

CENTRALE LANDBOUWCATALOGUS



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# Reproduction in *Spinacia oleracea* L.

Ultrastructural aspects of pistil development,  
pollination and fertilization

PLANTENTHIEK  
DER  
LANDBOUWHOGESCHOOL  
VAGENINGEN

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Promotoren: dr. J.L. van Went, hoogleraar in de plantkunde

dr. M.T.M. Willemse, hoogleraar in de plantkunde

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H.J. Wilms

REPRODUCTION IN SPINACIA OLERACEA L.

Ultrastructural aspects of pistil development,  
pollination and fertilization.

(with a summary in Dutch)

PROEFSCHRIFT

ter verkrijging van de graad  
van doctor in de landbouwwetenschappen  
op gezag van de rector magnificus  
dr. H.C. van der Plas,  
hoogleraar in de organische scheikunde  
in het openbaar te verdedigen  
op vrijdag 15 mei 1981,  
des namiddags te vier uur in de aula  
van de Landbouwhogeschool te Wageningen.

**BIBLIOTHEEK L.H.**

**0 6 MEI 1981**

**ONTV. TIJDSCHR. ADM**

Vakgroep Plantencytologie en -morfologie,

Landbouwhogeschool, Wageningen - 1981.

## STELLINGEN

- I. Waargenomen structurele verschillen tussen antipoden, die aanwezig zijn in de diverse embryozakken binnen de groep van dicotyle planten, berusten vooral op verschillen in snelheid van ontwikkeling en zijn daarom niet soort-specifiek.  
Dit proefschrift.
- II. Bij spinazie wordt de kieming van pollenkorrels en de groei van pollenbuizen in stempel en stijl niet gereguleerd door de embryozak in tegenstelling tot de groei van de pollenbuis in de nucellus.  
Dit proefschrift.
- III. De transportweg waarlangs de voeding van zaadknoppen geschiedt varieert en is afhankelijk van het ontwikkelingsstadium.  
Dit proefschrift.
- IV. De lichtmicroscopische fluorescentietechniek is niet geschikt voor het exact bepalen van de weg waarlangs pollenbuizen groeien.
- V. Voortplantings- en/of zaadzettingsonderzoeken, waarvoor gedurende het hele jaar deels met behulp van kassen plantmateriaal moet worden gekweekt, zijn wetenschappelijk niet verantwoord wanneer de seizoensinvloed daar niet bij betrokken wordt.
- VI. De veroudering en het afsterven, die in monocarpe planten optreedt na de bloei en de vorming van vruchten en zaden, kan niet zonder meer verklaard worden door aan te nemen dat zaden een senescence factor produceren.  
Noodén, L.D., D.C. Rupp en B.D. Derman (1978). *Nature* 271: 354-356.
- VII. "Door bestuiving worden stuifmeelkorrels bij de eicellen gebracht en vindt bevruchting plaats". De door deze formulering beoogde vereenvoudiging van de stof weegt niet op tegen de geïntroduceerde onjuistheden. Uit didactisch oogpunt zou het daarom beter zijn bepaalde onderwerpen uit de biologie in het basisonderwijs niet te behandelen.  
Meijs, P. (1981). *Onderwijstelevisie N.O.T.*, Den Haag, p. 41.
- VIII. Onderwijsmiddelen ter ondersteuning en stimulering van het individuele leerproces zijn lapmiddelen.

- IX. Informatie over nieuwe vakliteratuur dient in handbibliotheken voorhanden te zijn. Indien dergelijke informatie bij voortdurend eerst maanden na het verschijnen ter algemene kennisname komt, is het aan te bevelen de daarvoor verantwoordelijke vakgroepen in hun financiële middelen ten behoeve van tijdschriftabonnementen te korten.
- X. Democratie is gedoemd ten gronde te gaan, enerzijds aan het onvermogen tot besturen, anderzijds aan een intolerante houding van bepaalde groeperingen na genomen besluiten.
- XI. De voor en tegens van de "nationale kierenjacht" zijn nog onvoldoende onderzocht om nu reeds paniek te zaaien.  
Brunekreef, B. en J.S.M. Boleij (1981). *Intermediair* 17, nrs. 1-2: 1-7.



Ter nagedachtenis aan mijn vader  
aan mijn moeder  
aan Anja, Maarten en Janneke

---

## VOORWOORD

Het verschijnen van dit proefschrift schenkt mij veel voldoening. Velen hebben bijgedragen aan de tot standkoming ervan. Allen ben ik erkentelijk, enkelen wil ik hier noemen.

Mijn ouders, die mij in staat hebben gesteld in een goed klimaat een wetenschappelijke opleiding te volgen. Moeder, slechts aan U kan ik mijn dankbaarheid overbrengen.

Mijn promotoren, Prof.Dr. J.L. van Went en Prof.Dr. M.T.M. Willemse wil ik dankzeggen voor de inzet en tijd gegeven aan dit onderzoek. Jacques, jouw heldere en kritische benadering vanaf het eerste moment heeft mij duidelijk zicht doen houden. Jouw kernachtige formuleringen hebben de manuscripten duidelijk positief beïnvloed. Michiel, jouw komst op het laboratorium en je benadering van onderzoek hebben een zekere druk gelegd op de afronding van dit onderzoek. De gezamenlijke discussies heb ik als zeer bevruchtend beschouwd.

Tijdens zijn verblijf als gastmedewerker heeft Prof.Dr. R.N. Kapileen stimulerende invloed gehad op het opschrift stellen van de resultaten. Dear Prof. Kapil, I remember our discussions as valuable and pleasant. I am much indebted to you, especially for your inspiring way of correcting manuscripts.

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De overige mensen hebben mij op verschillende manieren duidelijk bijgestaan. De heren G. van Geerenstein, J. Verburg en A. van Ommeren voor de verzorging van het plantmateriaal, de heren T. Zaal en W. van Ooijen voor de hulpvaardigheid en kennis van zaken bij het vervaardigen en afwerken van het vele fotomateriaal en de heer E. Rothuis die ten alle tijde bereid is geweest assistentie te verlenen. Het omvangrijke tekenwerk is verricht door de heer A.B. Haasdijk. Alex, de kunstenaar, jouw kwaliteiten zijn aanleiding voor steeds meer gecompliceerde tekenopdrachten. Zo ook deze omslag. De heer J.S. de Block heeft de engelse teksten gecorrigeerd. Dank gaat ook uit naar Mw. R.J.J.R. Groot-Scholte en Mw. G.G. van de Hoef-van Espelo voor hun bijdrage aan het verzorgen van enkele manuscripten.

Tenslotte bedank ik Anja voor het opgebrachte geduld.

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## 1. INTRODUCTION

### 1.1. Reproduction

Plant species can multiply asexually, or vegetatively and sexually, or generatively. The generative propagation is accompanied by a change of the ploidy level, the diploid nuclear phase is succeeded by the haploid one and expressed in the alternation of the generation in which a sporophyte is followed by a gametophyte. The reduction of the ploidy level by meiosis implies a recombination of genes if fusion of nuclei occurs. Sexual reproduction maintains genetic viability.

In angiosperms the haploid phase, represented in the gametophyte, is highly reduced. The separation of sexes already is expressed on the sporophyte by the formation of macro- and micro-sporophylls. The macrosporophylls form a stamen with a stigma, style and ovary. In the ovule inside the ovary, a megaspore mother cell develops into an embryo sac of 7 cells, the megagametophyte. The microsporophyll is composed by a filament and the anther. In the anthers many pollen mother cells are formed, each of which gives after meiosis 4 microspores. Each microspore develops into a three-celled microgametophyte, a vegetative cell and two male gametes. Some plant species produce their sporophylls together on one organism (monoecious) or separated over two organisms (dioecious).

The male gametes are transported to the synergids and will fuse with the egg cell and the central cell. In angiosperms this double fertilization results in the formation of a zygote and endosperm. Subsequently a mature seed develops. Seed formation means for practical breeders and growers successful generative multiplication. What happens and what can go wrong in the processes of pollination, fertilization and seed setting has to be clarified before these processes can be regulated. This means that it is also necessary to study and analyse these processes morphologically and cytologically, to collect more information on the pistil, pollen-pistil interaction and the growth of the pollen tube to and into the embryo sac.

### 1.2. Pistil structure

The pistil consists of a stigma, style and ovary, in which one or more ovules with one female gametophyte each are present.

The morphology of the stigma shows a great diversity. Some comparative light microscopical studies (VASIL & JOHRI 1964) and several, mainly ultrastructural studies present detailed features

of some plants (KONAR & LINSKENS 1966a). On the basis of a study of the morphology of the stigma surface, the amount of secretion and the nature of the surface, cells of almost 1000 species of about 900 genera of 250 families (HESLOP-HARRISON et al. 1975), HESLOP-HARRISON & SHIVANNA (1977) give a general classification. The main classification divides the stigmas in types with copious fluid secretion, the wet stigmas and types with limited secretion, the dry stigmas.

The morphology and anatomy of the style is very variable. During development many changes take place. In relation to its transmitting function the style has either a canal or a solid core of transmitting tissue. A canal is lined with a glandular epidermis. Such a style is of the open type, which is mostly present in monocotyledons. Styles with a solid core of transmitting tissue, the solid type, are regular in dicotyledons. In some plants there is a half closed type in which the transmitting tissue is limited only to one side of the stylar canal (VASIL & JOHRI 1964; VASIL 1974).

Since the first description of the female gametophyte by STRASBURGER (1884) a large number of species have been studied, mainly by light microscopical methods. The results of these studies are well reviewed (MAHESHWARI 1950, 1963; RUTISHAUSER 1969). Since the early 1960's considerable new information has been obtained by means of transmitting electron microscopy and histochemistry. Recently KAPIL & BHATNAGAR (1981) reviewed these ultrastructural data.

### 1.3. Pollen-pistil interaction

The first interaction between pollen and stigma is the sticking. Adhesion results from the high viscosity and sticky nature of the exudate (KONAR & LINSKENS 1966b). Other factors as surface tension, wind force, the electro-statical force, the electro-dynamical force, the gravity and the inertial force, are involved too (WOITTIER & WILLEMSE 1979). HESLOP-HARRISON (1979, 1980) states that for grasses hydration occurs as long as the water potential of the stigma is less negative than that of the grain.

After sticking, pollen germination starts with the imbibition phase. Usually a lag phase, in which activation occurs, follows and leads to tube initiation and elongation (LINSKENS & KROH 1970).

From in-vitro studies (MASCARENHAS 1975; STANLEY & LINSKENS 1974; SHIVANNA et al. 1979) it appears that in many species pollen activation precedes the tube formation. This includes an initial high respiration, the development of mitochondria to an active state, the utilization of the pollen storage products and an enlargement of the membrane system as dictyosomes for the formation of the plasm membrane and pollen tube wall. HOEKSTRA (1979) reports

that rapidly germinating pollen must be equipped with fully-developed mitochondria already at dehiscence, which is in contrast to slowly-germinating types. The latter complete the structural and functional development of their mitochondria as a requirement for the outgrowth of their pollen tubes. The duration of the lag period is determined by the level and rate of mitochondrial development and not by the division of the generative cell.

The tube growth can start after a very short period of imbibition and activation. This includes the presence of a possibility to form new plasm membrane and a cell wall within the mature pollen grain. In quickly germinating pollen these processes are prepared by the presence of vesicles for the tip growth and another type of vesicles for the formation of the vacuoles by fusion and uptake of water in the mature pollen grain (VAN WENT 1974; DICKINSON & LAWSON 1975).

The formation of the pollen tube involves a growth zone, vacuolation and plasm streaming. The growth zone is the tip where a new plasm membrane is added and a wall with the characteristics of a primary wall appears. This tip region contains many golgi vesicles which fuse with the plasm membrane, excreting wall precursors and proteins (ENGELS 1974). In some species the ER is involved in this process.

In the first moment of tube formation the position of the vegetative nucleus is central, whereas that of the generative cell or sperm cells varies. In wheat CHANDRA & BHATNAGAR (1974) studied the beginning of pollen germination in detail. The sperm cells lay near the germination pore and come out before the vegetative nucleus. However, in the growing pollen tubes the vegetative nucleus lays normally near the tip and the generative cell or sperm cells follow (see also BROULAND 1980). The transport of the vegetative nucleus and sperm cells is related to the growth of the tube and the cytoplasmic streaming. This streaming involves microfilaments (FRANKE et al. 1972).

Germinating pollen excretes enzymes (STANLEY 1971; VASIL 1973; STANLEY & LINSKENS 1974) among other products probably at the tip zone mainly.

Pollen tube growth is directed. On the stigmatic surface the tube grows to the transmitting tissue or the stylar canal and in this tissue towards the ovary and here to the ovule. In studies on chemotropism a variety of data can be noticed in the review of ROSEN (1975), MASCARENHAS (1973, 1975), KAPIL & BHATNAGAR (1975) and LINSKENS (1976). The intercellular substance in the transmitting tissue of solid styles is considered as a secretion product of this transmitting tissue (SASSEN 1974). This secretion product is comparable with the mucilage that fills the canal cells of open styles. According to pollen tube growth conditions there is great similarity between the mucilage and the intercellular substance.

Complex is the situation in *Begonia* (PREIL & KEIJSER 1975) and in *Spinacia* (WILMS 1974) near the micropyle. In *Begonia* the

cells around the micropyle have a smooth surface. This is in contrast to the other cells, which are covered with deposits of waxy substances. In *Spinacia* attraction of one or a few tubes takes place whereas the mass of pollen tubes branches in the micropyle and do not penetrate the cells of the nucellar tissue.

In angiosperms the pollen tube has to enter the embryo sac for successful fertilization. The pathway of the pollen tube from the placenta to the embryo sac differs highly and is related to the structure and organization of the ovular tissues.

In most species the embryo sac is surrounded by one or two integuments, leaving a narrow opening called the micropyle. Between the integuments and the embryo sac there can be a varying amount of nucellar tissue, through which the pollen tube has to pass. Recently VAN WENT & WILLEMSE (1981) reviewed the entrance and discharge of the pollen tube in the embryo sac and the fusion of gametes in angiosperm plants, according to ultrastructural and histochemical reports.

The pollen tubes grow along the surface of the placenta, funiculus and ovules in order to reach the micropyle. Numerous authors assume that the synergids are the origin of chemotropic substances involved in the directed growth of the pollen tube towards the embryo sac. The chemotropic substances are thought to be secreted into the filiform apparatus, from which it leaches out into the micropyle or the neighbouring nucellar cells (COE 1954; JENSEN 1965; SCHULZ & JENSEN 1968a; VAZART 1969a, b; VAN WENT 1970a).

Penetration of the pollen tube in the embryo sac starts with the growth of the pollen tube into and through the filiform apparatus. After the passage of the filiform apparatus the pollen tube grows into one of the synergids. This synergid degenerates already before the arrival of the pollen tube, as in cotton (JENSEN 1965; JENSEN & FISHER 1968), mais (DIBOLL 1968), and oak (MOGENSEN 1972) or after pollen tube entry as in *Torenia* (VAN DER PLUYM 1964), *Capsella* (SCHULZ & JENSEN 1968a), and *Petunia* (VAN WENT 1970a). The question whether there is a preference for one synergid to degenerate or to be penetrated is still unanswered.

Shortly after the entrance of the pollen tube in the cytoplasm of the synergid, tube growth ceases (JENSEN & FISHER 1968; VAZART 1980; VAN WENT 1970b). The contents of the tube are discharged by a terminal or subterminal pore. In *Petunia* (VAN WENT 1970b) the pollen tube opens at its tip, probably by bursting. In *Gossypium* (JENSEN & FISHER 1968) a subterminal pore is formed on the side of the pollen tube towards the egg cell, and indicates a more complex system than simple rupturing of the pollen tube.

There is abundant evidence that the male gametes of angiosperms are cellular and complete descriptions of their ultrastructure are available (HOEFERT 1969a, b; JENSEN & FISHER 1968; VAZART 1970; CASS 1973; CASS & KARAS 1975). There are only few reports dealing with material of the sperm cells inside the synergid. With phase-contrast microscopy CASS & JENSEN (1970)

observed sperm nuclei surrounded by a distinct clear zone in the penetrated synergid in barley. This clear zone closely resembles the zone observed between the nucleus and the boundary of the sperm cell in the pollen grain, which represents the male cytoplasm.

The actual transfer of the sperm cells from the penetrated synergid into the egg cell and central cell still remains unresolved. Microscopical observations are limited mainly to identification of sperm cells in either the egg cell or central cell (JENSEN 1964, 1968b, 1972; JENSEN & FISHER 1967; CASS & JENSEN 1970; VAN WENT 1970b). Recently RUSSELL (1980) provided ultrastructural evidence for transmission of sperm cytoplasm into the zygote and endosperm of *Plumbago*. This species is rather exceptional since it lacks synergids. On the other hand, no synergids means in this case no mixing tube cytoplasm, since the pollen tube penetrates the cell wall between the egg cell and the central cell.

It is stated that fertilization in angiosperm plants includes a real cell fusion process (LINSKENS 1968; VAN WENT 1970b; JENSEN 1972). The plasm membrane of one sperm cell is thought to come into contact and fuse with the plasm membrane of the egg cell, whereas the plasm membrane of the second sperm cell fuses with the plasm membrane of the central cell. The fusion process leads to the formation of bridges between and gametes, through which the sperm nucleus and possibly sperm cytoplasm can pass. This fusion hypothesis is attractive, especially since in most species no ruptures or pores are found in the synergid-egg and synergid-central cell boundaries. However, in *Capsella*, a species unique in that it has real walls in between synergid-egg cell and synergid-central cell, SCHULZ and JENSEN (1968a) observed the presence of a pore in the common synergid-egg cell wall near the opened tip of the pollen tube. It is believed that one sperm cell has entered the egg cell through this pore.

Next in the process of double fertilization is the fusion of male and female nuclei. Ultrastructural studies (JENSEN 1964; VAN WENT 1970b; SCHULZ & JENSEN 1973) have shown that the nuclear fusion starts with local contact and fusion of the outer membranes of the two nuclear envelopes, which then become continuous. Next is the contact and fusion of the inner membranes forming bridges between the two nuclei. These bridges enlarge and coalesce.

Soon after fertilization is achieved, division of the endosperm nucleus occurs, leading to the formation of endosperm. The zygote remains undivided for some time, although considerable cellular and cytoplasmic changes can take place (JENSEN 1963, 1968b; SCHULZ & JENSEN 1968b, 1977; VAN WENT 1970b; ASHLEY 1972). In *Gossypium* zygote shrinkage up to 50% of the volume of the unfertilized egg is due to a decrease of the size of the vacuole (JENSEN 1968b). During the shrinkage period, which had been considered as a quiescent stage, the zygote is undergoing extensive reorganization. ASHLEY (1972) states that shrinkage and rearrange-

ment of the zygote cytoplasm is essential for normal embryonic differentiation.

#### 1.4. Research object

Development of new techniques on re-embedding and electron microscopy offers the possibility of studying material with the light microscope and the electron microscope simultaneously. This gives information on different levels. By comparing and quantifying the results, the changes in various cells and tissues can be estimated. This can contribute to a better comprehension of the process of generative reproduction and can give indications in what way embryo formation and seed setting can be influenced.

In ovaries possessing more ovules, which means more female gametophytes, many pollen tubes enter the ovary to fertilize subsequently the egg cell of each ovule. In an ovary with only one ovule the processes leading to fertilization can be regulated. Plants of the cultivated spinach, *Spinacia oleracea* L. are dioecious and each female flower has only one ovule. Parts of the flower biology of spinach as sex expression (ROSA 1925) and breeding (SNEEP 1957) were studied earlier. Fundamental questions in relation to the process of sexual reproduction are not included in these earlier studies of spinach. RAMANNA & MUTSAERTS (1971) reported that in spinach branching of pollen tubes occurs. This feature of pollen tube branching can be of importance for division and partition of nucleus and cytoplasm in the various branches. In this research project the structural aspects of the ontogeny of the pistil and the process of fertilization in spinach are studied on both light and electron microscopical level, including the process of gamete fusion in mature ovules. Qualitative and quantitative ultrastructural differences of the various cell-types made it necessary to study in detail the ontogeny of the ovule.

The present report is divided in two main parts:

##### A. Development of pistil tissues that are involved in the nutrition of the ovule and in the process of pollen tube growth.

Differentiation of the multicellular nucellus has been investigated and related to possible nutritional aspects. The ontogeny and the functioning of the different tissues are related to each other (WILMS 1980b). The ultrastructure of the developing embryo sac (WILMS 1981a) is followed, starting at the time of formation of the individual cells and ending with successful fertilization. The structures are related to possible functions. Storage and use of reserve material is taken into account (WILMS 1980b, 1981a).

B. The structural aspects of pollination and pollen tube growth through some of the tissues, penetration in the embryo sac and fertilization.

The structure and organization of cells and tissues that form the pathway to the embryo sac are studied on electron microscopical level (WILMS 1980a, 1981b). Morphological changes in the stigma and style are studied in relation to the pollen tube growth (WILMS 1980a). Pollen tube growth in young and mature pistils is compared (WILMS 1981b). Penetration in the nucellus and the embryo sac has been studied. The discharge of the pollen tube contents in the degenerated synergid, the process of fusion of sperm cells with egg cell and central cell, and the fusion of their nuclei are established (WILMS 1981b).

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## 2. ARTICLES

- 1980 a Development and composition of the spinach ovule. *Acta Bot. Neerl.* 29 (4):
- 1981 a Ultrastructure of the developing embryo sac of spinach. *Acta Bot. Neerl.* 30 (1/2):
- 1980 b Ultrastructure of the stigma and style in spinach in relation to pollen germination and pollen tube growth. *Acta Bot. Neerl.* 29 (1):
- 1981 b Pollen tube penetration and fertilization in spinach. *Acta Bot. Neerl.* 30 (1/2):

## DEVELOPMENT AND COMPOSITION OF THE SPINACH OVULE

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### SUMMARY

The ultrastructure and histochemistry of the developing spinach ovule have been examined. The development and differentiation of the integuments, nucellus and female gametophyte results in an ortho-amphitropous organisation of the ovule. In the nucellus four parts can be distinguished: the conductive part, the original chalazal part, the chalazal proliferating part and the lateral part. The cells of the various parts have common features as well as distinguishing characteristics related to their position and function. The inner and outer integuments show different features during their development. In the outer integument 3-5 cell layers develop, but the inner integument shows a development of two differing cell layers. Contact by plasmodesmata between the two cell layers diminishes and stops at maturity. The localization of different reserve substances (starch, other polysaccharides, proteins, lipids) have been studied in the developing ovules to determine the nutritional supply of the embryo sac and embryo. Extensive changes in the amount of reserve substances have been observed in the ovule right up to maturity of the embryo sac. In the outer integument storage of starch increases much up to maturity and after fertilization a gradual decrease occurs. The storage and transfer function of some cell types in relation to the nutrition of the embryo sac and embryo is discussed. The presence and location of polysaccharides during megasporogenesis and megagametogenesis is considered. Finally, the possible pathway of metabolites in spinach during different phases of the developing ovule is discussed.

### I. INTRODUCTION

The ovule of angiosperms consists of the nucellus enveloped by one or two integuments, and is attached to the placenta by a short stalk, the funiculus. Ovules can be classified depending upon the degree of curvature during ovule development (SCHNARF 1929 and MAHESHWARI 1950, 1963). Apart from this morphological variation, ovules also can highly vary in internal structure. The variation in size, dimensions and histology of the nucellus is much greater than the distinction between tenui- and crassinucellate type suggests (BOUMAN 1974). There is a strong variation in number of cells in the various tissues and in degree of differentiation of the various cells and tissues. A specific problem with respect to the formation and development of the ovule is its nourishment. Nutrition mainly takes place from the placenta through the funicular vascular tissue. Transport occurs in the ovule through nucellar cells to the developing female gametophyte or embryo and endosperm. According to JENSEN (1963, 1965a) in cotton the synergids play a role in the nutrition of the female gametophyte. VAN WENT (1970) for *Petunia*, MOGENSEN (1972) for *Quercus* and NEWCOMB (1973a, b) for *Helianthus* suggest different routes of metabolites to the embryo sac and developing embryo. However, in this discussion only the nutrition of the embryo

sac has been considered. No consideration has been given to the other parts of the ovule.

The present report deals with the study of the development and organisation of the cells of the *Spinacia oleracea* L. ovule, attempting to elucidate the way of bending and the pathway of some metabolites. It is part of an investigation of the early embryogenesis in spinach in terms of cellular ultrastructure and composition.

## 2. MATERIALS AND METHODS

Plants of *Spinacia oleracea* L., cv. Prévital, were grown in the greenhouse at approximately 25°C. Dissected ovules were fixed according to one of the following procedures.

A. Fixation for 1.5 hrs in 2.5%  $\text{KMnO}_4$  in 0.05 M phosphate buffer at pH 7.2 at room temperature. After fixation the tissue was washed in buffer, dehydrated in a graded ethanol series and embedded in ERL.

B. Fixation in 3.5% glutaraldehyde in 0.2 M phosphate buffer at pH 7.2 for 3.5 hrs at room temperature. After fixation the tissue was washed several times during 5 hrs in buffer and subsequently post-fixed overnight in 2%  $\text{OsO}_4$  in 0.2 M phosphate buffer at pH 7.2 at room temperature. The fixed material was then washed in buffer, dehydrated in a graded ethanol series and embedded in Epon. Post-staining with 2% uranylacetate occurred in the 70% ethanol for 1 hr. Sections were post-stained with 0.5% lead citrate for 1 minute (REYNOLDS 1963).

