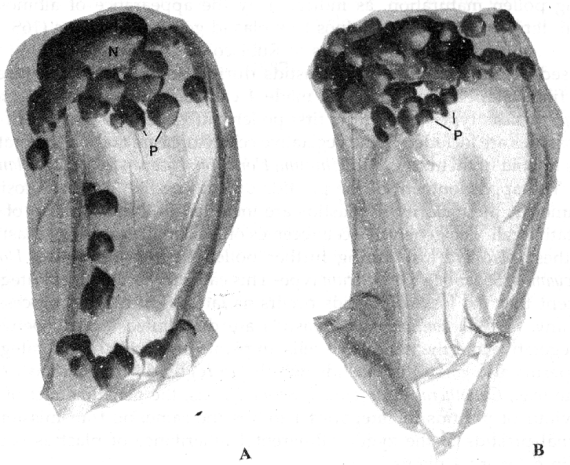


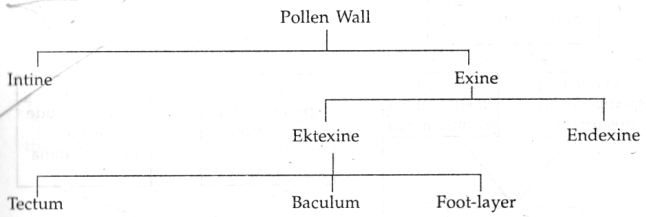
Fig. 4.11 Computer-generated 3-D reconstruction of alfalfa egg cells. **A.** Egg cell from genotype CUF-B, a weak female plastid transmitter, showing the plastids (*P*) distributed mostly below (toward the micropyle) the mid-region of the nucleus (*N*) which is the future division plane of the zygote. **B.** Egg cell from genotype 6-4, a strong female plastid transmitter, showing plastids (*P*) distributed essentially equally around the nucleus. (*courtesy*: Professor H.L. Mogensen, U.S.A.)

POLLEN WALL

The wall of the mature pollen grain is stratified. It comprises two principal layers: the inner layer is called intine and the outer layer, exine. The latter

comprises many sub-layers (for details *see* Table 4.1). The terms intine and exine were proposed by Fritsche (1837).

TABLE 4.1 Wall layers of pollen



The intine is pecto-cellulosic in nature, as is the primary wall of somatic cells. The cellulose component is microfibrillar, with the microfibrils oriented in a plane parallel to the surface. A special feature of the intine is the presence of beads, ribbons, or plates of enzymatic proteins, particularly in the vicinity of the germ-pores.

The exine is composed chiefly of a class of material called sporopollenin. It is derived from carotenoids by oxidative polymerization. Sporopollenin is resistant to physical and biological decomposition. Because of this property the pollen grain walls are often preserved for long periods in fossil deposits. The pollen wall also fulfills the important protective function during the hazardous journey of pollen from anther to the stigma. Different sculpturing patterns of the exine have proved of much taxonomic value. They usually permit the assignment of an individual grain to the family, often to genus and, rarely, even to species.

The pollen of the Scitamineae (Stone *et al.*, 1979, 1980) and several tropical Lauraceae (Erdtman, 1971; Lieux, 1978) are unusual in having a much thickened intine and a highly reduced exine, represented by a few spinules. This has been considered to be an adaptation to transportation of pollen in humid tropical rain forests.

Development

At the end of meiosis four haploid microspores are enclosed in a common callose wall. The individual spore lacks a wall of its own (Figs 4.12A, 4.13A) and is separated from other spores of the tetrad by callose partitions. While they are still enclosed in the callose wall the individual spore starts forming its wall. There are two phases in the formation of pollen wall (Table 4.2). In the first phase the wall material is contributed by the cytoplasm of the spore alone, and this is during the tetrad stage. In the second phase, which comes into operation after the release of the microspores through enzymatic degradation of the callose wall, the wall materials are contributed by the tapetal cells in addition to the spore cytoplasm.

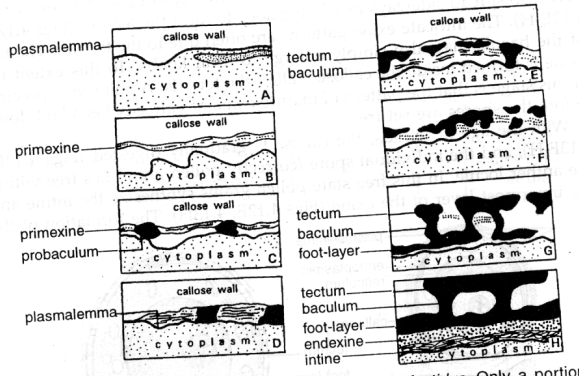


Fig. 4.13 Development of pollen wall in *Helleborus foetidus*. Only a portion of the spore/pollen (including portions of the plasmalemma, cytoplasm, and wall) has been magnified in all the diagrams. **A.** Plasmalemma is directly surrounded by the callose wall. **B.** Cellulosic primexine has appeared between the plasmalemma and the callose wall. **C, D.** Probacula have penetrated the primexine. **E.** Bacula and tectum are formed. **F, G.** The callose wall has disappeared and ektexine is well developed. **H.** The wall is fully developed. (*after* Echlin, 1968)

intine is by the activity of the dictyosomes in much the same way as that of the primary wall of the somatic cells. However, there are two special features of intine formation. Firstly, during the early growth of intine the thickening of the innermost layer of the exine (endexine) continues, and the lamellar material and sporopollenin precursors, which are contributed by the spore cytoplasm, must pass through the developing intine. Secondly, certain proteinaceous plates or ribbons are incorporated in the intine in the vicinity of the germ-pores. These proteins show enzymatic activity.

While the spore wall formation is going on inside the tetrad wall, some curious rounded bodies of lipid nature appear in the cytoplasm of the tapetal cells (Fig. 4.12A-C). These bodies, called pro-Ubisch bodies, migrate to the cell surface where they get coated with sporopollenin and are released into the anther cavity (Fig. 4.12D; also *see* Chapter 3). The sporopollenin-coated Ubisch bodies are involved in the external thickening of the exine. This deposition of sporopollenin from tapetum does not result in the appearance of a new structure. It simply contributes to the thickness of the pattern laid down in the tetrad stage.

Pollenkitt

The pollenkitt is an oily layer forming a thick viscous coating over the pollen grain surface of many insect-pollinated species. The stickiness, odour

and colour of the grains are because of the pollenkitt. Pollenkitt material is contributed by the tapetal cells and is later transferred to the pollen surface. It comprises chiefly of carotenoid or flavonoid pigments which impart the characteristic yellow or orange colour to the pollen. The pollenkitt or the surface pollen cement also contains glycoproteins, lipids, glycolipids and monosaccharides which are responsible for its sticky nature (Clarke *et al.*, 1979).

Hesse (1979) made a detailed study of the nature and function of pollenkitt in entomophilous and anemophilous species of *Acer*, and some other families. According to him, in the insect-pollinated species, the pollenkitt is electron-dense and homogeneous forming a coating on the exine rendering the pollen sticky. On the other hand, in the anemophilous taxa, the pollenkitt is electron-transparent and not homogeneous, and is in much less quantity. It becomes inactive in the pollen locule and settles into exine perforations, thus rendering the pollen powdery and non-sticky.

The biological function of the pollenkitt is not clear. However, the suggestions are that it may be contributing in the following ways:

1. Acting as an insect-attractant.
2. Protecting the pollen against the damaging effect of ultraviolet radiation.
3. Acting as an adherent to the insect body, because of the sticky nature.
4. Being hydrophobic, it might even be associated with the dispersal of pollen.
5. Functioning as the pollen-borne substances involved in sporophytic incompatibility.

ABNORMAL FEATURES

1. Development of Pollen in Cyperaceae

In the vast majority of angiosperms, as described in Chapter 3, the pollen mother cell divides by a meiotic division resulting in four haploid nuclei, each of which forms a functional spore. The members of the Cyperaceae exhibit a special mode of pollen development. Of the four nuclei formed after meiosis, only one functions. The functional nucleus remains in the centre and the three non-functional nuclei are cut-off on one side of the cell. In *Cyperus, Kyllinga,* and *Scirpus*, the non-functional nuclei are separated from the functional nucleus by a wall (Fig. 4.14A, B). Similar walls are also formed in between the non-functional nuclei. The functional nucleus divides to form a vegetative cell and a generative cell. The non-functional nuclei may also begin to divide but the divisions are abortive, and they eventually degenerate (Fig. 4.14C).

2. Pollen Grain Embryo Sacs

Normally, the number of nuclei in a mature pollen grain is two or three. Occasionally, grains with more nuclei, resulting in abnormal type of the

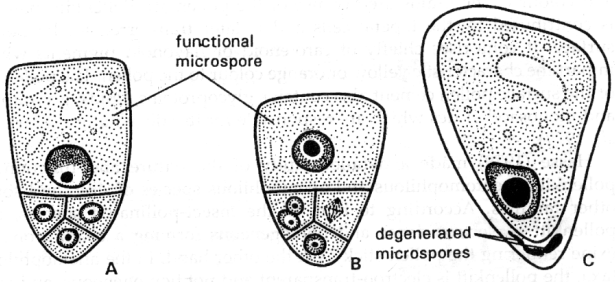


Fig. 4.14 Pollen development in *Cyperus*. **A.** The non-functional microspores have been cut-off on one side. The functional microspore is large and contains a prominent nucleus. **B.** Two of the non-functional spore nuclei have divided. **C.** The non-functional microspores have degenerated. (*after* Khanna, 1965)

male gametophyte are also met with. In some liliaceous members, however, the increase in the number of nuclei in the male gametophyte may lead to the formation of female gametophyte (embryo sac)-like structures. Nemec (1898) was the first to note embryo sac-like pollen grains in the pataloid anthers of *Hyacinthus orientalis*. This was confirmed by de Mol (1923), Stow (1930, 1934), and Naithani (1937). Similar abnormality has also been observed in *Ornithogalum nutans* (Geitler, 1941), and in a member of the Saxifragaceae, *Heuchera micrantha* (Vijayaraghavan and Ratnaparkhi, 1976), and *Leptomeria billardierii*, a member of the Santalaceae (Ram, 1959). Such embryo sacs may arise from the divisions of vegetative nucleus (Stow, 1930; Naithani, 1937; Geitler, 1941; Vijayaraghavan and Ratnaparkhi, 1976, 1977), or directly from microspore mother cells (Ram, 1959).

The work on *Hyacinthus* shows that under certain conditions, in appropriate genotypes, some of the microspores enlarge, escape from the spore wall and form sac-like structures. In some of the sacs the nucleus undergoes three mitotic divisions, like the nucleus of the functional megaspore, to form eight nuclei which organize like an 8-nucleate embryo sac. Some pollen embryo sacs with larger or lesser number of nuclei were also recorded. Stow observed the fusion of polar nuclei in these pollen embryo sacs. If the pollen embryo sacs were placed on an agar medium along with some normal pollen grains of another variety, the pollen tubes showed chemotropic response toward the pollen embryo sacs.

These observations suggest that the stimulus for the spore to develop into embryo sac is not unique to the confines of the ovule. Under certain treatments the free-floating microspores can organize into embryo sac-like structures.

3. Sporophytes from Pollen Grains

The first convincing demonstration of sporophytes arising from pollen grains was made by Guha and Maheshwari (1966) in *Datura*. Subsequently, many reports of this type have appeared (for details *see* Chapter 17). This feature, coupled with the phenomenon of pollen embryo sac formation, suggests that young microspores are not committed to any one pathway of development. They seem to have the potential of adopting any one of the following three modes of development, depending on the treatment:

A. Under normal conditions they would form male gametes.
B. Under special conditions, probably of temperature, they may form female gametophytes.
C. In cultures, on appropriate nutrient medium, they may grow to form apogamous sporophytes.

4. Pollen Sterility

The male sterility in angiosperms may arise due to several reasons:

A. Anther suppression, abortion, phyllody, petallody, and pistillody- this has premeiotic determination.
B. Aberrant meiosis or gametophytic development:
   (a) Determined by the individual meiocyte or gametophyte.
      (i) Meiotic irregularities- meiosis fails or produces genomically imbalanced spores.
      (ii) Irregularities in gametophyte development- meiosis is normal but gametophyte development is aberrant or incomplete.
   (b) Determined by the sporophyte- meiotic disturbances or arrest of gametophyte development.
C. Failure of anther dehiscence- normal pollen are formed but not released.
D. Premature dissolution of callose- abortion can occur at any stage during microsporogenesis.
E. Fungal or viral infection.

We are here concerned with only the second (*B*) cause of male sterility in which non-viable pollen grains are produced due to the malfunctioning of the spore mother cells or gametophyte, or it is due to some sporophytic factor. In the former (type *B*-a) an anther may contain a mixture of fertile and sterile pollen grains, whereas in the latter (type *B*-b) the entire population of pollen grains within an anther would be sterile.

Gametophyte-determined pollen sterility is usually due to meiotic abnormalities, such as meiosis in polyploids which often results in an unequal distribution of chromosomes in the daughter cells. Plants, heterozygous for a gene or minor deficiency which is lethal in the haplophase, will also produce non-viable pollen.

Sporophyte-determined pollen sterility is due to genic, cytoplasmic, or environmental factors. It may become operative at any stage of anther development. Genic and cytoplasmic pollen sterility is widespread among angiosperms. The latter is of considerable importance in plant breeding.

Genic pollen sterility is due to recessive genes. Maize shows genic as well as cytoplasmic pollen sterility.

Environmental factors such as photoperiodic treatment (*Glycine max, Silene pendula, Zea mays*) and temperature (*Oryza sativa*) are known to induce male sterility in otherwise fertile plants. Certain chemicals, such as auxins, maleic hydrazide, and Dalapon, selectively induce male sterility.

In most cases the pollen sterility has been attributed to the malfunctioning of tapetum. Some of these irregularities are:

1. Inhibition of normal RNA synthesis and increase in DNA content.
2. Hypertrophy of the tapetal cells. They enlarge acquiring supranormal cytoplasmic RNA and become multinucleate. The cells invade the anther locule, and the meiocytes or spores are crushed or even resorbed.
3. Premature degeneration of the tapetum depriving the developing spore of its nutrition.

It is now possible to induce male sterility in a wide range of angiosperms by inserting a bacterial gene BARNASE linked with a tapetum-specific promoter pTA29 from tobacco. This chimeric gene causes premature breakdown of tapetum resulting in arrest of microspore development. Introduction of another bacterial gene, BARSTAR, in a plant carrying the BARNASE gene can restore male fertility by inactivating the protein produced by the BARNASE gene (Fig. 4.15).

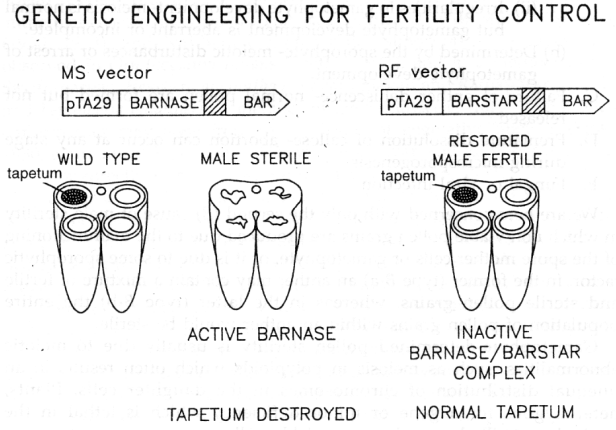


Fig. 4.15 Schematic representation of genetic engineering for male fertility control using *barnase* and *barstar* genes. (*courtesy*: Dr A. Reynaerts, Plant Genetic System, Belgium).

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more than 6 apertures (7 or more) are said to be polytreme and represented as N_7. When the apertures, irrespective of their number, are irregular or irregularly spaced, the pollen grains are described as anomotreme (N_8).

With regard to the position (P) of apertures, there are seven (P_0-P_6) groups (Fig. 5.2). The pollen are designated as catatreme (P_1) when the aperture is on the proximal face, and anatreme if it is on the distal face (P_3). When the centres of apertures are located on the equator, the pollen grains are referred to as zonotreme (P_4). The condition is said to be pantotreme when the apertures are more or less uniformly distributed all over the pollen surface (P_6).

The character-groups (Fig. 5.2), like the position-groups, are also seven (C_0-C_6). Pollen are designated C_0 (0 stands for a query) when the character of the aperture is not known. C_1 pollen have an aperture-like thin area, or leptoma. Pollen with one leptoma are called monolept. They may be catalept (leptoma on proximal face), or analept (leptoma on distal face). The grains with a 3-slit colpus belong to C_2 category, and are called trichotomocolpate. The remaining characters viz., C_3, C_4, C_5 and C_6 comprise colpate, porate, colporate, and pororate pollen grains, respectively.

Shape and Size

The shape of pollen grains can vary from very flat (peroblate), where equatorial diameter is more than twice the length of the polar axis, to very elongate (perprolate) where the diameter is less than half the length of the polar axis. Although the variations in the shape of pollen are quite characteristic for taxonomic and phylogenetic considerations, these are less important than apertures.

The size of pollen grains varies from nearly 10 µm in *Myosotis* to as much as 200 µm in some Cucurbitaceae and Nyctaginaceae.

POLLEN WALL FEATURES

It has been proposed by Walker and Doyle (1975) that pollen wall architecture should include pollen wall stratification, exine structure and sculpturing.

The two principal layers of the pollen wall are the intine and exine (Fig. 5.3). Of these, the intine is the inner, more or less uniform layer. It is pectocellulosic in nature, and usually destroyed during acetolysis. The exine is the outer, acetolysis-resistant layer. It is also resistant to physical and biological degradation. Because of this property of the exine, pollen grains are found well preserved for long periods in fossil deposits.

Exine Stratification

Morphologically, the exine is divisible into two distinct layers (Fig. 5.3): (a) an outer sculptured layer called sexine, and (b) an inner non-sculptured layer, nexine. The sexine further comprises an internal layer of upright rod-like elements, the columellae (bacula) covered over by a roof-like layer,

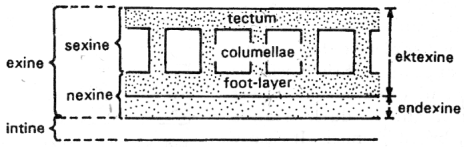

Fig. 5.3 Diagram showing wall stratification in a tectate-imperforate, non-acetolysed pollen grain, as seen in cross section. (*after* Walker, 1976)

the tectum. The exine is, thus, typically 3-layered consisting of tectum, columellae, and nexine. The tectum may be smooth, or have various types of processes.

Light microscopy as well as electron microscopy have revealed that, chemically, the exine is differentiated into two layers: an outer layer called ektexine and an inner layer, endexine (Fig. 5.3). These two layers may not always correspond to the two morphologically recognized layers, namely, sexine and nexine. Sometimes, the outer layer of nexine, called foot-layer, is chemically similar to sexine. In acetolyzed pollen it stains red with alcoholic fuchsin. In such cases the ektexine would include the sexine and the foot-layer. There are also on record such pollen grains where exine is undifferentiated into chemically distinct layers. Such homogeneous exine may be either ektexinous or endexinous.

Exine Structure

Structurally, the exine is of three basic types: tectate, semitectate, and intectate or atectate. In tectate and semitectate types the exine consists of nexine, columellae, and tectum. Whereas in the former type the tectum is continuous and roof-like (Fig. 5.3), in the latter it is perforated; the diameter of perforations is greater than the breadth of pollen wall between them, resulting in an open reticulum. If there is no tectum, and the columellae are free and exposed representing the sexine, the pollen are called intectate.

Exine Sculpturing

The exposed surface-details of the pollen wall constitute the sculpturing. Some of the more important types are: psilate (smooth), foveolate (pitted), fossulate (grooved), scabrate (very fine projections), verrucate (warty), baculate (rod-like elements), pilate (rod-like elements with swollen tips), gemmate (sessile pilar), echinate (spiny), rugulate (elongate elements irregularly distributed tangentially over the surface), striate (elongate, more or less parallel elements distributed tangentially over the surface), punctate (minute perforations) and reticulate (elements forming an open network).

LO-Analysis

The details of sporoderm patterns, which appear clear in thin sections, can

CHAPTER

5

MALE GAMETOPHYTE-MORPHOLOGY

The study of external morphological features of mature pollen grains is referred to as palynology. Since the first use of the term palynology by Hyde and Williams in 1845, several significant contributions have been made in this area, and it has emerged as an important discipline of fundamental and applied interests. In India, the chief centres of research in this field are : National Botanical Research Institute (Lucknow), Birbal Sahni Institute of Palaeobotany (Lucknow), Osmania University (Hyderabad) and Bose Institute (Calcutta).

Pollen grains are intitially formed in groups of four (tetrads). Each pollen grain has two poles (Fig. 5.1) at opposite ends of what is commonly described as the polar axis. The proximal pole (p.p.) is at the centre of the proximal face (toward the centre of the tetrad), whereas the distal pole is at the centre of the distal face (away from the centre of the tetrad). The polar axis must always be perpendicular with the distal pole at the apex and proximal pole at the base. The pollen grains are said to be heteropolar if their two faces are different, and isopolar if similar. In heteropolar grains one face

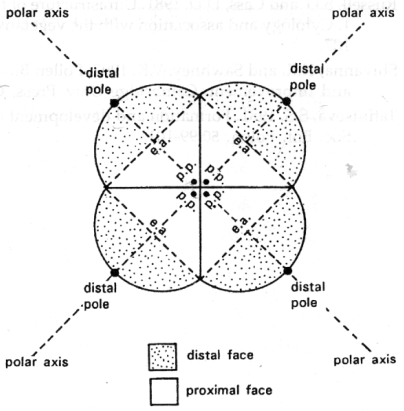

Fig. 5.1 Diagram of a pollen tetrad (all the pollen in the same plane), showing the polar axis, one equatorial axis (*e.a.*) of the equatorial plane, the distal face, the proximal face, the distal pole, and the proximal pole (*p.p.*) of each pollen grain in the tetrad. (*after* Walker, 1976)

has an aperture while the other has none. The equator runs round the surface of isopolar pollen grains midway between the poles. In order to illustrate the pollen grains in a uniform way and to facilitate comparison these terminologies and their definitions are essential. A schematic illustration (Fig. 5.1) of a pollen grain is called 'Palynogram'.

Apertures

An aperture is any weak area on the pollen surface which is directly or indirectly associated with its germination. Long apertures are called colpi, and short ones pores. The apertures may be simple or compound. Pollen grains with simple apertures are either colpate (with colpi) or porate (with pores). A compound aperture consists of a central region called oral, and an outer region called colpal in colporate pollen (with compound colpi), and poral in pororate pollen (with compound pores).

NPC-System

NPC refers to Number (*N*), Position (*P*), and Character (*C*) of apertures (Fig. 5.2). Under this system the usage of the term 'treme' has been recommended in place of 'aperture' for the purpose of preparing key for the classification of pollen grains (Fig. 5.2). Pollen grains without aperture are called atreme, also represented as N_0. Depending upon the number of apertures the pollen are monotreme (N_1), ditreme (N_2), tritreme (N_3), tetratreme (N_4), pentatreme (N_5), or hexatreme (N_6). Pollen grains with

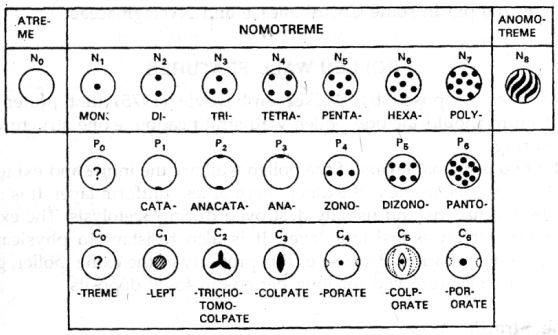

Fig. 5.2 The NPC-System. Diagram showing number (*N*), position (*P*), and character (*C*) of apertures. For details *see* text. (*after* Erdtman, 1969)

also be demonstrated by LO-analysis of entire pollen grains. The sporoderm pattern, at a higher plane of focus of the microscope, appears as bright islands separated by dark channels. On a lower adjustment it shows dark islands separated by bright channels. It clearly indicates that the picture at high adjustment is just the reverse of what is visible at the lower plane of focus. A comparative appraisal of the views under low and high planes of microscope adjustments is referred to as LO-pattern (Fig. 5.4).

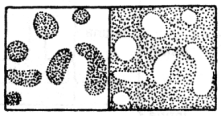

Fig. 5.4 LO-pattern in *Costus speciosus*. (*after* Nair, 1970).

SCOPE OF PALYNOLOGY

The palynological research can be either basic or applied. To the basic aspects belong the pollen morphology in relation to taxonomy and to the applied aspects belong geopalynology (fossil pollen grains), aeropalynology (pollen found in atmospheric air), iatropalynology (medical aspects such as hayfever, criminology, etc.), and melittopalynology (study of pollen in honey).

Applied Aspects of Palynology

The distribution of spore genera and species in coal is an important tool to investigate stratigraphical problems like correlation of coal seams and oil fields. It also helps in determining the age of rocks. The maximal use of applied palynology is in oil geology. Since many pollen and spore types serve as index fossils, these have helped in exploring oil-bearing strata. In fact, the most well-equipped palynological laboratories are those established by oil companies.

Aeropalynology is a subject of great importance because of its application in medicine, forestry, and palaeobotany. It is now established that airborne pollen cause allergies like hayfever and seasonal asthma. Information on the type of pollen in the air has been of great help in determining the allergens. Most of the plants that cause hayfever belong to weeds and grasses. Some of the allergenic taxa occurring in India are *Amaranthus spinosus, Artemisia scoparia, Chenopodium album, Cynodon dactylon, Prosopis juliflora, Ricinus communis*, and *Sorghum vulgare*. Seed production in forest trees is also closely linked with the occurrence of pollen in the air.

Pollen grains, to a great extent, reflect the vegetation of an area and its surroundings. In several cases it has been possible to locate the site of crime by studying pollen in the samples collected from dirt under the nails, and from mud stuck to shoes, clothes, etc.

Pollen samples from the stomach contents, or excrements, have provided a clue to several deaths in Brazil. The deaths were ascribed to the use of poisonous honey. The pollen of a poisonous plant, *Serjania lethalis* (Sapindaceae), were found in the stomach contents of one of the victims who died after taking poisonous honey.

There are several reports regarding the useful properties of certain pollen-tablets, tonics, creams, etc. The pollen tablets have been used in the treatment of prostatitis which has been confirmed by repeated experiments.

Plants are a source of both pollen and nectar collected by the honey-bee for making "bee-bread" (Nair, 1966). The pollen not only provides vitamins, minerals, and amino acids in the honey, but also information about the plants from which the nectar and pollen have been gathered. It also helps detect adulteration in the honey, and keeps in check the unscrupulous traders. Nair (1963) made an extensive study of Indian honeys collected from various places. Such an analytical study has provided useful data about pollen, and nectar-yielding plants. A trained melittopalynologist can easily distinguish between honey from two different localities. He can also tell the time when the particular honey was prepared.

Honey is said to be "unifloral" if it is dominated (50 per cent or more) by pollen of only one plant and "multifloral" if it contains various types of pollen in considerable percentages (Sharma and Nair, 1965). Seventy-six samples of Indian honey, examined microscopically showed the presence of pollen belonging to the following plants : *Eugenia, Nephelium, Sapindus, Putranjiva, Citrus, Plectranthus, Brassica* (all nectar-yielding plants), *Holoptelea, Alnus, Borassus* and other palms (all pollen-yielding plants; Nair and Chaturvedi, 1974). Such an analytical study provides useful data about pollen and nectar-yielding plants of a locality which should be helpful in establishing apiary gardens (Nair and Chaturvedi, 1974).

PREPARATION OF POLLEN GRAINS

For studying their external morphology, the pollen grains are acetolyzed using the following method (*see* Nair, 1960)*:

Pre-treatment

1. Collect fresh or fixed pollen material in 70% ethyl alcohol and crush with a glass rod. Sieve the dispersion through a fine mesh, and collect it into two separate tubes (*A* and *B*) in the ratio of 1:2.
2. Centrifuge the contents of tube *A* and add a few drops of 1% safranin. After 5 minutes add some water and centrifuge. Discard the supernatant and add more water. Continue washing with water until the supernatant is colourless. When washing is over, add 3 ml dilute glycerine (50% glycerine in water) and keep the tube aside.

*The National Botanical Research Institute, Lucknow, have prepared a pollenspore kit and a small information booklet which should prove to be a useful aid in teaching pollen morphology. The kit has 20 slides showing different pollenmorphotypes, and the booklet contains, besides the details of the slides, condensed information on pollen preparation, morphological characters, notes on morphological evolution, and vistas in palynology.

Acetolysis

3. Centrifuge portion *B*, pour out alcohol and add 5 ml glacial acetic acid. Centrifuge again and decant.
4. To the sediment add about 6 ml acetolysis mixture (1 ml concentrated sulphuric acid added drop by drop to 9 ml acetic anhydride).
5. Keep the tube in water bath and heat from 70°C to boiling point. Stir with a glass rod. Centrifuge and decant waste acetolysis mixture.
6. Add 10 ml glacial acetic acid and stir again. Divide the sediment in tube *B* into two equal portions (*B* and *C*).
7. Centrifuge the contents of tube B; remove acetic acid. Add water and shake well. Centrifuge again, and decant.
8. Add 2 ml dilute glycerine and keep tube *B* aside.

Chlorination

9. To tube *C* add one or two drops of saturated sodium chlorate solution in water, followed by one or two drops of concentrated hydrochloric acid.
10. Centrifuge and decant. Wash with water and add a few drops of methyl green. Wash again with water till it becomes colourless.

Mounting

11. Transfer the pollen from tube *A* to tube *B* and then from *B* to tube *C*. Mount pollen grains in glycerine jelly. Warm, and place a coverslip gradually. Seal with wax.

When seen through the microscope, the acetolysed pollen grains would look brown, the untreated ones red, and the chlorinated green.

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CHAPTER

6

MEGASPORANGIUM

The megasporangium together with its protective coats, the integuments, is called ovule. It is attached to the placenta, on the inner wall of the ovary (Fig. 2.1F, G), by a stalk called funiculus. An ovule ready for fertilization consists of nucellar tissue enveloped almost completely by one or two integuments, leaving a small opening at the apical end (Fig. 6.1). This opening, called micropyle, is the main passage for the entry of pollen tubes into the ovule. The basal region of the ovule, where funiculus is attached is called chalaza. In the nucellus is present the female gametophyte, also called embryo sac.

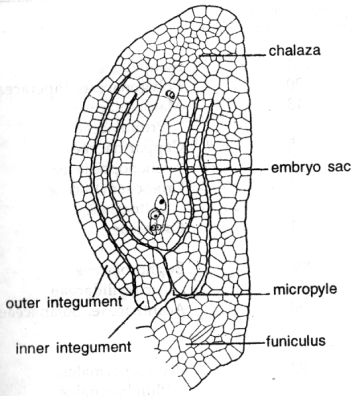

Fig.6.1 Ovule of *Limnocharis emarginata* as seen in median longitudinal section. (*after* Johri, 1938)

TYPES OF OVULES

Mature ovules are classified into five main types (Fig. 6.2). The classification

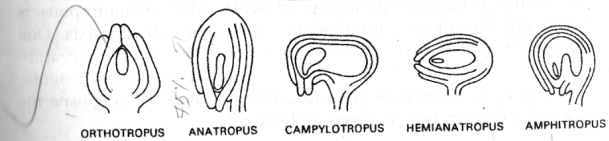

Fig.6.2 Types of ovules. (*after* Prantl; from Maheshwari, 1950)

is based principally on the position of the micropyle with respect to the funiculus. In orthotropous ovules (also called atropous) the micropyle and the funiculus lie in one line. The ovules where micropyle comes to lie close to the funiculus due to unilateral growth of the ovule are called anatropous (*see* Fig. 6.1). This is the most common type of ovule in angiosperms. According to Davis (1966) 82 per cent of the angiospermic families show anatropous ovules (Table 6.1). In campylotropous ovules the curvature is less than that in the anatropous ovules. Where the curvature of the ovule also affects the nucellus so that the latter becomes horse-shoe-shaped the ovule is called amphitropous. In hemianatropous or hemitropous ovules the funiculus is at right angles to the nucellus and the integuments.

TABLE 6.1 Data on the occurrence of various features of ovules in angiosperms. (*after* Davis, 1966)

Feature	*No. of families showing**	*Example*
OVULE		
Anatropous	204	Sympetalae
Orthotropous	20	Polygonaceae, Piperaceae
Hemianatropous	13	Malpighiaceae, Primulaceae
Campylotropous	5	Capparidaceae, Chenopodiaceae
Amphitropous	4	Crossosomataceae, Leitneriaceae
Circinotropous	1	Cactaceae
OVULE		
Bitegmic	208	Euphorbiaceae, Amaryllidaceae
Unitegmic	90	Acanthaceae, Solanaceae
MICROPYLE FORMED BY		
Inner integument	88	Centrospermales, Plumbaginales
Outer integument	4	Podostemaceae, Euphorbiaceae
Both integuments	74	Pontederiaceae

* Only those families are taken into account for which the feature is known to form a family character.

In the family Cactaceae a special type of ovule, called circinotropous, is found. Initially, the nucellar protuberance is in line with the axis. Due to unilateral growth it first becomes anatropous and, as the curvature continues, the micropyle again points upward in the fully formed ovule.

The frequency of occurrence of various types of ovules in angiosperms with examples of each type is given in Table 6.1.

INTEGUMENTS

Mostly an ovule has either one or two integuments. Ovules with one integument are called unitegmic, and those with two integuments are known as bitegmic. The Sympetalae predominantly show unitegmic condition. Bitegmic ovules occur in Polypetalae and monocots (Table 6.1). In some members of the Olacaceae (*Liriosma, Olax imbricata, Ptychopetalum*) the ovules lack an integument and are called ategmic (Davis, 1966).

Ontogenetically, an ovule arises as a small mound of homogeneous tissue (Fig. 6.3A) on the placenta. At this stage the ovule looks orthotropous. Integuments arise close to the base of this tissue (Fig. 6.3B) which forms the nucellus in a mature ovule. Except for Euphorbiaceae, where the inner integument is initiated sub-dermally (Bor and Bouman, 1974), in all others it is dermal in origin (*see* Bouman, 1984). The outer integument is initiated either dermally or sub-dermally. With the differentiation of integuments the ovule begins to curve (Fig. 6.3C, D), and by the megaspore tetrad stage it assumes its final shape (Fig. 6.3E). Although the integuments initiate later they grow faster than the nucellus and soon surround it almost completely, except in the region of the micropyle. Rarely, as in *Macadamia* (Proteaceae), the growth of the integuments is so slow that they do not enclose the nucellus until after fertilization.

In bitegmic ovules the inner integument differentiates earlier than the outer integument but the latter usually overgrows the former. In a fully formed ovule the outer integument is more massive and thicker as compared to the inner integument. Occasionally, the inner integument projects well beyond the outer integument (Annonaceae, Cactaceae, Proteaceae, Trapaceae).

The primordia of both the integuments arise independently at the base of the nucellus. However, in *Lannea* and *Rhus* the two integuments arise as a result of splitting of the single integumentary primordium. The unitegmic condition of ovules is considered to be derived from fusion of the two integuments, as in some Lecythidaceae and Myrtaceae, or by suppression of one integument.

Various degrees of fusion among its parts is a common feature of ovule;

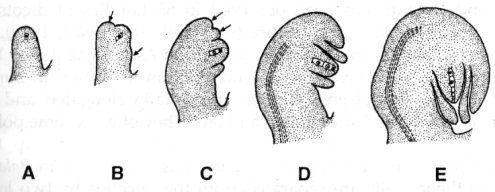

Fig. 6.3 **A-D.** Stages in ovule development in *Aquilegia vulgaris*. Arrows are pointing at the primordia of integuments. **E.** Fully developed bitegmic, anatropous ovule. (*after* Bhandari and Vijayaraghavan, 1970)

the two integuments in a bitegmic ovule may be fused along their length, or the inner integument may be fused with the nucellus up to various lengths. In anatropous ovules, very often, the outer integument on the side of the funiculus is almost indistinguishable from the funiculus because of their congenital fusion (Fig. 6.2).

In some taxa, especially belonging to the family Cactaceae, a prominent air space is present between the two integuments in the chalazal region. This feature is also shown by *Bassia* (Fig. 6.4), *Tetragonia tetragonioides* (Fig. 6.7) and *Trianthema argentea*. The occurrence of stomata on the outer integument has been reported in *Cleome, Isomeris* and *Magnolia*. In *Gossypium* stomata differentiate in the chalazal region of the outer integument two days before anthesis. In addition to stomata, abundant chlorophyll is present in the integuments of *Hymenocallis occidentalis*. Chlorophyllous integuments also occur in *Amaryllis belladonna, Gladiolus communis* and *Lilium martagon*.

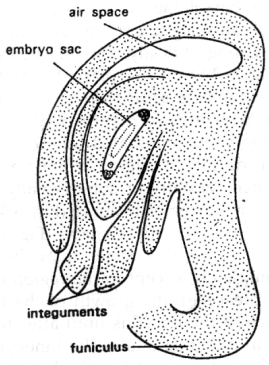

Fig. 6.4 Longitudinal section of an ovule of *Bassia* at mature embryo sac stage, to show the prominent air space between the two integuments. (*after* Hindmarsh, 1965)

ENDOTHELIUM. In most plants belonging to the Sympetalae with unitegmic, tenuinucellate ovules, the nucellus degenerates at an early stage of ovule development, and the innermost layer of the integument becomes specialized to perform the nutritive function for the embryo sac (Figs 6.5, 6.11). This specialized tissue, present around the embryo sac, is called endothelium. The differentiation of endothelial layer is also known in some plants with bitegmic ovules. In these cases it is derived from the inner epidermis of the inner integument. It may differentiate even before the disorganization of the nucellus. The occurrence of endothelium has been observed in 65 families of dicots. In 47 families it forms a diagnostic feature (*see* Kapil and Tiwari, 1978).

The endothelium is usually single-layered. In Asteraceae it may become multilayered; ten to twelve-layered endothelium is known in sunflower (*Helianthus*). The cells of endothelium are radially elongated and rich in cytoplasm (Fig. 6.5), and store starch and fats. They often become polyploid. In *Pedicularis palustris* the endothelial cells show ploidy up to 32n. Multinucleate condition of the endothelial cells is reported in *Balanites*

The endothelial cells are separated from the nucellus by two layers of cuticle, one belonging to the nucellus and the other to the integuments. The two cuticular layers later fuse. In addition to the cuticle, the cells of the endothelium also show wall thickenings. Normally, the endothelium covers

the entire embryo sac. However, sometimes it may remain confined to the lower two-thirds of the chalazal half, or to the micropylar end of the embryo sac. The time of differentiation of endothelium and the duration of its activity are also variable. In some families the endothelium persists as a distinct layer in the seed. It forms a pigment layer in Polemoniaceae, Plantaginaceae and Linaceae (*see* Bouman, 1984).

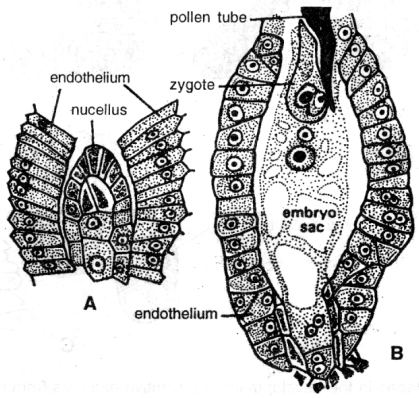

Fig. 6.5 Endothelium in the Asteraceae. **A.** *Volutarella ramosa*, at the megaspore tetrad stage. **B.** *Glossocardia bosvallia*, at fertilization. (*after* Deshpande, 1970)

At the ultrastructural level the endothelial cells exhibit features characteristic of meristematic and secretory cells. They possess high concentrations of proteins, RNA, carbohydrates, ascorbic acid and enzymes. The endothelial cells are in communication with each other and with those of the integument through plasmodesmata. Esser (1963) reported the occurrence of callose in the walls of endothelial cells of *Petunia hybrida*. It has been suggested that callose probably isolates the embryo sac from the influence of sporophytic tissues at the time of fertilization (*see* Kapil and Tiwari, 1978).

In some incompatible crosses, such as *Chrysanthemum makinoi* x *C. japonense*, the proliferation of endothelial cells results in the abortion of the embryo (Tanaka and Watanabe, 1972). This has also been observed in some incompatible combinations in *Datura, Lycopersicon, Nicotiana* and *Solanum*.

An interesting feature of the endothelial cells is the development of adventive embryos. Maheswari Devi and Pullaiah (1976) reported that in *Melampodium divaricatum*, sometimes, the endothelial cells look like egg

and undergo divisions forming structures resembling zygotic embryos. Such embryos lack suspensor and in this respect differ from the zygotic embryos (Fig. 6.6).

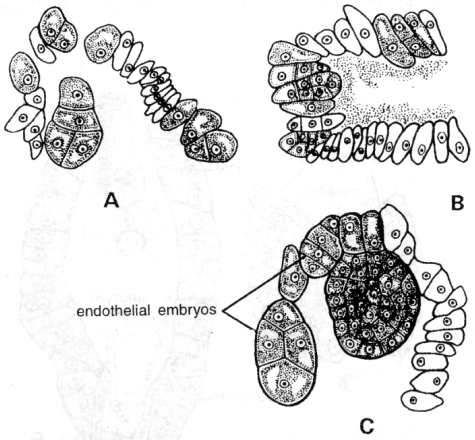

Fig. 6.6 Stages in the development of adventive embryos from endothelial cells in *Melampodium divaricatum*. (*after* Maheswari Devi and Pullaiah, 1976)

Although detailed structural and physiological studies concerning endothelium are lacking, its presence around the embryo sac and its cytological features suggest that it may be functionally similar to the anther tapetum which surrounds the sporogenous tissue. For this reason endothelium is also called integumentary tapetum.

In the Begoniaceae, Droseraceae, Elatinaceae and Fabaceae the persistent nucellar cells form endothelium-like tissue. Because of its different origin it has been called false-endothelium.

MICROPYLE. Depending upon the presence or absence of integument/s, the micropyle may or may not be organized. In bitegmic ovules the micropyle is generally formed by either both the integuments or only the inner integument. Only rarely does the outer integument alone constitute the micropyle. When both the integuments are involved the passage formed by the outer integument is called exostome and that by the inner integument is called endostome. In the Resedaceae, the exostome and endostome are not in the same line, so that a zig-zag path is formed.

The micropyle in *Ornithogalum* becomes filled with an exudate given out by the nucellar cap and the inner integument. An interesting feature is the formation of a thin sheet of material across the exostome, thus sealing the

latter. The exudate and the thin sheet (called "hymen" by Tilton, 1980) ensure a localized deposition of synergid-synthesized chemotropic agents in the micropyle. This may act as a stimulus for the pollen tube to enter the micropyle. In post-fertilization stages a plug is formed that occludes the micropyle. The plug probably has a protective role against desiccation and pathogen invasion.

OBTURATOR. Any ovular structure associated with directing the growth of pollen tube toward the micropyle is generally referred to as obturator. Obturators exhibit great variation in their origin, morphology, anatomy and extent of development. They may originate from placenta or funiculus, or both. The most common type of obturator is one formed by local swelling of the funiculus (Acanthaceae, Anacardiaceae, Lamiaceae, Magnoliaceae). In *Crinum* the funiculus simply becomes knee-shaped and functions as an obturator. In *Ceratocephalus* the cells of funicular epidermis, little above the

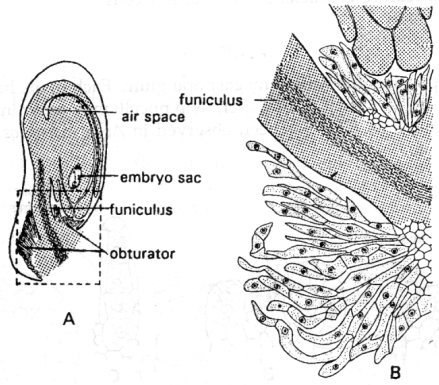

Fig. 6.7 *Tetragonia tetragonioides*. **A.** Longitudinal section of an ovule at the mature embryo sac stage. Note the presence of obturator on both sides of the funiculus. **B.** Enlarged view of the portion emblocked in **A**, to show multicellular, glandular hairs of the obturator. (*after* Prakash, 1967)

micropyle, elongate radially, become densely cytoplasmic and constitute the obturator. It degenerates after fertilization. In *Tetragonia tetragonioides* the obturator, comprising glandular epidermal hairs, arises from both sides of the long funiculus. However, it is better developed on the side away from the micropyle as compared to that on the side of the micropyle (Fig. 6.7).

Placental obturator occurs in the Euphorbiaceae and Cuscutaceae. In *Aegle* (Rutaceae) some epidermal cells of the funiculus as well as placenta

According to Davis (1966), of the 314 families for which information is available, 179 families show crassinucellate ovules, 105 families bear tenuinucellate ovules, and 11 families possess pseudo-crassinucellate ovules. In the remaining 19 families the nucellus forms only generic or specific character.

In several plants the cells of the nucellar epidermis form a cap in the micropylar region. In *Ornithogalum* these cells become columnar and lignified and are densely cytoplasmic with large amounts of RNA, smooth endoplasmic reticulum and microtubules with swollen cisternae. The high vesicular activity in these cells seems to be associated with the thickening of the proximal walls.

Generally, the nucellus remains within the confines of the inner integument. Rarely, however, it may project into the micropyle (Caryophyllaceae) or beyond it forming a nucellar beak (Euphorbiaceae). Nucellar beak has also been reported in members of the Cucurbitaceae, Nyctaginaceae, Polygonaceae and Salicaceae. Its cells may store starch and protein crystals.

The nucellus is mostly consumed by the developing embryo sac or endosperm. In some plants, it persists in the mature seed as a nutritive tissue. The persistent nucellus is called perisperm (for details *see* Chapter 12). There is the other extreme, where the nucellar tissue breaks down precociously. Consequently, a large cavity, called pseudo-embryo sac, is formed around the embryo sac (Fig. 12.15). This feature is unique to the family Podostemaceae (*see* Chapter 16). The pseudo-embryo sac may be formed at the megaspore mother cell stage (*Dicraea, Zeylanidium*) or after fertilization (*Indotristicha, Terniola*). In the absence of endosperm in the Podostemaceae the pseudo-embryo sac, which contains cytoplasm and free nuclei, nourishes the developing embryo.

HYPOSTASE AND EPISTASE. Hypostase refers to a group of cells present right below the embryo sac and above the vascular supply to the funiculus (Fig. 6.11). They become thick-walled due to lignification, and are poor in cytoplasmic contents. Occasionally, the cells of the hypostase may surround a portion of the embryo sac and may even extend into the micropylar half of the ovule (Tilton, 1980). Histochemical studies have shown that in *Agave* the cells of the hypostase accumulate starch, proteins and lipids. In *Ornithogalum* although starch is present, but no proteins or lipids could be localized. Hypostase occurs in many families such as Amaryllidaceae, Liliaceae, Zingiberaceae, Euphorbiaceae, Crossosomataceae, Theaceae and Apiaceae. In the Elaeagnaceae a prominent hypostase is organized after fertilization. In the Loranthaceae a hypostase (collenchymatous pad) is present below the archesporium. In *Aristolochia* the hypostase persists in the mature seed. Some of the functions attributed to hypostase are:

(1) Van Tieghem (1901), who was the first to identify this tissue and coin the term, suggested that hypostase forms a sort of barrier or boundary for the growing embryo sac and prevents it from pushing into the base of the ovule.

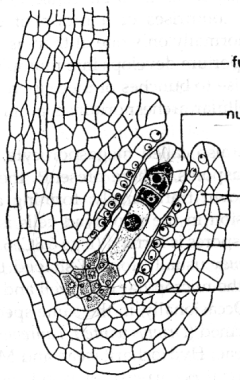

Fig. 6.11 *Bupleurum tenue.* Longitudinal section of ovule at the tetrad stage, showing hypostase and young endothelium. The three non-functional megaspores are on way to degeneration. (*after* Gupta and Gupta, 1964)

(2) It stabilizes the water balance of resting seeds over the long period of dormancy during the hot, dry seasons (Johansen, 1928).

(3) It serves to connect the vascular bundle in the funiculus with the embryo sac and, thus, facilitates the rapid transport of food materials (Venkata Rao, 1953). According to Tilton (1980), while the chief function of the hypostase is in the translocation of nutrients, it may, in some cases, act secondarily as a storage tissue.

(4) It may produce certain enzymes or hormones, or play a protective role in mature seeds (*see* Bouman, 1984).

While hypostase is derived from the nucellar cells below the embryo sac, the epistase is formed by the nucellar epidermis above the embryo sac (*Castalia, Costus*). It forms a cap-like structure of cutinized cells and is distinguishable even during advanced stages of embryo development.

MEGASPOROGENESIS

A single hypodermal cell in the nucellus functions as the archesporium (Fig. 6.8). It becomes more prominent than its surrounding cells because of its large size, denser cytoplasm and large nucleus. In tenuinucellate (Fig. 6.8) and pseudo-crassinucellate ovules the archesporial cell directly functions as megaspore mother cell, whereas in crassinucellate ovules it divides periclinally, cutting an outer primary parietal cell and an inner primary sporogenous cell. The latter functions as the megaspore mother cell (Fig. 6.9).

elongate into richly cytoplasmic, multicellular hairs reaching as far as the micropyle. In Thymelaeaceae the obturator is of stylar origin. Cells of the stylar canal elongate and extend up to the micropyle.

In some primitive genera cells at the apex of the ovarian locule (*Exospermum*) and some cells at the base of the funiculus (*Exospermum, Drimys*) proliferate and become secretory. The obturator may fill the micropyle to varying degrees; in *Arenaria* it shows a most prolific condition completely filling the locule (*see* Tilton and Horner, 1980).

The cells of the surface layer of the obturator in *Ornithogalum* are columnar and densely cytoplasmic being rich in ER, dictyosomes, vesicles and other organelles. The pollen tube grows along the obturator. The cells of the obturator produce a surface exudate and provide nutrition and mechanical and chemical guidance to the pollen tube (Tilton and Horner, 1980). In *Caltha* (Peterson *et al.*, 1979) the cells of the obturator show wall ingrowths, a feature characteristic of transfer cells.

NUCELLUS

Nucellus represents the wall of megasporangium. Each ovule has only one nucellus. As an abnormality, however, twin nucelli may occur in a common fold of integuments. This has been observed in *Aegle marmelos, Hydrocleis nymphoides*, etc.

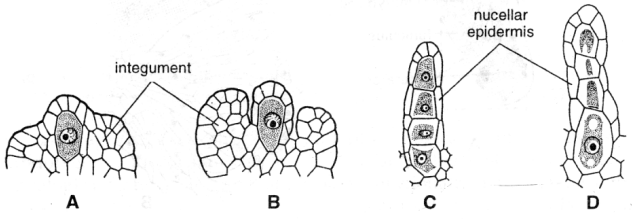

Fig. 6.8 Megasporogenesis in tenuinucellate ovule of *Elytraria acaulis*. Integument is not drawn in **C** and **D**. **A, B**. Megaspore mother cell stage. **C**. Tetrad stage. **D**. The functional, chalazal megaspore has enlarged while the other three megaspores have degenerated. (*after* Johri and Singh, 1959)

The archesporium differentiates immediately below the nucellar epidermis. In Sympetalae the archesporial cell directly functions as the megaspore mother cell so that the sporogenous cell is also hypodermal. Such ovules, where the sporogenous cell is hypodermal and the nucellar tissue around it remains single-layered (Fig. 6.8), are called tenuinucellate. In some other families the hypodermal archesporial cell divides transversely, cutting an outer parietal cell and an inner sporogenous cell. The parietal cell may either remain undivided or undergo a few periclinal and anticlinal divisions so that the sporogenous cell becomes embedded in the massive

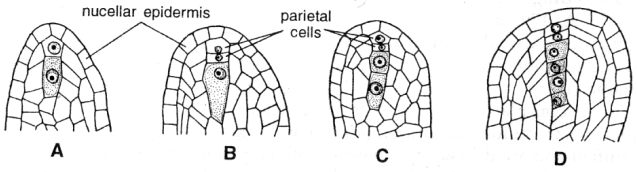

Fig. 6.9 Megasporogenesis in crassinucellate ovules of *Myriophyllum intermedium*. Stippled, nucleate cells are the megaspore mother cell and its derivatives whereas unstippled, nucleate cells are the primary parietal cell and its derivatives. **A**. After the division of the archesporial cell, forming primary parietal cell and primary sporogenous cell. **B**. The primary parietal cell has divided periclinally whereas the primary sporogenous cell has simply enlarged. **C**. Dyad stage. **D**. Tetrad stage. (*after* Bawa, 1969)

nucellus (Fig. 6.9). The sporogenous cell may also become embedded in the nucellar tissue by divisions in the nucellar epidermis (*Anemone*). In *Nigella damascena*, the primary parietal cell as well as nucellar epidermis contribute to the peripheral nucellar tissue (Fig. 6.10). All such ovules where the sporogenous cell becomes sub-hypodermal, either due to the formation of parietal cells, or due to divisions in the nucellar epidermis, or both, are called crassinucellate. Davis (1966) has, however, suggested that only those ovules should be referred to as crassinucellate where the sporogenous cell

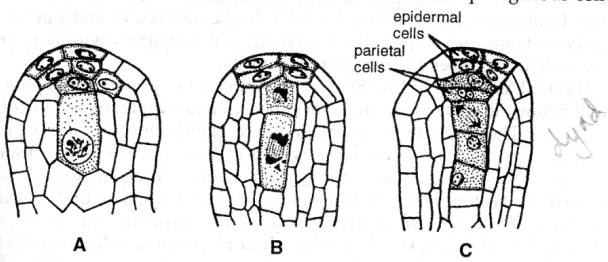

Fig. 6.10 Megasporogenesis in *Nigella damascena*. In this taxon the megaspore mother cell becomes embedded in the nucellar tissue due to divisions in the epidermal cells of the nucellus and the formation of parietal cells. (*after* Vijayaraghavan and Marwah, 1969)

becomes sub-hypodermal due to the occurrence of parietal cells. She has proposed the term pseudo-crassinucellate for all those ovules where divisions in the nucellar epidermis are responsible for the sub-hypodermal nature of the sporogenous cell. Tenuinucellate condition of ovule generally occurs in more advanced families.

Mostly, the female archesporium is single-celled but in the Paeoniaceae and Crossosomataceae it comprises of a group of cells, like the male archesporium. However, normally only one of them is functional. In *Sedum* sp. the multicellular archesporium develops into multiple megaspore mother cells which, in turn, give rise to bunches of megaspore tetrads arranged in filamentous rows. Multicellular archesporium is a family character of the Loranthaceae.

Megaspore mother cell, also called megasporocyte, undergoes meiosis to form four haploid megaspores. Where present[1], the wall after the first meiotic division is laid down transversely, forming a dyad (Fig. 6.9C). Similarly, the second division in the two dyad cells is transverse. In this way a linear tetrad of megaspores is formed (Figs 6.8C, 6.9D). *Orchis maculata* (Fig. 6.12), *Rheum* and *Rumex* usually form T-shaped tetrads, which arise due to vertical division in the micropylar dyad cell and transverse division in the chalazal dyad cell. Occasionally, in the same species both linear and T-shaped tetrads may be formed (*Drimys winteri, Laurembergia brevipes, Orchis maculata*). In the Crassulaceae, Hydrocharitaceae and Musaceae there have been reports of the occasional occurrence of isobilateral, tetrahedral and T-shaped tetrads, respectively.

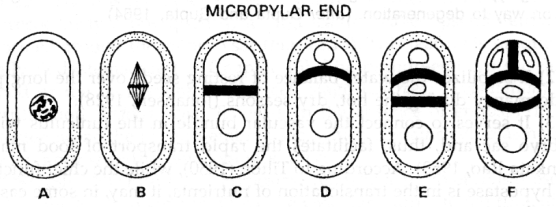

Fig. 6.12 Stages of megasporogenesis in *Orchis maculata*. **A.** Megaspore mother cell in diakinesis. **B.** Same, in metaphase-I. **C.** Young dyad. **D.** Older dyad with curved cross-wall. **E.** Triad. **F.** Tetrad. (*after* Rodkiewicz and Gorska-Brylass, 1968)

The second meiotic division in the two dyad cells may be asynchronous so that sometimes a triad, comprising two megaspores and one dyad cell, is formed. For example, in *Orchis maculata* the first meiotic division gives rise to two unequal cells (Fig. 6.12C, D). The chalazal cell of the dyad is generally two times larger than the micropylar cell. Nearly always, the chalazal cell divides first by a transverse division cutting a small micropylar cell and a large chalazal cell (Fig. 6.12E). In this way a triad is formed. Eventually, the micropylar dyad cell divides vertically, giving rise to a T-shaped tetrad (Fig. 6.12F).

[1]Formation of wall after each meiotic division, lack of wall formation after the second division, or after both divisions form the basis of classification of female gametophytes in angiosperms (*see* Chapter 7).

When both the meiotic divisions are followed by wall formation only one of the four megaspores functions and forms the female gametophyte; the other three megaspores degenerate. Generally, it is the chalazal megaspore of the tetrad that is functional (Fig. 6.8D). However, in the Onagraceae the embryo sac is formed by the micropylar megaspore. In *Schizomeria serrata*, as a rule, the sub-chalazal megaspore is functional.

In plants where the second meiotic division is not accompanied by wall formation, one of the dyad cells with two haploid megaspore nuclei contributes toward the formation of embryo sac, whereas the other dyad cell degenerates. Similarly, where no wall formation occurs throughout meiosis, all the four megaspore nuclei participate in the formation of embryo sac (for details *see* Chapter 7).

Physiological aspects of megasporogenesis have received very little attention as compared to parallel studies with microsporogenesis. This is because of some technical difficulties. There is just one megaspore mother cell in a megasporangium as compared to numerous microspore mother cells per microsporangium. Also, megasporocytes are not amenable to standard physiological and biochemical techniques as are the microsporocytes. Hundreds of microspore mother cells can be obtained almost free of anther wall tissue by cutting the anther at one end and pressing it at the other end. This is not possible with the megasporocytes. However, whatever little information is available it suggests a close similarity between microsporogenesis and megasporogenesis.

The wall of the megasporocyte is different from that of the adjacent nucellar cells in showing a rich distribution of randomly oriented fibrillar material. In *Lilium*, paramural bodies are present in the space between cell wall and plasma membrane (de Boer-de Jeu, 1978). These bodies are said to be involved in cell wall synthesis, and storage of polysaccharides.

The ultrastructural work on *Dendrobium* by Israel and Sagawa (1964) has revealed that the premeiotic megaspore mother cell is connected with its surrounding cells through plasmodesmatal connections. During late prophase of the first meiotic division these cytoplasmic continuities are lost. This has also been shown in maize (Diboll and Larson, 1966).

Another notable change that occurs during megasporogenesis is the deposition of callose in the wall of the megaspore mother cell (Figs 6.13, 6.14) which is originally cellulosic. Of the 43 species examined by Rodkiewicz (1970), 40 (39 species with monosporic embryo sac, and 1 species with bisporic embryo sac) showed the appearance of callose. The remaining 3 species, which bear tetrasporic embryo sac, did not show this feature.

In monosporic type of embryo sacs the functional megaspore in the spore tetrad may be the chalazal one (*Polygonum* type) or the micropylar one (*Oenothera* type). In *Epilobium palustre*, an example of *Oenothera* type of embryo sac, during the first meiotic prophase of megasporogenesis callose appears in the entire wall of the megasporocyte (Fig. 6.14A, B). Later the callose either disappears or becomes very feeble at the micropylar apex, and this is maintained throughout megasporogenesis (Fig. 6.14C-F). The cross-walls formed after each meiotic division are also richly callosic.

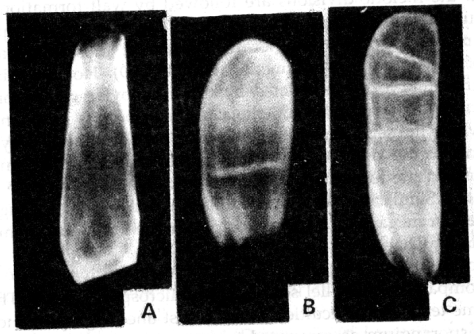

Fig. 6.13 Fluorescence photomicrographs of 3 stages in megasporogenesis in *Fuchsia hybrida*, taken after staining with aniline blue. The brighter areas represent the presence of callose. **A.** Megaspore mother cell. **B.** Dyad. **C.** Tetrad. (*Courtesy*: Professor B. Rodkiewicz, Poland)

Actually, the cross-walls are richer in callose than the outer wall (Fig. 6.14D-F). At the conclusion of the second meiotic division the callose first disappears from the side walls of the micropylar megaspore (Fig. 6.14F). The situation is reverse in *Polygonum* type of embryo sac. Here it is the chalazal end of the megasporocyte and, later, the chalazal megaspore which has a region with very little or no callose. In either case, in a megaspore tetrad the three non-functional megaspores are completely bound by callose-

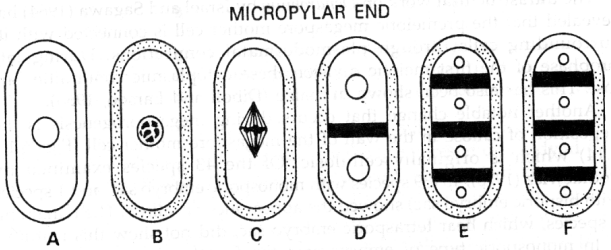

Fig. 6.14 Diagrams to show the appearance and disappearance of callose during megasporogenesis in *Epilobium palustre*. Regions with high concentration of callose are drawn dark, those with low concentration are stippled, and the regions lacking callose are drawn blank. **A.** Megaspore mother cell. **B.** Same, at a later stage when callose has appeared in its wall. **C.** Same, at metaphase-I. **D.** Dyad. **E.** Tetrad. **F.** An older tetrad; callose has almost completely disappeared from wall around the functional, micropylar megaspore. (Drawn *after* Rodkiewicz and Bednara, 1974)

rich walls whereas the functional megaspore, chalazal one in the *Polygonum* type and micropylar one in the *Oenothera* type, has throughout a region with least callose. A special situation exists in *Epipactis latifolia*, an example of *Polygonum* type of embryo sac. In this orchid the chalazal wall which appears continuously impregnated with callose, is actually highly porous and looks like a sieve plate (Fig. 6.15; Rodkiewicz, 1975).

Callose has at least two important properties: (a) it is highly impermeable,

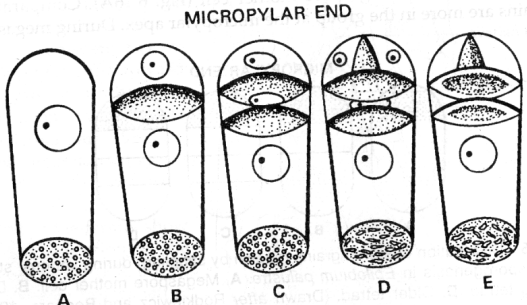

Fig. 6.15 Distribution of callose during megasporogenesis in *Epipactis latifolia*. Areas having callose are shown with thick lines or dense stippling. Although the chalazal wall remains impregnated with callose throughout, it has numerous small areas without callose giving the appearance of a sieve plate. **A.** Megaspore mother cell. **B.** Dyad. **C.** Triad. **D.** Early tetrad. **E.** Late tetrad, with callose partially disappeared from the cross-walls. (*after* Rodkiewicz, 1975)

and (b) it is very readily synthesized and degraded. Thus, the disappearance of plasmodesmatal connections and the appearance of callose all around the megasporocyte in the beginning of megasporogenesis brings about a sort of isolation of the megasporocyte. A comparison clearly suggests that the periods of appearance and disappearance of callose during megasporogenesis and microsporogenesis are almost the same. The temporary isolation of the sporocyte (micro- as well as mega-) may be connected with the process of differentiation of the sporocyte for basically a new type of development. During megasporogenesis, the polarization of the megasporocyte and megaspore tetrads is very obvious which results in the formation of an active megaspore. Invariably, the active functional megaspore has a callose-free wall or porous wall to allow the passage for the movement of substances into it. The non-functional megaspores, on the other hand, remain bound by callose wall for a long time and eventually degenerate. Noher de Halac and Harte (1977) suggested that the direction of nutritional supply in the ovule determines as to which of the four megaspores in a tetrad would give rise to the embryo sac.

Polarization of megasporocyte and megaspore tetrad can also be seen with respect to the distribution of organelles and starch grains (Rodkiewicz,

1974). In *Epipactis palustris* the chalazal wall of the megaspore mother cell is distinct from other walls in having ingrowths and plasmodesmata (Bednara *et al.*, 1981). The lack of pores in the micropylar part of the nuclear membrane, unlike the chalazal part, during prophase-I of megasporogenesis further suggests a polar differentiation of the megaspore mother cell along the micropylar-chalazal axis.

In *Epilobium palustre*, initially starch grains are grouped exclusively at the two poles of the megaspore mother cell (Fig. 6.16A). Comparatively, the grains are more in the group at the micropylar apex. During megasporo-

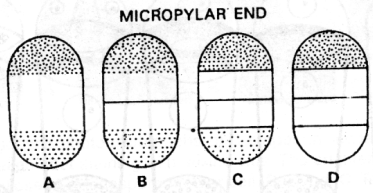

Fig. 6.16 Distribution of starch grains (shown by stippling) during various stages of megasporogenesis in *Epilobium palustre*. **A**. Megaspore mother cell. **B**. Dyad. **C**. Young tetrad. **D**. Older tetrad. (Drawn *after* Rodkiewicz and Bednara, 1974)

genesis the grains do not move from their position (Fig. 6.16B). Consequently, at the tetrad stage they are restricted to the two polar spores; the middle two spores lack starch grains (Fig. 6.16C). Subsequently, the number and size of the starch grains gradually decrease in the chalazal megaspore whereas in the micropylar megaspore new grains are synthesized. Eventually, the functional micropylar megaspore is filled with starch grains and the non-functional chalazal megaspores become completely free of them (Fig. 6.16D).

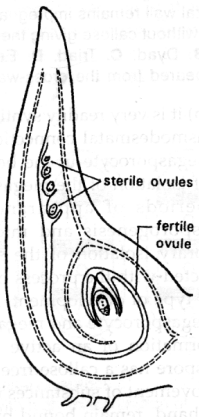

Fig. 6.17 Longitudinal section of a carpel of *Adonis flammea*. In addition to a fertile ovule, it has 4 sterile ovules. (*after* Bhandari, 1966)

SPECIAL FEATURES

Reduced Ovules:

In the achene-bearing genera of Ranunculaceae the carpels are usually uniovulate. In *Adonis flammea* (Fig. 6.17) and *Clematis* each carpel has 2-4 sterile ovules in addition to a fertile

one. Similarly, in *Anemone obtusiloba* there is one fertile ovule and two marginally elongated cylindrical structures. The latter represent the sterile ovules. In *Adonis chrysocyathus* and *Anemone rivularis* the sterile ovules are absent. Unlike the fertile ovules, the sterile ovules lack differentiation into nucellus and integuments, and do not have vascular supply. It has been suggested that the occurrence of sterile ovules, sometimes with 4 or 5 megaspore mother cells (*Anemone obtusiloba*), represent an evolutionary tendency toward the origin of uniovulate achenes from multiovulate follicles (Bhandari, 1968).

In the family Loranthaceae there is no ovule in the usual sense of the term. Many members of the family show a conical projection at the base of the ovary (Fig. 6.18A, B). The morphological nature of this structure, called mamelon or placenta, has been much debated. In genera such as *Lysiana* and *Nuytsia* the placenta is lobed, and in *Helicanthes* it is unlobed. In lobed placenta the archesporium differentiates hypodermally in each lobe whereas in unlobed placenta the entire sub-epidermal tissue functions as the archesporium.

Dendrophthoe, Helixanthera, and *Moquiniella* represent even a more obscure condition. In these genera there is no trace of any projection at the base of the ovary. The archesporial tissue differentiates in very young bud as a compact tissue in the hypodermis at the base of the ovarian cavity (Fig. 6.19A, B). The archesporial cells directly function as megaspore

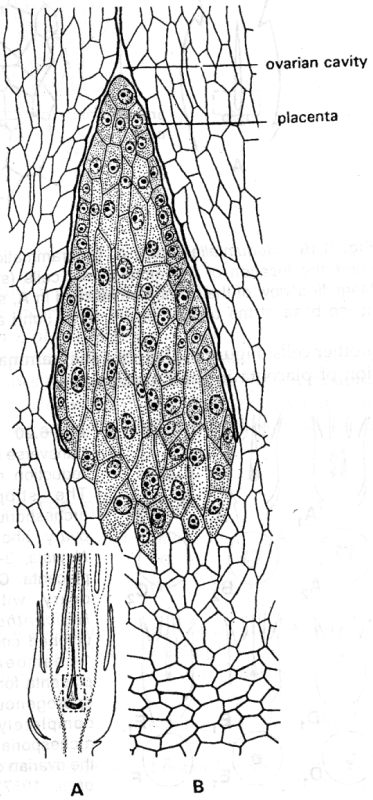

Fig. 6.18 *Helicanthes elastica*. **A**. Diagrammatized longitudinal section of the ovary to show the position of placenta or mamelon (stippled projection inside the block). **B**. Enlarged view of the placenta along with the adjacent ovarian tissue, from A. (*after* Johri *et al.*, 1957)

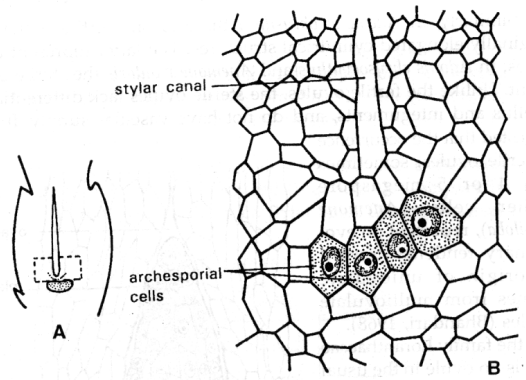

Fig. 6.19 *Moquiniella rubra*. **A**. Diagrammatic longitudinal section of ovary to show the location of the archesporial cells (stippled area inside the block). **B**. Magnified view of the portion emblocked in **A**, showing hypodermal archesporium at the base of the ovarian cavity. (*after* Johri and Raj, 1969)

mother cells. Figure 6.20 gives a diagrammatic representation of the reduction of placenta in the Loranthaceae.

