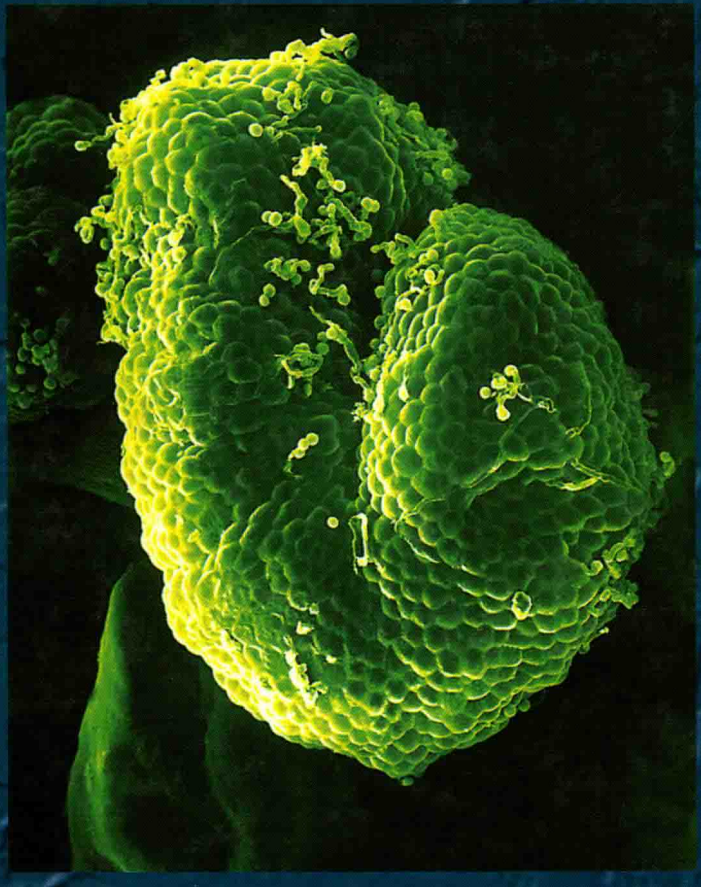
Reproductive Biology of Sexual Hybridization in Woody Plants : an Atlas

（木本植物有性杂交生殖生物学图谱）

LI Wendian　MA Fengshan



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Preface

Controlled sexual hybridization has been the most important approach for *Populus* breeding. Over the past decades, numerous hybrids have been generated, many of which have demonstrated desired features for forestry and/or ecology. However, there are several profound issues that are poorly understood. Here are some of the most frequently asked questions. What is the general mode of seed development in parent plants? What can we learn from an embryological perspective of successful cross combinations? And, most importantly, what are the barriers to other, unsuccessful combinations? Then, how to overcome these barriers? We have undertaken a research program on the hybrido-embryology of *Populus* since the early 1980s. Representative species from all five sections of the genus were included and trials covered as many as possible intersetional crosses. Our results showed that in intersectional crosses embryo abortion is a common barrier, although problems could happen at any stages of the hybridization process. Using an *in vitro* system for *in ovulo* embryo culture, we have successfully rescued hybrid embryos from abortion. Examples of hybrid plantlets obtained by this technique include *Populus simonii* × *P. pyramidalis*, *P. simonii* × *P. lasiocarpa*, and *P. simonii* × *P. euphratica.* We also worked on an intergeneric cross for the Salicaceae, *P. simonii* × *Salix matsudana*, and found that fertilization did take place in a few ovules, and embryo abortion was responsible for the small numbers of hybrid seeds.

In the recent years, we have extended our efforts into the embryology of distant hybridizations in conifers. *Cunninghamia lanceolata* is an important forestation species widely distributed in central and south China. In late 1950s, Professor Yieh Peichong reported successful distant crossings in both *Cunninghamia lanceolata* × *Cryptomeria fortunei* and *Cunninghamia lanceolata* × *Platycladus orientalis*, being intergeneric and interfamilial, respectively. Yet, this has raised a shadow of doubt among scientists. We thus decided to repeat the experiments. Our soft X-ray radiographic study has confirmed that the hybrid seeds from *Cunninghamia lanceolata* × *Cryptomeria fortunei* contained a few fully-developed embryos which could germinate into seedlings. On the contrary, investigation of *Cunninghamia lanceolata* × *Platycladus orientalis* revealed a post-fertilization failure.