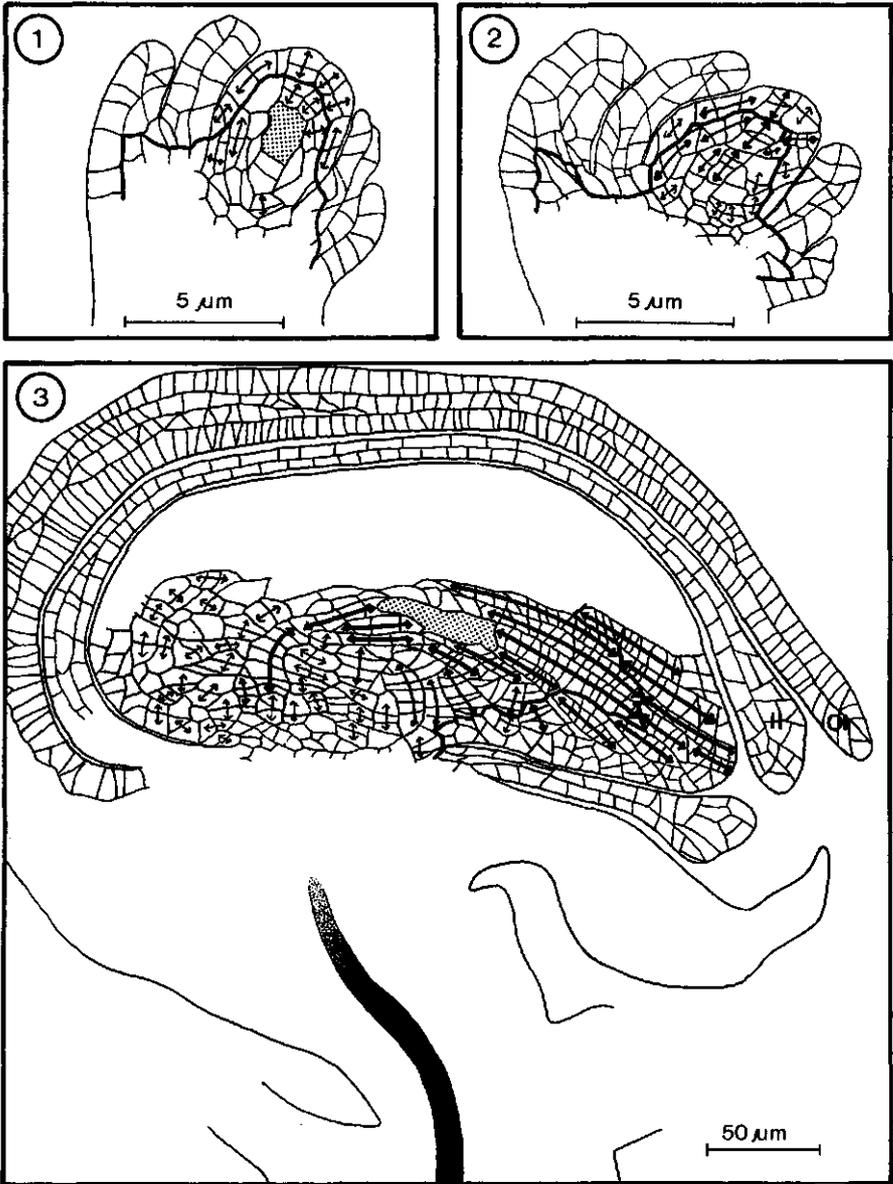
Tissue used for histochemical localization of starch was freeze-fixed in a glycol medium, cut in a IEC CTF cryo-microtome at  $-10^\circ\text{C}$  and stained with IKI (JENSEN 1962). Tissue for other histochemical localization procedures was FAA-fixed and sectioned in paraffin wax. For insoluble carbohydrates the periodic acid-Schiff (PAS) and for proteins the chloramine T-Schiff staining was used (JENSEN 1962).

## 3. RESULTS

The large megaspore mother cell of spinach is surrounded by multilayered nucellus tissue (figs. 1, 2). Round the nucellar tissue the integuments develop. Early in ovular development the nucellus points upward, but as it grows it gradually bends down and finally becomes ortho-amphitropous (fig. 4A).

### 3.1. Integuments

Formation of the integuments starts before the megaspore mother cell reaches the leptotene stage. The origin of both integuments is dermic. The inner integument initiates first, followed shortly by the outer. During its ontogeny the inner integument is two cells thick, except at the base and at the micropyle where it becomes thicker (fig. 3). In a young stage of development the cells of the outer layer are square and those of the inner layer are rectangular in longitudinal sections of the ovule (fig. 5). Plastids with few thylakoids are regular, in the outer



Figs. 1-3. Early stages in ovule development in *Spinacia*. The thick lines indicate the border between dermally and subdermally originating cells. Double ended arrows indicate growth pattern in the nucellus. The direction of the arrows indicates the dividing direction. The length of the arrows indicates division activity. Fig. 1. Cell pattern in the integuments and nucellus at megaspore mother cell stage. Fig. 2. Ovule at megaspore mother cell stage just beside the megaspore mother cell. Different division activities can be seen in the nucellus. Fig. 3. Ovule with the embryo sac in the eight nucleate stage.

layer sometimes with starch. Dictyosomes with 5–7 cisternae are relatively frequent. The cisternae have an average length of 0.6  $\mu\text{m}$  and form many small vesicles. The cell wall between the two layers of the inner integument is constant in thickness except at the places with plasmodesmata. The walls between the individual cells of each layer differ in thickness and all have plasmodesmata. Up to maturity of the ovule the cells of both layers elongate, while in the inner layer still some divisions occur. The number of plasmodesmata between the individual cells of each layer remains the same. Cells of the outer layer reach a length of about 30  $\mu\text{m}$ , which is twice the length of the cells of the inner layer. The thickness of the total inner integument remains about 8  $\mu\text{m}$ . The surface of the inner integument is covered with a cuticle. Where this cuticle borders the nucellus, it is less compact.

The outer integument is initially slightly shorter than the inner one (*fig. 1*). As the ovule grows the outer integument becomes equal in length by more frequent cell division (*fig. 3*). By this time the outer integument is composed of 2–5 layers of slightly radially elongated cells (*fig. 3*). The cells possess some lipid granules and many plastids (*fig. 6*). Initially no starch is observed in the plastids, but towards maturity the amount and size of starch grains increases in the plastids (*fig. 7*). Cells of the outer layer contain a larger amount of starch. Intercellular spaces appear between the subsequent layers (*figs. 4G, 7*). Their content shows a slight homogeneous electron-density. After maturity and until fertilization the amount of starch increases strongly in the outer integument.

After fertilization a decrease of starch in the outer integument occurs. The walls between the outer integument cells are relatively thin with many plasmodesmata, especially in the radial walls. The surface of the outer integument is covered with a distinct cuticle.

### 3.2. Nucellus

At the leptotene stage of the megaspore mother cell the growth of the nucellus becomes unequal. Unequal growth is achieved by a variation in both cell elongation and cell division activity (*fig. 1*). The cells close to the inner bending side are less elongated than the other cells. The latter also divide more frequently (*fig. 1*). At the outer bending side some periclinal divisions occur (*fig. 1*). Initially the expansion of the ovule is merely due to cell division. Obliquely orientated files of cells are produced (*fig. 2*). At this stage all nucellus cells show the same ultrastructure. At the megaspore stage the first differences between micropylar and chalazal cells are observed (*fig. 8*). The micropylar cells have amyloplasts, many dictyosomes and vacuoles, and the chalazal cells have many mitochondria. In the micropylar tissue the originally thin walls thicken (*fig. 8*). The general processes of cell elongation and division continue during the next developmental stages (*fig. 3*). The ultimate ovule shape is highly defined by the formation of files of cells at the chalazal side of the megaspore or young embryo sac. These files bend from the funiculus to the megaspore. This together with the division and extension of the cells at the micropylar side of the megaspore results in a long and curved ovule.

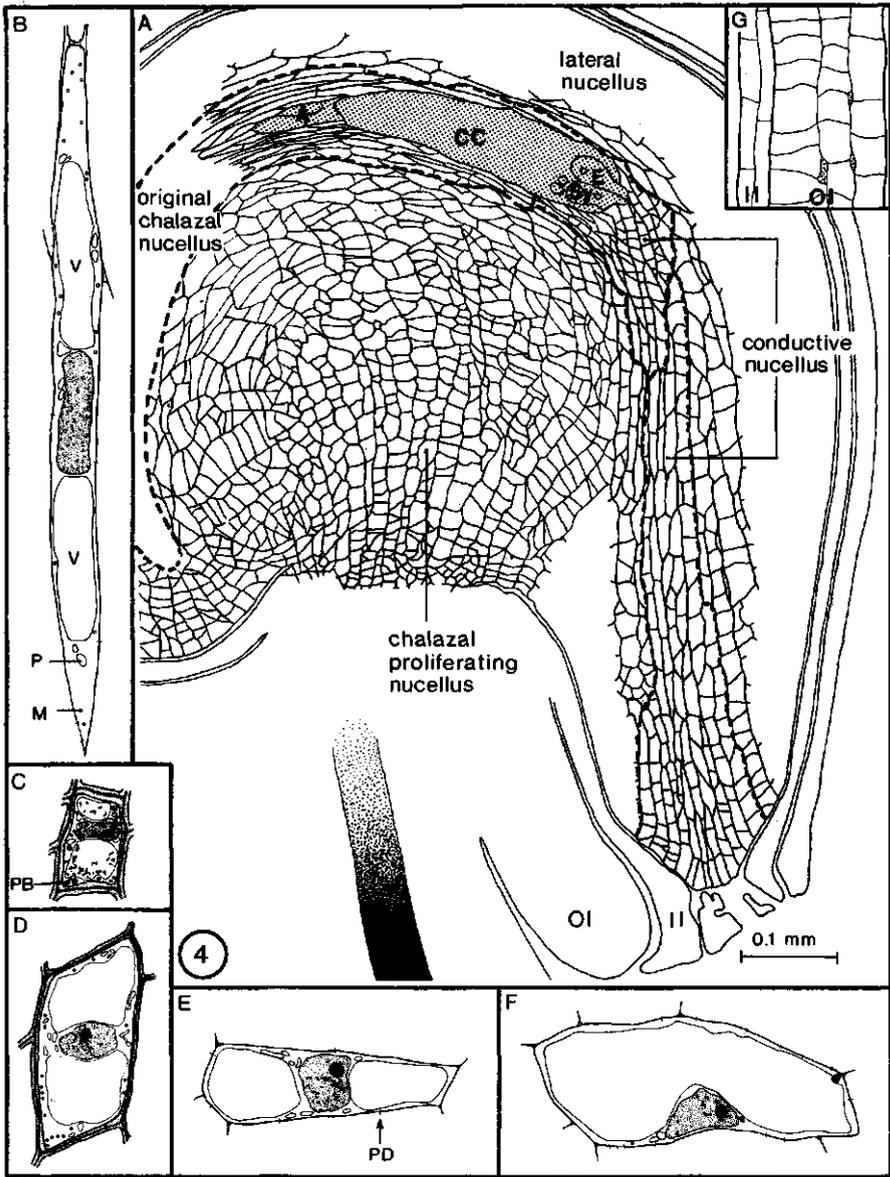


Fig. 4.A. Cell pattern in the mature ovule. The dotted lines mark the various parts of the nucellus. B-G: Cytological drawings of cell types of the nucellus and integuments. B. Original chalazal cell. C. Transmitting cell of the conductive tissue. D. micropylar cell of the conductive tissue. E. Chalazal proliferating cell. F. Lateral cell and G. Inner and outer integument.

During later stages in ovule development various parts of the nucellus show specific differentiations. Most of the elongation of the ovule is due to cell enlargement, which is especially prevalent in the micropylar part, in the central core around the embryo sac and in the original chalazal nucellus cells behind the embryo sac (*fig. 4A*). Cell division occurs in the nucellus cells perpendicular to the placenta and in the extension of the funiculus, which form many radial files of cells. These activities give the mature ovule its final shape (*fig. 4A*). According to the place, structure and organisation of the various parts of the mature nucellus the following tissues can be distinguished: a. the original chalazal tissue behind the antipodal side of the embryo sac, b. the conductive tissue between the micropyle and the embryo sac, c. the chalazal proliferating tissue in the extension of the funiculus and d. the lateral tissue. The relative position of these various tissues is indicated in *fig. 4A*.

*The original chalazal tissue.* The general form of the individual cells can be seen in *fig. 4B*. The cell walls are relatively thin and especially the short end walls show a high plasmodesmata density (*fig. 10*). The length of the cells in the young stage is 3–5  $\mu\text{m}$ , which is one quarter of the width (12–17  $\mu\text{m}$ ). In the mature stage however, the length is as much as 125  $\mu\text{m}$ , which is about 20 times the width. The nucleus remains located at the centre of the cell accompanied at both sides by vacuoles. Oval mitochondria with many well developed cristae are abundant (*fig. 10*). Oval to pear-shaped plastids are present with some single thylakoids. In the young stage many small starch accumulations are present (*fig. 9*), whereas at maturity there are hardly any (*fig. 17*). Endoplasmic reticulum (ER) is first observed as short cisterns, but later grouping of long cisterns with some branching is observed in the peripheral cytoplasm. The amount of dictyosomes decreases from abundant in the young stage to rare at maturity.

*The conductive tissue.* It consists of two cell types (*fig. 4A*). At the micropylar end are the micropylar cells with only slightly thickened walls (*fig. 4D*). Near the embryo sac are the small, so-called transmitting cells with thick cell walls (*fig. 4C*). The structural differentiation of the cells of the conductive tissue starts shortly before the ovule development has reached the functional megaspore stage (*fig. 8*). Then their walls thicken and the plastids accumulate starch for some time.

At maturity the transmitting cells (*fig. 11*) have plastids with plastoglobuli and only little starch, small oval mitochondria 0.3–0.5  $\mu\text{m}$  in length with short cristae, dictyosomes with 4–5 short cisternae (0.3–0.5  $\mu\text{m}$  length) and single strands ER. Also some lipid granules and protein bodies with a diameter of about 1  $\mu\text{m}$  are present (*fig. 12*). The observations strongly suggest that the latter are formed within ER. The cells of the transmitting tissue become loosely organized. Their middle lamellae appear disintegrated. Locally, some electron-dense material is accumulated (*fig. 11*).

The mature micropylar cells (*fig. 13*) have cell walls which are less thick than the transmitting cell walls. Plasmodesmata are common in all walls. The cell walls are initially homogeneous but at maturity the region of the middle lamellae as well as the outer wall parts beneath the cuticle bordering the micropyle

disintegrate. This cuticle is affected too (*fig. 13*). The large nucleus is located mostly central, with at both sides one vacuole. Oval plastids with few thylakoids and plastoglobuli but without starch are present in the peripheral cytoplasm, together with some mitochondria, dictyosomes and ER. Rarely some small lipid granules are observed.

*The chalazal proliferating tissue.* The increase of the chalazal proliferating tissue during the latest part of embryo sac development is mainly due to cell division and partly to cell enlargement. In the young stage (*fig. 3*) less than 10 cells can be counted in a file between the chalaza and the embryo sac. At maturity the files consist of over 30 cells (*fig. 4A*). The division activity is strongest near the chalaza. Eventually the cells have large vacuoles, a central nucleus with some cytoplasm and further peripheral cytoplasm (*figs. 4E, 15*). Plasmodesmata are common in all walls. The cells which are located near the embryo sac are storing starch (*fig. 14*).

*The lateral tissue.* This consists of vacuolate cells (*fig. 4F*), which show considerable variation in both size and shape. The large central vacuoles occupy the major portion of most of the cells. These vacuoles contain some ER, dictyosomes, mitochondria and several plastids (*fig. 16*). In the plastids large amounts of starch accumulate at maturity. Ribosome content is high in the lateral nucleus. The walls are thin and have some pit pairs (*fig. 16*).

### 3.3. The female gametophyte

The development of the female gametophyte corresponds to the monosporic, 8-nucleate *Polygonum* type of MAHESHWARI. During maturation of the embryo sac the area of the embryo sac in a median longitudinal section increases about 12 times. Only sporadically a neighbouring nucellus cell becomes resorbed.

At the end of the coenocytic period the egg and the antipodal region are formed first by centripetal wall formation. Next, walls separate the individual antipodes and the two synergids. In this young stage firstly vacuolation and polarisation take place in the egg and central cell. The egg nucleus and polar nuclei lose their central position. Vacuoles appear respectively at the micropylar side in the egg and chalazal in the central cell. All cells show the same cytoplasmic ultrastructure at this stage of development. The walls separating the various cells are thin and show plasmodesmata. Plasmodesmata are absent in the longitudinal and micropylar walls of the embryo sac. In the chalazal wall, plasmodesmata are observed frequently between the chalazal antipode and the bordering nucellus cells (*fig. 17*).

### 3.4. Histochemistry of the mature ovule

Polysaccharides occur in the form of cell wall, cytoplasmic polysaccharide and starch grains. The reaction of the different parts of the mature ovule after staining of all carbohydrates and separate staining of starch can be seen in *table 1*. The original chalazal tissue shows PAS-positive reaction for its cytoplasm. In the conductive tissue and in the chalazal proliferating tissue mainly the cell walls show PAS-reaction. The thickness of their walls is the cause of the different

amounts. All cells of the embryo sac are PAS-positive with slight differences according to their plasm/vacuole ratio and to the thickness of the walls with respective wall protrusions. The thin cell walls of the integuments cause the faint PAS-reaction. In the micropylar parts of the inner integument the PAS-reaction is stronger because of a positive reaction of the cytoplasm.

The presence of starch is localized (*table 1*). Red to brownish red and yellow colourings are observed. The outer integument gives a strong IKI-reaction with an accumulation in the outer layer. The reaction of the inner integument is faint except at the micropyle where it is more positive. A strong reaction occurs in the nucellus cells neighbouring the original chalazal tissue, embryo sac and transmitting part of the conductive tissue. The main part of the chalazal proliferating tissue colours yellow.

Table 1. Localization of reserve substances in the different parts of the mature ovule of *Spinacia oleracea* L. o = faint, + = positive and ++ = strong reaction. If no reaction is observed the sign - is used.

	Integument		Nucellus				Embryo sac			
	outer	inner	orig chal	chal prol	conductive transm	microp	ant	cc	syn	egg
Polysaccharides (PAS)	o	o/+	+	o	++	+	++	+	++	++
Starch (JKJ)	++	o	+	Yellow/ ++	++	-	o	o	+	++
Proteins (Chloramin-T-Schif)	o	++	+	-	++	+	o	++	o	++

\* ++ the layer of the outer cells surrounding the trace of original chalazal cells shows strong reaction.

The distribution of proteins is given in *table 1*. The total inner integument shows a positive reaction to protein staining with an accumulation in the micropylar parts. In the conductive tissue an increase in activity can be observed towards the embryo sac. In the embryo sac the egg and the central cell show a strong reaction. Of all parts of the ovule the nuclei react positively.

#### 4. DISCUSSION

GOEBEL (1933) considered the ovule of *Atriplex hortensis* L., belonging to the family Chenopodiaceae, as amphitropous. In the amphitropous ovule the bending of the nucellus is accompanied by the formation, beneath the ventral face, of a mass of cells. This classification is based on the cellular organisation of the mature ovule. On the other hand BOCQUET (1959) came to a different conclusion by considering the mode of development of the ovule and the organisation of its vascular tissue. He comes to two basic series: the orthotropous series and the anatropous series. In this view the amphitropous form is considered as an organisation which can appear in both developmental series; the central nucellus body develops independently. According to the classification of BOCQUET the bitegmic, crassinucellar ovule of *Spinacia oleracea* L. is ortho-amphitropous. In the first period of ovule development, expansion of the nucellus is as an extension

of the funicle, which is characteristic of the orthotropous series. In a later phase of ovule development the newly formed chalazal proliferating tissue causes a cellular organisation of the mature ovule agreeing with the term amphitropous.

According to DE BOER & BOUMAN (1972) some outer integuments originate sub-dermally, the other outer integuments and the inner integuments originate dermally. The initiation and development of the integuments of spinach is dermal and its final structure corresponds with many other dicotyledons (DE BOER & BOUMAN 1972; BROWN & MOGENSEN 1973; MOGENSEN 1973; CHEAH & STONE 1975). Usually the two cell layers of the inner integument develop differently (DE BOER & BOUMAN 1972; ROBERTSEN 1976). In spinach, both the rate of cell division and cell enlargement is different in the two layers. During the development of the integument the plasmodesmata between the two layers gradually disappear. The merely faint staining for reserve material suggests a limited supply and transport of metabolites.

Monocotyledons in general show a strong dividing activity of the nucellar cells between the chalaza and the embryo sac during the later stages of ovule development (MAZE & BOHM 1973; CAVE 1975; BERG 1978). In the dicotyledons many taxa have tenuinucellar ovules in which the single nucellus layer around the developing female gametophyte often degenerates before maturity of the embryo sac is reached. Few ovules of the studied taxa are crassinucellate. Most authors have not paid attention to a differentiation of the nucellar cells (COE 1954; MOGENSEN 1973; MUKKADA & CHOPRA 1973; NORSTOG 1974; MITCHELL 1975). More cell types are described for cotton (JENSEN 1965b) and partly for *Pandanus* (CHEAH & STONE 1975), and GUPTA & RAJESWARI (1977) have mentioned the presence of nucellar tracheids in the ovules of *Luffa*. Only MALIK & VERMANI (1975) suggest a supply route of metabolites to the embryo sac.

In the nucellus of spinach various cell types develop and four specialized tissues can be distinguished, although the tissues can not be sharply defined. In our opinion the specialized characters of the various tissues have to be related to various functions. The micropylar and transmitting cells of the conductive tissue certainly play a role in relation to the growth of the pollen tube. One can speculate about a function in attraction and guiding of the pollen tube. The original chalazal tissue likely serves in the young ovule as a direct transporting pathway from the chalaza to the developing embryo sac. These original chalazal cells have similar characteristics as the transfer cells of GUNNING & PATE (1969), especially the strongly developed ER system and the many plasmodesmata in the short transfer walls. Up to maturity the chalazal proliferating tissue develops many files and causes a structural cut off of the direct transport pathway of metabolites from the funicular vascular strand to the original chalazal tissue. The following supply of reserve metabolites is mostly stored as starch in the chalazal proliferating and lateral cells which are located around the original chalazal tissue. At maturity of the ovule and probably also during the development of the embryo and endosperm, the original chalazal cells possibly function as sink and transfer of nutrients, passed from the chalaza by the chalazal proliferating tissue.

The possible pathway of polysaccharides in spinach is proposed for three different phases of development (*diagram 1*). The young stage represents the developing ovule. In the mature stage the embryo sac is functional and the file of chalazal proliferating cells are formed. In the fertilized stage the zygote and endosperm start their development.

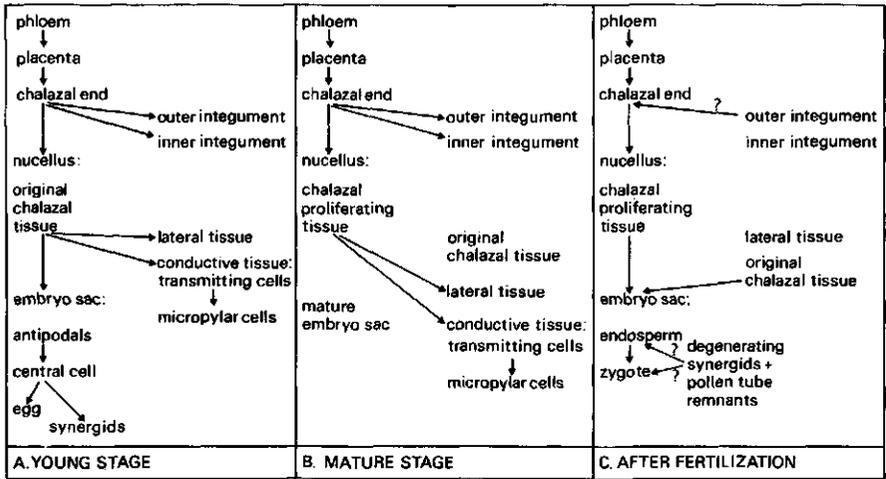


Diagram 1. The proposed pathway of polysaccharides in the spinach ovule during three stages of development, A. young stage; B. mature stage and C. after fertilization has taken place.

During successive stages of development and growth of the ovule there is substantial polysaccharide storing. In the young stage polysaccharides are stored in the file of nucellus cells beginning at the chalaza and ending at the micropyle. A concentration of storage material, starch grains, is observed in the transmitting cells of the conductive tissue. The file of original chalazal cells shows a strong PAS-positive cytoplasmic reaction. Near maturity metabolites are stored in the inner and outer integuments. With the process of fertilization insoluble polysaccharides are gradually depleted from the nucellus. The integuments and many nucellar cells are storage reservoirs for the main source of energy needed for subsequent embryo and endosperm development. The present data indicate that during ovular development the nucellus has only a minor nutritive role in relation to the relative small embryo sac.

The presence of protein in several parts of the mature ovule seems strongly related to the reproductive function of the female gametophyte. The positive to strong reaction in the tissues of the micropyle surrounding inner integuments and the conductive tissue of the nucellus can be related to pollen tube penetration. The presence of proteins in egg and central cell can be related to their future function. The observations in *Zephyranthus* of MALIK & VERMANI (1975) indicate the same direction. They found in the young antipodals a high amount of proteins, whereas at the approach of fertilization embryo sacs contain low amounts of proteins, mainly concentrated in the central cell and in the egg.