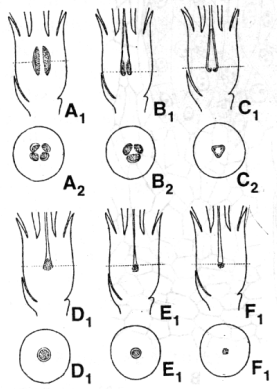

Fig. 6.20 Longitudinal sections (A_1-F_1) and transverse sections (A_2-F_2) of ovaries to show reduction of placenta in the Loranthaceae. The stippled regions represent the archesporium. A_1, A_2. *Lysiana*; 4-chambered ovary, showing 4-lobed placenta. B_1, B_2. *Nuytsia*; 3-chambered ovary, with 3-lobed placenta. C_1, C_2. *Macrosolen*; 1-chambered ovary, with 3-lobed placenta. D_1, D_2. *Helicanthes*; 1-chambered ovary, with unlobed conical placenta. E_1, E_2. *Amyema*; 1-chambered ovary, showing unlobed placenta formed as a result of elongation of sporogenous cells. F_1, F_2. *Helixanthera*; completely reduced placenta. The archesporial cells differentiate at the base of the ovarian cavity. (Modified *after* Maheshwari *et al.*, 1957)

Megaspore Haustoria

An uncommon phenomenon, seen in some species of Crassulaceae, Lauraceae, Rosaceae and Rubiaceae, is the formation of aggressive megaspore haustoria (*see* Mauritzon, 1933; Subramanyam, 1967; Rutishauser, 1969). In *Sedum chrysanthum* and *S. sempervivoides* each megaspore of the tetrad produces a subterminal protrusion which grows toward the apical region of the nucellus in the form of a tube (Fig. 6.21). Since 2-5 megaspore tetrads are present in a megasporangium, the upper part of the nucellus shows a tangle of megaspore tubes or haustoria. Only one of the haustorial tubes penetrates through the nucellar epidermis and enlarges into an extranucellar vesicle. The megaspore from which this tube arises alone forms the embryo sac.

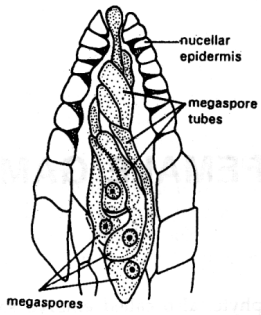

Fig. 6.21 Megaspore tetrad of *Sedum chrysanthum*. All the megaspores have given out tubes which are growing toward the micropyle. (*after* Subramanyam, 1967)

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CHAPTER

7

FEMALE GAMETOPHYTE

The female gametophyte, also called embryo sac, is mostly a 7-celled structure (Fig. 7.1). There is a large central cell with two polar nuclei which later fuse to form the secondary nucleus (Fig. 7.2E). The micropylar end of the central cell is occupied by the egg apparatus, comprising an egg cell and two synergids, and at its chalazal end three antipodal cells are present.

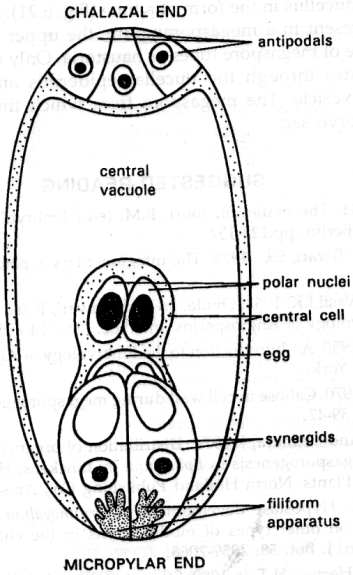

Fig. 7.1 Diagram of an organized embryo sac. (modified *after* Jensen, 1972)

Cells of the egg apparatus and the antipodal cells are uninucleate and haploid whereas the central cell is binucleate or diploid.

The development of the embryo sac begins with the elongation of the functional megaspore, which is usually the chalazal one of the tetrad. The elongation is largely in the micropylar-chalazal axis. Initially, the megaspore cytoplasm is non-vacuolate but later small vacuoles appear which may fuse together to form a large vacuole. The spindle of the first nuclear division in the megaspore is oriented along the long axis of the cell. Wall formation does not follow the nuclear division. A large central vacuole now appears between the two daughter nuclei and, as it expands, the nuclei are pushed toward opposite poles of the cell (Fig. 7.2A). Both the nuclei divide twice, forming four nuclei at each pole (Fig. 7.2B, C). At this stage all the eight nuclei are present in a common cytoplasm (Fig. 7.2C, D). After the last nuclear division the cell undergoes appreciable elongation, assuming a sac-like appearance. This is followed by cellular organization of the embryo sac (Fig. 7.2E). Of the four nuclei at the micropylar end of the embryo sac, three organize into egg apparatus and the fourth one is left free in the cytoplasm of the central cell as the upper polar nucleus. The egg apparatus is attached to the wall of the embryo sac only at the micropylar end, its major portion being surrounded by the central cell. Of the 3 cells, only the synergids are in direct contact with the wall of the embryo sac, the upper part of the egg being a little below the apices of the synergids. As all the 3 cells are of almost the same length, the egg cell extends a little more into the central cell than the synergids. Three nuclei of the chalazal quartet form three antipodal cells whereas the fourth one functions as the lower polar nucleus. Eventually, the latter comes to lie close to the upper

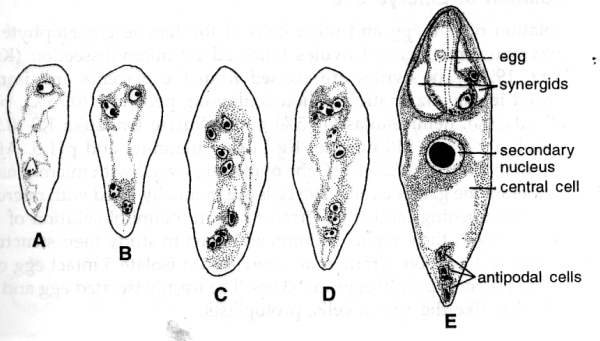

Fig. 7.2 Stages in megagametogenesis in *Morina longifolia*. **A.** Megaspore after first post-meiotic mitosis. **B.** 4-nucleate stage. **C.** 8-nucleate stage. **D.** Older 8-nucleate stage, showing 3+2+3 distribution of the nuclei. **E.** Mature embryo sac; it comprises 3-celled egg apparatus, 3 antipodal cells, and a large central cell with the secondary nucleus. (*after* Vijayaraghavan and Sarveshwari, 1968)

polar nucleus. This mode of embryo sac development occurs in the majority of flowering plants and is designated as the *Polygonum* type. According to Davis (1966), about 81 per cent of the families show *Polygonum* type of embryo sac development. Traditionally, the studies on the structure of egg and other cells of the female gametophyte are based on thin and semi-thin sections. Gross changes during megasporogenesis, gametogenesis and early post-fertilization development in the embryo sac can be rapidly studied by clearing the whole ovules to near transparent and observing them with phase contrast microscope. More recently, it has been possible to isolate live embryo sac and its constituent cells by enzymatic degradation to study their structure and for *in vitro* fertilization.

Clearing of Pistils/Ovules

The protocol described here is based on the work of Young *et al.* (1979). The ovules are fixed in FAA (95% ethanol:water:40% formalin:glacial acetic acid, 40:14:3:3 v/v) for 24 hrs and stored in 50% ethanol. For clearing, the fixed ovules are passed through a dehydration and clearing series in the following sequence: 70% ethanol, 85% ethanol, 100% ethanol (three changes), 1:1 ethanol-methyl salicylate (MESA), 1:3 ethanol-MESA and 100% MESA (two changes). The duration of each treatment may vary with the material and the number of ovules. For individual ovules of buffegrass (*Cenchrus ciliaris*) 1 ml of liquid and 30 min at each step were found suitable, but for processing 10-20 ovules together 2 ml of liquid and 2 hrs treatment at each step are recommended. The cleared material is stored in 100% MESA or mounted in it and observed with phase contrast microscope.

Isolation of Embryo Sac

Isolation of live egg and other cells of the female gametophyte involves enzymatic treatment of ovules followed by micro-dissection (Kranz and Lorz, 1993). The ovules are excised from the placenta and immediately placed in the enzymatic solution containing pectinase (0.75%), pectolyase 423 (0.25%), hemicellulase (0.5%) and cellulase Onozuka RS (0.5%) with osmolarity set at 540 mosmol Kg^{-1} using mannitol and pH 5. After 40-60 min of incubation at 24±0.5°C the ovules are teased with micro-glass needles to liberate the gametic cells. The cells are manually lifted with microcapillary (200 µm opening) and transferred to clean mannitol solution of the same osmolarity as the enzyme solution and used to study their structure or for *in vitro* fertilization. Kranz *et al.* (1991) could isolate 5 intact egg cells from 20 ovules and up to 40 eggs in 2-3 hrs. The freshly isolated egg and synergid cells are, like the sperm cells, protoplasts.

TYPES OF EMBRYO SACS

The *Polygonum* type of embryo sac is derived from only one of the four megaspore nuclei formed as a result of meiosis in the megaspore mother cell. Whereas it is the most common mode of embryo sac development in

angiosperms there are a substantial number of plants where two or all the four megaspore nuclei take part in the formation of female gametophyte. Depending on how many megaspore nuclei are involved in its formation, the embryo sac may be monosporic, bisporic or tetrasporic. Each group has more than one type of embryo sac named after the genus in which it was first described (Fig. 7.3).

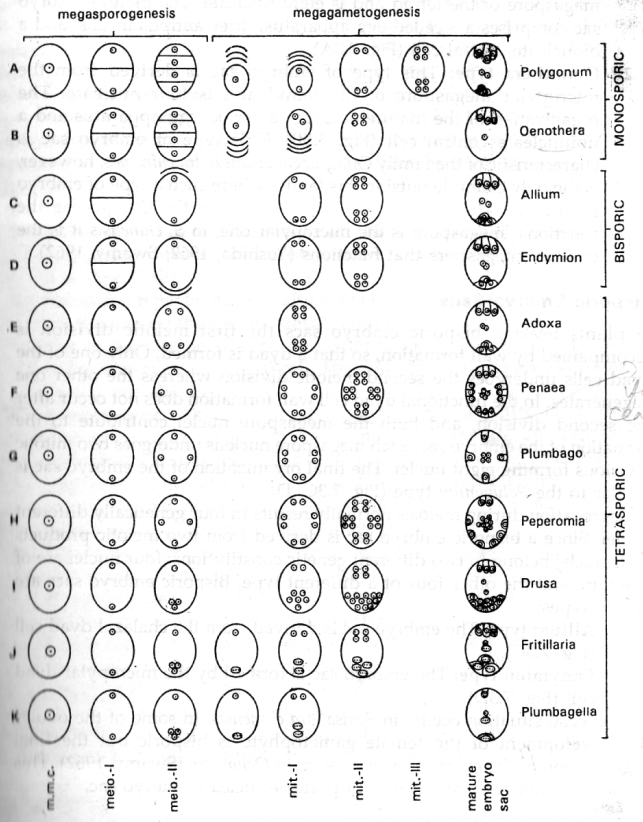

Fig. 7.3 Diagrammatic representation of various types of embryo sac development (*m.m.c.*, megaspore mother cell; *meio.*, meiosis; *mit.*, mitosis). (modified *after* Maheshwari, 1950)

coenomegaspore (one haploid and one triploid) divide twice forming four nuclei at each pole. The mature embryo sac comprises an egg apparatus of three haploid cells, three triploid antipodal cells, and a central cell with two polar nuclei - one haploid and one triploid (Fig. 7.3J).

7. **Plumbagella type**: After the nuclear fusion, by a single division of each nucleus four nuclei are formed; two at the micropylar end and two at the chalazal end. One of the haploid nuclei at the micropylar end forms egg, and the other one functions as the upper polar nucleus. Similarly, one of the triploid nuclei at the chalazal end forms a single antipodal cell whereas the other one functions as the lower polar nucleus (Fig. 7.3K). In this way, as in the *Fritillaria* type, one polar nucleus is haploid and the other triploid.

A special type of tetrasporic embryo sac, called *Chrysanthemum cinerariaefolium* type, has been described by Martinoli (1939). At the end of meiosis the four nuclei in the coenomegaspore are arranged in 1+2+1 fashion, one at the micropylar end, one at the chalazal end, and two in the centre of the cell. Based on the behaviour of the two central nuclei there are two variations within this type of embryo sac (Fig. 7.4).

(1) The two central nuclei remain closely associated but do not fuse. While the other two nuclei divide twice forming 4 nuclei at each pole of the cell, the central nuclei do not divide even once. Thus, the embryo sac is 10-nucleate. All the four nuclei in the chalazal quartet organize into four antipodal cells. Of the micropylar quartet three nuclei organize into egg apparatus while the fourth one, along with the two central nuclei, functions as a polar nucleus (Fig. 7.4A).

(2) The two central nuclei fuse to form a diploid nucleus. After fusion, all the three nuclei in the coenomegaspore undergo two mitotic divisions. In this way, three groups of four nuclei each are formed. At the time of cellularization of the embryo sac three nuclei of the micropylar quartet are cut-off as egg apparatus, and the fourth one is left free as a polar nucleus. All the four nuclei of the chalazal quartet and three nuclei from the middle quartet are cut-off as 7 antipodal cells. The central cell is left with a diploid nucleus from the middle quartet and a haploid nucleus from the micropylar quartet (Fig. 7.4B).

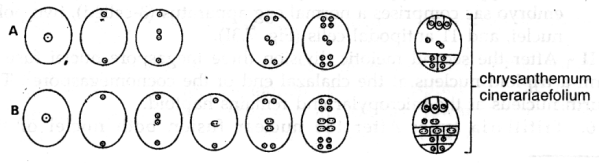

Fig. 7.4 Diagrammatic representation of *Chrysanthemum cinerariaefolium* type of embryo sac development. (*after* Maheshwari, 1950)

It is by no means rare to find more than one type of embryo sac in the same species or even in the same individual. For example, in *Delosperma cooperi* (Aizoaceae), Kapil and Prakash (1966) recorded 14 per cent ovules showing *Polygonum* type of embryo sac (monosporic), 18 per cent *Endymion* type (bisporic), and 68 per cent tetrasporic type. Of the tetrasporic types, 47 per cent conform to the *Drusa* type, 35 per cent to the *Penaea* type, and 18 per cent to the *Adoxa* type. Similarly, Abe (1971) reported *Allium* (31%), *Adoxa* (53%) and *Polygonum* (16%) types of embryo sacs in the orchid, *Oreorchis patens*. However, the mature embryo sac contained only six nuclei (egg apparatus, one polar and 2 chalazal nuclei) instead of eight nuclei normally present in these types of embryo sacs.

Hjelmqvist and Grazi (1965) have shown that, at least to some extent, temperature influences the type of embryo sac. *Ulmus glabra* bears *Adoxa* and *Drusa* types of embryo sacs, in addition to some other rare types. Hjelmqvist and Grazi cultured branches from one tree of this species in controlled environmental chambers in two groups and subjected them to different temperature regimes: 15-19°C and 26-27°C. It is quite evident from their data, presented in Table 7.1, that low temperatures (15-19°C) considerably enhanced the frequency of 8-nucleate *Adoxa* type of embryo sacs. Similar results have been obtained with some other species.

TABLE 7.1 Effect of temperature on the occurrence of two types of embryo sac in *Ulmus glabra*. (*after* Hjelmqvist and Grazi, 1965)

Temperature during embryo sac development	*Drusa type (%)*	*Adoxa type (%)*	*Other type (%)*
15°-19°C	81	18	1
26°-27°C	89	6	5

To explain the above observation, Hjelmqvist and Grazi have proposed that temperature affects nuclear division more than the cell growth and the vacuolation associated with it. At low temperatures the nuclear division is delayed in relation to cell growth so that the central vacuole appears after the first meiotic division separating the two megaspore nuclei to the two ends of the embryo sac. By a single mitosis after the second meiotic division an 8-nucleate *Adoxa* type of embryo sac is formed. At higher temperatures the nucleus divides rather rapidly in relation to cell growth so that vacuolation appears only after the second meiotic division separating a single nucleus at the micropylar end from three at the chalazal end. By two further nuclear divisions the 16-nucleate *Drusa* type of embryo sac is formed.

Dharamdhaj and Prakash (1978) reported that in *Capsicum* the embryo sacs formed in winter were bisporic and most of those formed in summer were of the monosporic type.

MATURE EMBRYO SAC

After the last nuclear division in the female gametophyte, the cleavages are

Monosporic Embryo Sacs

The chief characteristic of a monosporic embryo sac is that it is derived from only one of the four megaspores, as in the *Polygonum* type. All the nuclei in such an embryo sac are genetically identical because they are derived through mitoses of a single nucleus. There are two types of monosporic embryo sacs.

1. **Polygonum type**: The embryo sac is formed by the chalazal megaspore of the tetrad and is eight nucleate. The mature embryo sac comprises a 3-celled egg apparatus, three antipodal cells, and a binucleate central cell (Fig. 7.3A).
2. **Oenothera type**: This type of embryo sac is derived from the micropylar megaspore of the tetrad and is four nucleate. The organization of the mature embryo sac is: an egg apparatus and a uninucleate central cell (Fig. 7.3B). This type of embryo sac is characteristic of the family Onagraceae. *Schisandra chinensis*, however, is the only example outside this family where such a type of embryo sac occurs. The difference is that whereas in the Onagraceae the functional megaspore is the micropylar one, in *S. chinensis* it is the chalazal megaspore that functions (Yoshida, 1962; Swamy, 1962).

Bisporic Embryo Sacs

In plants bearing bisporic embryo sacs the first meiotic division is accompanied by wall formation, so that a dyad is formed. Only one of the dyad cells undergoes the second meiotic division whereas the other one degenerates. In the functional dyad cell wall formation does not occur after the second division, and both the megaspore nuclei contribute to the formation of the embryo sac. Each megaspore nucleus undergoes two mitotic divisions forming eight nuclei. The final organization of the embryo sac is similar to the *Polygonum* type (Fig. 7.3C, D).

Segregation during meiosis normally results in four genetically different nuclei. Since a bisporic embryo sac is derived from two meiotic products their nuclei belong to two different genetic constitutions; four nuclei are of one type and the other four of a different type. Bisporic embryo sacs are of two types:

1. **Allium type:** The embryo sac is derived from the chalazal dyad cell (Fig. 7.3C).
2. **Endymion type:** The embryo sac is formed by the micropylar dyad cell (Fig. 7.3D).

A special situation occurs in *Schisandra chinensis*. In some of the ovules the development of the female gametophyte is bisporic but the final organization of the embryo sac is as seen in *Oenothera* (Swamy, 1962). This is the only authentic report of a bisporic, 4-nucleate embryo sac.

Tetrasporic Embryo Sacs

In this group neither of the meiotic divisions is accompanied by wall formation so that at the end of meiosis all the four haploid nuclei remain

in a common cytoplasm forming a coenomegaspore. All the four nuclei of the coenomegaspore take part in the formation of the embryo sac. A tetrasporic embryo sac is more heterogeneous than a bisporic embryo sac because the four products of meiosis involved in its formation are genetically different.

Nuclear behaviour in tetrasporic embryo sacs is quite variable. The arrangement of the four nuclei in the coenomegaspore, before the beginning of postmeiotic mitosis, is of three types (Fig. 7.3): (a) 2+2 arrangement; two nuclei at the micropylar end and two at the chalazal end (*e.g.*, *Adoxa* type), (b) 1+1+1+1 arrangement; one nucleus at the micropylar end, one at the chalazal end, and two placed laterally, one on each side (*e.g.*, *Penaea* type, *Plumbago* type, *Peperomia* type), (c) 1+3 arrangement; one nucleus at the micropylar end and three at the chalazal end (*e.g.*, *Drusa* type, *Fritillaria* type, *Plumbagella* type). Depending on whether nuclear fusion occurs or not, number of the post-meiotic mitoses in the coenomegaspore, and final organization of the embryo sac, tetrasporic embryo sacs are of many types (Fig. 7.3E-K):

I - No nuclear fusion occurs.

1. **Adoxa type**: The embryo sac is eight nucleate, formed after a single post-meiotic mitosis, and its organization is similar to the *Polygonum* type (Fig. 7.3E).
2. **Plumbago type**: In this type also only one post-meiotic mitosis occurs, but the organization of the mature embryo sac is very different from that in *Adoxa* type. The mature embryo sac comprises an egg cell and a four-nucleate central cell. The other three nuclei are cut-off as peripheral cells (Fig. 7.3G).
3. **Penaea type**: As a result of two post-meiotic mitosis in the coenomegaspore 16 nuclei are formed. The mature embryo sac comprises 4 groups of 3 cells each; one group is at the micropylar end, one at the chalazal end, and two arranged laterally. The remaining four nuclei behave as polars (Fig. 7.3F). The micropylar triad usually functions as the egg apparatus.
4. **Peperomia type**: As in the *Penaea* type, the embryo sac is 16 nucleate. The organization of the mature embryo sac is: an egg apparatus comprising an egg and only one synergid, six peripheral cells and a central cell with eight polar nuclei (Fig. 7.3H).
5. **Drusa type**: This type of embryo sac is also 16 nucleate. The mature embryo sac comprises a normal egg apparatus (3-celled), two polar nuclei, and 11 antipodal cells (Fig. 7.3I).

II - After the second meiotic division three megaspore nuclei fuse to form a triploid nucleus at the chalazal end of the coenomegaspore[1]. The fourth nucleus at the micropylar end remains haploid.

6. **Fritillaria type**: After the nuclear fusion both nuclei of the

[1]The fusion of spindles of the three chalazal nuclei in the coenomegaspore, to form a triploid nucleus, was first reported by Bambacioni (1928), in *Fritillaria* and *Lilium*. Therefore, the phenomenon is often called "Bambacioni effect".

such that all the cells of the embryo sac are formed within the wall of the parent megaspore. The structure of the embryo sac wall is quite interesting. In cotton it is PAS-positive and rich in pectic substances. In maize it is multilayered and pectocellulosic (Chebotaru, 1975). Diboll and Larson (1966) believe that the innermost layer of the female gametophyte boundary represents the wall of the functional megaspore and the outer layers are the remains of the crushed nucellar cells. According to Maze and Lin (1975) the wall of the gametophyte in *Stipa* consists of three portions. The innermost portion is gametophytic, whereas the middle and outer portions represent the degenerated megaspore and nucellar cells, respectively. The walls separating cells within the embryo sac are traversed by plasmodesmata. The outer wall of the embryo sac lacks plasmodesmata and, at least, in some species it appears to be cutinised.

It is quite apparent from Fig. 7.3 that 7-celled organization of embryo sac (3-celled egg apparatus, three antipodal cells, and a binucleate central cell) is most common among angiosperms. Besides *Polygonum* type, it occurs in *Allium, Endymion, Adoxa*, and *Fritillaria* types of embryo sac. The occurrence of egg is universal. Except for *Plumbago* and *Plumbagella* types, the egg is always associated with two (rarely one, as in *Peperomia* type) synergids. The antipodals are almost always present, except in *Oenothera* type. Their number and ploidy is, however, variable. In the following few pages the structure and possible functions of various components of the mature embryo sac will be described.

Synergids

The synergids are elongated cells present at the micropylar end of the embryo sac. When two synergids are present they lie in contact with each other, and partly embrace the egg (Fig. 7.1). They are pointed or hooked toward the micropyle.

The wall around the synergids is incomplete. There is a distinct wall around the micropylar one-third of the cell which thins toward the chalazal end and, finally, disappears (Fig.7.9).

As a result, the chalazal one-third of the cell lacks a wall. In this region the protoplast of the synergid is separated from that of the central cell by double membranes; one of the synergid and the other of the central cell. While this description of wall is true for cotton and most other species investigated, in *Epidendrum scutella* the wall extends all over the synergid cells. Sometimes there may be deposits of cutin in the micropylar portion as in *Jasione* (Fig.7.5).

A prominent structure, called filiform apparatus (FA), is present at the micropylar end of each synergid (Figs 7.1, 7.6, 7.9). Its differentiation from the synergid wall was described as early as 1906 by Habermann. Electron microscopic studies have revealed that the filiform apparatus is a mass of finger-like projections of the wall into the cytoplasm (Fig. 7.9). Structurally, each projection of the filiform apparatus has a core of tightly packed microfibrils (possibly cellulosic) enclosed by a non-fibriller sheath. They

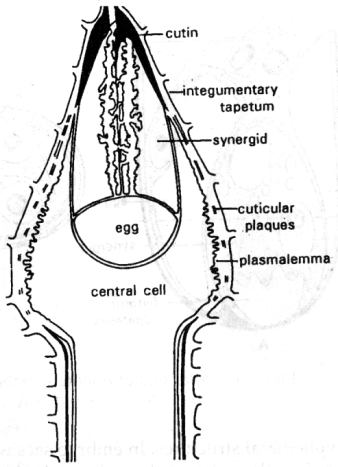

Fig. 7.5 *Jasione montana.* Longisection of micropylar half of the embryo sac (diagrammatic). There is a thick cuticle at the micropylar and middle portions. It is discontinuous in the region of egg apparatus. Plasmalemma of the central cell is highly folded, increasing surface area of absorption of nutrients. FA extends to the entire length of synergids. (*after* Berger and Erdelska, 1973)

are rich in polysaccharides. The form of filiform apparatus is variable. It may be spherical as in *Torenia* (Van der Pluijm, 1964), or wedge-shaped between the tips of synergids as in *Petunia* (Van Went, 1979) and *Helianthus* (Newcomb and Steeves, 1971). In *Jasione*, the FA extends irregularly along the entire length of the synergids (Fig. 7.5). The presence of abundant starch grains in a young synergid and their virtual absence from a fully developed synergid in *Paspalum* led Yu and Chao (1978) to suggest that the FA "seems to be formed mainly from substances transformed from starch grains". The filiform apparatus resembles the spongy wall of the so called "transfer cells" associated with short distance transport across the membrane. The synergids of *Crepis capillaris* lack filiform apparatus and invaginations of the wall (Kuroiwa, 1989).

The cytoplasm of the synergid is strongly polarized. The chalazal region of the cell is occupied by one large or many small vacuoles (Figs 7.6, 7.9). In cotton these vacuoles appear to be rich in calcium salts and carbohydrates. Large amount of cytoplasm and a prominent nucleus are present in the micropylar half of the cell (Fig. 7.6). The cytoplasm is rich in mitochondria, endoplasmic reticulum and dictyosomes, which are especially concentrated near the filiform apparatus (Fig. 7.9). Similarly, lipids, RNA and proteins are also abundant and concentrated in this region. The plastids are only few. They contain starch in cotton but not in maize and petunia.

doubts regarding the nutritive role of the synergids (Diboll, 1968; Godineau, 1969; Van Went, 1970). The presence of cuticle over the micropylar wall of the embryo sac in *Jasione* supports this view (Berger and Erdelska, 1973). The currently prevailing view is that the entry of metabolites into the embryo sac is mainly through its chalazal end (for details *see* under "Nutrition of embryo sac").

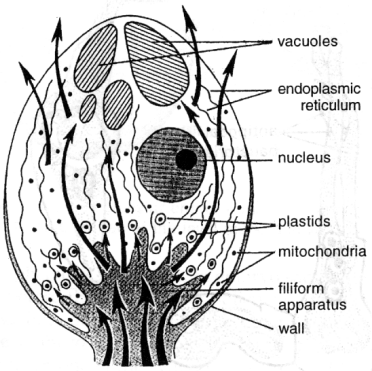

Fig. 7.9 A mature synergid of cotton (diagrammatic). Most of the cell organelles are concentrated around the filiform apparatus. The arrows depict the proposed direction of flow of nutrients into the embryo sac through the synergid. (*after* Jensen, 1965)

Although much has been said about the structure and function of synergids, yet several questions remain unanswered, such as the nature of metabolic activity of synergids, their differential behaviour, entrance of pollen tube through FA, and their role in cessation and opening of pollen tube.

Egg

The three cells of the egg apparatus (an egg and two synergids) are arranged in a triangular fashion (Fig. 7.6A, B). The egg shows common walls with the two synergids and the central cell. It has a wall similar in structure and extent to that of synergids. The wall is thicker in the micropylar region but becomes thinner toward the chalazal side. It is absent at the chalazal end in cotton, maize and *Torenia*. In *Capsella* PAS staining has indicated the presence of some wall material at the chalazal end but, in any case, a normal wall is lacking. Wall appears to be present all over the egg cell in *Epidendrum scutella*. At the micropylar end the lateral walls of the egg cell appear to join the central cell wall. Actually, the end wall of the egg cell is the wall of the central cell. The egg cell wall is traversed by

plasmodesmata on the sides of the two synergids and the central cell but not at its outer face.

The egg cell becomes highly polarized early in its development. The polarity is expressed by the aggregation of cytoplasmic elements at the chalazal end of the cell. The micropylar end of the cell is occupied by a large vacuole. Thus, the distribution of the vacuole and cytoplasm in the egg cell is just the opposite of that in the synergids. In *Epidendrum* and *Zea*, however, the nucleus is centrally located in the egg, and many small vacuoles are present along the periphery of the cell.

The young egg cell is richly endowed with organelles. At maturity, however, organelles become scarce indicating poor physiological activity. The mitochondria show only a few cristae. Except for *Zea*, dictyosomes are either absent (*Epidendrum*) or are only few in number. Where present, the dictyosomes exhibit an inactive state. A striking difference from the male gametes of most species is the occurrence of plastids in the egg cell. The egg may contain small or large amounts of starch, that is consumed during fertilization or early embryogeny. The egg cytoplasm is rich in ribosomes. The egg of *Nicotiana rustica* contains two strongly RNA-positive bodies that multiply and persist till the 4-celled stage of proembryo. Sehgal and Gifford (1978) associate these bodies with the synthesis of some enzymes that are important in early embryogeny.

In *Plumbago capensis* many finger-like wall projections arise at the micropylar end of the egg cell (Cass, 1972). In appearance and in being PAS-positive the projections resemble the filiform apparatus of the synergids. In the absence of synergids in this plant, the egg cell seems to have taken over the role of synergid in addition to its own gametic function.

In *Cortaderia jubata* (Philipson, 1981), rarely the egg may develop into an haustorium.

Antipodals

The antipodals exhibit the greatest variation amongst all the cells of the embryo sac. Usually they degenerate before or soon after fertilization, without any appreciable enlargement. In the Sapotaceae (except *Mimusops*) and Thismiaceae the antipodal nuclei degenerate even without organizing into cells.

In many plants the antipodals are persistent and show some structural and cytological features suggesting their possible role in the nutrition of the embryo sac. In *Caltha palustris* they persist up to the octant stage of the proembryo. In grasses they undergo a series of mitotic divisions leading to the formation of a large number of antipodal cells. The highest number of antipodal cells known is 300, recorded in *Sasa paniculata*. *Zea mays* has about 20 antipodal cells, each with 1-4 nuclei. Multinucleate condition of the antipodal cells also occurs in *Stackhousia*, *Tagetes*, etc. In *Zea mays*, during additional divisions in antipodals, the walls of many cells remain incomplete, leaving protoplasmic continuities between adjacent cells. This results in the formation of multinucleate protoplasm or syncytium (Diboll

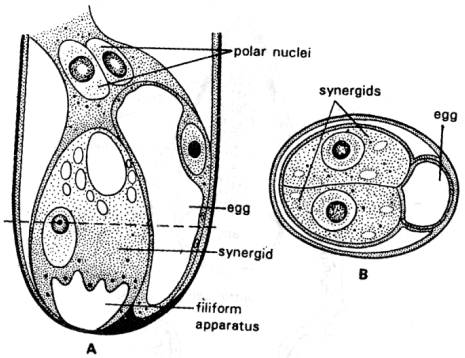

Fig. 7.6 **A.** Diagram of the micropylar end of a mature embryo sac of cotton. **B.** Transverse section of embryo sac at the level marked in **A.** (*after* Jensen, 1965).

Synergids are ephemeral structures. In embryo sacs with two synergids, one degenerates before the entry of the pollen tube into the embryo sac, whereas the other one, often called the persistent synergid, degenerates shortly after the embryo sac has received the pollen tube-discharge. Occasionally, however, one of the synergids may persist for a considerable period after fertilization. Its nucleus may enlarge and show polyploidy. In *Allium angulosum* and *A. pulchellum* the synergid nucleus becomes octaploid (Hasitschka-Jenschke, 1958).

As a rule, the synergids remain within the confines of the embryo sac. In some members of the Asteraceae, however, they break through the embryo sac and project into the micropyle. In *Cotula australis* one of the synergids becomes swollen and haustorial in nature. Synergid haustoria also occur in *Sedum sempervivoides* (Crassulaceae).

Quinchamalium chilense, a member of the family Santalaceae, shows the most extensive synergid haustoria (Fig. 7.7). In this species the synergids are conspicuously large. Their tips elongate and, breaking through the embryo sac, grow into the stylar tissue. They reach up to one-third the length of the style. The maximum length attained by them is 1,200 µm.

Extensive synergid haustoria have also been reported in *Cortaderia selloana* a member of the Poaceae (Philipson, 1977, 1981). The haustoria arise as finger-like projections from the FA and penetrate the micropyle (Fig. 7.8). The entire inner surface of the haustorium is provided with wall projections which are involved in the absorption and conduction of nutrients to the synergids.

FUNCTIONS OF SYNERGIDS. Looking at the structure and concentration of cell organelles the synergids appear highly active metabolically. Three functions have been ascribed to synergids:

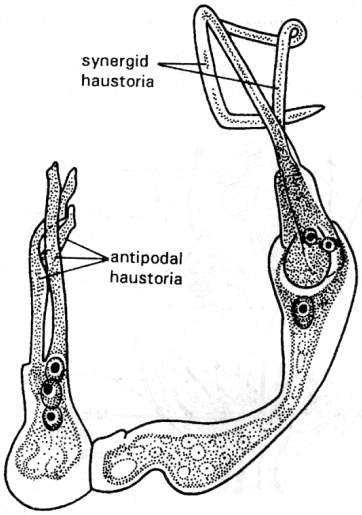

Fig. 7.7 Dissected whole mount of a mature embryo sac of *Quinchamalium chilense*, showing synergid and antipodal haustoria. (*after* Johri and Agarwal, 1965)

1. They play an important role in directing the pollen tube growth by secreting some chemotropically active substances (Ishikawa, 1918; Pluijm, 1964).
2. The degenerating synergid forms the seat for pollen tube discharge in the embryo sac.
3. Jensen (1965) suggested that the filiform apparatus may be aiding the synergid in the absorption and transportation of materials into the embryo sac from the nucellus (Fig. 7.9). He is of the opinion that the filiform apparatus forms the path of entry for these substances. However, many investigators have expressed

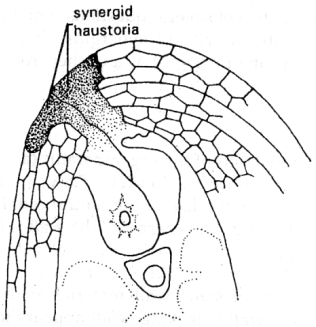

Fig. 7.8 *Cortaderia selloana*. Two synergid haustoria projecting through the micropyle. (*after* Philipson, 1977)

and Larson, 1966). Antipodal nuclei may also become polyploid due to endopolyploidy (*Chrysanthemum*) or polyteny (*Papaver*). Origin of various nuclear conditions in the antipodal cells is shown in Fig. 7.10.

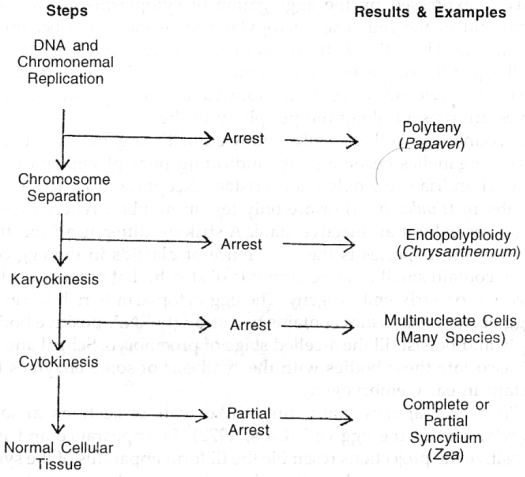

Fig. 7.10 Chart to show the origin of various nuclear conditions known to occur in antipodal cells. Arrest after different steps in the cell cycle may result in polyteny, endopolyploidy, multinucleate condition, or partial or complete syncytium. (*after* Heslop-Harrison, 1972)

Not much information is available regarding ultrastructural or histochemical aspects of antipodal cells. According to Diboll and Larson (1966) the antipodal cells in maize have abundant mitochondria, plastids and multicisternal dictyosomes. The cytoplasm is full of small vesicles derived from the endoplasmic reticulum or the dictyosomes. An interesting feature shown by some of these cells is the presence of papillate wall ingrowths projecting into the cytoplasm (Fig. 7.11A). These ingrowths, which are restricted to the cells bordering the nucellus appear similar to the filiform apparatus. Similar wall projections have been noticed in the antipodal cells of *Aquilegia* (Fig. 7.11B; Rifot, 1973), poppy (Jensen, 1972) and rice (Dong and Yang, 1989). Walls between the antipodals, and between antipodals and central cell are interspersed with plasmodesmata.

The antipodals are rich in ascorbic acid, oxidases, and sulfhydril compounds. Starch, lipids and proteins also occur in abundance. However, they show very low concentrations of RNA and polysaccharides.

Haustorial behaviour of antipodal cells is known in many plants (*Grindelia, Haplopappus, Quinchamalium*). In *Argemone mexicana* the antipodal

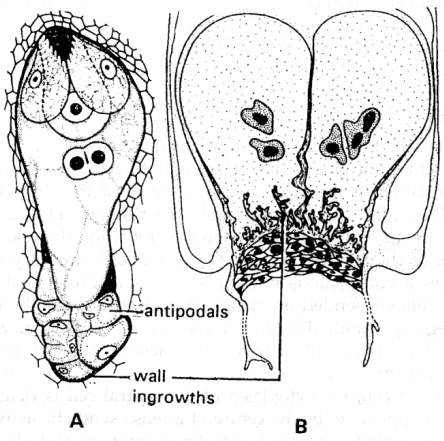

Fig. 7.11 **A.** *Zea mays.* Longisection of an organized embryo sac (diagrammatic). Note the prominent wall ingrowths and discontinuities in the walls of antipodals. **B.** *Aquilegia vulgaris.* Chalazal part of embryo sac, showing two antipodals with wall ingrowths. Central cell forms a skirt-like evagination overlying antipodal cells and degenerated nucellar cells. (**A.** *after* Diboll and Larson, 1966; **B.** *after* Rifot, 1973)

cells are much larger than either the egg or the synergids. After fertilization they continue to enlarge and persist up to heart-shaped stage of the embryo. They consume 8-10 layers of the nucellar cells at the chalazal end (Sachar, 1955). In *Quinchamalium chilense* the antipodal nuclei do not organize into individual cells. Instead, they are cut off from the rest of the embryo sac by a wall, forming a multinucleate antipodal chamber (Fig. 7.7). The latter grows through the funiculus, reaching up to the tip of the placenta where it branches profusely. The nuclei in the antipodal haustorium become hypertrophied and may even divide giving rise to 5-7 nuclei.

Three main functions have been attributed to the antipodal cells. Often a nutritive role has been proposed for the antipodal cells, especially where they are persistent. The nature of their cytoplasm characterizes them as highly active cells synthetically. The antipodal cells show similarities with other nutritive tissues, especially the glandular anther tapetum and integumentary tapetum, in having increased DNA content through multinucleate condition, endopolyploidy, or polyteny. Formation of wall projections in antipodal cells of maize, rice and poppy gives them the appearance of the so-called 'transfer cells', and support the suggestion that these cells may be associated with the nutrition of embryo sac.

The antipodal cells may also store large quantities of starch, lipids, and proteins which are utilized by the developing endosperm and embryo.

Another role ascribed to antipodal cells is to produce and secrete substances that control the growth and development of endosperm.

Central cell

It is the largest cell of the embryo sac, and the mother cell of the endosperm. The enlargement of the embryo sac after the last nuclear division is largely due to the inflation of the large central vacuole of the central cell. The vacuole in the central cell is presumably the reservoir of sugars, amino acids, and inorganic salts (List and Steward, 1965; Ryczkowski, 1964).

The nuclei of the central cell, called polar nuclei, are very large, and each possesses a conspicuous nucleolus. They are present either in the centre of the cell, suspended by cytoplasmic strands, or in the cytoplasm close to the egg apparatus (Fig. 7.1). In the latter case the chalazal portion of the embryo sac is occupied by a large vacuole. The two polar nuclei fuse, before or during double fertilization, to form the secondary nucleus.

Unlike the egg cell, the cytoplasm of the central cell is rich in all cell organelles and appears to be the centre of intense synthetic activity. There are plastids containing starch and, sometimes, proteins and phytoferretin. In *Capsella* the central cell possesses numerous sphaerosomes associated with glyoxysomes that probably convert fat into sugar. Interestingly, well developed chloroplasts have also been reported in this cell. Mitochondria are numerous. A strikingly large number of dictyosomes is usually associated with the cytoplasm of the central cell. They appear to be producing a large number of vesicles indicating their active state. There are numerous ribosomes, usually present as single ribosomes or small polysomes. The central cell contains sufficient food reserves that are available for use during fertilization and early stages of endosperm development.

The wall of the central cell is highly variable from one part of the embryo sac to the other. It is thickest in the regions against the nucellus. Where the central cell is in contact with the egg and synergids it shows the common feature of partial wall. It thins toward the chalazal end of the egg apparatus, and, finally, in the chalazal region there is no wall between the plasma membranes of the central cell and those of the egg and the synergids. The central cell is connected with the egg, synergids, and the antipodals through plasmodesmatal connections but no such connection exists between the central cell and the adjacent nucellar cells.

The presence of cell wall projections in the micropylar and/or chalazal region shows that central cell draws nutrition from the surrounding nucellus or integument (also *see* Kapil and Tiwari, 1979). In *Aquilegia vulgaris* skirt-like evaginations arise from the central cell (Fig. 7.11B). These haustorial extensions with parietal prolongations enter the ovular tissue. In addition to absorption, these extensions secrete enzymes that help in the digestion of the nucellar tissue (Rifot, 1973).

HAUSTORIAL BEHAVIOUR OF EMBRYO SAC

In addition to the synergid and antipodal haustoria (described earlier) the entire embryo sac may grow beyond the ovular tissue. It may also form micropylar or chalazal extensions or caeca from the central cell which grow into various carpellary tissues and function as haustoria.

Extra-ovular extension of the embryo sac at the micropylar end is quite common in the Santalaceae. In *Mida, Quinchamalium* and *Santalum* the embryo sac grows beyond the ovule at the 4-nucleate stage whereas in *Leptomeria* it occurs at the mature embryo sac stage.

In *Utricularia* (Lentibulariaceae) when the embryo sac is at the 2-nucleate stage the nucellar epidermis and the remains of the non-functional megaspores disappear completely. The tip of the embryo sac grows through the ovule and comes in direct contact with the placenta. At the 4-nucleate stage the embryo sac tip starts penetrating into the placental nutritive tissue and at the mature embryo sac stage it is buried in the nutritive tissue (Fig. 7.12). The embryo sac tip in this case possibly functions as an haustorium. In *Nelsonia campestris* (Acanthaceae) the 4-nucleate embryo sac protrudes out of the micropyle and lodges itself inside the placenta. The egg apparatus organizes in the embryo sac inside the placenta.

In the Loranthaceae the embryo sacs grow upward up to various heights. They reach the base of the style in *Macrosolen cochinchinensis*, up to one-fifth the length of the style in *Lepeostegeres gemmiflorus*, half the length of the style in *Dendrophthoe falcata*, and up to stigma in *Helixanthera ligustrina*. However, the longest embryo sacs occur in *Moquiniella rubra*. Here the tips of the embryo sacs sometimes curve backward into the style by 2-4 mm after touching the stigma (Fig. 7.13). The maximum length attained by such embryo sacs is about 48 mm. *Phthirusa pyrifolia* (Kuijt and Weberling, 1972) and *Struthanthus vulgaris* (Venturelli, 1981) are exceptions, where only one embryo sac develops in each ovary.

Most of the loranthaceous and santalaceous members show the extension of the chalazal end of the embryo sac in the form of a lateral caecum, leaving the antipodal cells *in situ* (Fig. 7.14). In *Scleropyrum* the antipodals migrate into the chalazal haustorium.

While chalazal caecum is quite frequent, the micropylar caecum is rare. The latter has been reported in *Nuytsia* (Fig. 7.16), *Atkinsonia* and *Comandra* (Fig. 7.15). In the first two genera it arises as a small lateral protuberance at about the level of the egg apparatus, elongates toward the stylar canal and, eventually, penetrates the stylar tissue. In *Nuytsia* the micropylar caecum may branch (Fig. 7.16). It usually lacks a nucleus.

In *Exocarpus strictus* numerous finger-like outgrowths arise from the middle of the embryo sac and hang over the lower portion like a skirt (Fig. 7.17). They probably provide additional absorptive surface (Ram, 1959). Tubular haustorial processes arising from the tip of the embryo sac and extending beyond the placenta occur in another species of *Exocarpus* (*E. menziesii*; Fagerlind, 1959).

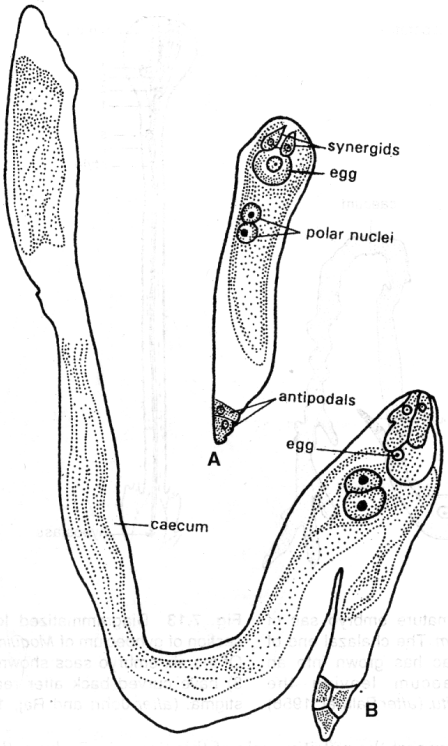

Fig. 7.15 *Comandra umbellata*. **A.** Young organized embryo sac. **B.** Mature embryo sac with a prominent lateral caecum arising at the level of the egg apparatus. (*after* Ram, 1957)

2. In *Quercus gambelii* the outer integument is rich in starch, and its cells are interconnected by numerous well-developed plasmodesmata, whereas the inner integument lacks both these features. Furthermore, vascular traces are restricted to the outer integument. A group of persistent nucellar cells, called postament, projects into the embryo sac at its chalazal end. On the basis of these observations, Mogensen (1973) suggested that in this plant the most likely pathway of food transport within the ovule is from the outer integument to chalaza (facilitated due to the occurrence of plasmodesmata), and through the postament to the embryo sac.

3. Ryczkowski (1971) had shown that in fertilized ovules the concentration

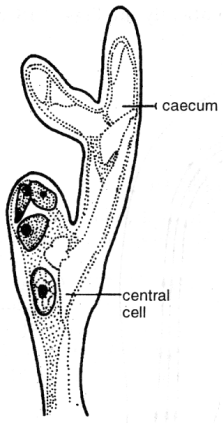

Fig. 7.16 Micropylar end of a mature embryo sac of *Nuytsia floribunda*, showing a branched caecum. (*after* Narayana, 1958)

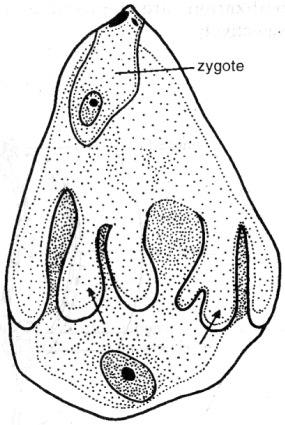

Fig. 7.17 Fertilized embryo sac of *Exocarpus strictus* (whole mount), showing a fringe of finger-like processes (arrow-marked). (*after* Ram, 1959)

of free amino acids is highest at the chalazal end of the embryo sac, and there exists a definite chalazal-micropylar gradient.

While the entry of food material into the embryo sac appears to be mainly through the chalazal end, the entire surface of the female gametophyte seems to be absorbing nutrients from its surrounding nucellar cells. In many species the nucellus is completely consumed by the developing embryo sac, as a result of which the latter comes in direct contact with the integument. In such cases the inner epidermis of the integument forms a glandular endothelium around the embryo sac. This specialized layer shows many similarities with the glandular type of anther tapetum, and is considered to be absorbing nutrients from the outer tissues and supplying them to the embryo sac (also *see* Chapter 6).

On the basis of their study of the cytochemistry of the ovule of *Linaria bipartita*, Kallarackal and Bhatnagar (1981) suggested that before fertilization synergids are involved in the nutrition of the egg, and the antipodals in the nutrition of the central cell, which later develops into the endosperm. For the nutrients to reach the micropylar end from the chalaza, the obvious pathway is the integumentary tapetum which, in *Linaria*, persists and is highly active metabolically. After fertilization the micropylar endosperm haustoria become active and take up the function of the synergids, and the chalazal endosperm haustorium, similarly, acts in the place of antipodals in providing nutrition to the developing endosperm (Bhatnagar and Kallarackal, 1980; Kallarackal and Bhatnagar, 1981). The possible pathways of the nutrients into the embryo sac in *L. bipartita*, before and after

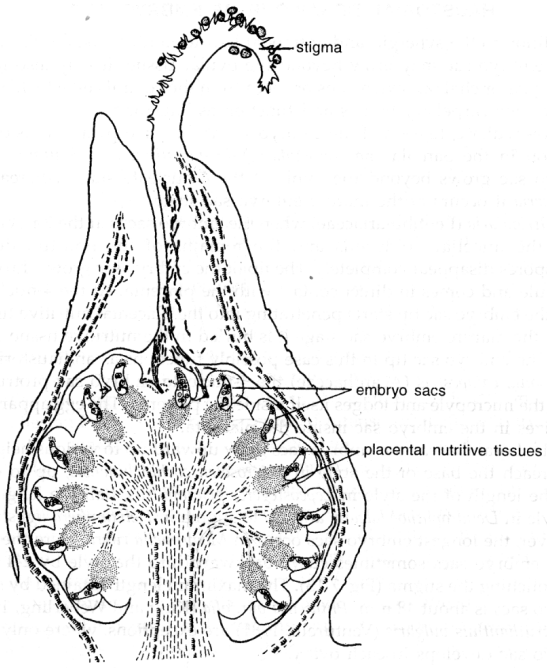

Fig. 7.12 Longitudinal section of gynoecium of *Utricularia flexuosa*. Note the vascular supply of placenta and groups of placental nutritive tissue with haustorial apices of embryo sacs embedded in this. (*after* Khan, 1954)

NUTRITION OF EMBRYO SAC

Nucellus is the obvious pathway for nutrients to enter the embryo sac because it surrounds the latter on all sides. The morphology of the ovule suggests that the main pathway of nutrients into the embryo sac is the chalazal end. The funicular vascular supply, which can be regarded as the normal channel for the supply of food materials to the ovule, terminates at the base of the integuments. From there the nutrients pass into the embryo sac through the nucellar tissue present between the vascular supply and the chalazal end of the embryo sac. Often a patch of specialized cells, called hypostase, is present in between the funicular vascular supply and the chalazal end of the embryo sac. It has been suggested by some authors that the hypostase may be involved in the transfer of food materials to the embryo sac (Venkata Rao, 1953). However, Coe (1954) did not find any

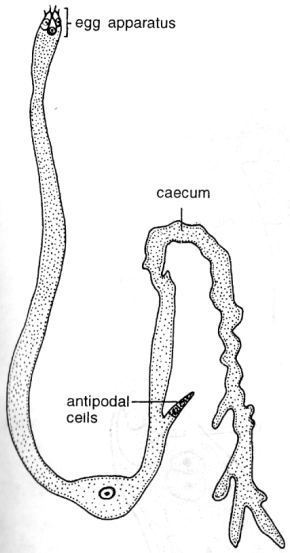

Fig. 7.14 A mature embryo sac of *Santalum album*. The chalazal end of the embryo sac has grown into an extensive caecum leaving the antipodals *in situ*. (*after* Paliwal, 1956)

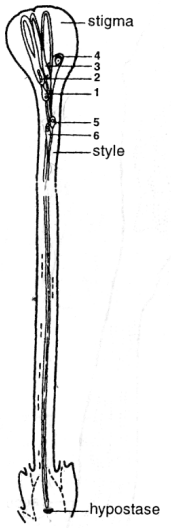

Fig. 7.13 Diagrammatized longitudinal section of gynoecium of *Moquiniella rubra*. Of the six embryo sacs shown, three (1-3) have curved back after reaching the stigma. (*after* Johri and Raj, 1969)

evidence to support the nutritive role of this tissue in *Zephyranthes*. Nonetheless, his experiments strongly endorse the suggestion of the chalazal entry of soluble assimilates into the embryo sac. When the plants of *Zephyranthes drummondii* were fed with ^{14}C-labelled assimilates through leaves and the distribution of its soluble and insoluble derivatives in the ovule was checked autoradiographically, the soluble derivatives were found mostly concentrated in the vascular supply, chalazal region of the nucellus (except the hypostase), and the cytoplasm and vacuole in the embryo sac.

Other evidences which support the suggestion for the chalazal entry of food materials into the embryo sac are as follows:

1. The occurrence of wall projections in the antipodal cells bordering the nucellus at the chalzal end of the embryo sac gives these cells the appearance of the so-called "transfer cells". The wall projections in the antipodal cells have been observed in maize (Diboll and Larson, 1966) and poppy (Jensen, 1972).

fertilization, are depicted diagrammatically in Figs 7.18 and 7.19, respectively.

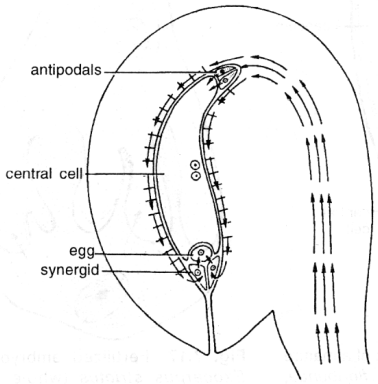

Fig. 7.18 Schematic diagram of the possible flow of nutrients in the ovule of *Linaria bipartita* before fertilization. (*after* Kallarackal and Bhatnagar, 1981)

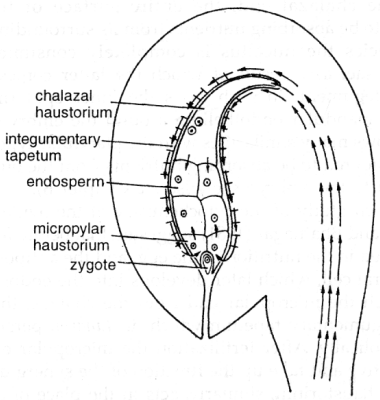

Fig. 7.19 Schematic diagram of the possible flow of nutrients in the ovule of *Linaria bipartita* after fertilization. (*after* Kallarackal and Bhatnagar, 1981)

Besides the above mentioned means of nutrition, in some plants the embryo sac grows out of the ovule, becomes haustorial, and obtains food materials from various ovular and carpellary tissues. The haustorial structures may also arise from individual cells of the embryo sac (for details *see* under the heads "Haustorial behaviour of embryo sac", "Synergids" and "Antipodals").

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From a geneticist's viewpoint geitonogamy is similar to self-pollination because all the flowers on a plant are, normally, genetically identical. To him cross-pollination makes sense only if two different plants, either of the same species or different species are involved in the process. However, pollination ecologists plead that since geitonogamy and xenogamy both require similar ecological factors for pollen transference they should be considered under one category. Unless mentioned otherwise, we shall follow the classification as accepted by ecologists.

Self-Pollination

Self-pollination is possible in only those plants which bear bisexual flowers, and only such bisexual flowers which achieve anther dehiscence and receptivity of stigma simultaneously.

The majority of angiosperms bear chasmogamous flowers, which means the flowers expose their mature anthers and stigma to the pollinating agents. This may be brought about: (a) by opening of the flower, or (b) the organs may protrude from a closed flower in such a manner as to expose themselves to the same agents. There is another group of plants which set seeds without exposing their sex organs. Such flowers are called cleistogamous, and the phenomenon is termed cleistogamy. This is the most efficient floral adaptation for promoting self-pollination. Cleistogamy may be constitutional (inherent) or ecological (induced by severe environmental conditions).

Commelina benghalensis (kankauoa) bears two types of flowers: (a) aerial flowers, which are brightly coloured (blue or violet), chasmogamous, and insect-pollinated; and (b) underground, dull and cleistogamous flowers, borne on the subterranean branches of the rhizome.

Under certain environmental conditions the plants which commonly form chasmogamous flowers start producing cleistogamous flowers. In Timbuctu, one of the world's hottest desert regions, the ground temperature throughout the year is 70-80°C, and it gradually decreases to 40-44°C at man's height (170 cm). In this part of the world the trees and shrubs of man's height or over are insect-pollinated whereas the ground flora, due to lack of any insect in the area, are self-pollinated. The latter is promoted by cleistogamy. The species of *Indigofera* growing in milder climates are pollinated by honey-bees whereas in the species which grow in the driest region of Timbuctu the anthers dehisce while the flower is still close and, thus, effect self-pollination.

Commelina forskalei, growing in Timbuctu, bears beautiful blue, aerial flowers which have only male sex organs and are, therefore, sterile. At the base of these flowers, enclosed in a hairy involucre, are 2 or 3 additional flowers. The cavity formed around these flowers by the involucre is filled with a viscid substance which protects the young buds from desiccation. These flowers are bisexual and fertilized by self-pollination. This plant also bears underground, fertile, cleistogamous flowers.

In okra (*Abelmoschus esculentus*), as in other members of the family Malvaceae, the androecium is monoadelphous. It has a staminal column to which are attached monothecous anthers by small filaments. The capitate stigma projects beyond the staminal column (Fig. 8.3A). Anthers dehisce about five hours before anthesis. At the time of anther dehiscence, the stigma is either in contact with the anthers by its lower surface and gets pollinated, or it may be slightly above the level of the anthers. In the latter case, the staminal column, carrying the dehisced anthers, grows upward to the extent that the anthers come to touch the lower surface of the stigma and impregnate it with pollen grains (Fig. 8.3B). Bristlebeetles are closely associated with the flowers of okra and feed upon its petals and pollen grains. While crawling inside the flower the beetles transfer pollen grains to the upper surface of the stigma.

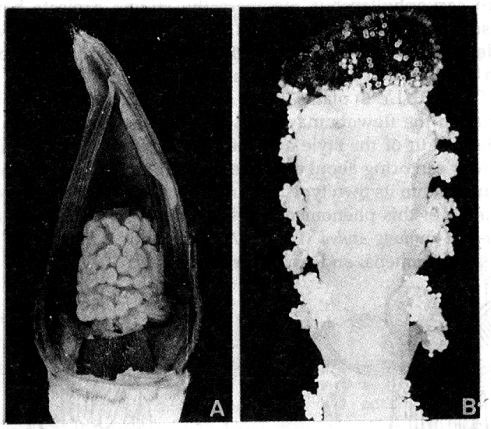

Fig. 8.3 Mechanism of self-pollination in okra. **A.** A portion of the floral envelope removed to show the disposition of stamens and stigma in bud condition. **B.** Sex organs at flower opening; the staminal tube has elongated and brought the dehisced anthers in contact with the lower surface of the stigma. (*Courtesy*: Dr. Sudhir Chandra, India)

Cross-pollination

Floral mechanisms favouring cross-pollination: In plants such as hemp and willow, where the flowers are unisexual, cross-pollination becomes obligatory. However, in plants with bisexual flowers legitimate self-pollination is prevented through the following adaptations:

(1) SELF-STERILITY. Landing of pollen on stigma is no guarantee for seed-set. In *Petunia axillaris*, for example, growth of the pollen tubes formed upon self-pollination is inhibited in the mid-part of the style. Failure of fertilization after self-pollination in self-sterile plants may also be due to

CHAPTER

8

POLLINATION

Pollination is commonly defined as the process of pollen transfer from anther to stigma of a flower. As described in Chapter 3, pollen grains are formed in the pollen sacs which are completely enclosed by multi-layered anther wall. Therefore, the first obvious requisite for pollination is the opening of anther sacs to release the pollen grains (anther dehiscence).

ANTHER DEHISCENCE

Maturation and desiccation of anther tissue are essential for anther dehiscence. Ripe anthers dehisce over a considerable range of temperatures and humidity. The dehiscence of anther involves two distinct processes: (a) During anther development thickened mechanical layers differentiate in its wall. In a mature anther these layers are present all around the locule except at special areas, called stomium. (b) In the second process, which comes into operation at the time of actual dehiscence, the mechanical layers become active and develop forces aiding in the rupture of anther wall along the stomium (Fig. 8.1).

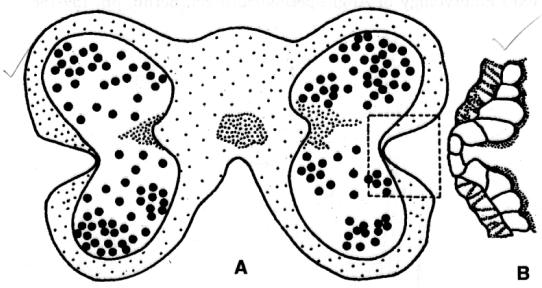

Fig. 8.1 **A.** Transverse section of a mature anther. **B.** The portion emblocked in **A** enlarged to show the stomium with a few wall cells on either side of it.

Usually it is the endothecium which serves the mechanical function in anther dehiscence by acquiring fibrous thickenings in its cells. In *Cassia* sp. the endothecium lacks the fibrous thickenings. Instead, the outer, inner and radial walls of the cells of this layer are unequally thickened, and it is the differential shrinkage of these walls which develops the mechanical pressure. Occasionally, as in *Dillenia*, the mechanical role in anther dehiscence is played by the cells of the epidermis.

In *Lilium*, as also in most of the angiosperms, the stomium is in the form of a narrow strip along the entire length of the anther lobe. Consequently, the dehiscence is by long slits (Fig. 8.2A). However, in other genera the

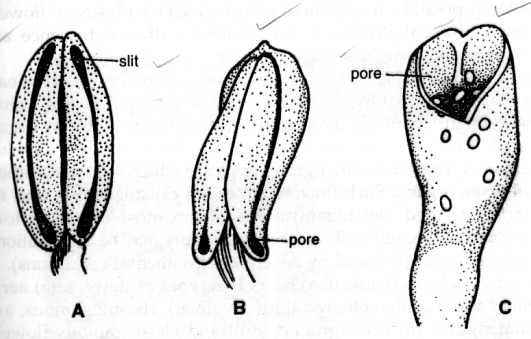

Fig. 8.2 Anther dehiscence. **A.** By slits (*Lilium*). **B.** By basal pores (*Cassia fistula*). **C.** By a single, large terminal pore (*Polygala chinensis*).

dehiscence zone may be variously restricted, and the anther may dehisce by means of a lid or valve (Berberidaceae), or empty themselves through pores (*Solanum*, *Cassia*, *Polygala*; Fig. 8.2B, C) or the theca may be detachable by the pollinators (*Garcinia* sp.). In *Najas* the anther wall breaks irregularly.

In the cleistogamous flowers of *Viola mirabilis* and *Oxalis acetosella* the endothecium is reduced, and the anthers do not dehisce at all. The pollen grains germinate within the pollen sacs, and the pollen tubes grow out through the anther wall.

POLLEN TRANSFER

Based on the destination of pollen grains, two types of pollination are recognized. When pollen grains are transferred from an anther to the stigma of the same flower the process is called self-pollination or autogamy. If they are transferred to the stigma of another flower, cross-pollination is said to have taken place. Cross-pollination is further classified depending on whether the pollination has occurred between two flowers on the same plant (geitonogamy) or between two flowers on different plants (xenogamy).

the inability of the pollen to germinate on its own stigma. All those plants in which pollen from a flower is incapable of bringing about fertilization in the same flower are said to be self-sterile, or self-incompatible.

(2) DICHOGAMY. This refers to maturation of male and female sex organs at different times. In *Saxifraga aizoides* and *Impatiens* the anthers dehisce much before the stigma of the same flower attains receptivity (protandry) and, therefore, self-pollination is not possible. Conversely, in the flowers of *Aristolochia* the stigma loses receptivity by the time the anthers dehisce (protogyny). This also safeguards against self-pollination. Protogyny is also common in Brassicaceae and Rosaceae.

(3) HERKOGAMY. In some bisexual flowers the structure of the male and the female sex organs itself proves a barrier to self-pollination. In flowers of caryophyllaceous members the stigma projects beyond the stamens so that the pollen do not fall on it. Conversely, in *Gloriosa* the anthers dehisce at a distance so that the stigma is out of reach of its own pollen. In orchids the pollen remain aggregated in pollinia.

(4) HETEROSTYLY. In plants which show this mechanism for preventing self-pollination the flowers may be of two or more types with regard to, mainly, the length of the style and the length of the stamens, and pollen from a flower can bring about effective pollination in flowers of a different type and not within its own type. Primrose (*Primula vulgaris*) is a commonly cited example of this phenomenon. It has two types of flowers (distyly):

(i). *Pin-eyed* or *long-styled* (Fig. 8.4A), with long style, long stigmatic papillae, short stamens, and small pollen grains.

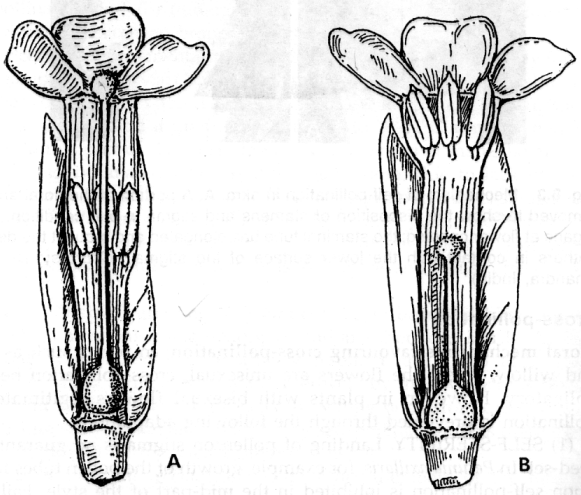

Fig. 8.4 Distyly in *Primula vulgaris*. **A**. Pin-eyed flower. **B**. Thrum-eyed flower.

(ii). *Thrum-eyed* or *short-styled* (Fig. 8.4B), with short style, small stigmatic papillae, long stamens, and large pollen grains.

The stigma in the thrum-eyed flowers is at the level of anthers in the pin-eyed flowers and vice-versa. As a rule, pollen from thrum-eyed flowers can bring about effective pollination only in pin-eyed flowers and, similarly, pollen from pin-eyed flowers can effect legitimate pollination in thrum-eyed flowers, but not in their own type.

Some flowers, such as *Lythrum* and *Oxalis*, show tristyly. They have three types of flowers with respect to the length of the style and stamens (Fig. 8.5A-C). Pollen from flowers of one type can effectively pollinate stigma of only the other two types of flowers, and not of its own type.

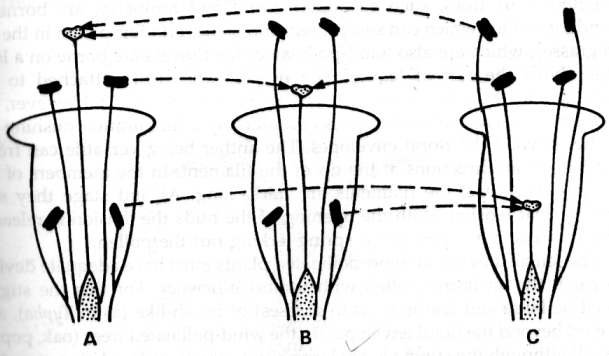

Fig. 8.5 Tristyly. **A**. Flower with long style, and normal and short stamens. **B**. Flower with mid-style, and long and short stamens. **C**. Flower with short style, and normal and long stamens. As shown by arrows, pollen from any one of these three types of flowers can bring about legitimate pollination in other two but not in its own type.

Agencies: Sexual reproduction in animals is brought about by the active participation of the male and the female individuals. Impelled by the urge to mate, the males and the females walk, crawl, swim, or fly to reach their respective counterparts. The plants are distinct in this regard. The male flowers or male organs have no internal device to reach the female organs in another flower. They are dependent for this on some external agency, which mostly transfers pollen grains from the male parent to the stigma in the female parent. Only rarely, as in *Vallisneria*, complete male flower may be transported to the female flower. Some of the agencies which have proved helpful to plants in this task are: wind (*anemophily*), water (*hydrophily*), insects (*entomophily*), birds (*ornithophily*), and bats (*cheiropterophily*).

1. ANEMOPHILY. It is an abiotic means of pollination and, being non-directional, a wasteful process. That the pollen would reach the right stigma

through wind is a hit-or-miss affair. During the transit of pollen through wind a considerable amount of pollen is lost because it never reaches a proper stigma. To stand this loss, anemophilous plants have to produce enormous quantities of pollen. A single plant of *Mercurialis annua* has been estimated to produce 1,352,000,000 pollen grains and that of maize 18,500,000.

Anemophilous plants bear small and inconspicuous flowers. The perianth lobes are quite reduced or absent. The pollen grains are small, light, smooth and dry, so that they can be carried by wind to long distances. Pollen of some plants are said to be blown to distances up to 1,300 km.

The structural adaptations in anemophilous flowers make it convenient for the wind to blow away the discharged pollen. The male flowers in anemophilous trees, such as poplar, hazel and mulberry, are borne in pendent catkins which can sway freely and shake-off their pollen in the air. In grasses, which are also wind-pollinated, the flowers are borne on a long axis above the tuft of leaves. The flowers are firmly attached to the inflorescence axis depriving it of free movement in the air. However, the filament of each stamen elongates considerably at maturation pushing the anther beyond the floral envelopes. The anther being versatile can freely oscillate in all directions at the tip of the filament. In the members of the family Urticaceae, the filaments are fairly long. At bud stage they stay under great tension. With the opening of the buds the tension is released and the filaments open like a spring jerking out the pollen.

The female flowers of wind-pollinated plants must have adequate devices to catch the air-borne pollen with utmost efficiency. For this the stigma must be large and feathery (as in grasses) or brush-like (as in *Typha*), and project beyond the floral envelopes. In the wind-pollinated trees (oak, poplar, hazel) although the stigma is not large but its entire surface being receptive offers considerable area to catch pollen.

Anemophily is associated with reduction in the number of ovules per ovary. Biological justification for this reduction is that chances for wind-borne pollen reaching the right stigma are remote. If the flowers of wind-pollinated plants were to produce many ovules most of them would have remained unfertilized. In grasses there is just one ovule per ovary.

Maize pollen are fairly large and heavy. They cannot be blown by light breeze. However, mild wind shakes the male inflorescence (tassel) to release the pollen. The latter fall down vertically. The distribution of male and female flowers on a maize plant (Fig. 8.6) overcomes the disadvantage of pollen being heavy. Clusters of male flowers are borne terminally, and the female inflorescences (cobs) are borne laterally at lower levels. The silk of the cobs which projects beyond the protective leaves comprises the stigmas. They measure up to 23 cm in length and are able to catch the pollen falling from the male flowers.

2. HYDROPHILY. Aquatic habitat of plant is no indication of its being pollinated through water. While some hydrophytes have taken advantage of water, most of them are pollinated through wind (*Myriophyllum, Potamogeton*) or insects (*Alisma, Nymphaea*).

As in anemophilous plants, floral envelopes of hydrophilous plants are highly reduced or absent. A male flower of *Callitriche* is represented by just one stamen, and the female flower comprises a naked carpel. Hydrophily is of two types:

(i) *Hyphydrophily*. It includes plants which are pollinated inside the water, *e.g.*, *Ceratophyllum, Najas* and *Zostera*. *Zostera marina* is a submerged marine perennial. It is a unique instance where pollen grains themselves have got adapted to promote water pollination. The pollen are exceptionally long (up to 250 μm) and needle-like. They look more like pollen tubes. The specific gravity of these pollen is almost the same as that of sea water. Consequently, they can freely float in water at any depth. The stigma is also very long in this plant. If the pollen comes in contact with the stigma, it coils around the latter.