In vitro fertilization has long been a dream for plant biologists. In 1991, Kranz, Bautor and Lörd reported the first successful case of *in vitro* fertilization with isolated, single gametes of maize. It was very encouraging for us to follow this "cell - cell" technique for overcoming fertilization failure in distant hybridization. We started out with the isolation of male and female gametes from both *Populus* and conifer species. We were very excited to obtain viable sperms and some female components (embryo sacs, egg apparates, or even single eggs). Fusion experiments were performed in the presence of polyethylene glycol, which revealed that the male and female gametes adhered to each other but had not fused yet. Further pursuit in this direction will definitely broaden the field of hybrido-embryology.

This book represents a pictorial summary of our research achieved over the past twenty

years. We have taken numerous photographs for publication. To write this book, we selected 700 images from which 80 plates were designed, including 34 in full-color. Some of the pictures were never published elsewhere. The information presented the sexual processes of controlled sexual hybridization, the secrets of hybridization barriers, the *in ovulo* embryo culture for overcoming hybrid embryo abortion, and the manipulation for isolating male and female gametes. This is an atlas of basic sexual reproductive biology of woody plants and experimental biotechnology for tree breeding. We hope this work will be helpful to both plant embryologists and tree breeders. It may also serve as a reference book for advanced students of plant biology and forestry.

Science is moving on at an unprecedented pace. Genome sequencing for *Populus trichocarpa* has recently been completed, and the *Pinus taeda* genome project is well underway. These two species, important economically and ecologically, are excellent models, one for angiospermatous trees and another for gymnospermatous trees. With these genomic resources, we are offered novel opportunities to study the molecular and genetic mechanisms of interspecific interactions during sexual hybridization. These opportunities represent a platform to display embryological and cellular events documented in this book in the context of molecular and genetic control and regulation. Discoveries in this direction will greatly improve our understanding of the recognition processes in compatible cross combinations. Fundamental progress is also anticipated in deciphering the machinery for the detected barriers in problematic combinations. These areas will be our interests for the many years to come.

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Beijing
July 2005

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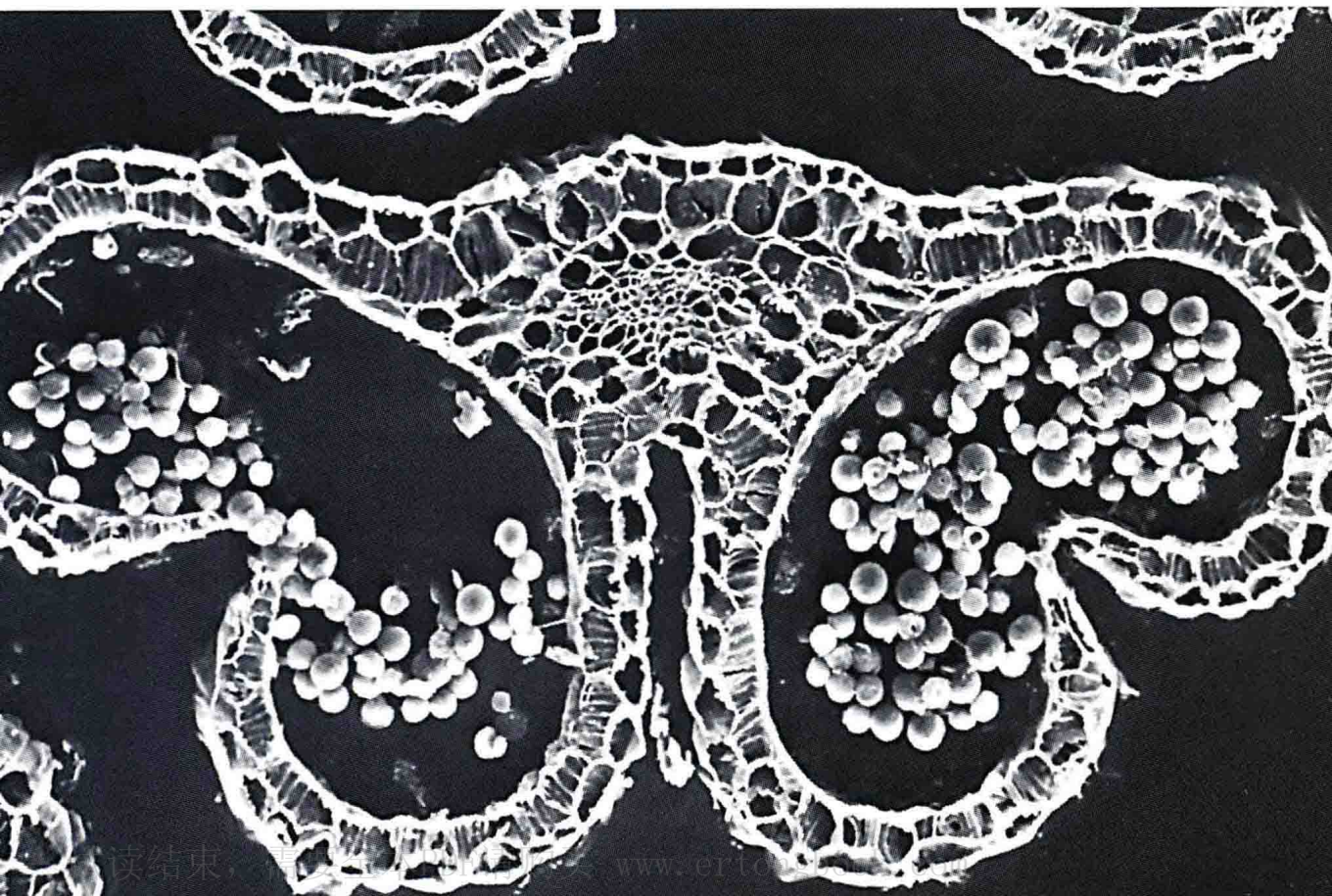
A List of Plates