According to MALIK & VERMANI (1975) the antipodals act as intermediate cells which help in the transit from the chalazal to the micropylar area of the embryo sac. This statement is supported by PANCHAKSHARAPPA & HEDGE (1972) and PANCHAKSHARAPPA & SYAMASUNDAR (1975), who found a strongly PAS-positive cytoplasmic staining of the mature antipodals. The PAS-reaction on the antipodals of spinach show similar features.

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#### ABBREVIATIONS

CN = conductive nucellus; CPN = chalazal proliferating nucellus; D = dictyosome; FM = functional megaspore; II = inner integument; IS = intercellular space; LG = lipid granule; LN = lateral nucellus; M = mitochondrium; ML = middle lamella; N = nucleus; OCN = original chalazal nucellus; OI = outer integument; P = plastid; PB = protein body; PD = plasmodesmata; PP = pit-pair; PER = rough endoplasmic reticulum; V = vacuole; W = cell wall; WP = wall protrusion.

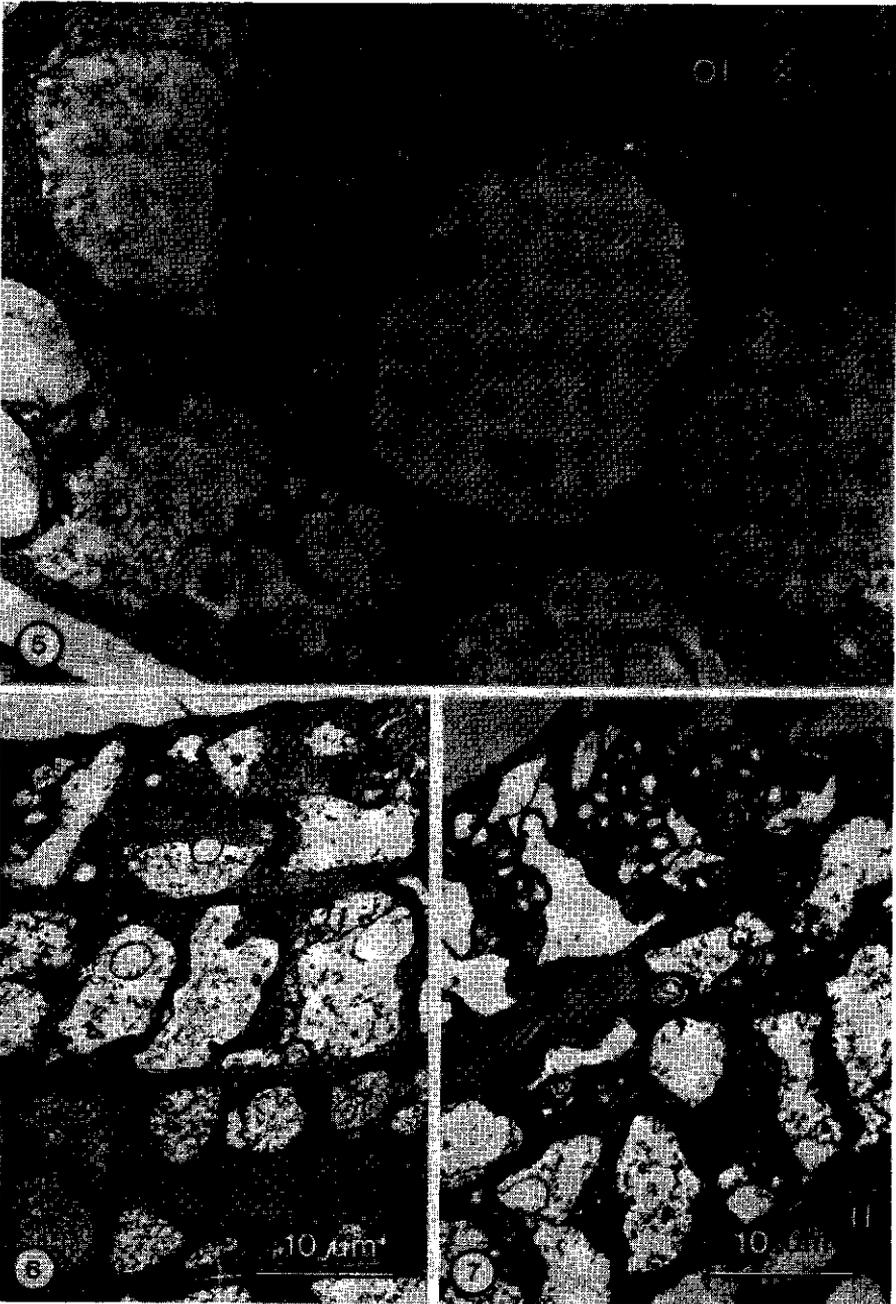


Fig. 5. Inner integument at the young embryo sac stage. Note the plasmodesmata between the two cell layers.  $\times 8000$ .

Fig. 6. Outer integument at young embryo sac stage.  $\times 2000$ .

Fig. 7. Outer integument at mature embryo sac stage.  $\times 2000$ .



Fig. 8. Functional megaspore with a surrounding core of nucellus. First differences between the chalazal and micropylar nucellar cells can be observed. Note the degenerating megaspore (asterisk).  $\times 3000$ .



Fig. 9. Young original chalazal tissue.  $\times 5000$ .

Fig. 10. Mature original chalazal tissue. Cytoplasm near the end wall between two cells.  $\times 32,000$ .

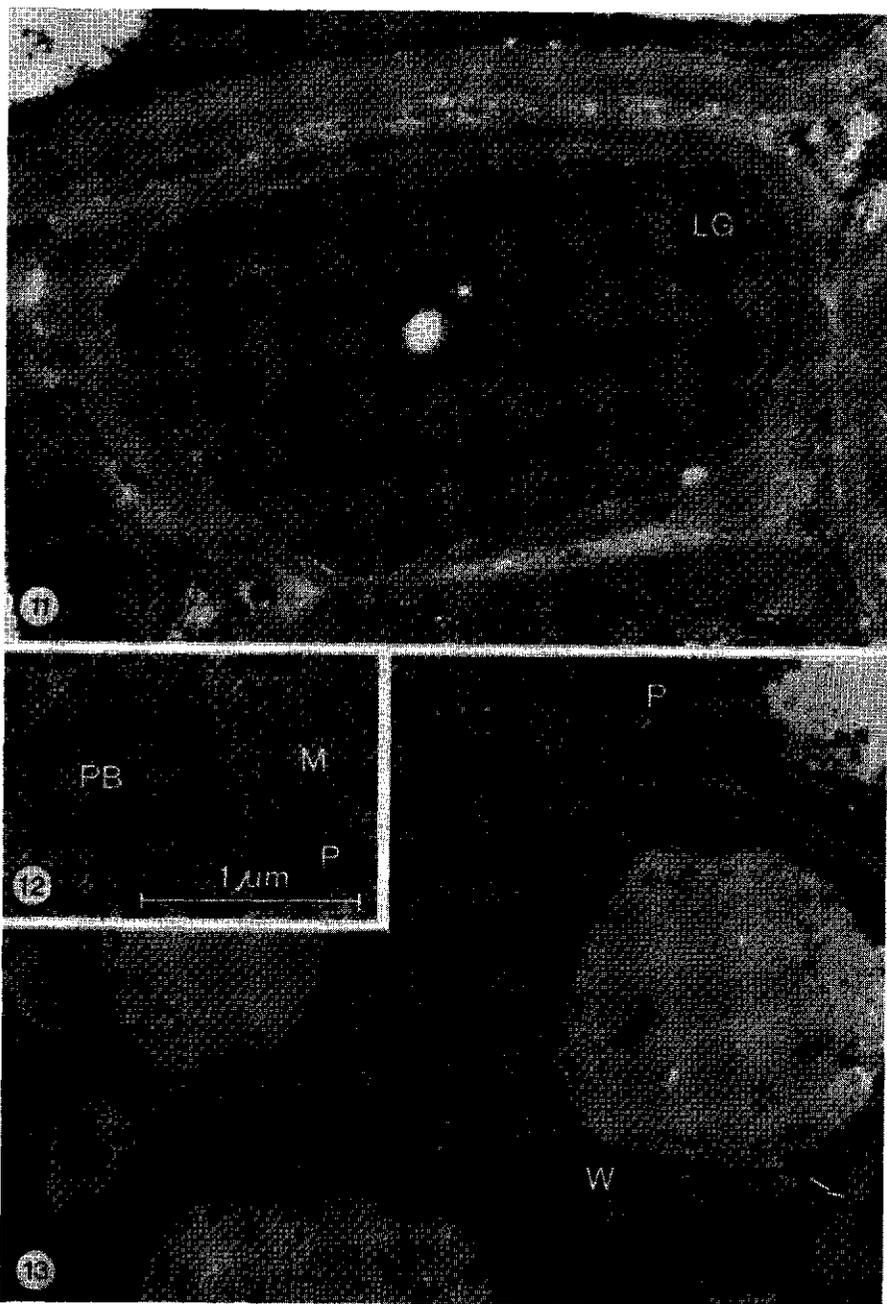


Fig. 11. Transverse section through the mature transmitting cells. The middle lamella has become disintegrated.  $\times 20,000$ .

Fig. 12. Detail of the cytoplasm of the mature transmitting cell, in which protein bodies are accumulated in the cisternal phase of the ER.  $\times 30,000$ .

Fig. 13. Mature micropylar cells. The cell walls at the outside and between two cells start to disintegrate, which can be seen as less electron-dense than the other cell wall parts.  $\times 10,000$ .

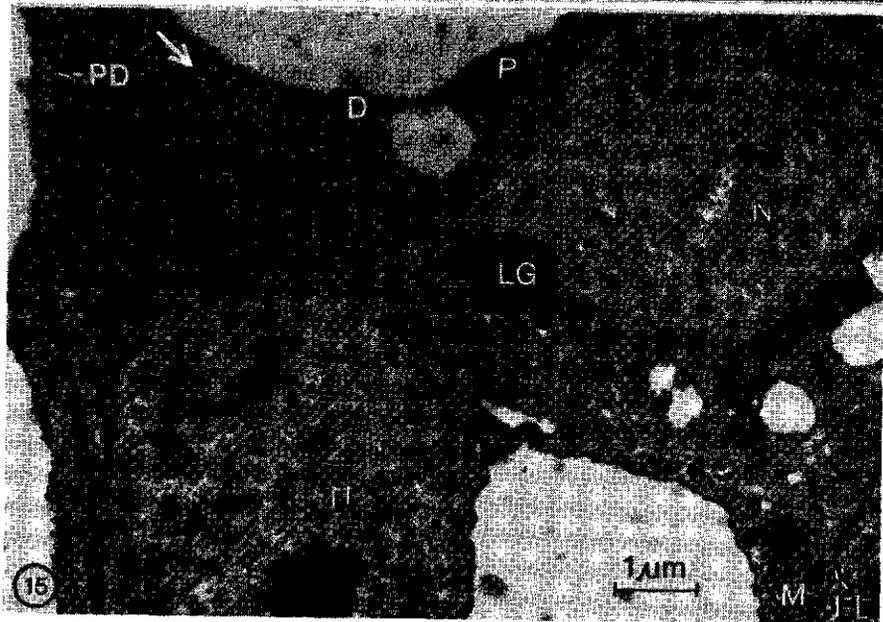
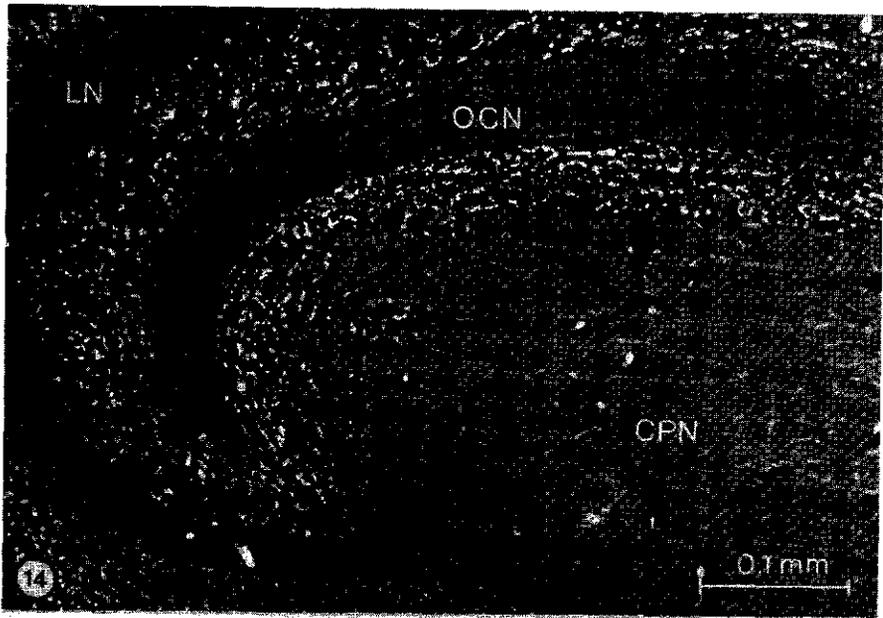


Fig. 14. Starch accumulation around the cells of the original chalazal tissue. Phase-contrast photograph.  $\times 220$ .

Fig. 15. Part of the chalazal proliferating tissue. Note the newly formed cell wall (arrows).  $\times 11,000$ .

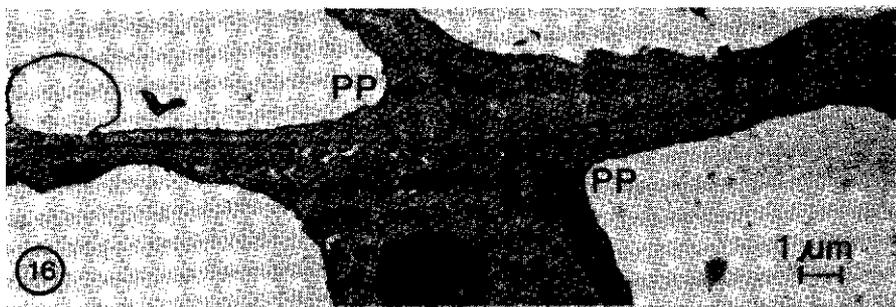


Fig. 16. Part of the lateral tissue.  $\times 5000$ .

Fig. 17. Chalazal part of the chalazal antipode surrounded by original chalazal cells. In the antipodal wall plasmodesmata are present.  $\times 12,000$ .

# ULTRASTRUCTURE OF THE DEVELOPING EMBRYO SAC OF SPINACH

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## SUMMARY

The ultrastructure of the embryo sac of spinach (*Spinacia oleracea* L. cv. Prévital) has been studied from the inception of the constituent cells to the time of fertilization. The development shows two phases. One is from cell formation until cell maturity and the second from maturity to the fertilizable stage. During the first phase the cell dimensions, areas and volumes of the various cells and cell parts have been measured and compared. The protoplasm of the antipodals hardly increases, whereas that of the egg and central cells multiplies 10 times. But the enlargement of all cells is mainly due to vacuolation. During the second phase the cells develop their final ultrastructure. Initially they are similar - have irregular nuclei, abundant ER, common mitochondria and dictyosomes, scanty plastids and no lipid. The antipodals attain their final structure sooner, are ephemeral and start degenerating. The egg cell differentiates fast, subsequently grows slowly and at maturity a renewed increase, in particular of mitochondria, occurs. The development of synergids and central cell is gradual. Before fertilization one of the synergids degenerates and in the central cell the polar nuclei form numerous long protrusions which fuse partly. The ultrastructural changes of each cell type have been related to their possible functions. Accumulation and degradation of reserve materials in various cells is discussed in relation with the nutritional supply to the differentiating female gametophyte.

## 1. INTRODUCTION

The embryo sac of spinach develops according to the *Polygonum* type. Earlier studies on other species have shown that each constituent cell of this embryo sac has its precise ultrastructure indicating specific functions. The antipodals are thought to be secretory and possibly synthesize enzymes, which digest the adjacent nucellus (DIBOLL & LARSON 1966; VAZART 1968; SCHULZ & JENSEN 1971; RIFOT 1973; ZHUKOVA & SOKOLOVSKAYA 1977). Many hypotheses have been postulated on the function of the synergids (VAZART 1958). It is obvious that their main function is related to the transfer of male gametes to the egg and central cells. Furthermore, most authors assume that the synergids direct the growth of the pollen tube (VAN DER PLUYM 1964; DIBOLL & LARSON 1966; VAN WENT 1970a). JENSEN (1965a) and SCHULZ & JENSEN (1968a), on the other hand, suggest that they have a nourishing function. The egg cell and also the central cell are the most important cells with respect to reproduction. They fuse with the sperm cells and develop into the embryo and endosperm, respectively.

The present paper is a part of the investigation on the early embryogenesis in spinach, starting with the formation of the ovule up to completion of fertilization. It describes and relates the ultrastructure of different cell types with their possible functions, from their inception to the time of fertilization.

## 2. MATERIAL AND METHODS

*Spinacia oleracea* L. cv. Prévital was grown in the greenhouse at approximately 25°C. The dissected ovaries were fixed and embedded according the GA-OsO<sub>4</sub> method, which has been described previously (WILMS 1980).

For detection of polysaccharides on electron microscopic level the method described by THIÉRY (1967) was used.

Measurement of length, width and area were taken with a Kontron MOP AM-2 on at least three ovules of each developing stage.

## 3. RESULTS

The cellular development of *Spinacia oleracea* embryo sac shows two phases. The first phase is from the formation of cells (young stage) until the attainment of their largest dimensions (mature stage). The latter stage is also determined by the presence of intact antipodals and identically structured synergids. The second phase starts at the mature stage and ends at the fertilizable stage during which cells develop their final ultrastructure. At the time of fertilization, however, the antipodals as well as one of the synergids degenerate.

The dimensions of various cell types and of the embryo sac at the young and mature stages are given in *table 1*. Since in transverse sections the cellular areas are nearly circular only length and width are included. The areas of protoplasm, vacuoles and total cell are measured from median longitudinal sections. The dimensions of constituent cells are also presented as percentage of the total area. The increase in volume is calculated in the following way: the volume of the mature embryo sac is nearly  $\pi r_m \times \text{median area}_m$ , in which  $r_m$  is the ray length in cross sections and for  $\pi r_m$  the width of the mature embryo sac is used. This volume is  $55 \times 11,400 \mu\text{m}^3$ . The volume of the young embryo sac, calculated in the same way, is  $17 \times 950 \mu\text{m}^3$ . The factor for the increase in volume of the embryo sac is  $55 \times 11,400 / 17 \times 950 = 39$ .

The development of the embryo sac from young to mature stage takes about 10 days, and from mature to fertilizable stage about 4 days. When young, the embryo sac is 69  $\mu\text{m}$  long and 17  $\mu\text{m}$  wide, but at maturity it measures  $350 \times 55 \mu\text{m}$  (*table 1*).

At the fertilizable stage there are considerable developmental differences between the persistent and the degenerated synergids. At the same time, the antipodals are also in various stages of degeneration.

### 3.1. Embryo sac at young stage

The ultrastructure of various cells of the young embryo sac is initially the same. The first structural differences become visible when vacuolation starts and the random distribution of the organelles in the cytoplasm becomes lost, first in the egg and central cells, next in the antipodals and finally in the synergids (see *diagrams 1-4*).

Table 1. Mean size and areas from median longitudinal sections at young and mature stages of embryo sac. Each number represents mean of measurements on three different ovules (prot = protoplasm, vac = vacuoles, tot = total).

	Young stage					Mature stage					Volume increasing factor		
	size		area in $\mu\text{m}^2$			size		area in $\mu\text{m}^2$					
	( $\mu\text{m}$ )	prot	vac	tot	%	( $\mu\text{m}$ )	prot	vac	tot	%	prot	vac	tot
embryo sac	69 × 17	720	230	950	100	350 × 55	2000	9400	11400	100	9	130	39
3 antipodals	24 × 17	210	10	220	23.2	65 × 18	200	200	400	3.5	1	21	2
synergid	16 × 10	70	-	70	7.4	64 × 22	200	500	700	6.1	6	>200	22
egg cell	18 × 14	80	50	130	13.7	70 × 28	400	400	800	7.0	10	16	12
central cell	42 × 17	360	170	530	55.7	300 × 55	1200	8300	9500	83.4	10	160	57

The nuclei are irregular in shape and approximately 5–6  $\mu\text{m}$  in diameter with homogeneous nucleoli of about 4  $\mu\text{m}$  in diameter. The size of the polar nuclei is, however, larger.

ER (endoplasmic reticulum) is abundant but its distinction as SER or RER is not clear. In the central cell most of the ER cisternae are interconnected or arranged in circular patterns which encircle small vacuoles.

Mitochondria are spherical and oval, are regularly spread in the cytoplasm, and have few tubular cristae, except for those in the synergids which have more cristae.

The number of plastids is low and they are oval to pear-shaped with a diameter of approximately 0.3  $\mu\text{m}$ . Their ultrastructure is rather simple. A small starch grain is observed in the plastids of the egg cell and of the synergids, whereas plastoglobuli sometimes occur in the plastids of the synergids.

Dictyosomes are frequent. Each consists of 4–6 mostly flat cisternae with an average length of 0.7  $\mu\text{m}$ . The dictyosomes of the egg cell have flat as well as circular cisternae. All cisternae form many small vesicles with an electron-transparent content.

Lipids are absent in the young stage but a very small amount is seen in the synergids.

### 3.2. Antipodals

The partitioning of the chalazal cytoplasm at the coenocytic stage results in the formation of three antipodals (*fig. 1*). Separating walls are formed from the periphery of the embryo sac to the centre. Dictyosomes are frequently at the places of wall formation. Wall synthesis also occurs in relation to the side walls of the antipodals. All walls of the antipodals possess wall projections (*fig. 2*) and have plasmodesmata (*figs. 3–5*). The longitudinal walls inbetween the antipodal cells and nucellus do not have plasmodesmata connections. In the degenerating phase no plasmodesmata are observed anymore.

The morphology of the antipodals, marked with A1, A2 and A3, at the mature stage is shown in *fig. 2*. A1 is the antipodal bordering the central cell and A3 is the

STAGE	DEVELOPMENT of the ANTIPODALS				
	YOUNG		MATURE		FERTILIZABLE
nucleus size (L×B)	6 × 5 μm	8 × 6 μm	6 × 6 μm	5 × 4 μm	4 × 2 μm
nucleus and nucleolus 1250×					
ribosomes polysomes and ER 12.500×					
mitochondria 12.500×					
plastids 12.500×					
dictyosomes 12.500×					
lipids 2.500×	—	—	•	•	•••

Diagram 1. Morphological changes of antipodal cell organelles during development of embryo sac. Sign — is used when no lipid granules are observed.

one bordering the chalazal nucellus. At maturity their average sizes ( $L \times B$  in  $\mu\text{m}$ ) in median sections are A1:  $24 \times 16$ , A2:  $36 \times 10$  and A3:  $40 \times 12$ . Soon after the mature stage has been reached degeneration starts successively in A1, A2 and finally A3.

At maturity the nucleus becomes almost spherical and has a diameter of approximately  $6 \mu\text{m}$ . A small amount of chromatin is spread over the entire nucleus. In a later stage it becomes irregular again and decreases in size. The nucleolus disintegrates and eventually disappears. The nuclear envelope often shows separated membranes with large electron-transparent spaces in between. The karyoplasm develops very condensed chromatin clumps.

During the development of the antipodals there is an increase in vacuolation whereas the organelles undergo a number of changes (*diagram 1*). In the first phase the amount of ribosomes per cytoplasm area diminishes. Long ER cisternae with attached ribosomes develop and become arranged parallel to each other. At the mature stage irregularly distributed cisternae, often with sacculate endings, are observed. The amount of attached ribosomes decreases as compared to the other polysomes as well as monosomes. When degeneration sets in RER is often observed as circular strands (*fig. 6*), sometimes encircling small vacuoles or cytoplasmic islands with accumulated ribosomes.

The mitochondria show an increase in diameter up to  $0.9 \mu\text{m}$  and many tubular cristae also develop. They begin to degenerate simultaneously with the antipodals. The number of cristae decreases and this is accompanied by the accumulation of strongly stained material in the surrounding membranes and perimitochondrial space.

Plastids are not common in the antipodals. A few long thylakoids and some short sacculate protrusions of the inner membrane develop. Sometimes single starch grains occur, which subsequently disappear.

The number of dictyosomes remains the same during cell maturation, whereas the number of cisternae becomes less. During degeneration the dictyosomes produce many vesicles, often large ones, with an electron-dense content.

A few droplets of lipid with a maximal diameter of  $0.6 \mu\text{m}$  appear when the antipodals become mature. During degeneration a slight increase in the amount of lipid occurs.

### 3.3. Synergids

The general topography of the micropylar part of the mature embryo sac is shown in *figs. 7 and 10*. The cells of the egg apparatus are only partly attached to the micropylar embryo sac wall. Because of this and because of the triangular arrangement of the cells, a number of cell walls are common or shared, including the common synergid wall, the egg cell-synergid wall, the synergid-central cell wall and the egg cell-central cell wall. The boundaries of the cells of the egg apparatus at the fertilizable stage vary considerably, from two membranes only at the chalazal side of the cells to a specialized thickened structure, the filiform apparatus (FA), at the micropylar pole of the synergids. In between these extremes the thickness of the remaining cell walls is irregular. The base of the egg

cell is attached at the side of the embryo sac, about 10  $\mu\text{m}$  from the most micropylar part (*fig. 7*).