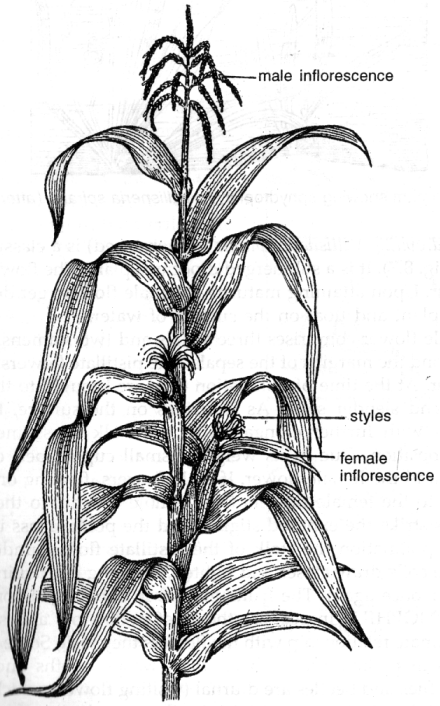

Fig. 8.6 A flowering plant of maize. Note the distribution of the male and female inflorescences.

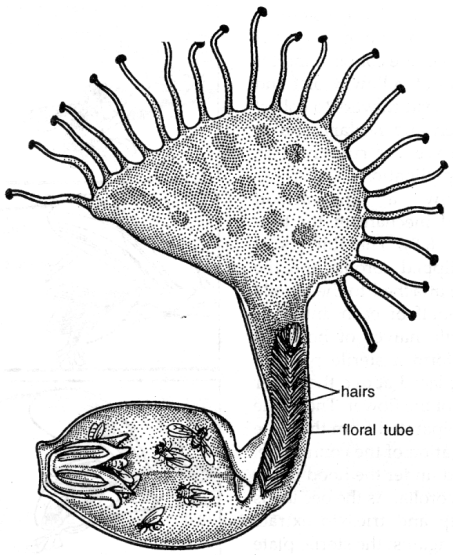

Fig. 8.9 Longitudinal section of a flower of *Aristolochia fimbriata* showing imprisoned flies in the dilated base of the floral tube. Also note the deflexed hairs lining the tube.

The small flies which are involved in its pollination are attracted by the flare and foetid smell of the flowers. The deflexed hairs facilitate the entry of the flies into the base of the tube but prevent them from escaping. During their futile attempts to escape the flies rub against the stigma and effect pollination if they are carrying some pollen. As the anthers dehisce the flies get laden with pollen. Concurrently, the hairs in the floral tube wither, the tube bends backwards and becomes flaccid making it convenient for the flies to come out.

For its pollination the orchid *Ophrys speculum* has picked on the most selective attraction in the entire animal kingdom. It is pollinated by a hairy wasp, *Colpa aurea*. The wasp has a fixed habit whereby its males leave the burrows for above-ground existence about four weeks before the females emerge for the open- air mating. The orchid opens its flowers about the same time the males appear, and they possess an appearance (Fig. 8.10) and odour similar to those possessed by the female wasps. The inexperienced males mistake the *Ophrys* flowers for their female counterparts and land to perform the act of pseudo-copulation. The insect repeats the act with a number of orchid flowers and carries pollinia from one flower to another. This insect-plant relationship is beneficial only to the plant.

Most of the moth-pollinated flowers open at or after dusk and are dull in colour. The moths are guided by their heavy fragrance. Unlike bees, moths do not land on flowers. They suck the nectar by inserting their long proboscis into the corolla tube while hovering over it. *Yucca* has developed an obligate symbiotic relationship with the moth, *Tegeticula*. The moth cannot complete its life-cycle without the association of *Yucca* flower and, in turn, *Yucca* has no other pollinator. The female moth visits the pendulous white flowers of *Yucca* at night and starts collecting pollen under its head, in the form of balls. When pollen collection is over the moth inserts its ovipositor into the ovary of the flower and lays eggs inside the ovarian cavity. It then climbs to the top of the style and pushes the pollen ball into the stylar canal which is lined with stigmatic tissue. Thus, pollination is brought about. As food for the larvae of moth, the young seeds of *Yucca* cannot be substituted by anything. While some seeds are eaten away by the larvae enough are spared to mature. The mature larvae escape from the ovary by eating away the ovarian wall and grow into adults underground. The time of emergence of the moth from the underground chrysalis coincides with the blooming of *Yucca*. It is one of the most striking examples of extreme specialization in nature.

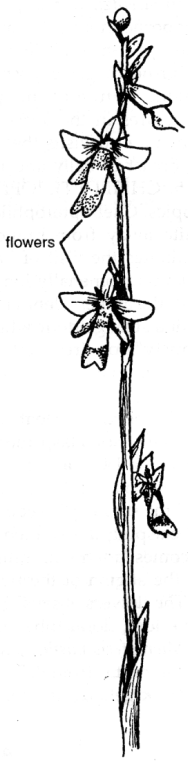

Fig. 8.10 A flowering twig of *Ophrys speculum*.

4. ORNITHOPHILY. In some parts of the tropics birds are more important pollinators than insects. Humming-birds, sun-birds, and honey-eaters are some of the birds which regularly visit flowers. General characteristics of the ornithophilous flowers are their tubular (*Nicotiana glauca*), cup-shaped (*Callistemon*), or urn-shaped (some members of Ericaceae) form, bright colour, large quantities of pollen, and plenty of nectar. They are, with a few exceptions, odourless. Their pistils and stamens usually project beyond the perianth lobes.

The birds have a powerful vision, with a preference for colours, in descending order being red, pink, orange, blue, yellow, white, green and maroon. The plants pollinated by humming-birds bear pendent flowers. These birds do not require any platform to land on the flower because they can extract nectar while hovering over it. The sun-birds, however, visit erect flowers because they can perch in any position and suck out nectar.

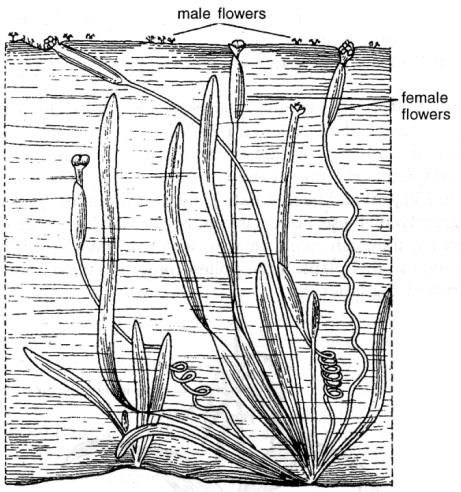

Fig. 8.7 Diagram showing ephydrophily in *Vallisneria spiralis*. (*after* Kausik, 1939)

(ii) *Ephydrophily*. *Vallisneria spiralis* (ribbon weed) is a classic example of this type (Fig. 8.7). It is a submerged dioecious plant. The flowers are borne under water. Upon attaining maturity the male flowers get detached from the parent plant and float on the surface of water.

Each male flower comprises three sepals and two stamens. The anthers project beyond the margins of the sepals. The pistillate flowers also develop under water. At the time of pollination they are brought to the surface by thin, long and slender stalk. As it arrives on the surface, the flower is vertical but with further elongation of the stalk it assumes an almost horizontal position. Due to its weight a small cup-shaped depression is formed around the female flower. If male flowers, floating on the surface, come close to the female flowers they tumble down into the depression. The anthers strike the exposed stigma, and the pollen mass is transferred to it. After pollination the stalk of the pistillate flower undergoes spiral torsion. The coils draw closer and tighten, bringing the pollinated flowers under water once again. The fruits mature in submerged condition.

3. ENTOMOPHILY. Insects are the chief pollinators and show various types of intimate relationship with the flowers they visit. Some of the insects which help in pollination are bees, flies, wasps, moths and beetles. Of these, bees, flies and beetles are diurnal (visiting flowers which open in the day time), and moths nocturnal (visiting flowers which open after sunset).

Of the various insects, bees are the main flower visitors. It is said: "Bees handle up to 80 per cent of all pollination done by insects". All the bee-pollinated flowers are brightly coloured, possess sweet fragrance, and/or produce nectar. Bees are colour-blind for red, and are fond of yellow, violet and purple. The bees visit flowers to collect their food (pollen and nectar), and in the process prove instrumental in bringing about pollination.

Salvias show a specialized "turnpipe" floral mechanism for bee-pollination (Fig. 8.8). The flowers of *Salvia* have a blipped corolla, and the two stamens are attached to the corolla tube. Only one-half of each anther is fertile. The sterile halves of both the anthers jointly form a sterile plate of tissue which is placed above the lower lip at the mouth of the flower. The fertile halves become separated from the plate due to the elongation of the connective. They are situated under the hood of the upper lip of the corolla. As the bee lands on the lower lip and tries to extract nectar, it pushes against the sterile plate which automatically brings down the fertile anthers to touch its back, depositing pollen thereon (Fig. 8.8A-D). In flowers whose anthers have already discharged their pollen contents the stigma hangs down. When a pollen-laden bee visits such a flower its back rubs against the stigma (Fig. 8.8E) and, thus, pollination is brought about.

Flies are mainly attracted by the unpleasant smell of flowers such as *Rafflesia* (rottening meat), *Arum* (human dung), and *Aristolochia* (decaying tobacco and humus). *Aristolochia, Arum* and *Ceropegia* have developed "fly-trap mechanism" for ensuring pollination. The flower of *Aristolochia* has a long perianth tube dilated at the base (Fig. 8.9). The latter encloses the sex organs. The lining of the tube is slippery and furnished with deflexed hairs. In this plant the pistil matures about ten days before anther dehiscence (protogyny).

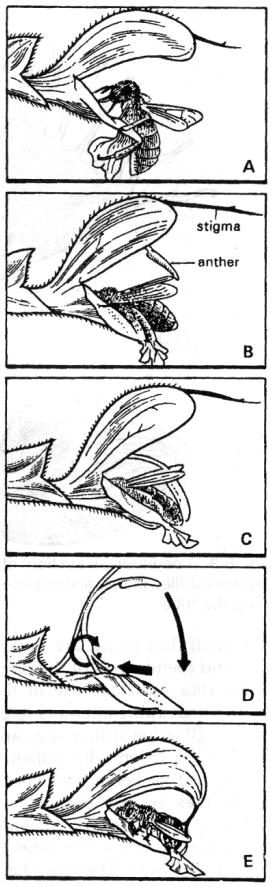

Fig. 8.8 Bee-pollination in *Salvia*. **A-C**. Stages in pollen transfer from anther to the bee. **D**. The structure of stamen in the flower. The arrows indicate the mechanism involved in the deposition of pollen from anther to the back of the bee. **E**. Deposition of pollen on the stigma from the bee.

In *Strelitzia reginae* pollination is brought about by sun-birds. The flowers are borne in a boat-shaped bract. Of the three sepals two form a long and stiff spear-head. The stamens and carpel, which are enclosed within the spear-head, get exposed only with the pressure of the visiting bird. A unique feature of this plant is the presence of certain sticky, thread-like structures in the anther cavity, each holding hundreds of pollen grains. When a bird visits these flowers, the pollen-laden threads get attached to its breast or belly and move with it from flower to flower.

5. CHEIROPTEROPHILY. Bats are instrumental as pollinators only in tropics. Cheiropterophilous plants have flowers borne singly or in clusters quite away from branches and foliage owing to their long stalk. This facilitates the visit of bats to the flowers. In *Mucuna gigantea* the flowers have phenomenally long stalks. These flowers open only at or after dusk, with the anthers dehiscing almost at the same time. Upon bloom the flowers emit a strong odour which has been variously described as "disagreeable", "musty", or "resembling sour milk". Besides the odour, they produce copious quantities of nectar. They are dull in colour. Being nocturnal the bats are attracted by the odour of the flowers.

The pollinating bats have a long and slender muzzle, and an extensive tongue. They clasp the flowers with their claws and lap nectar. *Eidolong helvum*, a large and strictly vegetarian bat, visits the flowers of *Adansonia digitata* to extract nectar. In the flowers of this species the "ball of stamens" and the stigma project beyond the floral envelopes. The bat holds the flower by clasping the stamen ball to its breast. While lapping nectar its breast becomes laden with numerous pollen grains, some of which get deposited on the stigma of the flower it visits next.

The flowers of sausage tree (*Kigelia africana*), another bat-pollinated plant, have long floral tube and collect nectar at its base. Only small bats, such as *Micropteris pusillus*, which can fold itself nearly into the floral tube can obtain nectar from it. The bat lands on the petals and during nectar lapping its back gets dusted with pollen powder.

ARTIFICIAL POLLINATION

In nature the useful characters are distributed among a large population of plants. The breeders are interested to bring them, as far as possible, in a single individual which can be multiplied to get a population of superior plants. The technique used for this purpose is called hybridization. It involves artificial pollination of the desired female parent with pollen from the desired male parent, taking all precautions to prevent contamination of the stigma with the pollen of any other type. For the plants with unisexual flowers the procedure is rather simple. Unopened buds (male as well as female) are covered with cellophane bags. When the stigma attains receptivity pollen from the male parent are dusted on it, and the pollinated flowers rebagged. In bisexual flowers, however, hybridization involves an additional step of emasculation. Before bagging the buds (in female parent) the undehisced anthers are carefully removed with the help of a sterilized

pair of forceps. This is done in order to eliminate the chances of self-pollination. Upon its maturation the stigma is impregnated with pollen from the desired male parent and rebagged.

A difficulty in hybridization arises when the two desired parents come into bloom in different seasons, or if they grow in different parts of the world. This is because pollen of most plants have a fairly short life-span. Normally, only in a few species does the pollen retain viability for over two days. In *Paspalum dilatatum*, a grass, the pollen fail to germinate 30 minutes after its shedding. In this respect extension of pollen viability period by storing them under special conditions is highly desirable.

Pollen Storage

The earliest reference on the handling and storage of pollen concerns the date-palm. There are indications that male inflorescence of the date-palm was an important commodity of commerce in the Middle East about 4,000 years ago. However, the methods of storage practised at that time are not well understood. Some of the methods currently used for pollen storage are described below:

1. DRY AND COLD STORAGE. During the past fifty years longevity of pollen has been attempted by controlling temperature and relative humidity (RH). Sub-freezing temperatures (-5° C to -10° C) and low RH (25 to 50%) have generally proved optimum for storing pollen in viable condition (*see* Table 8.1). Ordinarily, pollen of mango remains viable for eight days but at a temperature of 4.5 to 9°C, and 10, 25 or 50% RH they maintain viability for about 5 months. Viability of *Pyrus malus* and *P. communis* pollen could be prolonged up to 9 years by storing them at a temperature of -17°C. For grasses, except *Pennisetum typhoideum*, the relationship between RH and pollen viability is just the opposite. They can be stored longer at comparatively high RH (70-100%); low RH is detrimental.

TABLE 8.1 Extension of pollen viability by controlled temperatures and humidity. (*after* King, J.R., 1965)

Source of pollen	Storage conditions		Duration of storage
	Temperature °C	Relative humidity %	
Zea mays	4	90	9-11 days
Saccharum officinarum	5-13	85 & CO_2	12 days
Arachis hypogaea	3	40	15 days
Solanum tuberosum	5-8	31-40	2 months
Phoenix dactylifera	-13	-	12 months
Mangifera indica	-23	0	14 months
Cocos nucifera	5	40	18 months
Vitis vinifera	-12	28	48 months
Pyrus malus	-17	-	108 months

Mixing of certain pulverized, anhydrous substances, called diluents, with the pollen during storage under controlled conditions is beneficial. The diluents are probably effective through bringing about desiccation of the system. Of over 30 diluents, powdered *Lycopodium* spores and egg albumen are known to prolong viability substantially.

For sending pollen from one place to another the storage under controlled conditions of temperature and RH is both inconvenient and expensive. With this in mind, King (1959) perfected a new method called "Freeze Drying Method" for pollen storage. In brief, it involves the removal of water from pollen after freezing it (sublimation) and sealing them in vacuuo or in an inert gas, such as helium. The merit of this method is that the pollen can be stored under uncontrolled conditions and the shipment of pollen is inexpensive; conventional type of package is possible.

2. CRYOGENIC STORAGE. Application of cryogenic technique (storage in liquid nitrogen, at -196°C) to preserve pollen viability for prolonged duration has been found suitable for several economically important crops. At -196°C pollen undergo negligible metabolic changes in terms of physiological and biochemical processes which otherwise might render them inviable. This method has also been recommended for convenient and economical storage and transport of germplasm. At -196°C the pollen of *Secale cereale, Triticum aestivum* and *Zea mays* could be maintained in viable state for up to 10 years (Barnabas and Kovacs, 1997).

A reduction in the pollen moisture content below a threshold level prior to freezing at super-low temperatures (-70 to -196°C) seems to be crucial for achieving high survival, particularly in graminaceous plants where pollen at shedding contain a high amount of water (35-60%). Pearl millet (*Pennisetum glaucum*) pollen collected from field-grown plants lost viability after one day at room temperature. The viability period at room temperature could be extended to 186 days by mixing the pollen with *Lycopodium* diluent. However, the pollen dried to 3%-7.2% moisture content and stored at -73°C remained viable for 7-8 years (Hanna, 1990).

3. ORGANIC SOLVENTS. Acetone, benzene, ethanol, ether, chloroform and phenol, are generally regarded as toxic to organisms. However, it has been demonstrated beyond doubt that pollen grains kept in these organic solvents can germinate *in vitro* and even effect fertilization (Iwanami *et al.*, 1988). Of the 50 organic solvents tested to store *Camellia* pollen for one week, only acetic acid proved fatal (Iwanami *et al.*, 1988).

Normal, flowering plants of *Petunia* have been raised from pollinations made with the pollen grains stored in ether for one week. Hirata, at Chiba University, Japan, stored pollen grains of pear for one year in ether and used them for pollination in the following spring. The fruits obtained by these pollinations were bigger and sweeter than those obtained by fertilization with pollen stored under dry and cold conditions. Lily pollen stored in ethanol for 10 years were found capable not only of germination but also fertilization.

Some of the practical applications of pollen storage are:

1. To hybridize plants that flower at different times and locations or show non-synchronous flowering.
2. To provide a constant supply of short-lived (recalcitrant) pollen.
3. To facilitate supplementary pollination for improving yield.
4. To eliminate the need to grow male lines continuously in breeding programmes.
5. To ensure the availability of pollen throughout the year without using nurseries or artificial climate growth rooms.
6. To obviate the variability incidental to the daily collection of pollen samples.
7. To study pollen allergens and the mechanism of self-incompatibility.
8. To provide material for international exchange of germplasm.
9. Long-term germplasm storage, especially of unique genotypes.

Pollen Viability Tests: To test the efficacy of a pollen storage method, or to determine male fertility of a plant requires a reliable method to test pollen viability. The latter has been defined as the competence of individuals of a given pollen population to deliver male gametes to the embryo sac (Heslop-Harrison *et al.*, 1984). Most of the available tests of pollen viability, in actuality, measure particular cellular characteristics of a living cell. The methods currently available to test pollen viability fall under two categories, viz., germinability tests and histochemical tests.

(1) SEED-SET OR OVULE DEVELOPMENT AFTER POLLINATION OF COMPATIBLE LIVING PISTILS. This is probably the most reliable method but may be complicated, for some species, by the effect of pistil screening mechanisms. Moreover, it is not an instant test; one has to wait for a few days to see the results.

(2) POLLEN GERMINATION AND POLLEN TUBE GROWTH IN LIVING PISTILS. The most satisfactory method to test pollen tube growth employs decolourized aniline blue to detect callose plugs in pollen tubes. Pollinated pistils are fixed in a fixative such as acetic-alcohol (1:3) and cleared in 8N NaOH until the tissue becomes soft. These are then washed and mounted in 0.005% decolourized aniline blue prepared in 0.05M Na_2HPO_4. The tissues of the pistil are flattened by applying gentle pressure before observation under fluorescence microscope. The pollen tubes show bright yellow fluorescence due to the presence of callose.

(3) *IN VITRO* POLLEN GERMINATION. It is perhaps the simplest method to test pollen viability. It permits to score the percentage of germination and the rate of pollen tube growth. Media and methods for *in vitro* pollen germination are described on page 138

(4) TETRAZOLIUM TEST. Various tetrazolium salts have been used to test pollen viability, of which 2,3,5-triphenyltetrazolium chloride (TTC) is most popular. In practice, 0.5% to 10% solution of the salt is prepared in 12% sucrose solution (sucrose is used to prevent pollen bursting and, therefore, its concentration may vary with the material). The pH is adjusted to 5.8 (preferably buffered with 0.15M Tris HCl).

To test viability, the pollen are dispersed in a drop of the medium on a microscope slide and immediately covered with a coverglass to exclude oxygen, which inhibits the reduction of the dye. The slide is placed in a petriplate lined with moist filter paper and incubated at 35-60°C in dark for up to 30 min to 3 hrs and observed under microscope. The viable pollen grains turn red due to the reduction of the TTC to red dye formazan. As a precaution, only the pollen grains in the central region of the coverslip are scored, because those at the periphery are exposed to higher O_2 potential and do not respond to the test.

(5) FLUORESCEIN DIACETATE TEST. It is the most reliable cytochemical test to estimate pollen quality (Heslop-Harrison *et al.*, 1984). This test is dependent on two cellular parameters, viz., effectiveness of plasma membrane and presence of a non-specific esterase enzyme in the pollen cytoplasm. It is simple and takes only a few minutes to perform. A stock solution of fluorescein diacetate (2 mg per ml of acetone) is prepared that can be stored in fridge for several months. Immediately before use, few drops of the stock solution are added to sucrose solution of appropriate concentration (10-20%) until saturation is reached as indicated by the appearance of permanent cloudiness. Addition of 300 mgl^{-1} of calcium may improve the response in some cases. Pollen sample is dispersed in a drop of this medium on a microscope slide and the fluorochromatic reaction (FCR) is allowed to proceed for 10 min in a petriplate lined with moist filter paper. The pollen suspension is then covered with a coverglass and observed under a fluorescence microscope. The pollen grains with bright fluorescence are scored as viable. Pollen grains, killed by exposure to 80°C for 2-12 h, may be used as the control.

In viable pollen grains the non-fluorescent, polar compound FDR enters through the membrane, and in the cytoplasm it is cleaved by non-specific esterase enzyme to release the fluorescent, polar molecule fluorescine which cannot move across the plasma membrane and, therefore, accumulates in the pollen to give fluorescence. A drawback of this method is that it, sometimes, gives false positive and negative reactions. For example, mature viable pollen of plants such as *Secale* and *Populus*, which have an ineffective membrane before hydration, will lose the fluorescent molecules and give false negative reaction. In such cases the test should be performed after controlled hydration of the pollen which induces membrane repair.

(6) NUCLEAR MAGNETIC RESONANCE (NMR) METHOD. In tests 1-4 the pollen sample is used or destroyed. The NMR method is non-destructive and, therefore, has the advantage that the tested sample can be recovered for breeding experiments (Dumas *et al.*, 1983; 1984a, b, c). In this method comparative levels of free and bound water within the pollen are determined. Since a reproducible correlation has been found between bound water and pollen viability, this method provides a precise test for pollen quality. It has also been shown that during loss of pollen viability on ageing, a part of the water in the grain passes from the bound to the free state and the overall water content decreases.

Unfortunately, this test cannot be performed in most teaching laboratories because of lack of proper facilities.

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which takes from a few seconds to a few minutes. This induces the activation of the inactive cytoplasm of the pollen grains. Many cellular organelles undergo considerable changes in comparison to what has been observed in inactive ripe pollen. For example, more evident changes observed during the activation of the pollen grains of *Lycopersicon peruvianum* are: (1) cisternal or rough endoplasmic reticulum arranged in stacks in mature pollen are set free; (2) production of abundant vesicles (small ones concerned with the formation of pectocellulosic wall, and large ones concerned with the formation of callosic wall layer); (3) formation of a thin callosic layer, strictly related to dictyosome activity and developing first below the pore from which the tube originates; (4) aggregation of ribosomes into polysomes; and (5) formation of lamellae inside the plastids. Activation of pollen grain does not induce any change in mitochondria, lipidic bodies, generative cell and vegetative nucleus (Cresti *et al.*, 1977).

There is sufficient evidence to suggest that the pollen germination specific RNAs are transcribed before the morphological ripening of the pollen and are stored in an inactive form. As a consequence, the pollen can germinate and the tube can grow up to a certain length when incubated in the presence of a transcription inhibitor such as Actinomycin D (*see* Rosen, 1968; Mascarenhas, 1975).

In *Lycopersicon esculentum* the final organization of the pollen tube is achieved when it attains a length of 150 µm, and the generative cell and the vegetative nucleus have moved into it (Cresti *et al.*, 1977). At this stage the pollen tube shows four distinct zones: (1) *Apical zone* or *growth region* (2-4 µm long), is swollen and contains abundant vesicles (small and large) but is devoid of cell organelles; (2) *Sub-apical zone*, has granular cytoplasm rich in cell organelles. The vesicles are produced in this region; (3) *Nuclear zone*, houses the vegetative nucleus and the generative cell and later two sperms; and (4) *Vacuolization zone*, forms a transition between the active and the inactive cytoplasm of the pollen tube. It is highly vacuolated and ends with a callosic plug.

Growth and Structure of Pollen Tube

The pollen tubes, as a rule, emerge at the germ-pores on the pollen grains, which are generally covered with only a very thin layer of exine. The first event associated with germination is hydration and swelling of the outer pectic layer of the intine, which leads to the rupture of exine. Almost the entire contents of the grain move into the tube. Rapid growth of the tube is restricted to the tip region. In a growing tube most of the cytoplasm is confined to the apical region, and a large vacuole fills the grain and the older region of the tube (Fig. 9.4A, B). To restrict the cytoplasm to the apical region of the growing tube, a series of callose plugs are formed at a regular distance behind the tip. As a result, a fully grown pollen tube is subdivided into many compartments. The plugs originate as a ring on the inner side of the wall. They gradually grow toward the centre reducing the lumen and, finally, sealing the tube (Fig. 9.5A-C). The small amount of

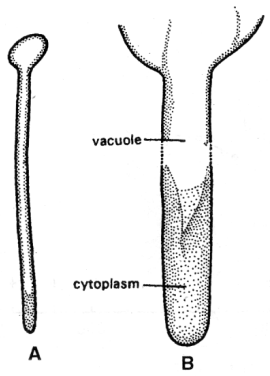

Fig. 9.4 **A**. A germinated pollen grain to show that in a growing pollen tube the cytoplasm is confined to the apical portion. **B**. Enlarged apical portion of pollen tube shown in **A**. (*after* Iwanami, 1959)

cytoplasm that is left behind the plug on the side of the grain, gradually degenerates. The partition in the pollen tube prevents the backflow of cytoplasm and nuclei.

Under the high power of a light microscope the extreme tip region of the tube appears hemispherical and transparent (Fig. 9.5A). The zone behind it looks granular. The transparent apical zone is called "cap block". It exists only as long as the tube is growing and disappears when the growth ceases (Fig. 9.6).

The cytoplasm behind the cap block is rich in the usual cell organelles, namely, mitochondria, Golgi bodies, rough and smooth endoplasmic reticulum, vesicles, amyloplasts, and lipid bodies. In the cap block region the aforementioned structures are absent, with the exception of Golgi vesicles which are present in

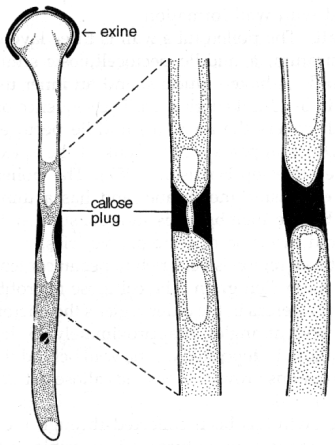

Fig. 9.5 **A-C**. Stages in callose plug formation in a growing pollen tube. **A**. Initiation. **B**. Intermediate stage. **C**. Lumen of the tube is completely sealed.

CHAPTER

9

FERTILIZATION

Fertilization involves the fusion of a male gamete with the female gamete. In angiosperms the female gametophyte is seated deep in the ovarian cavity, far away from the stigma. The pollen (male gametophyte) are, normally, held at the stigma, and there is no device for them to reach the egg inside the female gametophyte. To effect fertilization in this group of plants the pollen grains germinate on the stigma (Fig. 9.1) by putting forth tubes (pollen tubes) which grow through the style and find their way into the ovules (Fig. 9.2), where they discharge the sperms in the vicinity of the egg. One of the sperms fuses with the egg (forming zygote) while the other fuses with the polars or the secondary nucleus (forming primary endosperm nucleus). The distance a pollen tube has to travel in order to reach the egg depends on the length of the style which is quite variable in different species. For example, in sugar-beet it has to grow only a few millimetres

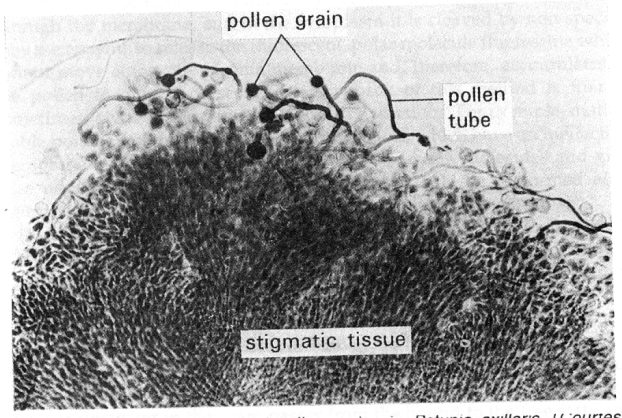

Fig. 9.1 Stigma with germinated pollen grains in *Petunia axillaris*. (*Courtesy*: Prof. K.R.Shivanna, India)

whereas in corn it grows as much as 450 mm. The rate of pollen tube growth is often extremely rapid (> 1 μm per sec).

POLLEN GERMINATION AND POLLEN TUBE GROWTH

Normally only one tube develops from a pollen grain. In polysiphonous grains, however, more than one tube may emerge from a grain; up to 10 pollen tubes have been observed in *Althaea rosea* and 14 in *Malva neglecta*. As a rule, only one of them grows further. In plants where pollen grains are united into tetrads (orchids) or pollinia (Asclepiadaceae) several pollen tubes are produced at the same time and all of them may grow (Fig. 9.3). Branching of pollen tubes is quite common in some members of the Amentiferae.

The first requirement for pollen to germinate is their hydration

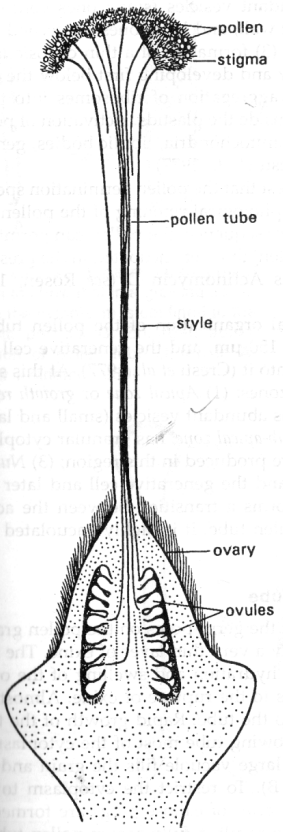

Fig. 9.2 Longitudinal section of a pollinated pistil of okra. The pollen tubes have grown to various lengths in the style; some have reached the ovules.

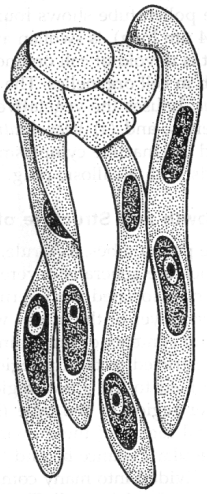

Fig. 9.3 A germinated pollen tetrad of *Cymbidium bicolor*. (*after* Swamy, 1941)

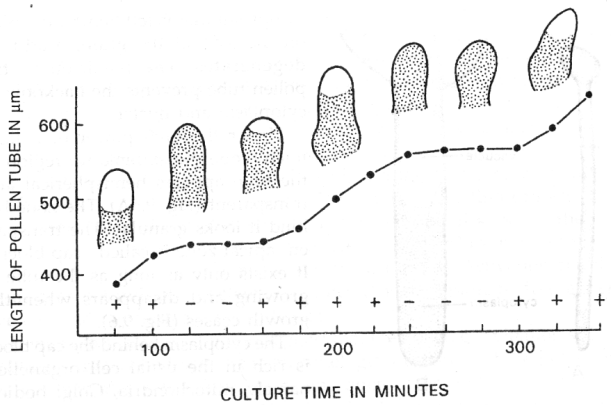

Fig. 9.6 The relationship between pollen tube elongation and the appearance of "cap block" in *Lilium longiflorum*. When the cap block disappears (indicated by –) the tube ceases to grow, and when the cap block re-appears (indicated by +) the tube starts growing. (*after* Iwanami, 1959)

abundance (Fig. 9.7). These vesicles are rich in polysaccharides or RNA, and are associated with wall formation.

Pollen tube wall: The pollen tube wall is three layered structure, with an outer pectin stratum, a middle pectocellulosic stratum with fibrillar component rich in β-1,4-linked glucans, and an inner usually amorphous callosic layer rich in β-1,3-linked glucans. The sequence of deposition is: (a) pectic layer, which covers the apex, (b) middle pectocellulosic layer, the first evidence of which appears on the flank of the apex, and then (c) the inner callosic layer (Heslop-Harrison, 1987). The pollen tube tip is not bound by a proper plasma membrane, and has a thin fibrillar wall. In actively growing tip the membrane is in a constant state of perturbation because of the rapid insertion of secretory vesicles.

Wall at the cap block region is very rich in pectin content which gradually decreases toward the pollen grain. The cellulose microfibrils are arranged at random at the tip whereas in the older regions the microfibrils are oriented in two directions, both at angles of approximately 45 degrees to the main axis of the tube. Callose is deposited on the wall behind the growing region. Only when the tube stops growing does the callose get deposited at the tip.

Most of the information on the factors influencing pollen germination and pollen tube growth has been collected through the culture of pollen grains in nutrient medium. In cultures it is possible to handle the pollen under controlled conditions and check their response to various substances. We shall, therefore, deal with pollen germination and pollen tube growth

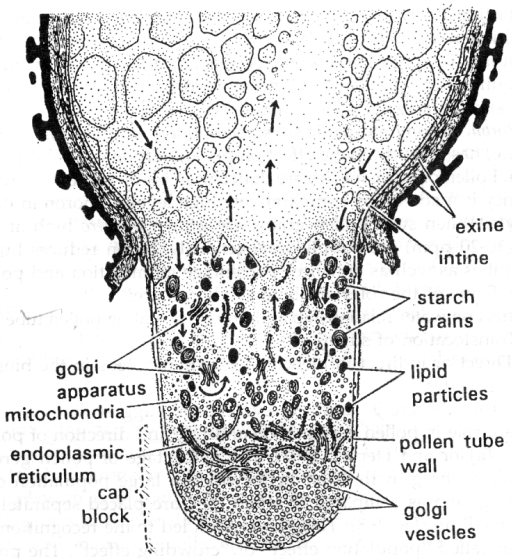

Fig. 9.7 Diagrammatic representation of the fine structure of an elongating pollen tube of lily. The cap block shows many golgi vesicles that have migrated to the pollen tube tip. The remaining part of the tube contains endoplasmic reticulum, mitochondria, golgi apparatus, starch grains and lipid particles. (*after* Iwanami *et al.*, 1988)

first in the nutrient medium (*in vitro*) and then in the stigma, style and ovary (*in vivo*).

IN VITRO. Pollen grains are resting plant organs. Uptake of water leads to swelling of the grains and their activation. Therefore, high relative humidity (RH) is the first essential requirement for pollen germination whether *in vitro* or *in vivo*. Pollen of some plants germinate readily in saturated atmosphere. Other factors which have been found important for pollen germination and pollen tube growth are as follows:

(1) *Carbohydrates*. For germinating pollen grains in nutrient medium a sugar is always necessary. Two roles have been ascribed to sugars: (a) control of osmotic pressure, and (b) to serve as the respiratory substrate.

Pollen of many species would burst when placed in water. Addition of a definite amount of sugar limits the diffusion rate of water into the pollen and, thus, prevents the bursting of pollen tube. Of the many sugars tested for their ability to promote pollen germination and tube growth, sucrose (the sugar present in most pollen) is most effective.

According to Iwanami (1959) the time taken between imbibition and tube initiation depends on the kind of sugar present in the grain at the time of shedding. The pollen grains of *Impatiens balsamina* contain mainly glucose and require only 2-3 minutes for germination, whereas *Lilium* pollen have largely sucrose and need 30-40 minutes for germination.

(2) *Boron*. Among inorganic substances, boron, in the form of boric acid or borate, has most dramatic effect on pollen germination and pollen tube growth. Pollen of most species are deficient in boron content. In nature this deficiency is made up by comparatively high levels of boron in the stigma and style. When such pollen grains are grown *in vitro* high amounts of boron (10-20 ppm) are supplied exogenously. Boron reduces bursting of pollen tubes as well as enhances percentage germination and pollen tube growth. Some of the roles attributed to boron are:

(i) Effect on water relationship and, thus, preventing pollen tube bursting.

(ii) Translocation of sugars.

(iii) Direct or indirect influence on enzymatic steps in the biosynthesis of carbohydrates.

(3) *Calcium*. There is sufficient evidence to suggest that Ca^{2+} plays an important role in pollen germination and rate and direction of pollen tube growth (Taylor and Hepler, 1997). The percentage of pollen germination and pollen tube growth is far better when a large population of pollen grains is grown as compared to when they are placed separately on the culture medium (Fig. 9.8). This observation led to the recognition of what has been called "population effect" or "crowding effect". The population effect is brought about by Ca^{2+} ions. Pollen grains contain very small amount of calcium. In aqueous medium calcium diffuses out of the pollen rapidly,

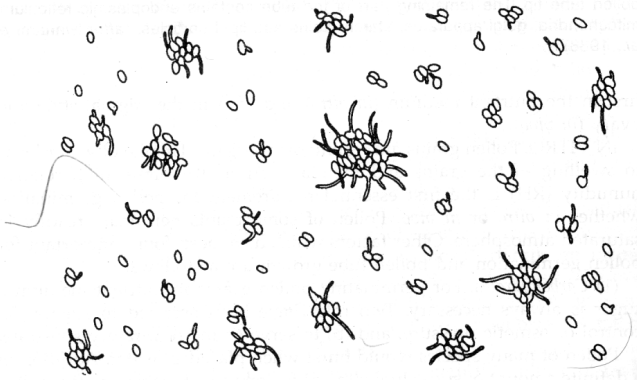

Fig. 9.8 Percentage pollen germination and the growth rate of pollen tube is better when a large number of pollen grains are grouped together as compared to when they are planted singly on the nutrient medium. (*after* Iwanami, 1959)

leaving a low endogenous level which is insufficient for pollen germination. When pollen grains are present in large groups on the surface of semi-solid medium the diffused out Ca^{2+} may be trapped between the pollen grains and, thus, bring about the population effect.

A growing pollen tube shows a tip-focused gradient of free Ca^{2+} ions, with values above 3 µM at the extreme tip and dropping to less than 0.2 µM within 20 µm from the tip; this feature is not shown by non-growing pollen tubes (Malho *et al.*, 1994,1995; Pierson *et al.*, 1994, 1996). Ca^{2+} influx from the outside into the pollen tube and the Ca^{2+} channels, through which the ions enter the tube, are restricted to the extreme apex of the tube where growth occurs and membrane deformation is maximum (Pierson *et al.*, 1994). The Ca^{2+} level at the high point of gradient fluctuates, with concentration changing as much as four fold, which is directly correlated with oscillation in pollen tube growth rates. Conditions which inhibit pollen tube growth block influx of Ca^{2+}, reducing the intracellular Ca^{2+} gradient at the tip. When conditions return to normal, the internal gradient is re-established. First the Ca^{2+} level increases throughout the apex and the tip swells. Thereafter, the sector in the swollen apex with the highest level of Ca^{2+} becomes the point where growth restarts (Pierson *et al.*, 1996).

(4) *Flavonols*. Virtually all pollen accumulate flavonols, often to very high levels. These aromatic compounds, which are derived from the anther tapetum, play an important role in pollen germination and pollen tube growth (Taylor and Hepler, 1997; Xu *et al.*, 1997). Flavonol-deficient plants of petunia and maize are self-sterile because their pollen are unable to germinate or produce a functional pollen tube. However, this defect is conditional. Application of nanomolar concentrations of flavonol aglycones to pollen suspension or to the stigma after pollination can induce germination of the deficient pollen (Taylor and Jorgensen, 1992). This reproductive defect could also be corrected by application of wild type stigmatic exudate. The bioactive compound from the stigmatic exudate has been identified as kaempferol, a flavonol aglycone (Mo *et al.*, 1992; Vogt *et al.*, 1994).

(5) *Enzymes*. Cellulase, pectinase and callase are present in pollen grains. Cellulase and pectinase are released immediately after the pollen are placed in the germination medium. When supplied exogenously, these enzymes increase the rate of tube elongation. Cellulase and pectinase cause tube elongation through an increase in the plasticity of the tip region.

(6) *Plant hormones*. Promotion of pollen tube growth by auxins and gibberellins has been recorded but the effect is not appreciable.

(7) *Physical factors*. Among the physical factors affecting the pollen tube growth, temperature is the most important. The growth rate is appreciably enhanced with an increase in temperature. An optimum range of temperature is 20-30°C. However, in some incompatible self-pollinations high temperatures have been reported to retard pollen tube growth.

A classroom exercise for *in vitro* germination of pollen: A simple method to demonstrate germination of 2-celled pollen is to grow them in a 'hanging drop'. It requires a cavity-slide and a coverglass. Take a clean

and dry coverglass and in the middle of it place a small drop of the nutrient medium (for composition *see* Table 9.1). Sprinkle pollen grains on the medium directly by shaking the dehisced anthers or transfer them with the help of a camel-hair brush. On the slide apply vaseline around the rim of the cavity. Lift the coverglass with a pair of forceps and turn it upside down. Place it on the slide in a manner that the culture drop hangs right above the middle of the cavity. Care must be taken that the drop does not touch the cavity wall.

When the objective is to study and compare the effects of some substances on percentage germination and pollen tube growth the above method is not satisfactory. This is because the distribution of pollen grains in the medium is not uniform. Consequently, the results are also not uniform (for explanation *see* population effect on page 132). In such a situation the method described by Iwanami (1959) may be used. In this method semi-solid medium (prepared by adding about 1% agar to the liquid medium) is employed instead of liquid medium. The medium containing agar is heated to dissolve the latter and poured on a slide or in a petriplate. On cooling a thin agar-nutrient-plate will be formed. Now spread pollen on a coverglass as evenly as possible. Scrape with the edge of another coverglass the pollen spread out and touch it on the surface of the medium. The pollen grains thus placed on the medium are in a perfect straight row. Pollen sown in this manner will show comparatively more uniform results with regard to percentage pollen germination and pollen tube growth.

In general, 3-celled pollen have proved difficult to germinate *in vitro*. However, a semi-solid medium, perhaps analogous to the stigma surface, was shown by Bar-Shalom and Mattsson (1977) to promote pollen tube growth in *Brassica* and other 3-celled pollen. They emphasized the importance of relative humidity in controlling germination. Roberts *et al.* (1983) independently confirmed the importance of relative humidity and showed that a new medium (for composition *see* Table 9.1) buffered at pH 8, using 1 mM Tris or methylamine, induced 80-90% germination of *Brassica oleracea* pollen.

TABLE 9.1 Composition of Nutrient Media for *in vitro* Germination of pollen Grains.

Constituents	*Amounts (mg/l)*	
	2-celled pollen[1]	*3-celled pollen*[2]
Sucrose	100,000	200,000
H_3BO_3	100	10
$Ca(NO_3)_2.H_2O$	300	-
$CaCl_2.6H_2O$	-	362
$MgSO_4.7H_2O$	200	-
KNO_3	-	100
pH	7.3	8

[1] *after* Brewbaker and Kwack (1963).

[2] *after* Roberts *et al.* (1983). This medium must be buffered at pH 8 using 1mM Tris or methylamine only.

IN VIVO. Stigma possesses a specialized surface for catching and holding the pollen. Konar and Linskens (1966) studied the development of stigma in *Petunia hybrida*. A young stigma shows a columnar tissue with a slight depression in the centre, and is divisible into two zones: an upper zone with epidermis forming the secretory zone and lower 1-3 layers of laterally extended cells constituting the storage zone. Many of the epidermal cells divide forming bicelled stigmatic papillae. The exudation accumulates in the schizogenous cavities (Fig. 9.9).

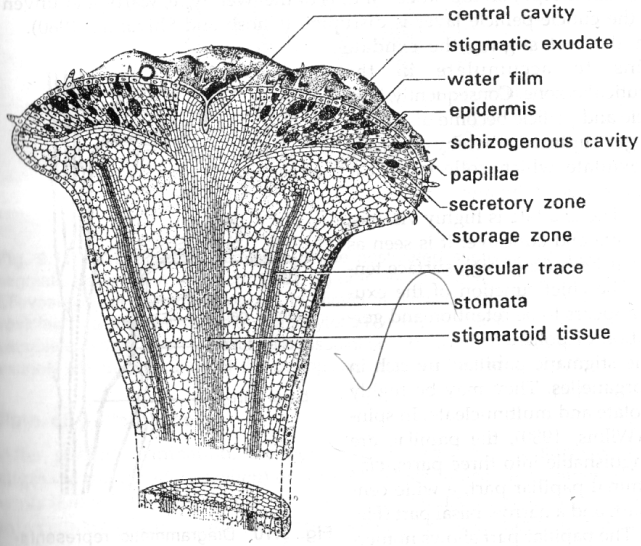

Fig. 9.9 Semi-diagrammatic reconstruction of the stigma of *Petunia hybrida* just before pollination. (*after* Konar and Linskens, 1966)

The main function of the stigma is to provide the pollen with water necessary for their germination. In many plants it also supplies the necessary medium for pollen germination in the form of exudates. The chief components of the exudates are of lipid and phenolic nature. In addition, small amounts of free sugars, amino acids, proteins, and peptides are also present. The composition of the exudates may vary from species to species. According to Ciampolini *et al.* (1981) in species having solid styles, the exudate consists of polysaccharides, proteins and lipids, whereas those with hollow styles show only polysaccharides.

The stigmas which secrete exudates are called wet stigmas (*Petunia*) and those which do not are called dry stigmas (cotton). Heslop-Harrison and

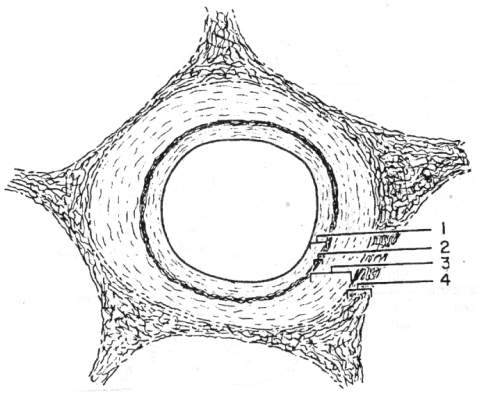

Fig. 9.12 The composition of the cell wall layers of the transmitting tissue in the solid style of cotton. The pollen tube grows through layer 3 which is very rich in pectic material. (*after* Jensen and Fisher, 1969)

plasmodesmata which, in later stages, get blocked (Cresti *et al.*, 1976; Bell and Hicks, 1976).

The cells of the transmitting tissue contain numerous mitochondria, plastids, RER, dictyosomes and ribosomes. Although the origin of the intercellular substance is not yet known, it is believed that ER and dictyosomes are involved in this secretion. The intercellular substance in many solid styled plants comprises of carbohydrates, proteins, glycoproteins and some enzymes such as acid phosphatases, peroxidases and esterases.

In the family Fabaceae, the style is hollow to different degrees. *Vigna unguiculata* shows a transition from solid to hollow condition (Ghosh and Shivanna, 1982). The stigma and a few mm of the upper part of style are solid. Below this a canal develops gradually by the dissolution of transmitting tissue, so that the base of the style is hollow and lined with canal cells. Some legumes such as *Crotalaria* and *Cajanus* (Ghosh and Shivanna, 1982) show a completely hollow style.

In *Gossypium hirsutum* and *Petunia hybrida*, where the style is solid, the pollen tubes grow by making a way through the pectin-rich wall layer (layer 3 in Fig. 9.12). In spinach the first tubes that reach the style grow through the intercellular spaces, but subsequently they may grow through the cell wall and plasma membrane (Fig. 9.10; Wilms, 1978).

In taxa with open or hollow styles (occurring predominantly in monocots), there is a canal which runs throughout from stigma to the base of the style. The canal is lined by a single layer of glandular canal cells (Fig. 9.13) which may become multinucleate and polyploid (Vasil and Johri, 1964).

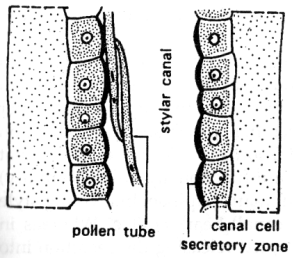

Fig. 9.13 A portion of hollow style in longisection. Note the large cells lining the stylar canal.

The canal in a mature style may contain secretion fluid as seen in *Amaryllis* and *Lilium* or may remain dry as in *Crocus* and *Gladiolus*.

The most striking structural feature of the canal cells in *Lilium* is the presence of 8-14 µm thick, domed "secretory zone" on the side facing the canal (Fig. 9.13). The secretory zone consists of three regions (Fig. 9.14).

(1) An outer 1 µm thick wall layer (L_1). It chiefly consists of cellulose fibrils.

(2) 7-13 µm thick granular-fibrillar wall layer (L_2) which extends as irregular projections into the adjacent cytoplasm. The cellulose microfibrils in this layer are randomly dispersed and are continuous with the cellulose fibrils in the L_1. The granules in this region are pectic in nature, probably complexed with proteins.

(3) An irregular interphase between L_2 and the adjacent cytoplasm. It contains aggregates of tubules and vesicles. The aggregations have been termed "paramular bodies".

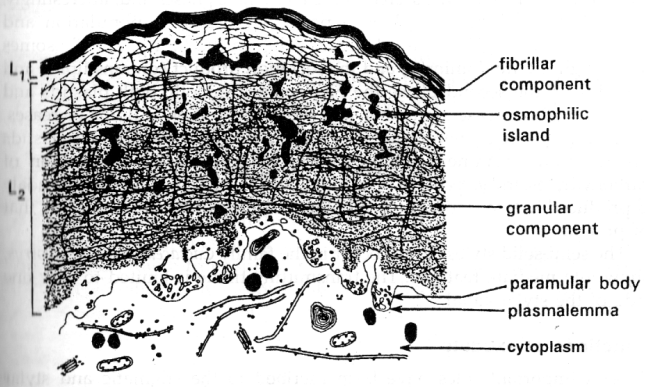

Fig. 9.14 Diagrammatic representation of the secretory zone and adjacent cytoplasm of a typical canal cell of *Lilium*. (*after* Dashek *et al.*, 1971)

In *Citrus limon* also the inner tangential walls of the canal cells become very thick and consist of two layers. The outer layer, facing the canal, is

Shivanna (1977), who investigated nearly 1000 species of both monocots and dicots, have further subdivided the 'wet' and 'dry' stigmas. The groupings are based on disposition of receptive cells, and the papillate and non-papillate nature of cells.

Structural differences have been noted in the stigmatic papillae of floral morphs in many heterostylous plants (Dulberger, 1975). In *Linum grandiflorum* the stigma of the pin-morph is of the 'dry' type and smooth, and the papilla is covered by a distinct cuticle-pellicle layer. The stigma of the thrum-morph, on the other hand, is of the 'wet' type, warty and unven and the cuticle-pellicle layer is disrupted (Ghosh and Shivanna, 1980).

In the wet stigma, the exudate begins to accumulate in the subcuticular zone. Consequently, both cuticle and pellicle become distended and get ruptured, thereby releasing the exudate which collects on the stigma surface (Shivanna and Sastri, 1981). The exudate is highly viscous, refractive and adhesive. It is seen as droplets because of high surface tension. The chief function of the exudate appears to be retention and germination of pollen.

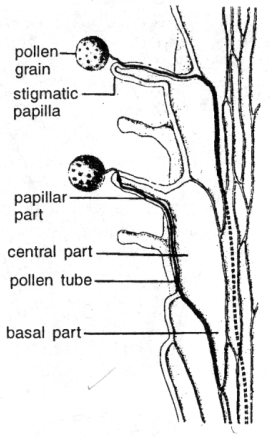

Fig. 9.10 Diagrammatic representation of the growth of pollen tube on and in the stigma of spinach. (*after* Wilms, 1980)

The stigmatic papillae are rich in cell organelles. They may be highly vacuolate and multinucleate. In spinach (Wilms, 1980), the papillae are distinguishable into three parts, *viz.*, a terminal papillar part, a wide central part, and a narrow basal part (Fig. 9.10). The papillar part shows numerous mitochondria, ER, ribosomes and dictyosomes, whereas plastids are concentrated in the basal part.

In *Aptenia cordifolia* (Kristen *et al.*, 1979), the stigmatic papillae contain numerous vesicles with fibrous material. These vesicles probably originate from ER because of their continuity with the latter, and are responsible for the secretion of the exudate. A diagrammatic representation of the secretory pathways is shown in Fig. 9.11.

In certain plants, such as *Diplotaxis tenuifolia* (Brassicaceae), the stigma is covered with cuticle. The pollen grains degrade the cutin enzymatically in order to obtain water necessary for germination. Pollen grains contain an elaborate set of enzymes. Some of these are present in the wall and are available as soon as the pollen grain makes contact with the stigma.

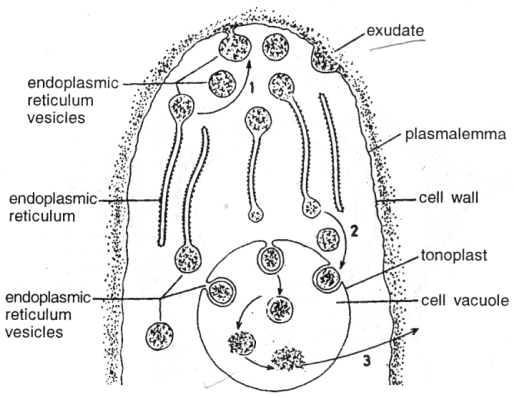

Fig. 9.11 Diagrammatic summary of the postulated secretion pathways in the stigmatic papilla of *Aptenia cordifolia*. (1) granulocrine secretion of the exudate by ER-vesicles via exocytosis occurs in immature papillae. (2) incorporation of ER-vesicles into the cell vacuole phagocytotically; vesicle contents are mixed with vacuole substances after membrane dissolution. (3) holocrine excretion of a mixed exudate via degeneration of the protoplast. (*after* Kristen *et al.*, 1979)

Path of pollen tube

After pollen germination, the pollen tubes grow on the surface of the stigmatic papillae (*Gossypium*) or through the cellulose-pectic layer of their walls (*Lilium*). Upon reaching the base of the papillae the tubes grow in the intercellular spaces of the stigmatic tissue. The subsequent course of the tubes depends on the nature of the style.

Based on morphological features, three types of styles have been described : solid, open and semi-solid. The solid style, which occurs mainly in dicots, is characterized by the presence of a central strand of elongated, specialized cells that constitute the 'conducting tissue' or the 'transmitting tissue'. The transmitting tissue is either single-stranded, as in *Withania* (Garg and Bhatnagar, 1988), *Solanum nigrum* (Bhatnagar and Uma, 1969), *Petunia* (Konar and Linskens, 1966), *Lycopersicon* (Cresti *et al.*, 1976) and *Nicotiana tabacum* (Bell and Hicks, 1976), or multistranded, as in *Solanum melongena* (Bhatnagar and Uma, 1969). In a transection the cells of the transmitting tissue appear circular and separate with exceptionally thick walls (Fig. 9.12) surrounded by intercellular substance which is mainly pectinaceous. In cotton the cells of the transmitting tissue are not surrounded by secretion. The cells of the transmitting tissue are generally uni-nucleate and densely cytoplasmic. The transverse walls of these cells are traversed by

fibrillar and homogeneous, whereas the inner layer is granular and non-homogeneous (Ciampolini *et al.*, 1981).

It has been suggested that in *Lilium* the parenchyma cells underneath the canal cells synthesize some mucilaginous substances and transport them to the canal cells which, in turn, secrete them into the stylar canal. After pollination the canal is filled with the secretion which serves as nutrition for the growing pollen tubes. Rosen and Thomas (1970) have shown that in lily the stigmatic cells and canal cells are functionally alike (secretory) but a secretory zone is not found in the stigmatic papillae. In a young style the inner wall of canal cells (facing the canal) bears cuticle. Whereas in *Lilium* the cuticle in later stages gets disrupted releasing the secretion into the stylar canal, in *Crocus* and *Gladiolus*, it remains intact so that the secretion gets accumulated between the canal cells and the distended cuticle.

The secretion from the canal cells contains proteins, carbohydrates and lipids and shows activity for esterases and acid phosphatases. In *Ornithogalum caudatum* the secretion occurs in three phases and, interestingly, each phase is characterized by a particular organelle population and secretory product (Tilton and Horner, 1980). In the first phase ribosomes and rough ER are dominant, and the secretion product contains lipids and proteins. In the second phase the ER becomes smooth, both plastids and mitochondria increase in number, and the concentration of lipids increases. In the final phase numerous large dictyosomes become visible and plastids are no more prominent. This phase is marked by the production of carbohydrates. In *Lycopersicon* (Cresti *et al.*, 1976), however, the stylar exudate is produced in two steps. The synthesis of carbohydrate is followed by that of protein.

The semi-solid styles, which occur in the family *Cactaceae*, and *Artabotrys*, show intermediate features. The transmitting tissue is limited to only one side of the stylar canal.

Functions of exudate

Several important roles have been ascribed to the stigmatic and stylar exudates.

1. Help in adhesion, hydration and germination of pollen on the stigma.
2. Protect the pistil from insect attacks and/or microbial infection.
3. Act as a liquid cuticle to save stigma from dehydration.
4. Serve as food for vectors during pollination.
5. Serve as nutrition for the growing pollen tubes through stigma and style.

With regard to the fact that the pollen tubes always grow in the direction of the ovary, Strasburger (1887) stated that the path of pollen tubes in the pistil is guided by the secretion from the ovule. Subsequently, it was demonstrated that the ovules, placenta, the inner epidermis of the ovary and the stigma attract pollen tubes (Fig. 9.15). Moreover, if a slice of the pistil is placed on the medium and later removed the tubes grow toward the spot where it was lying. Schildknecht and Benoni (1963) suggested that

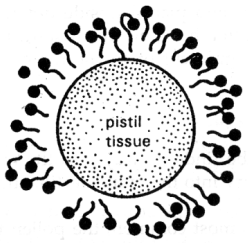

Fig. 9.15 "Surface Test" to demonstrate the chemotropic attraction of pollen tubes by pistil tissue. All pollen tubes are growing toward the pistil tissue.

amino acid and amino mixture coupled with sugars is responsible for the chemotropic attraction of pollen tubes in *Oenothera* and *Narcissus*. Rosen (1964) and Mascarenhas and Machils (1962), however, did not observe such a response with *Lilium* and *Antirrhinum*, respectively. Mascarenhas and Machils suggested that Ca^{2+} ions are a naturally occurring chemotropic agent in the pistils of *Antirrhinum majus*. They also suggested that calcium-controlled unidirectional growth of pollen tubes in the pistil may be of universal occurrence. However, the distribution of Ca^{2+} ions in the pistil of *A. majus*, as measured cytochemically, does not correlate well with the distribution of chemotropic activity (Mascarenhas, 1966). The concentration of Ca^{2+} throughout the length of the style is very low and almost constant. It is slightly higher in the stigma and the ovary. In the ovary the concentration of Ca^{2+} is extremely high in the placenta and the ovary wall but the ovules have comparatively low Ca^{2+} concentration, and there is no increase in it in the micropyle or the embryo sac. A similar distribution of calcium ions occurs in the pistils of *Oenothera* (Glenk *et al.*, 1967). This anatomical distribution of calcium ions in the pistil would not be expected if increasing Ca^{2+} gradient was necessary for directing pollen tube growth from stigma to ovules (Mascarenhas, 1975). Moreover, Ca^{2+} is chemotropically inactive with the pollen tubes of lily (Rosen, 1964), corn (Cook and Walden, 1967), and *Clivia* and *Crinum* (Kwack, 1969). These two observations argue against the suggestion that Ca^{2+} alone controls the unidirectional growth of the pollen tubes in the pistil.

Mascarenhas (1975) proposed an altogether a new hypothesis for the mechanism controlling unidirectional growth of pollen tubes in the style. According to this theory, for straight growth of pollen tube a gradient of chemotropic substance/s is not necessary. It would only require the tropic factor to be present along the path of pollen tube at a concentration above a certain threshold value. A steep gradient of the tropic factor is necessary only in localized regions of the pistil where pollen tube has to take a sharp turn, *e.g.*, at the placenta for the pollen tube to turn toward the ovule. This hypothesis of Mascarenhas is supported by early work of Iwanami (1959), which clearly demonstrates the lack of a gradient of chemotropic factor in the pistils of lily. Iwanami took different segments of the style and stigma and studied the direction of pollen tube growth. If the pistil was placed horizontally and pollen grains germinated on the inner surface of the style by making a hole in it about 55 per cent pollen tubes grew toward the ovary while the remaining 45 per cent tubes grew toward the stigma.

Similarly, if a part of the style was cut-off and placed upside down and pollen germinated at the top almost all pollen tubes grew downward. These observations clearly suggest the lack of a gradient of chemotropic factor within the style.

Entry of pollen tube into the ovule

After arriving in the ovary, the pollen tube finds its way into the ovule. Depending on the place of pollen tube entry into the ovule, fertilization is of three types (Fig. 9.16).

(1) POROGAMY. In this type, which is most common, the pollen tube enters through the micropyle.
(2) CHALAZOGAMY. This refers to a situation where pollen tube enters the ovule at the chalazal end. This type is found in *Casuarina*.
(3) MESOGAMY. In this type the entry of pollen tube into the ovule is through the funiculus (*Pistacia*) or through the integuments (*Cucurbita*).

With respect to porogamy it has been suggested by some workers that the entry of pollen tube into the ovule and its subsequent growth toward the embryo sac is regulated by a chemotropic substance secreted by the filiform apparatus into the micropyle. The idea of chemotropic guidance of pollen tube growth toward the embryo sac is also supported by Rosen (1965). However, he holds the view that the substance is secreted by the micropyle itself rather than the synergid. Chao (1972) carried out a detailed cytological investigation on *Paspalum orbiculare* and demonstrated that the distal part of the integuments by dissolution of its cells *in situ*, secretes a mucilaginous substance into the micropyle which provides a way of least resistance for the pollen tube and guides it toward its ultimate destination. The mucilaginous secretion is largely a water soluble carbohydrate and it aids the pollen tube growth both mechanically and chemotropically.

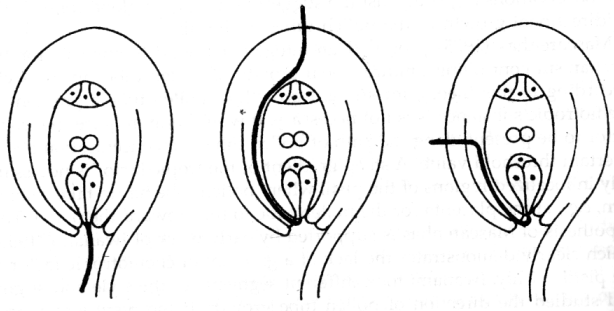

Fig. 9.16 Modes of pollen tube entry into the ovule.

A special structure which facilitates the entry of pollen tube into the ovule is the obturator (*see* Chapter 6). It forms a sort of bridge for the pollen tube to reach the ovule. After fertilization the obturator shrinks and disappears. Unfortunately, there is no explanation available for the mechanism of pollen tube entry into the ovules showing chalazogamy or mesogamy.

In Loranthaceae there is no structure like an ovule. Here the embryo sacs undergo remarkable elongation and meet the pollen tubes at some point in the stylar canal or stigma.

Entry of pollen tube into the embryo sac

Irrespective of the place of entry of pollen tube into the ovule, it invariably enters the embryo sac at the micropylar end (Fig. 9.16). The tube enters at the apex of the filiform apparatus and, after growing through it, arrives in the cytoplasm of the synergid (Fig. 9.17). Which one of the two synergids a pollen tube would enter seems to be predetermined. Mostly the penetrated synergid starts degenerating before the arrival of the pollen tube, but after pollination. This receptive synergid is readily recognized after it degenerates because it lacks plasma membrane and a tonoplast (Russell, 1993). However, in *Petunia* the synergid visited by pollen tube does not show any visible change until the arrival of the tube. Increase in GA_3 concentration in the ovules as a consequence of pollen tube-pistil interaction has been suggested as the possible cause of the breakdown of synergid in cotton. In the cultures of cotton ovules from unpollinated pistils in a medium containing GA_3, synergid degenerates in a manner similar to that in pollinated flowers (Jensen and Ashton, 1981).

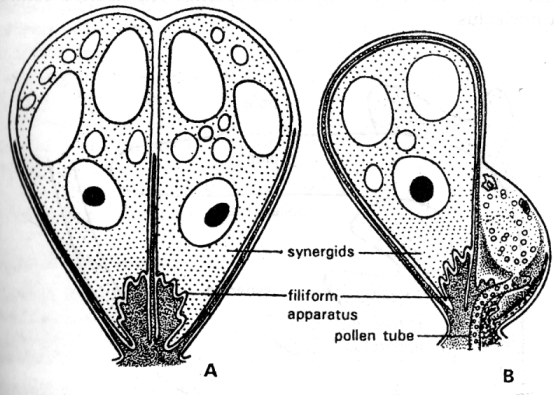

Fig. 9.17 Diagrammatic summary of the changes in the synergids after pollen tube discharge. **A.** Synergids in a pollinated flower. **B.** Synergids after pollen tube discharge. (*after* Jensen and Fisher, 1968)

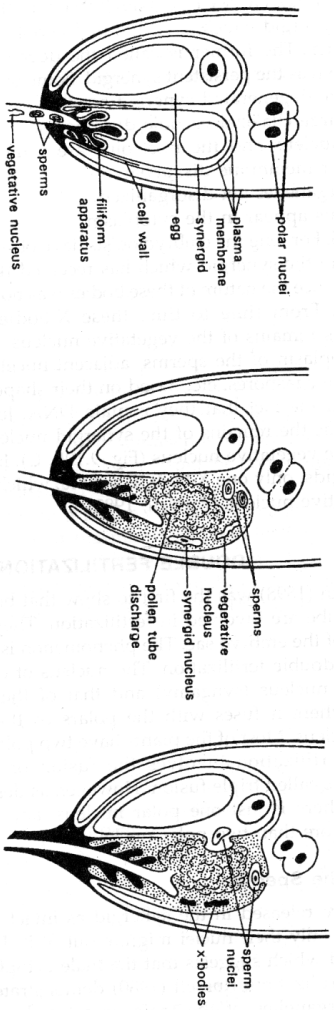

Fig. 9.19 Summary diagram to show sperm transfer in the embryo sac. (*after* Jensen, 1973)

1976), and biparental inheritance of plastids in some plants (Hagemann, 1981; Russell and Cass, 1981; also *see* page 47).

The mode of sperm transfer is a matter of speculation at present. According to Jensen (1973), one of the sperms comes in contact with the plasma membrane of the egg cell while the other contacts the plasma membrane of the central cell. The membranes at the points of contact dissolve and the sperm nuclei are released, one in the egg and the other in the central cell (Fig. 9.19A-C). After entering their destined cells the male nuclei are passively carried along the cytoplasmic stream to the egg nucleus or to the polars as the case may be. Belayeva (1981) has reported that the sperms are released from the synergid one after another. The first sperm is attracted by the egg cell, whereas the second sperm moves to the central cell through cytoplasmic current. The entry of one of the sperms into the central cell is preceded by the entry of the other sperm into the egg. Thus, the first sperm nucleus reaches the egg nucleus before the second sperm nucleus reaches the polars. However, the fusion of the egg nucleus and the sperm nucleus takes much longer than the fusion of the male nucleus and the polars. The reason for this is the active state of central cell as compared to that of egg which is inert (Jensen and Fisher, 1967).

Syngamy

Fusion of the egg nucleus with sperm nucleus is called syngamy. Gerassimova-Navashina (1960) has described three types of syngamy (Fig. 9.20). According to her, at the time when the two nuclei come in contact with each other the egg nucleus is in a state of deep mitotic rest whereas the male nucleus is at the telophase of the previous mitosis.

Type 1. *Premitotic*. The sperm nucleus fuses immediately on coming in contact with the egg nucleus, and the zygote nucleus divides subsequently; *e.g.*, Poaceae, Asteraceae.

Type 2. *Postmitotic*. The sperm nucleus and the egg nucleus remain in contact for a while and fuse only after both the nuclei have entered into divisions (zygotic mitosis); *e.g.*, *Lilium, Fritillaria*.

Type 3. *Intermediate*. The sperm nucleus fuses with the egg nucleus after completing its previous mitosis. Even after the fusion of the nuclear membranes the contents of the two nuclei show incomplete mixing. At the prophase of zygotic mitosis often the two sets of chromosomes can be seen separate; *e.g.*, *Impatiens*.

UNUSUAL FEATURES

Polyspermy

This refers to a situation where more than two sperms are released in an embryo sac. This may result because of the formation of more than two sperms in a pollen tube or due to the penetration of an embryo sac by more than one pollen tube. Polyspermy occurs only as an abnormality. Normally, an embryo sac receives only two sperms. Polyspermy may bring about

The contents of pollen tube are discharged in the synergid, and the tube does not grow beyond it in the embryo sac. However, in *Plumbago* the pollen tube enters the synergidless female gametophyte through the filiform apparatus at the tip of the egg (Russell and Cass, 1981; Russell 1982). It penetrates the egg wall, and discharges the sperms and the tube nucleus between the egg and the central cell. The process of discharge takes place in seconds. In cotton the contents of the tube are discharged through a subterminal pore which is invariably on the side facing the chalaza (Figs 9.17-19). In *Epidendrum* and *Plumbago*, however, the pore is described as terminal.

The pollen tube discharge

It includes two sperms, the vegetative nucleus and a fair amount of cytoplasm. A portion of the cytoplasm is retained in the pollen tube where it rapidly degenerates. The cytoplasm released by the pollen tube and that of the synergid hardly show any mixing. They remain as two separate entities. The synergid cytoplasm is confined to the micropylar end and that of pollen tube is restricted to the chalazal end of the cell. The latter can be easily recognized by the presence of thousands of tiny polysaccharide spheres (0.5-1.0 µm). The cytoplasm released from the pollen tube undergoes immediate degeneration (Russell, 1982).