Contents

I Hybrido-embryology in *Populus*



1 Development of Sex Organs

1.1 Flowers and inflorescences (Plates: 1, 5①, 27①, 28①, 29①, 30①, and 31①)

Populus, together with *Salix* and *Chosenia*, belongs to the family Salicaceae. There are two characteristic features of the flowers of *Populus*. First, they are unisexual, the plants dioecious, with pendulous or rarely erect catkins. Only a few species were documented to be bisexual, including *P. glauca* (Haines 1906) and *P. deltoides* (Campo 1963). Campo (1963) also reported that self-fertilization happens in *P. deltoides* leading to the formation of a high percentage (more than 65%) of fertile seeds. Sometimes, both unisexual and bisexual flowers are borne on the same tree (polygamous) with either pistillate flowers or staminate flowers being predominant. Second, it is generally held that the flower is "naked", that is, lacking a perianth, and only has a rudimentary floral organ, commonly known as the "disc", which bears the pistil or stamens. Haines (1906) considered that *P. glauca* frequently possesses "bisexual flower with undoubted perianth" and Hong's (1987) scanning electron microscopic studies confirmed the presence of a well-differentiated, bowl-shaped perianth in *P. lasiocarpa*, an endemic poplar in central China. Based on these unique characteristics, there are two contradictory opinions concerning the phylogenetic position of the Salicaceae. In one, the family is considered primitive and should be placed at or near the beginning of the dicotyledonous line. Alternatively, the family is considered highly evolved, and its apparently primitive characters are due to simplification and reduction of the floral parts. For revealing additional evidence in support of the opposed views, Fisher (1928) carried out investigations into the morphology and anatomy of the flowers of Salicaceae and concluded that "the simplicity of the flowers of the Salicaceae is largely due to extreme reduction and not a retention of archaic features".

Since the publication of Treub's (1891) magnificent treatise on the chalazagamous fertilization in *Casuarina*, botanists had put a lot of effort on the embryology of the Amentiferae (Benson 1893; Chamberlain 1897). Chamberlain (1897) first made a drawing for *P. monilifera* to illustrate the "lenticular cell cut from the rest of the spore", or, as known later, the formation of the generative cell from the microspore. Nagaraj (1952) did an excellent study on the floral morphology of *P. deltoides* and *P. tremuloides*. He produced many nice figures for micro-and mega-sporogenesis, micro-and mega-gametogenesis, fertilization, and development of embryo and endosperm. Davis (1966), in his book entitled "Systematic Embryology of the Angiosperms", summarized the work on the Salicaceae, among other families. Yet, a detailed account on the embryology of the majority of *Populus* species has been lacking. For species, such as *P. simonii, P. euphratica* and *P. lasiocarpa*, chosen for their ecological and economical importance in our projects, no or little embryological information is available for their

hybridization. Over the past 20 years, we have obtained a large amount of data for these and several other *Populus* species (Li *et al.* 1982; Li and Zhu 1988; Zhu and Li 1988, 1989). Here below is an overview of these studies.