The major part of the synergid cytoplasm and the nucleus are located in the micropylar region of the cell. The chalazal portion is filled with a number of vacuoles. One of the synergids disorganizes before fertilization (*fig. 10*) whereas the other (psy) does so soon after double fertilization. In a few older but unfertilized ovules the synergids start degenerating simultaneously.

At the young stage each synergid is completely surrounded by a cell wall of equal thickness (about 0.1  $\mu\text{m}$ ) over the entire length (*fig. 1*). With the formation of the FA, the remaining parts of walls at the micropylar side become thicker (*fig. 8*). Both the FA and other parts of the cell wall show an increase in density during development (*fig. 9*). At the chalazal side there is less increase in wall thickness and this is accompanied by decrease in density.

The differentiation of FA starts simultaneously with the formation of wall protrusions at different places of the merged synergid-embryo sac wall. Rod-, club-, finger- and plate-like extensions of the wall grow into the cytoplasm. As a result of their fusion and formation of new wall protrusions finally a complex striated filiform apparatus develops. A plasmalemma separates the wall material from the cytoplasm but the interface is so convoluted that the FA appears as a sponge-like mass of wall material interpenetrated by cytoplasm.

In one and sometimes both synergids a discontinuity in FA wall formation is observed close to the common synergid wall. On these places the original wall is still present in close connection with the cytoplasm and the FA shows a slit (*fig. 12*). The other small cytoplasmic inclusions in the FA are round and show all organelles. Initially the FA has the same homogeneous ultrastructure (*fig. 9*). After the mature stage however, different wall material is deposited. At the time of fertilization, a number of layers are already deposited (*fig. 11*). The electron density of the successively formed layers gradually decreases (*fig. 11*). In  $\text{GA-OsO}_4$  fixed material the density of the later deposits corresponds with the density of the content of the dictyosome vesicles present at that time (*fig. 11*).

The FA gives a strong PAS positive reaction for insoluble carbohydrates and also a positive reaction on the Thiéry-test for polysaccharides.

Plasmodesmata connect the two synergids and also the synergids with the egg cell and the central cell. They are observed only in the micropylar half of the synergid.

The various organelles show ultrastructural changes during the course of differentiation and maturation (*diagram 2*) accompanied by an ultimate polar distribution. The nucleus elongates towards the FA and then the shape changes to oval. It is approximately 12  $\mu\text{m}$  long and about 4  $\mu\text{m}$  in thickness. The total length of the nuclear envelope in median sections increases about twice. The nuclear envelope has many pores. Contact between the nuclear envelope and the ER is rare. The outer nuclear membrane is partly covered with ribosomes. At maturity numerous small concentrations of chromatin are regularly spread over the entire nucleus. During the development to the fertilizable stage the nucleolus of the degenerated synergid disintegrates almost totally, whereas in the persistent

STAGE	DEVELOPMENT OF THE SYNERGID				
	YOUNG		MATURE	PERSISTENT	DEGENERATED
nucleus size (L × B)	6 × 5.4 μm	7 × 5.4 μm	12 × 7.4 μm	12 × 6.4 μm	12 × 6.4 μm
nucleus and nucleolus 1250 ×					
ribosomes polysomes and ER 12.500 ×					
mitochondria 12.500 ×					 Chalazal Microcyllar
plastids 12.500 ×					
dictyosomes 12.500 ×					
lipids 2500 ×	 NUC				 NUC

Diagram 2. Synergid cell organelles as seen during embryo sac development. Lines in lipid columns represent microcyllar parts of synergid walls (NUC - nucellus).

synergid its diameter decreases to about 1  $\mu\text{m}$ .

The number of mitochondria increases tremendously up to maturity. They are elongated and their average diameter is 0.5  $\mu\text{m}$ . The tubular cristae become well developed. When degeneration of the synergid starts, electron-dense material accumulates at the membranes. The cristae collapse and then disappear whereas a thick electron-dense layer accumulates at the perimitochondrial space. The inner and outer membranes cannot be distinguished any more.

The plastids are distributed mostly in the cytoplasm around the nucleus and partly in between the nucleus and the FA. They become cup-shaped and subdense. In the persistent synergid they appear translucent, and have some electron-dense material. In the degenerated synergid very few vesicles, all without electron-dense material, are visible.

The distribution of dictyosomes in the cytoplasm increases at random, except near the nucleus where they are absent. The cisternae of each dictyosome increase in number to 4 or 5 and in length to 1.0  $\mu\text{m}$ . Associated with the cisternae are vesicles of different sizes and of various degrees of density according to the developmental stage of the synergid. Shortly after the coenocytic stage electron-transparent vesicles are observed and at maturity most of these become electron-dense. In the persistent synergid they appear translucent, and have some electron-dense material. In the degenerated synergid very few vesicles, all without electron-dense material, are visible.

The RER cisternae become dilated and rearranged towards maturity, each resulting in single ER cisternae and having short and swollen lamellae, both covered with ribosomes. The number of ribosomes greatly decreases at maturity. Lipid bodies increase during the development of the synergids, both in number and diameter. Intensification of this process occurs in the degenerating synergid.

The thin plasma strands surrounding the vacuoles contain only a few organelles. The plasma membrane of the degenerated synergid is no longer distinguishable at the chalazal side.

### 3.4. Egg cell

The egg cell is a polarized cell (*fig. 1*), with its cytoplasm and nucleus located at the chalazal end, and vacuoles at the micropylar side. The mature egg cell is approximately 70  $\mu\text{m}$  long, 30  $\mu\text{m}$  wide at the chalazal and 18  $\mu\text{m}$  wide at the micropylar part (*table 1*). The micropylar half is surrounded by a wall with intermediate density whereas at the chalazal half the membrane is in close contact with that of the central cell. During the development from mature to fertilizable stage the wall between the egg and central cells in the region of the degenerated synergid gets an irregular and unusual morphology. In this area the plasma membranes of the egg and the central cells become intermittently separated by gaps which contain randomly distributed granular dense material (*fig. 15*). In between the gaps the membranes of the egg and the central cells remain close together.

The partial egg cell wall only contains plasmodesmata where it borders the synergids and the central cell.

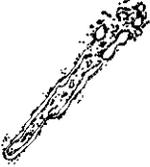
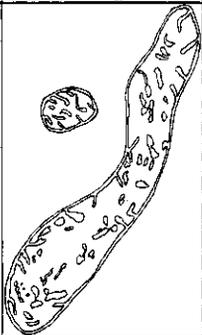
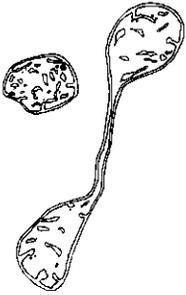
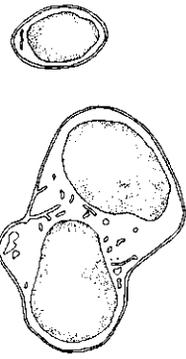
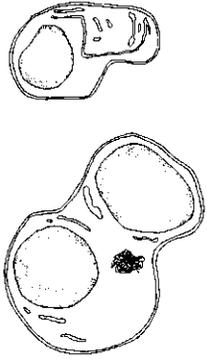
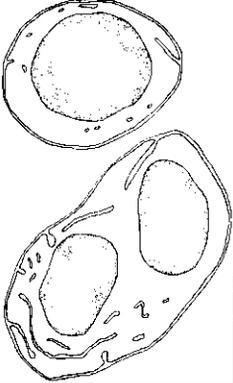
STAGE	DEVELOPMENT of the EGG CELL			
	YOUNG		MATURE	FERTILIZABLE
nucleus size (L x B)	6 x 5 μm	13 x 8 μm	14 x 14 μm	18 x 13 μm
nucleus and nucleolus 1250 x				
ribosomes polysomes and ER 12.500 x				
mitochondria 12.500 x				
plastids 12.500 x				
dictyosomes 12.500 x				
lipids 2.500 x	—	•	•	••

Diagram 3. Egg cell organelles during development of embryo sac. Sign - indicates absence of lipid granules (NE - nuclear envelope).

The nucleus is located at the chalazal tip of the egg cell. At maturity the nucleolus has an electron-dense granular structure with some electron-opaque vacuoles. Then the karyoplasm often contains some multi membrane-like structures resembling myeline (*fig. 13*) and some electron-translucent vacuolar material (*fig. 14*).

The changes in the organelles during the course of differentiation and maturation of the egg cell are depicted in *diagram 3*.

Mitochondria elongate and their number increases. At the fertilizable stage many mitochondria become clustered in the micropylar part of the egg cell.

The plastids are located near the nucleus and possess single starch grains. These amyloplasts grow to an average diameter of 2.5  $\mu\text{m}$  at maturity. Also the amount of starch increases, whereas usually more than one large starch grain develops.

The number of dictyosomes remains low during the maturation of the egg cell. Their ultrastructure stays simple.

The number of ribosomes gradually decreases during development. Single strands of RER and free mono- and polyribosomes are present at mature stage. Some small lipid droplets are also present.

### 3.5. Central cell

The central cell comprises all the original megaspore cytoplasm that is not included in the antipodals, synergids and egg cell. A large central vacuole restricts the cytoplasm to peripheral areas whereas in the vicinity of the egg apparatus an accumulation of cytoplasm, including the two polar nuclei, is observed. A few strands run through the vacuole, connecting the cytoplasm associated with polar nuclei to the peripheral cytoplasm of the cell. The mature central cell is approximately 300  $\mu\text{m}$  long and 55  $\mu\text{m}$  wide (*table 1*).

The central cell is surrounded by a rather irregular wall. At the place of contact with the antipodals the wall shows projections alternated with plasmodesmata. The wall bordering the nucellus is equally thin, except for the micropylar region near the egg apparatus, where projections are present (*fig. 7*). Both parts are without plasmodesmata. The central cell shares a wall with plasmodesmata with the synergids and egg cell at the micropylar part of their contact.

The two polar nuclei approach each other and the chalazal tip of the egg apparatus during the development of the embryo sac. In the nuclei electron-opaque vacuoles appear and the nucleolar diameter increases to 8  $\mu\text{m}$ . The size of the polar nuclei also increases to about  $20 \times 14 \mu\text{m}$ . After maturity the overall shape still remains spherical but numerous long protrusions are formed into the surrounding cytoplasm (*fig. 16*). The protrusions are enveloped by both nuclear membranes. Meanwhile the nuclear size diminishes to about  $14 \times 12 \mu\text{m}$ . The polar nuclei fuse partly before fertilization, but only after pollination has taken place. The partial fusion of the nuclei takes several hours and usually begins by fusion of the membrane of the nuclear protrusions, occasionally by the joining of the nuclear membranes directly.

While the number and shape of mitochondria hardly changes, their length

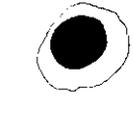
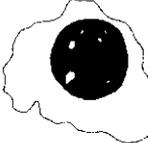
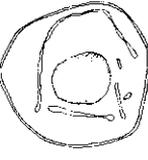
STAGE	DEVELOPMENT of the CENTRAL CELL				
	YOUNG		MATURE		FERTILIZABLE
nucleus size (L × B)	7 × 6 μm	11 × 8 μm	18 × 14 μm	20 × 14 μm	14 × 12 μm
nucleus and nucleolus 1250 ×					
ribosomes polysomes and ER 12.500 ×					
mitochondria 12.500 ×					
plastids 12.500 ×					
dictyosomes 12.500 ×					
lipids 2500 ×	—	•	•	•	•

Diagram 4. Cell organelles of central cell during development of embryo sac. Sign — shows absence of lipid granules.

increases to 2.5  $\mu\text{m}$  at maturity (*diagram 4*) and a large number of short tubular cristae develops. Ribosome-like bodies also are present at maturity.

The plastids, restricted in number and position, grow and develop during central cell maturation. They become almost spherical (2–3  $\mu\text{m}$  in diameter) and usually possess one small starch grain, some long thylakoids, and plastoglobuli. In a later stage a sacculate reticulum develops.

The decrease of ribosomes in a cytoplasmic area results in a distinction between ribosomes associated with the ER and those lying free. Whorls of RER are commonly found when the mature stage is reached. Near the egg apparatus usually 2 or 3 parallelly arranged RER cisternae are visible along the plasma membrane.

The dictyosomes develop differently depending on their location. In the peripheral cytoplasm surrounding the central vacuole they are similar to those at the young stage. Sometimes larger vesicles with and without electron-dense material are formed. The dictyosomes in the micropylar part of the developing central cell first decrease in number and then become more elongated, to about 2.5  $\mu\text{m}$ , showing an increasing number of accompanying vesicles containing some electron-dense material.

#### 4. DISCUSSION

The development of the spinach embryo sac from the formation of the individual cells to the fertilizable stage can be seen in two phases: 1) enlargement and differentiation of the cells; 2) realisation of the final functional situation.

Cell enlargement ends at the mature stage. The area in median longitudinal sections, which is occupied by the antipodals in the young stage is about the same as that of the egg apparatus, whereas that of the central cell is more than twice. During maturity the central cell increases maximal, the egg cell and the synergids enlarge relatively much less, whereas the antipodals increase very little. The enlargement is mainly the result of an increase of vacuoles and partly of protoplasm. The egg and central cells produce relatively more new protoplasm as compared to the other cells of the embryo sac. (see *table 1*). In fact the protoplasm of the antipodals hardly increases. This may be considered as a first indication of differences in the developmental stages of the various cells of the embryo sac.

Realisation of the final functional situation, starting with the degeneration of the antipodals and one of the synergids, seems to be related to pollination. Degeneration of one synergid in general starts after pollination, but further study is needed to confirm this. The functional differentiation of the cells is also indicated by the changes in wall structure and their protoplasmic ultrastructure.

##### 4.1. Antipodals

Wall protrusions occur mainly in the chalazal antipodal cell(s) (MASAND & KAPIL 1966; FISHER & JENSEN 1969; D'ALASCIO-DESCHAMPS 1973; GODINEAU 1973; NEWCOMB 1973a RIFOT 1973; KAPIL & BHATNAGAR 1978). Initially the

wall between the micropylar antipodal and the central cell is relatively thin, at maturity this wall thickens (D'ALASCIO-DESCHAMPS 1973; NEWCOMB 1973a; RIFOT 1973). Plasmodesmata are present in the walls of the chalazal antipodal, in between the antipodals and in the walls between the micropylar antipodal and the central cell (SCHULZ & JENSEN 1971; GODINEAU 1973; NEWCOMB 1973a). In spinach all antipodals have wall protrusions. The location of plasmodesmata is similar to that described for other species. That means that plasmodesmatal contact between sporophytic and gametophytic tissue is restricted to the extreme chalazal position. During megasporogenesis and megagametogenesis the entire development is turned towards the elimination of plasmodesmatal contact between the sporophyte and gametophyte. On the other hand, the absence of plasmodesmata means an obstruction in the supply of metabolites. The maintenance of a limited area (the extreme portion of the chalazal antipodal) with plasmodesmata can be considered as an intermediate situation between the isolation mechanism and the maintenance of required nutrition at this time of development. In a later stage these plasmodesmata are not observed any more.

The final degeneration of the antipodals starting with the micropylar one can be an effect of the continuing contact between genetically different sporophytic and gametophytic protoplasm. But it can also be due to a definite break of the protoplasmic contact in view of the future fertilization, to prevent direct contact between the two, genetically different, sporophytes. From this stage on contact and nutrition have to go via cell walls and plasma membranes. This results in a shift of transport routes and confirms earlier statements (WILMS 1980). Transport no longer takes place through the original chalazal nucellus tissue, but through the chalazal proliferating tissue and subsequently through the entire embryo sac wall.

The ultrastructure of the cytoplasm of antipodals of spinach differs in various stages of development. During the process of development they have many mitochondria, ribosomes and RER, whereas the number of dictyosomes is variable (GODINEAU; 1973; RIFOT 1971, 1973; D'ALASCIO-DESCHAMPS 1973; ZHUKOVA & SOKOLOVSKAYA 1977; DUMAS 1978; NEWCOMB 1973a), although poorly developed antipodals are often reported during embryogenesis (BANNIKOVA 1971; SCHULZ & JENSEN 1971). In *Spinacia* initially the dictyosomes generate vesicles which are likely to be related to the formation of irregular wall thickenings. At maturity the mitochondria develop many tubular cristae, which suggests a high activity of the cells. The antipodals function till the embryo sac is mature but in the following period, to the fertilizable stage, degeneration sets in, beginning with the micropylar antipodal. The ephemeral antipodals do not secrete enzymes which can digest the enclosing nucellus as is suggested in other taxa (SCHULZ & JENSEN 1971; RIFOT 1973), because no degeneration of the surrounding nucellus is observed.

#### 4.2. Synergids

In spinach the FA is an elaboration of the wall as in other species. Its material is

synthesized within the synergids. The FA consists of a homogeneous osmiophilic structure, much more osmiophilic than the cell wall parts of the surrounding nucellus (WILMS 1980), indicating that this consists of a major non-cellulosic compound. The FA of *Petunia* (VAN WENT 1970a) or *Helianthus* (NEWCOMB 1973a) is only a thickening of the cell wall. In other species such as *Capsella* (SCHULZ & JENSEN 1968a), cotton (JENSEN 1965a), *Zea mays* (DIBOLL & LARSON 1966), barley (CASS & JENSEN 1970), *Linum* (VAZART 1971), *Aquilegia* (FOUGERE-RIFOT 1975), *Plantago* (VANNEREU 1978) and in *Spinacia* too the formation of the FA implies an increase of the plasma membrane area. These synergids might be considered as 'transfer cells' (GUNNING & PATE 1969; PATE & GUNNING 1972).

It is of interest that the time of synergid degeneration varies from plant to plant: from before pollen tube entry, as in cotton (JENSEN 1965a), *Hordeum vulgare* (CASS & JENSEN 1970), *Stipa elmeri*, *Epidendrum scutella* (COCUCCI & JENSEN 1969), *Linum* (VAZART 1971), *Acer* (HASKELL & POSTLETHWAIT 1971), *Helianthus* (NEWCOMB 1973b) and perhaps *Zea mays* (DIBOLL 1968), to after pollen tube entry, as in *Capsella* (SCHULZ & JENSEN 1968a) and *Petunia* (VAN WENT 1970a). The degeneration of one synergid in spinach seems a direct response to pollination as reported for *Gossypium* (JENSEN & FISHER 1968). An indication for this is that in some older unfertilized ovules both synergids are equally degenerated.

According to MAZE & LIN (1975) the FA in each of the two synergids has specific functions. In the penetrated synergid it controls the pollen tube growth or the transfer of pollen-tube-growth-directing substances out of the synergid. The FA of the persistent synergid transfers material into the megagametophyte (GUNNING & PATE 1969; JENSEN 1965a; SCHULZ & JENSEN 1968a; NEWCOMB 1973b). Neither VAN WENT (1970a) nor MOGENSEN (1972) interpret the persistent synergid as having a transference function. In spinach no supply route goes this way till fertilization (WILMS 1980) and after fertilization the persistent synergid starts degenerating quickly. This indicates that the assumed transference function of the persistent synergid may be of minor importance. The FA of the degenerated synergid seems to control and regulate its molarity. After degeneration this synergid probably has a lower molarity than the persistent synergid and also lower than the intercellular spaces of the bordering conductive nucellar tissue.

The synergids show in the young stages many well developed mitochondria and dictyosomes and long strands of RER, which are indicatives of a high metabolic activity. The increase in the number of dictyosomes and vesicles with an osmiophilic content indicates production of secretory compounds.

When one synergid degenerates, its organelles lose their internal structure and an accumulation of lipid in between the membranes occurs. Lipid granules accumulate and aggregate in the cytoplasm. This can possibly be related to growth stimulating or growth inhibiting effects of fatty acids in pollen tubes (IWANAMI 1980), since in *in vitro* experiments the monocarboxylic acids inhibit pollen germination and pollen tube growth, whereas dicarboxylic acids stimulate pollen tube growth, both related to IAA activity.

The very thin cell walls at the chalazal part of the young synergid disappear, and during the following stages only a plasma membrane can be seen. Finally, when pollen tube penetration is about to occur this membrane disappears and the chalazal part of the cell collapses, possibly due to a decrease in molarity of this cytoplasm. Absence of starch, or presence of only small amounts, and presence of much lipid suggest a small amount of water-soluble and molarity-increasing compounds.

#### 4.3. Egg cell

The changes in size, shape and ultrastructure of the differentiating egg cell occur mainly in the first days after formation. At the end of the differentiation the number of mitochondria increases much in the micropylar part. Such a large number of well-formed mitochondria is also recorded in *Zea mays* (DIBOLL & LARSON 1966). According to RUNNSTRÖM, HAGSTRÖM and PERLMAN (1959) this does not necessarily mean that a high rate of respiration is taking place; rather it might indicate a potential for a high metabolic rate, generally associated with postfertilization activity. The relatively small number of other organelles and their internal morphology indicate a low rate of activity in the mature egg cell. The occurrence of starch in *Spinacia* egg cells seems to be common as it is also in other angiosperms (JENSEN 1965b; DIBOLL & LARSON 1966; SCHULZ & JENSEN 1968b; VAN WENT 1970b; NEWCOMB 1973a). This is probably needed shortly after fertilization for the development of the proembryo, since it disappears then.

The occurrence of myeline- and vacuole-like structures in the karyoplasm during a short period of the mature stage is exceptional for interphase nuclei. Similar membranous bodies are reported in megaspore mother cells at the meiotic prophase of *Allium* (DE BOER-DE JEU 1978) and for microspore mother cells at the zygotene stage in *Pinus sylvestris* (WILLEMSE 1971). In *Pinus banksiana* DICKINSON & BELL (1976) report myeline-like figures in the meiotic nuclei of the microspore mother cells, whereas they suppose that their initiation is by invagination of the inner membrane of the envelope. In the development of the egg nucleus there seems a moment which corresponds with the meiotic stage of the spore mother cell. In the egg cell the nucleus is preparing for fusion while the nucleus of the spore mother cell is still in the stage of division.

#### 4.4. Central cell

The structural changes during development of the central cell of *Spinacia* are rather slow and spread over the total developing period. In the cytoplasm around the polar nuclei an increasing ER system develops. At the same time nuclear extensions, visible as long thread-like runners, develop and get connected with each other. The tremendous increase in surface area of the nuclear envelopes may be of importance for the fusion of the nuclei with the sperm nucleus, but is not yet understood. The fusion of the polar nuclei occurs in a similar way as reported in cotton (JENSEN 1964, 1965b) *Zea* (DIBOLL 1968) and *Petunia* (VAN WENT 1970b). They partially fuse before fertilization, whereas in other species total nuclear fusion is completed by this time (GODINEAU 1973).

At the fertilizable stage partly fused nuclei, newly formed ER-cisternae, free ribosomes and many mitochondria with tubular cristae in the neighbourhood of the egg apparatus suggest that the central cell is awaiting fertilization.

The structural development of the cell types of the embryo sac of spinach can be summarized and correlated with the developing time of the embryo sac (*diagram 5*). The ultrastructure at the formation is the reference. The degree of changes is considered as degree of development, either positive as it reflects increase of complexity, or negative as it is interpreted as degeneration. When the organelles are optimal in development and maximal in number the cell type gets a development degree of 100, and each cell type seems to have its own specific metabolic activity.

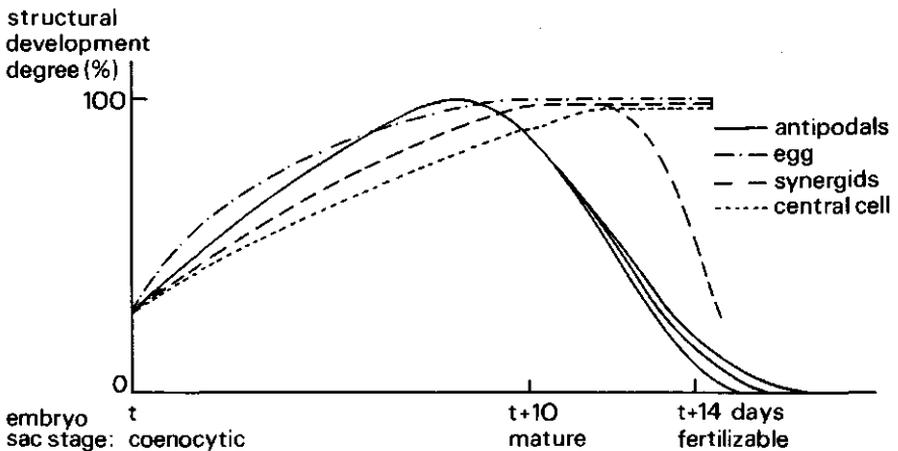


Diagram 5. Correlation between development of various cell types of embryo sac and development, differentiation and degeneration of their organelles.

In the young stage of development of the embryo sac the main supply of metabolites is via the antipodals to the central cell to the egg and synergids (WILMS 1980). Storage of reserve material starts in the egg as starch, and increases during its entire development until the fertilizable stage. At the same time a little amount of starch and some lipid are stored in the synergids. During the first phase of cell enlargement and differentiation lipid granules are formed in the central cell, egg cell and synergids, whereas the storage of starch stops in the synergids.

In the latter some plastoglobuli appear for a short while in their plastids. Near maturity starch is formed also in the central cell and the antipodals, suggesting more metabolic supply needed for further cell development. At maturity this metabolic supply stops and in the antipodals the starch is broken down gradually whereas lipid formation continues. In the central cell and also in the synergids a similar but slower process occurs.