SPERMS. The two sperms in a pollen tube often change their shapes. They are cells, each bound by a plasma membrane. In the tube the two sperms are usually placed close to each other. The sperm cytoplasm contains the usual cell organelles (*see* Chapter 4). Microtubules have been suggested to be associated with the motility of the sperms. The nucleus contains a distinct nucleolus.

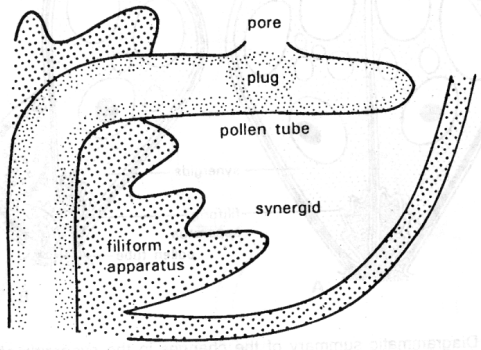

Fig. 9.18 A portion of the penetrated synergid in Fig. 9.17 enlarged to show subterminal pore on the pollen tube. (*after* Jensen and Fisher, 1968)

POST-POLLINATION CHANGES IN THE EMBRYO SAC. As mentioned earlier, the synergid which is visited by the pollen tube degenerates faster than the other. The former is called the degenerating synergid and the latter is known as the persistent synergid. Some of the characteristic features shown by the degenerated synergid are :

(1) The large chalazal vacuole disappears.
(2) The nucleus and the nucleolus are somewhat flattened, and the nuclear membrane disappears.
(3) The organelles get disorganized.
(4) Crystals appear in the cytoplasm.

X-BODIES. For long the embryologists have observed two darkly staining oval bodies in the synergid which has received the pollen tube discharge (Fig. 9.19C). Since the nature of these bodies was not clear they were denoted as X-bodies. From time to time these X-bodies have been variously interpreted as remains of the vegetative nucleus, remains of the synergid nucleus, cytoplasm of the sperms, adjacent nucellar cells, remains of the degenerated megaspores, etc. Based on their shape and distribution in the synergid, and the fact that they contain DNA, Jensen (1972) interpreted one of them as the remains of the synergid nucleus and the other as the remains of the vegetative nucleus (Fig. 9.19B, C). In *Plumbago*, where there are no synergids, only one X-body is visible, which represents the remains of the vegetative nucleus (Russell, 1982).

DOUBLE FERTILIZATION

S.G.Nawaschin (1898) was the first to show that both the sperms released by a pollen tube are involved in fertilization. They fertilize two different components of the embryo sac. The phenomenon is unique to angiosperms and is called double fertilization. The nucleus of one of the sperms fuses with the egg nucleus (syngamy) and that of the other migrates to the central cell where it fuses with the polars or their fusion product, the secondary nucleus. Most of the plants have two polar nuclei. Consequently, the second fertilization involves the fusion of three nuclei, and the phenomenon is called triple fusion. However, as described in Chapter 7, in some plants there is just one polar nucleus, and there are some others which have more than two polar nuclei.

Passage of the Sperms

The sperms are released in the synergid as intact cells but, according to general belief, only their nuclei migrate out of it. However, evidence has been presented which suggests that the male cytoplasm is also sometimes involved in fertilization. Russell (1980) demonstrated the transmission of cytoplasmic organelles of the male gametes during gametic fusion in *Plumbago*. Similarly, Cass (1981) has shown the entry of male cytoplasm into the egg in barley. This observation is supported by transmittance of cytoplasmic virions through sperm cells in barley (Carroll and Mayhew,

fertilization of egg by more than one male nucleus or the supernumerary sperms may fertilize other components of the embryo sac, such as synergids or antipodals. In the embryo sac which receives two or more pollen tubes the sperm nucleus fusing with the egg nucleus may be derived from one pollen tube and the one fusing with the polars may be derived from another (hetero-fertilization).

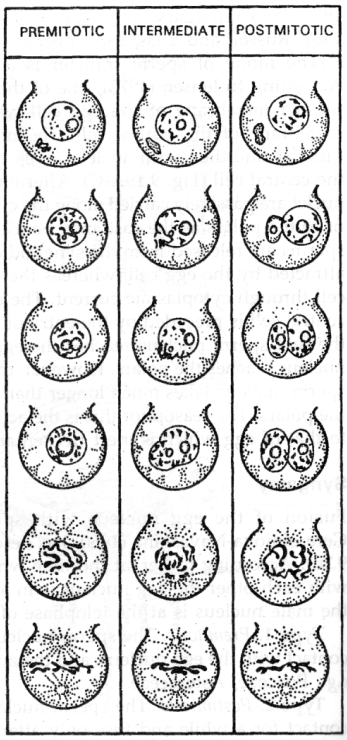

Fig. 9.20 Types of syngamy. (*after* Gerassimova-Navashina, 1960)

Persistent and Branched Pollen Tubes

In angiosperms the pollen tube is normally an unbranched structure which collapses soon after fertilization. Some reports, however, claim that rarely the pollen tube may persist for as long as three weeks. In *Cucurbita* and some members of the Onagraceae branching of the terminal portion of the pollen tube has been observed. Ramanna and Mutsaerts (1971) reported that in spinach 10-12 per cent of the pollen tubes branch in the stylar region and near the micropyle, and the tubes grow like fungal mycelium producing haustoria-like structure. A reinvestigation of pollen tube growth in spinach by Wilms (1974) has revealed that branching of the pollen tube occurs mainly in the micropyle, and the branches grow between and around the inner and outer integuments. According to Wilms, branching of pollen tubes is probably a post-fertilization phenomenon. Initially, many pollen tubes may enter the nucellus but once a pollen tube has entered the embryo sac further entry of pollen tubes into the nucellus is checked at the micropyle where they branch. Ramanna and Mutsaerts described that the pollen tube branches penetrate into the integuments, nucellus, and other ovular tissues and ascribed them a haustorial function. Wilms does not agree with this. According to him the branches of pollen tube do not penetrate the

Post fertilization changes in flower: After fertilization the ovule develops in to seed. The integuments of the ovule develop in to seed coat. In the meantime the ovary enlarges

integument or other tissues. The pollen tube branches simply ramify between the nucellus and the surrounding tissue. He could not detect any haustorial structure. Consequently, the haustorial function of the pollen tubes is doubtful.

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CHAPTER

10

SEXUAL INCOMPATIBILITY

In nature the stigma receives a variety of air-borne and/or insect-carried pollen but not all pollen that reach the stigma succeed in effecting fertilization. The pistil is adequately equipped with devices to allow the pollen of only the right mating type to function normally; others are discarded. If a pistil carrying functional female gamete/s fails to set seed/s following pollination with viable and fertile pollen, capable of bringing about fertilization in another pistil, the two are said to be incompatible, and the phenomenon is known as sexual incompatibility. Sexual incompatibility may be interspecific (between individuals of different species) or intraspecific (between individuals of the same species). The latter is also called self-incompatibility. The biological significance of the two types of incompatibility would be described later in this chapter.

Interspecific incompatibility is heterogenic, i.e., controlled by more than one gene at different loci on the chromosomes (Linskens, 1975). It prevents free cross-pollination, and is the basis of creation of new races and species. In incompatible interspecific crosses either fertilization does not occur, or syngamy is followed by abortion of hybrid embryo due to inadequate development of endosperm or embryo-endosperm incompatibility. The physiology and biochemistry of incompatibility have been investigated almost exclusively with reference to self-incompatible systems. Whatever little information is available on these aspects of interspecific incompatibility suggests that the basic mechanism controlling the two types of incompatibility is grossly similar. Some instances of interspecific incompatibility and the ways to overcome them have been described while dealing with practical applications of embryo culture (*see* page 329).

SELF-INCOMPATIBILITY

A large number of flowering plants are outbreeders, which means that they are successfully fertilized only by the pollen of other plants and not by their own. In nature different floral adaptations, such as dichogamy, herkogamy and unisexuality (*see* page 112) have evolved to prevent self-pollination but the most widespread and effective natural device to enforce outbreeding is self-incompatibility, which refers to the inability of a plant

producing functional male and female gametes to set seeds when self-pollinated. Self-incompatibility has been reported in 66 families (McCubbin and Dickinson, 1997). Morphologically, self-incompatibility is of two types:

1. HETEROMORPHIC. It is characterized by the occurrence of two (distyly) or three (tristyly) morphologically distinct mating types* within a species, which can be easily recognized without a breeding test. The differences in the mating types are generally with respect to the relative lengths of the stamen and style (*see* Chapter 8). In these systems the incompatibility reaction may be controlled by one gene with two alleles, as in dimorphic species (*Primula*), or by two genes with two alleles each, as in tristyly (*Lythrum*). Other features of heteromorphic incompatibility are:
   (a) the incompatibility reaction is determined sporophytically;
   (b) the growth of incompatible pollen tubes is inhibited in the style; *Armeria maritima* is an exception where inhibition occurs on the surface of the stigma (Baker, 1975); and
   (c) dominance between alleles of the incompatibility genes is expressed both in the pollen and the style.
2. HOMOMORPHIC. In this category all the mating types within a species are morphologically indistinguishable and require proper breeding tests for their recognition. A species with this type of incompatibility has numerous mating types.

Depending on the origin of factors determining the mating types on the pollen side two categories of self-incompatibility have been recognized:

(i) *GAMETOPHYTIC SELF-INCOMPATIBILITY(GSI)*.The incompatibility process is determined by the genotype of male gametophyte (pollen) itself, *e.g.*, Liliaceae, Poaceae, Solanaceae, *Trifolium*.

(ii) *SPOROPHYTIC SELF-INCOMPATIBILITY*(SSI). The incompatibility process is controlled by the genotype of the sporophytic tissue of the plant from which the pollen is derived, *e.g.*, Asteraceae, Brassicaceae.

GENETIC BASIS OF SELF-INCOMPATIBILITY

Incompatibility is a gene-physiological process. Therefore, before dealing with the physiological aspects of the phenomenon, it would be appropriate to acquaint ourselves with the genetics of self-incompatibility.

The most popular hypothesis about the genetic control of self-incompatibility is the one of "opposition *S*-alleles", first proposed by East and Mangelsdorf in 1925. According to this, incompatibility reactions are controlled by a single gene, called *S*-gene, which has several alleles. Pollen grains that possess the *S*-allele common to any one of the two alleles present in the cells of the pistil will not be functional on that particular pistil. To illustrate it further, let us consider a plant having S_1S_2 alleles in its

*Mating type refers to a group of individuals showing similar breeding behaviour. In the present context, legitimate pollination can occur only between two individuals belonging to different mating types; pollinations within a mating type would not favour seed-set.

sporophytic cells, including the pistil (Fig. 10.1). During microsporogenesis such a plant will produce two types of pollen, one-half carrying the S_1 allele and the other half S_2 allele. According to the oppositional S-allele concept, neither of these pollen types (S_1 or S_2) would be able to bring about fertilization in the style of this particular plant (Fig. 10.1A) because both the alleles are present in the stylar cells. On the other hand, if this plant is pollinated with the pollen from a plant of S_2S_3 genotype, 50 per cent pollen grains, carrying S_3 allele, would be functional whereas the remaining 50 per cent, carrying S_2 allele, would be non-functional (Fig. 10.1B). However, every pollen grain from a S_3S_4 plant would be functional on the pistil of a S_1S_2 plant (Fig. 10.1C) as none of the alleles is common between these two plants. It should be emphasized at this stage that in all the situations described so far it is the S-allele of the pollen or the male gametophyte which determines the incompatibility reaction (GSI).

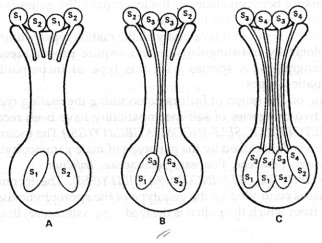

Fig. 10.1 Response of pollen on a pistil of S_1S_2 plant, showing gametophytic self-incompatibility. **A.** None of the pollen from a S_1S_2 plant is able to effect fertilization. **B.** From a S_2S_3 plant only S_3 pollen succeed in fertilizing the ovules. **C.** All the pollen from S_3S_4 plants bring about fertilization.

In SSI-systems all the pollen of a plant behave similarly, irrespective of the S-allele they carry. For instance, from a plant carrying S_1S_2 alleles the pollen carrying S_1 or S_2 allele would behave as S_1 if S_1 is dominant, or as S_2 if S_2 is dominant; if there is no dominance both will behave as S_1 plus S_2. In other words, the presence of even one of the alleles of the stylar tissue in the sporophytic tissue of the male parent would render all the pollen of that plant non-functional with respect to that particular style (Fig. 10.2). A S_1S_2 plant would, therefore, be completely incompatible to plants carrying S_1S_2 (Fig. 10.2A), S_1S_4, S_1S_5 or S_2S_3 (Fig. 10.2B), S_2S_4, S_2S_5, and so on, but would show 100 per cent compatibility with a plant carrying S_3S_4 (Fig. 10.2C), S_3S_5, and so on.

Grasses exhibit two-locus or bifactorial GSI, and the two genes, termed *S* and *Z*, are polyallelic. There appears to be co-operation between *S* and *Z* in the pollen but they act independently in the pistil (McCubbin and Dickinson, 1997). This bifactorial GSI-systems differ from other GSI-systems in having 3 nucleate pollen and the rejection reaction on the stigma, as in the SSI-systems.

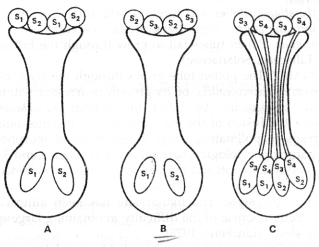

Fig. 10.2 Response of pollen on a pistil of S_1S_2 plant, showing sporophytic self-incompatibility. None of the pollen from a S_1S_2 (**A**) or S_2S_3 (**B**) plant can bring about fertilization, but every pollen of a S_3S_4 plant is capable of it (**C**).

Heteromorphic self-incompatibility systems exhibit diallelic SSI. In this case the two alleles are called *S* and *s*. In distyly the allele for short style (*S*) is dominant over the allele for long style (*s*). The long-styled plants are homozygous (*ss*) whereas the short-styled plants are heterozygous (*Ss*). Besides the length of the style and stamen, various other phenotypic characters, including pollen size and sculpturing, cell shape in the stylar conducting tissue, and stigma morphology, often vary with the two morphs. Recombination between the incompatibility phenotype and heteromorphs suggests that in most cases heteromorphic self-incompatibility is controlled by two linkage groups, one comprising genes coding for morph associated characters and the other *S/s* incompatibility locus, and the two groups are themselves closely linked in a supergene.

Tristyly is determined by two genes (*M* and *S*) with two alleles each (Shivanna and Johri, 1985). Long-styled plants are homozygous recessive (*mmss*), medium-styled plants are homozygous recessive for *S* gene and homozygous dominant or heterozygous for *M* gene (*MMss/Mmss*), and short-styled plants are heterozygous for *S* gene and any form for *M* gene (*MMSs/MmSs/mmSs*).

BARRIERS TO FERTILIZATION

In incompatibility systems barriers to fertilization may occur at different levels:

(1) POLLINATION. Methods adopted by plants to check illegitimate self-pollination, such as dichogamy, herkogamy, and unisexuality, have been described in detail on pages 111–113.

(2) PROGAMIC PHASE. In this category the inhibition occurs at any stage from pollen germination to the discharge of male gametes in the vicinity of egg.

(a) *On the stigma*: Pollen fail to germinate, or the pollen tubes cannot penetrate into the stigma (*e.g.*, Brassicaceae, Poaceal).

(b) *In the style*. Pollen tubes fail to grow through the full length of the style (*e.g.*, Liliaceae, Solanaceae).

(3) SYNGAMY. The pollen tube grows through the style, but it fails to enter the ovary (*Hemerocallis*), or its growth is arrested within the ovule (*Acacia, Lotus corniculatus*), or within the embryo sac (*Theobroma cacao*). Consequently, the fusion of the male and female gametes fails to occur.

The barriers to pollination (stage 1) are more of morphological and ecological nature; physiological barriers occur at stages 2 and 3. During the past 40 years physiological and biochemical studies on self-incompatibility have been mainly restricted to the reaction of male gametophyte on the stigma and in the style. The ovular site has been almost completely neglected, chiefly because of the difficulty in obtaining large quantities of material for study (Linskens, 1975).

With only a few exceptions, a very reliable correlation exists between the type of self-incompatibility, pollen cytology, and the site of inhibition (Brewbaker, 1957). GSI is common in species with 2-celled pollen whereas SSI is associated with species shedding pollen at the 3-celled stage. Moreover, in GSI-systems the pollen germinate normally, the pollen tubes penetrate through the stigma but fail to reach the base of the style. Thus, in these cases the rejection of the incompatible male gametophyte occurs in the style. On the contrary, in SSI-systems the rejection reaction occurs on the stigma; either the pollen do not germinate or the pollen tubes fail to penetrate the stigma. However, if the stigma is bypassed, and the pollen grains are directly introduced into the style, the pollen tubes grow normally and effect fertilization. These and some other distinguishing features of the two types of the self-incompatibility systems are listed in Table 10.1.

TABLE 10.1 Some features of sporophytic (SSI) and gametophytic (GSI) self-incompatible systems.

Features	*Type of Incompatibility*	
	SSI	*GSI*
Pollen		
Cytology	3-celled	2-celled
In vitro germination	Difficult	Easy
Viability	Short-lived	Long-lived
Respiration rate	High	Low
Pistil		
Stigma	Dry	Wet
Rejection Reaction	On the stigma	In the style

Some of the well studied systems which can be cited as exceptions to the correlation drawn above are the Poaceae, *Oenothera organensis* and *Theobroma cacao*. The Poaceae, characterized by 3-celled pollen with high respiratory rate, are an established example of GSI. Similarly, in *Oenothera organensis* and *Papaver rhoeas,* undoubtedly GSI-systems, inhibition occurs on the surface of stigma. The correlation is also not applicable to heteromorphic systems (*Forsythia, Lythrum, Oxalis* and *Primula*) where incompatibility is under sporophytic control, but the barrier to fertilization may occur in the style (Baker, 1975; Stevens and Murray, 1982).

PHYSIOLOGY AND BIOCHEMISTRY OF INCOMPATIBILITY

In an incompatible process two reactions can be distinguished which are described below:

(1) RECOGNITION REACTION. It occurs at the molecular level, and by this pistil recognizes whether a particular pollen grain is to be accepted (compatible) or rejected (incompatible). In SSI-systems the recognition reaction sets in almost immediately after the pollen comes in contact with the stigma. This also seems to be true for certain GSI-systems (Bredemeijer and Blass, 1975; Shivanna, 1982). In *Prunus avium*, for example, the presence of stigma is necessary for the recognition of pollen. Pollen grains deposited on artificial stigma made on cut stump of the style (after removing the original stigma) are able to germinate and the pollen tubes grow through the style and reach the ovary even in incompatible pollinations. Thus, in such cases the recognition and the rejection reactions are separated by time and space (Raff and Knox, 1977). Generally, however, in GSI-systems the recognition and rejection reactions occur inside the pistil (*see* Dickinson *et al.*, 1982).

(2) REJECTION REACTION. The recognition reaction sets in physiological and biochemical processes in the pistil specific to the type of pollen received. In incompatible pollination the phenotypic manifestations of these changes bring about the rejection reaction. It may occur right on the stigma, inhibiting pollen germination or preventing the entry of pollen tube into the stigma (SSI-systems), or it may occur in the style, inhibiting pollen tube growth or leading to bursting of the tube in the style (GSI-systems).

Most studies on the physiology and biochemistry of intra- and interspecific incompatibility have highlighted the importance of the pollen wall and its protein contents in the pollen-stigma interaction. As early as 1894, Green reported that intact pollen upon hydration release various hydrolytic enzymes, and suggested that these enzymes played some role in the pollen-stigma interaction. However, the importance of the pollen wall as a storage site of mobile proteins was first brought to light by the work of Tsinger and Petrovskaya-Baranova (1961). Employing cytochemical methods, these workers located definite concentrations of proteins in both intine and exine of *Paeonia* and *Amaryllis* pollen and concluded that the wall "... perforated by protoplasmic strands, appears to be a living, physiological structure playing a very responsible role in the process of

fraction remains on the surface of the tectum forming the pollenkitt. A similar mode of protein transfer from tapetal cells to the sexine cavities has been described for non-tectate pollen of *Raphanus* by Dickinson and Lewis (1973).

In contrast to the intine, in which a host of enzymes has been detected (Knox and Heslop-Harrison, 1970), the exine-held proteins exhibit only one enzymatic activity (Heslop-Harrison *et al.*, 1973).

RELEASE OF WALL-HELD PROTEINS. The sporophytically, or gametophytically derived proteins held in the pollen wall-layers are available for immediate release. Hydration of the pollen, whether on the stigma or on an agar-plate, stimulates the release of these proteins (Heslop-Harrison *et al.*, 1974). Whereas the exine-held proteins (of sporophytic origin) pass out within seconds (maximum of 30 seconds), those in the intine (of gametophytic origin) take a couple of minutes (Howlett *et al.*, 1973; Heslop-Harrison *et al.*, 1975). This step-wise release of the two types of proteins is extremely important for it allows the collection of pure samples of the two fractions separately for experimental purposes. The immediate release of sporophytic protein may partly explain the much faster occurrence of the rejection reaction in SSI-systems as compared to GSI-systems.

Stigma Surface Inhibition

In SSI-systems (barring heteromorphic systems) and some gametophytic systems (Poaceae, *Oenothera*) the recognition and rejection reactions occur on the stigma; the pollen fail to germinate or the malformed tubes are unable to penetrate the stigma. The fact that, in these cases bypassing stigma and introducing the pollen directly into the style can bring about normal fertilization clearly suggests that the barrier is restricted to the stigma.

In *Arabis* and *Brassica* (both members of the Brassicaceae) the stigma is covered with a cuticular layer. According to Kroh (1966), in compatible pollinations a pollen tube is able to penetrate the cuticular layer, whereas in incompatible pollinations it fails to achieve this. However, if the pollen grains are left on a compatible stigma for sometime before transferring them to an incompatible stigma the pollen tubes are able to penetrate the cuticular layer. On the basis of these observations it was suggested that cutinase, the enzyme necessary to erode the cuticle, is activated only by the compatible stigma, and once activated it is effective even on the incompatible stigma. However, electron microscopic observations do not support such a differential behaviour of pollen on compatible and incompatible stigmas (Kanno and Hinata, 1969; Dickinson and Lewis, 1973). According to Dickinson and Lewis erosion of the cuticle occurs following compatible as well as incompatible pollinations.

Most species, where the rejection reaction occurs on the surface of the stigma, are characterized by a dry stigma (lacking copious exudate). However, the so-called dry stigma does possess a hydrated layer, called pellicle, over the cuticle. The pellicle is a dynamic system. It consists of a

lipid film in which float a mosaic of proteins, many of them capable of rapid renewal under artificial conditions (*see* Dickinson and Roberts, 1981). Some of these proteins possess esterase activity (Mattsson *et al.*, 1974). Consequently, the pellicle layer can be localized histochemically by its non-specific esterase activity and digestion by pronase. The exact origin of this layer is not known but it has been suggested that the pellicle is extruded on to the surface of the papillae through discontinuities in the cuticle (Shivanna, 1982; Fig. 10.4). Soon after receiving the pollen the stigmatic surface becomes moist due to water exuded by the papillate cells of stigma. Stimulated by this, the exine-held proteins flow out within seconds and bind with the proteins of the pellicle. In the Brassicaceae this happens within 10-15 minutes of the pollen alighting on the stigma. The pellicle has been suggested as the receptor site for exine proteins on the stigma, and the interaction between the two protein fractions is responsible for the incompatibility reaction.

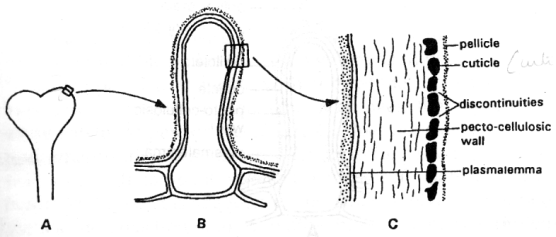

Fig. 10.4 A-C The structure of stigmatic papilla. Note the discontinuity of the cuticle in **C**. (*after* Shivanna, 1977)

In incompatible pollinations a range of abnormal behaviours of the male gametophyte has been recorded: (a) the pollen may fail to germinate, (b) if germination occurs, the tube is very short, (c) the inhibition of one tube leads to the emergence of another tube, (d) appressoria-like swellings develop at the tip of pollen tubes, etc. The most distinct response of the stigma to an incompatible pollen is the development of callosic plug between the plasma membrane and pectocellulosic layer of the stigmatic papillae, just below the point of contact with the pollen (Fig. 10.5); a callose plug also appears at the tip of the pollen tube. With the appearance of the callose-reaction-body the growth of the pollen tube ceases. The reaction between the pollen and the stigma is extremely localized, and does not interfere with the germination of other pollen lying on the same stigma. The deposition of callose in the stigmatic cells can be employed as a reliable bioassay to detect incompatible pollen particularly where the rejection occurs on the stigma. This bioassay has served as a useful aid in providing direct evidence for the involvement of exine-borne proteins in incompatibility reaction. Extracted exine-held proteins when applied to the stigma bring

interchange between the pollen grain and the substrate". Subsequent electron microscopic studies of pollen wall development have clearly demonstrated the incorporation of proteinaceous substances (Fig. 10.3) in the exine (Afzelius, 1955; Chambers and Godwin, 1961) and the intine (Heslop-Harrison, 1963; Larson and Lewis, 1961; Rowley *et al.*, 1959).

The idea that the wall-held proteins may be associated with inter- or intra-specific incompatibility emerged from the studies of D.Lewis and his co-workers. Makinen and Lewis (1962) demonstrated that in *Oenothera organensis*, which shows gametophytic type of self-incompatibility, and where the inhibition of male gametophyte occurs on the surface of the stigma, a substantial amount of the mobile proteins of the pollen grains was S-gene specific. The subsequent observations of Lewis *et al.* (1967) suggested that the recognition proteins of incompatibility are held extra-

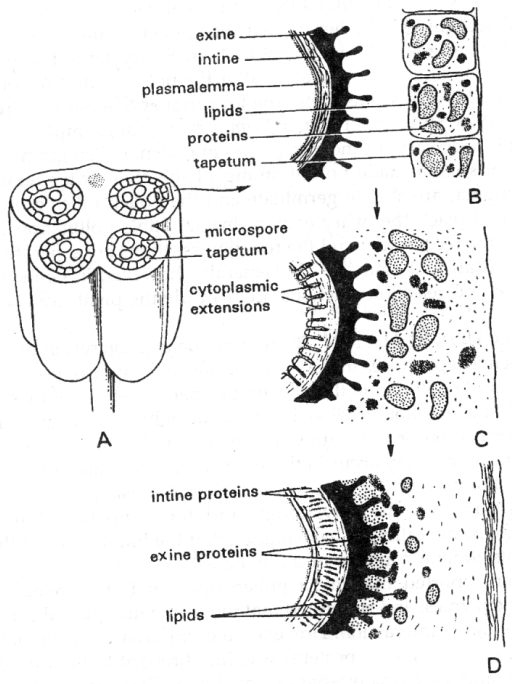

Fig. 10.3 Origin of the intine and exine proteins. **A.** Anther cut transversely to show young microspores surrounded by a layer of tapetum. **B-D.** Note the origin of intine proteins in the pollen cytoplasm and of exine proteins in the tapetum. (*after* Shivanna, 1977)

cellularly in the pollen wall. It has been suggested that in GSI-plants these proteins are present in the intine, and in the SSI-systems in exine (Heslop-Harrison, 1968, 1975).

Since the pollen wall and its proteins have gained so much importance with respect to incompatibility reactions it would be worthwhile to describe the mode of incorporation of these proteins in the two domains of pollen wall, viz., the intine and exine (Fig. 10.3), before discussing the pollen stigma interaction.

Intine. It is the inner layer of the pollen wall laid down only after the release of spores from the tetrad. In the polysaccharide matrix of the intine are embedded proteinaceous lamellae which are generally concentrated around the germpore (Knox and Heslop-Harrison, 1970; Larson and Lewis, 1961). The studies on intine development have clearly demonstrated that the proteins incorporated into the intine are contributed by the cytoplasm of the gametophyte (Heslop-Harrison, 1975).

Knox and Heslop-Harrison (1970) traced the insertion of proteinaceous material in the intine of *Cosmos bipinnatus* (Asteraceae) pollen. They have described a 4-phasic development of the intine in the following sequence: (a) deposition of an early intine layer, (b) a thin pectocellulosic layer without included proteins, (c) a layer in the mesocolpial region in which tangentially disposed protein-lamellae alternate with polysaccharide layers and, finally (d) the innermost polysaccharide layer without the protein lamellae. Thus, the protein-containing layer is neither in contact with the exine, nor with the pollen cytoplasm. Similar dispersion of proteinaceous lamellae in the intine has been described in the Malvaceae and several other plants. Whereas in polyporate pollen (Malvaceae) the proteins are generally included around the germ-pore (Heslop-Harrison *et al.*, 1973), in certain inaperturate monocots, such as *Canna*, the entire width of the intine is impregnated with proteinaceous material. The intine has been shown to exhibit enzymatic activities (Heslop-Harrison, 1970) associated with the proteins derived from the pollen cytoplasm (Knox and Heslop-Harrison, 1971).

Exine. In the large majority of flowering plants the pollen grains have a well-developed exine which, as described in Chapter 4, consists of a continuous inner layer called nexine and an outer sculptured layer called sexine. The latter comprises rod-like bacula showing terminal expansion to various degrees. In extreme cases these terminal expansions of the adjacent bacula fuse forming a roof-like tectum perforated by micropores. In the final stages of pollen development the tapetum releases into the thecal cavity a mixture of substances (sometimes referred to as tryphine), of which two important components are: (a) carotenoid-containing lipid droplets, and (b) fibro-granular proteins. The latter are enclosed in single membrane-bound cisternae. In the Malvaceae, having tectate pollen, following the breakdown of the tapetum its contents settle down on the surface of pollen grains. The cisternae membranes rupture, and the enclosed proteins enter the tectum through the micropores and get accumulated in the spaces between the bacula (Heslop-Harrison *et al.*, 1973). The pigmented lipid

about a similar reaction as do intact pollen (Dickinson and Lewis, 1973). Identical reaction can also be reproduced by placing isolated fragments of anther tapetum on the stigma confirming the sporophytic origin of the exine-held proteins already suggested by the studies on pollen wall development.

In GSI-systems, such as grasses, where the rejection reaction occurs on the stigma rather than in the style, there is no callose deposition in the stigmatic papillae following incompatible pollination. However, callose deposition is very conspicuous in the pollen tube. In the extreme cases the pollen germination itself is inhibited by the deposition of callose in the germpore. The recognition reaction in the Poaceae is completed within a few minutes after pollination, and subsequent acceptance or rejection becomes evident in less than 10 minutes.

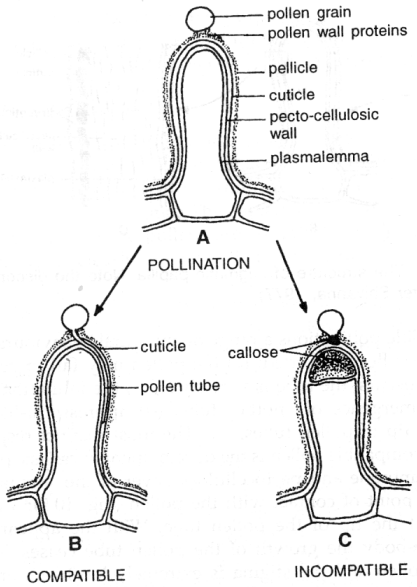

Fig. 10.5 Pollen-stigma interaction in Brassicaceae and Asteraceae. **A.** Pollen-wall proteins are released onto the pellicle where recognition reaction occurs. **B.** The compatible pollen tube penetrates the cuticle and grows down the papilla. **C.** The incompatible pollen tube, although penetrates the cuticle, is inhibited from further growth. Note the deposition of callose in the stigmatic papilla below the incompatible pollen. (*after* Shivanna, 1977)

Stylar Inhibition

In GSI-systems (excluding the Poaceae and *Oenothera*) the recognition and rejection reactions occur in the style after the pollen tube has grown to about two-thirds the length of the style. The genetic studies have revealed that the recognition factor on the male side is contributed by the male gametophyte itself, and not by the tapetum (Pandey, 1979). Possibly, the proteins incorporated in the intine during pollen wall development are involved. However, since the rejection reaction is considerably delayed in these cases, as compared to the SSI-systems, it is equally possible that the synthesis or activation, and release of the recognition substances are delayed in GSI-systems and they occur in the pollen tube (Heslop-Harrison, 1975; Pandey, 1979).

In GSI-systems the incompatibility reactions occur due to the production of S-allele-specific proteins secreted by the stylar cells. The polypeptide, apparently most closely linked with recognition of pollen incompatibility, has been identified as a highly polarized glycoprotein (Dickinson *et al.*, 1982).

Bredemeijer and Blass (1975) reported that in *Nicotiana alata* the style with incompatible pollen tubes shows a much higher activity of peroxidase-10 as compared to the style with compatible tubes which exhibits only a very low activity of this enzyme. Moreover, the capacity of the style to show enhanced peroxidase-10 activity does not exist at the young stages of bud when selfing is successful. Its appearance coincides with the sudden shift from self-compatible to self-incompatible nature of the pistil shortly before anthesis. This suggests the possible involvement of peroxidase-10 in the rejection reaction of incompatible tubes. Changes in protein metabolism in the style and ovary following self and cross-pollinations have also been reported by Linskens (1975) and Deurenberg (1976). According to Deurenberg the differences in the protein metabolism in the ovary appear much before the pollen tube reaches the site, indicating the transmission of some signal from the stigma or style to the ovary to induce this change. Similarly, Linskens pointed out that the recognition reaction in GSI-systems seems to occur very early during the contact between pollen tube and stylar tissue or even on the stigma.

Van der Donk (1975) and Herrero and Dickinson (1979) had also reported that in *Petunia hybrida* the polypeptides specific to the incompatibility (S) alleles are synthesized only after pollination. However, the studies of Sharma and Shivanna (1982), employing an *in vitro* bioassay, have shown that in this taxon the self-incompatibility factor/s is/are present in the style even before pollination. This is also true of *Nicotiana*. Even in *Lilium longiflorum*, another GSI-system, no new proteins involved in self-incompatibility reaction are secreted into the stylar canal on pollination (Dickinson *et al.*, 1982). Thus, the synthesis of *S*-allele-specific molecules is not a pollination induced response.

Following recognition, the pollen tube growth in the incompatible styles may be arrested in different ways.

In *Petunia hybrida* the incompatibility reaction is accompanied by a thickening of the pollen tube walls, and degeneration of the tube cytoplasm. These observations and a number of other genetical and biochemical considerations led van der Pluijm and Linskens (1966) to suggest that the incompatibility proteins have their site of action on the surface of the pollen tube, and not in the style. This hypothesis, initially put forth by Makinen and Lewis (1962), has been further substantiated by fine structural studies of pollen tube following self-compatible and self-incompatible pollinations in *Lycopersicon peruvianum* (de Nettancourt *et al.*, 1973). A pollen tube growing through a compatible style possesses a cylindrical apical region, and its wall is distinctly 2-layered; an outer layer consisting of microfibrils and an inner layer which is homogeneous and less electron-dense. In an incompatible style the tubes initially show normal structure but, after they have grown about two-thirds the length of the style, their inner wall gradually thins down and eventually disappears. Disappearance of the inner wall is associated with the appearance of small particles (0.2 μm in diameter) in the tube cytoplasm. As the inner wall disappears and the tube gets filled with these particles the outer wall becomes thickened and gives a swollen appearance at the tip. At this stage the pollen tube bursts liberating its contents. These observations clearly indicate that the incompatibility reaction is an active process; bursting of the incompatible pollen tubes in the style is similar to that of compatible tubes in the vicinity of egg. The small particles appearing only in incompatible tubes are presumed to be consisting of a mixture of incompatibility proteins and constituents of the tube wall (de Nettancourt *et al.*, 1973).

In *Lilium longiflorum*, Rosen and Gawlik (1966) noted striking differences in the tips of pollen tubes growing through compatible and incompatible styles. In compatible styles the tip of the pollen tube shows a morphology characteristic of a transfer cell which is missing in the tube growing through an incompatible style. On the basis of these observations, the authors suggested that compatible tubes grow uninhibited because they are able to absorb the pistil secretions through their tips. On the other hand, the inability of the incompatible tubes to do so is responsible for their retarded growth.

OTHER ABNORMAL BEHAVIOURS OF THE POLLEN TUBES IN AN INCOMPATIBLE STYLE ARE: (a) retardation of pollen tube growth, (b) abnormal behaviour of nuclei in the pollen tube; generative cell does not divide or the vegetative nucleus disappears within the first few hours, and (c) the pollen tube shows a branched tip, increased number of callose plugs, or a high density of cellulose microfibrils in the thickened walls (Linskens and Esser, 1957; Muhlethaler and Linskens, 1956).

As in stigma-surface-inhibition, here also the rejection reaction appears to be highly localized between a pollen tube and the closely contiguous cells of the style. There is no interaction between the tubes of different genotypes growing through a common style.

McClure *et al.* (1990) have suggested that in *Nicotiana alata* the gametophytic self-incompatibility is mediated through the *S*-gene-encoded glycoproteins in the style which show ribonuclease (RNase) activity (*see* also Huang *et al.*, 1994). They observed high RNase activity in the styles of self-incompatible species, such as *N. alata* and *Lycopersicon peruvianum*. In comparison, their pollen grains and pollen tubes contained only traces of this enzyme. According to the proposed hypothesis, in incompatible pollinations RNase present in the style penetrates the pollen tubes and degrades RNA in the cytoplasm, thus inhibiting the pollen tube growth. In compatible pollinations in these species the pollen tubes carry the *S*-gene product which either rejects or inactivates the stylar RNase and are, therefore, able to effect fertilization. The styles of self-compatible species, such as *N. tabacum*, lack RNase activity.

We have so far considered only homomorphic systems. The heteromorphic systems (with SSI) show certain distinguishing features from the homomorphic SSI-systems (Stevens and Murray, 1982). In the former, pollen wall proteins, derived from the tapetum, have no role to play in the incompatibility reaction. Moreover, in heteromorphic systems there is no callose formation in the stigmatic papillae following self-pollination.

In *Primula obconica* no difference was observed in the rate of pollen germination between pin and thrum plants or between compatible and incompatible matings. The self-incompatibility reaction in this taxon occurs only after pollen germination, and the sites of rejection reaction in the two morphs are different. In thrum-flowers the recognition as well as rejection reactions take place on the stigma so that pollen tubes grow only over the stigma surface and very few tubes penetrate the stigma. On the other hand, in pin-flowers the pollen tubes penetrate the stigma equally rapidly following self and cross-pollinations, but the number of tubes reaching the tip of the style in self-pollination is fairly low as compared to that in intermorph pollinations. According to Stevens and Murray (1982) in thrum-flowers also recognition reaction occurs on the stigma surface but the major rejection reaction occurs within the pistil tissue.

The physiological basis of self-incompatibility and the promotion of outbreeding in the heteromorphic system - *Linum grandiflorum* - have been explained on the basis of variations in the osmotic pressures of stylar cells and the pollen grains in the two types of flowers (Lewis, 1943). In thrum-eyed flowers the osmotic pressure of the stylar cells is equivalent of 11 per cent sucrose, and that of its pollen 80 per cent sucrose. On the other hand, in pin-eyed flowers the osmotic pressure of stylar cells is equivalent of 20 per cent sucrose, and that of its pollen 50 per cent sucrose. According to Lewis, in compatible crosses (thrum x pin, or pin x thrum) the ratio of osmotic pressures of pollen and style is about 4:1 and a significant deviation from this ratio leads to incompatibility. For example, if the pistil of a thrum-eyed flower is pollinated by its own pollen the osmotic pressure of the pollen would be seven-times higher than that of stylar cells. Consequently, the pollen tubes will absorb excess water and burst in the style. In self-pollination of pin-eyed flowers, on the other hand, the pollen grains completely fail to germinate because their osmotic pressure is so low as compared to the stigma that they are unable to absorb water.

BIOLOGICAL SIGNIFICANCE OF INCOMPATIBILITY

In nature a balanced inbreeding and outbreeding of the plants is regulated by intraspecific and interspecific incompatibility. Extensive selfing of the plants leads to highly homozygous individuals which have a very low survival value. To circumvent this and ensure outbreeding and the maintenance of heterozygosity within a species, nature has imposed self-incompatibility. On the other hand, interspecific incompatibility brings about reproductive isolation and is, thus, responsible for the emergence of new races and species through the process of mutation and open hybridization. If free flow of genes between two populations of a species stops they are regarded as two variants, and if the differences in the populations are fairly large they are considered to be two distinct races or species.

Despite its natural value sexual incompatibility may turn out to be a serious hurdle in a plant improvement programme. The importance of homozygous plants in plant breeding and basic genetic studies has been highlighted in Chapter 17. Before the introduction of anther culture technique selfing was one of the chief methods to obtain homozygous individuals. For several plants self-pollination continues to be an important approach to achieve this. In this context self-incompatibility is a serious problem. Equally frustrating may be interspecific incompatibility because it prevents distant hybridization and, thus, the synthesis of better types by combining good characters from different plants. The knowledge of the mechanisms controlling incompatibility should be helpful in evolving effective methods to overcome it. Some of the methods that have been tested to overcome intra- and interspecific incompatibility are described here.

METHODS TO OVERCOME INCOMPATIBILITY

1. MIXED POLLINATION. In this method the stigma is disguised from the incompatible pollen by pollinating it with a mixture of incompatible and compatible pollen (termed Mentor pollen or Recognition pollen). To prevent fertilization by the mentor pollen they are either inactivated by irradiation or killed by treating them with chemicals (*e.g.*, methanol) or subjecting them to repeated freezing and thawing. These treatments do not disturb the wall-held proteins.

Stettler and Ager (1984) have listed several examples where the use of mentor pollen had facilitated overcoming interspecific (*Cucumis, Nicotiana, Passiflora, Populus*), sporophytic self- (*Cosmos bipinnata, Theobroma cacao*) and gametophytic self- (*Malus* sp., *Nicotiana alata, Petunia hybrida*) incompatibility.

The mechanisms implicated in overcoming incompatibility by the mentor pollen methods are summarized in Table 10.2. Mechanisms A and B have been proposed to explain events at the stigmatic surface. In mechanism A, exine or intine-held recognition proteins from compatible pollen mask the inhibition reaction and permit incompatible pollen to germinate and

penetrate the stigma (Knox *et al.*, 1972). Mechanism B underrates the role of protein recognition and, instead, considers n-hexane soluble compounds (presumably mainly lipoidal) from the exine to be more important (Willing and Pryor, 1976). Both these hypotheses are based on experiments demonstrating the effectiveness of mixing incompatible pollen with extracts of respective substances derived from compatible pollen. Mechanism C was formulated by Pandey (1977, 1978) to account for mentor effects manifested in the style, notably in gametophytic self-incompatibility. He postulated that non-specific pollen growth substances (PGS) or regulatory substances are released from the intine or exine of the mentor pollen and these substances provide extra growth potential for incompatible pollen tubes. In mechanism D the mentor pollen, presumably, acts indirectly in facilitating both fertilization event and successful seed maturation. The proposed mechanism involves contribution of stimulatory signals by developing mentor pollen tubes for fruit retention and ovule enlargement, and induce parthenocarpy (Stettler and Ager, 1984).

TABLE 10.2 Proposed mechanisms to explain mentor pollen effects. (*after* Stettler and Ager, 1984)

Site of mentor effect		*Proposed mechanism*
At stigmatic surface	A.	Mentor pollen provide recognition proteins which permit incompatibile pollen to germinate
	B.	Mentor pollen provide P-factor which interacts with S-factor from stigma to render it accessible to incompatible pollen.
In style	C.	Mentor pollen provide a pollen growth promoting (PGS) or regulating substance which permits incompatible pollen to sustain tube growth.
In distal tissues	D.	Mentor pollen, after tube penetration of pistil, provide substances critical for sustained growth of ovules, ovary, and other fruit tissues.

2. BUD-POLLINATION. In some plants pollination at an early bud stage has proved very effective in overcoming intraspecific incompatibility. In *Petunia axillaris* delayed pollination or pollination with stored pollen could not overcome self-incompatibility, but self-pollination of buds, 2 days before anthesis (corolla length 2.5-3.0 cm), gave seed-set (Shivanna and Rangaswamy, 1969). At the bud stage the stigma lacks the exudate which appears only at anthesis. However, this difference cannot explain why buds allow normal fertilization after selfing because in *Petunia* the inhibition occurs in the style and not in stigma. Moreover, the application of the stigmatic exudate collected from an open flower promotes seed-set after self- as well as cross-pollination, irrespective of the fact that the exudate was collected from the same plant or a different plant. Evidently, at least

in this system, the incompatibility factor does not reside in the stigmatic exudate (Shivanna and Rangaswamy, 1969).

It is likely that in the style the factor complementary to the pollen factor for incompatibility reaction appears only at or just before anthesis. Therefore, if the stigma is self-pollinated at bud stage when this factor has not appeared the pollen tubes will grow normally and effect fertilization.

3. STUB-POLLINATION. Where the incompatibility reaction is restricted to the stigma (Hecht, 1960), or the length of the style of the female parent is more than the maximum length attained by the pollen tubes of the male parent (Swaminathan and Murty, 1957), removing the stigma and a part of the style has often proved helpful in overcoming incompatibility.

In *Ipomoea trichocarpa* the primary site of self-incompatibility is the stigmatic surface which inhibits pollen germination. If the stigmatic lobes are removed or the terminal half of the style is trimmed and the cut surface of the style pollinated, fairly good pollen germination occurs and the pollen tubes grow uninhibited into the ovary (Charles *et al.*, 1974).

Swaminathan and Murty (1957) had observed that the crosses *Nicotiana tabacum* x *N. rustica* and *N. tabacum* x *N. debney* fail whereas their reciprocal crosses are successful. This is because the style in *N. tabacum* being longer than that in the other two species, the pollen tubes fail to reach the ovary. To overcome this barrier, Swaminathan and Murty removed a large part of the style of *N. tabacum* and smeared its cut surface with an agar-sucrose medium to provide a substrate for the pollen to germinate on. When this artificial stigma was pollinated with the pollen of *N. rustica* or *N. debney* fertilization occurred normally.

4. INTRA-OVARIAN POLLINATION. Where the zone of incompatibility lies on the stigma or in the style, direct introduction of pollen suspension into the ovary would be helpful. In this technique (Fig. 10.6), known as intra-ovarian pollination, the ovary is surface sterilized with ethanol and two punctures are made in the wall, one for introducing the pollen suspension and the other to permit the escape of air present in the ovarian cavity (Kanta *et al.*, 1962). A pollen suspension is prepared in distilled water and injected into the ovary with the help of a hypodermic syringe. Subsequently, both the holes are sealed with petroleum-jelly. Pollen grains germinate inside the ovary and bring about fertilization. If the pollen grains require some special substance/s for germination, this/these can be added to the pollen suspension before injection.

Viable seeds following intra-ovarian pollination have been obtained in several members of the Papaveraceae, such as *Argemone mexicana, A. ochroleuca, Papaver somniferum* and *P. rhoeas*. Using this technique, Kanta and Maheshwari (1963) developed an interspecific hybrid between *Argemone mexicana* and *A. ochroleuca*.

A limitation of this technique is that it is applicable to only such plants which have large ovarian cavities.

5. *IN VITRO* POLLINATION AND FERTILIZATION. *In vitro* pollination is yet another method to overcome prezygotic barriers to fertility. This technique was developed by Kanta *et al.* (1962) using *Papaver somniferum* as

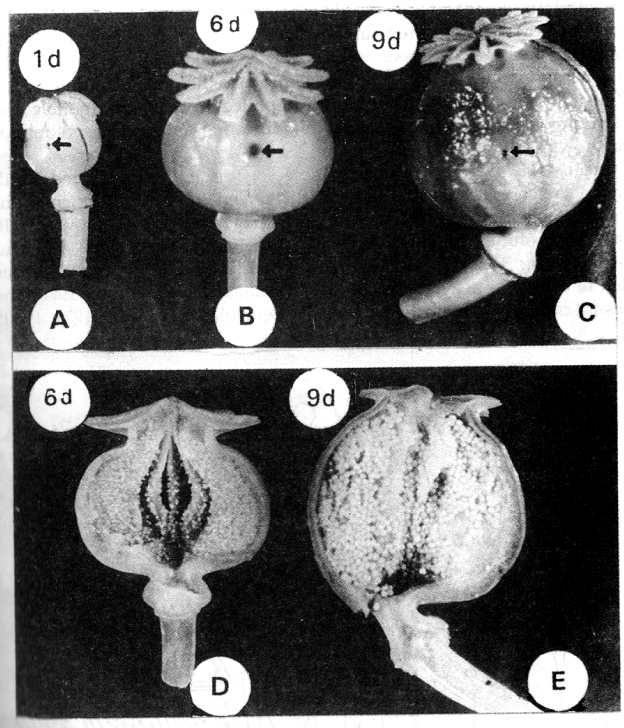

Fig. 10.6 Intra-ovarian pollination in poppy. Arrow shows the prick. **A-C**. Ovaries 1,6 and 9 days after injection of pollen suspension. **D, E**. Longisection of the fruits shown in **B** and **C**, respectively. Note the large number of seeds formed. (*from* Maheshwari and Kanta, 1962)

the experimental system. The stigmatic, stylar and ovary wall tissues were completely removed from the path of pollen tube, and the exposed ovules directly dusted with pollen grains. The pollinated ovules were cultured on a suitable nutrient medium which favoured pollen germination as well as the development of fertilized ovules. The pollen grains lodged on the ovules germinated within 15 min, and fertilization was effected within 1-2 days after pollination. Within 5 days the fertilized ovules enlarged, became turgid and opaque, and contained a 4-celled proembryo and free nuclear endosperm. A fully differentiated dicotyledonous embryo developed after

22 days. The technique is more effective if the ovules are cultured with the placental tissue intact (also called placental pollination). Kanta and Maheshwari (1963) reported similar success with other taxa belonging to the Papaveraceae (*Argemone mexicana, Eschscholtzia californica*) and the Solanaceae (*Nicotiana rustica, N. tabacum*). Since then the technique of test tube pollination has been successfully applied to many compatible and incompatible combinations. Various modifications of *in vitro* pollination are shown in Figure 10.7.

Petunia axillaris is a self-incompatible species. In self-pollinated pistils the pollen germination is good but the pollen tube does not enter the ovary. Rangaswamy and Shivanna (1967, 1971) have shown that when self-placental pollinations are performed, fertilization and seed-set occur normally (Fig. 10.8). The technique of placental pollination has also been used to overcome self-incompatibility in *Petunia hybrida* (Niimi, 1970) and *Brassica campestris* (Zenkteler *et al.*, 1987). Zenkteler (1967, 1969, 1971, 1980),

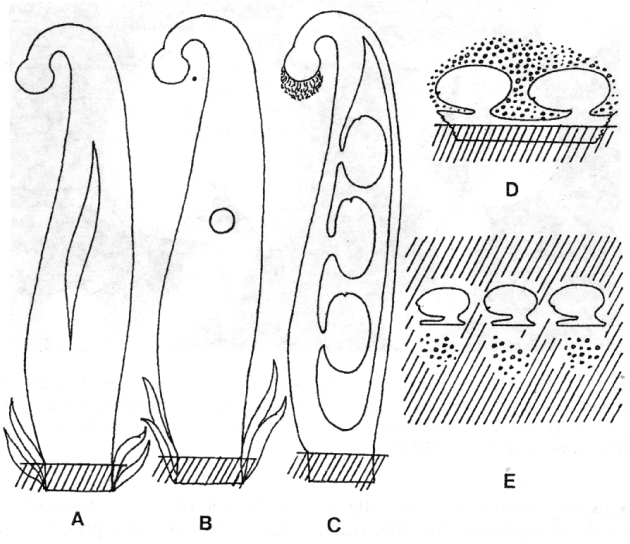

Fig. 10.7 Diagrams depicting various types of *in vitro* pollination. **A, B**. Intra-ovarian pollination through a slit or a pore, respectively. **C**. Stigmatic pollination; pollen are applied to the stigma of a cultured pistil. **D**. Placental pollination; pollen are dusted on the ovules cultured along with a piece of placental tissue. **E**. Ovular pollination; individual ovules are cultured and pollinated. (*after* Bhojwani and Razdan, 1996)

Zenkteler *et al.* (1975) and Zenkteler and Melchers (1978) attempted several interspecific, intergeneric and interfamily crosses through placental pollination (*see* Zenkteler, 1981). In the crosses *Melandrium album* x *M. rubrum*, *M. album* x *Viscaria vulgaris*, *M. album* x *Silene schafta* and *Nicotiana alata* x

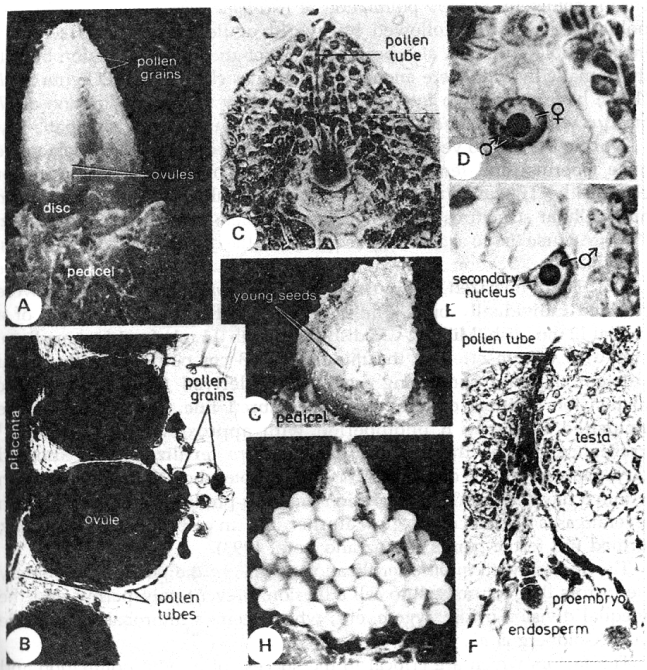

Fig. 10.8 *In vitro* placental pollination to overcome self-incompatibility in *Petunia axillaris*. **A**. Both the placentae of an ovary, with their entire mass of ovules covered with pollen, ready for culture. **B**. Free-hand section of placentae 24 hours after self-pollination; the picture shows a portion of a placenta bearing 3-4 ovules. Pollen grains have germinated and the pollen tubes are seen all over and in between the ovules. **C**. Longisection through micropylar region of ovule 24 hours after self-pollination; note the entry of pollen tube into the embryo sac. **D, E**. Sections of ovules 2 days after selfing, showing syngamy and triple fusion, respectively. **F**. Micropylar end of a 7-day-old seed in longisection; note extra-micropylar part of pollen tube, remnants of pollen tube in micropyle, testa, filamentous proembryo and cellular endosperm. **G, H**. Five and 24-day-old cultures, respectively; numerous mature seeds are formed in **H**. (**A, B, F-H**, *after* Rangaswamy and Shivanna, 1967; **C-E.** *after* Rangaswamy and Shivanna, 1971)

N. debney seeds containing viable embryos developed. When ovules of *Nicotiana tabacum* were pollinated with the pollen of *Hyoscyamus niger* the maximum stage attained by the hybrid embryos was pre-heart shape. Dhaliwal and King (1978) raised interspecific hybrids by pollinating the exposed ovules of *Zea mays* with the pollen grains of *Z. mexicana*.

Marubashi and Nakajima (1985) produced fertile hybrids with 96 chromosomes by *in vitro* pollination of *Nicotiana tabacum* pistils with the pollen of *N. rustica*, followed by hybrid ovule culture. This sexually incompatible cross has also been made through protoplast fusion but all the somatic hybrids were aneuploids (Douglas *el al.*, 1981). D'Verna *et al.* (1987) have raised two interspecific hybrids by *in vitro* pollination of *N. tabacum* with *N. amplexicaulis*. This cross exhibits unilateral incompatibility.

During the last 10 years it has become technically feasible to isolate live male (sperms) and female (egg) gametes and fuse them chemically or electrically. The *in vitro* formed zygotes are able to divide and form multicellular structures or, as in maize, complete plants (Kranz and Lorz, 1993). A nurse tissue is generally required for the division of naked zygotes.

In vitro fertilization in maize, as described by Kranz and Dresselhaus (1996), is summarized in Figure 10.9. The *in vitro* fertilized eggs were cultured individually on a semi-permeable, transparent membrane of a special 12 mm dish (Millicell-CM dish) filled with 0.1 ml of nutrient medium. This dish was inserted in the middle of a 3 cm petridish with 1.5 ml nutrient medium containing feeder cells derived from embryogenic suspension cultures of another maize inbred line. The cultures were incubated under 16 h photoperiod with approximately 50 $\mu Em^{-2}s^{-1}$ irradiance. Under these conditions, *in vitro* fertilized eggs showed karyogamy within 1 h of fusion, 92% of the zygotes showed unequal division within 3 days, and 90% of the fusion products produced mini-colonies. In several cases a mini-colony grew further into an embryo and, eventually, formed full fertile plants (Kranz and Lorz, 1993).

The success with *in vitro* fertilization opens out the possibility to study the factors that control cell to cell adhesion, prevent polyspermy, and the unequal division of the zygote, etc., which seemed improbable a decade back (Goldberg *et al.*, 1989).

The factors which are critical to the success of *in vitro* pollination and fertilization and their practical applications are discussed by Bhojwani and Raste (1996).

6. MODIFICATION OF STIGMATIC SURFACE. As mentioned earlier, in plants with dry stigma and sporophytic self-incompatibility the integrity of pollen surface proteins and of the stigma surface pellicle appears to be essential for determination of compatible and incompatible pollen. In such cases the application of the lectin Concanavalin A to the stigma, which binds and apparently masks the pellicle, blocks the self-incompatibility reaction in *Brassica* (Kerhoas *et al.*, 1983). Similar pre-treatment of stigma with detergents, such as Triton X-100, and organic solvents, such as hexane (Willing and Pryor, 1976), modifies or partially solubilizes the pellicle and blocks the incompatibility reaction. Extracting materials from exine of

Populus alba with n-hexane also inhibited the incompatibility reaction with *P. deltoides* (Willing and Pryor, 1976).

This approach to overcome incompatibility is not practical. While most

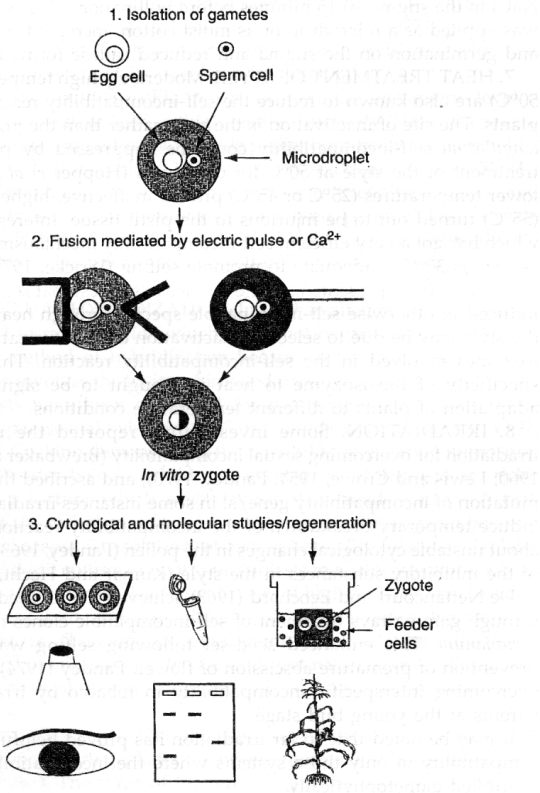

Fig. 10.9 Diagrammatized summary of the technique of *in vitro* fertilization in maize. **1.** Freshly isolated egg and sperm cells are placed in a microdroplet and covered with a thin layer of mineral oil. **2.** The gametes, which are naked cells or protoplasts, are fused electrically (left) or chemically (right). **3.** The fusion product (zygote) is characterized cytologically (left) or biochemically (centre) or co-cultured with feeder cells to induce division and plant regeneration (right). (*after* Kranz and Dresselhaus, 1996)

N. debney seeds containing viable embryos developed. When ovules of *Nicotiana tabacum* were pollinated with the pollen of *Hyoscyamus niger* the maximum stage attained by the hybrid embryos was pre-heart shape. Dhaliwal and King (1978) raised interspecific hybrids by pollinating the exposed ovules of *Zea mays* with the pollen grains of *Z. mexicana*.

Marubashi and Nakajima (1985) produced fertile hybrids with 96 chromosomes by *in vitro* pollination of *Nicotiana tabacum* pistils with the pollen of *N. rustica*, followed by hybrid ovule culture. This sexually incompatible cross has also been made through protoplast fusion but all the somatic hybrids were aneuploids (Douglas *el al.*, 1981). D'Verna *et al.* (1987) have raised two interspecific hybrids by *in vitro* pollination of *N. tabacum* with *N. amplexicaulis*. This cross exhibits unilateral incompatibility.

During the last 10 years it has become technically feasible to isolate live male (sperms) and female (egg) gametes and fuse them chemically or electrically. The *in vitro* formed zygotes are able to divide and form multicellular structures or, as in maize, complete plants (Kranz and Lorz, 1993). A nurse tissue is generally required for the division of naked zygotes.

In vitro fertilization in maize, as described by Kranz and Dresselhaus (1996), is summarized in Figure 10.9. The *in vitro* fertilized eggs were cultured individually on a semi-permeable, transparent membrane of a special 12 mm dish (Millicell-CM dish) filled with 0.1 ml of nutrient medium. This dish was inserted in the middle of a 3 cm petridish with 1.5 ml nutrient medium containing feeder cells derived from embryogenic suspension cultures of another maize inbred line. The cultures were incubated under 16 h photoperiod with approximately 50 $\mu Em^{-2}s^{-1}$ irradiance. Under these conditions, *in vitro* fertilized eggs showed karyogamy within 1 h of fusion, 92% of the zygotes showed unequal division within 3 days, and 90% of the fusion products produced mini-colonies. In several cases a mini-colony grew further into an embryo and, eventually, formed full fertile plants (Kranz and Lorz, 1993).

The success with *in vitro* fertilization opens out the possibility to study the factors that control cell to cell adhesion, prevent polyspermy, and the unequal division of the zygote, etc., which seemed improbable a decade back (Goldberg *et al.*, 1989).

The factors which are critical to the success of *in vitro* pollination and fertilization and their practical applications are discussed by Bhojwani and Raste (1996).

6. MODIFICATION OF STIGMATIC SURFACE. As mentioned earlier, in plants with dry stigma and sporophytic self-incompatibility the integrity of pollen surface proteins and of the stigma surface pellicle appears to be essential for determination of compatible and incompatible pollen. In such cases the application of the lectin Concanavalin A to the stigma, which binds and apparently masks the pellicle, blocks the self-incompatibility reaction in *Brassica* (Kerhoas *et al.*, 1983). Similar pre-treatment of stigma with detergents, such as Triton X-100, and organic solvents, such as hexane (Willing and Pryor, 1976), modifies or partially solubilizes the pellicle and blocks the incompatibility reaction. Extracting materials from exine of

Populus alba with n-hexane also inhibited the incompatibility reaction with *P. deltoides* (Willing and Pryor, 1976).

This approach to overcome incompatibility is not practical. While most

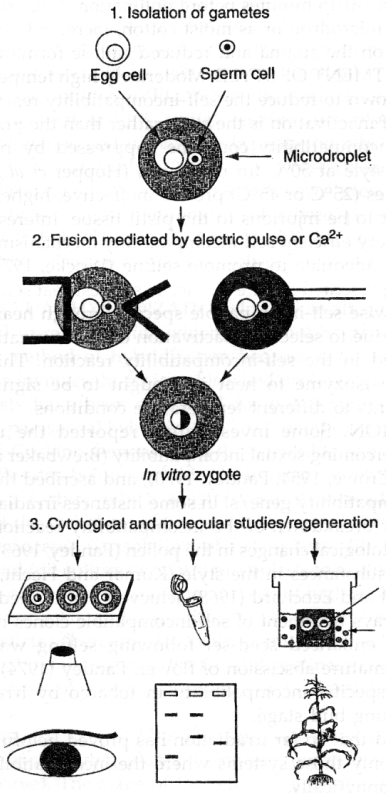

Fig. 10.9 Diagrammatized summary of the technique of *in vitro* fertilization in maize. **1.** Freshly isolated egg and sperm cells are placed in a microdroplet and covered with a thin layer of mineral oil. **2.** The gametes, which are naked cells or protoplasts, are fused electrically (left) or chemically (right). **3.** The fusion product (zygote) is characterized cytologically (left) or biochemically (centre) or co-cultured with feeder cells to induce division and plant regeneration (right). (*after* Kranz and Dresselhaus, 1996)

of these treatments applied to the stigma prevent incompatibility reaction as indicated by lack of callose formation they adversely affect pollen adhesion and pollen germination.

Monteiro *et al.* (1988) have reported that self-incompatibility in *Brassica campestris* could also be overcome by the application of 1.5% solution of NaCl to the stigma 10-15 minutes before pollination. NaCl solution, which was applied as a microdrop or as moist cotton, increased pollen adhesion and germination on the stigma and reduced callose formation.

7. HEAT TREATMENT OF STYLE. Moderately high temperatures (up to 50°C) are also known to reduce the self-incompatibility reaction in certain plants. The site of inactivation is the style rather than the pollen. In *Lilium longiflorum* self-incompatibility could be suppressed by pre-pollination treatment of the style at 50°C for 6 minutes (Hopper *et al.*, 1967). While lower temperatures (25°C or 45°C) proved ineffective, higher temperature (55°C) turned out to be injurious to the pistil tissue. Interestingly, in rye, which has got a very efficient self-incompatibility mechanism, temperature as low as 30°C is adequate to promote selfing (Wricke, 1974).

Pandey (1973) presented some evidence to suggest that self-compatibility induced in otherwise self-incompatible species through heat treatment of the style may be due to selective inactivation or denaturization of specific isozymes involved in the self-incompatibility reaction. This remarkable specificity of the isozyme to heat is thought to be significant in the adaptation of plants to different temperature conditions.

8. IRRADIATION. Some investigators reported the usefulness of irradiation for overcoming sexual incompatibility (Brewbaker and Natarajan 1960; Lewis and Crowe, 1954; Pandey, 1970), and ascribed the effect to the mutation of incompatibility gene/s. In some instances irradiation is said to induce temporary breakdown of the incompatibility reaction by bringing about unstable cytological changes in the pollen (Pandey, 1963), or alteration of the inhibitory substances in the style (Kumar and Hecht, 1965).

De Nettancourt and Ecochard (1968) achieved higher seed-set per plant through gamma rays treatment of self-incompatible clones of *Lycopersicon peruvianum*. This enhanced seed-set following selfing was due to the prevention of premature abscission of flower. Pandey (1974) succeeded in overcoming interspecific incompatibility in tobacco by irradiating male parents at the young bud stage.

It may be noted that so far irradiation has proved helpful in achieving compatibility in only those systems where the incompatibility process is controlled gametophytically.

9. CHEMICAL TREATMENT. If the failure to set seeds following self- or cross-pollination is due to premature abscission of flowers, the application of certain chemicals which suppress or delay abscission may be helpful. Chaudhury (1965) could prolong the life of arrowroot flowers by applying 100 mg/l of p-chlorophenoxyacetic acid to the pedicel and, thus, succeeded in obtaining seeds in crosses. Similarly, in *Ipomoea batatas* there is no inhibition of pollen germination or pollen tube growth in the style but abscission of the ovary 1-3 days after anthesis does not allow seed setting after selfing. Charles *et al.* (1974) extended the flower life by treating them with 100 mg/l of 2,4-D. This facilitated the occurrence of fertilization and early embryo development. However, embryo culture was essential to obtain full plants.

A significant promotion of pollen tube growth in the incompatible style of *Oenothera organensis* by the application of p-chloromercuribenzoate ($5x10^{-4}$-$2x10^{-3}$M) has been reported by Hodgdon and Hecht (1975). Henry and Ascher (1975) reported that self-incompatibility reaction in detached styles of *Lilium longiflorum* could be overcome by kinetin.

10. INCREASED CO_2 LEVEL. When the normal atmospheric level of CO_2 is raised 100-fold, to 4-6%, at high relative humidity (100%) for several hours after pollination, self-incompatibile pollinations behave as compatible pollinations in *Brassica* species (O'Neill *et al.*, 1984; Palloix *et al.*, 1985). This method has become an important tool in *Brassica* breeding since it saves considerable time in the maintenance of inbred parental lines for the production of F_1 hybrid seeds (Knox *et al.*, 1986). Formerly, inbreeding of self-incompatible plants required bud self-pollination by insects; normally flies are used as honey bees are adversely affected by elevated CO_2 levels.

11. PARASEXUAL HYBRIDIZATION. In 1969 an altogether new approach was proposed to raise hybrids which could not be produced through the conventional method of hybridization because of sexual incompatibility. This technique involves the fusion of isolated protoplasts. Since only somatic cell protoplasts have been employed for this purpose, the technique is described as "parasexual hybridization" or "somatic hybridization". In 1972 Bhojwani and Cocking demonstrated the possibility of isolating microspore protoplasts. Besides being haploid, the microspore protoplasts are richly cytoplasmic, a feature which must favour their ready fusion. A brief description of the technique of parasexual hybridization is given below. It involves three steps: (i) isolation of protoplasts, (ii) fusion of isolated protoplasts, and (iii) culture of hybrid protoplasts to regenerate whole plants.

(i) *Isolation of protoplasts.* The somatic cells of higher plants are bound by a rigid cellulosic wall, and the adjacent cells are cemented together by a pectin-rich matrix. Therefore, to obtain plant protoplasts the cells/tissues are treated with a mixture of cellulase and pectinase enzymes (Fig. 10.10). The concentrations of the two enzymes, and the duration of treatment vary with the tissue.

Bhojwani and Cocking (1972) isolated protoplasts from microspore tetrads of a number of flowering plants, using one per cent solution of helicase (snailgut enzyme). The stage of the tetrad is crucial. Protoplasts could be isolated only from very young tetrads, when the microspores were either naked or vested with a thin cellulosic wall. Once the deposition of sporopollenin started the enzyme could not release the protoplasts. In 1973 he described the technique for isolating meiotic protoplasts from microspore mother cells of some liliaceous plants, using cellulase and macerozyme. Under optimal conditions it takes only 30 minutes to convert all the tetrads or meiocytes into protoplasts. With somatic cells it may generally take anything from 4 to 12 hours.

Zimmermann and his co-workers, in West Germany, developed an electrically stimulated protoplast fusion method which is claimed to be 10,000 times more efficient than the chemical method (Zimmermann and Vieken, 1982; Zimmermann and Greyson, 1983). It is a two-step process. In the first stage the membranes of the neighbouring protoplasts are brought into contact by exposing them to AC field (10-200 V/cm of 500 KHz) for about 10 minutes. This is followed by a 10-20 µsec DC pulse of 600-700 V/cm, which leads to fusion at the point of contact of membranes.