1.2 Floral morphology

The pistillate flower consists of a pistil and a cupulate or bowl-shaped perianth, subtended by a bract. Occasionally, 2 or 3 pistillate flowers occur within a bract, as in *P. simonii*. Pistil consists of 2 to 3 (less often 4) carpels. Ovary is unilocular with parietal or basal placentas, bearing 2 (*P. adenopoda*) to numerous (*P. euphratica*) ovules. Styles are short, connate, except those of *P. lasiocarpa* that are rather long and separate. Stigmas are usually 2 to 4: 2-lobed and horseshoe-shaped in *P. simonii* and *P. nigra*, 2-parted and linear-forked in *P. alba*, and 3-valved and petaloid in *P. lasiocarpa* and *P. euphratica* (Li *et al.* 1991). The staminate flower also consists of a reduced perianth, disc-shaped, and several to numerous stamens (up to 110 in *P. lasiocarpa*), red or yellow, borne in the axil of a bract. The connective in *P. lasiocarpa* distinctively extends beyond the pollen sac to form a protrusion while those in other species are less protrusive or even flat (Hong 1987). The diagnostic features of flowers from representatives for the five sections of the genus *Populus* are listed in Table 1-1.

Table 1-1 Floral morphology of *Populus*

Species	Section	Pistillate flower	Staminate flower
P. lasiocarpa Oliv.	Leucoides Spach	Perianth well-developed, cupulate or bowl-shaped; carpels 3; styles separate; stigmas 3, petaloid	Stamens 41-110; connective protrusive
P. euphratica Oliv.	Turanga Bge.	Perianth well-developed, cupulate or bowl-shaped; carpels 3; styles connated or upper part separate; stigmas 3, petaloid	Stamens 22-36; connective more or less protrusive
P. nigra Linn.	Aigeiros Duby	Perianth bowl-shaped; carpels 2; styles connated; stigmas 2, horseshoe-shaped	Stamens 40-60; connective flat
P. alba Linn.	Leuce Duby	Perianth bowl-shaped, crenulated; carpels 2; styles short, connate; stigmas 2, parted and linear-forked	Stamens 8-20; connective flat
P. simonii Carr.	Tacamahaca Spach	Perianth bowl-shaped, entire or wavy incised; carpels 2 or 3; styles short, connated; stigmas 2 or 3, horseshoe-shaped	Stamens 6-12; connective flat

1.3 Development of pollen (Plates 2 to 4)

Staminate flower buds and flowers of *P. simonii* and *P. euphratica* were collected at various developmental stages. Some samples were fixed in the Farmer's fluid (anhydrous ethanol : acetic acid, 3 : 1, *V/V*), stained with carbol fuchsine, and processed to make squash preparations. Other samples were fixed in FAA and subjected to paraffin embedding and serial sectioning, counter-stained with safranin-fast green or hematoxylin-fast green. Specimens from

both treatments were examined with a bright field microscope. Some paraffin sections were stained in 0.0036% acridine orange in McIlvaine's buffer, pH 5.0 for observation of Übisch bodies (Abrams 1962; Franklin *et al.* 1981), and with 0.005% decolorized aniline blue in phosphate buffer pH 8.2 (Currier 1957) for observation of callose variations by using incident light fluorescence microscope. Scanning electron microscopy was employed for observation of anther structure, particularly the Übisch bodies. There, the paraffin section was adhered to a small piece of cover slip. After being deparaffinized with xylene, it was mounted on a supporting stub with bi-faced adhesive tape ready for routine critical drying method.

1.3.1 The anther

The anther is tetrasporangiate. The mature anther wall comprises an epidermis, a layer of endothecium, two middle layers and a single-layered tapetum. The epidermis is persistent. The endothecium develops fibrous bands (thickenings) and the two middle layers are ephermeral of which one becomes flattened and is eventually crushed during the microspore stage, and the other is disintegrated prior to anther dehiscence.

The tapetum is of the glandular type. Tapetal cells are uninucleate at the early stage and become bi-nucleate or multi-nucleate when pollen mother cells are at their meiotic stage. They begin to degenerate at uninucleate pollen stage and disintegrate at binucleate pollen stage.

There are some minute spherical granules, the Übisch bodies (Echlin and Godwin 1968), lining on the inner tangential walls of tapetal cells, manifesting a bright green, acridine orange induced fluorescence. In SEM, Übicsh bodies of *Populus* appear as cloud, star or flower like, sometimes with an orbicular center. Occasionally, sporopollenin "bridges" are seen between Übisch bodies and the exine of pollen grains.