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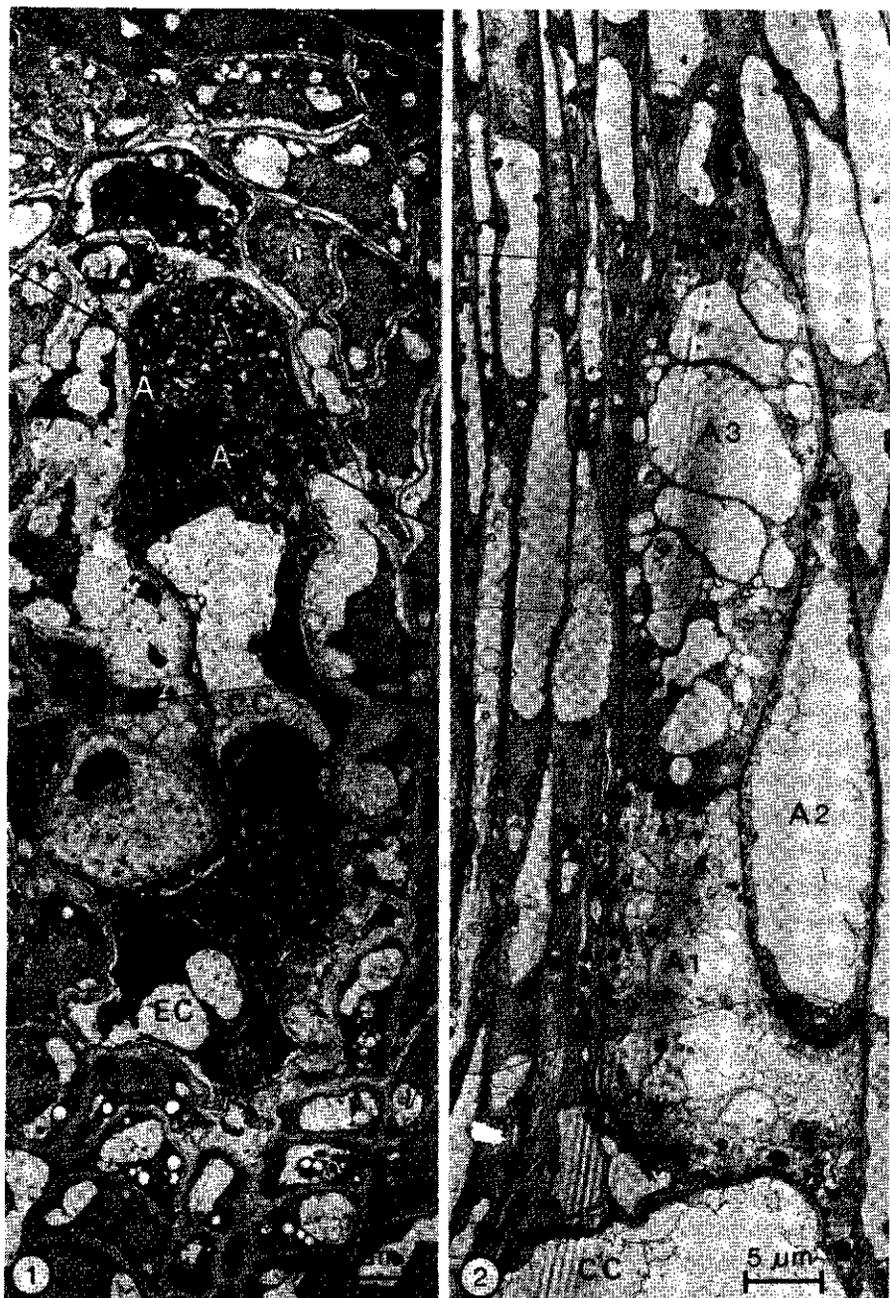


Fig. 1. Young embryo sac just after coenocytic period. Cell wall of antipodals (A) and synergids (Sy) is not yet formed (arrows). 2,000  $\times$ . Fig. 2. Antipodals (A1, A2, A3) at fertilizable stage. 2,000  $\times$ . (CC - central cell, EC - egg cell).

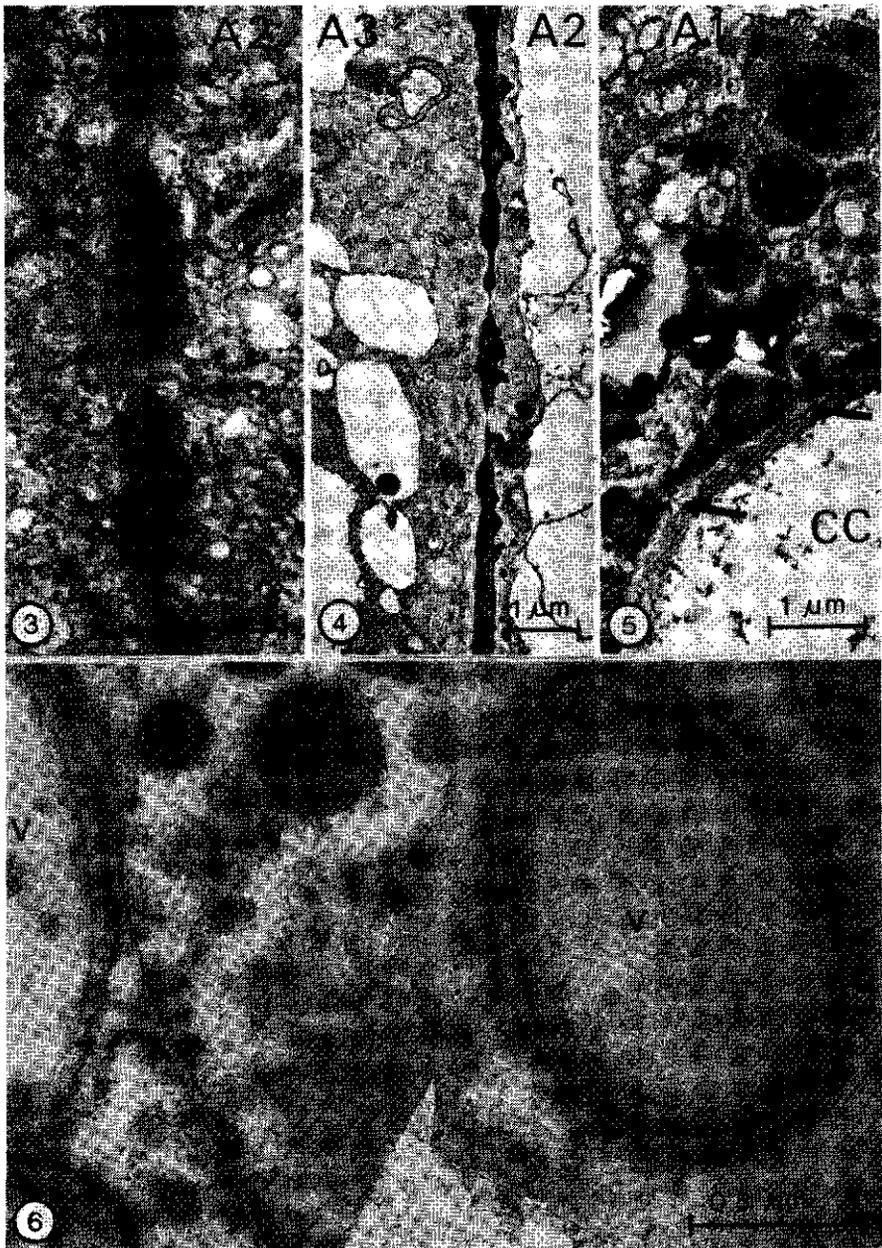


Fig. 3. Part of antipodals (A) when mature showing wide plasmodesmatal connections between common cell wall. 22,000 $\times$ . Fig. 4. Portion of A3-A2 enlarged from fertilizable stage depicted in fig. 2. 9,000 $\times$ . Fig. 5. Portion of the A1-central cell (CC) magnified from fig. 2. 13,000 $\times$ . Fig. 6. Cytoplasm of A3 during fertilizable stage exhibiting circular strands of RER around vacuoles. 45,000 $\times$ . (M - mitochondrium, X - cluster of degenerating ribosomes, V - vacuoles).



Fig. 7. Longitudinal section through egg apparatus and polar nuclei (PN) at fertilizable stage. 2,200 $\times$ . (CC - central cell, EC - egg cell, N - nucleus, Sy - synergid).

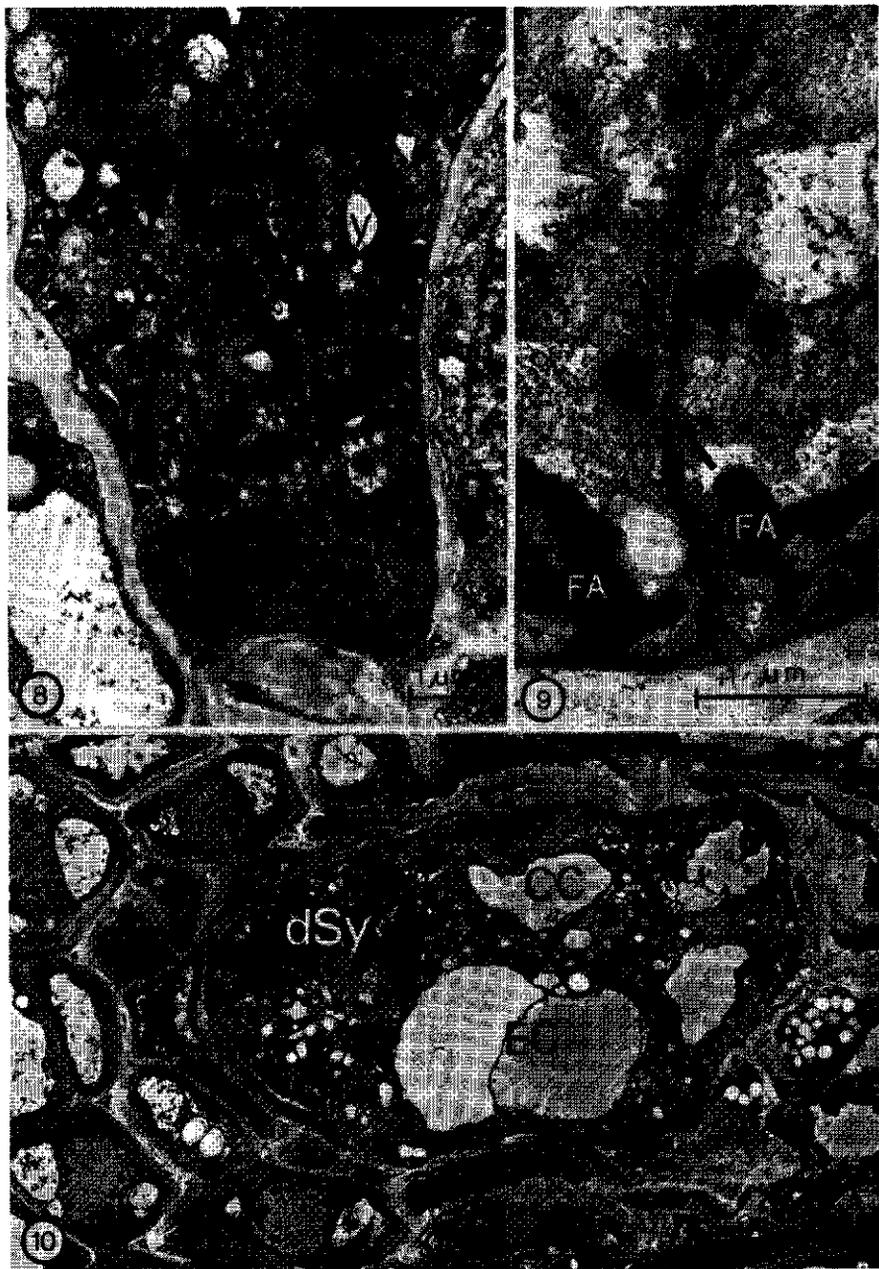


Fig. 8. Formation of the cell wall protrusions (FA) in basal part of synergids (Sy). 9,000 $\times$ . Fig. 9. Enlarged view of a part of FA and common synergid cell wall with plasmodesmata (arrows). 22,000 $\times$ . Fig. 10. Cross section through basal part of embryo sac at fertilizable stage. 1,800 $\times$ . (CC - central cell, dSy - degenerated synergid, EC - egg cell, N - nucleus, pSy - persistent synergid).

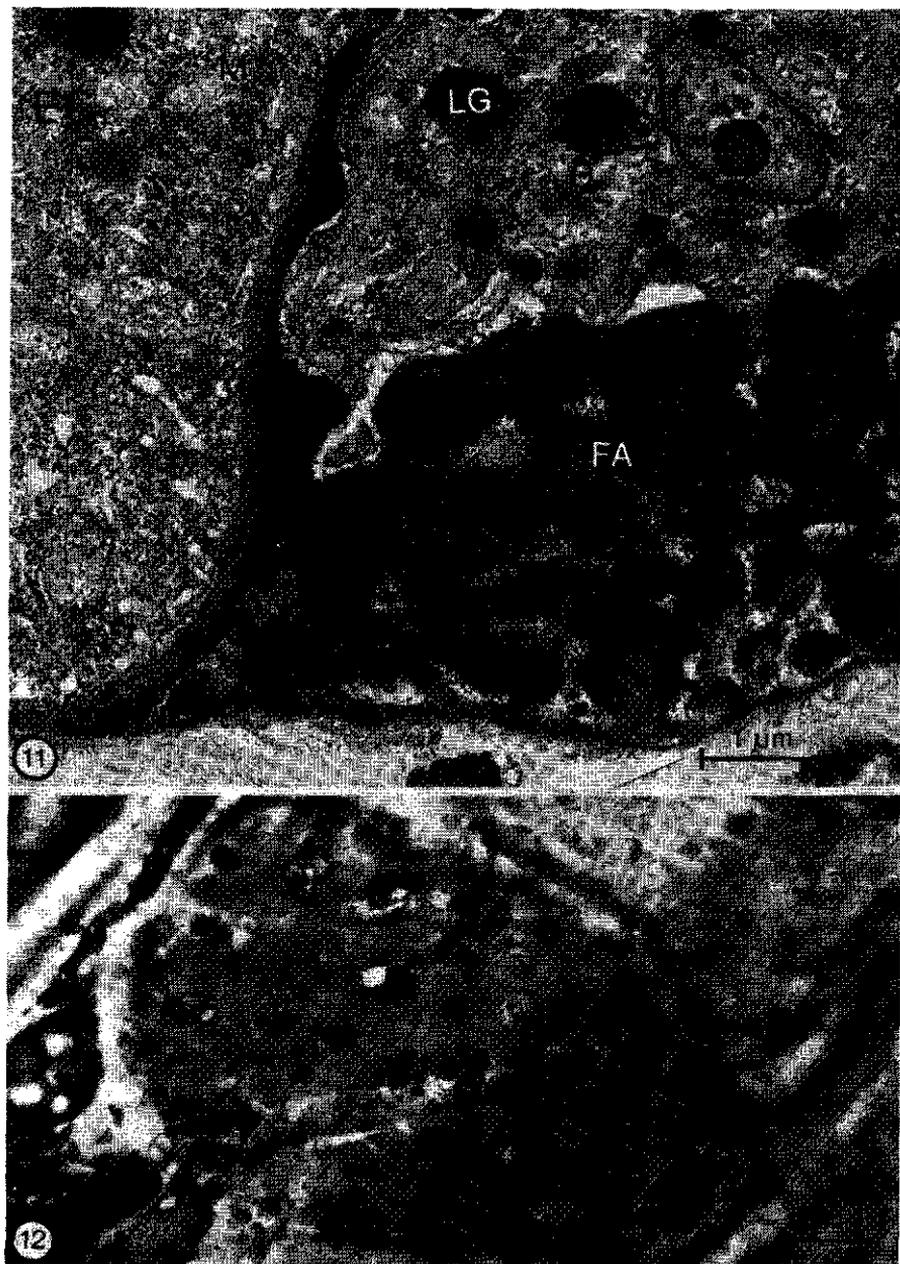


Fig. 11. Enlarged part of a synergid with FA and egg cell (EC). Note cell wall thickening at synergid side of common synergid-egg cell wall.  $20,000\times$ . Fig. 12. Cross section through FA. Long slit with cytoplasm is seen between arrows.  $10,000\times$ . (D - dictyosome, LG - lipid granule, M - mitochondrion, P - plastid).

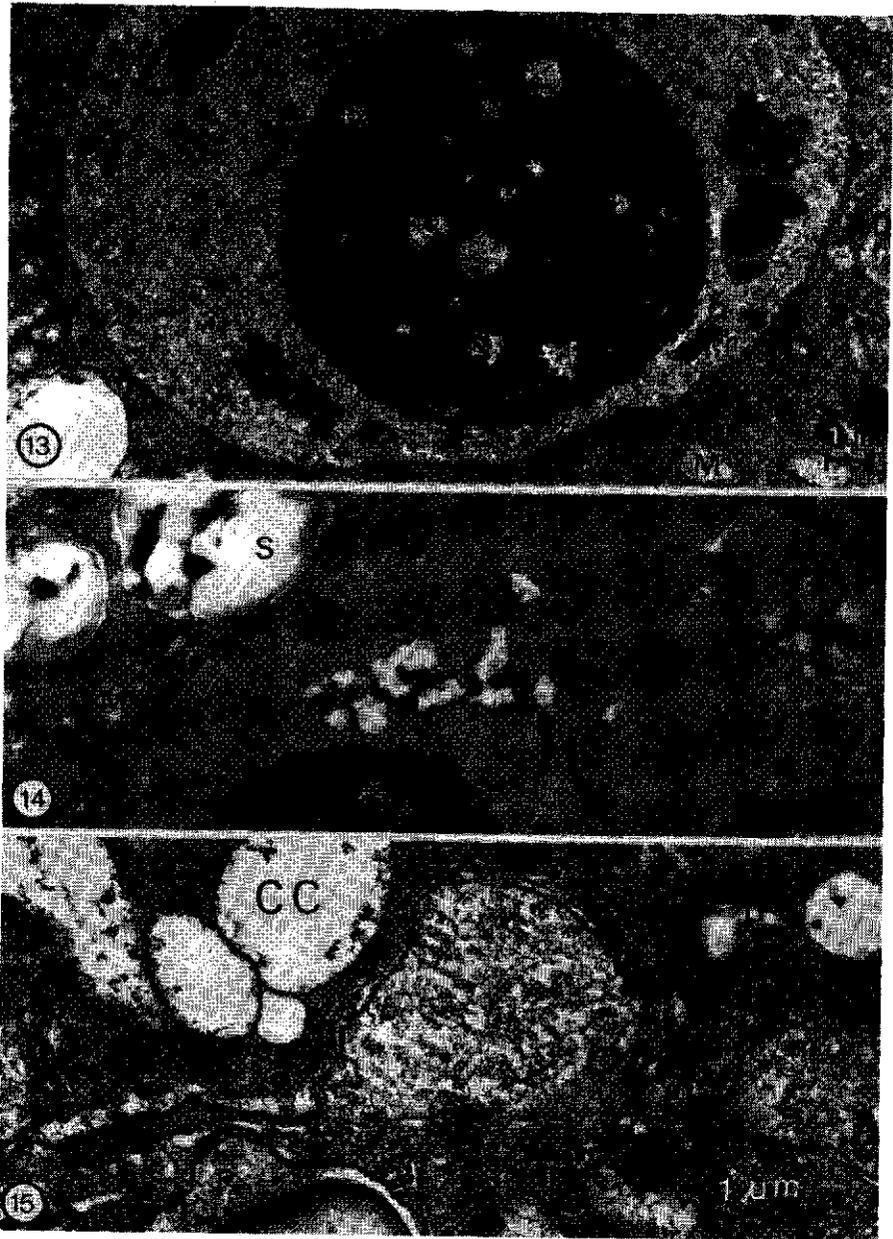


Fig 13. Egg cell nucleus with multi membrane-like structures. 6,000  $\times$ . Fig. 14. Egg cell nucleus with electron-transparent vacuole-like structures (arrow). 8,000  $\times$ . Fig. 15. Contact between chalazal part of egg cell (EC) and bordering central cell (CC). Dissolution of thin cell wall causes disintegration, while 'puffs' develop. 20,000  $\times$ . (D - dictyosome, M - mitochondrium, P - plastid, S - starch).

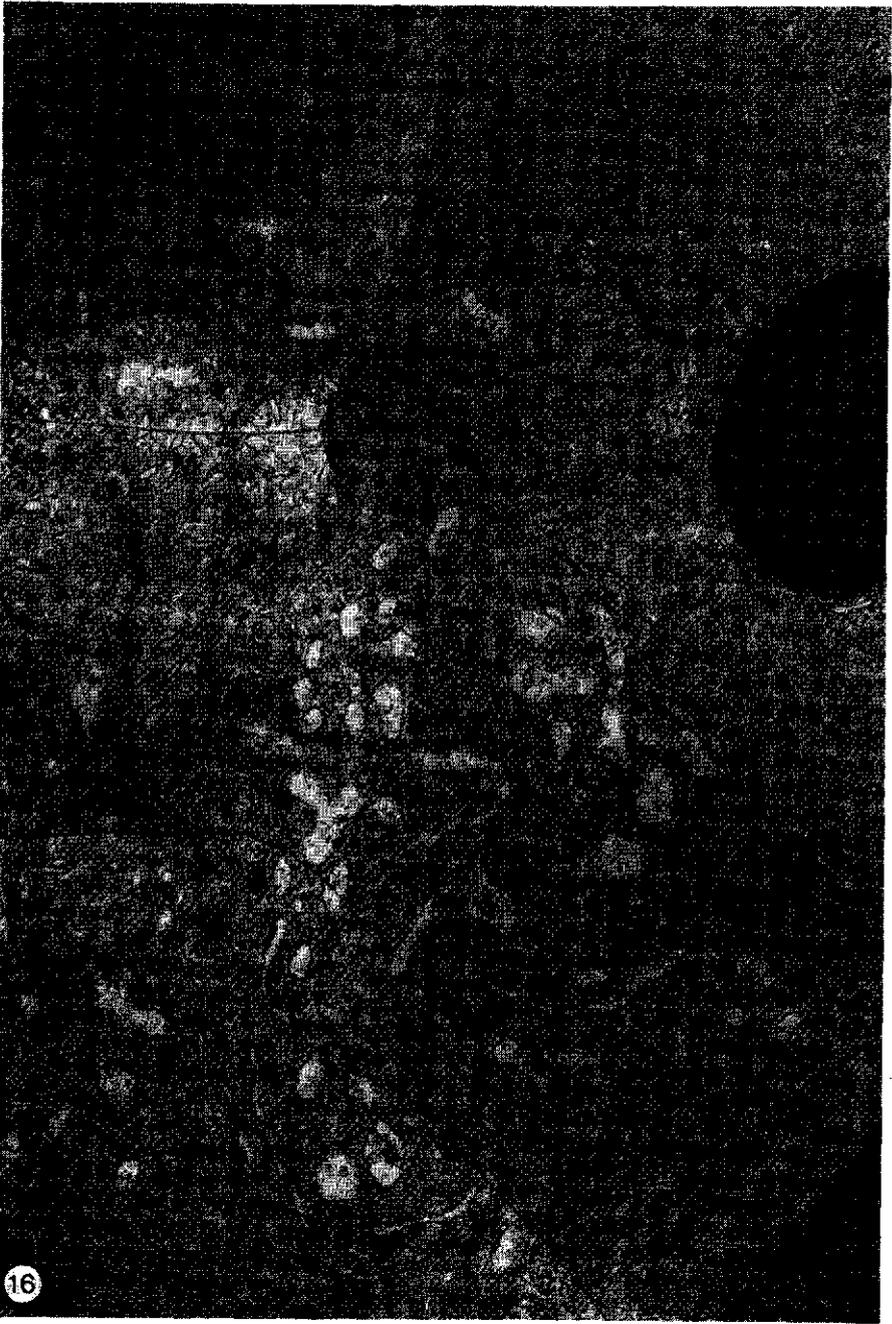


Fig. 16. Enlarged view of polar nuclei (N) with long nucleoplasm runners (NR) which fuse at several places. Nuclear membrane fusion is normal (arrow). 35,000 $\times$ . (ER - endoplasmic reticulum, M - mitochondrium).

# ULTRASTRUCTURE OF THE STIGMA AND STYLE OF SPINACH IN RELATION TO POLLEN GERMINATION AND POLLEN TUBE GROWTH

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## SUMMARY

Structural events in the stigma and style of spinach pistils have been investigated in detail with regard to pollination and pollen tube growth. The upper portion of the stigma contains only papillae, papillate cells with a wide central part and a narrow spiral tail part. The lower portion of the stigma also has cylindrical parenchymatous cells. In each stigma a central core is formed, initially by the tail parts, later together with the small inner parenchymatous cells. In the style these central cores join and fuse to one central core of transmitting tissue. At the base of the style the central core bends towards the location of the ovular micropyle. In the various portions of the stigma and style the intercellular spaces differ in sizes and also in electron-density of their matrix.

Pollen germinates within 10-20 minutes after pollination. Within 7-10 minutes the pollenkit fuses with the pellicle of the papillar wall. The pollen tube penetrates the pellicle and the eroded cuticle and grows through the outer cell wall layer downwards. Tube growth in the stigma and style is initially intercellular, but following tubes can follow various pathways. In all cases they come out in the space between the carpel and the outer integument. The interactions between pollen/pollen tube and stigma/stylar transmitting tissue are discussed.