Microspore protoplasts and meiocyte protoplasts can fuse in the absence of a fusogen. Ito (1973) used cavity slides for fusing meiocyte protoplasts of some liliaceous members. By gentle tapping of the slide the isolated protoplasts were aggregated at the base of the cavity, in the enzyme solution, where they readily fused. In Fig. 10.11 fusion bodies can be easily distinguished from individual protoplasts by their larger size. Fusion occurred between two or more protoplasts.

(iii) *Culture of protoplasts*. For a full appreciation of the consequences of protoplast fusion, it is essential to grow the fused protoplasts into whole plants. However, before attempting this with hybrid protoplasts, it is desirable to test the potentiality of parental protoplasts to form whole plants in cultures. Following the pioneering work of Takebe *et al.* (1971) in raising whole plants from isolated protoplasts of *Nicotiana tabacum* the list of plant species in which this phenomenon has been reported has steadily increased. It includes 288 dicotyledons and 32 monocotyledons (Roest and Gilissen, 1993).

In cultures a protoplast first synthesizes a new wall around it and reconstitutes a cell which undergoes sustained division forming a callus. Regeneration of a proper wall, which is essential for regular divisions, usually occurs readily. In general, the composition of nutrient media used for protoplast culture is not much different from those used for cell and tissue culture. The composition of the nutrient medium used for the successful culture of tobacco mesophyll protoplasts by Nagata and Takebe (1971) is given in Table 17.1(D).

The fusion of plasma membranes to form a heterokaryon is only the beginning in the formation of a stable proliferating hybrid cell line. Several factors may operate between heterokaryon formation and the formation of a hybrid cell proper, leading to the loss of many of the heterokaryons. Consequently, the final frequency of stable hybrid cells capable of continued development is very low. Moreover, when protoplasts are cultured after PEG-treatment the heterokaryons are accompanied by large numbers of parental protoplasts which may overgrow the hybrid protoplasts causing further complications. It is, therefore, of key importance in somatic hybridization to be able to sort out and follow the development of rare hybrid component. One approach to achieve this goal is to use some visual markers, such as the pigmentation of the parental protoplasts, to identify the fusion products and mechanically remove them from mixed population before the distinguishing features are lost, and grow them in isolation. This, indeed, has been possible. Gleba and Hoffmann (1978, 1979) produced

intergeneric hybrids between *Arabidopsis thaliana* and *Brassica campestris* by growing individual hybrid cells isolated using a micropipette.

In situations where fusion products are indistinguishable from other components of the fusion mixture an alternative method of selecting the hybrids is to be adopted. In the past a range of selection systems has been used which are based on: (a) complementation of natural differences in the sensitivity of the two parents to media constituents, antimetabolites, temperatures, etc., (b) complementation of recessive genes, and (c) mutant lines whose metabolisms have been blocked at different sites by irreversible biochemical modifications.

The first parasexual hybrid between *Nicotiana glauca* and *N. langsdorffii* was produced by Carlson *et al.* (1972). Whereas the protoplasts of the two species exhibit an obligate requirement for growth substances, those of the sexual hybrid between the two species can grow even in the absence of the growth substances. Therefore, when the protoplasts of the two species, after fusion-treatment, were planted on a medium without the growth substances, only hybrid colonies grew. Power *et al.* (1976) exploited the naturally-occurring differential sensitivities of the protoplasts of *Petunia parodii* and *P. hybrida* to actinomycin-D for raising somatic hybrids between the two species.

Using the techniques of protoplast fusion several somatic hybrids between sexually incompatible parents (*Solanum tuberosum* x *S. brevidens*, *Oryza sativa* x *O. officinalis*, *Brassica napus* x *B. juncea*) have been raised.

In sexual hybridization the plastome is mostly contributed by only one of the parents (female), whereas in somatic hybridization the hybrid receives plastomes from both the parents. Consequently, the latter approach to crossing plants offers a unique opportunity to study the interaction of the cytoplasmic organelles from two parents. On the practical side, it has been possible through protoplast fusion to transfer cytoplasmic male sterility inter- and intraspecifically without transferring the nuclear characters of the donor plant (Belliard *et al.*, 1977, 1978; Zelcer *et al.*, 1978; Izhar and Power, 1979). Medgyesy *et al.* (1980) transferred streptomycin resistance (controlled by chloroplast DNA) from *Nicotiana tabacum* to *N. sylvestris* by fusing iodoacetate-treated, non-dividing protoplasts of streptomycin-resistant *N. tabacum* with normal protoplasts of streptomycin-sensitive *N. sylvestris*.

Alloplasmic male sterile lines of B. *napus* and B. *oleracea* produced by substituting their cytoplasm by the Ogura cytoplasm of male sterile *Raphanus sativus* could not be utilized for hybrid seed production because of their yellowing at low temperature (Jourdan *et al.*, 1985). By fusing the protoplasts of these lines with those containing normal cytoplasm of the respective species, Pelletier *et al.* (1983), Robertson *et al.* (1985) and Menczel *et al.* (1987) replaced the sensitive chloroplasts by the insensitive ones. The new alloplasmic lines retained the useful male sterility trait while acquiring functional chloroplasts.

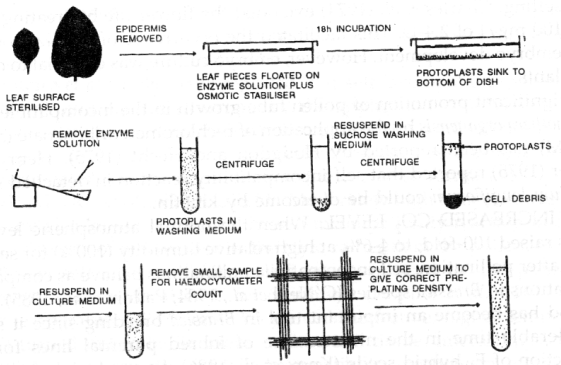

Fig. 10.10 Flow diagram for isolation of mesophyll protoplasts (*Courtesy:* Professor E.C. Cocking, U.K.)

A fundamental property of the isolated protoplasts is their osmotic fragility. Therefore, a suitable osmotic stabilizer is essential to obtain viable and stable protoplasts. For this purpose, mostly a metabolically inert sugar (mannitol) is used at an appropriate concentration in the enzyme solution, washing medium, fusion medium and culture medium. In a solution of proper osmolarity (slightly hypertonic) the freshly isolated protoplasts appear completely spherical (Fig. 10.11).

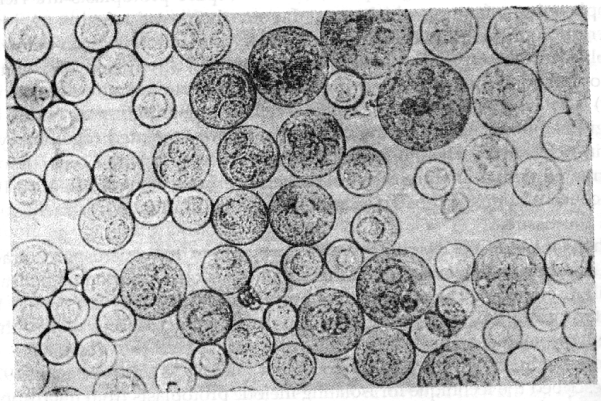

Fig. 10.11 Meiocyte protoplasts of *Trillium kamtschaticum* after fusion treatment. Smallest spherical structures of uniform size are individual protoplasts whereas larger structures are fusion bodies showing two to many nuclei. (*Courtesy:* Dr. M. Ito, Japan)

(ii) *Fusion of isolated protoplasts.* Power *et al.* (1970) reported intra- and interspecific fusion of higher plant protoplasts under the influence of sodium nitrate. The chief problem associated with sodium nitrate induced protoplast fusion is the low frequency of heterokaryon formation. This prompted workers to look for a better fusing agent (fusogen). Since 1974 polyethylene glycol (PEG; molecular weight higher than 1500) has found widespread acceptance as a fusogen because it induces high frequency of heterokaryon formation, most of which are binucleate. Using PEG, fusion has been reported between protoplasts from as diverse taxa as soybean-corn, soybean-barley, and soybean-pea. PEG is equally effective in fusing animal cells, and animal cells with plant protoplasts. In practice, equal quantities of dense suspensions of the protoplasts of two types are mixed, and to this is added 15 per cent PEG solution (molecular weight 6000). The ratio between the protoplast suspension and the solution is of the order of 1:3. After 10-30 min the PEG solution is gradually diluted with the medium in which the PEG solution is prepared and, finally, washed off completely (for detailed procedure *see* Bhojwani and Razdan, 1996).

PEG treatment brings about rapid aggregation of the protoplasts (Fig. 10.s12A, B) which is a prelude to fusion. During aggregation the plasma membranes of the adjacent protoplasts are tightly adpressed over a significant portion of the protoplast surface. Mostly, the fusion of adpressed membranes occurs during the dilution of PEG rather than during the treatment period. In membrane fusion plasmodesmata-like interconnections are formed between fusing partners (Fig. 10.12 C). These cytoplasmic continuities gradually expand and fuse together to form broader connections (Fig. 10.12D). Eventually, the fusion bodies assume a completely spherical structure (Fig. 10.12E, F). Fusion may occur between two or more protoplasts (Fig. 10.11). The fusion of two dissimilar protoplasts results in the formation of a heterokaryon. Upon culture the nuclei in the heterokaryons may fuse to establish a true hybrid cell.

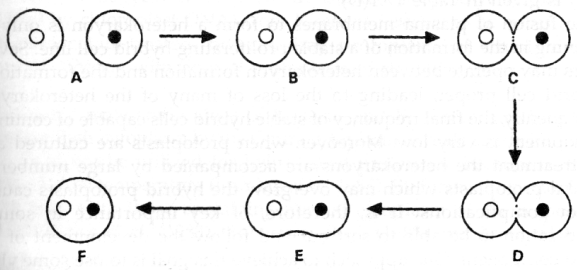

Fig. 10.12 Diagrams showing the sequential stages in protoplast fusion. **A.** Two separate protoplasts. **B.** Agglutination of two protoplasts. **C, D.** Membrane fusion at localized sites. **E, F.** Formation of spherical heterokaryons. (*after* Bhojwani and Razdan, 1996)

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CHAPTER

11

ENDOSPERM

Endosperm is the most common nutritive tissue for the developing embryos in angiosperms. Functionally, it is comparable to the female gametophyte in gymnosperms but has a unique origin. Whereas the female gametophyte in gymnosperms differentiates before fertilization and is haploid, the endosperm is the product of fertilization and is usually triploid. After double fertilization the egg is called zygote, and the fusion product of polars and the second male gamete is termed primary endosperm nucleus. The former develops into an organized embryo whereas the latter gives rise to an almost formless tissue, the endosperm. For normal seed development, the genetic balance between embryo, endosperm and maternal tissue seems to be critical. The *indeterminate gametophyte* (*ig*) mutation of maize produces accessory polar nuclei which, upon pollination and fertilization, form endosperm with ploidy level 2n to 8n. However, only 3n endosperm develops fully (Lin, 1984).

The only angiosperms that do not form endosperm are the members of the families Orchidaceae, Podostemaceae and Trapaceae. Where present, the endosperm may either be consumed by the developing embryo, so that the seeds are non-endospermous (pea, beans), or it may persist in mature seeds and continue to support the growth of embryo during seed germination. Common examples of endospermous seeds are cereals, castor-bean and coconut. Endosperm forms the edible part of cereals and coconut and it is the source of commercial castor-oil in castor-bean.

Brachiaria setigera, an apomict species, is the only example where endosperm has been reported to produce triploid embryos and seedlings (Muniyamma, 1977).

DEVELOPMENT OF ENDOSPERM

The primary endosperm nucleus is normally located directly below the egg cell and undergoes division almost immediately after its formation. During triple fusion only the sperm nucleus fuses with the polar nuclei, while the male cytoplasm does not take part in this process. The membrane of the primary endosperm nucleus is contributed by both the secondary nucleus and the male nucleus.

Following fertilization several changes occur in the fine structure of the central cell that are indicative of increased metabolic activity and organization of the protein-synthesis machinery for the differentiation of the primary endosperm cell (for a detailed account *see* Bhatnagar and Sawhney, 1980). Depending upon its mode of development, three types of endosperm have been recognized. These are Nuclear, Cellular and Helobial.

According to the data of Davis (1966), of the 288 families of Angiosperms for which information was available at that time, 161 families show Nuclear endosperm, 72 Cellular endosperm, and only 17 Helobial endosperm. Cellular endosperm is largely restricted to dicotyledonous families. In monocots it occurs only in Araceae and Lemnaceae. Similarly, of the 17 families showing Helobial endosperm 14 are monocotyledonous.

Nuclear Endosperm

In this type of endosperm the division of the primary endosperm nucleus and a few subsequent nuclear divisions are not accompanied by wall formation. This results in a condition where the central cell of the embryo sac has formed a few to several thousand nuclei freely suspended in its sap (Fig. 11.1A-D). Such a condition of endosperm may persist until it is

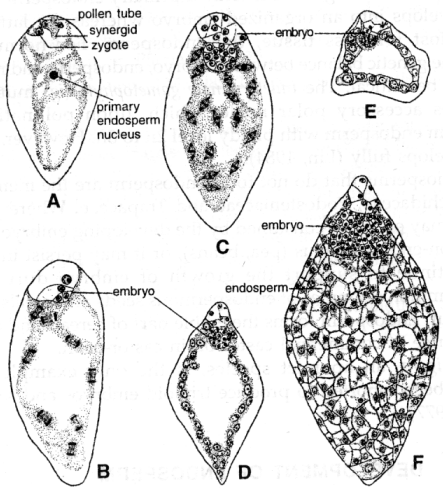

Fig. 11.1 Nuclear endosperm in *Acalypha indica*. **A.** Embryo sac after fertilization; the primary endosperm nucleus and the zygote have not yet divided. **B, C.** Embryo sac, showing synchronous divisions of the endosperm nuclei. **D.** The endosperm nuclei have moved to the periphery. **E.** The peripheral part of the embryo sac has become cellular. **F.** Completely cellular endosperm. (*after* Johri and Kapil, 1953)

consumed by the developing embryo (*Floerkea, Limnanthes, Oxyspora*) or it may become cellular at a later stage. When latter is the case, which is more common, the wall formation is mostly centripetal, *i.e.*, from the periphery toward the centre (Fig. 11.1E). The degree of cellularization varies a great deal. Mostly the endosperm becomes completely cellular (Fig. 11.1F) but in *Phaseolus* cellularization occurs only around the embryo. In *Crotalaria* the wall formation is confined to the upper region of the embryo sac; the chalazal region remains free-nuclear, and it often elongates and behaves like an haustorium (Fig. 11.2).

Using the technique of dissections, Kausik (1938, 1941), for the first time, reported the presence of a vermiform appendage at the chalazal end of the endosperm in *Grevillea robusta* (Fig. 11.3). Since then endosperm haustoria have been reported in several members of the Cucurbitaceae, Fabaceae and Proteaceae. Whereas in *Grevillea* the chalazal endosperm haustorium remains free-nuclear throughout, in *Coccinia* and *Citrullus fistulosus* it becomes partitioned into multinucleate chambers. The longest endosperm haustorium is reported in *Echinocystis lobata* of the Cucurbitaceae. It measures up to 16 mm in length (Chopra and Seth, 1977).

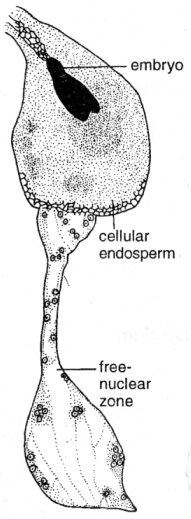

Fig. 11.2 In *Crotalaria* the upper-half of the endosperm becomes cellular whereas the lower-half remains free-nuclear. (*after* Rau, 1951)

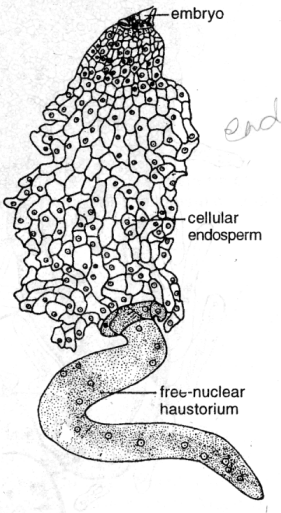

Fig. 11.3 Endosperm in *Grevillea robusta*. Note the free-nuclear, vermiform appendage (haustorium) at the chalazal end of the cellular part of the endosperm. (*after* Kausik, 1938)

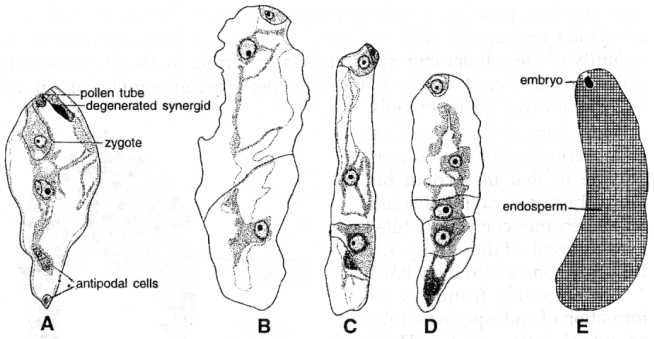

Fig. 11.6 Cellular endosperm in *Drimys winteri*. **A.** Embryo sac after fertilization. **B-D.** Two-celled, 3-celled and 4-celled endosperm, respectively. **E.** Older embryo sac completely filled with cellular endosperm. (*after* Bhandari and Venkataraman, 1968)

basal two or three cells of the tail elongate to form an haustorium (Fig. 11.7D) which penetrates into the chalazal part of the nucellus.

A very aggressive chalazal haustorium is formed in *Iodina rhombifolia* (Bhatnagar and Sabharwal, 1969). The haustorium is actually formed before fertilization. The chalazal end of the unfertilized embryo sac forms an extensive caecum, the lower end of which extends into the placenta and branches. After fertilization, the division of the primary endosperm nucleus is followed by transverse partitioning of the central cell, resulting in the formation of a micropylar chamber and a chalazal chamber. The endosperm proper is derived from the micropylar chamber alone. The chalazal chamber functions as an aggressive, uninucleate haustorium. The nucleus migrates into the caecum and becomes much hypertrophied (about 600 µm). Profuse branching at the free-end gives the haustorium a coralloid appearance (Fig. 11.8 A,B).

Melampyrum lineare and *Blumenbachia insignis* show micropylar as well as chalazal endosperm haustoria. In *Melampyrum* the micropylar haustorium comprises a single, 4-nucleate cell with many tubular processes (Fig. 11.9A,B). One of the processes enlarges and enters the funiculus . The chalazal haustorium is a binucleate cell, broader above and narrow below (Arekal, 1963).

In the Acanthaceae the endosperm development is asymmetric, and it shows characteristic micropylar and chalazal haustoria. The general pattern of endosperm development in this family is as follows. The primary endosperm nucleus moves to the chalazal end of the embryo sac and divides forming a smaller chalazal chamber and a larger upper chamber (Fig. 11.10A). The upper chamber again divides transversely, so that a linear row of three cells is formed (Fig. 11.10B). The chalazal chamber and the

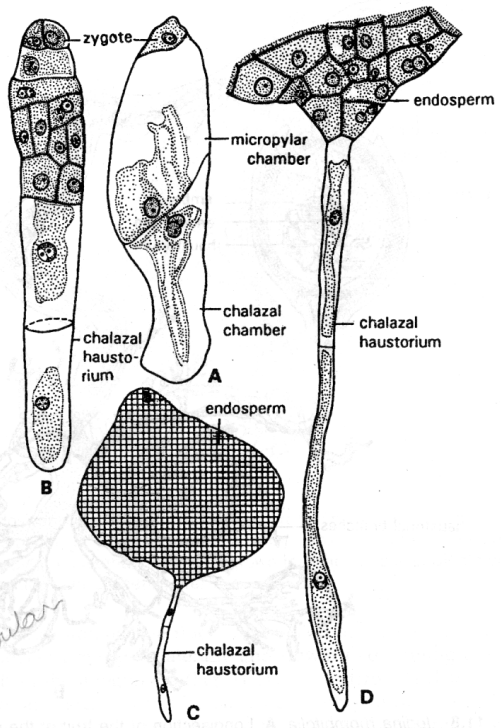

Fig. 11.7 Endosperm development in *Magnolia obovata*. **A.** Two-celled endosperm. **B.** Thirteen-celled endosperm; note that the zygote has not yet divided. **C.** Diagram of endosperm at the globular stage of embryo to show 2-celled chalazal haustorium. **D.** A portion from C enlarged to show the chalazal haustorium with a few cells of the endosperm proper. (*after* Kapil and Bhandari, 1964)

micropylar chamber develop into haustoria and the central cell forms the endosperm proper (Fig. 11.10C). Variation exists in the behaviour of the central cell. Nuclear divisions in the central cell may be accompanied by wall formation right from the beginning so that the endosperm is *ab initio* cellular (*Barleria prionitis, Elytraria*), or wall formation may occur after a few free-nuclear divisions. When latter is the condition, cellularization of the central cell may be complete (*Barleria cristata, Schaueria*) or partial (*Ruellia tuberosa*), the lower free nuclear portion is termed 'basal apparatus' (*see* Mohan Ram and Wadhi, 1964).

In *Lomatia*, besides the main chalazal haustorium, numerous single-celled, finger-shaped projections are present all over the endosperm (Fig. 11.4). This increases the absorbing surface of the endosperm.

Nijalingappa and Devaki (1979) have reported the presence of both micropylar and chalazal endosperm haustoria in *Scleria foliosa*, a member of the Cyperaceae. This is the only report of the occurrence of endosperm haustoria in this family.

Development of endosperm in coconut deserves special mention. The primary endosperm nucleus undergoes a number of free nuclear divisions. When the fruit is about 50 mm long the embryo sac gets filled with a clear fluid in which float numerous nuclei of various sizes. At a later stage (about 100 mm long fruit) the suspension shows, in addition to free nuclei, several cells each enclosing a variable number of nuclei. Gradually these

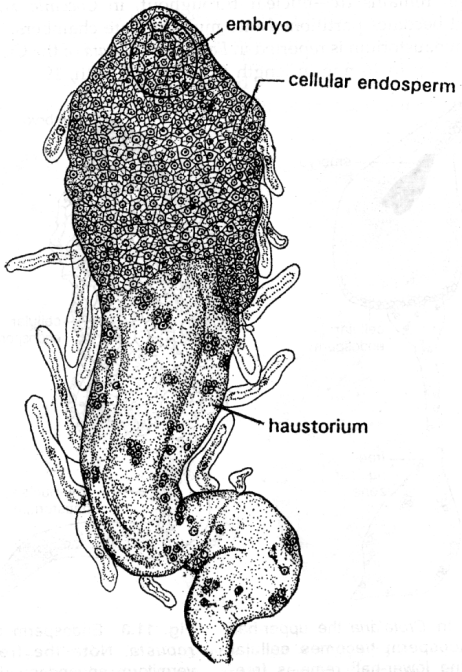

Fig. 11.4 *Lomatia polymorpha.* Endosperm enclosing the embryo dissected out from a young seed. Several uninucleate projections are present on the surface of the endosperm as well as the haustorium. (*after* Venkata Rao, 1963)

cells and free nuclei start settling at the periphery of the cavity, and layers of cellular endosperm start appearing. This forms the coconut meat. The quantity of the cellular endosperm increases further by the divisions of the cells. In mature coconuts the liquid endosperm becomes milky, and it does not contain free nuclei or cells.

In *Areca catechu* (areca nut) the endosperm development is comparable to that in coconut but the embryo sac cavity is small, and it becomes completely filled by the growth of the endosperm. It later becomes extremely hard.

An interesting feature is the formation of endosperm nodules or cytoplasmic vesicles. These originate from the peripheral lining of the embryo sac. The nodules may be nucleate as in *Salix, Cinerea, Carica* and *Capsella*, or may be enucleate as in *Stackhousia* (Fig. 11.5), *Pennisetum* and some cucurbits. The nodular cytoplasm is rich in various organelles. The function of the nodules is uncertain, but as to their fate they may either merge with the endosperm proper or degenerate.

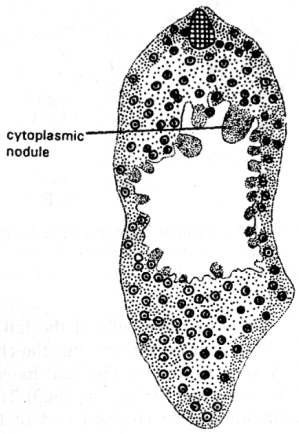

Fig. 11.5 Endosperm of *Stackhousia*, showing cytoplasmic nodules. (*after* Narang, 1953)

Cellular Endosperm

The Cellular endosperm is characterized by the absence of free-nuclear stage. The division of the primary endosperm nucleus and a few subsequent nuclear divisions are followed regularly by wall formation (Fig. 11.6A-E). In avocado, the wall formed after the first division of the primary endosperm nucleus is probably callosic (Sedgley, 1979). The occurrence of haustoria is a common feature of this type of endosperm; it is more varied than that in the Nuclear endosperm. The haustoria may be micropylar or chalazal. Occasionally, both types of haustoria are present in the same plant.

Micropylar haustoria are known to occur in *Impatiens roylei* and *Hydrocera triflora*. Development of 2-celled chalazal haustorium in *Magnolia obovata* is shown in figure 11.7. The first division of the primary endosperm nucleus is followed by a transverse wall resulting into two chambers of almost equal size (Fig. 11.7A). Divisions in the micropylar chamber are rapid and in all directions (Fig. 11.7B). The chalazal chamber divides transversely and at a comparatively slow rate. This results in a tail-like chalazal part attached to the more massive tissue at the micropylar end. Further divisions occur in the upper part of the tail and add to the endosperm tissue. The

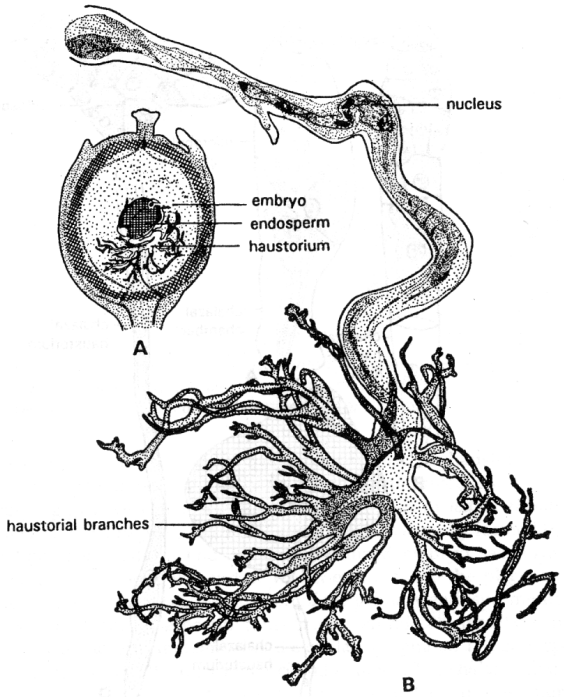

Fig. 11.8 *Iodina rhombifolia*. **A.** Longisection of the fruit at the globular stage of the embryo; note the aggressive nature of the chalazal haustorium. **B.** Enlarged view of the haustorium with branched lower end and hypertrophied nucleus. (*after* Bhatnagar and Sabharwal, 1969)

Deviation from the general pattern of endosperm development in Acanthaceae is shown by *Blepharis* and *Thunbergia*. The linear three-celled stage, present in all other members of the family, does not occur in these plants. They also lack the chalazal haustorium. After the division of the primary endosperm nucleus a micropylar chamber and a chalazal chamber are formed. The former grows into an haustorium whereas the latter gives rise to the endosperm proper. In *Thunbergia* the micropylar haustorium is a branched, coenocytic structure.

Klugia notoniana shows an interesting pattern of haustorium development (Arekal, 1961). Both chalazal and micropylar haustoria occur in this plant (Fig. 11.11). The chalazal haustorium is initially a binucleate cell but,

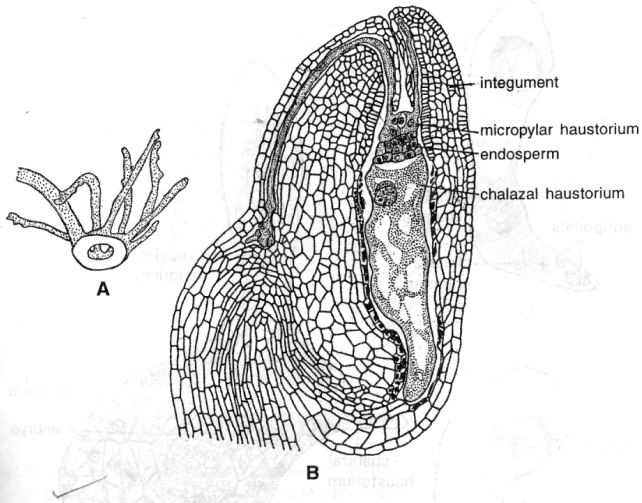

Fig. 11.9 Endosperm haustorium in *Melampyrum lineare*. **A.** Dissected out micropylar haustorium. **B.** Longisection of a young seed, showing extension of one of the micropylar haustorial processes grown into the funiculus. Single-celled chalazal haustorium is also seen. (*after* Arekal, 1963)

eventually, becomes uninucleate due to the fusion of the two nuclei. The uninucleate haustorium grows laterally and upward consuming the sub-epidermal cells of the integument (Fig. 11.11). The micropylar haustorium comprises two uninucleate cells. It becomes active only during later stages of seed development, when the activity of the chalazal haustorium begins to decline.

Fine structural studies on the endosperm haustoria of *Lobelia dunnii* have provided a strong evidence of their absorptive function (Torosian, 1971). The haustorial cells, with wall ingrowths causing invaginations of the plasma membrane, act as transfer cells. Finger-like wall projections extending into the cytoplasm of the micropylar chamber and chalazal endosperm haustorium have been observed in *Vaccinium macrocarpon* (Brisson and Peterson, 1975). However, in *Plantago lanceolata* (Vannereau and Mestre, 1975) the walls are smooth and no such projections are seen in the micropylar haustoria.

The development of endosperm in the Loranthaceae is unique. There being no true ovule, all the embryo sacs in an ovary lie close to each other. After fertilization, the primary endosperm nucleus moves to the basal part of the embryo sac where it divides. During their development, the endosperms of all the embryo sacs in an ovary fuse to form a composite

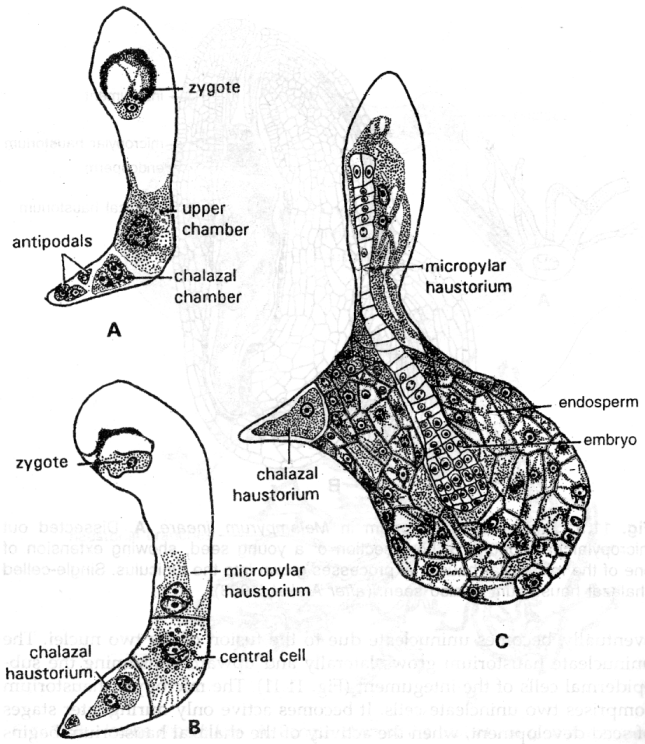

Fig. 11.10 Endosperm development in *Barleria cristata*. **A.** Two-celled stage; the chalazal chamber is binucleate. **B.** Three-celled stage. **C.** The central cell has formed cellular endosperm; also note the micropylar and chalazal haustoria. (*after* Mohan Ram and Wadhi, 1964)

endosperm (Fig. 11.12A, B). *Struthanthus vulgaris* stands out alone in the Loranthaceae. In this taxon the endosperm develops from a single embryo sac and, thus, a composite structure is not formed (Venturelli, 1981).

Helobial Endosperm

This type of endosperm is restricted largely to the monocotyledons. The primary endosperm nucleus moves to the chalazal end of the embryo sac where it divides forming a large micropylar chamber and a small chalazal chamber (Fig. 11.13A). In the micropylar chamber, as a rule, free-nuclear

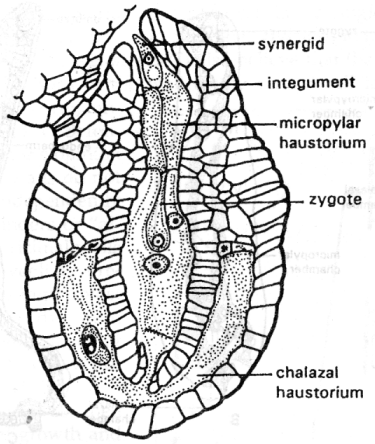

Fig. 11.11 Endosperm haustoria in *Klugia notoniana*. Longisection of the ovule, showing laterally upward growing chalazal haustorium, and the micropylar haustorium. (*after* Arekal, 1961)

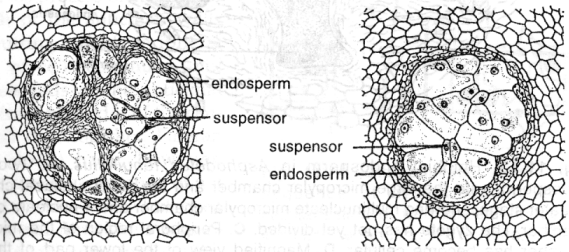

Fig. 11.12 Composite endosperm in *Tolypanthus involucratus*. **A.** Transverse section of ovary, showing four embryo sacs, each with 4-seriate endosperm and a biseriate suspensor. **B.** Same, at a later stage of development. All the endosperms in the ovary have fused and formed a composite structure. (*after* Dixit, 1961)

divisions (Fig. 11.13B) and cell formation, if any, start at a much later stage (Fig. 11.13C). In the chalazal chamber the nucleus either remains undivided or divides only a few times. If latter is the situation, the divisions are usually free-nuclear (Fig. 11.13D). However, sometimes, as in *Phylidrum lanuginosum*, it may become cellular.

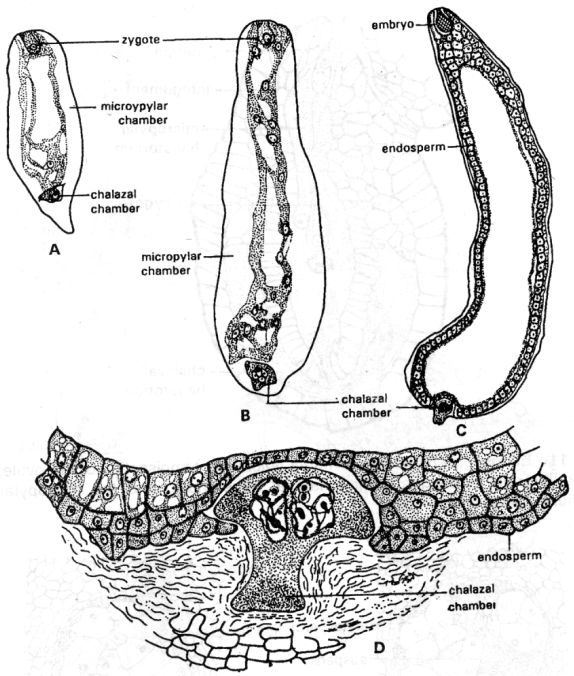

Fig. 11.13 Helobial endosperm in *Asphodelus tenuifolius*. **A**. Two-celled endosperm; note the large micropylar chamber and the small chalazal chamber. **B**. Later stage, showing multinucleate micropylar chamber and 4-nucleate chalazal chamber. The zygote has not yet divided. **C**. Peripheral region of the micropylar chamber has become cellular. **D**. Magnified view of the lower part of the seed showing multinucleate chalazal chamber. (*after* Eunus, 1952)

Cellularization of Endosperm

Ultrastructural studies have provided useful information on the cellularization of the nuclear endosperm. Reports on the initial stages of wall formation in wheat are at variance. Morrison and O'Brien (1976) reported that the formation of the first layer of endosperm cells does not involve phragmoplast. According to them, the process of cellularization starts with the development of centripetally growing walls from the central cell wall. The free ends of these anticlinal walls branch and the branches from the adjacent walls fuse on the side nearest to the large central

vacuole. This turns the free nuclear central cell into a single layer of uninucleate cells. Regular anticlinal and periclinal divisions in these cells complete cellularization. Mares *et al.* (1977) also believe that the first anticlinal walls arise as projections of the wall of the central cell independent of mitotic spindle. However, unlike Morrison and O'Brien, these authors are of the opinion that the anticlinal walls do not branch but grow independently forming open cylinders or "alveoli". The first periclinal walls arise within the alveoli as a result of normal phragmoplast following nuclear division, forming a peripheral layer of cells and the new inner layer of alveoli (Fig. 11.14). The free ends of the anticlinal walls then resume centripetal growth and after some time the open cylinder undergoes another periclinal division cutting off the second peripheral layer of cells. This process of centripetal growth of the anticlinal walls and cutting off of peripheral layers of cells continues until the free ends of the anticlinal walls from two sides of the central cell fuse together. Fineran *et al.* (1982) have, however, shown that the formation of not only the cross walls but even the first anticlinal walls is associated with cytokinesis (Fig. 11.15). According to them during early stages of cellularization of the endosperm typical phargmoplasts develop between the free nuclei in the pheripheral cytoplasm of the central cell. The outer ends of the cell plate formed by them fuse with the wall of the central cell. The anticlinal walls develop on all sides of the nu-

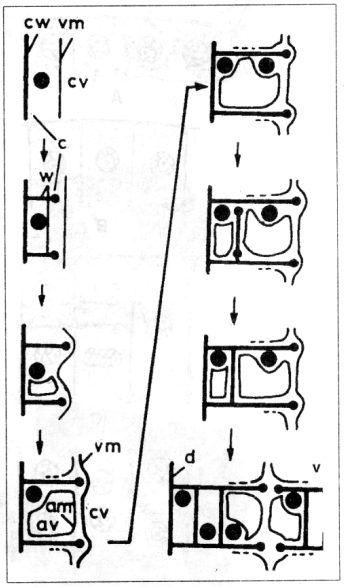

Fig. 11.14 Diagrammatic sketch of the sequence of events leading to the cellularization of free nuclear endosperm in wheat. •, endosperm nucleus; —•, free end of a growing wall partition or a phragmoplast, which consists of a planar array of vesicles and transversely oriented microtubules. For explanation *see* text. (*am*, alveolar vacuolar membrane; *av*, alveolar vacuole, *c*, dorsal cytoplasm of central cell; *cv*, central cell vacuole; *cw*, central cell wall and plasma membrane; *d*, dorsal surface of the endosperm; *v*, ventral surface of the endosperm; vm, central cell vacuolar membrane; *w*, wall partition and accompanying plasma membrane; *from* Mares *et al.*, 1977)

RUMINATE ENDOSPERM

Mature endosperm with any degree of irregularity and unevenness in its surface contour is called ruminate endosperm (Fig. 11.17). Rumination starts at a late stage of endosperm development, and it may belong to any one of the three categories described above. Ruminate endosperm is known to occur in about 32 families of angiosperms.

Rumination is caused by the activity of the seed coat or the endosperm itself. In the former case, the irregularities on the inner surface of the seed coat may arise by: (a) unequal radial elongation of any one or the only layer of the seed coat (*Passiflora calcarata*), or (b) definite ingrowth or infolding of the seed coat. The second cause is more common and is found in the Annonaceae and Aristolochiaceae.

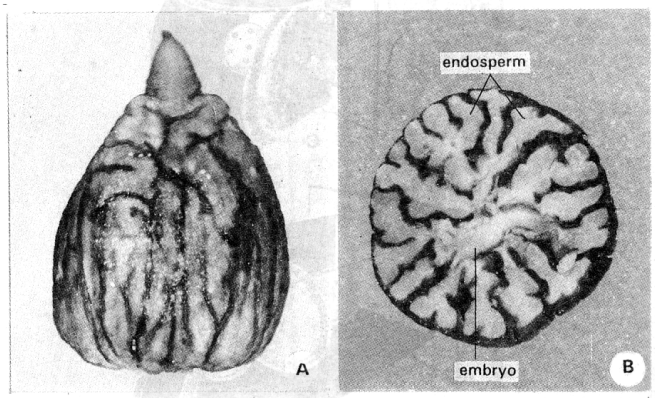

Fig. 11.17 Ruminate endosperm of *Antigonon*. **A**. Surface view. **B**. Same in longisection; note the irregular structure of the endosperm.

Rumination of endosperm by its own activity occurs in *Myristica, Coccoloba*, etc. In these plants the endosperm begins to increase in volume simultaneously with the increase in volume of the seed. It soon absorbs the nucellus and comes in direct contact with the seed coat. During further growth of the endosperm the irregular inner surface of the seed coat makes it ruminate. On the other hand, in *Andrographis* and *Elytraria* the endosperm exhibits unequal peripheral activity during late stage of its development and causes the seed coat to attain an irregular configuration.

CYTOLOGY OF ENDOSPERM

In the majority of plants the endosperm is triploid because it is derived from the fusion product of three haploid nuclei - one from the male gametophyte and two from the female gametophyte. Whereas the number of nuclei contributed by the male gametophyte in the formation of the endosperm is constant throughout the angiosperms, the number of nuclei contributed by the female gametophyte varies with the type of embryo sac (*see* Chapter 7); in *Oenothera* it is just one, and the endosperm is diploid, whereas in *Peperomia* it is eight, and the endosperm is 9n.

The endosperm tissue is well known for a high degree of polyploidization of its cells during development. Erbrich (1965) studied endosperm cytology in many flowering plants. The ploidy of the nucleus of the endosperm haustorium in *Thesium alpinum* is up to 384n. The highest ploidy, however, has been reported in *Arum maculatum* where the nucleus becomes 24576n. In *Melampyrum pratense* each nucleus of the chalazal haustorium attains a ploidy of 1536n. In maize endosperm, after the syncytial stage, there is dramatic increase in the size and DNA content of nuclei (Kowles and Phillips, 1988). The most significant change occurs between 10-20 days after pollination, when the DNA content of these cells increases from 3C to as much as 690C. In the inbread line A188 most of the cells range from 10C to 50C with a maximum of 90-100C. The purpose of DNA increase is not well understood. It had been thought that it may result in enhanced starch and protein synthesis but it is not only the starch synthesis genes that are amplified but the entire DNA goes through repeated cycles of DNA synthesis.

Endomitosis and nuclear fusion are some of the methods of polyploidization of endosperm cells (Kapoor, 1962). The occurrence of various mitotic irregularities, such as chromosome bridges, lagging chromosomes, spontaneous breakage of the chromosomes, and fragmentation of the nuclei (Fig. 11.18 A, B, D-G), is quite common in the endosperm tissue (Kapoor, 1962). Size of the nuclei and the number of nucleoli per nucleus also exhibit great variation (Fig. 11.18C).

Exception to the rule is *Dendrophthoe falcata* where the endosperm cells show remarkable stability in their chromosome number; all the cells of mature endosperm are triploid (Johri and Nag, 1974).

The endosperm is usually non-chlorophyllous. In *Crinum*, during seed development, the seed coat as well as fruit wall are absorbed and the endosperm is exposed to sunlight. Consequently, it becomes green. Chlorophyllous endosperm also occurs in *Mathiola, Raphanus* and *Viscum*.

ALEURONE TISSUE

In cereals one or few outermost layers of the endosperm become highly specialized morphologically as well as physiologically, and constitute the aleurone tissue. In barley this tissue is 3 or 4-layered. In mature grains the aleurone cells are alive whereas the starchy part of the endosperm, surrounded by the aleurone cells is dead (Verner, 1972).

Several morphologically distinct types of aleurone cells occur in cereals. In *Setaria* the aleurone cells near the placental vascular supply are columnar and show wall ingrowths. Since these cells help in transferring substances

clei and soon fuse with each other in such a way that an open cylinder with free inner end is formed around each nucleus (Fig. 11.16). The further cellularization is largely same as described by Mares *et al.* (1977).

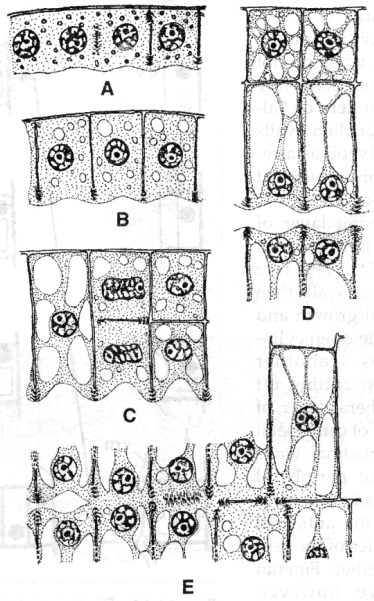

Fig. 11.15 Initial formation of walls in the endosperm of wheat (semi-diagrammatic). **A.** Free endosperm nuclei (left), appearance of phragmoplast between nuclei (middle), and formation of cell plate (right). **B.** Stages of cell plate fusion to the central cell wall. Distal margins of the anticlinal walls continue to grow freely toward the central vacuole. **C.** First layer of open cylinders or alveoli is seen on left. The alveolus divides periclinally (middle) forming a complete endosperm cell toward central cell wall and another alveolus toward the central vacuole. **D.** Stage showing peripheral layer of endosperm cells and an inner layer of alveoli. Alveoli from opposite sides are approaching each other. **E.** Stage showing fusion of alveoli from opposite sides of the grain. On the left the opposite alveoli are about to fuse followed by fusion of their 'naked' cytoplasms (middle left). Phragmoplast has arisen (middle) and within this the plate appears (middle right). Cell plate extends to meet the approaching anticlinal walls and other developing periclinal walls between apposed alveoli (right). The alignment of the apposed radial wall is opposite (left) or alternate (right). (*after* Fineran *et al.*, 1982)

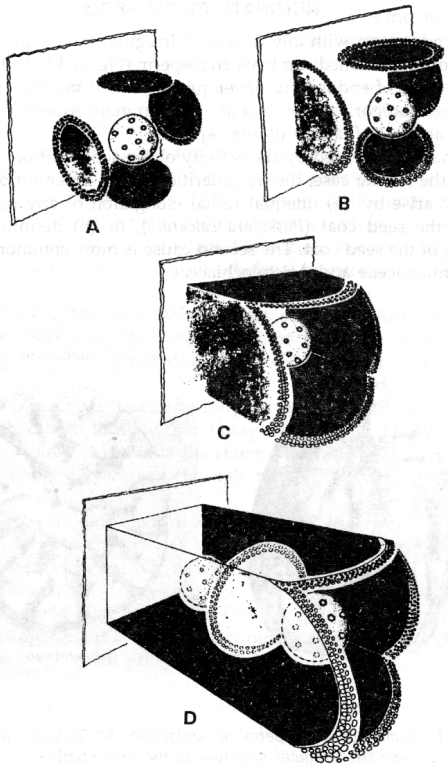

Fig. 11.16 Three-dimensional representation (perspective exaggerated) of the events during the initial cellularization of the free nuclear endosperm of wheat. Only individual compartments and completed cells are depicted with closely related stages of wall formation combined into the same diagram. Central cell wall is shown at the rear. **A.** Phragmoplasts and their first-formed cell plates, grouped around the nucleus, perpendicular to the central cell wall. **B.** Later stage; cell plates are meeting and have fused with the central cell wall. **C.** Cell plates have completely fused with the central cell wall. Anticlinal walls are growing freely by their inner margin toward the central vacuole. **D.** A compartment or alveolus is fully grown and is undergoing periclinal division (top and near side walls are not shaded) resulting in a complete cell centrifugally (toward central cell wall) and another compartment or alveolus centripetally (toward central cell vacuole). (*after* Fineran *et al.*, 1982)

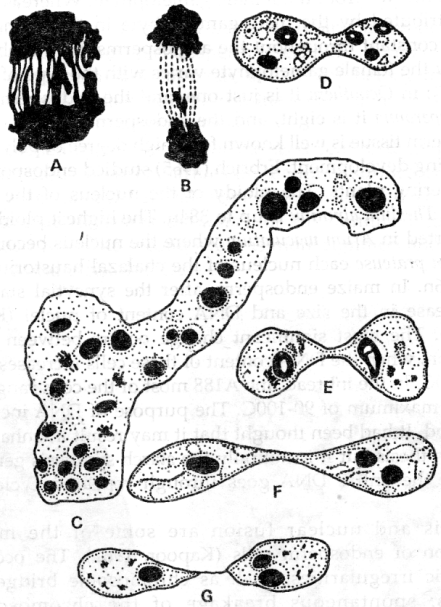

Fig. 11.18 Cytology of the endosperm. **A.** *Nothoscordum*; **B, C.** *Zephyranthes*, and **D-G.** *Vicia*. **A.** Late anaphase, showing chromosome bridges. **B.** Late anaphase, showing lagging chromosomes. **C.** An exceptionally large nucleus with 23 nucleoli. **D-G.** Stages in the fragmentation of the nucleus. (*after* Kapoor, 1962)

from the vascular tissue to the embryo and endosperm proper, they have been termed 'transfer aleurone cells' (TAC). TAC have also been observed in *Echinochloa* and *Zea mays*, but they are absent in wheat and rice. Two types of aleurone cells occur in rice: (a) rectangular cells with less dense cytoplasm surrounding the embryo, and (b) cuboidal cells with dense cytoplasm around the starchy endosperm. It is believed that because of their different structure, the two types of aleurone cells may have different functions (Bechtel and Pomeranz, 1977).

The aleurone cells are characterized by the presence of thick walls and non-vacuolated cytoplasm (Fig. 11.19A). They are interconnected by the plasmodesmata. The most prominent organelles of these cells are aleurone grains, followed by spherosomes (Fig. 11.19 A, B). Aleurone grains are surrounded by a single unit membrane which is closely associated with spherosomes (Fig. 11.19B). The main components of the aleurone grains are protein, phytin, phospholipids, and some carbohydrates. Each of these

components is localized in discrete particles (Jacobson *et al.*, 1971). Structurally, the aleurone grains possess two kinds of inclusions, besides the ground substance (Fig. 11.19B): (a) Globoids, present within the globoidal cavities, contain phytin and lipids; (b) Protein-carbohydrate bodies, which are 1-1.5 μm in diameter. The ground substance also contains high concentration of protein, but it is less than that in the protein-carbohydrate bodies.

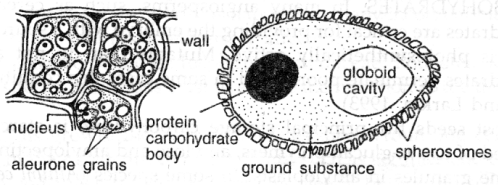

Fig. 11.19 **A.** Aleurone cells of barley. **B.** A single aleurone grain enlarged. (**A.** diagrammatized from Jones, 1969 and **B** from Jacobson *et al.*, 1971)

Protein-storage organelles were first identified in the aleurone tissue and thus called aleurone grains. Subsequently, similar organelles were observed in endosperm, embryo and cotyledons of several plants and variously termed by different authors (protein vacuoles, protein granules, protein bodies, aleurone vacuoles, aleurone bodies, aleurone grains). Indiscriminate use of these terms has created some confusion. Some authors consider protein bodies different from aleurone grains. Rest (1972) has classified protein bodies into 3 categories: (i) protein bodies without inclusion, (ii) protein bodies with globoid inclusion, and (iii) protein bodies with globoid and crystalloid inclusions. According to Rest and Vaughan (1972) protein-storage organelles with inclusions are the aleurone grains and those without inclusions are the myrosin grains. In our opinion it is best to call any protein-storage organelle as protein body, unless ontogenetically it is something else.

Aleurone tissue has received considerable importance in studies of plant hormone action. During seed germination the reserve food (starch and protein) in the endosperm cells is digested by the activity of certain hydrolytic enzymes (amylases and proteases) which are secreted by the aleurone cells. Gibberellins have been shown to activate these enzymes or induce their *de novo* synthesis.

RESERVE FOOD SUBSTANCES

Where endosperm persists in mature seeds, it is rich in reserve food materials in the form of carbohydrates, fats and/or proteins. During seed germination these substances are digested and utilized for the growth of the seedling, until the latter develops chlorophyll and is able to manufacture its own food. It has been suggested that storage substances do play an

important role in embryo development. The mutations affecting deposition of storage proteins and starch biosynthesis in several cereals lead to dramatic changes in the composition and physical structure of endosperm. Storage protein mutants often have a fragile and lustreless endosperm and are usually described as opaque or floury. Starch mutants usually have shrunken or collapsed seeds. These mutants germinate poorly and are susceptible to insects and pathogens.

CARBOHYDRATES. In many angiosperms, such as cereals, reserve carbohydrates are critical for providing the embryo with a source of energy until it is photosynthetically active. Mutants that do not accumulate carbohydrates germinate poorly, and in some cases this condition is lethal (Lopes and Larkin, 1993).

In most seeds the principal storage carbohydrate is starch which is composed of two α-glucan polymers, amylose and amylopectin, packed as crystalline granules in amyloplasts. In some species (*Allium cepa, Phoenix dactylifera, Washingtonia filifera*) the reserve carbohydrates are mannose and xylocans deposited in endosperm cell walls. Ivory nut (*Phytelephas macrocarpa*) has mannose as the major storage carbohydrate (79% of the endosperm dry weight; Bewley and Black, 1978).

PROTEINS. In general, two types of storage proteins, globulins and certain albumins, occur in all species, and prolamines are unique to the cereals. The globulins are soluble in saline solutions whereas prolamines are soluble only in alcoholic solution or denaturing solvents. Prolamines, which contain glutamine and proline, account for 50-60% of the total proteins in cereal endosperm. Two basic types of prolamines can be distinguished; those found in wheat (gliadins and glutenins) and its relatives (barley and rye), and those found in maize (zeins) and its relatives (millets and coix).

In maize, storage proteins are synthesized on RER membrane and are deposited in an organized fashion within the lumen of this organelle. In wheat, barley and oats the storage proteins are found both in RER (prolamines) and vacuoles (globulins).

LIPIDS. Oilseeds are among the most ancient crops to be domesticated. Oils have higher calorific value (38 kJ/g) than proteins and carbohydrates (17kJ/g) (Jones, 1992). However, only a few species store oil in endosperm. Castor-bean, oil palm, opium poppy and spurge accumulate oil up to 50% of the seed DW. These reserves are packed into discrete organelles, known as lipid bodies, spherosomes or oleosomes. They usually consist of triacylglycerols surrounded by a half-unit phospholipid membrane and a few proteins (Huang, 1991; Bednarek and Raikhel, 1992).

FUNCTIONS OF ENDOSPERM

In plants with albuminous seeds the endosperm reserves support early seedling growth. However, a more important contribution of endosperm is in the nutrition of embryo during its early stages of development (from zygote to globular embryo). This conclusion is based on the following observations (also *see* Maheshwari and Rangaswamy (1965):

1. At the time of fertilization the embryo sac has very little nutritive material. After the endosperm advances in development it stores enough food substances to ensure an adequate supply for the developing embryo.
2. In the majority of angiosperms the zygote divides only after the endosperm has reached a reasonable stage of development. Even in those cases where the zygote divides before or simultaneously with the endosperm the latter soon surpasses the embryo in growth.
3. Generally the embryo grows only when the endosperm develops properly. If the endosperm aborts, as happens in many incompatible crosses, the growth of the embryo is adversely affected.
4. In the absence of the endosperm (Orchidaceae, Podostemaceae and Trapaceae) special provisions exist to ensure the nutrition of the developing embryo.
5. During its growth the embryo depletes the surrounding cells of the endosperm of their contents. In many plants, such as legumes and cucurbits, the embryo consumes the endosperm completely before the seed attains maturity.
6. In some cereals several layers of endosperm at the base differentiate into transfer cells, with extensive wall ingrowths. This specialized tissue helps in transport of metabolites from the maternal tissue into the endosperm. Most of the sucrose arriving at the base of the endosperm from maternal tissue is cleaved to glucose and fructose by the invertase activity. The two hexoses then move to other portions of endosperm where sucrose is partially resynthesized and used (Shannon *et al.*, 1986). In *Miniature-1 (mn1)* mutant of maize, loss of invertase activity in the basal tissue of the endosperm leads to the destruction of maternal cells transferring sucrose. Thus, the endosperm fails to accumulate starch and protein (Miller and Chourey, 1992).

During early stages the endosperm, as a nurse tissue, does not show specificity for its own embryo. This has been shown experimentally. Liquid endosperm or endosperm extract from immature fruits not only supports the growth of its own embryo but can also nourish the embryos of other angiosperms. Coconut milk, collected from green fruits, has been successfully employed for growing embryos of many plants. Immature endosperm is also potent for inducing divisions in highly differentiated and mature cells, such as those of the secondary phloem in carrot. Besides coconut, such a property has been demonstrated for the extract of corn endosperm in the milk stage, and liquid endosperm of horse-chestnut and walnut. When added to the basic nutrient medium, coconut milk also induces the differentiation of embryos and plantlets from various plant tissues. Older endosperm, as a rule, lacks these stimulatory properties; it may even prove inhibitory. This may be correlated with the fact that young endosperm is rich in various growth hormones such as auxins, cytokinins, and gibberellins whose concentration decreases after a certain age of the tissue. Zeatin, a very potent cytokinin, is extracted from the young endosperm of maize.

many workers but organogenesis was never observed. The first convincing demonstration of organ formation from endosperm was by Johri and Bhojwani (1965) in *Exocarpus cupressiformis*. Seeds (lacking seed coat) cultured on White's medium containing casein hydrolysate (CH)+IAA+ kinetin developed shoot buds all round the endosperm (Fig. 11.20). In a single explant as many as 8 buds developed. These buds originated from the peripheral cells of the endosperm and possessed distinct shoot apices and well differentiated vasculature. Since then organogenesis has been induced in the endosperm cultures of many species (*see* Table 11.1).

TABLE 11.1 Species which have been reported to form shoots or plantlets from endosperm tissue. (*after* Bhojwani and Razdan, 1996).

Species	*Reference*
Actinidiaceae	
Actinidia chinensis	Gul *et al.* (1982)
Apiaceae	
Petroselinum hortense	Masuda *et al.* (1977)
Euphorbiaceae	
Codiaeum variegatum	Chikkannaiah and Gayatri (1974)
Jatropha panduraefolia	Srivastava (1971)
Putranjiva roxburghii	Srivastava (1973)
Loranthaceae	
Dendrophthoe falcata	Nag and Johri (1971, 1974)
Scurrula pulverulenta	Bhojwani and Johri (1970)
Taxillus vestitus	Nag and Johri (1971)
Moraceae	
Morus alba	Thomas *et al.* (1999)
Poaceae	
Oryza sativa	Nakano *et al.* (1975) Bajaj *et al.* (1980)
Rosaceae	
Prunus persica	Shu-quiong and Jia-qu (1980)
Pyrus malus	Mu *et al.* (1971)
Rutaceae	
Citrus grandis	Wang and Chang (1978)
Santalaceae	
Exocarpus cupressiformis	Johri and Bhojwani (1965)
Santalum album	Lakshmi Sita *et al.* (1980)

In loranthaceous and santalaceous parasites shoot buds differentiate either directly by the activity of the peripheral cells of the endosperm, or the endosperm may first proliferate to form a callus and then exhibit organogenesis. In mistletoes the callused endosperm forms shoot buds as well as haustoria. In euphorbiaceous members the organogenesis is almost always preceded by callusing of the endosperm.

For shoot bud differentiation from endosperm tissue an exogenous cytokinin is always required. In *Scurrula pulverulenta* and *Taxillus vestitus* cytokinin alone induces shoot differentiation, whereas in *Dendrophthoe falcata*

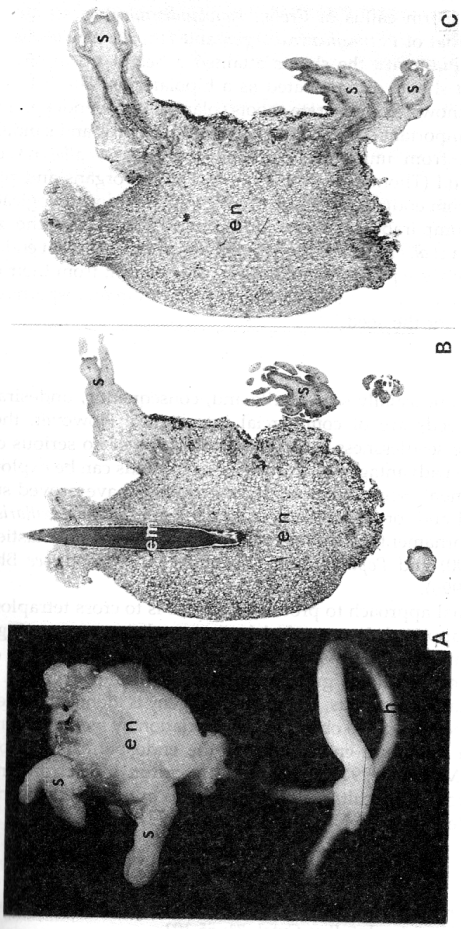

Fig. 11.20 Endosperm morphogenesis in 'seed' (endosperm + embryo) cultures of *Exocarpus cupressiformis*, on White's medium + casein hydrolysate + IAA + kinetin. **A**. 5-week-old culture showing germinated seed and shoot buds formed by the endosperm. **B, C**. Longisections of the seed shown in A; the shoot buds have arisen from the peripheral cells of the endosperm. (*em*, embryo; *en*, endosperm; *h*, hypocotyl; *s*, shoot bud; *after* Johri and Bhojwani, 1965)

Apart from being a nutritive tissue, endosperm also regulates the precise mode of embryo development. Removed from the influence of the endosperm, very young embryos often fail to mature even in the richest artificial nutrient medium. Moreover, the isolated older embryos often skip the normal stages of embryogeny and show precocious germination in cultures.

MORPHOGENETIC STUDIES

In nature the endosperm grows as a formless mass of cells and shows negligible differentiation, if any. It has a short life-span; it is either consumed by the time the seed attains maturity or shortly after its germination. The question often asked in this regard is: as to why do the two products of double fertilization (the zygote and the primary endosperm nucleus) follow such diverse modes of development that one forms a well organized embryo and the other an amorphous mass of cells?

Following the discovery of syngamy (Strasburger, 1884) and triple fusion (Nawaschin, 1898), embryologists regarded endosperm as a second embryo modified to serve as a nutritive tissue for the embryo (LeMonnier, 1887; Sargant, 1900). Sargant suggested that in triple fusion "... the third nucleus may have been introduced to maim the second embryo from the beginning and secure the survival of the first without struggle". That the triploid state of the tissue is not the cause for the formless nature of the endosperm tissue is suggested by the fact that in the Onagraceae, where embryo as well as endosperm are derived from the fusion products of one male and one female nucleus, the diverse destinies of the zygote and the primary endosperm nucleus are maintained. Actually, the question posed above is not unique to the endosperm and embryo. A similar question can be asked with wider implications: as to why are the derivatives of a zygote so different as mesophyll cells, companion cells, or, say, root hairs? An indirect approach to this problem is to isolate the specialized cells in question and grow them on a nutrient medium under aseptic conditions to see whether the potentiality for forming complete plants is retained by these cells or it has been lost in the process of differentiation to perform a particular function.

Lampe and Mills (1933) grew young corn endosperm on a medium containing an extract of potato or young corn and observed slight proliferation of endosperm layers adjacent to the embryo. After many years of work, LaRue (1949) was able to raise, for the first time, continuously growing tissue from the immature endosperm of corn. Subsequently, tissue cultures were established from the immature endosperm of *Asimina triloba* (Lampton, 1952), *Lolium perenne* (Norstog, 1956) and *Cucumis sativa* (Nakajima, 1962). A common feature of all these studies was that the endosperm grew in cultures only if it was excised at the right stage of development; 7-10 DAP (days after pollination) for *Cucumis*, 9-10 DAP for *Lolium*, and 8-11 DAP for *Zea*. In other words, endosperm younger than 7 DAP or older than 11 DAP failed to proliferate. Tamaoki and Ullstrup (1958) suggested that certain physiological changes occur in the corn endosperm 12 DAP which render it incapable of responding to the treatments in cultures. Sturani and Cocucci (1965) have mentioned that there is marked decrease in almost all enzyme activities in the endosperm of maturing seeds.

Unlike the above mentioned observations, Rangaswamy and Rao (1963) demonstrated that it is possible to induce divisions in mature endosperm of *Santalum album* and raise continuously growing tissues from them. This report was followed by similar observations on *Croton, Jatropha, Osyris, Putranjiva, Ricinus* and a host of other plants. The trick involved in successfully growing the mature endosperm was the initial presence of the embryo; if only endosperm pieces are cultured they fail to proliferate. The exact role of embryo in the proliferation of mature endosperm is at present a matter of speculation. However, it is evident from the work of Brown *et al.* (1970) that some factor/s is/are contributed by the germinating embryo which is/are essential for the activation of mature and dried endosperm of castor-bean. These investigators have reported that endosperm pieces from dried seeds of castor-bean do not grow in cultures. However, if endosperm pieces are excised from germinated seeds they exhibit proliferation, and there is a direct relationship between the number of days for which the seeds have germinated and the number of cultures showing endosperm proliferation. In *Croton* it is possible to replace the 'embryo factor' by gibberellin. It is well known that during germination the embryo releases gibberellin-like substances. Loranthaceous parasites, however, do not show dependence of mature endosperm on embryo for callusing or organogenesis.

LaRue (1949) grew corn endosperm on nutrient medium containing besides its basic constituents (minerals, vitamins, sugar), tomato juice, green corn juice, yeast extract, or cow's milk. Of these, tomato juice supported maximum growth but on this medium the results were not consistent. Yeast extract could fairly substitute tomato juice in supporting the growth of the callus tissue, and the results were also consistent. Subsequently, Straus (1960) showed that asparagine (1.5×10^{-2}M) could suitably replace yeast extract. Nakajima (1962) carried out a series of experiments and concluded that for satisfactory growth of the immature endosperm of *Cucumis* a nutrient medium containing an auxin, a cytokinin, and a source of organic nitrogen was necessary. Nutrient medium with similar composition has proved satisfactory for the culture of mature endosperm of various plants.

Differentiation

As mentioned earlier, the endosperm tissue, in nature, lacks differentiation; it does not even show vascular elements. However, in the cultures of this tissue the differentiation of tracheidal elements is quite common. It was first reported in *Ricinus* by Satsangi and Mohan Ram (1965).

In 1947, LaRue reported that in corn endosperm cultures "... less than one in thousand developed roots and a single one formed a root-shoot axis and miniature leaves ...". Corn endosperm has since then been cultured by

it does so when added to the medium along with a low concentration of IAA. Of the various cytokinins tested for shoot bud differentiation from the endosperm of *Scurrula* and *Taxillus*, 6-g,g-dimethylallylamino purine proved most effective and triacanthine the least. Among euphorbiaceous members, endosperm callus of *Croton bonplandianum* has so far formed only roots and that of *Putranjiva roxburghii* and *Jatropha panduraefolia* roots and shoots. In *Putranjiva* the shoots attained a height up to 15 cm and, often, roots and shoots differentiated as a bipolar axis. The list of plants (*see* Table 11.1) known to regenerate shoots/plantlets from endosperm tissue includes some important crop plants, such as apple, citrus and sandalwood. Plants derived from immature endosperm of *Morus alba* have been established in soil (Thomas *et al.*, 1999). Most of the organs and plantlets differentiated from endosperm tissue are triploid and morphologically and anatomically comparable to their counterparts formed by the zygotic embryo. Masuda *et al.* (1977) reported that plants originated from endosperm callus of parsley were predominantly diploid. However, from their work it is not clear whether the callus actually developed from endosperm or from some other part of the seed.

Applications

Triploid plants are usually seed-sterile and, consequently, undesirable for plants where seeds are of commercial importance. However, there are instances where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. In such cases triploids can be exploited for plant improvement. Some examples where triploids have proved superior to their diploid and/or tetraploid counterparts are: *Petunia axillaris* (more vigorous and ornamental; Gupta, 1982), tomato (larger and tastier fruit; Kagan-zur, 1990) and *Populus* (more desirable pulp wood; *see* Bhojwani and Razdan, 1996).

The traditional approach to produce triploids is to cross tetraploids and diploids. This approach is not only laborious and time consuming but in many cases it may not be possible. Thus, regeneration of triploids directly from endosperm cells holds great promise in plant improvement.

In pseudogamous apomicts, where seed progeny is identical to the female parent, it may be possible to obtain genetic variation by *in vitro* regeneration of plants from immature endosperm, as the endosperm is a product of fertilization. Matzk (1991) succeeded in callus induction and root regeneration from such cultures of *Poa pratensis* but so far no plants have been regenerated (*see* den Nijs and van Dijk, 1993).

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CHAPTER

12

EMBRYO

The fertilized egg is called zygote. Following a predetermined mode of development (embryogeny) it gives rise to an embryo, which has the potentiality to form a complete plant. A typical dicotyledonous embryo, as seen in median longitudinal section, is drawn in Figure 12.1A (also *see* Fig. 15.1E). It comprises an embryonal axis with two cotyledons attached to it laterally. The portion of embryonal axis above the level of cotyledons is called epicotyl (distinctly seen in Fig. 15.1E), and the portion below the level of cotyledons is known as hypocotyl. The epicotyl terminates into plumule (embryonic shoot), and the hypocotyl at its lower end bears radicle (embryonic root). The embryo of a monocotyledon differs from that of a dicotyledon mainly in having only one cotyledon (Fig. 12.1B). The embryos

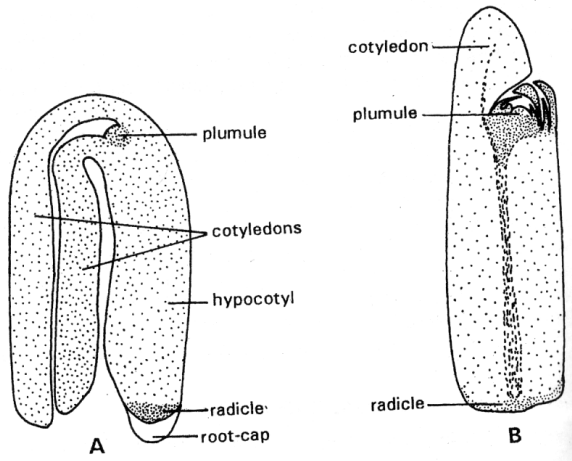

Fig. 12.1 Median longitudinal section of: **A.** Mature dicotyledonous embryo of *Capsella bursa-pastoris*, and **B.** Mature monocotyledonous embryo of *Hydrilla verticillata*. (**B.** *after* Maheshwari and Johri, 1950)

of grasses show a highly specialized structure (Fig. 12.6M). In this chapter the chief structural changes associated with principal types of embryogeny in angiosperms are described. At the end some aspects of the nutrition of embryo are also discussed.