1.3.2 Microsporogenesis

Simultaneous cytokinesis takes place after pollen mother cell meiosis, and the resultant microspore tetrads are tetrahedral and decussate. The meiotic division does not exhibit a high degree of synchrony; it can be asynchronous within one pollen sac or among the four pollen sacs of the same anther. Callose deposition occurs at the onset of meiosis of pollen mother cells, reaches a peak at metaphase II or anaphase II by enveloping the pollen mother cells or microtetrads, and disappears at the end of meiosis. It is considered that the cycling of callose during microsporogenesis plays an important role in the protection of the pollen mother cells and release of microspores from the microtetrads. In addition to the nucleolus, it is found that some smaller supernumerary nucleoli may appear during microsporogenesis.

1.3.3 Formation of vegetative and generative cells

The microspore tetrads soon separate from each other and release to form the microspores. The newly formed microspore is the first cell of the male gametophyte that is referred to as pollen. The young pollen cell is uninucleate which undergoes a mitotic division to give rise to a large, central vegetative cell and a small, lenticular generative cell that is adjacent to the wall of the vegetative cell. Next, the generative cell is detached from the wall of the vegetative cell, lying free in the cytoplasm, forming a 2-celled pollen grain. Most *Populus* pollen grains are

shed at 2-celled stage. Accordingly, Brewbaker (1967) listed *Populus* as the binucleate type. Hamilton and Langridge (1976) reported that 80%-90% pollen grains were tricellular in *P. alba*, but this was not supported by Villar *et al.* (1987) who documented less than 10% as tricelllular in *P. alba* and *P. nigra*. The timetables of pollen development in *P. simonii* and *P. euphratica* are provided in Table 1-2 and Table1-3 respectively.

Table 1-2 Timetable of the development of pollen grains in *P. simonii*

(Collected from Beijing, cultured in a water jar, 10-18℃, 1982)

Date(day/month)	Developmental stage	Morphological features
20/02-24/02	Pollen mother cells	Pollen mother cells from compactly packed to loosely arranged
24/02-25/02	Meiosis of pollen mother cells	Simultaneous cytokinesis; supernumerary nucleoli present; chromosome minute, $n = 19$; synchrony / asynchronous
25/02-26/02	Microspore tetrads	Tetrahedral and decussate
27/02-28/02	Uninucleate pollen	Contractive stage present
29/02-01/03	Two-celled pollen	Pollen grains spherical, mean diameter 28.10 μm, acolpate, inaperturate
01/03-03/03	Dehiscence of pollen sac	Two-celled pollen at shedding

Table 1-3 Timetable of the development of pollen grains in *P. euphratica*

(Collected from Inner Mongolia, open field, 1985)

Date(day/month)	Developmental stage	Morphological features
10/04	Pollen mother cells, at early stage	Cells arranged compactly
15/04	Prophase of meiosis I	Cells arranged loosely
20/04	Meiosis finished; release of microspore tetrads	Microspore tetrads tetrahedral, rarely isobilateral
24/04	Mitosis of uniculeate pollen	Formation of vegetative and generative cells
27/04	Initiation of pinch-off of the generative cell from the wall of the vegetative cell	Generative cell lenticular
28/04	Pinch-off of the generative cell into the cytoplasm of the vegetative cell	Generative cell spherical and in a more central position
29/04	Two-celled pollen, fully mature	Pollen grains spherical, mean diameter 21.82 μm, acolpate, inaperturate
30/04	Dehiscence of pollen sac	Two-celled pollen when shed

1.4 Development of embryo sac (Plates 5 to 8)

Nagaraj (1952) gave a detailed description with nice figures for the megasporogenesis and megagametophyte development in *P. tremuloides* and *P. deltoides*. He reported that the megaspore tetrads are linear or T-shaped, the chalazal megaspore is functional and the embryo

sac is of the *Polygonum* type that has penetrated quite deeply into the micropyle at maturity. Rodkiewicz and Góriska-Brylass (1967) first noticed the callose accumulation during megasporogenesis in *Orchis maculata*. Kuran (1972) pointed out that for different species the pattern of callose distribution in cell walls during megasporogenesis is a constant specific trait.