## 1. INTRODUCTION

The flower biology of spinach was studied earlier with special interest being devoted to sex expression and breeding (ROSA 1925; SNEEP 1957). Less attention has been paid to the fundamental process of sexual reproduction and the ultrastructure of the pistil with regard to pollination and pollen tube growth. The stigmas are long with a papillate structure and belong to the "dry" stigma type (WOITTEZ & WILLEMSE 1979). Dry stigma surfaces were studied in a number of species of the Cruciferae (KROH 1964; KROH & MUNTING 1967; DICKINSON & LEWIS 1975) and Malvaceae (HESLOP-HARRISON et al. 1975). Such stigmas are dry only in a relative sense, since the surface is known to bear a hydrated proteinaceous pellicle (MATTSON et al. 1974). This pellicle lies over the cuticle of the stigma, the cuticle regulating the passage of water into the pellicle from the underlying protoplasts. The pistil of spinach has a single ovule. The solid style contains no vascular tissue and has specifically structured transmitting tissue (WILMS & VAN AELST 1978). This paper deals with the cellular organisation and ultrastructure of the stigma and style. Mature pistils have been studied before and after pollination. Morphological changes in relation to the penetrating pollen tubes are described and discussed.

## 2. MATERIALS AND METHODS

Unpollinated and hand-pollinated pistils of the spinach (*Spinacia oleracea* L.) cv. Prévital were used.

*Scanning electron microscopy*: both fresh and prepared material was studied. The prepared material was fixed in 3.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2 for 3 hrs at room temperature, rinsed in buffer and dehydrated in a graded ethanol-amylacetate series. The material was critical point dried and sputter-coated with gold.

*Transmitting electron microscopy*: the pistils were fixed in 3.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2 and 0.2 M saccharose for 3.5 hrs at room temperature. They were post-fixed in 2% OsO<sub>4</sub> in 0.2 M phosphate buffer pH 7.2 and in a graded ethanol-propylene oxide series and embedded in Epon. Sections were post-stained with uranylacetate and leadcitrate and observed in a Philips E.M. 300.

## 3. RESULTS

### 3.1. Stigma and style

The morphology of the pistil can be seen in *fig. 1*. Ontogenetically the pistil results from one carpel and consists of a round ovary with a single ovule and a short style, which usually forks into 4 stigmas. The style has a length of approximately 0.4 mm. The stigmas are 6–7 mm in length and are covered with papillae over their entire surfaces (*fig. 2*). The extension of the papillae marks the maturation. They then reach a length of 0.02 mm, which can increase a little if pollination is delayed.

The composition of the upper portion of the stigma can be seen in *figures 3* and *4*. In transverse sections of it (*fig. 3*) the cells gradually decrease in diameter from the outside to the centre. In none of the longitudinal sections (*fig. 4*) a complete lengthwise view of a cell can be obtained, since the cells have a spiral form. The upper portion of the stigma consists of only papilla cells with three parts. These are the papillar part extending into the air, a wide central part and a narrow tail part directed towards the centre of the stigma. The cell wall, which is in contact with the air, is relatively thick and covered with a distinct cuticle and pellicle (*figs. 5* and *10*). These walls reveal a layered structure. The other walls are thin and have some plasmodesmata. The cells are highly vacuolated.

The ultrastructure of the cytoplasm is different for the successive parts of the stigmatic cells. These differences concern the quantity rather than the quality of the constituents (*figs. 5, 6* and *7*). In the papillar part mitochondria, endoplasmic reticulum (ER), ribosomes and dictyosomes are more frequent, whereas plastids are concentrated in the tail part. Mitochondria are uniformly distributed over the papillar cytoplasm. Their shape is in sections round to oval, their average diameter being 0.7  $\mu\text{m}$ . They contain short cristae, which partly appear to be tubular.

There is no special arrangement of the cristae. At the tip of the papillar part

dictyosomes are concentrated. Each dictyosome consists of 4–5 flat cisternae, of which the middle ones are longest. The average length of the cisternae is 0.9  $\mu\text{m}$ . Associated with the dictyosomes are some small vesicles, about 0.1  $\mu\text{m}$  in diameter, located at the ends of the cisternae. Many ribosomes are present (*figs. 6 and 13*). Some are observed as free ribosomes, some are attached to the ER and others, especially near the plasma membrane, are seen as polyribosomes (*fig. 6*). Near the plasma membrane of the papillar part electron-dense material is accumulated (*fig. 6*). From the top to the base of the stigma the width of the central part of the papillae diminishes gradually from 20 to 10  $\mu\text{m}$ . The intercellular spaces between these parts are initially filled with an electron-dense matrix (*fig. 8A*), which gradually decreases downwards (*fig. 8B*). The nucleus usually located at the base of the papillar part is flattened and has a clear nucleolus (*fig. 5*). The plastids (*fig. 7*), mostly located in the tail part of the cells, are large and have well developed thylakoids with small grana. They contain an extensive electron-transparent peripheral reticulum. The stroma reveal some plastoglobuli. In the tail part of the cell both smooth and rough ER are present, together with free ribosomes and polysomes. The intercellular spaces are filled with an electron-dense substance (*fig. 8C*). The basal part of the stigma consists of two types of cells: the papilla and the parenchymatous cells, the latter being cylindrical in shape. The inner parenchymatous cells have few peripheral cytoplasm and large vacuoles. The lobed nucleus (*fig. 14B*) is located at the centre of the cell. The intercellular spaces are filled with an electron-dense substance (*fig. 14A*).

The transition from stigma to style is not sharp. The various cores of small inner parenchymatous cells of the stigmas gather and fuse. The result of this fusion is a central core of transmitting tissue in the style (*fig. 9*). The electron density of the intercellular substance of the central core increases towards the base of the style (*figs. 14B and 16*). At a number of places in the central core the cell walls are thicker and contain a "cuticle"-like structure. These electron-dense structures strongly resemble the epidermal cuticle (*figs. 9 and 17*). From stigma to style the epidermal cells lose their papillate extension (*fig. 9*). Between the epidermis and the central core of transmitting tissue there are several layers of large parenchymatous cells. They have less peripheral cytoplasm with an irregular lobed nucleus (*fig. 9*). At the base of the style the central core of transmitting tissue bends towards the location of the ovular micropyle. The inner surface of the carpel at the ovary is not covered by a cuticle (*fig. 19*).

### 3.2. Growth of the pollen tube in the stigma and style

The pollen lands on the surface of the stigma and sticks to the papillar part of a papilla (*fig. 2*). Within 10–20 minutes germination starts at a pore close to the place of contact. From scanning electron microscopic observation one gets the impression that the tube growth is over the surface towards the base of the papillar part and downwards to the style. The tube seems to follow the surface of the epidermal cell and seems to penetrate into the stigma at the place where the epidermal cells border each other (*fig. 2*). From transmission electron microscopic (TEM) observations it is obvious that within 7–10 minutes there has been a

fusion between the pollenkitt and the pellicle of the papillar wall while the cuticle is still intact (fig. 10). The pollen tube penetrates the pellicle and cuticle of the papillar wall and grows within and along the outer cell wall layer (figs. 11 and 12). The cuticle near the place of penetration has eroded. The ultrastructure of the papillar wall has changed. The outer papillar wall layer becomes more electron-dense near the penetrating pollen tube (figs. 12 and 13) and looks strongly swollen at other places (fig. 12). As the diameter of the tube increases the papillar part is crushed, the total circumference remaining about the same.

After the pollen tubes have entered the stigma, they continue their growth within the intercellular spaces which are filled with an electron-dense substance. The shape of the tubes changes from round to triangular (fig. 14). More to the base and in the centre of the stigma the intercellular spaces get larger and most tubes appear to be roundish. The intercellular growth of the pollen does not alter the ultrastructure of the adjacent parenchymatous cells (fig. 15).

Whereas the first arriving pollen tubes grow intercellularly in the style, the next can follow a different pathway (figs. 16 and 17). Somewhere in the top of the style some tubes grow through the cell walls and continue their growth between the cell wall and the plasma membrane. Near the "cuticle"-like structures the pollen tubes grow through the outer layer of the cell walls. When the pollen tubes grow inside the cell walls, it often happens that the surrounding intercellular spaces are not affected by pollen tubes at all (fig. 16). Sometimes the cytoplasm of the affected cell degenerates (fig. 18). The nucleus becomes more lobed and the nucleoplasm stains more intensively. The plastids and mitochondria deform till only the outer membranes are present with a homogeneous electron-dense content. Vacuolar membranes, ER and dictyosomes get disorganized, ribosomes are not distinguished anymore. The total cytoplasm becomes even much more electron-dense than the pollen tube cytoplasm (fig. 18).

The loosely organized cells below the base of the style facilitate the pollen tubes to grow further towards the micropyle through the space between the carpel and the outer integument (fig. 19).

#### 4. DISCUSSION

##### 4.1. Pollen-stigma interactions

Each female flower of spinach has long stigmas with many unicellular papillae. Such a great receptive surface enables the contact and attachment of the wind transported pollen. Pollen germination on these "dry" stigmas is rapid. Fusion of the pellicle with the pollenkitt, which is released from the exine of the pollen-grain, occurs almost immediately after contact. Soon after this fusion, pollen germination and tube growth start. However, spinach has no incompatibility system; recognition of the pollen by the stigma apparently needs only a very short time. It is likely that the pellicle is involved in recognition, as is proposed for *Populus* (KNOX et al. 1972) and for *Raphanus* (DICKINSON & LEWIS 1973b).

The cuticle and outer layer of the papillar wall are quickly affected, suggesting either that the enzymes involved are already present or that they are rapidly

synthesized. In certain Caryophyllaceae the entry of the tube in incompatible pollinations is prevented or greatly delayed when the proteinaceous pellicle of the stigma is removed enzymatically by pronase (HESLOP-HARRISON & HESLOP-HARRISON 1975). According to these authors the pellicle carries a factor, possibly a protein, which enhances the activity of a pollen-borne sporophytic or gametophytic originating cutinase. HEINEN & LINSKENS (1961) have established the presence of an active cutinase in developing pollen tubes of Cruciferae. In pollen of *Petunia hybrida*, however, no cutinase is present (LINSKENS & HEINEN 1962). This species represents the "wet" type stigma, which lacks a cutin layer at the stigma surface.

In spinach the pollen grain germinates and the tube penetrates the eroded cuticle and wall layer beneath the cuticle. The electron-dense substance, which is observed near the place of penetration, indicates a disturbance of the organisation of the outer wall layer. It might be a product of enzymatic degradation of the cell wall components. KROH (1964) suggested that for Cruciferae it is a product of degradation of pectins. According to Kroh the cellulose microfibrils of the stigma wall are displaced by the growth of the pollen tube, while the pectic substances are degraded by enzymes secreted by the tube. Secretion of pectinases by pollen tubes was also observed by PATON (1921) and DICKINSON & LEWIS (1973a).

In the Cruciferae the pollen tubes grow through the wall of the papilla to enter the style (CHRIST 1959). In spinach the pollen tube starts similarly but has to pass much more stigmatic tissue before entering the style. The pathway of the pollen tube through the stigmatic tissue is shown in *fig. 20*.

#### 4.2. Pollen tube growth in stigma and style

After the growth of the pollen tube within and along the papillar cell wall, it enters the more massive stigmatic tissue by penetrating the intercellular spaces. These intercellular spaces are small and the tubes appear to adapt themselves to the available space. From TEM observations it is clear that the characteristics of the content of the intercellular spaces gradually changes from the stigma to the base of the style. There is initially a decrease in electron-density, followed by a gradual increase. The electron-dense substance in the intercellular spaces closely resembles the intercellular substances as observed in the mature *Lycopersicum* styles (CRESTI et al. 1976). It is likely that in spinach the electron-dense substance also consists of pectins and proteins as is shown for *Lycopersicum*.

During the passage of the pollen tubes no changes in ultrastructure of the stigmatic cells are observed. Spinach pollen is trinucleate and both pollen and pollen tubes are rich in reserve material of all types: starch, lipid and proteins. This suggests the spinach pollen in a stage of development which needs few metabolic substrates to give germination and pollen tube growth. Where the pollen tubes grow through the intercellular spaces the electron-density of the intercellular substance has disappeared. Digestion of parts of this material by pollen tubes is probable. In cotton with empty intercellular spaces in the stigma and pollen tubes also rich of reserve material, the pollen tube growth in the

intercellular spaces causes some crushage but no visible ultrastructural changes in the adjacent cytoplasm (JENSEN & FISHER 1969).

The transition from the stigmatic tissue to the stylar tissue is gradual. The growth of the pollen tube in the stylar tissue is initially the same as in the stigma, which is through the intercellular substance. In all angiosperm plant species studied the pollen tubes always follow one specific pattern of growth in the style. In *Petunia* (VAN DER PLUYM & LINSKENS 1968; SASSEN 1974), *Lycopersicon* (CRESTI et al. 1976), *Nicotiana* (BELL & HICKS 1976), *Capsella*, *Lythrum* (SASSEN 1974) and *Diplotaxis* (KROH & MUNTING 1967) the tubes grow through the intercellular substance. In cotton (JENSEN & FISHER, 1969) the cells of the transmitting tissue have thickened cell walls and the pollen tubes penetrate and grow in layer 3 of these walls.

In spinach the first tubes continue their growth through the intercellular substance. The next tubes can follow various pathways, namely a. intercellular ones, b. through the outer part of the cell wall and c. after having passed the cell wall between the plasma membrane and the cell wall. The pathway of the pollen tubes through the style is determined by the structure of the cell walls and the morphology and distribution of the central core of the transmitting tissue in the style. In what way the tubes penetrate and pass the cell wall is not clear. It does not result from a changing anatomy of the stylar tissue or from a shortage of intercellular spaces. The growth through the outer layer of the cell wall is similar to the growth at the stigma papilla. This indicates that the tube has enzymes which can affect cell walls. The strands of cuticles in the basal parts of the style are not affected by passing pollen tubes. This indicates that pollen tubes at this stage do not produce cutinases.

When the tubes grow within the cell walls, the cytoplasm of the penetrated cells has to give way to expanding tubes, which results in a slow degeneration of the cytoplasm. The impression gained from the present study of the ultrastructure and composition of the tissues of the stigma and style in relation to pollen germination and tube growth in spinach is that these tissues function primarily as a guiding route for the growth of the tube and, possibly, as active agents in the control of tube development.

#### ACKNOWLEDGEMENTS

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## ABBREVIATIONS

CP = central part of the papilla cell	PG = pollen grain
CU = cuticle	PK = pollenkitt
D = dictyosome	PP = papillar part of the papilla cell
EP = epidermis	PT = pollen tube
II = inner integument	RER = rough endoplasmic reticulum
IS = intercellular space	S = starch
OI = outer integument	TP = tail part of the papilla cell
M = mitochondrium	TW = wall of the pollen tube
PD = plasmodesmata	W = cell wall
PE = pellicle	

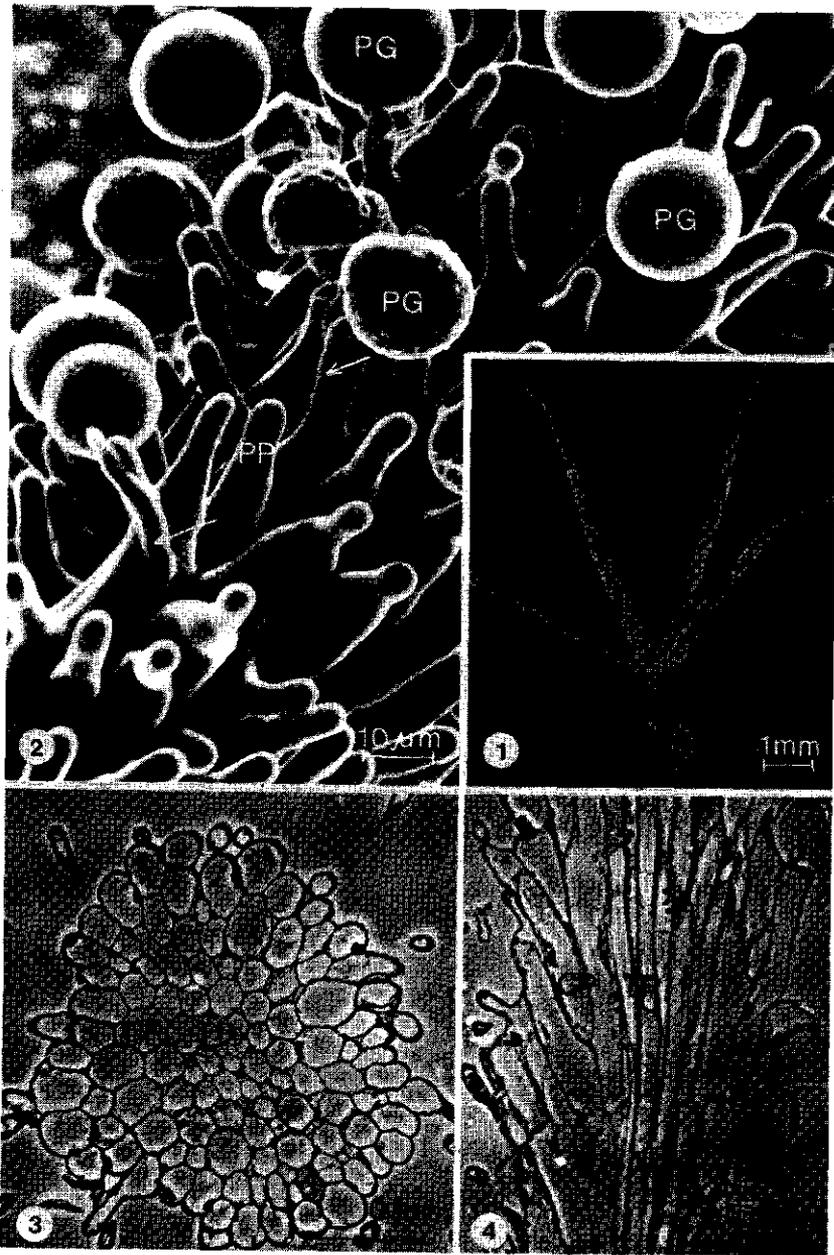


Fig. 1. The pistil of *Spinacia oleracea* L. showing ovary, style and four long stigmas.  $\times 6$ .  
 Fig. 2. Stigma papillae and germinating attached pollen, 4 hrs after pollination. Note the pollen tubes (arrow).  $\times 900$ .  
 Fig. 3. Cross section of the upper part of the stigma. Only papillae are present. GA-OsO<sub>4</sub> fixation.  $\times 160$ .  
 Fig. 4. Longitudinal section of the stigma shows the three parts of the papillae, the papillar parts, the wide central parts and the narrow tail parts. GA-OsO<sub>4</sub> fixation.  $\times 160$ .

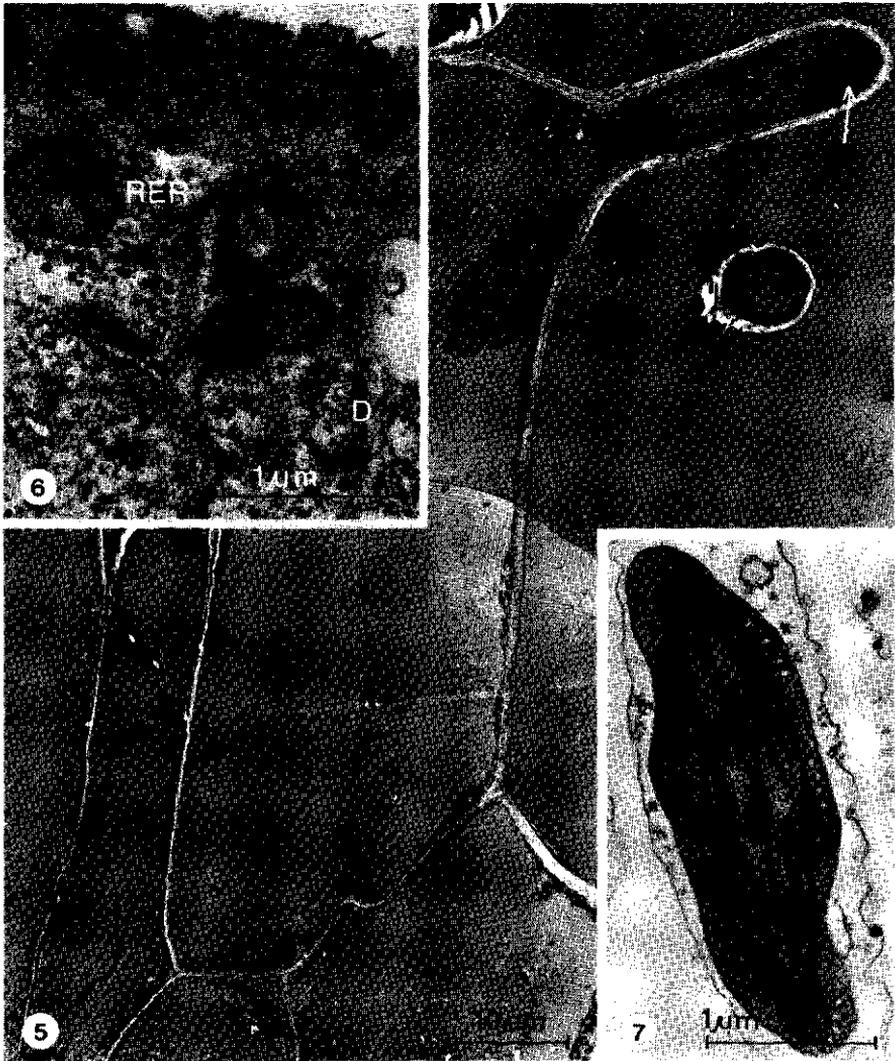


Fig. 5. Part of a papilla cell with the nucleus and peripheral cytoplasm. The tail part of another papilla cell shows some plastids. The cross section is of the upper papillar part. Note the presence of several cell wall layers at the papillar part.  $\text{Ga-OsO}_4$  fixation.  $\times 1600$ .

Fig. 6. Enlarged portion of the papillar cytoplasm in cross section showing RER cisterns and numerous free ribosomes. Most free ribosomes are present as polyribosomes. Note the presence (arrow) of electron-dense material near the plasma membrane.  $\text{Ga-OsO}_4$  fixation.  $\times 27,000$ .

Fig. 7. Plastid of the tail part of the papilla cell showing well developed thylakoids, plastoglobuli and abundant peripheral reticulum (arrow).  $\text{Ga-OsO}_4$  fixation.  $\times 22,000$ .

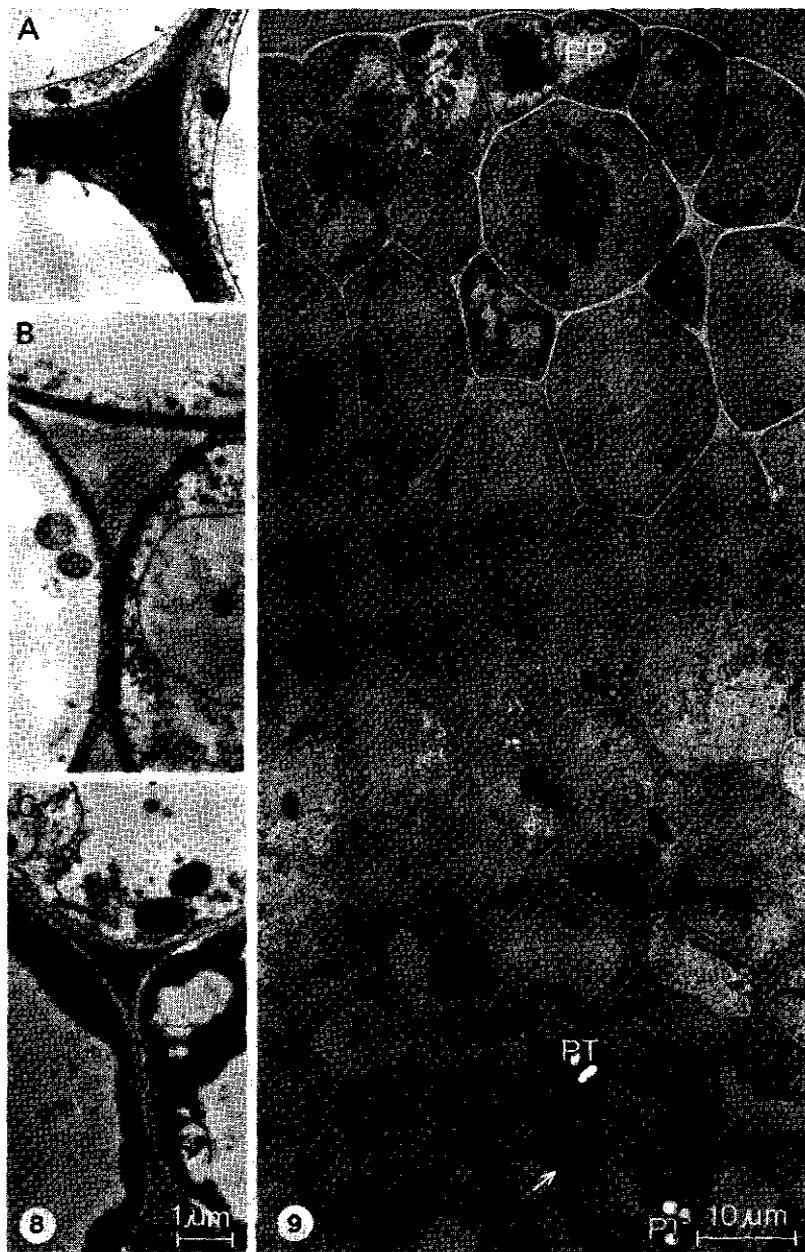


Fig. 8. Intercellular spaces in the stigma. A. Between the wide central parts of the upper papillae. B. Between the wide central part of the lower papillae. C. Between the narrow tail part of the papillae. GA-OsO<sub>4</sub> fixation.  $\times 7000$ .