ZYGOTE

From the time of its formation the zygote undergoes a period of dormancy, which varies greatly with species. Generally, this period is shorter where the endosperm is Cellular than where it is Nuclear. During the dormancy period many striking changes occur, as a result of which the zygote at the end of the dormancy period shows more pronounced polarity than the egg.

Soon after syngamy the large vacuole in the zygote starts shrinking. As a result, the cell size is also reduced. This reduction may be minor as in *Capsella*, or highly significant as in cotton. In the latter, the size of the zygote is reduced to half the original size of the egg within 24 hours after pollination. The decrease in cell size causes additional accumulation of cytoplasm at its chalazal end where first division of the zygote will take place. Another immediate response to syngamy is increase in the number of active dictyosomes which is related to wall synthesis around the zygote. The ribosomes aggregate to form polysomes, indicating the beginning of metabolic activities.

The zygote, ready to divide, is a highly polarized, isolated cell. The wall, which was restricted to the micropylar end of the egg before fertilization, is now complete around the zygote. It becomes an isolated cell in the sense that the plasmodesmatal connections with the surrounding cells are blocked. The nucleus, surrounded by a large number of plastids and mitochondria, is located at the chalazal region of the cell (apical pole). The micropylar end of the zygote (basal pole) is occupied by one or more vacuoles, and the number of cell organelles in this region is extremely small.

EMBRYOGENY

In the majority of angiosperms the zygote divides transversely, resulting in a small apical cell (conventionally designated *ca*) toward the interior of the embryo sac and a large basal cell (conventionally designated *cb*) toward the micropyle. Rarely, the division of the zygote may be vertical (Ranunculaceae) or oblique (*Triticum* sp). The variations in the developmental pattern of embryo during early embryogeny are common to monocotyledons and dicotyledons. Differences appear when the initials of plumule and cotyledon(s) are laid down. From the 2-celled stage until the initiation of organs the embryo is commonly called proembryo.

PROEMBRYO

In a 2-celled proembryo, the basal cell (*cb*) either remains undivided, or it

Ceratocephalus falcatus: The zygote (Fig. 12.3A) divides transversely, forming a small apical cell (*ca*) and a large basal cell (*cb*; Fig. 12.3B). Cell *cb* divides transversely, forming two superposed cells *ci* and *m* (Fig. 12.3C), and cell *ca* undergoes a vertical division giving rise to two juxtaposed cells (Fig. 12.3D). Thus, a T-shaped, 4-celled proembryo is formed. Of the two daughter cells of *cb*, cell *ci* divides transversely giving rise to *n* and *n'* (Fig. 12.3D). These two cells divide further forming a linear row of 3 or 4-celled suspensor*. Cell *m* and its derivatives divide by a vertical division to form 4-6 cells. Oblique periclinal divisions in each of these cells result in an inner set of cells (the initials of root apex) and an outer set of cells (the initials of root cap; Fig. 12.3H-K).

In the meantime the daughter cells of the apical cell divide by another vertical division at right angles to the first division (Fig. 12.3E), forming a quadrant *q*. A transverse division of the quadrant results in an octant arranged in two tiers (l, l_1) of four cells each (Fig. 12.3F, G). Vertical divisions in tiers l and l_1, give rise to a globular proembryo (Fig. 12.3H). Periclinal divisions in the pripheral cells of the globular proembryo demarcate a single-layered dermatogen, the future epidermis (Fig. 12.3I). Cells of the tier l differentiate the initials of plumule and the two cotyledons. The latter flank the former on either side (Fig. 12.3 I-K). Growth in the cotyledonary zones is much faster than in the plumular zone. As a result, in the mature embryo the plumule is enclosed at the base of the two cotyledons (Fig. 12.3L). The tier l_1 finally forms the hypocotyl-radicle axis.

Embryogeny in Monocotyledons

As mentioned earlier, the development of embryo up to the octant stage is almost similar in monocotyledons and dicotyledons. The differences appear later.

The main difference between the mature embryos of monocotyledons and dicotyledons is in the number of cotyledons. The single cotyledon in monocotyledons has been regarded by many authors as a terminal structure. Wardlaw (1955) remarked: "In the dicotyledonous embryo the plumule is typically distal and is situated symmetrically between two equivalent cotyledons: in the monocotyledonous embryo the shoot apex occupies a lateral position in the somewhat cylindrical embryo and cotyledon is terminal." However, extensive ontogenetic work on monocot embryos by Swamy and his co-workers (*see* Lakshmanan, 1972) has established that the epicotyl in monocotyledonous embryos is truly a terminal structure; both epicotyl and cotyledon arise from one and the same terminal tier. The apparent lateral position of the epicotyl is due to early growth of the cotyledon; the epicotyl, after initiation, shows slow growth. This is well illustrated by the embryogeny in *Najas* (*see* below).

According to Lakshmanan (1972) the chief difference between embryos of the two groups lies in the number of cells of the terminal quadrant of

*Suspensor is an ephemeral structure, chiefly concerned with nutrition of the proembryo. It is described on pages 215-222.

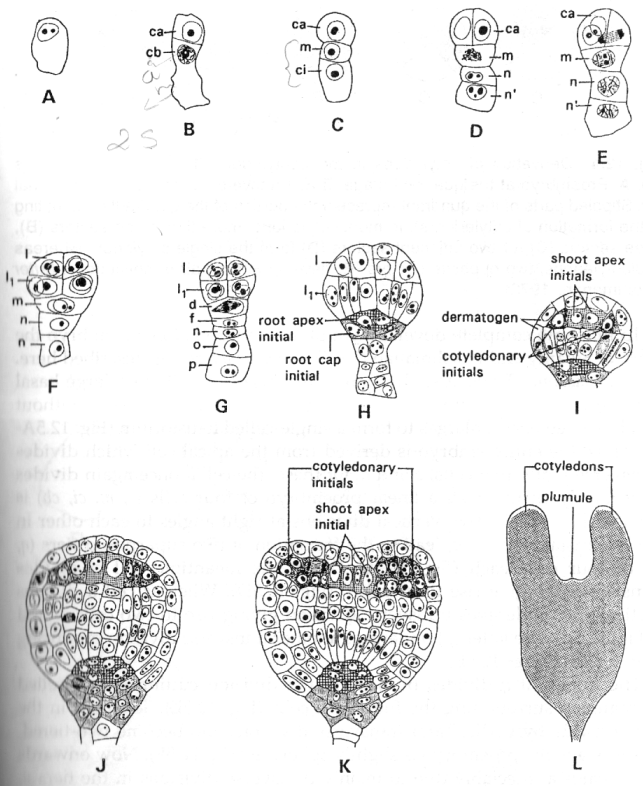

Fig. 12.3 Development of embryo in *Ceratocephalus falcatus*. For details refer to the text. (*after* Bhandari and Asnani, 1968)

a proembryo (Fig. 12.4 A) which contribute to the formation of cotyledon(s) and epicotyl; where the number of cells forming cotyledon(s) in the two types of embryos is same, the relative position of the cells in the quadrant may be different. In dicotyledons it is the two opposite cells of the terminal quadrant that give rise to the two cotyledons (Fig. 12.4E). In monocotyledons the number of cells involved in cotyledon formation is variable, practically all the four cells (except a few cells derived from one of the quadrant cells) in the Philydraceae (Fig. 12.4B), three cells of the quadrant in the Iridaceae, Pontederiaceae and Sparganiaceae (Fig. 12.4C), and two adjacent cells in the Amaryllidaceae, Hydrocharitaceae and Potamogetonaceae (Fig. 12.4D).

undergoes a transverse division to form two cells, *m* and *ci*. In the latter case, depending on whether the division of the apical cell (*ca*) is transverse or vertical, the 4-celled proembryo is linear or T-shaped, respectively (Fig. 12.2A-C). In the linear proembryo the two daughter cells of *ca* (l,l_1), by two vertical divisions at right angles to each other, give rise to an octant with two superposed tiers (l,l_1) of four cells each (Fig. 12.2A). An octant of similar configuration is formed by the T-shaped proembryo by one transverse division and one vertical division (Fig. 12.2B). The T-shaped proembryo can also form an octant of a different configuration, in which all the eight cells are included in the same tier (*q*); an axial quadrant is surrounded by four peripheral cells (Fig. 12.2C). Thus, in angiosperms two types of octant configurations occur : (a) the component cells are arranged

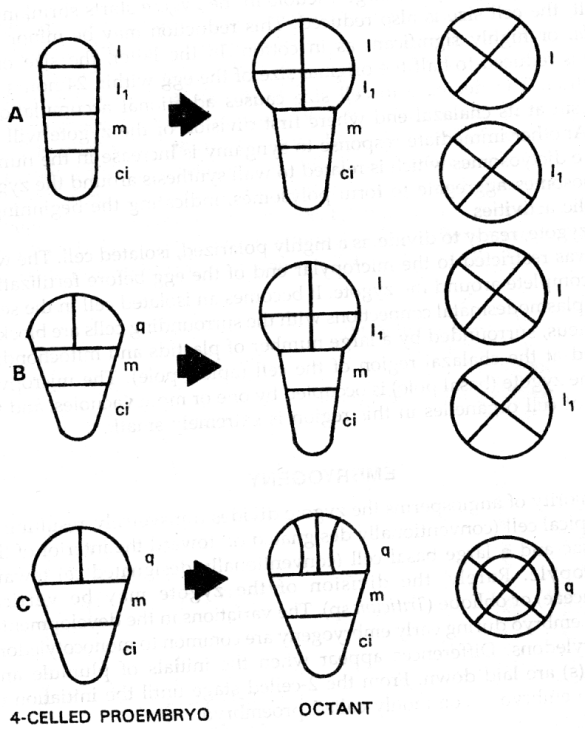

Fig. 12.2 Formation of two different types of octants. On the extreme right are transverse sections of the tiers *l*, l_1 and *q* of the octants. For details refer to the text. (*after* Swamy, 1962)

in two superposed tiers of 4-cells each (*Beta, Capsella, Poa, Sagittaria*), for (b) all the 8-cells occur in a single tier (*Lactuca, Muscari*). As is evident from the cited examples, both types of octants occur in monocotyledons as well as in dicotyledons. It is at the octant stage of the proembryo that the destinies of various cells become determined.

Embryogeny in Dicotyledons

Based on the plane of division of the apical cell in the 2-celled proembryo, and the contribution of the basal cell (*cb*) and the apical cell (*ca*) in the formation of embryo proper, five chief types of embryogeny have been recognized by Maheshwari (1950)*.

A. The apical cell of the 2-celled proembryo divides longitudinally.
   (1) The basal cell plays only a minor role or none in the subsequent development of the embryo proper - *Crucifer Type* or *Onagrad Type* (*e.g.*, Annonaceae, Brassicaceae, Onagraceae, Pedaliaceae, Ranunculaceae, Scrophulariaceae).
   (2) The basal cell and apical cell both contribute to the development of embryo - *Asterad Type* (*e.g.*, Asteraceae, Balsaminaceae, Violaceae, Vitaceae,

B. The apical cell of the 2-celled proembryo divides transversely.
I- The basal cell plays only a minor role or none in the subsequent development of the embryo proper.
   (3) The basal cell usually forms a suspensor - *Solanad Type* (*e.g.*, Campanulaceae, Linaceae, Solanaceae, Theaceae).
   (4) The basal cell undergoes no further division, and the suspensor, if present, is always derived from the apical cell - *Caryophyllad Type* (*e.g.*, Caryophyllaceae, Crassulaceae, Haloragaceae).
II- (5) The basal and apical cells both contribute to the development of embryo - *Chenopodiad Type* (*e.g.*,Boraginaceae, Chenopodiaceae).

These five types of embryogeny refer to those plants where first division of the zygote is transverse, so that an apical cell and a bsal cell are formed. Johansen (1950) has recognized a sixth type of embryogeny, called *Piperad Type*, which includes those cases where first division of the zygote is vertical (Loranthaceae, Piperaceae).

Often the type of embryogeny is constant throughout a family. Rarely, however, the same species may show more than one well established trend of embryo development. For example, in *Anemone rivularis, Solanad Type* as well as *Crucifer Type* of embryogeny occur regularly.

To illustrate complete development of a dicotyledonous embryo the work of Bhandari and Asnani (1968) on *Ceratocephalus falcatus* (Ranunculaceae) is described. In this species the embryogeny is of the *Onagrad Type.*

*Following Maheshwari (1950) this classification of embryogeny is given under dicotyledons but it appears to be equally applicable to monocotyledons. For example, the embryogeny in *Najas lacerata*, described under "Embryogeny in Monocotyledons" in this chapter, conforms to the *Caryophyllad Type* (also *see* Comparative Embryology of Angiosperms, INSA, New Delhi, 1970).

The portion of the embryonal axis below the level of scutellum is the radicle, which bears an apical meristem and a root cap at the lower end. The radicle and its cap are enclosed in coleorhiza, which is the undifferentiated lower part of the proembryo. The portion of the embryonal axis above the level of scutellum is the epicotyl. It comprises a shoot apex with some leaf primordia, enclosed in a hollow foliar structure, the coleoptile. The latter has an apical pore. On one side the coleorhiza gives out a small outgrowth called epiblast.

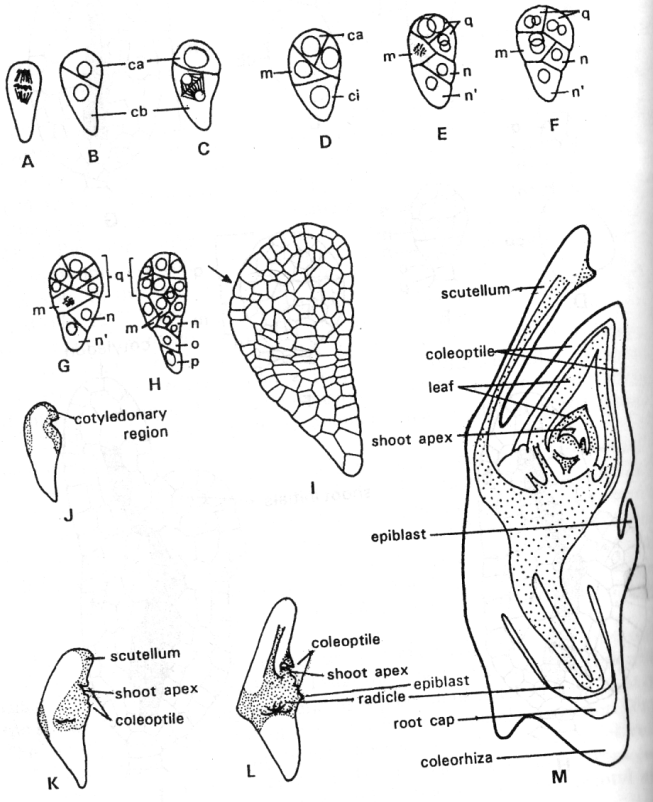

Fig. 12.6 Development of embryo in *Triticum*. For details refer to the text. (after Batygina, 1969)

Batygina (1969) has described in great detail the mode of embryo development in *Triticum*. All the four species of *Triticum* examined by her show a fixed pattern of embryogeny, which is so different from that in dicotyledons and other monocotyledons that she remarked: "... the unique mode of embryogenesis in the Gramineae may allow the separation of a new type of embryogeny - *Graminad Type*". The early part of embryogeny in *Triticum* is characterized by the regular occurrence of oblique divisions.

The first nuclear division of the zygote (Fig. 12.6A) is followed by laying down of an oblique wall, cuttting a small apical cell (*ca*) and a large basal cell (*cb*; Fig. 12.6B). Cell *cb* again divides obliquely forming cells *ci* and *m* (Fig. 12.6C, D). The upper end of the wall formed during this division connects with the wall separating *ca* and *cb*. The third division occurs in the cell *ca*, in a plane perpendicular to the first division of the zygote. Thus, a T-shaped proembryo (4-celled) is formed (Fig. 12.6D). However, the orientation of the walls is very different from the T-shaped tetrads in either dicotyledons or other monocotyledons. This characterizes the wheat embryogeny.

Cell *ci* divides by a wall at right angles to the wall between *ci* and *m*, resulting in the formation of cells *n* and *n'* (Fig. 12.6E). Divisions of the daughter cells of *ca* are in the same plane as the first division in *ca* but at right angles to it, forming the typical quadrant *q* (Fig. 12.6E, F). The cell *m* divides vertically into two cells (Fig. 12.6 E-H). Further divisions occur in various planes (Fig. 12.6G-I).

Organogenesis sets in at 16-32 celled stage of the proembryo. The first organ to be initiated is the single cotyledon or scutellum. Its differentiation starts with growth in the apical-lateral region of the proembryo (Fig. 12.6I; arrow marked), involving sectors, *q*, *m*, and *n*. With further development a constriction appears opposite the scutellum (in the sector *ca*) demarcating it from the rest of the embryo (Fig. 12.6J). This is followed by the appearance of primordia of coleoptile and then the shoot apex close to the notch (Fig. 12.6K, L). The radicle differentiates endogenously in the central zone of the embryo (Fig. 12.6L, M). As in *Najas*, in *Triticum* also the epicotyl is formed by the terminal tier (*q*). Its apparent lateral position in the mature embryo is a secondary feature. It arises due to active growth of the cotyledon leaving behind the epicotyl.

SUSPENSOR

The suspensor is an ephemeral structure, found at the radicular end of the proembryo. The suspensor grows much faster than the embryo during early stages of embryo development and usually attains its maximum size by the globular or early heart-shaped stage (Fig. 12.7). In a mature seed only remnants of the suspensor may be seen. The suspensor shows great variation with regard to its size, shape and the number of its component cells. The variations are usually related to its chief function of the nutrition of embryo.

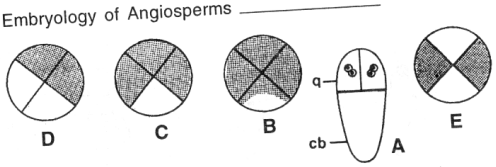

Fig. 12.4 Derivation of cotyledons in monocotyledons (**B-D**) and dicotyledons (**E**). **A.** Proembryo at the quadrant stage. **B-E.** Transverse sections of the terminal tier. Stippled parts of the quadrant represent the portion of the quadrant contributing to the formation of cotyledon(s). In monocotyledons, more than three sectors (**B**), three sectors (**C**), or two adjacent sectors (**D**) form the single cotyledon, whereas in dicotyledons, two opposite sectors (**E**) develop into a pair of cotyledons. (*after* Lakshmanan, 1972)

To illustrate complete development of a monocotyledonous embryo the work of Swamy and Lakshmanan (1962) on *Najas lacerata* is described here.

Najas lacerata: Transverse division of the zygote results in a large basal cell (*cb*) and a small apical cell (*ca*; Fig. 12.5A). The basal cell, without dividing even once, enlarges to form a single-celled haustorium (Fig. 12.5A-D). Thus, the entire embryo is derived from the apical cell which divides transversely into two cells, *c* and *d*. Of these, the cell *d* once again divides transversely. In this way a linear proembryo of four cells (*c, m, ci, cb*) is formed (Fig. 12.5B). Two vertical divisions at right angles to each other in the two distal cells (*c, m*) lead to the formation of two superposed tiers (*q, m*) of four cells each (Fig. 12.5C, D). In the meantime cell *ci* divides transversely to give rise to *n* and *n'* (Fig. 12.5D). Whereas cell *n* divides vertically, *n'* undergoes transverse division giving rise to two cells, *o* and *p* (Fig. 12.5E). The latter (*p*) undergoes another transverse division producing cells *h* and *s* (Fig. 12.5F).

The quadrant *q* divides by a periclinal division cutting a four-celled dermatogen surrounding the four axial cells (Fig. 12.5E). The cells in the tier *m* divide by vertical and transverse divisions and become two-tiered. At this stage the proembryo is slightly spherical (Fig. 12.5F). Now onwards it elongates appreciably due to mainly transverse divisions in the tiers *m* and *n*. At the stage when embryo becomes oval the central core of cells in the tiers *q, m,* and *n* differentiates into plerome initials (stippled cells in Fig. 12.5G).

At the eight-celled stage of the tier *q* (4 axial cells and 4 circumaxial cells) three of the axial cells divide faster than the fourth one. This disturbs the symmetry of the proembryo, and its top becomes notched (Fig. 12.5H). The rapidly growing portion of the tier *q* forms the single cotyledon (Fig. 12.5I), and the slow growing tissue, derived from the fourth axial cell, gives rise to the initials of epicotyl. The radicle is organized from the derivatives of *n*. Thus, in *Najas* the single cotyledon and the epicotyl are distinctly terminal structures, ontogenetically.

Grass embryo. The monocotyledonous embryo of grasses is strikingly different from that of other monocotyledons in its mature structure as well

as development. A mature embryo of *Triticum* (Fig. 12.6M), for example, has a single cotyledon, called scutellum. In a median longitudinal section of the mature embryo it appears laterally attached to the embryonal axis.

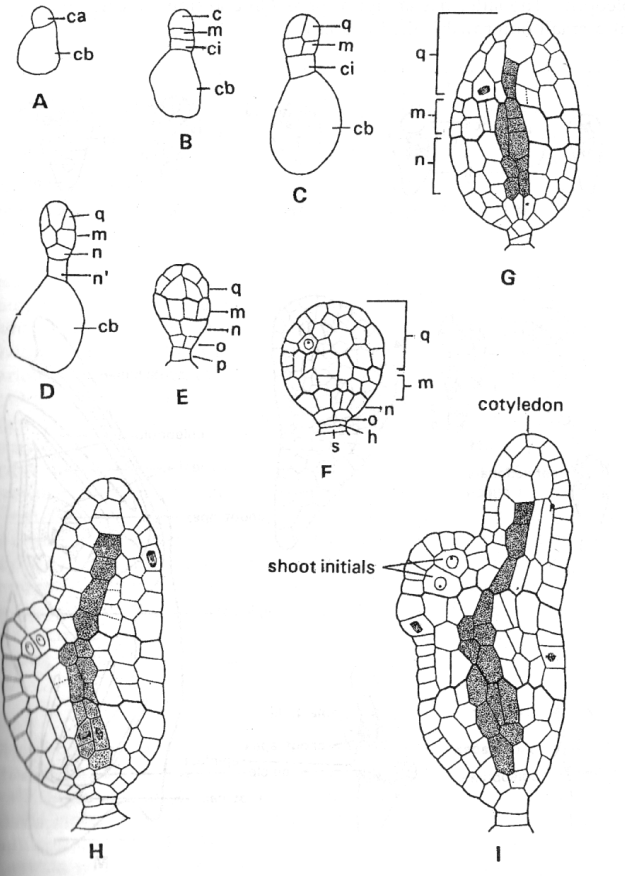

Fig. 12.5 Development of embryo in *Najas lacerata*. For details refer to the text. (*after* Swamy and Lakshmanan, 1962)

The suspensor anchors the embryo to the embryo sac and pushes it deep into the endosperm so that the embryo lies in a nutritionally favourable environment. In the Loranthaceae (where the embryo sac grows up to various heights in the style), the egg is fertilized at the tip of the embryo sac in the style, and the endosperm is formed inside the ovary. In these plants the suspensor is exceptionally long so that it is able to bring the embryo down into the endosperm.

As early as 1902, Lloyd had shown the occurrence of suspensor haustoria in some Rubiaceae, and remarked: "The function of the suspensor in these forms is, therefore, not alone to bring the embryo into favourable position with relation to the food supply in a mechanical sense, but to act as a temporary embryonic root". The suspensor haustoria are of wide occurrence in angiosperms. They show structural similarities with `transfer cells' involved in short distance transport of materials across the cell membrane. These features support the idea that the suspensor is actively involved in the absorption of nutrients from various ovular and extra-ovular tissues and translocating them to the embryo proper.

In the families Orchidaceae, Podostemaceae and Trapaceae, where the endosperm is absent, the embryo possesses extensively developed suspensor haustoria. A large variety of suspensor organizations is met within the Orchidaceae (Swamy, 1949). It may be: (a) single-celled; enlarged to become sac-like, conical, tubular, or cyst-like (Fig. 12.8 A; *e.g., Cypripedium,*

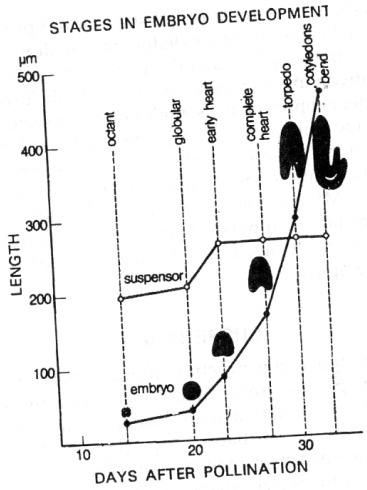

Fig. 12.7 Comparative growth of suspensor and embryo in *Diplotaxis erucoides* (*after* Simoncioli, 1974)

Dendrobium); (b) uniseriate filament of 5-10 cells (Fig. 12.8B) which grows beyond the micropyle, and upon reaching the placenta penetrates it by issuing haustorial branches (*Habenaria, Ophrys, Satyrium*); (c) looking like a bunch of grapes (Fig. 12.8C; *e.g., Epidendrum, Sobralia*); (d) the suspensor initial divides by three vertical divisions and the eight cells thus formed elongate downward enveloping more than half of the embryo (Fig. 12.8D; *e.g., Cottonia, Lusia, Vanda*); or (e) the zygote divides to produce an irregular mass of 6-10 cells, some of which, situated toward the micropylar end, elongate and form tubular structures (Fig. 12.8E; *e.g., Cymbidium, Eulophia*).

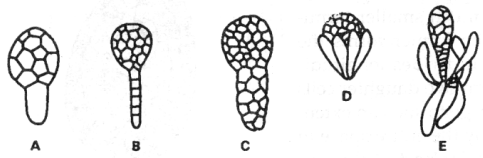

Fig. 12.8 Some suspensor types found in orchids. (*after* Swamy, 1949)

In *Dicraea stylosa* (Podostemaceae) the basal cell (*cb*) enlarges and contains two hypertrophied nuclei. During further development it gives out several thin-walled haustorial branches (Fig. 12.9) which grow in between the two integuments and are larger than the embryo proper. Nuclei do not migrate into these branches.

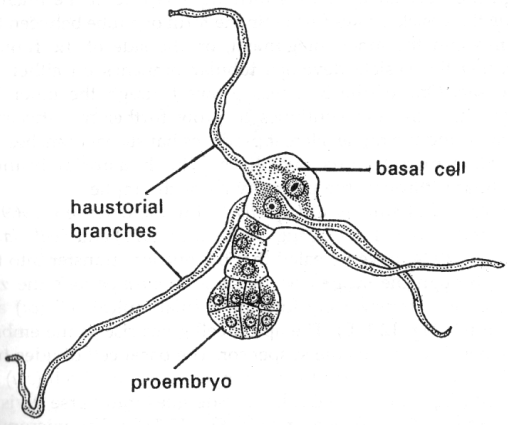

Fig. 12.9 Globular proembryo of *Dicraea stylosa*. The basal cell has enlarged and given out haustorial branches. (*after* Mukkada, 1962)

Suspensor haustoria are also commonly found in the families Crassulaceae, Fabaceae, Fumariaceae, Haloragaceae, Tropaeolaceae, etc. In the Haloragaceae, the suspensor haustoria appear like synergids. In *Myriophyllum*, a member of this family, the 2-celled proembryo comprises a large basal cell and a much smaller terminal cell. The former forms the haustoria. It divides longitudinally to form two daughter cells which enlarge to such an extent as to occupy the entire space in the micropylar part of the embryo sac. The nuclei are extremely hypertrophied (Fig. 12.10).

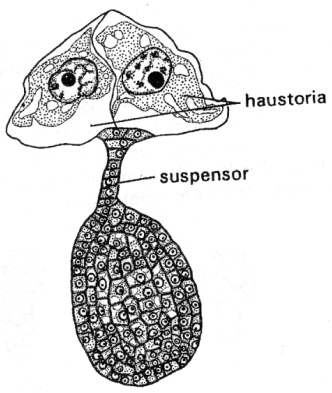

Fig. 12.10 Globular proembryo of *Myriophyllum intermedium*. The two daughter cells of the basal cell (*cb*) have enlarged and their nuclei hypertrophied to assume haustorial structure. (*after* Bawa, 1969)

In *Sedum* (Crassulaceae) the apical cell of the 2-celled proembryo gives rise to the embryo proper as well as the 4-celled suspensor whereas the basal cell enlarges without any division. The nucleus in the latter becomes prominently hypertrophied, and the cell grows through the nucellar epidermis forming a vesicle at the micropyle. In *S. ternatum* the vesicle grows further in the form of a tube between the nucellar epidermis and the inner integument, on the side of the funiculus. In *S. ochroleucum* the vesicle develops tubular branches on either side of the embryo sac. One of the branches grows through the inner integument digesting its cells, and continues to grow further in between the inner integument and the raphe. Here it produces haustorial branches, resembling the hyphae of a fungus mycelium. The haustorial branches grow intracellularly through the parenchyma of the raphe.

Fine structural studies in *Capsella* (Schulz and Jensen, 1969), *Phaseolus* (Clutter and Sussex, 1968; Yeung and Clutter, 1979) and *Stellaria* (Newcomb and Fowke, 1974) have revealed that the nutrient transfer into the embryo may be through the suspensor. In *Capsella bursa-pastoris* the zygote (Fig. 12.11A) divides transversely forming a small apical cell (*ca*) and a large basal cell (*cb*; Fig. 12.11B). The apical cell gives rise to the embryo proper, and the basal cell forms the suspensor. The basal cell divides transversely into a large cell (*ci*) toward the micropyle and a small cell (*cm*) toward the apical cell (Fig. 12.11C). The cell *cm* undergoes transverse divisions giving rise to a row of 6-10 suspensor cells (Fig. 12.11D). The micropylar cell (*ci*) simply enlarges without any division and forms the haustorial cell of the suspensor (Fig. 12.11D). It is anchored to the micropylar end of the embryo sac. Schulz and Jensen (1969) have shown that almost from the beginning of embryo development certain ingrowths of the wall, projecting into the cytoplasm, are present at the micropylar end of the basal cell. With the development of the embryo these finger-like projections become more prominent, and by the globular stage they extend over the entire micropylar half of the haustorial cell; highest concentration of the projections is always maintained at the micropylar tip of the haustorial cell (Fig. 12.11E).

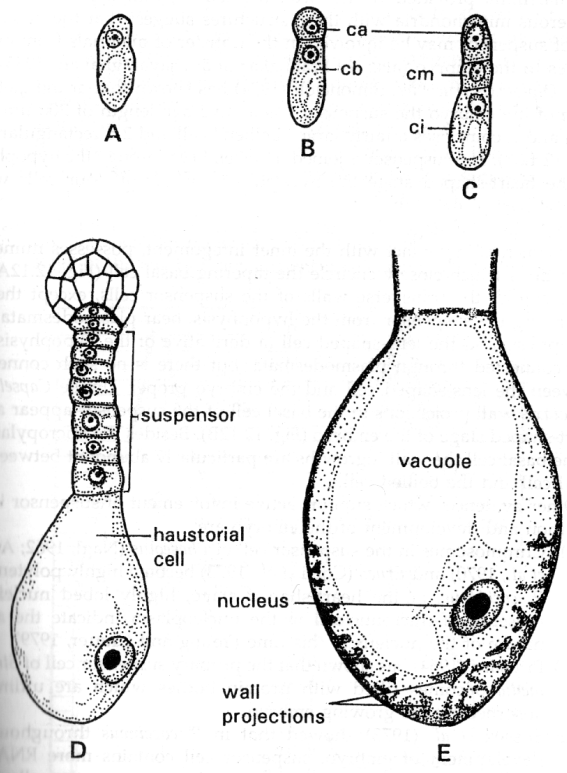

Fig. 12.11 Early stages in the development of embryo in *Capsella bursa-pastoris*. The basal cell of the suspensor becomes haustorial. **A**. Zygote. **B**. Two-celled stage. **C**. Three-celled stage. **D**. Globular proembryo. **E**. Diagrammatised ultrastructure of the haustorial cell to show wall projections at the micropylar end. (**A-D**, *after* Soueges 1914, 1919; **E**, *after* Schulz and Jensen, 1969)

times higher than the embryo proper. After the formation of cotyledons, when the suspensor is in the initial stages of degeneration, the level of gibberellin in suspensor cells drops dramatically and there is a considerable increase in its level in the embryo proper which is presumably due to the transfer of gibberellin from suspensor cells to the embryo proper.

In vitro studies have also shown that suspensor plays an important role in the development of young embryos (Corsi, 1972; Cionini *et al.*, 1976; Yeung and Sussex, 1979). Cionini *et al.* (1976) observed that older embryos (5 mm or over in length) of *Phaseolus coccineus* grew equally well when cultured with intact suspensor or without it. However, in the cultures of younger embryos (heart-shaped and early cotyledonary stage, 0.5-1.5 mm in length), removal of the suspensor significantly reduced the frequency of plantlet formation. Confirming the importance of suspensor for the growth of young embryos of *P. coccineus*, Yeung and Sussex (1979) showed that 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 12.1). The growth promoting activity of the suspensor was maximum at the early heart-shaped stage of the embryo. Of the various growth regulators tested, gibberellin at a concentration of 5 mg l^{-1} most effectively substituted suspensor. This is in accordance with the observations of Alpi *et al.* (1975) that gibberellin activity in the suspensor of *P. coccineus* is maximum at the heart-shaped stage.

UNUSUAL FEATURES

Embryo Development in *Paeonia*

The development of embryo in *Paeonia*, a dicot, is very peculiar (also *see* Chapter 16). According to Yakovlev and Yoffe (1957) and Yakovlev (1969) it is very similar to some gymnosperms. Initially the divisions in the zygote are free-nuclear giving rise to a coenocytic structure (Fig. 16.3 A-D). Subsequently, the nuclei assume a peripheral position, and the centre is occupied by a large vacuole (Fig. 16.3D). At a later stage the nuclei become delimited by walls, and a cellular mass is formed (Fig. 16.3E). A few embryonal primordia differentiate from the peripheral cells (Fig. 16.3F) but only one of them matures into a normal dicotyledonous embryo. These observations are supported by Cave *et al.* (1961), Matthiessen (1962) and Moscov (1964).

Under-developed and Reduced Embryos

Irrespective of its mode of development, a mature embryo generally possesses an embryonic root (radicle), an embryonic shoot (plumule), and one or two cotyledons. However, some groups of plants are characterized by the presence of reduced embryos, lacking the differentiation of these

TABLE 12.1 The effect of suspensor on *in vitro* growth and development of *Phaseolus coccineus* embryos. (*after* Yeung and Sussex,1979).

Initial stage[a] *(fresh weight in mg)*	*Treatment*	*Fresh weight*[b] *± standard error (N) (mg)*	*per cent*[d] *embryos forming plants (No. of embryos cultured)*
Early heart-shaped (0.87±0.02)	Embryo proper only	3.19±0.52(10)	41.5(89)
	Embryo proper with suspensor attached	8.91±1.16[c](10)	88.4(95)
	Embryo proper with detached suspensor in direct contact	6.22±0.78[c](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 1cm away		33.3(30)
Late heart-shaped (1.07±0.07)	Embryo proper only	17.2±2.84(5)	94.4(18)
	Embryo proper with suspensor attached	15.4±1.41(6)	94.4(18)
Early cotyledon (3.92±0.19)	Embryo proper only	20.3±2.5(7)	100(18)
	Embryo proper with suspensor attached	24.4±2.75(11)	100(19)

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

organs (Balanophoraceae, Burmanniaceae, Gentianaceae, Orchidaceae, Orobanchaceae, Pyrolaceae, Rafflesiaceae). This feature is generally found in saprophytes and parasites.

The seeds of *Eranthis hiemalis* (Ranunculaceae), at shedding, contain a pear-shaped embryo (*see* Fig. 13.9), without a radicle, plumule, or cotyledons. After the seed has fallen on the ground the embryo undergoes differentiation and attains the conventional morphology of dicotyledonous embryo.

In the Orobanchaceae and Orchidaceae the embryos never differentiate a true radicle, plumule and cotyledons. A mature seed of broomrape

The wall ingrowths of the haustorial cell of suspensor are similar to the filiform apparatus in the synergids, and the wall projections found in the transfer cells associated with short distance transfer of materials in the glandular tissues, nectaries, haustoria of parasitic plants, etc. In all these cases the wall projections are PAS-positive (rich in insoluble polysaccharides), and consist of a central electron dense core bordered by an electron translucent zone. The greatly increased surface area of the plasmalemma provided by the wall projections, and the association of numerous mitochondria with these structures suggest that the haustorial cell of suspensor may be involved in the transfer of materials from ovular tissues to the embryo (also *see* Prabhakar and Vijayaraghavan, 1983).

In *Diplotaxis erucoides*, Simoncioli (1974) has shown that at the globular stage of the embryo the suspensor has an average length of 200 µm (Fig. 12.7) and it comprises a fairly large "bellied" cell and 2-5 rectangular cells (Fig. 12.12A). The suspensor is joined to the embryo through the hypophysis. By the heart-shaped stage the hypophysis has cut off four cells which become part of the embryo.

At the globular stage of the embryo the micropylar part of the endosperm wall, which is in contact with the inner integument, possesses numerous projections which closely encircle the tapering basal cell (Fig. 12.12A). At this stage all the transverse walls of the suspensor cells, except the one separating the suspensor from the hypophysis, bear plasmodesmata. The hypophysis and the lens-shaped cell (a derivative of the hypophysis) are also connected through plasmodesmata, but there is no such connection between the lens-shaped cell and the embryo proper. Unlike *Capsella*, in *Diplotaxis* wall projections in the basal cell of the suspensor appear at the heart-shaped stage of the embryo (Fig. 12.12B). Besides the micropylar end of the basal cell the wall ingrowths are particularly abundant between the basal cell and the bellied cell.

Other evidences which support active involvement of suspensor in the nutrition and development of the embryo are:

(i) Chromosomes in the suspensor cell of *Phaseolus* (Nagl, 1962; Avanzi *et al.*, 1970) and *Eruca* (Corsi *et al.*, 1973) become highly polytenic. In *P. coccineus*, at the heart-shaped stage, highly lobed nuclei and presence of micronucleoli in the nucleoplasm indicate the active nature of the nucleus at this time (Yeung and Clutter, 1979).

(ii) Prichard (1964) has shown that the primary suspensor cell of *Stellaria media* becomes filled with protein bodies which are ultimately absorbed by the growing embryo.

(iii) Sussex *et al.* (1973) showed that in *P. coccineus* throughout the development of embryo, suspensor cell contains more RNA and protein, and synthesizes them at a higher rate than the cells of the embryo proper at the corresponding stage.

(iv) According to Yeung and Cluttter (1979), abundance of plastids in the suspensor cells of *P. coccineus* at the late globular and heart-shaped stages, and their disappearance with the maturity of the embryo suggest that they have a specific role in the development of the embryo.

(v) Yeung (1980) has demonstrated that if the developing pods of *P. coccineus* are fed with ^{14}C-sucrose the radioactivity appears first in the suspensor and later in the embryo proper. This suggests that suspensor is the site of nutrient uptake particularly at the heart-shaped stage. In maturing embryos the cotyledons themselves take up the nutrients (also *see* Brady and Combs, 1988).

(vi) Enormous development of smooth endoplasmic reticulum, a characteristic feature of many glandular cells in plants, has been observed in the suspensor cells of *Ipomoea purpurea* by Ponzi and Pizzolongo (1972). These suspensor cells also exhibit the presence of a special type of tubules with a uniform diameter and resistance to digestion by pepsin. Such tubules have been observed in the leaf gland cells of *Phaseolus vulgaris* (Steer and Newcomb, 1969). These features of suspensor cells prompted Ponzi and Pizzolongo to ascribe a secretory function to this organ. On the basis of some indirect evidences the nature of the substance/s secreted by these cells was suggested to be gibberellin-like. This view is supported by Alpi *et al.* (1975). They have shown that in *P. coccineus*, at the heart-shaped stage of the embryo, the suspensor cells have gibberellin activity 30

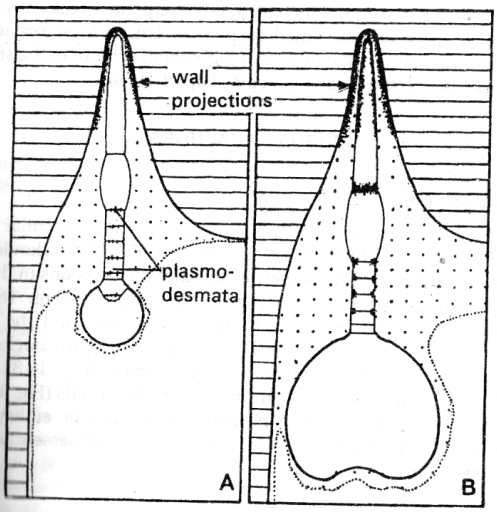

Fig. 12.12 *Diplotaxis erucoides.* Diagrammatic representation of globular (**A**) and heart-shaped (**B**) embryos. In **A**, note the presence of plasmodesmata on cross walls of suspensor cells and wall projections on the micropylar part of endosperm wall. In **B** the wall projections have also developed in the basal cell of suspensor. (Modified *after* Simoncioli, 1974)

(*Orobanche*) contains a sub-globose embryo. The pole of the embryo toward the micropyle is the morphological radicular pole with small cells, and that away from the micropyle is the morphological plumular pole. During seed germination, in nature, the plumular pole remains inactive, cotyledons never differentiate, and the entire plant is derived by the sole activity of the radicular pole. Similarly, in the Orchidaceae the mature embryo is an ovoidal mass of cells, and its germination is monopolar. However, unlike *Orobanche*, in the Orchidaceae it is the plumular pole which is active (also *see* Chapter 17).

Mature embryo of *Cuscuta* is highly coiled. It bears a few plumular scales but there is no differentiation of cotyledons and radicle. Actually, this plant does not possess true roots at all.

PHYSIOLOGICAL AND GENETIC CONTROL OF EMBRYOGENESIS

The location of plant embryo deep within the maternal tissue renders it difficult to access during early embryogenesis. Therefore, for long not much progress could be made in the areas of physiological and genetic control of embryo development in higher plants. Recently, plant embryogenesis has received renewed attention and advances are being made in our knowledge about regulation of gene expression during embryo development (Goldberg *et al.*, 1989, 1994; Wang and Cuming, 1996). Genetic approaches have begun to reveal genes required for pattern formation, cell differentiation and organ development during embryogenesis. Some information has also become available on hormonal control of morphogenesis during embryo development. The nutrition of embryo, based on *in vivo* and *in vitro* studies, is described under a separate head in this chapter.

Physiological Aspects

A major step during early embryogenesis is the transition from the radial symmetry of the globular embryo to bilateral symmetry, leading to the differentiation of cotyledons and embryonal axis. Polar transport of auxin has been implicated in this morphogenic process. Interference with this transport by application of an auxin polar transport inhibitor, such as N-1- naphthylphthalamic acid (NPA), quercitin, TIBA and cinnamic acid, to globular embryos either blocks the transition from the globular to the heart stage or induces abnormalities such as differentiation of a ring of cotyledonary tissue rather than two distinct cotyledons in *Brassica juncea* (Liu *et al.*, 1993) and differentiation of additional shoots in wheat (Fischer *et al.*, 1997).

Based on their studies on the effect of *in vitro* treatment of globular embryos of wheat with polar auxin transport inhibitors, Fischer *et al.* (1997) have proposed a model of regulation of shoot meristem differentiation (Fig.12.13). According to them, auxin is synthesized in the lower part of the embryo and is tansported along the longitudinal axis to the site where

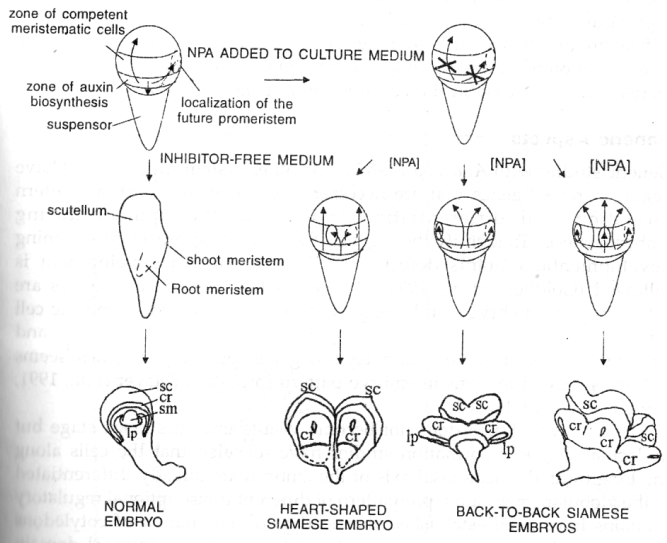

Fig. 12.13 Model for auxin fluxes regulating shoot meristem differentiation in wheat embryos (the arrows depict auxin fluxes). Normally, the auxin is synthesized in the lower part of the globular embryo and is transported along the longitudinal axis to the area where scutellum differentiates and laterally to the area where shoot differentiates. Application of the auxin polar transport inhibitor, NPA, reduces the polar transport of the auxin leading to accumulation of the auxin in the cells in which it is transported. Diffusion of the auxin to the neighbouring cells results in differentiation of additional shoot meristems. At low level, the inhibitor reduces but does not disrupt the polar transport of auxin which results in accumulation of auxin. Due to diffusion of the auxin to neighbouring cells, two adjacent shoot meristems differentiate. Such treatments result in the development of two heart-shaped Siamese embryos whose shoot meristems are adjacent. With a higher concentration of the inhibitor, the polar transport of the auxin would be more reduced, causing higher accumulation of auxin. Consequently, a second auxin flux may appear and might be perceived by the neighbouring cells as a signal by polar cells in the ring leading to differentiation of second shoot meristem at 180° from the first one. This would result in back-to-back Siamese phenotype with an extra shoot. At still higher concentration, NPA may result in more than one additional auxin flux, and 2-4 extra shoot meristems may differentiate in a ring. (*cr*, coleoptile ring; *lp*, first leaf primordium; *NPA*, N-1-naphthylphthalamic acid (NPA); *sc*, scutellum; *sm*, shoot meristem; *after* Fischer *et al.*, 1997)

during embryogenesis and produces seedlings without leaves. The *embryonic flower* mutant, on the other hand, develops a shoot meristem at the top of the embryonal axis but instead of leaves it produces flowers, suggesting that the fate of the shoot meristem was altered during embryogenesis.

The deletion of mature embryo regions can be traced back to histological defects at the proembryo and globular stages, suggesting that at least some of the genes responsible for establishment of embryo body plan are expressed during early embryogenesis and regulate early cell division. The division of a *emb30/gnom* zygote results in two similar-sized cells instead of unequal-sized terminal and basal cells, as found in wild type embryos. Later divisions in *emb30/gnom* embryos are also highly variable and abnormal. On the other hand, in *monopteros* mutants the division of zygote and a couple of following divisions are normal but at the octant stage it has four tiers of cells compared to the two tiers in wild type embryos. In the mutant, derivatives of all the four tiers of cells divide during subsequent stages in a pattern similar to the upper tier of the wild type embryos. Consequently, the hypocotyl and embryonic root, which in wild type embryos are derived from the basal tier, are missing in the mutant.

Agrobacterium T-DNA tagged *emb30/gnom* gene has led to the isolation of the EMB30/GNOM gene (Shevell *et al.*, 1994). This gene codes for a protein that is active throughout the plant life cycle and is involved in cell division, elongation and adhesion events required at many stages of sporophyte development, including embryogenesis. Thus, the EMB30/GNOM gene does not seem to establish the embryonic cell division pattern directly. Most likely it facilitates a pattern set by other genes (Goldberg *et al.*, 1994).

For modular organization of plant embryos, promoters of embryo-specific genes are required to regulate the expression of genes unique to each autonomous region of the embryo. It has been suggested that these genes interact with specific promoter elements, and their combinations give rise to the transcriptional pattern of the whole embryo (Goldberg *et al.*, 1994).

stm seedling looks essentially normal, except that it lacks a shoot meristem and, therefore, will not produce a shoot. In the wild type embryo, shoot meristem can be identified only during the torpedo stage long after all the other pattern elements are present (Burton and Poethig, 1993). Until early torpedo stage the shoot tip region of the *stm* mutant appears similar to that of the wild type embryo. It implies that the early development of *stm* mutant embryo is normal and the *stm* mutation blocks shoot meristem development only during the torpedo stage, much later than the GNOM and MP genes.

NUTRITION OF EMBRYO

There are two approaches to understand the nutrition of embryo. One is to examine the structures possibly involved in the nutrition of embryo inside the ovule, and their chemical analysis (*in vivo* studies). This would give an idea about the substances that nurture the embryo in nature. The other approach is to culture the embryos excised from ovules at various stages

of their development and investigate the optimal conditions of nutrition for their growth (*in vitro* studies).

In vivo Studies

ENDOSPERM. The chief source of nutrition of embryo inside the ovule is the endosperm. There is ample evidence to suggest that the endosperm, which mostly forms the immediate environment of the embryo, is rich in nutritive and growth regulating substances.

Smart and O'Brien (1983) examined the development of wheat embryo in relation to the neighbouring tissues, and concluded that the developing embryo is a powerful sink of nutrients. Within 7 days after anthesis cellularization of endosperm around the embryo is completed. The endosperm cells so formed surround most of the embryo. Unlike the normal endosperm the cells in this part of the endosperm (modified endosperm) are densely cytoplasmic with only a few vacuoles and accumulate no starch or protein. Whilst the embryo is less than 15 days old, the nucellar parenchyma cells and the modified endosperm cells are hydrolysed and completely digested. Soon after, the nucellar epidermis around the basal part of the embryo forms specialized cells which show PAS positive tips and wall ingrowths, giving the appearance of transfer cells.

Chemical analysis of coconut milk (liquid endosperm) has revealed that it is rich in inorganic ions and amino acids. It also contains sugars and growth regulators. Similarly, corn endosperm at the milk stage has been shown to contain large quantities of carbohydrates, amino acids, and protein complexes. The amino acid concentration shows a decreasing trend with the age of the endosperm. Corn milk is also rich in IAA. Several purine compounds, active in inducing cell divisions, have been identified in immature corn endosperm. Zeatin, a cytokinin, has been crystallized from the young corn endosperm. Endosperm of many cereals (wheat, rye, rice, barley) is known to contain auxin. Endosperm extracts of *Aesculus californica, Echinocystis macrocarpa, Persea americana, Pyrus amygdalus*, and some other plants have been reported to show gibberellin-like activity (Phinney *et al.*, 1957).

There is also direct evidence of the nutritive qualities of the endosperm tissue. Milk from immature coconuts is able to support the growth of isolated young embryos of many plants. Endosperm extracts of coconut as well as many other plants possess the ability to induce divisions in highly mature and differentiated cells.

Another feature of endosperm which enables it to nourish the embryo is its capacity to develop haustorial structures. They penetrate into various ovular tissues, absorb nutrients from there and transfer them to the embryo (for details *see* Chapter 11).

In the absence of endosperm, or when it is not sufficiently well developed, the embryo depends for its nutrition on some other structures. Mostly it is the suspensor haustorium which substitutes the endosperm. These haustoria obtain nutrients from various parts of the ovule and supply them to the

scutellum differentiates and laterally to the site where the shoot primordium differentiates. Disruption of polar transport by the inhibitors results in accumulation of auxin in the cells transporting auxin. The cells accumulating highest amount of auxin would be closest to the site of auxin synthesis. Diffusion of the auxin into neighbouring cells would result in the differentiation of additional shoots. The number of additional shoots would increase with increase in the concentration of the inhibitor.

Genetic Aspects

Genetic studies with *Arabidopsis* and a few other systems (maize, rice) have begun to reveal genes that are necessary for the events, such as pattern formation, cell differentiation and organ development during embryogenesis. To identify these genes, the classical approach of screening developmental mutants defective in normal embryo development is followed (Goldberg *et al.*, 1994). Approximately 1500 diverse genes are active in plant embryos, and many of these are expressed in specific cell types, regions and organs of embryo (Goldberg *et al.*, 1989; Perez-Grau and Goldberg, 1995). However, relatively few genes, probably 50 genes, seems to have specific functions in embryo pattern formation (Mayer *et al.*, 1991; Jurgens *et al.*, 1991).

The embryonal axis does not become visible until the heart stage but molecular and transformation studies have revealed that the cells along the longitudinal apical-basal axis of the embryo are already differentiated at the globular stage, and a pre-pattern of different transcriptional regulatory domains have been established that lead to differentiation of cotyledons and axis region at the heart stage. Probably each transcriptional domain sets in motion a cascade of events leading to the differentiation of specific embryo regions later in embryogenesis (Goldberg *et al.*, 1994).

Longitudinal axis of mature embryos is made of three regions that can be designated as apical region (includes cotyledons and shoot meristem), central region (includes hypocotyl) and basal region (includes radicle) (Fig. 12.14). Genetic studies have uncovered *Arabidopsis* mutants that alter the embryo body. Most of these mutants fall in three categories: (1) deletion of apical-basal pattern events, (2) affecting radial symmetry, and (3) inducing changes in the shape of the seedlings. The mutants of the category 1, that have been studied in some detail, include those causing deletion of: (a) the apical region (*gurke*), (b) the central region (*fackel*), (c) the central and basal regions (*monopteros, mp*), and (4) the apical and basal regions (*emb 30/gnom*). These mutant genes, specifying the embryo body plan, act after fertilization. Moreover, the loss of a specific region does not affect the adjacent region. For example, a *gurke* embryo lacks apical region but has normal central and basal regions. Similarly, the *mp* embryos are missing the central and basal regions but possess a normal apical region. Certain mutants of *Arabidopsis* affect only apical meristems, such as shoot and root meristems, and have no other effect on embryo development. For example, *shoot meristemless* (*stm*) mutant fails to differentiate the apical meristem

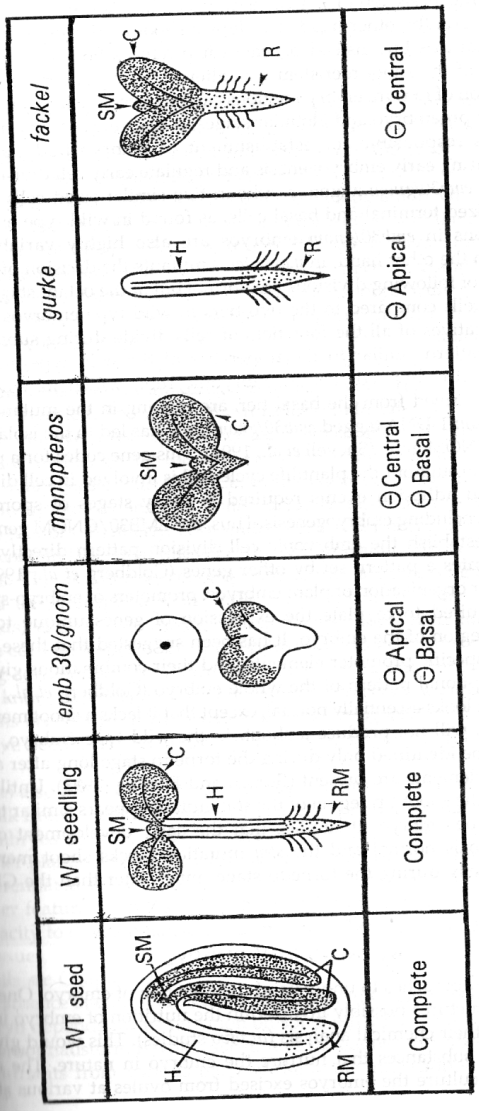

Fig. 12.14 Schematic representation of *Arabidopsis* pattern mutants. The densely stippled, blank, and lightly stippled areas represent the apical, central and basal regions, respectively. Strong (upper) and weak (lower) *gnom* phenotypes are depicted. (*WT*, wild type; *RM*, root meristem; *SM*, shoot meristem; *C*, cotyledon; *H*, hypocotyl; *R*, root; after Goldberg *et al.*, 1994)

developing embryo. The suspensor haustoria have been shown to be structurally well adapted for absorbing and short distance transportation of substances (for details *see* pages ...).

PSEUDO-EMBRYO SAC. In the Podostemaceae, the absence of endosperm is substituted by suspensor haustoria as well as pseudo-embryo sac, a structure found exclusively in this family. The nucellar cells below the megaspore mother cell enlarge longitudinaly, and their walls become thin and fragile. At the 4-celled stage of the proembryo the nucellar cells break down forming a large cavity, called pseudo-embryo sac (Fig. 12.15 A, B). In some genera (*Dicraea, Hydrobryum, Zeylanidium*) the pseudo-embryo sac develops as early as when the megaspore mother cell is undergoing meiosis.

The pseudo-embryo sac contains several free nuclei and dense cytoplasm which are gradually consumed by the developing embryo.

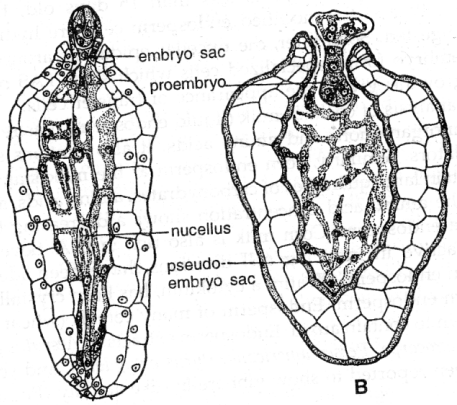

Fig. 12.15 Formation of pseudo-embryo sac in *Indotristicha ramosissima*. **A** Mature embryo sac stage with intact nucellus. **B.** Proembryo at the quadrant stage; the nucellar cells have broken down to form pseudo-embryo sac. (*after* Chopra and Mukkada, 1966)

PERISPERM. The nucellus is usually consumed by the developing endosperm. However, in certain families, such as Amaranthaceae, Cannaceae, Capparidaceae, Piperaceae, Portulacaceae and Zingiberaceae, the nucellar tissue surrounding the embryo sac persists in the seed and becomes densely packed with food materials. The persistent nucellus is called perisperm. In such cases the endosperm functions as an intermediary tissue in between the perisperm and the embryo, absorbing food stored in the former and passing it on to the latter.

In *Piper nigrum* (Piperaceae) the nucellar cells below the embryo sac divide faster than the rest of the tissue. Their activity increases enormously following fertilization. Walls between some of these cells break down and their nuclei fuse. These composite cells are large and densely cytoplasmic, and store oil globules. Starch appears in the nucellar cells surrounding the embryo sac and the cells of the nucellar epidermis. The quantity of starch increases from the chalazal end toward the base of the embryo sac. Ninety-five per cent of a seed in this plant is perisperm (Fig. 12.16). The endosperm, present around the embryo, is highly reduced as compared to the perisperm.

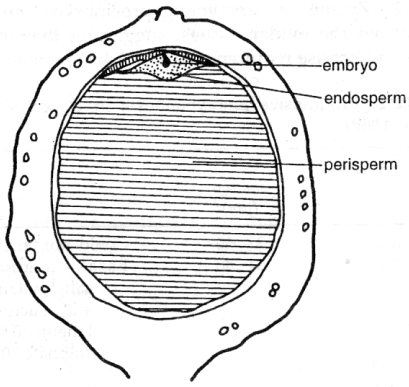

Fig. 12.16 *Piper nigrum*. Longitudinal section of the fruit at pre-globular stage of the proembryo; note the massive perisperm and extremely reduced endosperm. (*after* Kanta, 1962)

CHALAZOSPERM. In *Cyanastrum* the endosperm and the major part of the nucellus disappear during seed development. However, the nucellar cells in the chalazal region of the ovule, above the vascular bundle, divide actively forming a prominent tissue called chalazosperm. The cells of this tissue, which are loosely arranged, get filled with fat and starch, and serve as a substitute for the endosperm.

In vitro Studies

With respect to its nutrition, two phases of embryo development have been recognized by Raghavan (1966): (a) heterotrophic phase - during this phase, which may last up to the globular or even later stage, the embryo is dependent and draws upon the endosperm for its nutrition, and (b) autotrophic phase- during this phase, which starts at the late heart-shaped stage, the embryo is fairly independent for its nutrition. Consequently, isolated older embryos can be grown on a simple nutrient medium whereas

younger embryos require an elaborate medium for their successful culture. This point can be illustrated with the observations of Van Overbeek *et al.* (1941, 1942) on *Datura*: (a) mature embryos form normal seedlings on mineral salts and dextrose; (b) young torpedo-shaped embryos (up to 0.5 mm) require a medium containing minerals, dextrose, glycine, thiamine, ascorbic acid, nicotinic acid, vitamin B_{12}, adenine, succinic acid, and pantothenic acid; and (c) embryos smaller than 0.5 mm in length could be cultured successfully only with the addition of coconut milk to the medium required by torpedo-shaped embryos. A similar increasing autonomy of embryo with age is evident from the data for *Capsella bursa-pastoris* (*see* Table 12.2). Zygote or very young proembryos could not be grown in isolation from the ovular tissues, suggesting that they may have more elaborate and precise requirements which have not been worked out so far.

TABLE 12.2 Progressive embryogenesis in *Capsella bursa-pastoris*. (*after* Raghavan, 1966)

Developmental stage	*Length of embryo (μm)*	*Nutritional requirements*
Early globular	21- 60	Could not be cultured
Late globular	61- 80	Basal medium (macronutrient salts[1]+vitamins[2] + trace elements[3] + 2% sucrose) + IAA (0.1mg l^{-1}) + kinetin (0.002 mg l^{-1}) + adenine sulphate (0.001 mg l^{-1})
Heart-shaped	81- 450	Basal medium alone
Torpedo-shaped	451-700	Macronutrient salts[1]+ vitamins[2] + 2% sucrose
Walking stick-shaped and mature embryos	700 and larger	Macronutrient salts[1] + 2% sucrose

1. Macronutrient salts (mg/l): 480 $Ca(NO_3)_2.4H_2O$, 63 $MgSO_4.7H_2O$, 63 KNO_3, 42 KCl and 60 KH_2PO_4.
2. Vitamins (mgl^{-1}): 0.1 Thiamine hydrochloride, 0.1 Pyridoxine hydrochloride, 0.5 Niacin.
3. Trace elements (mgl^{-1}): 0.56 H_3BO_3, 0.36 $MnCl_2.4H_2O$, 0.42 $ZnCl_2$, 0.27 $CuCl_2.2H_2O$, 1.55 $(NH_4)_6Mo_7O_{24}.4H_2O$, and 3.08 Ferric tartrate.

It may be concluded from the data on *Datura* and *Capsella* (presented here) that at some stage during embryogenesis the embryo becomes metabolically adapted to carry out the synthetic processes necessary for its growth and morphogenesis and draws upon its own metabolites. Younger embryos lack one or more of these substances that will sustain their normal growth.

Osmotic value of the nutrient medium seems to be an important factor for the proper growth of the embryo. Ryczkowski (1960-1972) has shown

that in monocotyledons as well as in dicotyledons the values of osmotic pressure, viscosity, specific gravity, and concentrations of sugar and amino acids of ovular sap decrease with the age of the ovule. Accordingly, isolated mature embryos grow fairly well with 2% surcose in the culture medium but younger embryos require higher levels of the carbohydrate for normal embryogenic development. With lower levels of sucrose proembryos directly develop into weak seedlings displaying only those structures already present at the time of embryo excision (precocious germination). It has been suggested that the beneficial influence of the high osmotic pressure may be mediated through its effect on endogenous pool of growth regulators. Raghavan and Torrey (1969) had noted a stimulatory effect of high concentration (12-18%) of sucrose on *in vitro* development of excised globular embryos (smaller than 80 μm) of *Capsella bursa-pastoris*. However, a combination of IAA, kinetin and adenine sulphate added to the medium with only 2% sucrose provided the best medium for continued division and growth of proembryos. Similarly, abscisic acid has been shown to replace high level of osmoticum in preventing precocious germination of excised immature embryos of cotton (Dure, 1975).

Changing growth requirements of developing embryo necessitates the transfer of embryos from one medium to another in order to achieve their optimal *in vitro* growth. Monnier (1976, 1978) has described a culture method which allows complete development of 50 μm long embryos (early globular stage) of *Capsella* up to germination in the same culture plate without moving the embryo from its original position (for details *see* Fig. 12.17). The compositions of the two media used in the culture dish are given in Table 12.3. A similar technique was used by Liu *et al.* (1993) for *ex ovulo* culture of 8-celled proembryos of *Brassica juncea*.

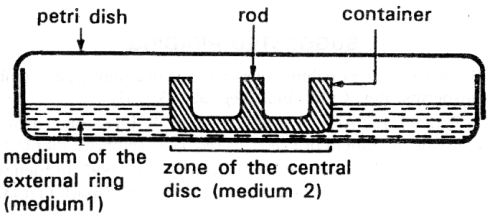

Fig. 12.17 A device allowing the juxtaposition of two media with different compositions. The agar medium 1 is liquefied by heating and poured in the petri plate around a central glass container. This medium will give the external ring. After cooling and solidification of this medium, the container is removed. In the central ring, thus formed, the medium 2, 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)

TABLE 12.3 Compositions 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 (*after* Monnier, 1976)

Amount (mgl^{-1})	*Constituents*	
	Medium 1 (external ring)	*Medium 2 (central zone)*
KNO_3	1900	1900
$CaCl_2.2H_2O$	484	1320
NH_4NO_3	990	825
$MgSO_4.7H_2O$	407	370
KCl	420	350
KH_2PO_4	187	170
Na_2.EDTA	37.3	-
$FeSO_4.7H_2O$	27.8	-
H_3BO_3	12.4	12.4
$MnSO_4.H_2O$	33.6	33.6
$ZnSO_4.7H_2O$	21	21
KI	1.66	1.66
$Na_2MoO_4.2H_2O$	0.5	0.5
$CuSO_4.5H_2O$	0.05	0.05
$CoCl_2.6H_2O$	0.05	0.05
Glutamine	-	600
B_1-B_6	0.1	0.1
Sucrose	-	180,000
Agar(Difco)	7000	7000

The nutritional requirements of zygote remain undefined. *In vitro* culture of excised zygotes of maize and barley has been possible only by supplementing the culture medium with actively growing nurse cells (Kranz and Lorz, 1993; Holm *et al.*, 1994; see page 170).

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maturity. Rarely, two germinable embryos may be formed in a seed. In *Zygophyllum fabago* the suspensor embryos may develop up to the heart-shaped stage.

Embryos from Cells of the Embryo Sac Other Than the Egg

In this category the most common source of additional embryo is the synergid. Depending on whether it arises from fertilized synergid or unfertilized synergid, the embryo may be diploid or haploid.

In *Aristolochia bracteata, Poa alpina* and *Sagittaria graminea* besides the egg and the polars, one or both the synergids may get fertilized. This can be brought about either by the entry of more than one pollen tube into the embryo sac or by the presence of additional sperms in the same pollen tube. In such a situation the zygotic as well as the synergid embryos are diploid. Embryos arising from unfertilized synergids are known in *Argemone mexicana* and *Phaseolus vulgaris*. In such cases the zygotic embryo can be distinguished from the synergid embryo (haploid) by its diploid nature.

Formation of embryos from antipodals is rather rare. It has been observed in *Paspalum scrobiculatum, Ulmus americana* and *U. glabra*. The antipodal cells may divide a few times to form proembryo-like structures (Fig. 13.4). However, they fail to grow into an adult embryo and there is no suggestion of antipodals forming germinable embryos.

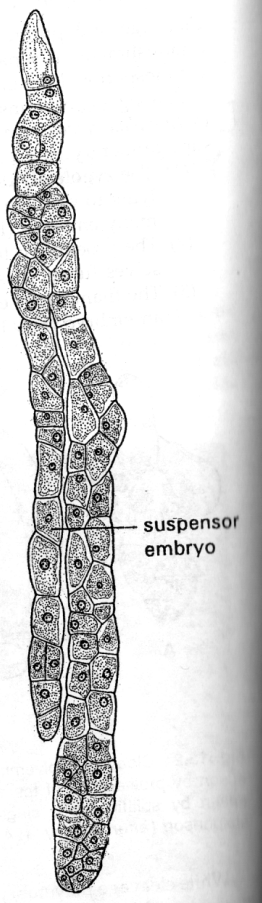


Fig. 13.3 Suspensor polyembryony in *Exocarpus sparteus*. (*after* Ram, 1959)

Most of the reports concerning endosperm cells forming embryos are doubtful. However, *Brachiaria setigera*, an apomict species, is the only example where endosperm has been reported to produce triploid embryos and seedlings (Muniyamma, 1978).

More Than One Embryo Sac in the Same Ovule

Multiple embryo sacs in an ovule may arise from:

(1) Derivatives of the same megaspore mother cell,

(2) Derivatives of two or more megaspore mother cells, or

(3) Nucellar cells.

Formation of twin embryo sacs within an ovule is known in *Casuarina equisetifolia, Citrus* and *Poa pratensis*. In *Pennisetum ciliare* 22 per cent seeds contain twin embryos. The normal embryo sac develops only up to the 4-nucleate stage, and the multiple embryos are formed by aposporous embryo sacs.

The members of the family Loranthaceae lack a conventional ovule. Numerous embryo sacs develop concurrently in the same ovary and their tips, carrying the egg apparatus, grow up to various heights in the style. After fertilization, the embryos grow downward and enter the composite endosperm (formed as a result of fusion of endosperms of all the embryo sacs in the ovary) in the ovarian cavity (Fig. 13.5).

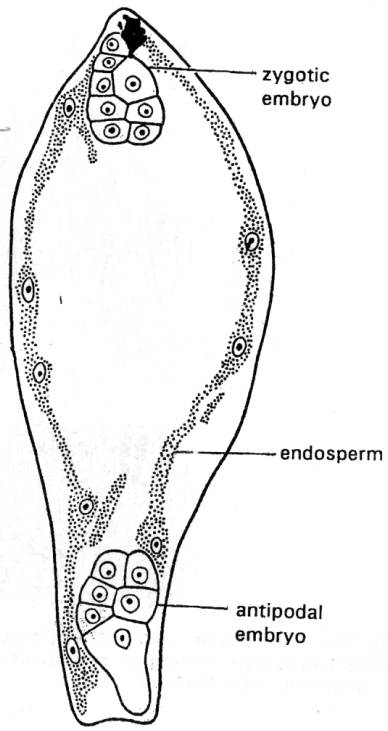


Fig. 13.4 Embryo sac of *Ulmus glabra* showing zygotic and antipodal embryos. (*after* Ekdahl, 1941)

All embryos but one collapse during seed development, resulting in monoembryonate seeds. Occasionally, however, two or more embryos may mature. In *Scurrula pulverulenta* about 2 per cent seeds are polyembryonate (Bhojwani, 1968). In such seeds there are either two fully developed green embryos, or there are more than two embryos of which only one is well developed, and the other under-developed and non-green. The only exception in this family is *Struthanthus vulgaris* which has only one embryo sac and thus does not exhibit polyembryony (Venturelli, 1981).