Pistillate flower buds and flowers of *P. simonii*, *P. euphratica* and *P. lasiocarpa* were collected at various developmental stages. Serial paraffin sections were prepared to observe the development of embryo sacs and Aniline blue staining (Currier 1957) was used to study callose deposition during megasporogenesis.

1.4.1 Megasporogenesis

The ovule is anatropus, unitegemic (*P. simonii*) or bitegemic (*P. euphratica* and *P. lasiocarpa*) at the megaspore mother cell stage. The archesporial cell is of hypodermal origin. Nucellus is crassinucellar, containing usually one or sometimes two megaspore mother cells, juxtaposed or superposed (*P. euphratica*). Megaspore mother cell undergoes meiosis to form four linear or T-shaped tetrads.

In *P. euphratica*, callose deposits appear in the transverse walls (linear type) or in both transverse and vertical walls (T-shaped type) during megasporogenesis, but never appear in the external walls.

1.4.2 Megagametogenesis

The chalazal or subchalazal megaspore (functional megaspore) develops into a *Polygonum* type embryo sac. A mature embryo sac comprises a 3-celled egg apparatus, 3 antipodal cells and a large central cell with 2 polar nuclei. The latter fuse before fertilization to form a secondary nucleus. Occasionally, the polar nuclei remain apart even though a sperm has adhered to one of them. The filiform apparatus, a mass of finger-like projections, is present at the micropylar end of each synergid. Owing to the degeneration of the nucellus at the micropyle pole during megagametogenesis, the egg apparatus pole of the mature embryo sac comes in direct contact with the integument and penetrates into the micropyle. The epidermis of the integument and the nucellus are covered by cuticular layers. The major events of embryo sac development in *P. simonii* and *P. euphratica* are documented in Table 1-4 and Table 1-5, respectively.

Table 1-4 Timetable of the development of embryo sac in *Populus simonii*

(Collected from Beijing, cultured in water jar, 10-18℃, 1982)

Date(day/month)	Developmental stage	Morphological features
07/03	Meiosis of megaspore mother cells, forming 4 megaspore tetrads	Megaspore tetrads, linear or T-shaped
08/03	Functional megaspore determined	Functional megaspore at the chalazal end
09/03	Mitosis of functional megaspore; various stages of embryo sac	2-, 4-, and 8-nucleate embryo sacs
10/03-12/03	Organization of mature embryo sac	*Polygonum* type embryo sac: 3-celled egg apparatus, 1 secondary nucleus, 3 antipodals

Table1-5 Development of embryo sac in *Populus euphratica*

(Collected from Inner Mongolia, open field, 1985)

Date(day/month)	Developmental stage	Morphological features
03/04-05/05	Megaspore mother cells; prophase of meiosis I	Megaspore mother cells 1-2, juxtaposed or superposed
05/05-06/05	Meiosis reached its peak; metaphase I to anaphase II, forming 4 megaspore tetrads	Megaspores, linear or T-shaped functional megaspores, chalazal or subchalazal
06/05-07/05	Mitosis of functional megaspore, forming 2-, 4-, and 8-nucleate embryo sacs	3-celled egg apparatus + 2 polar nuclei + 3 antipodals
07/05-08/05	Organization of mature embryo sac	*Polygonum* type: 3-celled egg apparatus + 1 secondary nucleus + 3 antipodals

1.5 Summary

Flowers of *Populus* are unisexual, occasionally bisexual; dioecious or sometimes polygamous. The pistillate flower is characterized by a reduced cupulate or bowl-shaped perianth, along with 2 to 4 lobed, parted or petaloid stigmas. Pistil usually consists of 2 to 4 carpels. The ovary is unilocular, with 2 to 4 parietal or basal placentas bearing 2 to numerous ovules. The staminate flower is also very simple, with just a disc-shpaed perianth and a few or many stamens.

Pollen grains are two-celled when shed, being spherical, acolpate, and inaperturate. Callose appearance and disappearance are constant features across the genus during both micro- and mega-sporogenesis. Übisch bodies are visualized with scanning electron microscopy by using our improved procedure on paraffin sections. Embryo sac is of the *Polygonum* type organization. The egg apparatus is in direct contact with the integument and penetrates into the micropyle. The cuticular layers on the epidermis of integument and embryo sac are protective coverings; they offer a great difficulty for our trials in isolating embryo sacs and their components.

[This chapter contains information extracted from Hong *et al.* 1987; Li *et al.* 1982, 1988, 1991; Zhu *et al.* 1988, 1989].

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