Fig. 9. Cross section of the style with epidermis, large parenchymatous cells and the cells of the central core through which pollen tubes grow. Note the cuticle strands in the central core (arrow). GA-OsO<sub>4</sub> fixation.  $\times 1300$ .

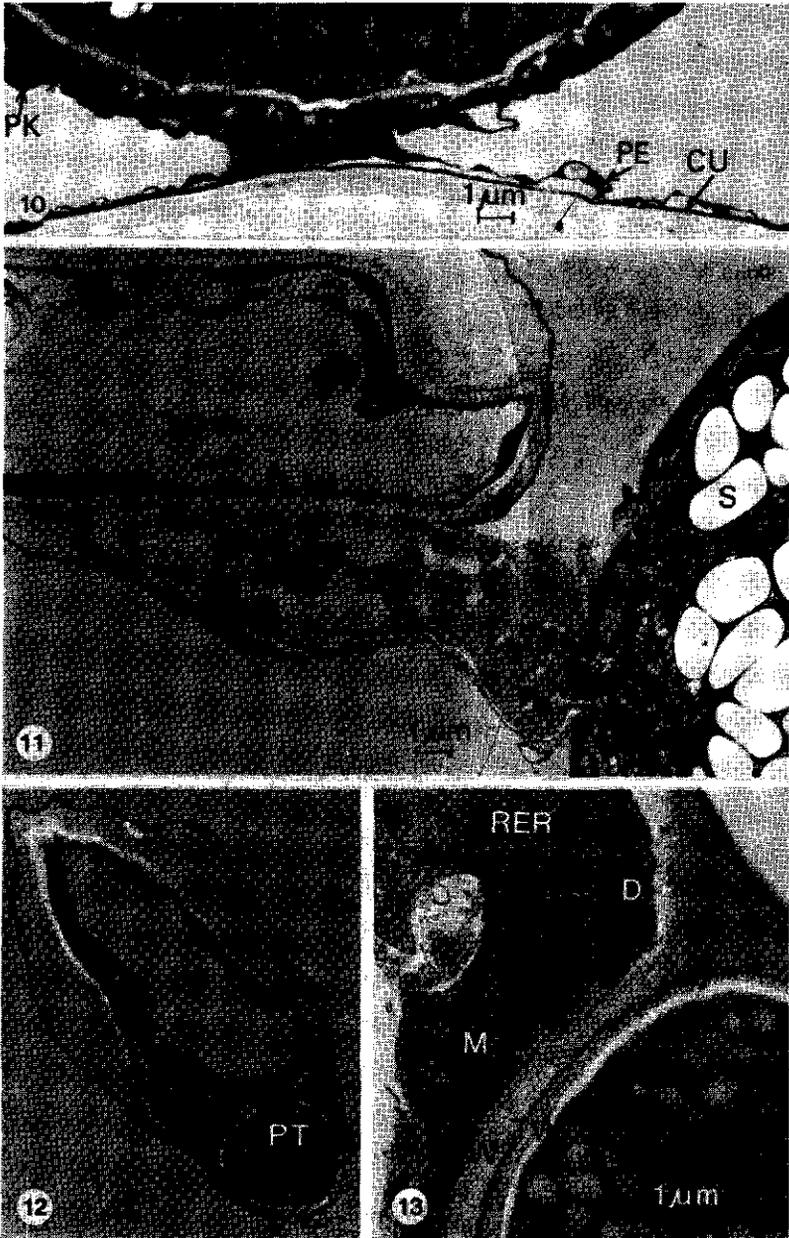


Fig. 10. Portion of the stigma papilla and attached pollen grain. Note the fusion of pellicle and pollenkitt. GA-OsO<sub>4</sub> fixation.  $\times 4500$ .

Fig. 11. Longitudinal section of a germinated pollen grain on a papilla showing the ingrowth of the tube in the outer cell wall layer. The molarity of the fixative has been adapted to the pollen tube cytoplasm and not to the papillar cytoplasm. GA-OsO<sub>4</sub> fixation.  $\times 4500$ .

Fig. 12. Cross section of the papillar tip and the tube tip. Note the electron-dense material near the tube tip in the outer cell wall layer of the papilla cell. GA-OsO<sub>4</sub> fixation.  $\times 4500$ .

Fig. 13. Enlarged portion of the papillar tip and the tube tip. GA-OsO<sub>4</sub> fixation.  $\times 18,000$ .

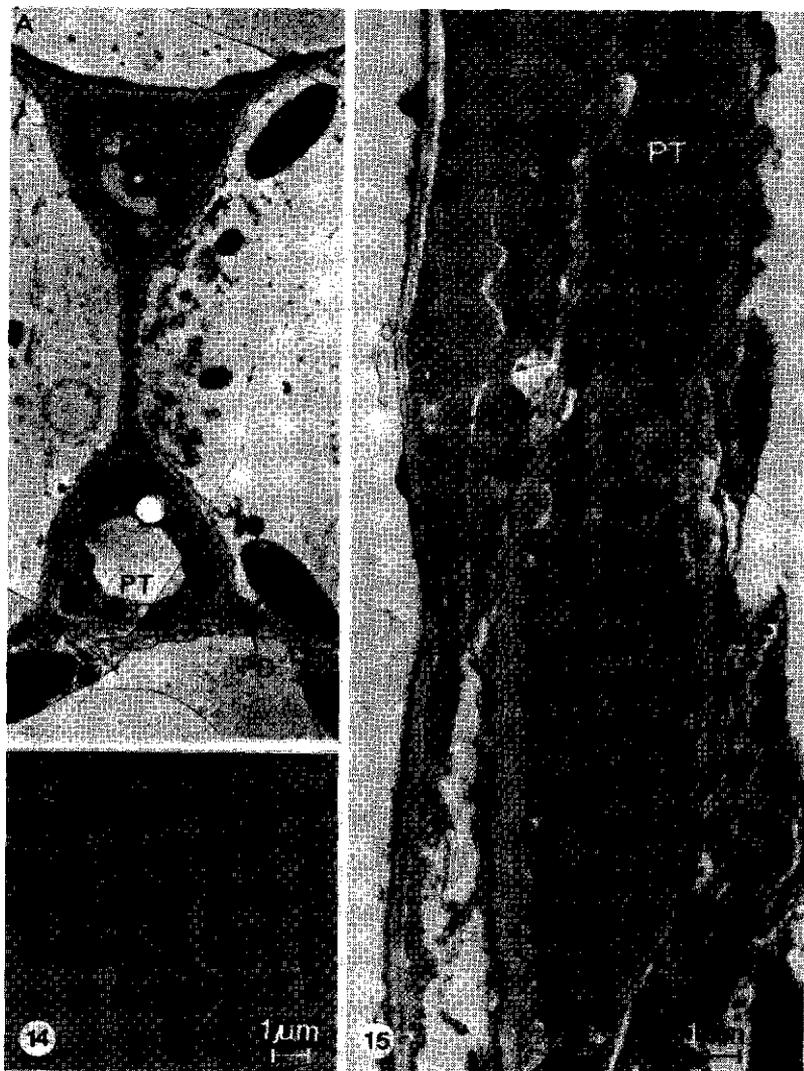


Fig. 14. Cross section of the central part of the stigma, 3 hrs after pollination. A. The pollen tubes grow through the intercellular spaces, which are filled with an electron-dense substance. B. Lobed nucleus of the inner parenchymatous cells. GA-OsO<sub>4</sub> fixation.  $\times 4500$ .

Fig. 15. Longitudinal section of the central part of the stigma with a pollen tube. Stigmatic cells near the tube are not changed by the immediate presence of the pollen tube. GA-OsO<sub>4</sub> fixation.  $\times 6000$ .

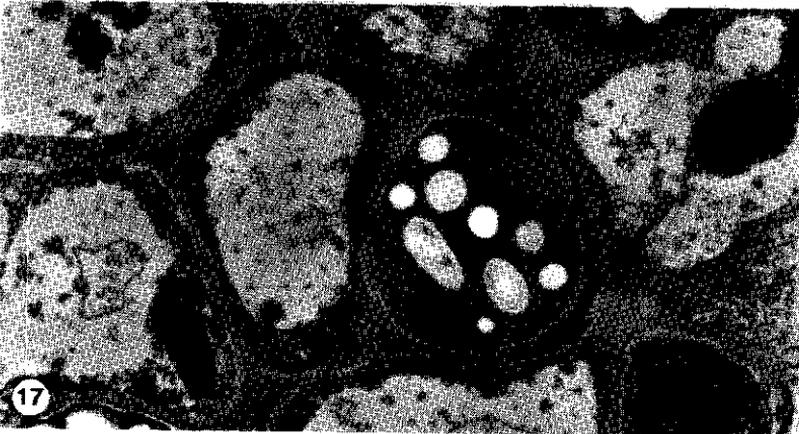
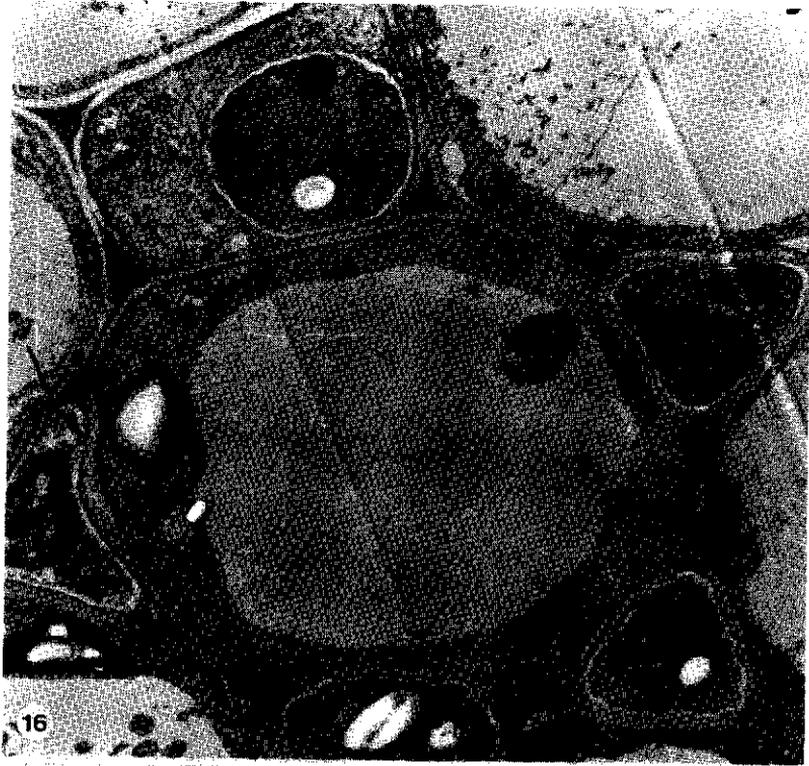


Fig. 16. Pollen tubes in the central core of the style, 5 hrs after pollination. Several tubes grow intercellular and some grow inside the cell wall. The triangular pollen tube (arrow) is cut near the tip of the tube. GA-OsO<sub>4</sub> fixation.  $\times 7000$ .

Fig. 17. Cross section of a pollen tube in the style, 4 $\frac{1}{2}$  hrs after pollination. This tube grows in the cell wall layer beneath the cuticle strands. GA-OsO<sub>4</sub>.  $\times 9000$ .

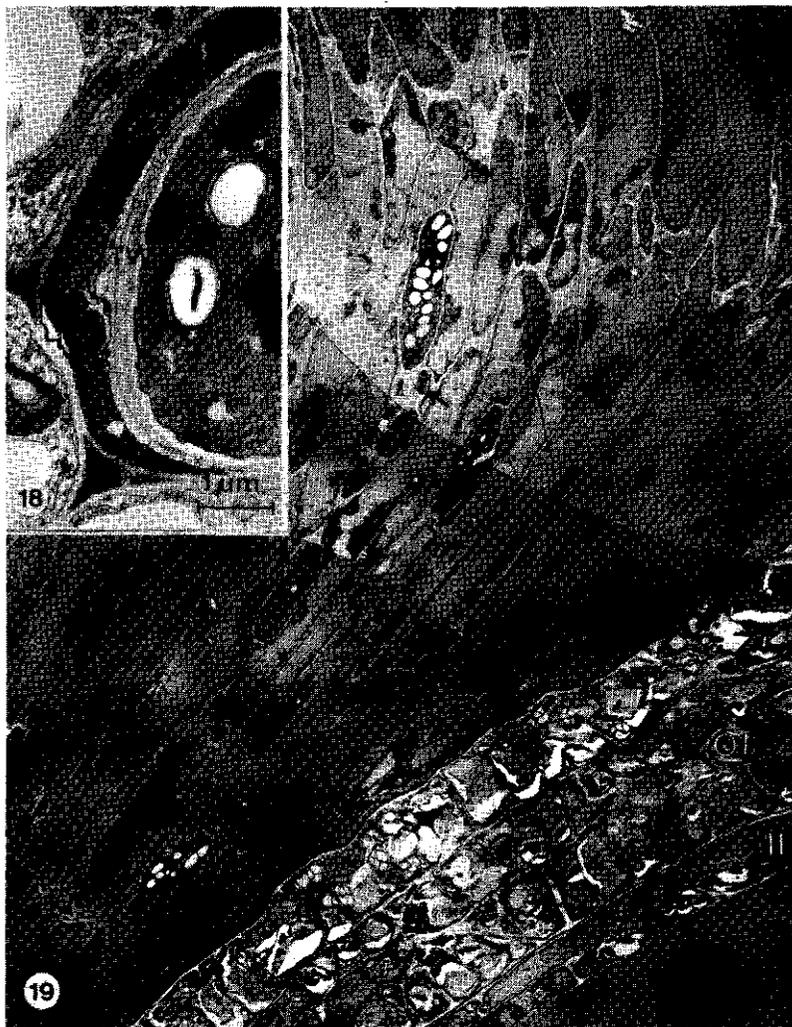


Fig. 18. Pollen tube inside the cell wall in the style, 5 hrs after pollination. The tube occupies most of the volume of the affected cell, resulting in a strong degeneration of the cytoplasm of that cell. GA-OsO<sub>4</sub> fixation.  $\times 10,000$ .

Fig. 19. Basal part of the stylar tissue, 3 hrs after pollination. The cells of the central core bend to one side. The pollen tubes follow this direction. Note the cuticle strands near the pollen tubes (arrow). GA-OsO<sub>4</sub> fixation.  $\times 900$ .

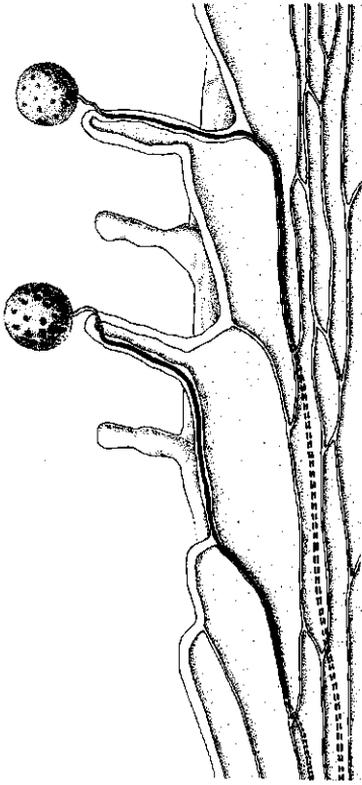


Fig. 20. Diagrammic summary of the pollen tube growth at and in the stigma.

# POLLEN TUBE PENETRATION AND FERTILIZATION IN SPINACH

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Key-words: *Spinacia oleracea*, pollen tube growth, ovule penetration, fertilization, ultrastructure

## SUMMARY

The nucellus of immature crassinucellate ovules of *Spinacia* cannot be penetrated by pollen tubes, which cluster together in and around the micropyle. After maturation of the ovule, pollination activates the embryo sac, i.e. the synergids, to excrete substances which dissolve the middle lamellae of the nucellus tissue in front of the synergids. Eventually the nucellar cuticle near the micropyle becomes interrupted. At that developmental stage pollen tubes can penetrate into the nucellus, initially growing intercellularly, and subsequently following various pathways. More than one tube can reach the embryo sac, but only one pollen tube penetrates the degenerated synergid via the filiform apparatus. Sperm cells, vegetative nucleus and tube cytoplasm with many amyloplasts are discharged into the degenerated synergid. The vegetative nucleus and the sperm cells are pressed to the chalazal part of the synergid, of which the plasma membrane is degenerated previously. The discharge and the fusion processes of the sperm cells with the egg cell and central cell (7-9 hrs after pollination) are described and discussed. Apparently no or very few organelles of the sperm cells enter the female gametes.

## 1. INTRODUCTION

The ovule of spinach, *Spinacia oleracea* L., is crassinucellate and the embryo sac develops inside a multi-layered nucellus tissue. During maturation the nucellus differentiates into various tissues (WILMS, 1980b). The cells of the nucellus near the micropyle become elongated with slightly thickened cell walls (micropylar cells), whereas those near the embryo sac are small with strongly thickened cell walls (transmitting cells). It is through this conductive tissue that pollen tubes have to grow before reaching the embryo sac.

Only few crassinucellate ovules have been studied. In cotton (JENSEN 1969) a column of nucellar cells extending from the embryo sac to the micropyle degenerates before the arrival of the pollen tube. The pollen tube grows between the radial walls of degenerated nucellar cells, crushing some of them but not entering them. In *Reseda lutea* the cell walls of the nucellar epidermis in the apical region become mucilaginous making a convenient passage for the penetration of the pollen tube (CHABRAN & YAKOVLEV 1974).

In this paper pollen tube growth into the ovule of *Spinacia* is described in terms of ultrastructure. It includes the tube growth to and into the embryo sac, the discharge of its contents and the process of double fertilization.

## 2. MATERIAL AND METHODS

Dissected ovules of *Spinacia oleracea* L., cv. Prévital, were fixed in glutaraldehyde and osmiumtetroxide at various stages before and after artificial pollination and embedded according to the GA-OsO<sub>4</sub> method described earlier (WILMS 1980a). To obtain good results the molarity of the solutions had to be adjusted. For fixation of the pollen tubes in the region from style to micropyle 0.2 M saccharose was added. Pollen tubes that had entered the nucellus were treated with solutions without saccharose.

Several stages of fertilization were studied using the re-embedding method described by WILMS (1980c).

The rate of pollen tube growth was estimated with UV-fluorescence microscopy (WILMS 1974). Pollination occurred in the middle of the stigma and the subsequent pollen tube growth was measured by estimating the position of the tip of the fastest growing pollen tube.

## 3. RESULTS

### 3.1. Ovule penetration

In spinach the first pollen tubes reach the micropyle in about 6 hrs after pollination (*diagram 1*). They mainly grow in the space between the carpel and the outer integument (*fig. 1a*). At the base of the style they occasionally penetrate the carpellary cells and grow downwards through these cells, i.e. between their plasma membranes and the cell walls (*fig. 1b*). Many pollen tubes penetrate the micropyle (*fig. 2*). No penetration into integuments was observed. The distance from micropyle to embryo sac is about 0.6 mm and the first tube can reach the embryo sac within little over an hour. Growth through the nucellus and penetration of the embryo sac usually takes up to three hours.

*Diagram 1* shows the growth of pollen tubes in mature and in immature pistils. This growth is measured by estimating the position of the tip of the fastest growing pollen tube. The velocity of the growth depends on the stage of pistil development. In immature pistils pollen tube growth is less fast than in mature pistils. However, the pollen tubes reach the micropyle in a shorter time because of the shorter pathway. No growth into the nucellus tissue was observed whereas the tubes continue their growth in the micropylar region. In both stages of pistil development the rate of pollen tube growth decreases from stigma to micropyle or to embryo sac.

The nucellus tissue of the immature pistil is covered with a thick continuous cuticle (*fig. 7a*). The pollen tubes are in close contact with the nucellus as well as with the integuments but do not penetrate the cuticle (*fig. 3*). In some cases local breakdown of the cuticle was observed after several hours of contact (*fig. 4*). At this place of contact there is an increased thickness of the inner cell wall layer of the pollen tube.

At maturity of the ovule a structural change of the middle lamellae in the

Mean Length „mature”

Mean Length „immature”

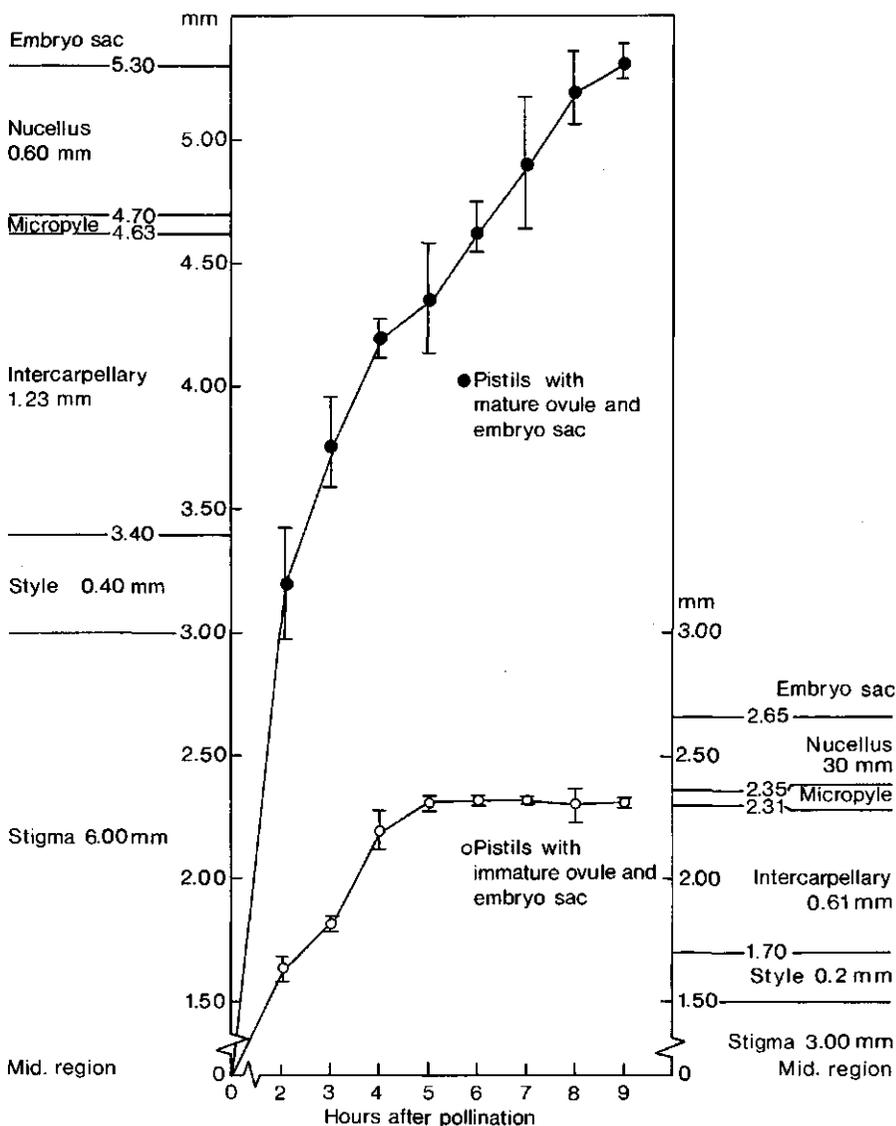


Diagram 1. Position of tip and length of fastest growing pollen tube in pistils of *Spinacia* at two stages of development. Points represent means of at least 12 measurements with standard error.

nucellus tissue between the embryo sac and the micropyle has occurred. After development of the nucellus and the embryo sac till maturity only the radial middle lamellae are changed (*fig. 6*), especially the middle lamellae that are parallel to the embryo sac-micropyle axis. Initially, the middle lamellae are homogeneously electron-dense (*fig. 5*). Later they become broader and irregular in density. This change begins near the synergids and extends to the micropyle. Near the micropyle the cuticle begins to change and to become thinner, and the outer cell wall layer of the nucellus cells in this region is affected as well (*fig. 7b*). Pollen tubes reaching this stage of ovule development directly penetrate the nucellus and continue to grow.

Initially the pollen tubes grow through the outer wall part (*fig. 8*), and then via the middle lamellar region. Basically this growth is intercellular (*figs. 12, 13*) but after the pollen tube reaches the thick-walled transmitting nucellar cells, they also can grow through the cell wall or between the wall and the plasma membrane. In the last two cases, especially near the embryo sac, the nucellus cells degenerate and the pollen tube occupies the gained space (*fig. 14*). A limited number of pollen tubes can grow into the nucellus. Pollen tubes growing in or near the micropyle, frequently branch (*fig. 8*). Sometimes two branches of one pollen tube penetrate the nucellus. Regularly one of them develops faster and the other stops growing shortly after its formation. In one case a pollen tube is observed with two branches which are oriented in opposite direction (*fig. 12*).

### 3.2. Structure of the pollen tube

The growing pollen tube can be divided in various zones, each with a specific cytoplasmic ultrastructure. Some characteristics are given that are necessary for a good understanding of the process of fertilization.