Activation of Some Sporophytic Cells of the Ovule

The embryos arising from the maternal sporophytic tissues (outside the

CHAPTER

13

POLYEMBRYONY

Polyembryony has been defined as the occurrence of more than one embryo in a seed (Fig. 13.1). However, it would be quite clear from the examples described in this chapter that the additional embryos do not always mature. They may become arrested at very early stages or may degenerate during the course of seed development. Therefore, if mature seeds are taken into account the percentage of polyembryony in a species would be far less than its actual frequency. Strictly speaking, polyembryony includes all those instances in which there is a clear indication of the actual occurrence of two or more proembryos or embryos in a developing ovule. Except for a few taxa (*Citrus*, *Mangifera*), polyembryony occurs only as an abnormal feature.

The first case of polyembryony was reported in certain orange seeds by Antoni van Leeuwenhoek (1719). Braun (1859) gave a survey of 58 cases of polyembryony recorded in the botanical literature at that time and referred them to four categories on the basis of the origin of the additional embryos. Polyembryony in angiosperms may arise by:

(1) Cleavage of proembryo,
(2) Formation of embryos by cells of the embryo sac other than the egg,
(3) Development of more than one embryo sac within the same ovule, or
(4) Activation of some sporophytic cells of the ovule.

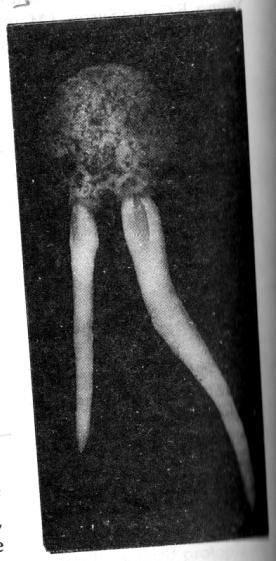


Fig. 13.1 Picture of germinated bi-embryonate seed of *Oxyra wightiana*. (*after* Bhojwani, 1966)

Cleavage Polyembryony

Cleavage and proliferation of zygote or its derivatives leading to the establishment of separate embryonal primordia is widespread among gymnosperms. In angiosperms this feature is less frequent.

Among angiosperms cleavage polyembryony is quite common in orchids. In *Eulophia epidendraea*, Swamy (1943) recorded three different modes of supernumerary embryo formation:

(1) The zygote divides irregularly to form a mass of cells of which those lying toward the chalazal end grow simultaneously and give rise to many embryos (Fig. 13.2A).
(2) The proembryo forms small buds or outgrowths which may themselves function as embryos (Fig. 13.2B).
(3) The filamentous embryo becomes branched, and each branch forms an embryo (Fig. 13.2C).

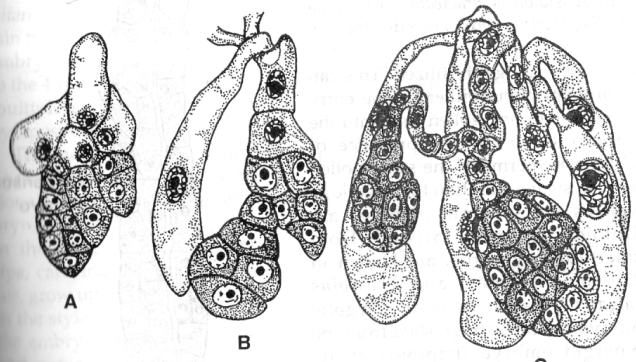


Fig. 13.2 Cleavage polyembryony in *Eulophia epidendraea*. **A.** Three embryos arisen by proliferation of the zygote. **B.** Budding of the embryo. **C.** Two embryos arisen by splitting of a single embryo. Large, vacuolated cells belong to the suspensor. (*after* Swamy, 1943)

While cleavage polyembryony arising during seed development is known in many orchids, the formation of plural embryos during seed germination is known only in *Vanda*. In this genus the apical promeristem of the embryo divides into a number of primordia (3-9) each of which further organizes into an embryo (Rao, 1965).

Suspensor polyembryony is a common feature in the genus *Exocarpus* (Fig. 13.3), a member of the Santalaceae. As many as six embryos may develop simultaneously in an ovule by the proliferation of the suspensor cells. Eventually, however, only one of them takes the lead and reaches

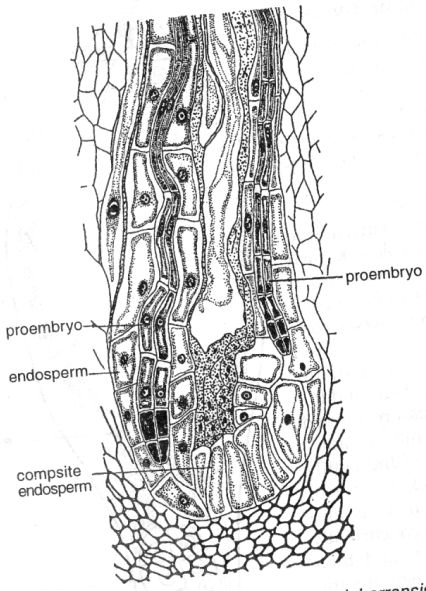


Fig. 13.5 False polyembryony in *Dendrophthoe neelgherrensis*. Longisection of central part of ovary, showing two proembryos embedded in the composite mass of endosperm. (*after* Narayana, 1954)

embryo sac) are called adventive embryos. The only maternal tissues which are known to form adventive embryos are the nucellus and the integuments. Besides the more popular examples of *Citrus* and *Mangifera*, nucellar polyembryony occurs in *Opuntia dillenii* and *Trillium undulatum*. Some species of *Citrus* are monoembryonate (*C. grandis, C. limon*) while others are polyembryonate (*C. microcarpa, C. reticulata*). Seeds with as many as 40 embryos have been recorded in *C. unshiu*. In polyembryonate species the adventive embryos arise by the proliferation of the nucellar cells. With rare exceptions (*Trillium undulatum*) nucellar embryos arise from the micropylar half of the nucellus. In *Mangifera* the nucellar cells destined to form adventive embryos can be distinguished from other cells of the nucellus by their dense cytoplasm and starchy contents. The inception of nucellar embryos takes place outside the embryo sac but they are gradually pushed into the embryo sac cavity (Fig. 13.6A-C) where they divide and differentiate into mature embryos (Fig. 13.7). The adventive embryos do not show synchronous development. A single seed may show embryos at various

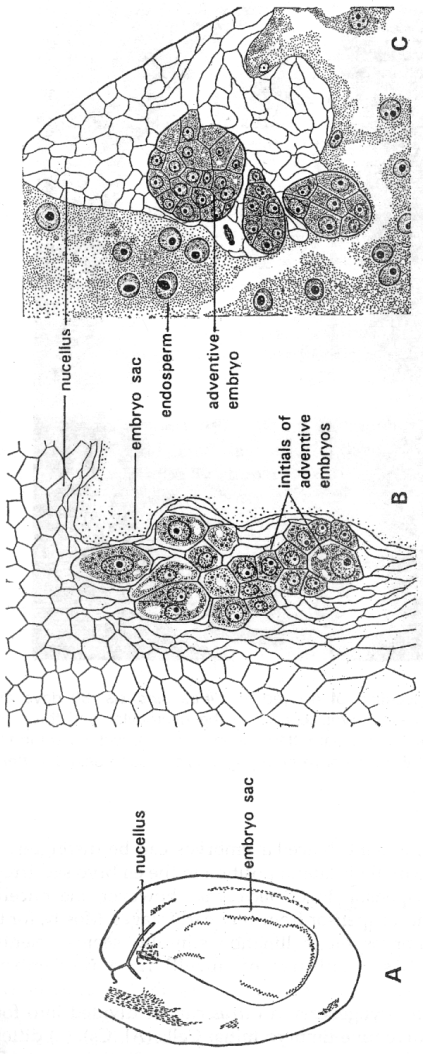


Fig. 13.6 Nucellar polyembryony in *Mangifera*. **A**. The emblocked region of the ovule shown in this diagram has been enlarged in **B** and **C**. **B**. *M. indica*; differentiation of embryo-forming cells. **C**. *M. odorata*; nucellar embryos growing into the embryo sac cavity. (*after* Sachar and Chopra, 1957)

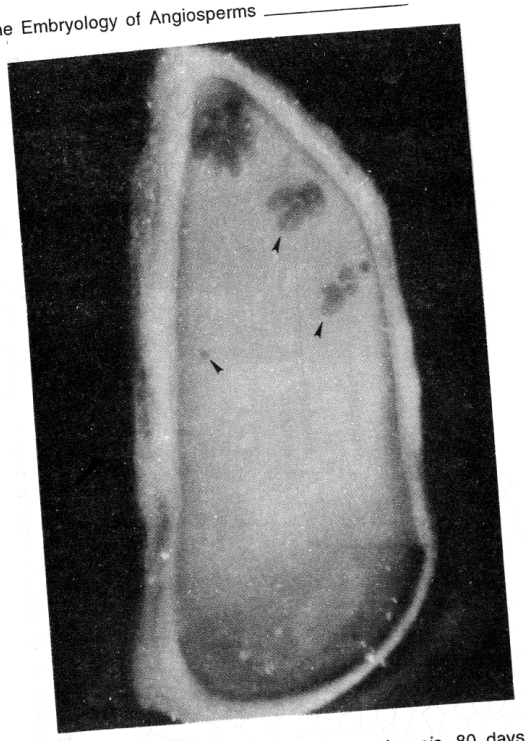


Fig. 13.7 Longisection of a seed of *Citrus sinensis*, 80 days after anthesis, showing adventive embryos located in the micropylar and three lateral islands (arrowhead). There is a clear difference in embryo development in four islands. In the chalazal half the adventive embryogenesis is suppressed. (*after* Wakana and Uemoto, 1988)

stages of development. Nucellar embryos can be distinguished from the zygotic embryo by their lateral position in the embryo sac, irregular shape, and lack of suspensor. In *C. microcarpa*, however, the nucellar embryos possess a distinct suspensor from very early stages. Mostly, for the initiation of nucellar embryos the pollination stimulus seems essential. Embryo formation in tissue cultures of nucelli from polyembryonate and monoembryonate species of *Citrus* is described in Chapter 17.

Adventive embryogenesis in *Citrus* can be divided into four steps: (1) formation of Adventive Embryo Initial Cells (AEICs), (2) differentiation of AEICs to acquire the appearance of a zygote, (3) division of AEICs, and (4) development of adventive embryos (Kobayashi *et al.*, 1978, 1981; Wilms *et al.*, 1983; Wakana and Uemoto, 1987, 1988). AEICs generally appear before anthesis and are characterized by their homogeneous cytoplasm, a large and spherical nucleus, irregular plastids with single thylakoids, numerous oval mitochondria (0.1-0.2μm in length) with short cristae, some single strands of rough ER together with many free ribosomes and polysomes and some dictyosomes with 4-5 cisternae and associated vesicles. The walls of these cells stain intensely and show the presence of granular, heterogeneously dispersed, electron dense material between the plasma membrane and the cell wall. Before dividing, the AEICs show complete change in their shape, composition and ultrastructure, and attain zygote-like appearance. The initials become spherical and are isolated from the surrounding degenerating nucellar cells by blocking of plasmodesmata, formation of a completely new cell wall, and ultimate breakdown of the original cell wall. After pollination their cytoplasm shows strong reduction in the number of proplastids and increase in the number of ER, free polysomes, dictyosomes and their associated vesicles, and the amount of lipid. In fertilized seeds the division of the AEICs generally occurs before the division of the zygote but after free nuclear endosperm has developed to some extent (Esen and Soost, 1977; Wilms *et al.*, 1983). In unfertilized seeds the AEICs may divide 40-50 days after anthesis.

The recent studies have clearly shown that the initiation of adventive embryos in *Citrus* occurs autonomously all around the embryo sac (Fig. 13.8); it is not affected by pollination, fertilization or the development of

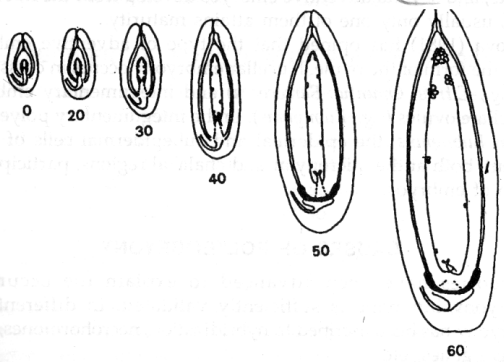


Fig. 13.8 A summary of the major events in the ontogeny of adventive embryos in a diploid seed of an apomictic *Citrus* cultivar, up to 60 days after anthesis (DAA; the number below each figure indicates DAA). Adventive embryo initial cells (AEICs) are formed all around the embryo sac before fertilization (O). During post-fertilization development of seed the AEICs split into several islands (20-50 DAA) due to cell division in endosperm and enlargement in the chalazal portion of the nucellus. While the micropylar AEICs develop normally into adventive embryos, those toward the chalazal end do not grow beyond the initial stage (60 DAA). (*after* Wakana and Uemoto, 1988)

zygotic embryo or endosperm (Kobayashi *et al.*, 1978, 1981; Wilms *et al.*, 1983; Wakana and Uemoto, 1987, 1988). However, the development of adventive embryos is strongly influenced by endosperm development. Lack or poor development of endosperm results in poor development of adventive embryos. Therefore, in unfertilized seeds the nucellar embryos fail to develop beyond the globular or early dicotyledonous stage and are incapable of normal germination under natural conditions (Wakana and Uemoto, 1988). The presence of endosperm promotes the development of adventive embryos at the micropylar end, but suppresses their development towards the chalazal end; the degree of suppression is directly related to the distance of embryos from the micropylar end. In normal seeds the AEICs at the chalazal end generally do not develop beyond the initial-celled stage (Fig. 13.8). The suppressive effect of endosperm on the development of adventive embryos toward the chalazal end is supported by the observations that in abnormal seeds with 3n embryo and 5n endosperm or where endosperm either aborts or fails to develop altogether the adventive embryos at the chalazal end may develop up to the early cotyledonary stage. Moreover, the AEICs from normal seeds if removed from the inihibitory effect of endosperm and cultured on simple, hormone-free medium give rise to normal embryos. The differential effect of endosperm on the adventive embryos at the micropylar end and at the chalazal end awaits explanation.

In *Opuntia dillenii* the egg apparatus, antipodals and polars invariably degenerate, and several adventive embryos develop from the nucellar cells. However, usually only one of them attains maturity.

Naumova (1981) has opined that the type of adventive embryony is related to the form of the ovule. Nucellar embryony occurs in crassinucellate ovules (*e.g.*, *Citrus*, *Opuntia*, *Sarcococca*) and integumentary embryony in tenuinucellate ovules (*e.g.*, *Euonymus*). In the integumentary polyembryony, as seen in *Euonymus*, the epidermal and subepidermal cells of the inner integument, both at the micropylar and chalazal regions, participate in the formation of embryos.

CAUSES OF POLYEMBRYONY

Many theories have been advanced to explain the occurrence of polyembryony but none is sufficiently validated. In different theories polyembryony has been ascribed to hybridization, necrohormones, the effect of recessive genes, etc.

Haberlandt (1921, 1922) proposed the "necrohormone theory". He regarded the degenerating cells of the nucellus as source of stimulus for the adjacent cells to divide and form adventive embryos. Haberlandt attempted to induce adventive polyembryony in *Oenothera* by damaging cells by pricking the ovules with a fine needle and by gently squeezing the ovary. In one ovule he got two embryos which he considered to be of nucellar origin. Attempts by subsequent workers to induce adventive polyembryony using Haberlandt's method yielded only negative results.

Leroy (1947) thought that polyembryony in mango was caused by one or more recessive genes. He states that while in the primary centre of origin (Eastern India) only monoembryonate forms with dominant genes occur, the secondary centres of origin (China, Philippines, Sudan) have forms with recessive genes and polyembryonate seeds. Actually, it is not true. At least some Indian varieties of mango *viz.*, 'Bambai', 'Fazli' and 'Langra' are polyembryonate.

According to Frusato *et al.* (1957), the embryo number in *Citrus* seeds may be influenced by the following factors:

(1) Age of the tree; increasing in older trees.
(2) Fruit-set; being higher in years of higher fruit- set.
(3) Nutritional status of the plant; decreasing with reduced food supply.
(4) Orientation of the branch of the tree; being higher on northern than on southern branches.

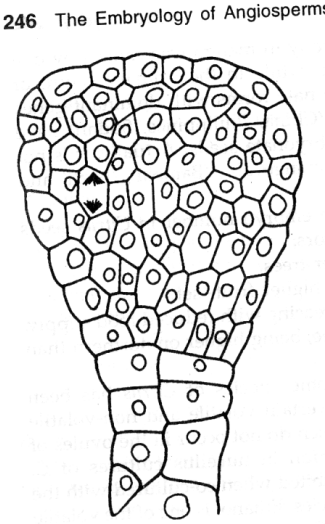
The monoembryonate condition in some species of *Citrus* has been ascribed to the synthesis and release of certain volatile and non-volatile embryogenic inhibitors in their ovules which do not occur in the ovules of polyembryonate species. Embryo formation in nucellus cultures of *C. reticulata*, a polyembryonate species is inhibited when co-cultured with the ovules of *C. medica*, a monoembryonate species. Ethanol is one of the volatile inhibitors produced by the ovules of *C. medica*. When applied at a concentration equal to that produced by the ovules of *C. medica*, ethanol markedly inhibited embryogenesis in carrot tissue cultures. The non-volatile component of the inhibitors has been identified with IAA, ABA and GA_3.

EXPERIMENTAL INDUCTION OF POLYEMBRYONY

In *Eranthis hiemalis*, a member of the family Ranunculaceae, a seed at shedding encloses an undifferentiated embryo which is pear-shaped and possesses a long suspensor (Fig. 13.9). The radicle as well as the cotyledons differentiate several weeks later while the seed is in the soil. Treatment of the freshly harvested seeds with substances such as 2,4-D, 2,4,5-T or NAA at a concentration of 0.1% induces many abnormalities. Some of the seeds (up to 8%) develop twin embryos (Fig. 13.10 A-C; Haccius, 1955). These treatments damage the plumule, and the cells which are destined to produce cotyledons give rise to additional plumules.

The potential to reconstitute additional embryos is also retained by mature embryos. When the older embryos are treated with acidic buffer (pH 4) the embryonal body is selectively killed and the surviving suspensor cells develop into a new adventive embryo (Fig. 13.11A, B). If the treatment is now repeated, the adventive embryo-1 is killed and a second adventive embryo arises (Fig. 13.11C).

In nature the formation of embryos is restricted to the ovular tissues. For long it was assumed that, for their development, the embryos require a special physical and chemical environment which is available only inside the "magic bath" of the embryo sac. However, it is now well established that by providing suitable nutritional and environmental conditions inside



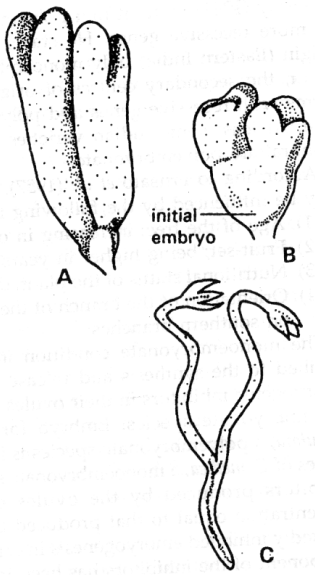


Fig. 13.9 Pear-shaped embryo excised from a freshly harvested seed of *Eranthis hiemalis*. (*after* Haccius, 1957)

Fig. 13.10 Induction of polyembryony in seeds of *Eranthis hiemalis* treated with auxins. **A, B**. Formation of twin embryos. **C**. Twin seedlings. (*after* Haccius, 1955)

culture vials, any cell of the plant body can be stimulated to give rise to viable embryos. Embryos formed in tissue cultures are called adventive embryos. Depending on their origin these embryos are termed somatic embryos (arising from any somatic tissue) or pollen embryos. *In vitro* adventive embryogenesis has been reported in the cultures of zygotic embryos (bamboo, cotton, oilpalm, soybean, wheat), nucellus (mono- and polyembryonate species of citrus and mango), stem segments (tobacco), root segments (carrot), fruit tissue (pumpkin), and anthers/pollen (grape, mustard, rice). In this chapter only somatic embryogenesis is discussed. For pollen embryogenesis *see* Chapter 17.

Somatic embryogenesis has been observed in over 100 species, which include many important crop plants, such as alfalfa, apple, coffee, cotton, grape, rice, soybean and wheat (Rangaswamy, 1986). *Ranunculus sceleratus* (buttercup) and *Daucus carota* (carrot) have proved especially suitable for somatic embryogenesis. Any part of these two plants will form embryos in tissue culture. The other plants in which this phenomenon has been studied in detail are *Citrus* sp., *Coffea* sp. and *Macleaya cordata*. For detailed reviews

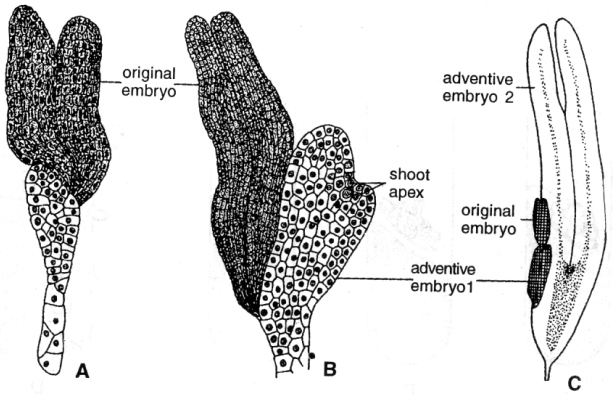


Fig. 13.11 *Eranthis hiemalis*. Formation of adventive embryos from suspensor cells of the mature embryos treated with acidic buffer solution. (*after* Haccius, 1965)

on the subject consult Gray and Purohit (1991), Michaux-Ferriere and Schwendiman (1992), Zimmerman (1993), de Jong *et al.* (1993) and Emons (1994).

The work on buttercup was originally done at the Department of Botany, University of Delhi by Konar and Nataraja. Very young flower buds of *Ranunculus sceleratus* were excised and planted on White's nutrient medium (which contains mainly inorganic salts of major and minor elements, vitamins, and sucrose, and is jelled with 0.8% agar). The buds proliferated to form an amorphous mass of tissue, called callus (Fig. 13.12A). After six weeks numerous embryos differentiated from the callus (Fig. 13.12B). Addition of coconut water from young nuts to the nutrient medium enhanced the formation of adventive embryos.

The differentiation of somatic embryos in cultures is not synchronous. Squashes of a differentiated callus show various stages of embryo development. It has not been possible to compare very early stages of embryo development with their parallel stages in the development of zygotic embryo because of the difficulty in recognizing the embryo initials. However, the post-globular stages in somatic embryo development showed normal histogenic differentiation. The somatic embryos in buttercup were attached to the callus by a distinct suspensor and were virtually indistinguishable from the seed embryos. They possessed a radicle-plumule axis and 2 (rarely 3 or 4) well developed cotyledons. If left attached to the callus, the embryos germinated *in situ* to form complete plantlets (Fig. 13.12C). A striking feature of the plantlets thus formed was the activation of the intact epidermal cells of their hypocotyl to form embryos. In a young seedling one could se-

The other factor which has been suggested to be essential for somatic embryogenesis is a reduced form of nitrogen, such as ammonium nitrate, ammonium chloride, glycine, yeast extract, etc. (Halperin, 1965; Kato and Takeuchi, 1966). However, Tazawa and Reinert (1969) have shown that if high concentration of nitrate is added to the medium embryo formation occurs to the same extent as in the presence of a low concentration of NH_4^+. Although the occurrence of NH_4^+ in the medium is not necessary for embryo formation, it appears that a certain level of intracellular NH_4^+ is a prerequisite for the process. There is a positive correlation between embryo formation and the concentration of soluble and insoluble organic nitrogen in the callus.

Besides growth regulators, the physiological state of the explant and the genotype of the experimental material have proved critical in the induction of somatic embryogenesis. In cereals and several other crop plants immature embryos yield highly embryogenic cultures while older tissues do not exhibit such a morphogenic potential. Alfalfa is an excellent example to illustrate the role of genotype in somatic embryogenesis. Of the 20 cultivars of alfalfa, tested by Brown (1988), 7 did not produce embryogenic cultures either from cotyledon or hypocotyl. In the remaining 13 cultivars the percentage of genotypes which produced embryogenic cultures varied from 9 to 80.

Very young somatic embryos of carrot (Browley *et al.*, 1984) exhibit electric gradient along the future longitudinal axis. Exposure of freshly isolated protoplasts of alfalfa to low voltage electric field considerably promoted their embryogenic potential (Dijak *et al.*, 1986; Dijak and Simmonds, 1988).

Somatic embryogenesis has been studied extensively from morphological and physiological viewpoints, but the molecular mechanisms that determine and control the process are not well understood. It has been known for some time that changes in gene expression occur when somatic cells embark on embryogenic development, as indicated by the synthesis of embryo-specific proteins (Sung and Okimoto, 1981), increased organic decarboxylase activity (Montague *et al.*, 1979), increased enzyme activities in the pyrimidine pathway (Ashihara *et al.*, 1981) and the acquisition of a capacity to inactivate cyclohexamide (Sung *et al.*, 1981). However, it is only during the past five years or so that several molecular markers indicating the transition of somatic cells into embryogenic cells have been recognized, and about 21 "embryo specific" or "embryo enhanced" genes have been cloned from embryogenic cultures of carrot (Zimmerman, 1993).

Nomura and Komamine (1985) demonstrated that suspension cultures of carrot in 2,4-D containing medium exhibit some morphologically distinct cells (Type 1 cells) which are capable of developing into proembryogenic masses upon transfer to low levels of the auxin. However, according to de Vries *et al.*, (1988) cells competent to form embryos do not exist in the explant. They acquire the competence 19 days after the initiation of fresh cultures in a medium containing 2,4-D. Most of the embryogenic capacity is acquired after 50 days of culture initiation, and it reaches a maximum after 75 days. The acquisition of embryogenic potential in fresh cultures is considerably accelerated by supplementing the medium with cell-free medium conditioned by established embryogenic suspension cultures.

Analyses of the conditioned media have revealed that a number of extracellular proteins (EPs) are excreted by the embryogenic cells into the medium which can act as molecular markers to distinguish between embryogenic and non-embryogenic cultures. Some of these proteins play an important role in the induction and development of somatic embryos. For example, the expression of EP2 gene, which encodes a secretory lipid transfer protein, is enhanced during embryogenesis *in vivo* and *in vitro*. It is probably involved in chitin synthesis. Another extra-cellular protein EP3, purified from the conditioned medium, has been identified as glycosylated acid endochitinase which is required for the formation of normal protoderm. In the mutant deficient in this protein the somatic embryos are unable to form a proper protoderm and, consequently, remain arrested at the globular stage. Certain arabinogalactan proteins (AGPs) secreted by embryogenic cultures seem to be associated with transition of somatic cells into embryogenic cells. Addition of specific AGPs from embryogenic cultures to fresh medium at a very low concentration (10-100 µM) enhanced the frequency of proembryogenic masses from 30% to 80% and could also restore the embryogenic potential of old cultures which had lost it.

CLASSIFICATION OF POLYEMBRYONY

Broadly speaking, polyembryony is of two types:

(1) *Spontaneous*: Includes instances of naturally occurring polyembryony.
(2) *Induced*: Includes instances of experimentally induced polyembryony.

Spontaneous polyembryony has been subdivided by Ernst (1901, 1910; also adopted by other leading embryologists) into two categories:

(1) *True polyembryony*: Two or more embryos arising in the same embryo sac from; zygote or embryo (*Eulophia, Vanda*), synergid (*Sagittaria*), antipodal cell (*Ulmus*), or nucellus or integument (*Citrus, Spiranthes*).
(2) *False polyembryony*: Development of embryos in more than one embryo sac in the same ovule (*Fragaria*) or placenta (Loranthaceae).

Yakovlev (1967) proposed a classification of polyembryony on genetic basis. He distinguished two types of spontaneous polyembryony:

(1) *Gametophytic*: Arising from any gametic cell of the embryo sac after or without fertilization.
(2) *Sporophytic*: Arising from the zygote, proembryo or the initial sporophytic cells of the ovule (nucellus, integuments).

Bouman and Boesewinkel (1969) put forth another classification based on the origin of the additional embryos. According to them, spontaneous polyembryony should be divided into four categories:

(1) Supernumerary embryos arising from sporophytic cells of the parental generation (integuments, nucellus).
(2) Supernumerary embryos arising from the cells of the gametophyte (by one or more embryo sacs in the same ovule).

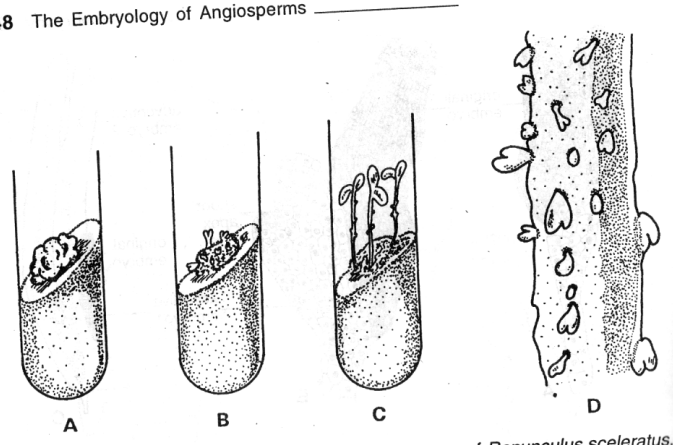


Fig. 13.12 Somatic embryogenesis in floral bud cultures of *Ranunculus sceleratus*. **A**. Callused floral bud. **B**. Six-week-old culture, showing numerous embryos arising from the callus. **C**. Five-week-old plantlets formed by the somatic embryos; note numerous embryos at the hypocotyledonary region. **D**. Portion of the hypocotyl enlarged from **C** to show the somatic embryos. (*after* Nataraja, 1968)

numerous exposed embryos hanging from the hypocotyl (Fig. 13.12 C,D). Embryos from epidermal cells of the hypocotyl have also been observed in carrot.

In vitro development of somatic embryos in carrot is a two-step process, each requiring a different medium (*see* Fig. 13.13). The callus is initiated in a medium containing 0.5-1.0 mg l^{-1} of 2,4-D. On such a medium, called "proliferation medium" (PF-medium), callus differentiates localized groups of meristematic cells called "embryogenic clumps" (ECs). In repeated subcultures on the PF-medium the ECs continue to multiply without the appearance of mature embryo. However, if the ECs are transferred to a medium with a very low level of the auxin (0.01-0.1 mg l^{-1}) or without 2,4-D ("embryo development medium", ED-medium) they form mature embryos. PF-medium is considered as the "induction medium" because ECs do not develop if the callus is continuously maintained in auxin-free medium (Sung and Okimoto, 1981).

Regarding the factors controlling somatic embryogenesis, a clear picture has not emerged yet. The work to-date highlights the importance of two media constituents, viz., auxin and source of nitrogen. Role of 2,4-D in somatic embryogenesis in carrot has been described earlier; it is essential for the appearance of ECs. The importance of auxin in somatic embryogenesis is also suggested by the detailed work on habituated tissue of *Citrus sinensis* (Spiegel-Roy and Kochba, 1973; Kochba and Button, 1974; Kochba and Spiegel-Roy, 1977a,b,c). Initially, the nucellar callus of *C. sinensis* required IAA and kinetin for its growth and embryo differentiation. In

repeated subcultures the callus showed a gradual decline of the embryogenic potential. After about two years some tissue lines appeared which were phytohormone autonomous. In these habituated tissues the presence of as low as 0.001 mg l^{-1} of IAA in the medium inhibited embryogenic differentiation, and any treatment that checks auxin concentration in the cells, such as auxin synthesis inhibitors and irradiation (breaks down IAA) significantly improved embryo differentiation. In the tissues exposed to irradiation levels higher than 16 kR the auxin, which otherwise inhibited embryo formation, turned out to be promotory. These studies and those of Fujimura and Komamine (1979) on carrot clearly indicate that a minimal level of endogenous or exogenous auxin is necessary for somatic embryogenesis.

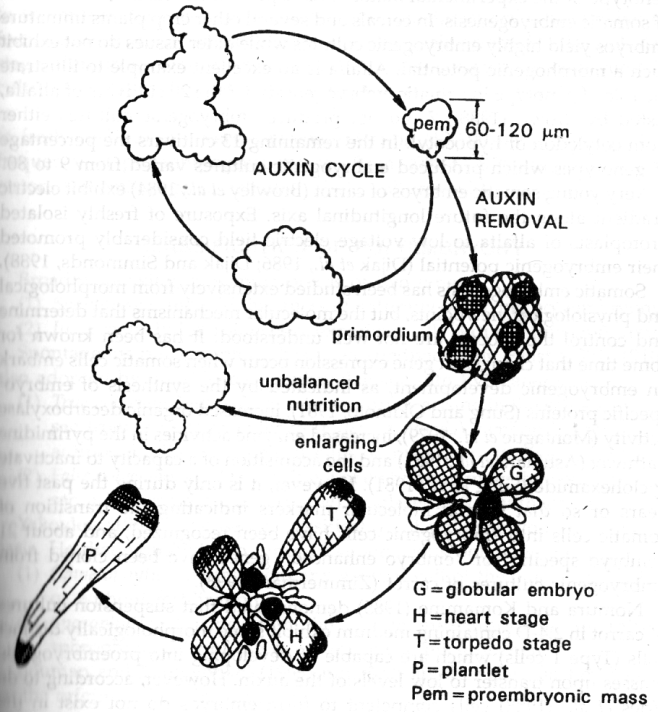


Fig. 13.13 Diagrammatic representation of somatic embryogenesis in suspension cultures of wild carrot (not drawn to scale). (*Courtesy*: Professor D.W.Wetherell, U.S.A.)

CHAPTER

14

APOMIXIS

The normal sexual cycle (*amphimixis*) involves two important processes: (a) *Meiosis*; this transforms a diploid sporophytic cell (megaspore mother cell; MMC) into four haploid gametophytic cells, and (b) *Fertilization*; in which two haploid gametes of opposite sex fuse re-establishing the diploid sporophytic generation. Thus, in a sexual cycle a diploid generation (sporophytic) alternates with a haploid generation (gametophytic). In angiosperms the gametophytic generations are very short and are represented by embryo sac on the female side and microspores or pollen grains on the male side. The remaining part of the life-cycle belongs to the sporophytic generation.

In some plants meiosis and syngamy are interrupted and still a viable embryo is formed within the confines of the seed coat. Such asexual seeds, which produce progeny identical to the female parent, are called apomictic seeds, and the phenomenon is known as apomixis (*Apo* = away from + *mixis* = act of mixing or mingling).

At the turn of this century it was known that certain male sterile biotypes of *Taraxacum* and *Hieracium* set ample seeds even when no pollen plants were growing far and wide, suggesting their apomictic nature. This was experimentally confirmed simply by cutting off the upper half of unopened flower buds with a razor so that the anthers, stigma and most of the corolla were removed. Even after this brutal emasculation seed-set and seed germination were as good as from intact and amply pollinated heads (Ostenfeld and Raunkiaer, 1903), and the plants derived from such seeds were genetically identical to the respective mother plant. This explains the unexpected results obtained by Mendel, when he extended the work to *Hieracium* to corroborate his laws of genetics based on studies with *Pisum*. In the progeny of the crosses he obtained numerous maternal plants, besides some hybrids. Moreover, the sister hybrids in the F_1 generation were not uniform, in contrast to his 1st law (the law of uniformity in F_1 generation), and the F_2 progenies of these hybrids were uniform in contrast to his 2nd law (Nogler, 1994).

To-date apomixis has been reported in more than 300 species belonging to 35 families (Khush *et al.*, 1994). It is most common in the families

Asteraceae, Poaceae, Rosaceae and Rutaceae (Bashaw, 1980; Hanna and Bashaw, 1987). As a rule, apomixis is more common in polyploids than diploids. Following Winkler (1908), who coined the term, plant breeders restrict the scope of apomixis to asexual seed formation (Bashaw, 1980; Khush *et al.*, 1994). However, many authors favour a broader concept of apomixis, according to which apomixis refers to substitution of the usual sexual reproduction by a form of asexual reproduction which does not involve meiosis and syngamy. Accordingly, there are two main categories of apomixis (Fig. 14.1).

1. VEGETATIVE REPRODUCTION. In this category the plants propagate by a part of their body other than the seed. The structural units employed for this purpose are called propagules.

2. AGAMOSPERMY. The plants belonging to this category have retained seed as the agent of propagation but the embryo is formed by some process in which normal meiosis and syngamy have been eliminated (*see* Fig. 14.1). Two different types of agamospermy are recognized, based on whether the embryo develops from a cell of the unreduced female gametophyte (gametophytic) or directly from diploid sporophytic cells of the ovule, such as nucellus and integuments (sporophytic or adventive embryony).

In gametophytic apomixis an unreduced embryo sac develops from the MMC by circumvention of meiosis (*diplospory*) or directly from a cell in the

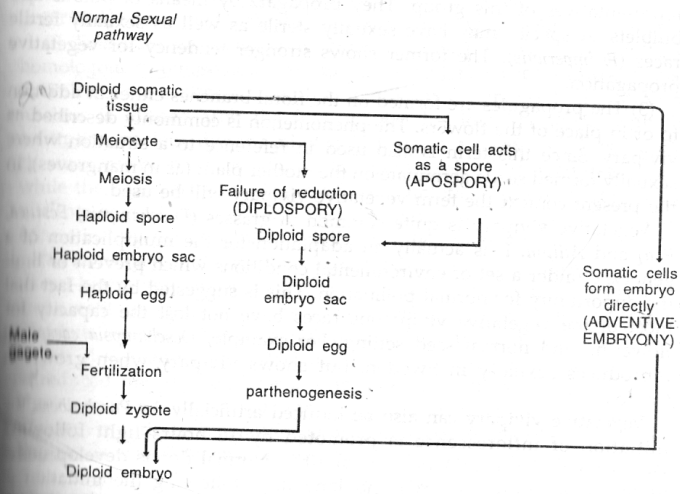


Fig. 14.1 Developmental pathways of various types of agamospermy compared with the normal sexual cycle. According to this chart diplospory shows the least departure from the normal sexual pathway, and the adventive embryony the greatest. (*modified from* Heslop-Harrison, 1972)

nucellus (*apospory*). The embryo is formed by the unfertilized egg (*parthenogenesis*) or some other cell of the embryo sac (*apogamety*). Certain species of *Potentila* show both apospory and apogamy. In gametophytic apomixis alternation of gametophytic and sporophytic generations is maintained.

VEGETATIVE REPRODUCTION

Some form of vegetative reproduction is generally found associated with the seminiferous plants (reproducing by means of normal seeds). However, only those plants are regarded as apomictic where vegetative reproduction has replaced the sexual method completely or essentially so.

Vegetative propagation includes reproduction by means of bulbils, bulbs, runners, suckers, and so on. These propagules are formed by the sporophyte only. Gustafsson (1946) has distinguished three types of vegetative reproduction in higher plants:

(1) The propagules are formed outside the floral regions, and despite the occurrence of functional sex organs no fertilization or seed setting takes place. *Agave americana* and *Elodea canadensis* may be cited as examples of this type.

(2) The propagules are formed outside the floral regions, and the plants are sexually sterile. *Fritillaria imperialis* and *Lilium bulbiferum* are typical representatives of this group. They propagate by means of bulbils and bulblets. A species may have sexually sterile as well as sexually fertile races (*F. imperialis*). The former shows stronger tendency for vegetative propagation.

(3) The propagules are formed on the floral branches either in addition to or in place of the flowers. The phenomenon is commonly described as vivipary. Since this term is also used in reference to a situation where sexually formed seeds germinate on the mother plant (as in mangroves), in the present context the term vegetative vivipary will be used.

Vegetative vivipary is quite common in grasses (*Deschampsia*, *Festuca*, *Poa*) and *Allium*. It is actually an adaptation for the multiplication of a genotype under a set of environmental conditions which prevent or limit the opportunity for normal pollination. This is suggested by the fact that most of the vegetative viviparous races have not lost the capacity for flowering and normal seed setting. For example, *Deschampsia caespitosa* reproduces sexually in Sweden, but shows vivipary when grown in California.

Vegetative vivipary can also be induced artificially. In *Poa bulbosa* the initiation of inflorescence is promoted by 16 hours light following vernalization for a week or more at 10°C. Normal florets develop under high temperatures (21-27°C) and long days following the initiation of inflorescence. However, if short days and low temperatures (20°C or below) are given after the initiation of inflorescence, bulbils develop in place of normal florets.

AGAMOSPERMY

Adventive Embryony

In this type of agamospermy the gametophytic generation is completely eliminated. In this respect it is close to the vegetative propagation, but differs from it essentially in two ways: (a) it has retained the seed habit, and (b) one or more diploid sporophytic cells undergo the stages of embryogeny and grow into mature embryos inside the normally developed sexual embryo sac. The zygotic embryo either degenerates or competes with the apomictic embryos.

Adventive embryony usually leads to the formation of more than one embryo in a seed. Besides the well known example of citrus, adventive embryony is known to occur in Buxaceae, Cactaceae, Euphorbiaceae, Myrtaceae and Orchidaceae (for further details and illustrations see Chapter 13).

Gametophytic Apomixis

DIPLOSPORY. In this category of apomicts, the MMC differentiates as in sexual ovules but it does not enter meiosis. It produces diploid embryo sac through mitotic divisions. As early as 1896, Juel described diplospory in *Antennaria alpina*. Since then this phenomenon has been reported in several species. Four types of diplosporic embryo sac development have been described, and named after the genera in which they were first observed.

(i) *Taraxacum type*. The MMC enters meiotic prophase and pairing of homologous chromosomes occurs to some extent. However, due to asynapsis univalents are scattered over the spindle at metaphase-I. A restitution nucleus is formed after the first meiotic division. The MMC with the restitution nucleus divides mitotically to form a dyad with somatic (2n) chromosome number. Usually the micropylar cell of the dyad degenerates while the chalazal cell undergoes further mitotic divisions to form an 8-nucleate embryo sac (Fig 14.2). Besides *Taraxacum*, this type of diplospory occurs in *Erigeron* sp., *Arabis holbellis* and *Agropyron scabra*

(ii) *Ixeris type*. In the triploid races of *Ixeris dentata* the MMC divides by a semi-heterotypic (asyndetic) meiotic prophase, in which there is no pairing and a restitution nucleus results. The restitution nucleus undergoes 3 mitotic divisions leading to the formation of an 8-nucleate embryo sac (Fig. 14.3).

(iii) *Antennaria type*. MMC does not enter meiosis and acts as an unreduced megaspore. After a long interphase, it increases in size and shows pronounced vacuolation to appear like functional megaspore. Through mitotic divisions it forms an unreduced 8-nucleate embryo sac of *Polygonum* type. This type of diplospory is of wide taxonomic distribution. It has been reported in *Tripsacum dactyloides*, *T. zopilotense* and *Eupatorium glandulosum* (Fig. 14.4) (Leblanc and Savidan, 1994).

(iv) *Allium Type*. In this type of diplospory premeiotic chromosome doubling is the cause of unreduced embryo sac formation (Hakansson and Levan, 1957; Kojima and Nagato, 1992). Chromosome number in MMC is

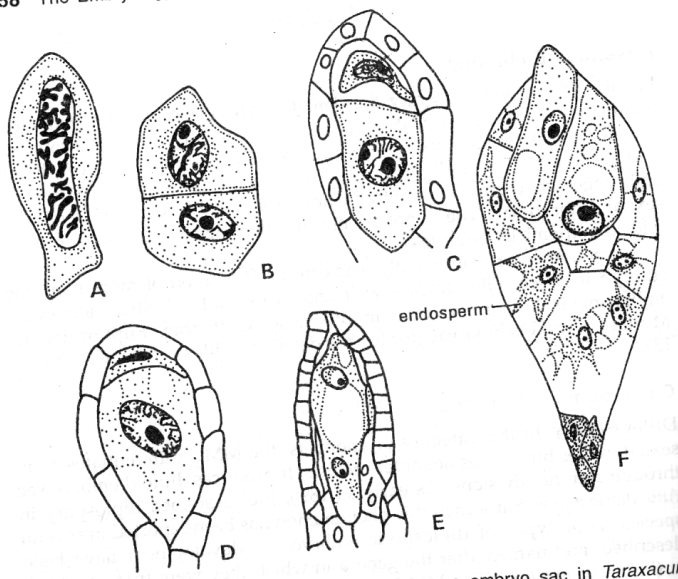


Fig. 14.2 Stages in the formation of diplosporous embryo sac in *Taraxacum albidum*. **A.** Megaspore mother cell with restitution nucleus. **B.** Dyad stage derived by a mitotic division of the restitution nucleus. **C,D.** Upper cell of the dyad is degenerating, and the lower cell functions and gives rise to embryo sac. **E.** Two-nucleate embryo sac. **F.** Mature embryo sac; endosperm development has started. (*after* Osawa, 1913)

doubled by endomitosis and the ensuing meiosis results in a dyad of unreduced cells. Two subsequent mitoses in the chalazal dyad cell results in an 8-nucleate embryo sac. *Allium nutans* and *A. odorum* show *Allium* type of diplospory. Kojima and Nagato (1992) reported 76%-98% diplospory in six cultivars of *A. tuberosum*, based on the percentage of reduplicated MMC.

APOSPORY. Apospory is by far the most common mechanism of apomixis in higher plants (Bashaw, 1980). It was first reported by Rosenberg (1907) in *Hieracium* sp. Obligate nucellar apospory with pseudogamy has been observed in seven species of *Pennisetum*, viz. *P. macrourum*, *P. mezianum*, *P. orientale*, *P. pedicellatum*, *P. polystachion*, *P. setaceum*, and *P. squamulatum* (Dujardin and Hanna, 1984, 1994). In aposporous plants a normal MMC differentiates in the same manner as in sexual ovules and begins to enter meiosis which may or may not complete both divisions to form a linear tetrad. At some stage during this period one or more adjacent nucellar cells become meristematic and appear like MMC. These cells undergo mitotic divisions to form unreduced embryo sacs. In obligate apospory the sexual

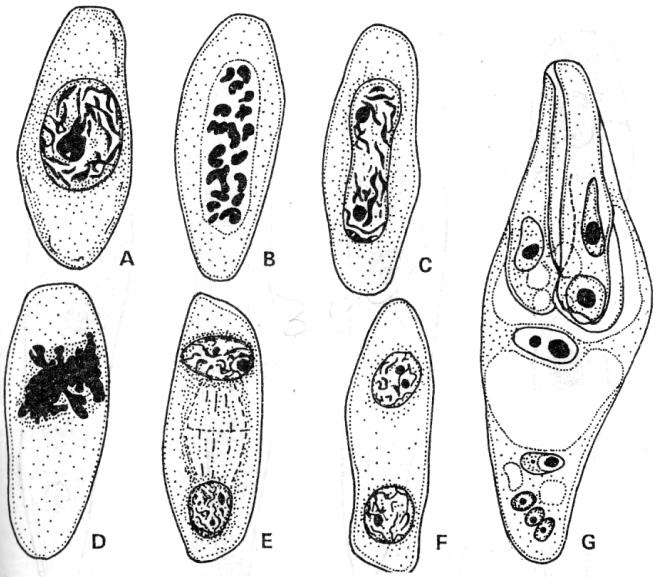


Fig. 14.3 Stages in the formation of diplosporous embryo sac in *Ixeris dentata*. **A.** Megaspore mother cell with prophasic nucleus. **B.** Later stage; meiosis with univalents only. **C.** Restitution nucleus. **D.** Mitosis in restitution nucleus. **E,F.** Two-nucleate embryo sac. **G.** Mature, 8-nucleate embryo sac. (*after* Okabe, 1932)

MMC or its products abort and the aposporous embryo sac occupies the entire area of the ovule. The timing of degeneration of the sexual embryo sac depends on the time of initiation of aposporous embryo sacs. The sooner an aposporous initial is induced earlier the degeneration of the developing megaspore occurs. There is considerable variation, even within the same species, with respect to the number of fully developed aposporous embryo sacs formed within an ovule. In the aposporous members of the Asteraceae, only one nucellar cell acts as the mother cell and gives rise to structurally normal, 8-nucleate embryo sac. In grasses, on the other hand, more than one embryo sac may develop in the same nucellus with 4-nucleate monopolar organization. Occasionally, aposporous and sexual embryo sacs may co-exist. Apospory is mainly of two types.

(i) *Hieracium type*. In *Hieracium* subg. *Pilosellae* the MMC undergoes the usual meiotic division and forms a tetrad. At this stage a nucellar cell at the chalazal end of the tetrad becomes activated and starts developing into an aposporous, unreduced embryo sac. All the four megaspores gradually

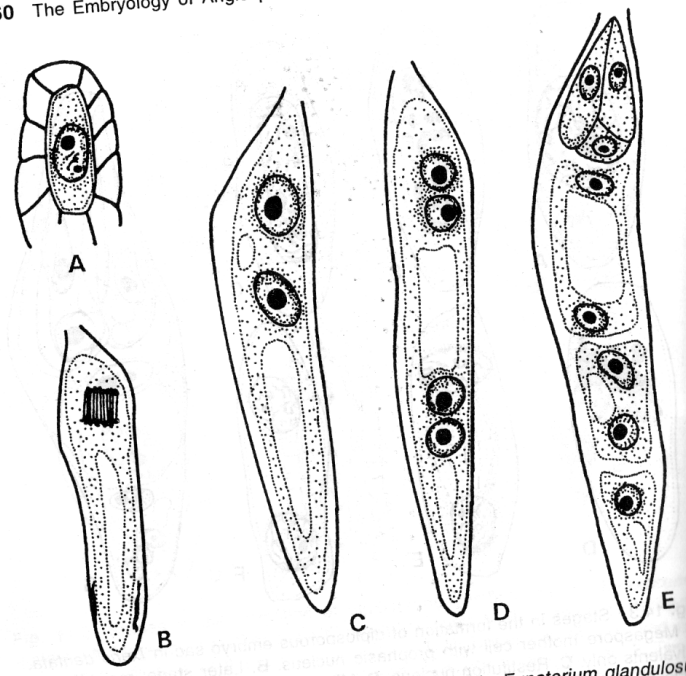


Fig. 14.4 Development of aposporous embryo sac in *Eupatorium glandulosum*. **A**. Megaspore mother cell. **B**. Megaspore mother cell with its nucleus in the late anaphase of mitosis. **C-E**. Two, four and eight-nucleate embryo sacs, respectively. (*after* Holmgren, 1919)

degenerate and the aposporic embryo sac matures (Fig. 14.5). Occasionally, aposporous embryo sac may develop in addition to the reduced embryo sac. This type of aposporic embryo sacs are 8-nucleate.

(ii) *Panicum type*. In *Panicum* and some other grasses, the aposporous embryo sac is 4-nucleate, organized into a 3-celled egg apparatus and a polar nucleus. Antipodals are absent. In *Poa arctica* and *P. pratensis* the number of apomictic embryo sacs in an ovule may be over 12 (Grazi *et al.*, 1961).

Embryo and Endosperm Development

Diplospory as well as apospory produce diploid embryo sacs. To complete the apomictic cycle without altering the chromosome number of the sporophytic generation the diploid egg must develop into embryo without the participation of the male nucleus. Formation of embryo from an

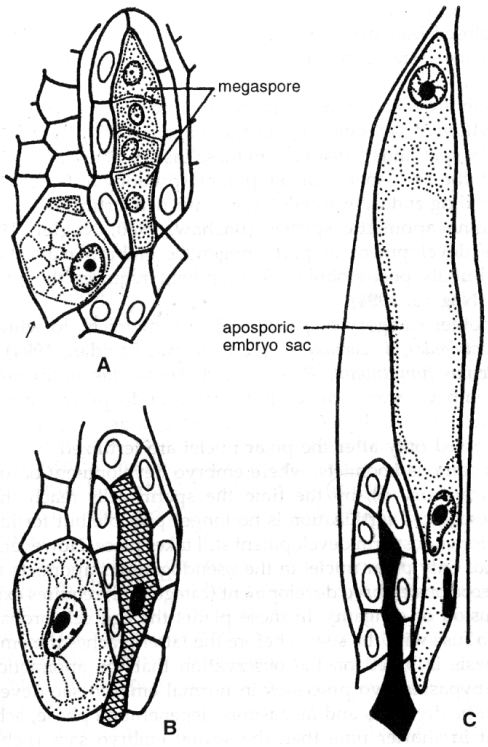


Fig. 14.5 Development of aposporic embryo sac in *Hieracium flagellare*. **A**. A nucellar cell (arrow marked) near the base of the megaspore tetrad has enlarged to form an aposporic embryo sac. **B**. The megaspore tetrad has degenerated and the aposporic embryo sac mother cell has further enlarged. **C**. Two-nucleate stage of the aposporic embryo sac; the dark area represents the degenerated megaspores. (*after* Rosenberg, 1907)

unfertilized egg is called parthenogenesis. Although the embryo development in apomicts occurs without the union of male and female gametes, pollination is often necessary for full development of the embryo and endosperm. Based on the timings of embryo and endosperm development the apomicts can be divided into three categories:

(i) In some apomicts embryo and endosperm development are autonomous, not requiring a pollination stimulus. For example, in *Alchemilla*

CHAPTER

17

EXPERIMENTAL AND APPLIED EMBRYOLOGY

Embryology began largely as a descriptive science, in which developmental details of various structures associated with fertilization and embryo development were studied through sections and dissections of fixed materials. In the later part of the 19th century it was realized that embryological data could profitably be applied for taxonomic considerations. This led to the emergence of a new trend in embryological studies, namely, comparative embryology. Since 1960 embryology has become an experimental science. The two main objectives of experimental embryology are: (a) to understand factors that control the various embryological processes, and (b) to manipulate the embryological processes by altering the environmental conditions of the whole plant or its excised parts for practical applications. Chapters 3 to 15 deal mainly with descriptive embryology, and Chapter 16 is concerned, exclusively, with comparative embryology. In this final chapter some aspects of experimental embryology are described.

Experimental embryology has established intimate contacts between embryology and other disciplines of botany, especially genetics, physiology and biochemistry. It has also made embryology of considerable practical application in plant improvement. Some aspects of experimental embryology, *viz. in vitro* pollination and fertilization (Chapter 10), pollen germination and pollen tube growth (Chapter 9), endosperm culture (Chapter 11), nutrition of embryo (Chapter 12) and somatic embryogenesis (Chapter 13), have been discussed elsewhere in the book. The readers are advised to consult them in order to have a fuller appreciation of the subject. In this chapter the following aspects of experimental embryology are discussed:

1. Haploid production
2. Embryo culture
3. Nucellus culture
4. Ovule and seed culture
5. Ovary culture
6. Effect of young seeds on fruit growth
7. Parthenocarpy
8. Genetic transformation

The notable progress in the field of experimental embryology has been possible through the techniques of "Plant Tissue Culture", in which isolated plant cells, tissues, organs, or even whole plants are grown in nutrient medium, in glass or plastic containers (tubes, flasks, jars or petri-plates), under aseptic conditions. Since this technique may not be very familiar to many, it is considered necessary to briefly describe it before dealing with the actual subject matter. There are three important aspects of the technique of plant tissue culture, namely nutrient medium, maintenance of aseptic conditions, and aeration of the cultured tissues (for details *see* Bhojwani and Razdan, 1996).

TISSUE CULTURE TECHNIQUES

Nutrient Medium

Many recipes of nutrient media have been developed from time to time. Every tissue and organ has its special requirements for optimal growth, and these need to be worked out when starting work with a new system. However, most of the plant tissue culture media contain inorganic salts of major and minor elements, vitamins, and sucrose (*see* Table 17.1). A medium with these ingredients will be referred to as the basal medium. Some growth regulators, such as auxins, cytokinins, and gibberellins may also be added to the basal medium either alone or in various combinations. Natural plant extracts, such as coconut milk, various fruit juices, casein hydrolysate, and yeast extract have also been used as supplements to the basal media for growing some tissues.

All constituents of the medium are dissolved in distilled water. If necessary, the medium is solidified with 0.8% agar. The pH of the medium is adjusted around 5.8. Now, equal quantities of the medium are dispensed in culture vials, which are usually screw cap bottles, glass tubes, petri-plates or flasks. The culture tubes and flasks, containing medium, are plugged with non-absorbant cotton wrapped in cheese cloth. Such a closure allows the exchange of gases but does not permit the entry of microorganisms into the culture vials.

The nutrient media of the compositions given in Table 17.1, especially when they contain sugar, would support a luxuriant growth of many microorganisms, like bacteria and fungi. Reaching the medium, these organisms grow much faster than the cultured tissue and, finally, kill it. It is, therefore, extremely important to maintain a completely aseptic environment inside the culture vials. There are three major sources of contamination of the medium.

Aseptic Conditions

1. The microorganism may be present in the medium right from the beginning. To destroy such microorganisms the culture vials containing the medium are properly capped and autoclaved (steam heating under pressure). The medium can be completely sterilized by maintaining it at 120°C for 15-20 minutes.

TABLE 17.1 Composition of some nutrient media used for *in vitro* culture of plant tissues.

Constituents	*Modified White's Medium 'A' mgl^{-1}*	*Murashige and Skoog's Medium 'B' mgl^{-1}*	*Nitsch's Medium 'C' mgl^{-1}*	*Nagata & Takebe's Medium 'D' mgl^{-1}*
Inorganic				
KNO_3	80	1,900	950	950
NH_4NO_3	-	1,650	720	825
$Ca(NO_3)_2.4H_2O$	260	-	-	-
$CaCl_2$	-	440	166	220
KCl	65	-	-	-
$MgSO_4.7H_2O$	360	370	185	1,233
Na_2SO_4	200	-	-	-
$NaH_2PO_4.2H_2O$	165	-	-	-
KH_2PO_4	-	170	68	680
$MnSO_4.4H_2O$	3	22.3	25	22.3
H_3BO_3	0.5	6.2	10	6.2
$ZnSO_4.4H_2O$	0.5	8.6	10	8.6
$Na_2MoO_4.2H_2O$	0.05	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.05	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	-	-
$CoSO_4$	-	-	-	0.03
KI	-	0.83	-	0.83
$Fe(C_6H_5O_7).3H_2O$	10	-	-	-
$FeSO_4.7H_2O$	-	27.8	27.8	27.8
Na_2EDTA	-	37.3	37.3	37.3
Organic				
Niacin	1.25	0.5	5	-
Glycine	7.5	2	2	-
Pyridoxine HCl	0.25	0.5	0.25	-
Thiamine HCl	0.25	0.1	0.5	1
Folic acid	-	-	0.5	-
Biotin	-	-	0.05	-
Calcium Pentothenate	0.25	-	-	-
Myo-inositol	-	100	100	-
Meso-inositol	-	-	-	100
Sucrose	20,000	30,000	20,000	10,000
IAA	-	1.30	-	-
NAA	-	-	-	3
Kinetin	-	0.4-10	-	-
Benzylaminopurine	-	-	-	1

Medium 'A'- Rangaswamy (1961) Phytomorphology **11**: 109-127.
Medium 'B'- Murashige & Skoog (1962) Physiol.Plant. **15**: 473-497.
Medium 'C'- Nitsch (1969) Phytomorphology **19**: 389-404.
Medium 'D'- Nagata & Takebe (1971) Planta **99**: 12-20.

2. The microorganisms may also be carried along with the tissue that is being cultured. To prevent this, the plant material from which the tissue is to be excised is surface-sterilized. The material is first washed with a detergent and then surface-sterilized with saturated chlorine water or any other solution which has nascent chlorine, such as a dilute solution of sodium hypochlorite and mercuric chloride. After treating with disinfectant the material is thoroughly washed with sterilized distilled water to remove all traces of chlorine. If the material is fairly hard, as are some fruits and seeds, it may be surface-sterilized by rinsing in alcohol.

3. Finally, precautions must also be taken to prevent the entry of microorganisms while the closure of a culture vial is removed to transfer the tissue to the nutrient medium (inoculation). For this, all operations from surface-sterilization of the tissue up to inoculation are done in an aseptic environment, inside a laminar airflow cabinet where the air is constantly replaced by fresh, filter-sterilized air. The table inside the inoculation chamber is swabbed with alcohol before use. During inoculation the neck of the culture vial is flamed and, in quick succession, the closure of the vial removed, the tissue transferred to the medium, and the closure replaced. It is equally important to sterilize all the instruments used for inoculation by dipping them in alcohol and flaming, and used only after cooling.

Aeration

The methodology of plant tissue culture should also account for proper aeration of the cultured tissue. If the tissue is grown on the surface of a solid medium it acquires enough aeration without a special device. However, if liquid medium is employed the tissue would get submerged. In such a situation a special device is to be adopted for proper aeration of the tissue. One possibility is to use a "filter paper bridge" whose two legs remain dipping in the medium, and the horizontal part carrying the tissue is above the level of the medium. Agitation of the medium by passing through it filter-sterilized air also serves the purpose. More commonly, however, aeration is provided by shaking the flasks or tubes on an automatic shaker. Besides supplying oxygen to the tissue, shaking of the medium also brings about dissociation of the tissue into single cells and small cell clumps. The importance of single cell culture in higher plant genetics is well recognized.

HAPLOID* PRODUCTION

In vitro culture of anthers was first undertaken by a Japanese cytologist, Shimakura (1934) with the objective of understanding the physiology of meiosis. Guha and Maheshwari (1964) also cultured mature anthers of *Datura innoxia* with a similar aim, but, accidentally, made a more significant

*With reference to higher plants, haploids are defined as sporophytes with gametic chromosome constitution (Kimber and Riley, 1963).

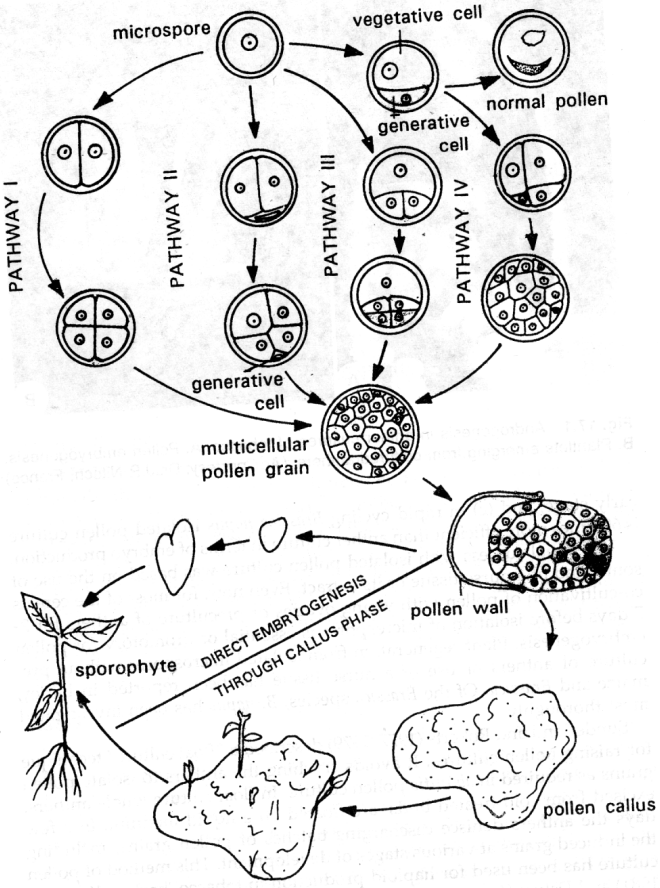


Fig. 17.2 Diagram showing the origin of sporophytes from pollen grains in anther cultures. A microspore may follow any one of the four pathways to form a multicellular pollen grain. The latter may directly form an embryo or produce sporophytes through a callus phase. (*after* Bhojwani and Razdan, 1996)

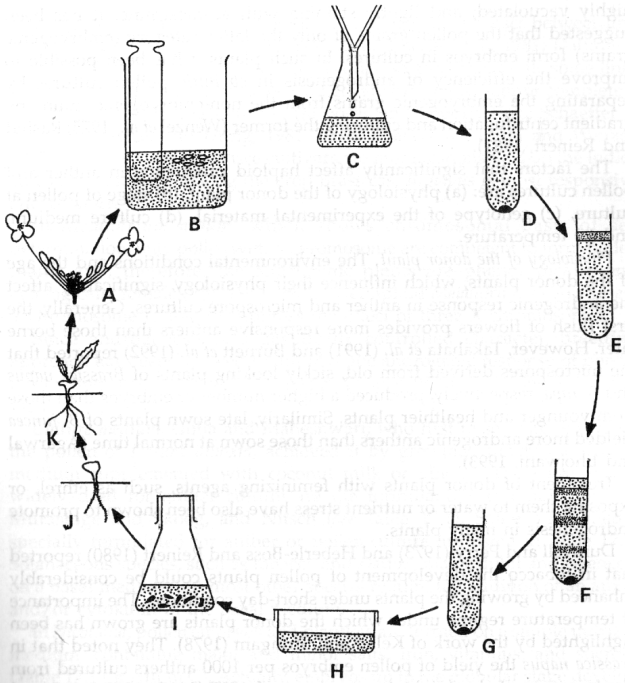


Fig. 17.3 Summary diagram of a protocol for isolated pollen culture of *Brassica napus*. The surface-sterilized buds (**A**) of suitable size are crushed to release the pollen grains in B_5 medium containing 13% sucrose (B_5-13) in a glass homogenizer (**B**) and the medium is filtered through 42 µm nylon mesh to remove the large debris (**C**). The filtrate is centrifuged at 1000 rpm for 3 min (**D**) and, after discarding the supernatant solution, the pellet is suspended in B_5-13 medium and gently loaded on the 24%/32%/40% Percoll gradient solution (**E**) and centrifuged at 1000 rpm for 5 min. The two upper layers (**F**) are pipetted out and mixed with the B_5-13 medium. The suspension is again centrifuged at 1000 rpm for 5 min (**G**) and the supernatant medium is pipetted out and the pollen grains are suspended in NLN medium adjusting the plating density of the pollen grains to 2-5 x 10^4 ml^{-1}. The suspension is plated as a thin layer in petri plates (**H**) and incubated in the dark at 32°C for 3-5 days and then at 25°C. The regenerated tissue/embryos are transferred to 18 ml of hormone-free NLN medium in conical flasks (**I**) and maintained on a shaking machine at 60 rpm at 32°C. Finally, the mature embryos are transferred to solidified B_5 medium containing 2% sucrose for germination (**J,K**). Fertile plants can be obtained by diploidization of pollen plants. (*after* Ohkawa, 1988)

observation. They noticed that on nutrient medium containing kinetin, coconut milk, or grape juice numerous embryos appeared from the inside of anthers which, eventually, developed into plantlets. In 1966, these workers confirmed the origin of the embryos from pollen grains. As expected, the plantlets of pollen-origin were haploid. This report aroused a worldwide interest to develop the technique of anther and pollen culture for producing androgenic haploids of a wide variety of crop plants because of their potential significance in basic and applied genetics and plant breeding (*see* page 323). In nature, haploids occur with an extremely low frequency (.001-.01%) and there is no reliable method to produce them artificially.

To-date *in vitro* androgenesis has been reported in over 134 species and hybrids distributed over 35 families (Bhojwani and Razdan, 1996). This list includes many economically important crop plants, such as barley, mustard, maize, potato, rice, tomato and wheat. *In vitro* gynogenesis and distant hybridization followed by hybrid embryo culture are two other approaches to produce haploids.

ANDROGENESIS

Anther Culture

TECHNIQUE. For raising haploids from microspores or pollen grains, generally the entire anther is cultured. Buds are surface-sterilized with alcohol or mercuric chloride, and the anthers excised and planted on the nutrient medium. By selecting buds at the right stage of pollen development and appropriate nutritional conditions, Nitsch (1972) obtained up to 75% of the cultured anthers of tobacco to yield plantlets at the rate of 1 to 100 plants per anther. In anther cultures, depending on the species and the composition of nutrient medium, the pollen may either directly develop into embryos (Fig. 17.1), or first form a callus which would regenerate plants (Fig. 17.2).

Isolated Pollen Culture

There is a serious problem with raising haploids through anther culture. The pollen population within an anther is genetically heterogeneous. Therefore, the plants arising from an anther constitute a heterogeneous population. Moreover, where the formation of haploid plants is preceded by callusing of pollen, the tissue arising from an anther is actually a chimera because numerous pollen in the anther contribute to the callus. A solution to this problem lies in the possibility of raising plants by isolated pollen culture (Fig. 17.3). The other advantages of isolated pollen culture are: (a) homogeneous population of pollen grains at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation, (b) isolated microspores can be genetically modified by mutagenic treatment or genetic engineering before culture and new genotypes selected at an

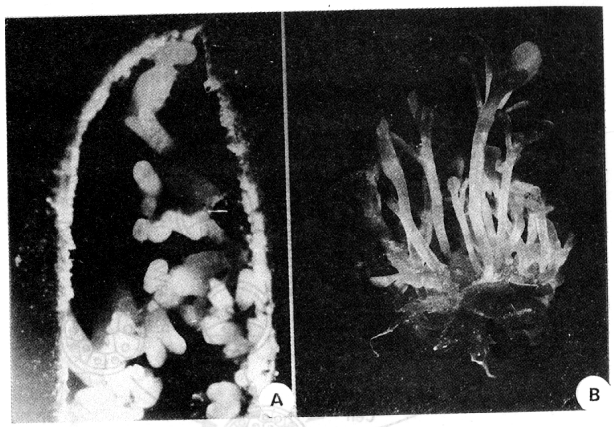


Fig. 17.1 Androgenesis in anther cultures of tobacco. **A.** Pollen embryogenesis. **B.** Plantlets emerging from cultured anthers. (**A.** *Courtesy:* Dr. J.P. Nitsch, France)

early stage, and (c) in rapid cycling *Brassica napus* isolated pollen culture is 60 times more efficient than anther culture in terms of embryo production.

The initial success with isolated pollen culture was based on the use of some kind of a nurse tissue or its extract. Even now, for most of the cereals co-cultivation of pollen with young ovaries or preculture of anthers for 2-7 days before isolation of microspores is essential or promotory for pollen embryogenesis. Plant regeneration from isolated microspores without preculture of anthers or use of a nurse tissue has been reported in barley, maize and *Brassica*. Of the *Brassica* species, *B. napus* has been investigated most thoroughly.

Sunderland and Roberts (1977, 1979) described a "float culture" technique for raising pollen cultures. It avoids crushing the anthers to isolate pollen grains as required in *ab initio* pollen culture. In float culture whole anthers, excised from cold-treated buds, are floated on a liquid medium. In a few days the anthers dehisce discharging batches of pollen grains, including the induced grains at various stages of development. This method of pollen culture has been used for haploid production in tobacco, barley (Xu *et al.*, 1981) and *Datura* (Tyagi *et al.*, 1979). For *Datura*, float culture method proved better than *ab initio* pollen culture.

Several plants exhibit the phenomenon of pollen dimorphism, which refers to the occurrence of two types of pollen grains within the same anther. In tobacco while most of the pollen grains are large, starch-filled, and deeply staining with acetocarmine, some are comparatively small,

highly vacuolated, and lightly staining with acetocarmine. It has been suggested that the pollen grains of only the latter category (embryogenic grains) form embryos in cultures. In such plants it has been possible to improve the efficiency of androgenesis in *ab initio* pollen cultures by separating the embryogenic grains from the non-embryogenic grains by gradient centrifugation and culturing the former (Wenzel *et al.*, 1975; Rashid and Reinert, 1981).

The factors that significantly affect haploid production in anther and pollen cultures are: (a) physiology of the donor plant, (b) stage of pollen at culture, (c) genotype of the experimental material, (d) culture medium, and (e) temperature.

Physiology of the donor plant. The environmental conditions and the age of the donor plants, which influence their physiology, significantly affect the androgenic response in anther and microspore cultures. Generally, the first flush of flowers provides more responsive anthers than those borne later. However, Takahata *et al.* (1991) and Burnett *et al.* (1992) reported that the microspores derived from old, sickly looking plants of *Brassica napus* and *B. rapa*, respectively, produced a higher number of embryos than those from younger and healthier plants. Similarly, late sown plants of *B. juncea* yielded more androgenic anthers than those sown at normal time (Agarwal and Bhojwani, 1993).

Treatment of donor plants with feminizing agents, such as ethrel, or exposing them to water or nutrient stress have also been shown to promote androgenesis in many plants.

Dunwell and Perry (1973) and Heberle-Boss and Reinert (1980) reported that in tobacco the development of pollen plants could be considerably enhanced by growing the plants under short-day conditions. The importance of temperature regime under which the donor plants are grown has been highlighted by the work of Keller and Stringam (1978). They noted that in *Brassica napus* the yield of pollen embryos per 1000 anthers cultured from plants grown at day/night temperatures of 15°C/10°C, 25°C/15°C and 25°C/20°C was 979, 579 and 267, respectively.

Stage of pollen development. For the induction of androgenesis the stage of pollen development at which the anthers are cultured is often more crucial than the composition of the nutrient medium. There is a staging optima for each species. Generally, the most responsive stage of anther is when pollen are just before, at, or just after the pollen mitosis (*e.g.*, *Datura innoxia, Nicotiana tabacum, Petunia hybrida*). The early binucleate stage is best for *Nicotiana sylvestris*, but absolutely essential for *N. knightiana*. Late uninucleate stage is most suitable for androgenesis in rice (Raghavan, 1990) and for most *Brassica* species (Leelavathi *et al.*, 1984; Dunwell *et al.*, 1985; Sharma and Bhojwani, 1985).

In practice, it is always convenient to use some external marker to pick up anthers of the right stage without testing each one of them before culturing. The commonly used marker for this purpose is the length of flower buds which, under fixed environmental conditions, shows a reasonably good correlation with the developmental stage of the anther.

Genotype. Striking variation occurs in androgenic responses between and within species (Jacobsen and Sopory, 1978; Brettell *et al.*, 1981; Datta and Wenzel, 1987). Intraspecific variation is often so great that while some lines of a species exhibit good response, others are extremely poor or completely non-responsive androgenetically (Jacobsen and Sopory, 1978; Schaeffer *et al.*, 1979; Wenzel and Uhrig, 1981; Lazer *et al.*, 1984; Datta and Wenzel, 1987). In general, japonica rice cultivars are more responsive than the indica cultivars. Similarly, among the crop Brassicas, *B. napus* is most responsive and *B. juncea* the least.

In *Melandrium album*, which shows chromosomal basis of sex determination, only pollen with X chromosome are competent to form pollen plants and, therefore, the androgenic plants are phenotypically and cytologically female (Wu *et al.*, 1990). In tetraploid *Melandrium* even a single Y chromosome is able to suppress the effect of three X chromosomes.

Since androgenesis is a genetically controlled character, breeding approach has been followed to improve the androgenic response of poorly responding lines of potato (Jacobsen and Sopory, 1978) and barley (Foroughi-Wehr *et al.*, 1982).

Culture medium. Guha and Maheshwari, who first raised haploids from the pollen of *Datura innoxia*, achieved it by growing anthers on a basal medium supplemented with coconut milk or kinetin. The basal media widely used for anther culture are the formulations recommended by Murashige and Skoog, and Nitsch (*see* Table 17.1). Some other media specially formulated for anther or pollen culture are listed in Table 17.2. Solanaceous species, such as tobacco and *Datura*, may exhibit androgenesis on a basal medium but most other species require the addition of an auxin and/or cytokinin to the basal medium.

In *Nicotiana*, pollen embryos and plantlets are formed on a simple nutrient medium, containing major and minor salts, iron, vitamins, and sucrose (Table 17.1 - medium C). Actually, embryos up to the globular stage develop simply on agar-sucrose medium (Nitsch and Nitsch, 1969). Addition of an auxin to the nutrient medium C (Table 17.1) is slightly promotory. *Datura* pollen also form embryos on medium C alone but the addition of a cytokinin, especially kinetin or zeatin, brings about a substantial enhancement of the response (Sopory, 1972). Except for a couple of reports, sucrose has been recognized as an essential ingredient of the nutrient medium. It is generally used at a concentration of 2 per cent. However, higher levels of sucrose (6-12%) have proved beneficial in potato (Sopory *et al.*, 1978), cereals (Clapham, 1971; Ohyang *et al.*, 1973) and *Brassica* (Bhojwani *et al.*, 1996). High concentration of sucrose tends to suppress callusing of somatic tissues and promote pollen embryogenesis. According to Dunwell and Thurling (1985), high sucrose concentration (16-20%) favours better survival of microspores, thus improving the frequency of embryo induction in B. *napus*. Similarly, in anther cultures of wheat 6 per cent sucrose promoted pollen callusing and inhibited the proliferation of somatic tissues (Ohyang *et al.*, 1973). Iron is another substance whose presence in the medium has been described as obligatory for pollen embryo formation.

exhibit sporophytic division within the first two days of culture (Cordewener *et al.*, 1994; Custers *et al.*, 1994). About 90% of these grains acquire irreversible commitment to embryogenesis within 8 hours of high temperature treatment.

Cytology of Androgenesis

Based on the first few divisions in the microspores/pollen four pathways of *in vitro* androgenesis have been identified (Bhojwani and Razdan, 1996; Fig. 17.2):

(i) As commonly observed in *Brassica napus* (Zaki and Dickinson, 1992), the microspores divide by an equal division, and the two identical daughter cells contribute to sporophyte development. In this case distinct vegetative and generative cells are not formed (pathway I).

(ii) The uninucleate microspores divide by a normal unequal division, and the sporophytes arise through further divisions in the vegetative cell only. This mode of development is commonly encountered in *Capsicum annuum, Hordeum vulgare, Nicotiana tabacum, Triticale* and *Triticum aestivum* (pathway II).

(iii) In *Hyoscyamus niger* the pollen embryos are predominantly formed from the generative cell alone. The vegetative cell either does not divide at all or does so only to a limited extent (pathway III).

(iv) As in pathway II, vegetative and generative cells are formed but both the cells divide further and participate in the formation of sporophyte (*e.g.*, *Datura innoxia*) (pathway IV).

Irrespective of the early pattern of divisions, the responsive grains finally become multicellular and burst open to release the tissue, which has an irregular outline. In *Atropa, Datura* and *Nicotiana*, this cellular mass gradually assumes the shape of a globular embryo and undergoes normal embryogenic differentiation. By this method a single haploid plant is derived from a grain. However, in several species (most of the cereals) where androgenesis has been reported the multicellular mass released by bursting of pollen undergoes further proliferation and forms a callus. From such calli numerous sporophytes differentiate (*see* Fig. 17.2).

Where pollen grains directly form embryos the plants derived from them are mostly haploid. However, where sporophyte formation is preceded by callus formation the plants derived from them are mixoploid (2n, 3n or higher). This is because of the well-known phenomenon of polysomaty (change in ploidy level of somatic cells) in callus cultures.

Using an improved technique of paraffin sectioning (Bhandari, 1976), in which the pollen embryos are retained in their original positions during processing of the material, Haccius and Bhandari (1975) made detailed ontogenetic studies on pollen embryos of tobacco. These studies have revealed that the site of exine rupturing determines the polarity of pollen embryos. When the exine bursts open the exposed end of the proembryonal tissue develops into the plumular end of the future embryo. These investigators also noted that about 82 per cent of the well developed pollen

embryos had their basal end adhering to a supporting tissue (thecal wall, other embryos, or clusters of dead pollen) suggesting that for the normal development of pollen embryos at least a temporary support is essential. Moreover, further development of globular embryos and differentiation of protoderm occurs only if the exine ruptures in such a way that the plumular end of the proembryonal mass of tissue is not in direct contact with the supporting, underlying tissue (Fig. 17.4).

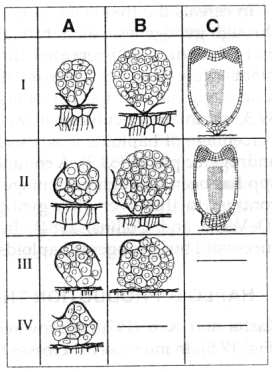


Fig. 17.4 Diagram to show the relationship between the place of exine bursting (**A**) and the fate of the pollen embryos (**B, C**) in *Nicotiana*. Mature embryos develop only from class I and class II pollen grains. For details *see* text. (*after* Haccius and Bhandari, 1975)

GYNOGENESIS

Gynogenetic development of plants from unfertilized cells of female gametophyte (embryo sac) in ovary/ovule cultures is an alternative to anther/pollen culture for haploid production. Since the first report of gynogenesis in barley by San Noeum (1967), this approach has been successfully applied to raise haploid embryos/plants of about 20 species, including *Allium cepa, Beta vulgaris, Gerbera jamesonii, Helianthus annuus, Lilium davidii, Morus alba, Nicotiana tabacum, Oryza sativa, Petunia axillaris, Triticum aestivum and Zea mays* (Yang and Zhou, 1982; DeVerna and Collins, 1984; Yang *et al.*, 1986; Zhou *et al.*, 1986). This method of haploid production is more tedious than androgenesis, but it may be useful where anther culture has been unsuccessful, plants are male sterile, or androgenesis is confronted with the problem of albino or non-haploid formation.

For *in vitro* gynogenesis unfertilized ovaries/ovules are cultured, preferably attached to the pedicel and other accessory parts of the flower which are known to promote the response. The most responsive stage of embryo sac at culture may vary with the species. It was uni- to four-nucleate stage for *Oryza sativa* (Zhou *et al.*, 1986) and 8-nucleate stage for *Helianthus annuus* (Yang *et al.*, 1986).

The culture media used for gynogenesis vary considerably. However, the Chinese workers, who have taken the lead in this field, have consistently used the auxin MCPA (2-methyl-4-chlorophenoxy acetic acid) as one of the supplements.

Gynogenic haploids may arise through parthenogenesis, as in *Helianthus annuus* (Yang *et al.*, 1986) or synergid-apogamy, as in rice (Zhou *et al.*, 1986).

TABLE 17.2 Composition of some of the media used for anther and pollen culture. Unless mentioned otherwise, all concentrations are in mgl^{-1}.

Constituents	*Media*					
	N&N[a]	N_6^b	*Potato*[c]	*Nitsch*[d]	*KA*[e]	*NLN*[f]
KNO_3	950	2830	1000	950	2500	125
NH_4NO_3	725	-	-	725	-	
$NaH_2PO_4.2H_2O$	-	-	-	-	150	-
KH_2PO_4	68	400	200	68	-	125
$(NH_4)_2SO_4$	-	463	100	-	134	-
$MgSO_4.7H_2O$	185	185	125	185	250	125
$CaCl_2.2H_2O$	166	166	-	166	750	-
$Ca(NO_3)_2.4H_2O$	-	-	100	-	-	500
KCl	-	-	35	-	-	-
$FeSO_4.7H_2O$	27.8	27.8	-	27.8	-	27.8
$Na_2.EDTA$	37.3	37.3	-	37.3	-	37.3
Fe-EDTA	-	-	37	-	-	-
Sequestrene 330Fe	-	-	-	-	40	-
KI	-	0.8	-	-	0.75	-
H_3BO_3	10	1.6	-	-	3	10
$MnSO_4.4H_2O$	25	4.4	-	-	-	25
$MnSO_4.H_2O$	-	-	-	-	10	-
$ZnSO_4.7H_2O$	10	1.5	-	-	2	10
$Na_2MoO_4.2H_2O$	0.25	-	-	-	0.25	0.25
$CuSO_4.5H_2O$	0.025	-	-	-	0.025	0.025
$CoCl_2.6H_2O$	-	-	-	-	0.025	0.025
m-Inositol	100	-	-	4502	100	100
Thiamine HCl	0.5	1	1	-	10	0.5
Pyridoxine HCl	0.5	0.5	-	-	1	0.5
Nicotinic acid	5	0.5	-	-	1	0.5
Glycine	2	2	-	-	-	2
Glutamine	-	-	-	730	800	800
Glutathione	-	-	-	-	-	30
Serine	-	-	-	105	100	100
Folic acid	5	-	-	-	-	0.5
Biotin	0.5	-	-	-	0.1	0.5
NAA	-	-	-	-	0.1	-
2,4-D	-	2	1.5	-	-	0.05
BAP	-	-	-	-	-	-
Kinetin	-	0.5	0.5	-	-	-
Potato extract	-	-	-	10%[g]	-	-
Sucrose	2%	5-12%	9-10%	2%	10%	13%
Agar	0.8%	0.8%	0.55-0.7%	-	0.8%	-

a Nitsch & Nitsch (1969) Science **163**: 389-304. (tobacco anther culture)
b Chu (1978) Proc. Symp. Plant Tissue Culture, Peking, pp 45-50. (cereal anther culture)
c Chuang *et al.* (1978) Proc. Symp. Plant Tissue Culture, Peking, pp 51-56. (wheat anther culture)
d Nitsch (1977) Fundamental and Applied Aspects of Plant Cell, Tissue and Organ Culture. Springer pp 268-278. (isolated pollen culture of tobacco)
e Keller and Armstrong (1977) Can. J. Bot. 55:1383-1388. (*Brassica* anther culture)
f Polsoni et al. (1988) Can. J. Bot. 66:1682-1685. (isolated pollen culture of *Brassica*)
g 100g diced potato tuber boiled in distilled water for 25-30 min and then strained and filtered.

Incorporation of activated charcoal (0.5-2%) into the nutrient medium has been shown to stimulate androgenesis in anther cultures of some plants (Kohlenbach and Wernicke, 1978; Keller and Stringam, 1978).

Physical factors. Several physical treatments, such as temperature shock, centrifugation and γ-irradiation, to the flower buds or cultured anthers/microspores promoted androgenesis in different systems. Of these, promotion by temperature shock is most widely observed.

In many species (*Nicotiana tabacum, Hyoscyamus niger*) incubation of anthers/microspores at low temperature (4-5°C) for various periods before shifting them to 25°C (standard culture room temperature) enhanced the androgenic response. The optimum duration of the treatment may vary with the temperature, stage of pollen development and the genotype. Cold treatment seems to delay the degeneration of the anther wall and, thereby, enhance the proportion of the surviving microspores.

In some other plants (*Brassica* species, *Capsicum annuum, Avena sativa*) an initial high temperature shock has proved beneficial. The efficiency of pollen embryogenesis in anther cultures of *B. campestris* treated at 35°C for 24 hours was 20 times higher than the untreated control (Hamaoka *et al.*, 1991). For most *Brassica* species a high temperature treatment (30-35°C) for the initial 1-4 days of culture is essential to induce androgenesis. In the cultures of late uninucleate microspores of *B. napus* the minimum duration of high temperature (32°C) required to induce pollen embryogenesis is 2 hours, and the maximum response (70% grains forming embryos) is achieved after 4 days of heat shock (Pechan *et al.*, 1991). In *B. napus*, exposure to 41°C for one or two hours followed by incubation at 32°C for 4 days induced androgenesis even in the cultures of late binucleate pollen grains which normally do not respond (Hause *et al.*, 1996).

Induction of Androgenesis

For most species a suitable stage for androgenesis lies between just before and just after first pollen mitosis. During this period the microspores are noncommittal in their developmental potential. After the first mitotic division the cytoplasm gets populated with gametophytic information and it gradually becomes irreversibly committed to form male gametophyte. A variety of stresses applied during the liable developmental period can wash the gametophytic programme and induce the expression of sporophytic genes and, thus, induce the pollen to switchover from gametophytic to sporophytic mode of development.

In *Brassica napus* the late uninucleate microspores are most suitable for androgenesis. When these microspores are cultured at 18°C they show only gametophytic development. At 32°C at least 40% of the microspores

In cereals the frequency of green plant formation is significantly higher through gynogenesis than through androgenesis. For example, San Noeum and Ahmadi (1962) reported that in barley *cv* 'Bernice' 100% gynogenic plants were green as against 1% androgenic plants. Similarly, in rice *cv* 'Zao Geng No. 19' the percentage of green plants in ovary cultures was 89.3, but in anther cultures it was only 36.4% (Zhou *et al.*, 1986). In rice the percentage of haploids was also higher in gynogenic plants (77.5) than in androgenic plants (63.9). A common problem with anther culture of *Petunia* spp has been the high percentage of polyploid plants that are produced. In contrast to this over 93% gynogenic plants of *P. axillaris* were haploid (DeVerna and Collins, 1984). In *Morus alba* androgenesis has not been successful but gynogenic haploids could be produced (Thomas *et al.*, 1999).

HAPLOID PRODUCTION THROUGH DISTANT HYBRIDIZATION

Kasha and Kao (1970) discovered a new method for obtaining haploids (Fig. 17.5). In interspecific crosses between the tetraploids of *Hordeum vulgare*

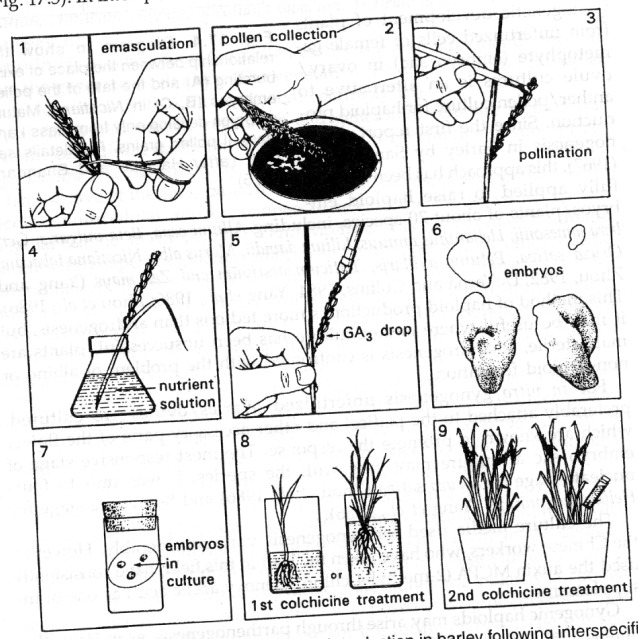


Fig. 17.5 General procedure for haploid production in barley following interspecific hybridization. (*Courtesy*: Dr. N.C. Subrahmanyam, India)

and *H. bulbosum* nearly all the plants formed were dihaploids. Similarly, in the crosses of the diploids of these species the progeny comprised haploids. The progeny was like *H. vulgare* in morphology and chromosome complement. Cytological examinations have revealed that in this cross double fertilization occurs normally but selective elimination of *bulbosum* chromosomes during early stages of development of embryo, which initially contains 1 *vulgare* and 1 *bulbosum* genome, leads to haploid embryo formation (Subrahmanyam and Kasha, 1973). In this cross the embryo normally aborts after 10 days of pollination, but it is possible to raise whole haploid plants through embryo culture. Barclay (1975) obtained high frequency haploids of wheat (*Triticum aestivum*) by crossing it with *Hordeum bulbosum*, followed by embryo culture (also *see* Zenkteler and Straub, 1979).

Diploidization of Haploids

The haploids may grow normally up to the flowering stage but in the absence of homologous chromosomes the meiosis is abnormal and, consequently, viable gametes are not formed. To obtain fertile homozygous diploids the chromosome complement of the haploids must be duplicated. For long, colchicine has been used for this purpose.

Another method followed to diploidize the haploids (Fig. 17.6) utilizes the tendency of *in vitro* growing callus cells to undergo endomitosis. Segments from vegetative parts of haploid plants are grown on a suitable culture medium and made to proliferate into calli. After some time many of the callus cells become diploid due to endomitosis. By transferring such calli to an appropriate medium fertile diploids can be obtained.

Importance of Haploids in Higher Plants

1. Mutations are difficult to detect in higher organisms because they are usually recessive and do not express themselves in the presence of their dominant alleles on the homologous chromosomes. Repeated selfing of the plants carrying the mutations is essential to bring out the recessive traits. However, this is not always possible because certain species, such as tea, are self-incompatible. Moreover, where selfing is possible one individual out of four will bear the recessive character, and where more recessive mutations are present the possibility of getting individuals showing all the mutations is extremely low. On the other hand, mutations induced in haploids can be easily detected because they have only a single set of genes and, consequently, there is no interference by their dominant alleles. Haploids with desirable mutations can be picked up and their chromosomes duplicated to get fertile diploids with all desirable mutations in a single generation. This approach has yielded an improved variety of rice (DAMA) in Hungary (Heszky *et al.*, 1992).

2. Haploid plants provide a source of haploid tissue which can be maintained *in vitro* in undifferentiated state and can then be dissociated into free haploid cells. In this way it will be possible to obtain real suspensions of haploid cells and to carry out new genetic researches on the

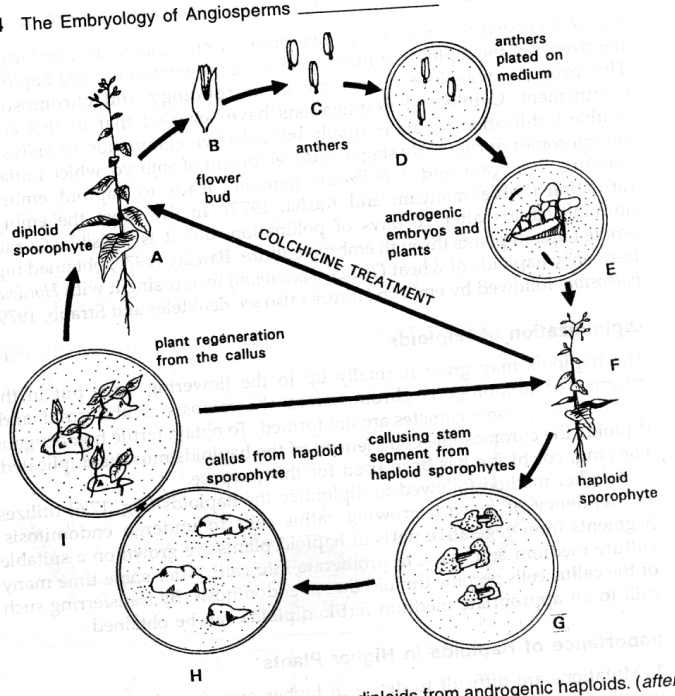


Fig. 17.6 Method to obtain homozygous diploids from androgenic haploids. (*after* Bhojwani and Razdan, 1996)

higher plants by applying the techniques used for microorganisms, such as mutation studies at the physiological level, biochemical analysis, etc.

3. For using hybrid vigour as a method of crop improvement, homozygous, true breeding cultivars are extremely important. Obtaining such cultivars through the conventional method of plant breeding, especially in out-breeding crops, requires many years of patient work. It involves selfing for many generations. Moreover, this technique cannot be applied to self-incompatible plants. In this respect, haploids can be easily treated with colchicine to produce homozygous diploids in a single generation.

4. The commercially desirable features of *Asparagus officinalis* are uniform, male plants with inflorescences (spears) having a low fibre content. In this dioecious crop plant an inbred population is produced through sibcrosses between pistillate (XX) and staminate (XY) plants. Consequently, 50 per cent plants are male and 50per cent female. Thevinin (1974) and Tsay *et al.* (1982) cultured anthers from the male plants and raised homozygous super

males (YY). When such males are crossed with female (XX) plants the entire progeny consists of males. Corriolis *et al.* (1990) announced the release of the first homogeneous all male F_1 hybrid of *A. officinalis*, called 'Andreas', using homozygous supermales derived from pollen embryos and a homozygous female parent obtained by diploidizing a spontaneous parthenogenetic haploid. Andreas is a very regular, high yielding variety with large spear diameter and very tight head.

5. Anther culture is also a good source of genetic variation (gametoclonal variation) and enables analysis of gametic variation at plant level. Pollen grains within an anther form a highly heterogeneous population because they are formed as a result of meiosis which involves recombination and segregation. Therefore, each pollen-plant is genetically different from the other. Variation in pollen-plants is especially high when the parent plant is a recent hybrid.

Schaeffer *et al.* (1984) obtained rice plants with longer grains, higher level of seed storage proteins, shorter stature and more tillers than the parents by selfing pollen-plants. The Chinese have developed and released about 20 new improved varieties of wheat and 61 varieties of rice from pollen-plants (Hu, 1985). Of the 40,000 plants obtained through selfing of temperature sensitive strain of *Nicotiana tabacum* none survived 13°C day/ 8°C night temperature regime but 2 out of 366 dihaploids produced through anther culture survived the cold treatment (Matzinger and Burk, 1984). Siebel and Pauls (1989) observed that some of the pollen-derived spontaneous diploids from crosses between low and high erucic acid lines of *Brassica napus* produced high erucic acid oil than either of the parents; some erucic acid-free lines also occurred in these androgenic plants. Both the variants are economically useful for different purposes.

EMBRYO CULTURE

The first systematic attempt to grow the embryos of angiosperms *in vitro*, under aseptic conditions, was made by Hannig in 1904. He cultured mature embryos of two Brassicaceae members, *Cochlearia* and *Raphanus*. Subsequently, many other workers raised plantlets by culturing embryos excised from mature seeds. Dietrich (1925) recorded that on semi-solid medium containing minerals and 2.5-5% sucrose mature embryos showed normal growth, but the embryos 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. Dietrich described this phenomenon of "precocious germination" of embryos as "kunstliche Fruhgeburt".

Stimulus for further progress in the field of embryo culture came from the work of Laibach (1925, 1928) who demonstrated the practical application of this technique. In certain interspecific crosses of the genus *Linum*, Laibach noted that the seeds were greatly shrivelled, very light and incapable of germination. By excising embryos from such seeds and growing them on a moist filter paper or on cotton wadding containing sucrose or glucose, he

Practical Applications of Embryo Culture

1. SHORTENING OF THE BREEDING CYCLE. Occasionally the breeding work on horticultural plants, such as deciduous fruit trees, is delayed due to long dormancy periods of their seeds. By growing excised embryos on nutrient medium this period can be considerably cut down.

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 embryos, Platnikov (1983) could reduce the life cycle of sunflower to half (60-70 days). 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 days old embryos, respectively (Fig. 17.8).

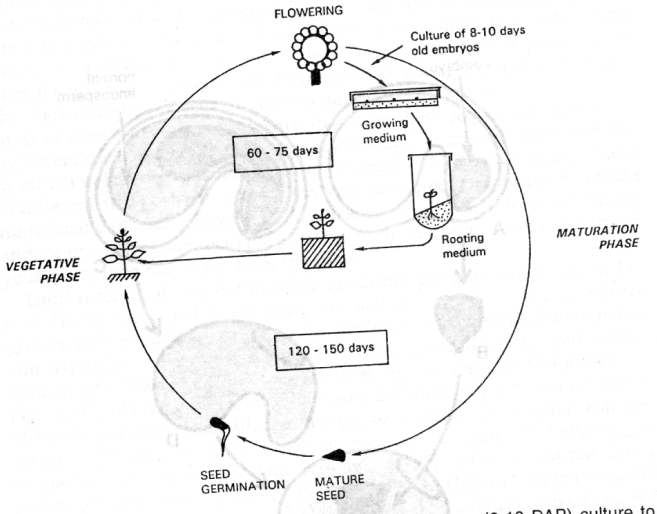


Fig. 17.8 Diagrammatized summary of immature embryo (8-10 DAP) culture to shorten the life cycle duration of sunflower from 120-150 days to 60-75 days. (*after* Serieys, 1992)

Slow, poor and erratic (in a seed batch germination may continue up to 3 years) seed germination in olive has been a serious handicap in the breeding of this crop. Early harvesting and removal of sclerified endocarp have helped in promoting germination but even with these measures many cultivars exhibit slow and low rate of germination. In the 10 cultivars of olive studied by Acebedo *et al.* (1997) the frequency of and the mean time required for germination of endocarp-lacking seeds ranged from 5-26% and 8-15 weeks, respectively. In contrast, all the excised embryos germinated

within 2 weeks (synchronously) with a mean frequency of 75% (3-15 fold increase over seed germination). The plants raised by seed germination and embryo culture showed normal growth in glasshouse although the root growth in the seed-derived plants was slightly faster (Acebedo *et al.*, 1997).

2. RAPID SEED-VIABILITY TEST. The possibility of breaking seed dormancy by embryo culture also allows the use of this technique as a rapid test for seed-viability. According to Barton (1961), germination of excised embryos is reliable and capable of more exact interpretation than the commonly used staining tests for seed-viability.

3. HAPLOID FORMATION. Haploid production with the aid of embryo culture following distant hybridization is described on page 322 (also *see* Fig. 17.5).

4. PROPAGATION OF RARE PLANTS. As an abnormality, some coconuts develop soft, solid, fatty tissue in place of the liquid endosperm. Such nuts are termed "makapuno" and, being rare, are very expensive; served only at special banquets in the Philippines. It had been felt that plants propagated from makapuno nuts would be truly makapuno-bearing (Torres, 1937; 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) succeeded in raising plants from makapuno nuts. Eighty-five per cent of the nuts borne by these plants were makapuno (De Guzman *et al.*, 1976).

5. OBTAINING RARE HYBRIDS. In plant breeding programmes all desirable crosses are not successful. A cross fails due to some pre-fertilization barriers (described in Chapter 10), or fertilization occurs normally but the embryo dies at an early stage of development due to poor or abnormal development of the endosperm. In the latter case, where hybrid embryo capable of normal development aborts due to inadequate supply of nutrition inside the seed or due to endosperm-embryo incompatibility, it is possible to raise complete hybrid plants through embryo culture. This method has been profitably used for many interspecific crosses.

Emsweller and Uhrig (1962) crossed *Lilium speciosum-album* and *L. auratum*. The seeds obtained in this cross were larger in size due to large amount of endosperm. The embryo was also very long. When these seeds were sown the embryo degenerated and no seedlings were formed because of embryo-endosperm incompatibility. However, by growing excised immature embryos on nutrient medium the authors could raise full hybrid plants.

The caryopses formed in the crosses between *Hordeum vulgare* and *H. bulbosum* are frequently devoid of endosperm. As a result of this the embryo dies prematurely. By excising the embryos 14-28 days after pollination, Davis (1960) was able to raise seedlings capable of transplantation to soil. Similarly, Inomata (1967) crossed diploid *Brassica chinensis* and tetraploid *B. pekinensis*, but the triploid hybrid embryo failed to mature because of early endosperm degeneration. By culturing hybrid embryos on a medium

was able to raise the hybrid plants. Now this technique is widely used by plant breeders for raising many more such hybrids which could not be obtained due to the abortion of embryos. Besides its practical applications, the technique of growing embryos outside the environment of the ovule (*ex ovulo*) provides an excellent opportunity to study the nutrition and physical and chemical regulation of morphogenesis during embryogenesis. Using this technique the regeneration potentialities of whole embryos and their parts have also been investigated.

Nutrition of the embryo *in vitro* has been discussed in Chapter 12. To recount the salient features, the nutritional requirements of differentiated embryos are simple. They can be grown successfully in a medium containing only a few mineral salts and sucrose. This is because their synthetic ability is well developed and, consequently, they are able to fulfill most of their growth requirements. At this stage, which is also called the "autotrophic phase" of embryo development, cultured embryos of most plants show almost similar requirements. On the other hand, younger embryos represent the "heterotrophic phase" of embryo development and are dependent for most of their growth requirements. In nature these requirements are met by the endosperm or some other maternal tissue. For growing such embryos *in vitro* an elaborate nutrient medium is required. Globular embryos and undifferentiated post-globular embryos can be reared to maturity by adding nutritionally rich substances (coconut milk, casein hydrolysate, yeast extract) and/or growth regulators to the basal medium. The requirement of cultured young embryos may vary with the plant.

Until recently, it was not possible to culture pre-globular embryos. Liu *et al.* (1993) succeeded in culturing as small as 8 to 36-celled proembryos (35 µm) of *Brassica juncea* on a complex medium containing, besides minerals and vitamins, 10 sugars, 4 organic acids, coconut water and casein hydrolysate. They used the doubled-layer culture system of Monnier (*see* Fig. 12.17), involving two semi-solid media differing only in their osmolarity. The embryos were embedded in the upper medium with higher osmotic pressure (63 mol l^{-1}) than the lower medium (45 mol l^{-1}). Following a similar procedure, Fischer and Neuhaus (1995) obtained normal direct embryogenesis in the cultures of globular embryos (100-160 µm in diameter) of wheat. Full fertile plants have also been raised by culturing naked zygotes formed *in vivo* (Holm *et al.*, 1994) and *in vitro* (Kranz and Lorz, 1998). Nurse cells derived from embryogenic suspension cultures or microspores undergoing embryogenesis were essential for the cultivation of isolated zygotes.

Embryo-nurse Endosperm Transplant

Despite considerable progress made in the improvement of embryo culture technique one is still confronted with the problem of rescuing hybrids when embryo abortion occurs at a very early stage of development. To some extent this barrier to crossability can be overcome by implanting the excised immature embryos on cultured endosperm. In the cross *Hordeum x Secale* the survival of immature hybrid embryo when planted directly on the culture medium was one per cent. This could be increased to 30-40 per cent by implanting the embryo on cultured endosperm (Kruse, 1974).

A slightly modified endosperm transplant technique for young embryo culture was described by de Lautour *et al.* (1978), Williams (1978, 1980) and Williams and de Lautour (1980). They inserted the excised hybrid embryo into cellular endosperm dissected out from a normally developing ovule (Fig. 17.7) 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 nutrient medium.

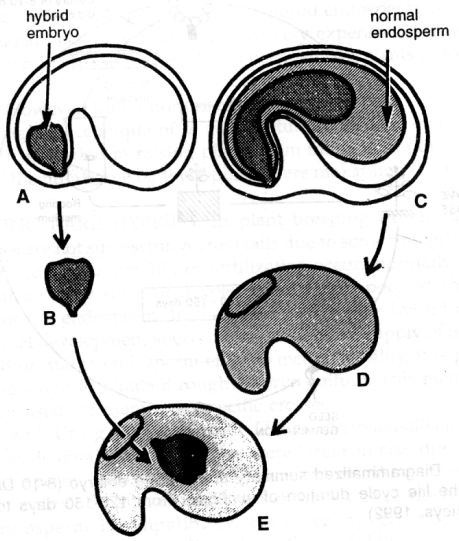


Fig. 17.7 Endosperm-transplant for culture of hybrid embryos in *Trifolium, Lotus* and *Ornithopus*. **A, B.** The hybrid embryo is removed from the ovule in which endosperm development has failed. **C.** To provide transplant endosperm a normally developing intra-specifically pollinated ovule is dissected at a stage when it contains cellular endosperm enclosing torpedo-shaped to heart-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. (*Courtesy*: Dr. E. Williams, New Zealand)

containing diffusate of young seeds of *Lupinus luteus* Inomata was able to raise complete hybrid plants.

Through embryo culture Iyer and Govila (1964) raised hybrids from several interspecific crosses of *Oryza* which are otherwise impossible to obtain. Excised embryos or whole ovaries 10-25 days after pollination were cultured on semi-solid medium supplemented with 10% coconut milk and 0.1% malt extract. On this medium up to 88% embryos germinated. After the seedlings had grown fully in the test tubes they were transplanted to soil. Crossing cultivated tomato (*Lycopersicon esculentum*) with wild tomato (*L. peruvianum*) has been considered desirable from the point of view of transferring pests and disease-resistance from the latter to the former. The cross *L. peruvianum* × *L. esculentum* does not succeed due to the 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). Thomas and Pratt failed to 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 from this cross. The poorly developed hybrid embryos were dissected out from ovules 35 days after pollination 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 crosses *L. esculentum* × *L. chilense* and *L. esculentum* × *Solanum lycopersicoides* in which embryos capable of direct plant formation do not develop (Scott and Stevens; *cited in* Thomas and Pratt, 1981).

NUCELLUS CULTURE

The natural occurrence of nucellar polyembryony and its practical value in raising disease-free clones of *Citrus* is described in Chapter 13. The genus *Citrus* includes monoembryonate as well as polyembryonate species. In the latter, the adventive embryos from nucellar cells arise only after pollination, and they are confined to the micropylar-half of the nucellus.

Rangaswamy (1961) and Sabharwal (1963) reported that nucellar tissue excised from post-pollinated carpels, when the adventive embryos are already in torpedo-shaped stage, and cultured on White's medium (medium-A, Table 17.1), supplemented with casein hydrolysate, it proliferates and forms a callus. From the callus differentiate numerous tumeroid outgrowths, called pseudobulbils. Eventually, many of the pseudobulbils develop into embryos which germinate to form seedlings (Rangaswamy, 1962; Sabharwal, 1962). Button and Bornman (1971) demonstrated, for the first time, that nucellus tissue from unfertilized ovules (when there is no adventive embryo present) can also form embryos under suitable culture conditions. The nucelli from pre-fertilized ovules exhibit embryogenesis only if the medium is supplemented with malt extract and adenine. On this medium fully differentiated embryos are formed but they fail to germinate. If these embryos are excised and planted individually on a medium supplemented with GA_3, they sometime form plantlets. Button and Bornman could successfully transplant these saplings to soil.

Rangan *et al.* (1968) extended the studies on nucellus culture to monoembryonate species of *Citrus (C. grandis, C. limon, C. reticulata, C. sinensis)* so that, in the absence of natural adventive embryony in these species, this technique could be employed for clonal propagation of their disease-free stocks. They cultured nucelli only from post-fertilized ovules. The nucelli excised 100-120 days after pollination did not grow on White's medium supplemented with casein hydrolysate. However, on White's medium containing malt extract 10-12% of the cultured nucelli formed embryos (Fig. 17.9A). According to these authors, the embryos developed directly from the nucellar cells, without any callusing or the formation of pseudobulbil-like structures as noted in nucellus culture of polyembryonate species. The embryos germinated *in situ* and, within 6-7 weeks, grew into plantlets with well developed roots and leaves (Fig. 17.9B). However, Kobayashi *et al.* (1981) did not obtain adventive embryogenesis in nucellus cultures of any of the 23 monoembryonate cultivars of *Citrus* they tried, but polyembryonate cultivars exhibited good embryogenesis. In the latter case adventive embryos were derived by the activity of the preformed adventive embryo initial cells (AEICs, *see* page 242) and not from any other cell of the nucellus. According to Kobayashi *et al.* the lack of AEICs in the monoembryonate cultivars is the cause for their failure to produce adventive embryos in tissue cultures.

Besides *Citrus, in vitro* nucellar polyembryony has been reported in *Cynanchum vincetoxicum* (Haccius and Hausner, 1976), *Malus domestica* (Eichholtz *et al.*, 1979) and *Vitis vinifera* (Mullins and Srinivasan, 1976).

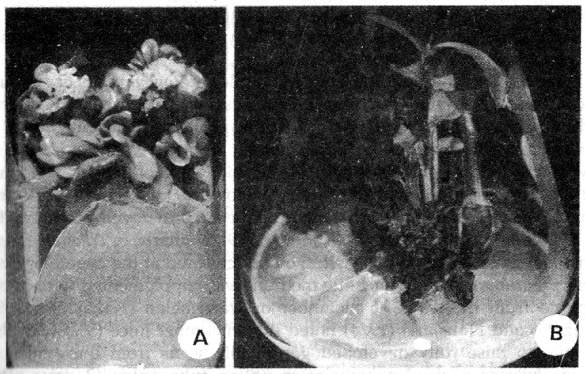


Fig. 17.9 Nucellus cultures of *Citrus limon* ("ponderosa limon"). **A.** Six-week-old culture showing polyembryonal mass. **B.** Some of the embryos have developed into plantlets. (*Courtesy*: Dr. T.S.Rangan, U.S.A.)

OVULE AND SEED CULTURE

Ovule Culture

Ovule culture is especially valuable when the objective is to study the behaviour of zygote or very young embryos which are difficult to excise as well as to culture. The first attempt to isolate ovules and culture them under aseptic conditions was made by White (1932) in *Antirrhinum majus*. However, the technique of ovule culture was perfected by N. Maheshwari (1958).

Raising mature seeds by culturing ovules at the globular or a later stage of embryo development is comparatively easy and has been achieved with many plants, such as *Gynandropsis* and *Impatiens* (Chopra and Sabharwal, 1963), *Nicotiana tabacum* (Dulieu, 1966), and *Allium cepa* (Guha and Johri, 1966). On the other hand, success in obtaining viable mature seeds from ovules excised soon after fertilization has been limited. Younger ovules often require a complicated medium.

Maheshwari (1958), and Maheshwari and Lal (1961) could raise viable seeds of *Papaver somniferum* starting with ovules excised 6 days after pollination when they contained zygote or 2-celled proembryo and a few endosperm nuclei. Seeds formed in cultures germinated *in situ*. On Nitsch's medium (without any growth supplement) the growth of embryos was initially slower than that in nature but after the globular stage they grew very rapidly. Twenty six days after pollination the embryos in cultured ovules measured 0.93 mm as compared to the maximum length of 0.65 mm attained by the embryos *in vivo*.

Poddubnaya-Arnoldi (1959, 1960) successfully grew ovules from the pollinated ovaries of several orchids on simply 10% sucrose solution. In her studies she was able to trace events from entry of the pollen tube into the ovule up to the development of embryo.

Unlike *Papaver* and orchids, when the ovules of *Zephyranthes* containing zygote and the primary endosperm nucleus were cultured on Nitsch's medium alone the embryo grew up to the globular stage, but failed to exhibit differentiation (Sachar and Kapoor, 1959). Even the addition of growth substances, such as kinetin, IAA, and gibberellic acid could not promote further growth of the embryo. However, if coconut milk and casamino acids were added to the medium the embryo developed normally and the seeds germinated *in situ* (Kapoor, 1959). Similarly, in *Trifolium repens* mature seeds could be obtained from ovules cultured at the zygote or two-celled proembryo stage only if the medium was supplemented with juices prepared from the fruits of cucumber or watermelon (Nakajima, 1969). Wakizuko and Nakajima (1974) noted that cucumber juice (5%) was also essential to raise fully developed germinable seeds from the ovules of *Petunia hybrida* excised shortly after pollen tube entry (2 days after pollination). Thus, the growth requirements of very young ovules are quite diverse.

Chopra and Sabharwal (1963) noted a beneficial effect of placental tissue on the growth of cultured ovules of *Gynandropsis*. This has also been observed by Kanta and Maheshwari (1963), and Rangaswamy and Shivanna (1967) in their studies on *in vitro* pollination and fertilization of excised ovules.

Joshi (1962), and Joshi and Johri (1972), cultured the ovules of *Gossypium hirsutum*. Maximum growth of the embryo was noted on White's medium supplemented with casein hydrolysate (1000 ppm) or an extract prepared from its ovules. In all the treatments the cotton-fibre initials accumulated tannin and collapsed. Beasley and Ting (1973) conducted detailed studies on the physiology of fibre cell elongation in cultured ovules of this plant. If the ovules were excised on the day of anthesis or a day later the fibres either failed to develop or only a few fibres developed. However, most of the ovules cultured two days after anthesis showed the development of fibres. The ovules floating on the surface of the liquid medium produced more fibres than those submerged. Fibre development was better at 30°C than at 20°C or 25°C. Gibberellic acid induced marked elongation of the fibres whereas kinetin and abscisic acid caused inhibition.

Pundir (1967) cultured the ovules of cotton with an entirely different objective. In the cross *Gossypium arboreum* × *G. hirsutum* the embryo developed only up to 8-10 days after pollination. Subsequently, numerous abnormalities occurred in the endosperm, leading to the failure of embryo development. Beasley (1940), Miller (1950) and Weaver (1958) cultured the young hybrid embryos to raise hybrid plants but the excised embryos failed to grow. Pundir (1967) excised the ovules 3 days after pollination, when they contained zygote or 2-celled proembryo, and cultured them on Murashige and Skoog's medium (medium-B; Table 17.1). By the 5th week fully differentiated embryos were formed and, by the 7th week, 70-80% ovules developed hybrid seedlings. Following similar technique, Steward and Hsu (1978) raised four different interspecific hybrids in the genus *Gossypium* which are otherwise unknown.

Seed Culture of Plants with Reduced Embryos

Some plants are characterized by the presence of embryos that lack differentiation into various embryonic organs, namely radicle, plumule and cotyledons. In *Eranthis* (Ranunculaceae) the seeds at shedding enclose an undifferentiated, pear-shaped embryo (Fig. 13.9). After the seed has fallen on the ground the embryo undergoes intraseminal growth and achieves the usual morphology of a mature dicotyledonous embryo. In orchids and the members of the Orobanchaceae also the seeds contain an unorganized embryo. However, in these plants the intraseminal growth is absent, and the seedlings are formed directly by the globose embryos. In such cases the embryonal pole proximal to the micropyle is called the radicular pole and that distal to the micropyle is termed the plumular pole.

According to Avdhani (1962), during seed germination in orchids the plumular pole of the embryo enlarges to form a spherule-like structure

fruits containing viable seeds. Recently, Applewhite *et al.* (1997) reported *in vitro* development of red, ripe fruits of tomato with normal flavour from pre-anthesis flower buds. On MS medium (3% sucrose, pH 5.8) supplemented with 1 µM BAP and 10 µM IBA, 100% of the buds formed red berries. However, the size of the *in vitro*-developed fruits was smaller (< 2 g F.W.) than those formed in nature, as also reported earlier (Teitel *et al.*, 1985; Varga *et al.*, 1988). The reduced fruit size *in vitro* is due to reduction in both cell division and cell enlargement. It is interesting to note that the DNA content per cell increases 15 fold during the growth of tomato fruits on the plants as compared to only 2-3 fold in cultured fruits (Teitel *et al.*, 1985). These differences in the fruit growth *in vivo* and *in vitro* could be due to insufficient supply of carbohydrates under latter conditions (Varga *et al.*, 1988). Until this bottleneck is removed, the *in vitro* system cannot be recommended as a model for *in vitro* fruit development. The largest fruits formed in ovary cultures of *Linaria marocanna* (Sachar and Baldev, 1958) and *Tropaeolum* (Sachar and Kanta, 1958) were smaller than their natural fruits.

Maheshwari (1958) succeeded in rearing the ovaries of *Iberis amara* excised from flowers one day after pollination. At this stage the ovules contained a zygote and a few endosperm nuclei. On a simple medium containing mineral salts and sugar the growth of ovaries was good but the embryos remained smaller than those formed in nature. With the addition of B-vitamins to the above medium normal healthy fruits, matching the natural fruits, were obtained. *In vitro*-formed fruits, were even larger than the *in vivo*-formed fruits when IAA was added to the mineral salts-sugar-vitamins medium (Maheshwari and Lal, 1958). In cultures normal development of ovaries, excised at the zygote or 2-celled embryo stage, has also been reported in *Anethum graveolens* (Johri and Sehgal, 1966) and *Hyoscyamus niger* (Bajaj, 1966). On a medium supplemented with coconut milk the ovaries of *Anethum* surpassed the size of natural fruits.

Whereas ovaries excised from pollinated flowers are able to grow on simple mineral salts-sugar-vitamins medium, ovaries from unpollinated flowers fail to grow on such a medium. An auxin can replace the pollination stimulus. Nitsch (1951) obtained seedless fruits of tomato from unpollinated ovaries cultured on a medium supplemented with 2,4-dichlorophenoxyacetic acid or 2-naphthoxyacetic acid. In *Althaea rosea* the addition of indolebutyric acid to the nutrient medium resulted in the parthenocarpic development of fruits attaining natural size (Chopra, 1958).

Most apomicts require pollination stimulus for the growth of ovary and seeds. *Aerva tomentosa* is an obligate apomict. In Delhi, only pistillate plants of this taxon are found. It is, therefore, thought that seed-set in this plant occurs without the pollination stimulus. Puri (1963) cultured ovaries, flowers and portions of spikes of this apomict and studied the effect of IAA, casein hydrolysate, coconut milk, and yeast extract on the growth of the embryos. The best response was noted when portions of spikes were cultured instead of individual flowers or ovaries. In the presence of IAA and yeast extract

the seed-set was comparable to that in nature. The development of endosperm and embryo was also fairly normal.

Effect of Calyx and Roots on Fruit Growth

In vitro culture of ovary has revealed that the perianth lobes, especially sepals, function not only as protective structures for the sex organs but play an important role in the development of fruits. Culture of ovaries after removing the calyx results in poor development of fruits (Table 17.3). This has been demonstrated for *Allium, Althaea, Hordeum, Hyoscyamus, Iberis* and *Triticum*.

TABLE 17.3 Effect of calyx and corolla on the growth of fruits and seeds of *Hyoscyamus niger*. Ovaries were cultured for 6 weeks on White's medium. (*after* Bajaj, 1966)

Treatment	*Fruit diameter mm*	*Size of seed (length x breadth) mm*
1. Control *in vivo*	12.2	1.5 x 1.2
2. Ovary cultured with intact calyx and corolla	9.5	1.5 x 1.1
3. Ovary cultured with intact calyx (without corolla)	9.5	1.5 x 1.1
4. Ovary cultured without calyx and corolla	5.5	0.4 x 0.2

Guha and Johri (1966) noted that in *Allium* the perianth extract improved the growth of ovaries cultured without the perianth but it did not match the effect of intact perianth. On the basis of his studies on tomato, Nitsch (1963) has proposed that the calyx supplies some nitrogenous substances which are indispensable for fruit growth.

According to Nitsch (1963) roots have a stimulatory effect on fruit growth. He cultured the ovaries of tomato with a small pedicel and induced rooting of the pedicel by adding tomato juice in the medium. He observed that the growth of the ovaries after root formation was very rapid and they formed small fruits which matured and turned red. Similar other studies to confirm this observation are, however, lacking.

Development of Seeds in Cultured Ovaries

Satisfactory development of fruits is no indication of normal development of seeds in it. Nitsch (1951) stated that in ovary cultures of tobacco and tomato, on the basal nutrient medium, although fruit development was fairly good, seeds failed to develop. In gherkin also most of the *in vitro*-formed fruits showed only a few seeds. In *Tropaeolum* ovary cultures on basal medium the embryos showed some growth and differentiation but were invariably smaller than those formed *in vivo* (Sachar and Kanta, 1958). However, in some plants, such as *Hyoscyamus, Iberis* and *Linaria*, embryo

called protocorm. It is initially white but gradually turns green. After attaining a certain size the protocorm differentiates roots and shoots. In this way in orchids the entire seedling is derived by the activity of the plumular pole of the embryo.

Monopolar seedling formation is also known in Orobanchaceae. As described by Kadry and Tewfic (1956), during seed germination in *Orobanche crenata* the radicular pole forms a "germ-tube" whose tip penetrates the host-root. The portion of the germ-tube that remains outside the host-root proliferates into an irregular mass of tissue, called tubercle, from which differentiates a shoot. The plumular pole of the embryo remains inside the testa. Rangan and Rangaswamy (1968) germinated the seeds of *Cistanche tubulosa in vitro* and recorded essentially the same pattern of germination as described for *Orobanche crenata* by Kadry and Tewfic. Similarly, Rangaswamy (1963) reported monopolar seedling formation in embryo cultures of *Orobanche aegyptiaca*. However, Usha (1968) demonstrated that in seed cultures of *O. aegyptiaca* seedling formation may be monopolar or bipolar depending on the composition of the culture medium. On nutrient medium supplemented with coconut milk or yeast extract the seedling formation is monopolar, but if gibberellic acid, kinetin, IAA, or strigol is added to the medium seedling formation is bipolar; the radicular pole forms root and the plumular pole forms shoot. This suggests that in nature the activity of the plumular pole in these plants is arrested due to lack of some essential factors or that some specific inhibitors do not allow the plumular pole to function normally.

Seed Culture of Parasites

In obligate root parasites such as *Orobanche* and *Striga* the seeds germinate close to host roots, and it is only after the parasite establishes contact with the host-roots that it develops shoot. In seed cultures of these parasites it is possible to substitute the host stimulus with some known chemicals. The seeds of *Orobanche* germinate *in vitro* in the absence of a natural host if the nutrient medium is enriched with a cytokinin, gibberellin, scopoletin or strigol.

Most of the root parasites do not form haustoria in cultures without a host. Aseptic seedlings of *Striga senegalensis* formed haustoria only if grown with the seedling 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 (Riopel and Musselman, 1979; Sahai and Shivanna, 1981), cotton strings and soybean (Alssatt *et al.*, 1978). None of the common sugars were effective in haustorial induction.

Unlike root parasites, loranthaceous stem parasites frequently form distinct haustorial structures, comparable to those formed in nature, without host or a plant extract (*see* Johri and Bhojwani, 1971). The seeds of *Scurrula pulverulenta* germinate on plain White's basal medium. However, the

seedlings grow better when casein hydrolysate is added to the medium. In either case the seedlings bear well differentiated haustoria (Fig. 17.10) which are structurally comparable to the haustoria formed in nature, inside the host tissue. Haustoria differentiation has also been reported from the callused embryo and endosperm of *Scurrula* and *Dendrophthoe* (Johri and Bhojwani, 1971). In *Scurrula* the differentiation of haustoria is inhibited in the presence of coconut milk or watermelon juice.

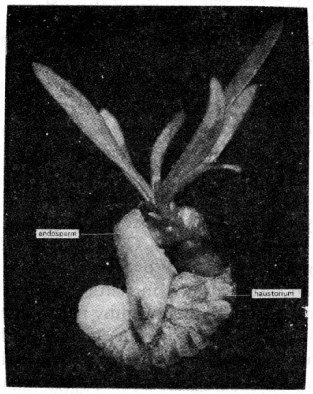


Fig. 17.10 Nine-week-old seedling of *Scurrula pulverulenta* formed in cultures. Note the worm-like haustorium and several plumular leaves. (*after* Bhojwani, 1969)

Ovary Culture

The knowledge of fruit physiology is of great value to horticulturists in improving the quality of fruits. When the effect of various chemicals on fruit growth is studied using entire plants, it remains questionable whether the chemical tested is acting in its original form or is getting modified in the plant system before reaching the ovary. In this regard the technique of excised ovary culture is highly promising. It allows direct feeding of the ovary with the chemicals. Nitsch (1951) and, subsequently, many other workers have shown that the general pattern of *in vitro* development should allow a reasonable comparison with the *in vivo*-developed fruits.

LaRue (1942) was the first to raise aseptic cultures of angiosperm flowers. He obtained rooting of the pedicel and a limited growth of the ovaries. The technique was further developed by Nitsch (1951) who grew detached ovaries of *Cucumis anguria, Fragaria* sp., *Lycopersicon esculentum, Nicotiana tabacum* and *Phaseolus vulgaris* on synthetic medium. The ovaries of *Cucumis* and *Lycopersicon* excised from pollinated flowers developed into mature

and endosperm development was normal on the basal medium. In *Anethum*, normal development of endosperm and embryo occurred only when ovaries were cultured on a medium enriched with coconut milk.

In *Ranunculus sceleratus* achenes produced in nature remain dormant for about an year. Sachar and Guha (1962) observed that the achenes formed in ovary cultures germinated without any dormancy period.

Practical Applications

Besides being useful for understanding the physiology of fruit development, ovary culture has been used as an aid to produce haploids and rare hybrids.

Hess and Wagner (1975) raised parthenogenetic haploids of *Mimulus luteus cv. tigrinus grandiflorus* by pollinating their exposed ovules in ovary cultures. Anther cultures of this plant did not form haploids. Parthenogenetic development of haploid plants in the cultures of unfertilized ovaries has also been reported in about a dozen species (see page 321).

EFFECT OF YOUNG SEEDS ON FRUIT GROWTH

Young seeds play a significant role in the development of fruits. This is borne out by the following observations:

1. *Seeds and furit-drop*. It has been seen that the apple fruits that fall off the tree contain, on an average, lesser number of seeds than the ones which remain attached to the tree. Accordingly, anything which interferes with the development of young seeds (parasites or thinning chemicals) will promote fruit-drop.

2. *Shape and size of the Fruit*. The development of seeds has been shown to control the shape of fruits. In strawberry, if the carpels in a flower are selectively pollinated so that some remain unpollinated, or after open pollination some of the carpels are removed, the receptacle shows only localized growth around the developing achenes. This gives the fruit an abnormal shape (Fig. 17.11A-D; Nitsch, 1965). There is also a linear relationship between the number of developing achenes and the weight of the fruit (Fig. 17.12). A similar relationship has been observed between seed development and fruit growth in apples, stone fruits, and cucurbits.

3. *Effect of seed removal*. Removing seeds at various stages of fruit development has revealed that seeds are necessary for fruit growth only during the early phase of their development. For example, in stone fruits and apples, if seeds are removed after a definite period following pollination the growth of fruits is not affected.

There is now ample evidence to suggest that seeds control fruit growth through growth regulators. The evidences fall under two categories: (a) of the total amount of growth regulators synthesized in the fruit, the major portion is present in the seeds, and (b) some growth regulators applied exogenously are able to stimulate fruit growth in the absence of seeds.

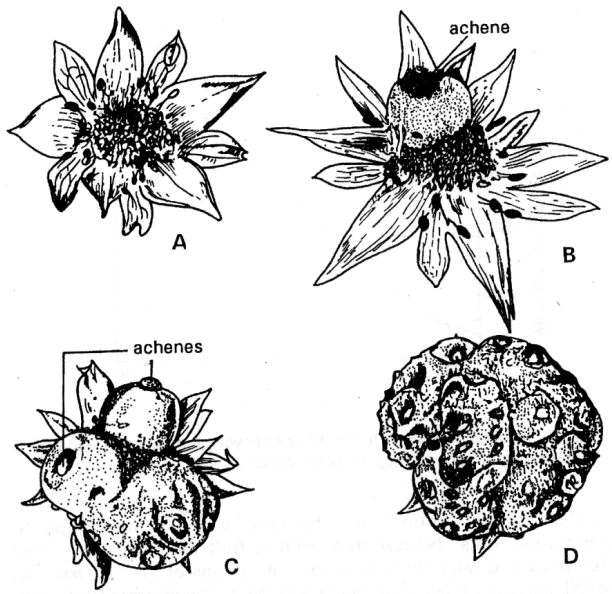


Fig. 17.11 Effect of developing achenes on the growth of strawberry receptacle. **A**. Unpollinated flower; the receptacle has not developed at all. **B**. Only one pollinated achene has developed; the growth of receptacle has occurred only around the achene. **C**. Several pollinated achenes have developed; several areas of receptacular growth are seen. **D**. The number of pollinated achenes is larger than that in **C**; the receptacle is approaching the shape of a normal strawberry fruit. (*after* Nitsch, 1965)

PARTHENOCARPY

Parthenocarpy is of widespread occurrence, especially among species which have a large number of ovules per ovary, such as banana, pineapple, tomato, melons and figs. According to Noll (1902), who introduced the term, parthenocarpy means the development of fruits without pollination or any other stimulus. Since then the definition of parthenocarpy has undergone slight modification and, according to the present concept, it refers to "the formation of fruits without fertilization" (Nitsch, 1965). The parthenocarpic development of fruit may require the pollination stimulus (stimulative parthenocarpy) or it may occur in unpollinated flowers (vegetative parthenocarpy).

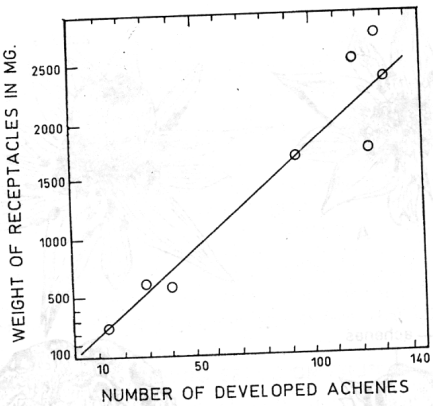


Fig. 17.12 Relationship between the number of developing achenes and weight of the fleshy receptacle of strawberry. (*after* Nitsch, 1950)

"Seedless fruits" should not be considered synonymous to "parthenocarpic fruits" because in a seedless fruit the ovules may have been fertilized and later aborted, as in some strains of *Vitis vinifera* var. concor (Nitsch *et al.*, 1960). Also, there may be parthenocarpic fruits with seeds in them. Auxin treatments are known to produce seeded parthenocarpic fruits in *Citrus* sp., grapes, watermelons and pineapples. The seeds in such fruits are really pseudo-seeds, lacking a sexual embryo; parthenogenic or adventive embryo may, however, be present.

Nitsch (1963) had recognized three types of parthenocarpy, namely: (a) genetic, (b) environmental, and (c) chemically induced. Recently, parthenocarpic plants have been developed by genetic engineering.

Genetic Parthenocarpy

Many of the plants cultivated for their fruits show seeded as well as parthenocarpic varieties. This type of parthenocarpy is known to arise due to mutations or hybridization. The famous navel oranges arose from a normal seeded *Citrus* variety through mutation in an axillary bud which grew out into a branch bearing seedless oranges. Besides navel oranges, genetic parthenocarpy occurs in *Citrus, Cucurbita, Eugenia, Musa, Punica,* and *Vitis*.

Wellington and Hawthorn (1929) obtained a parthenocarpic hybrid cucumber by crossing an "English forcing" variety with "Arlington White spine".

Environmental Parthenocarpy

Variations in environmental conditions such as frost, fog, and low temperatures, interfere with the normal functioning of sexual organs and bring about parthenocarpy. Campbell (1912) observed that a heavy fog in the month of June caused the formation of seedless olives. Lewis (1942) obtained parthenocarpic pears by exposing the flowers to freezing temperatures for 3-19 hours. Cochran (1936) increased fruit setting and obtained parthenocarpic fruits in *Capsicum* by shifting the plants from temperatures of 32°-38°C to 10°-16°C at the time of anthesis. Osbrone and Went (1953) were able to induce parthenocarpy in tomatoes with low temperatures and high light intensity. Under these conditions pollination is poor.

Chemically Induced Parthenocarpy

Auxins and gibberellins at low concentrations (about 10^{-7}-10^{-6}M) have been successfully used to induce parthenocarpy in a number of plants which normally bear seeded fruits. These substances are applied to flowers in the form of a lanolin paste, or as sprays. The latter is more convenient for commercial purposes.

Balasubramanyam and Rangaswamy (1959) noted that as a result of artificial pollination most of the varieties of *Psidium guajava* developed into seeded fruits but the variety "Allahabad Round" yielded seedless parthenocarpic fruits. In this variety parthenocarpy could also be induced by treating the emasculated flowers with an aqueous extract of pollen. This "pollen hormone"-effect could be duplicated by the application of indoleacetic acid and indolebutyric acid. Other plants where auxins (IAA, IBA, NAA, NOAA, 2,4-D, 2,4,5-T) could bring about parthenocarpic development of fruits include tomato, blackberry, strawberry, figs, cucurbits, *Citrus* and *Rosa*. Gibberellic acid has been reported to induce parthenocarpy in a number of rosaceous fruit trees, grapes, figs and tomato.

It has been suggested that for the induction of parthenocarpy through auxin treatment, the growth substance should be applied sometime after anthesis because an early application may damage the flowers and cause seed abortion and consequently, fruit drop. On the contrary, where gibberellic acid is used for this purpose it should be applied as early as possible, preferably at anthesis (Nitsch, 1963).

Genetically Engineered Parthenocarpy

By inserting a chimeric gene *DefH9-iaaH*, Rotino *et al.* (1997) were able to induce parthenocarpy in tobacco and eggplant. The *iaam* gene from *Pseudomonas syringae* pv *savastanoid* increases auxin synthesis in the tissues and organs of the transgenic plants and the ovule-specific promoter and regulatory gene *DefH9*, from *Antirrhinum majus*, regulates the expression of *iaam* only in the ovules without affecting vegetative growth.

The transgenic plants of eggplant carrying the gene *DefH9-iaam* produced fruits with viable seeds when pollinated and parthenocarpic fruits in the

absence of pollination. Thus, in glasshouses where non-transformed plants produce normal fruits only during summer, the transgenic plants continue to produce fruits with normal appearance throughout the year which is commercially very attractive. The parthenocarpic character was transmitted to the progeny in a Mendelian fashion as a dominant trait.

Parthenocarpy finds its importance in horticulture because seedless fruits are ideal for consumption either as such or in the juice and jam industries. One can imagine the annoyance caused by seeds while eating a watermelon. Parthenocarpy may also increase proportion of edible part of the fruit.

GENETIC TRANSFORMATION

While the conventional methods of breeding require 6 to 8 generations to produce a new variety, the same could be achieved in only 3 to 4 generations by introducing DNA fragments (genetic transformation). Several methods of genetic transformation are being currently tried. Initially, isolated protoplasts, cells or tissues were used to introduce desired genes, which required that the recipient cells should express the potentiality to regenerate whole plants. Unfortunately, several important cultivars of crop plants lack this potentiality. Here two embryological systems are described in which DNA pieces or foreign genes are delivered to the recipient egg, and the resulting embryo develops in a natural fashion. In comparison to the systems based on protoplasts or callus transformation, these methods are much simpler because they avoid the time consuming cell culture step and are not, in principle, limited by the ability to regenerate whole plants from protoplasts or calli. Therefore, these techniques are of wider applicability.

Gametic Transformation

While attempting to overcome self-incompatibility in *Nicotiana alata* and *N. forgetiana* by the technique of "mentor pollen" (described on page ...), Pandey (1975) made an intriguing observation. He noted that pollination with highly irradiated (100 Krad) pollen can cause certain genes from pollen to be transferred to the egg. The implications of this finding are that only one or a few desirable genes can be transferred from one plant to another following an almost natural method of crossing plants. The feasibility of this approach to transformation in higher plants, termed "gametic transformation", was confirmed by a group of British scientists (Jinks *et al.*, 1981; also *see* Davies, 1981).

Transformation of higher plants by the techniques used with prokaryotes (introduction of chemically extracted DNA) into free cells has several limitations. Firstly, it is difficult and expensive to obtain pure and active preparations of DNA. The major problem, however, is of protecting the naked DNA from enzymatic degradation during and after its uptake into the alien cytoplasm. In this respect gametic transformation is most safe, as in this technique the DNA fragments are delivered to the egg by pollen tube in a natural fashion. Once this technique is developed to a stage of

practical application it might, owing to its simplicity in technical requirements, become an important tool for plant breeders and, as Pandey (1981) remarked, it would be a "poor man's genetic engineering".

The series of events involved in a gametic transformation are as follows. A high dose of irradiation (20-100 Krad) given to pollen pulverizes the generative cell nucleus to produce a mass of fine chromatin fragments and the nucleus does not divide to form sperms. However, the treated pollen grain retains its ability to germinate and deliver the fragmented DNA into the embryo sac (Fig. 17.13A,B). Transfer of DNA pieces to the egg, called "pseudo-fertilization", causes the egg nucleus to divide. The chromosome number of the egg or early embryo then doubles ("parthenogenetic diploidy"), and during chromosome replication certain pollen-DNA fragments are incorporated into, or associated with, the maternal genome.

Using the technique of gametic transformation, Pandey (1978) demonstrated intra- and interspecific transfer of self incompatibility and/or flower colour alleles from paternal genome in the genus *Nicotiana*. Jinks *et al.* (1981) reported that 6 out of 96 plants of M_2 progeny of *Nicotiana rustica* generated after pollination of cultivar V27 with the pollen of cultivar V12, exposed to 20 Krad of γ-irradiation, closely resembled the maternal parent and yet had the black ovary character of the paternal parent. This is indeed fairly high rate of transformation by any standard. Using this approach partial transfer of genome has been successfully achieved in *Nicotiana* (Caligari *et al.*, 1981; Yamamoto, 1984), *Hordeum* (Powell *et al.*, 1983), *Triticum* (Snape *et al.*, 1983) and *Capsicum* (Daskalov, 1984).

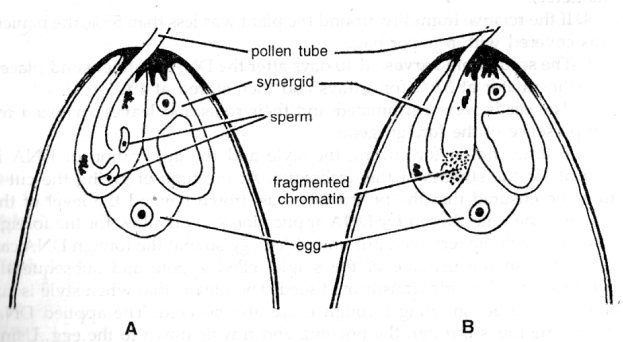


Fig. 17.13 Diagram of the upper-half of embryo sac at the time of fertilization. **A**. After pollination with normal pollen; note the two sperms. **B**. Following pollination with irradiated pollen; the pollen tube-discharge contains fragmented chromatin instead of normal sperm cells. (Based on the work of Dr. K.K. Pandey, New Zealand, *after* Kumar and Bhojwani, 1983)

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SUBJECT INDEX

Pollen-Tube Pathway Method

This new approach to genetic transformation of plants was first reported by Duan and Chen (1985). Considering the simplicity of approach, Luo and Wu (1989) re-examined the efficiency of this method in the transformation of rice and offered molecular evidence to confirm the integration of the donor DNA into the genome of the transformed plants. Essentially, the pollen-tube pathway method consists of cutting the upper portion of a recently pollinated pistil and applying a drop of DNA solution to the cut end of the style. The DNA presumably reaches the ovule by flowing down the pollen tube and interacts with the genome of the egg, leading to the formation of transformed seed.

Following the protocol summarized below, Luo and Wu (1989) observed that 20% of the treated florets of rice set seeds of which 20% were transgenic. Thus, 4% of the treated florets yielded transgenic plants.

Protocol

1. Rice plants were potted in soil and grown in a greenhouse at 26°C, under 55-60°C relative humidity and 14 hr photoperiod. The florets whose glumes had just opened were marked by putting a dot at their base.
2. After one to two hours the top two-thirds or three-quarters of the florets was removed with a pair of scissors so that stigma was cut off and the style had a severed end.
3. A 2-3 ml drop of the DNA solution (50 mg/ml in TE buffer) was placed at the cut end of the style using a capillary tube (0.2 mm inside diameter).
4. If the relative humidity around the plant was less than 55%, the panicle was covered with a paper bag.
5. The seeds were harvested 40 days after the DNA treatment and placed in an incubator at 37°C for 4 days and then stored at 4°C.
6. The seeds were germinated and their roots and leaves analyzed for the presence of the foreign gene.

The time chosen for cutting the style and the application of DNA is critical for the success of this technique. At the time of giving the cut it must be ensured that the pollen tube has grown beyond the level of the cut, and the time chosen for DNA application should allow for the foreign DNA to reach the fertilized but undivided egg so that the foreign DNA can interact with the genome of the single-celled zygote and subsequently produce non-chimeric transformed seed. The idea is that when style is cut the pollen tubes growing through it are also severed. The applied DNA enters the tubes through the opening and travels down to the egg. Using this protocol Luo and Wu (1989) have introduced gene for neomycin phosphotransferase into rice. Transformed seeds and plants of rice and cotton, using this method, were obtained by Zhou *et al.* (1988).

This method is simple and relatively efficient because transgenic rice seeds can be obtained in about 40 days. Moreover, each transformed seed is expected to give a non-chimeric transgenic plant.

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