First there is the tip (*fig. 9*) showing many small electron-transparent vesicles at the outmost end, and vesicles, small mitochondria, dictyosomes and compact SER in the adjacent region. The following part shows a second type of vesicles in which some electron-dense material has accumulated (*fig. 10*). This density is identical to that of the main component of the outer part of the pollen tube wall. In this and the following parts longitudinally oriented strands of RER, polyosomes, small but well-developed mitochondria and small active dictyosomes are frequent. Some lipid droplets and sometimes plastids with starch grains can be seen (*fig. 10*).

The following region contains the vegetative nucleus and the two sperm cells. From their formation in the pollen grain till their discharge into the degenerated synergid the sperm cells of spinach are in close proximity of the vegetative nucleus. Both sperm cells are in intimate contact with each other (*fig. 10*). They are connected with each other by plasmodesmata and small cytoplasmic canals and they are both surrounded by a common plasma membrane of the vegetative cell. In the region behind the vegetative nucleus the tube is crowded with plastids containing single large starch grains (*fig. 12*).

### 3.3. Embryo sac penetration

After its passage through the transmitting nucellar tissue, the pollen tube reaches the filiform apparatus (FA), grows through it and enters into the cytoplasm of the degenerated synergid (*fig. 15*). Then pollen tube growth stops, and the tube opens by a terminal pore and discharges its contents. The opening apparently does not result from simple bursting. Since the pollen tube cytoplasm contains organelles as plastids with a specific structure, the presence of pollen tube cytoplasm within the synergid can be traced easily. Plastids with single, large starch grains and the cytoplasmic vesicles retain their ultrastructure for some time (*fig. 16*). The other organelles of the ejected tube cytoplasm very soon lose their regular ultrastructure, in which they behave the same as the organelles of the degenerated synergid. The thin, long vegetative nucleus also degenerates but remains recognisable. Frequently this nucleus is observed in the most chalazal part of the degenerated synergid (*fig. 15*), partly pressed between the plasma membranes of egg cell and central cell (*fig. 18*). After discharge a plug is formed in the pollen tube at the nucellus near the FA.

In one case two sperm nuclei are observed within one cell in the chalazal region of the degenerated synergid (*fig. 17*). This cell is surrounded by a single plasma membrane which at one side is in contact with the plasma membrane of the egg cell and at the other side in the vicinity of that of the central cell. Between the two nuclei some vacuoles are located. The number of organelles in the cell is lower than in the sperm cells of the grain and tube.

Nuclear fusion in the egg cell is completed very rapidly (*fig. 18*), whereas nuclear fusion in the central cell is rather slow (*figs. 18, 19, 20*).

Although the ovary contains only one ovule, many pollen tubes penetrate the ovary. Sometimes additional pollen tubes arrive at the embryo sac after the latter has already been penetrated successfully by a pollen tube. In that case the additional pollen tube does not penetrate the embryo sac. We observed in one case that an additional tube has passed the FA and has arrived at the persistent synergid, but penetration of this synergid did not occur. Instead of opening, an increased thickness of the inner cell wall layer of the tube tip is observed (*fig. 21*).

Shortly after completion of nuclear fusion the first nuclear division in the endosperm begins. The metaphase is reached 7-8 hrs after fertilization whereas the following interphase is reached 16-17 hrs after fertilization (*fig. 22*).

Around these nuclei firstly the number of mitochondria and plastids increases strongly, and next starch grains in the plastids are formed (*fig. 22*). The zygote remains undivided during this period and structural changes of the cytoplasm are minimal.

The persistent synergid degenerates soon after fertilization similarly to the earlier degenerated synergid. Its organelles lose their internal structure and an accumulation of lipid between the membranes occurs. Lipid granules accumulate and aggregate in the cytoplasm. At 16 hrs after fertilization only the FA and some degenerated cytoplasm remnants are present.

## 4. DISCUSSION

In *Spinacia*, pistils with young ovules – ovules with immature embryo sacs – can be pollinated and pollen germination and pollen tube growth occurs. The pollen tube reaches and enters the micropyle in about 5 hrs, but there is no ingrowth in the nucellus tissue. The tubes cluster together in the micropyle, around the nucellus and between the integuments. The conductive nucellar tissue consists in this stage of development of a compact tissue, while adjacent to the micropyle the cell walls are cutinized and a cuticle is present. Pollen tubes can partly affect this cuticle after hours, indicating that they do not produce enough cutinase. The thickening of the inner cell wall layer corresponds with the behaviour of pollen tubes after an incompatibility reaction (CRESTI et al. 1979).

The rate of pollen tube growth is different in immature and mature pistils. In the mature pistil the tube growth in a particular tissue is always faster than in the corresponding tissue of the young pistil. Only when tubes grow outside a tissue, in this case intercarpellary, the growth rate is about the same. Obviously the stage of development of the female tissues influences the rate of tube growth.

In mature ovules pollen tubes reach the micropyle after about six hrs and after another 1–3 hrs the embryo sac is reached. Ultrastructural observations indicate a gradual dissolution of the longitudinal middle lamellae of the nucellus tissue extending from the embryo sac to the micropylar region. Ultimately the nucellar cuticle is attached and disintegrated.

The pollen tube growth in *Spinacia* is initially intercellular. In the transmitting nucellar cells the pollen tubes can grow in different ways, which are the same as in the stylar tissue (WILMS 1980a). This is a) intercellularly, b) through the cell wall and c) between the cell wall and the plasma membrane (diagram 2). The relative thin strand of transmitting tissue is surrounded by the compact lateral nucellar tissue, the cells of which have thin walls and small electron-dense intercellular spaces.

There is no relation between ways of growing and of reaching the embryo sac. In each way they can reach the FA. The pollen tube penetration into the cytoplasm of the degenerated synergid through the FA is similar to that in other species: *Torenia* (VAN DER PLUYM 1964), *Gossypium* (JENSEN 1965a, b, JENSEN & FISHER 1967), *Capsella* (SCHULZ & JENSEN 1968a, b), *Zea* (DIBOLL 1968), *Epidendrum* (COCUCCI & JENSEN 1969a, b), *Petunia* (VAN WENT 1970), *Hordeum* (CASS & JENSEN 1970), *Linum* (VAZART 1971), *Quercus* (MOGENSEN 1972), *Proboscidea* (MOGENSEN 1978a, b), *Nicotiana* (MOGENSEN & SUTHAR 1979) and *Helianthus* (NEWCOMB 1973).

In spinach the pollen tube extends into the degenerated synergid for a short distance and the tube cytoplasm, containing the two sperm cells and the vegetative nucleus, is discharged by a terminal pore. After its discharge the pollen tube forms a plug near the penetration place of the FA. This plug consists of the same material as the inner layer of the pollen tube, which is callosic in nature (WILMS 1974). Plugging of a discharged pollen tube has been reported earlier for cotton, in which a plug was formed near the opening pore (JENSEN & FISHER

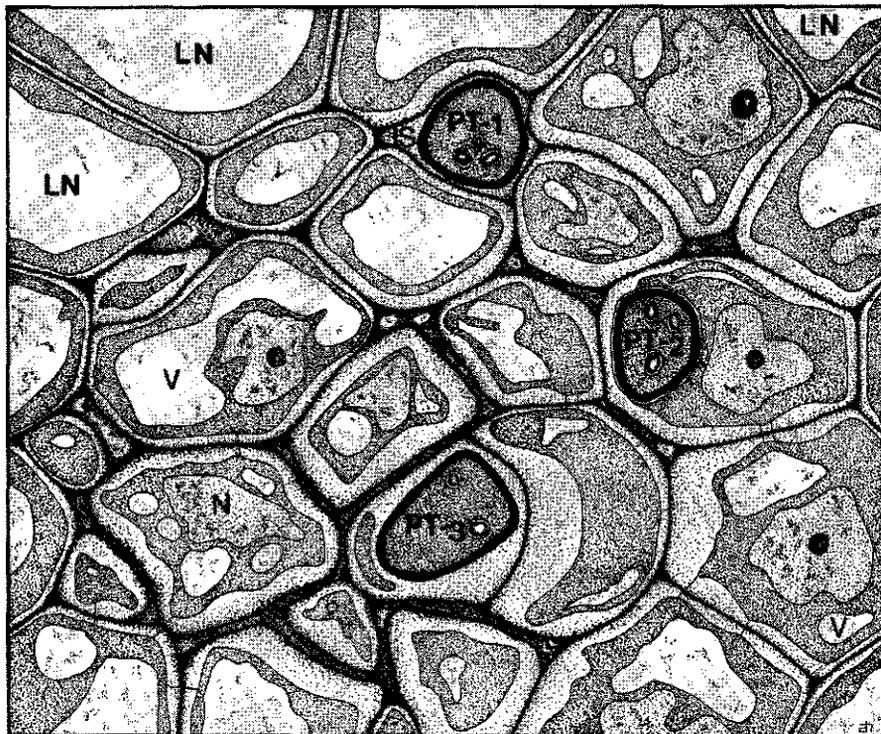


Diagram 2. Pollen tube growth in transmitting nucellus tissue of *Spinacia*. Pollen tube PT-1 grows intercellular, PT-2 between cell wall and plasma membrane and PT-3 in the cell wall. Lateral nucellus cells (LN) with characteristic small electron-dense intercellular spaces enclose transmitting nucellus cells (IS - intercellular space, N - nucleus, V - vacuole).

1968). Both species, cotton and spinach, have multilayered crassinucellate ovules.

In tenuinucellate and thin-layered crassinucellate ovules the micropyle is in contact or almost in contact with the egg apparatus of the embryo sac. In these types of ovules the integuments probably function as a closing gate after the discharge of a pollen tube, to prevent the penetration of additional tubes. In multi-layered crassinucellate ovules, however, the transmitting nucellus tissue is normally penetrated by more tubes. Obviously the integuments do not prevent effectively the penetration of additional pollen tubes.

Additional pollen tubes which reach the persistent synergid, show a thickening of the inner cell wall layer at their tip. Since they do not branch or grow sideways it is concluded that their growth ceases. Thickening of the end wall and cessation of growth is similar to what occurs in incompatible pollen tubes. This indicates that incompatibility reactions are involved in *Spinacia* too.

In one case a small cell containing two nuclei and surrounded by one plasma membrane was observed within the penetrated synergid. It was concluded from

the cytoplasmic constitution that it was a sperm cell, containing both sperm nuclei. Especially, since no sperm cells were found inside the pollen tube and both central cell and egg cell were not fertilized. It is possible that one sperm cell containing two nuclei is a fixation artefact or an aberrant situation. When not, the situation can be explained with the following hypothesis.

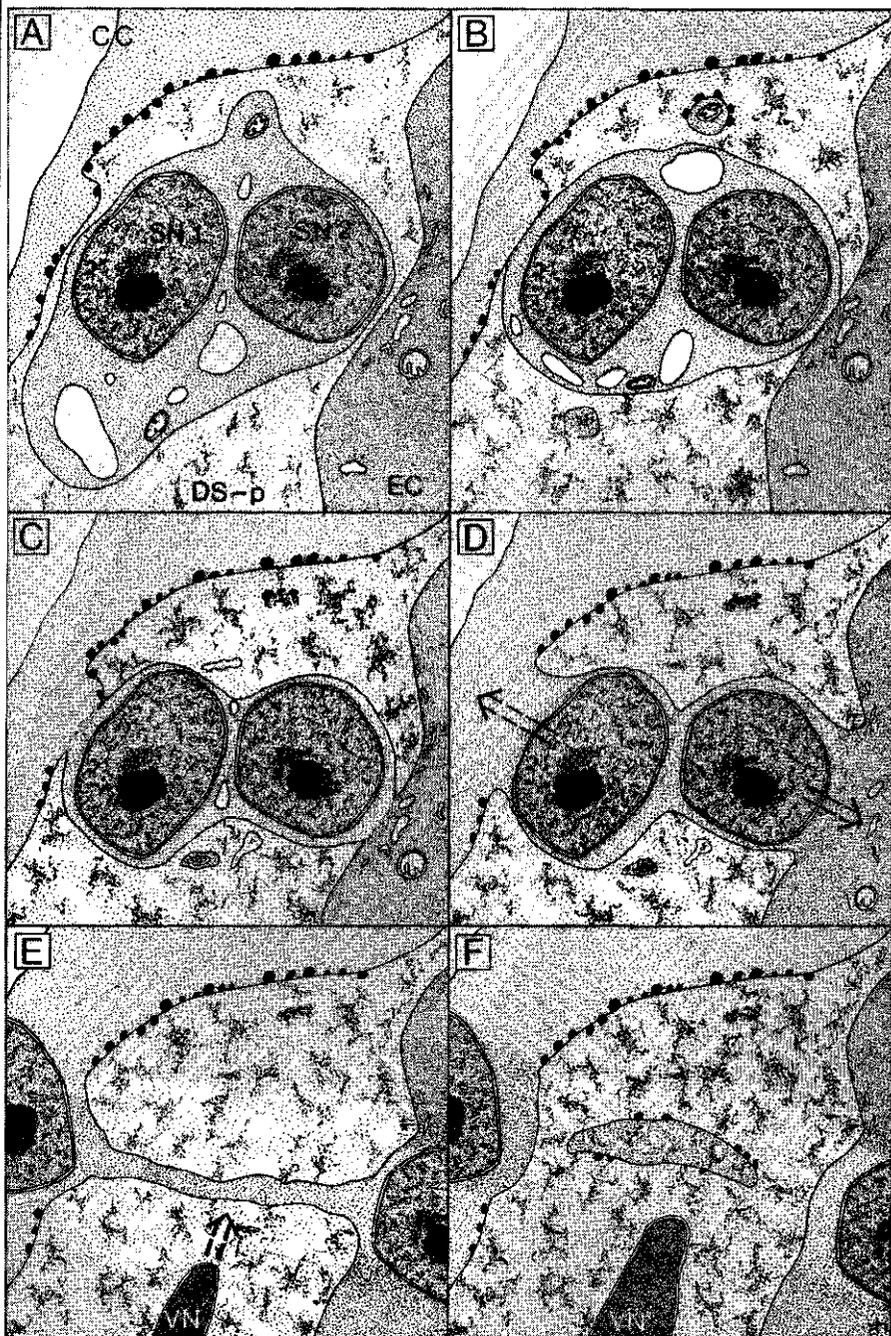
In the growing pollen tube sperm cells are always in close proximity with each other and near the elongated vegetative nucleus. In pollen tubes which grow in the styler tissue sperm cells frequently are seen connected with each other by small plasma canals whereas the plasma membrane of the vegetative cell is enclosing both sperm cells simultaneously (*fig. 11*). After the discharge into the synergid the plasma membrane of the vegetative cell degenerates and disappears. The cytoplasmic canals between the two sperm cells become larger, resulting in one sperm cell containing both nuclei and some cytoplasm. *Diagram 3* represents the proposed process. A gradual extension of cytoplasm parts occurs (*diagram 3A-D*). This results in two sperm nuclei with very little cytoplasm and held within one plasma membrane.

Since the plasma membrane of the degenerated synergid has disappeared previously, the membrane of the sperm cell can come into direct contact with the plasma membrane of the egg cell, followed by fusion of both membranes. One sperm nucleus then enters the egg cell cytoplasm (*diagram 3B-E*). Identical membrane fusion occurs between the plasma membranes of the sperm cell and the central cell and the second sperm nucleus enters the central cell cytoplasm (*diagram 3B-E*). Subsequently the plasma membranes are closed again (*diagram 3F*). The membranes of the fusing sperm cells can be incorporated in the membrane of the egg cell and central cell respectively. The fusion of the nuclei is similar to what has been described for *Gossypium* (JENSEN 1964) and for *Petunia* (VAN WENT 1970). First the nuclear outer membranes fuse and become continuous in several places, followed immediately by the fusion of the inner membranes. The nuclear bridges formed by this way enlarge and coalesce.

The fusion of the nuclei in the egg cell is completed more rapidly than in the central cell. The fusion of the sperm nucleus with the polar nuclei triggers the completion of fusion of the two polar nuclei.

In *Plumbago* organelles of the sperm cells are involved in the fusion process (RUSSEL 1980). After cell fusion a few male plastids and mitochondria are observed inside the fertilized egg cell and central cell. The data from spinach indicate that the sperm nuclei are devoid of cytoplasm before they merge with the cytoplasm of the egg and central cells.

**Diagram 3.** Fusion process of a sperm cell containing two sperm nuclei with egg cell and central cell. A. In chalazal part of degenerated synergid one plasma membrane surrounds two sperm nuclei and some cytoplasm. B. Cytoplasm extrusion and fusion of plasma membranes. C - D - E. Transport of  $S_1$ -nucleus into central cell and  $S_2$ -nucleus into egg cell. F. Closing plasma membranes exclude remnants of sperm cytoplasm.



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The author is much indebted to Prof. Dr. J. L. van Went for his stimulating interest and discussions and to Prof. Dr. M. T. M. Willemse for useful suggestions and discussions. The author thanks Mr. A. B. Haasdijk for making the drawings, Mr. W. van Ooyen for the photography and Mr. J. S. de Block for correction of the English text.

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## ABBREVIATIONS IN THE FIGURES

CA = carpel, CC = central cell, CU = cuticle, CW = cell wall, DS-p = degenerated synergid with discharged pollen tube contents, EC = egg cell, EN = endosperm, ES = embryo sac, FA = filiform apparatus, II = inner integument, L = lipid, M = mitochondrion, MC = micropylar nucellus cells, N = nucleus, NUC = nucellus, OI = outer integument, P = plastid, PM = plasma membrane, PN = polar nuclei, PS = persistent synergid, PT = pollen tube, RER = rough endoplasmatic reticulum, S = starch SC = sperm cell, SER = smooth endoplasmatic reticulum, SN = sperm nucleus, TC = transmitting nucellus cells VE = vesicle, VN = vegetative nucleus.

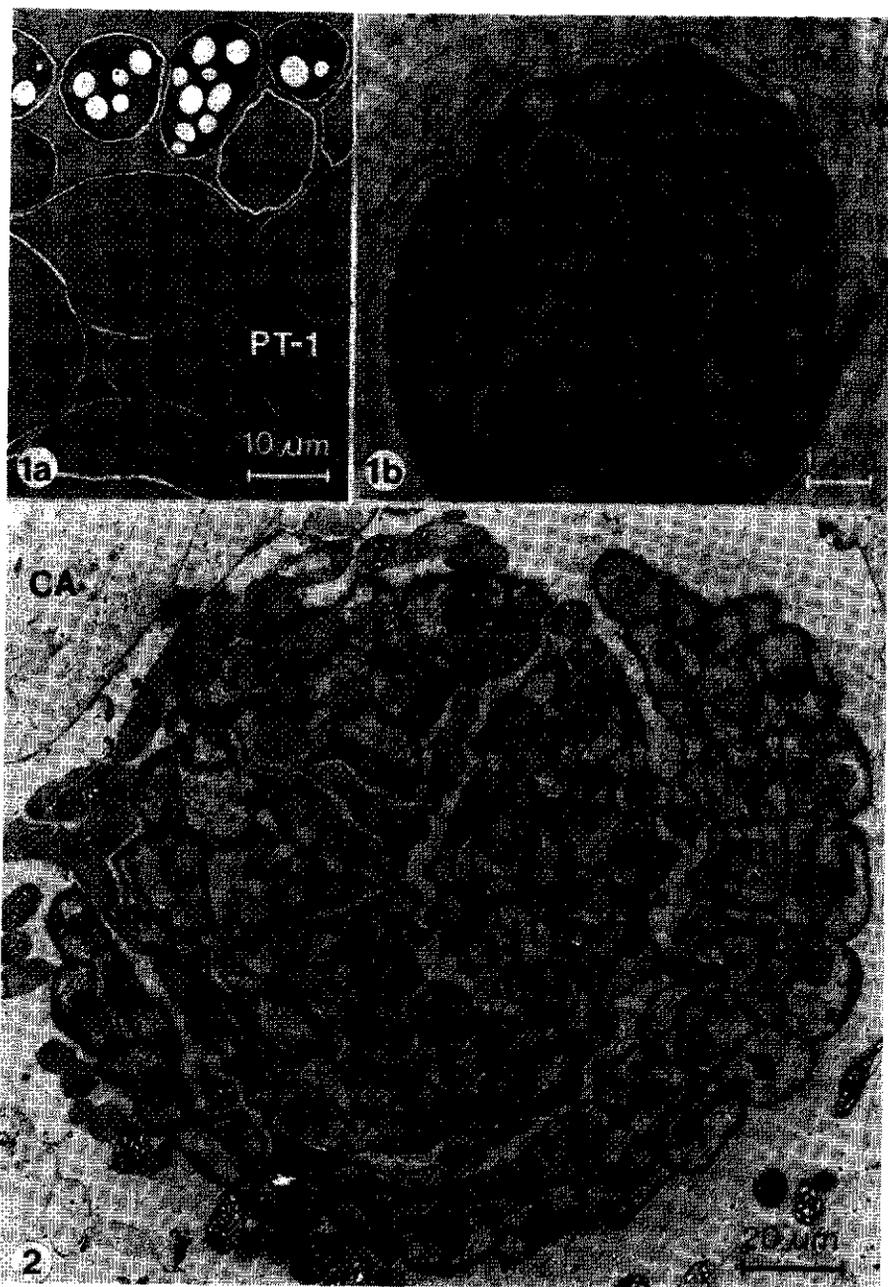


Fig. 1a. Pollen tube growth between carpel and outer integument. One, PT-1, grows inside a cell wall. 1100 $\times$ . Fig. 1b. Enlarged view of PT-1 growing between plasma membrane and cell wall. 9000 $\times$ . Fig. 2. Transverse section of the micropylar region, 6 hrs after pollination. 650 $\times$ .

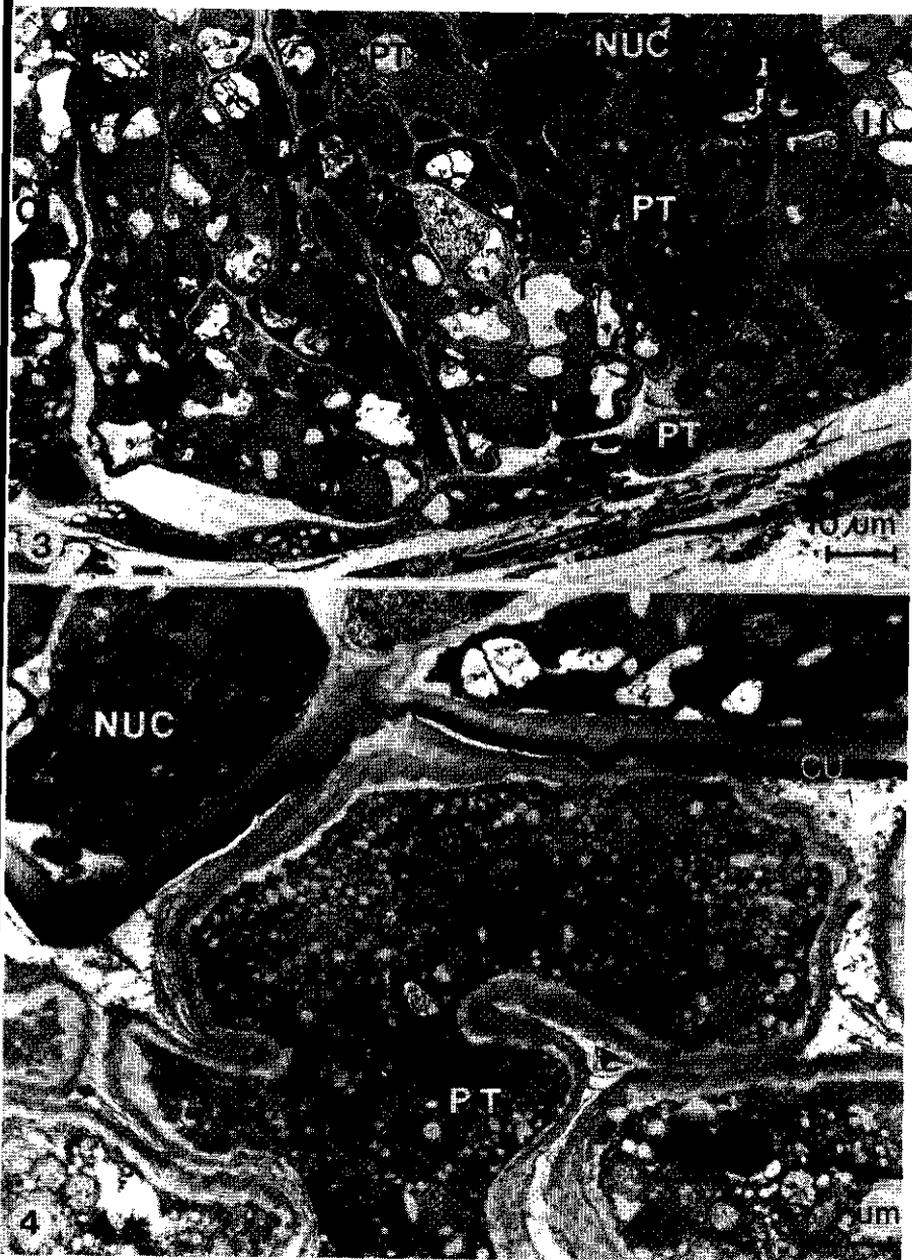


Fig. 3. Pollen tube growth in micropyle of immature ovule, 9 hrs after pollination. 900 $\times$ . Fig. 4. Enlarged view of *fig. 3*; pollen tube is unable to penetrate the cuticle of nucellus tissue. 5500 $\times$ .

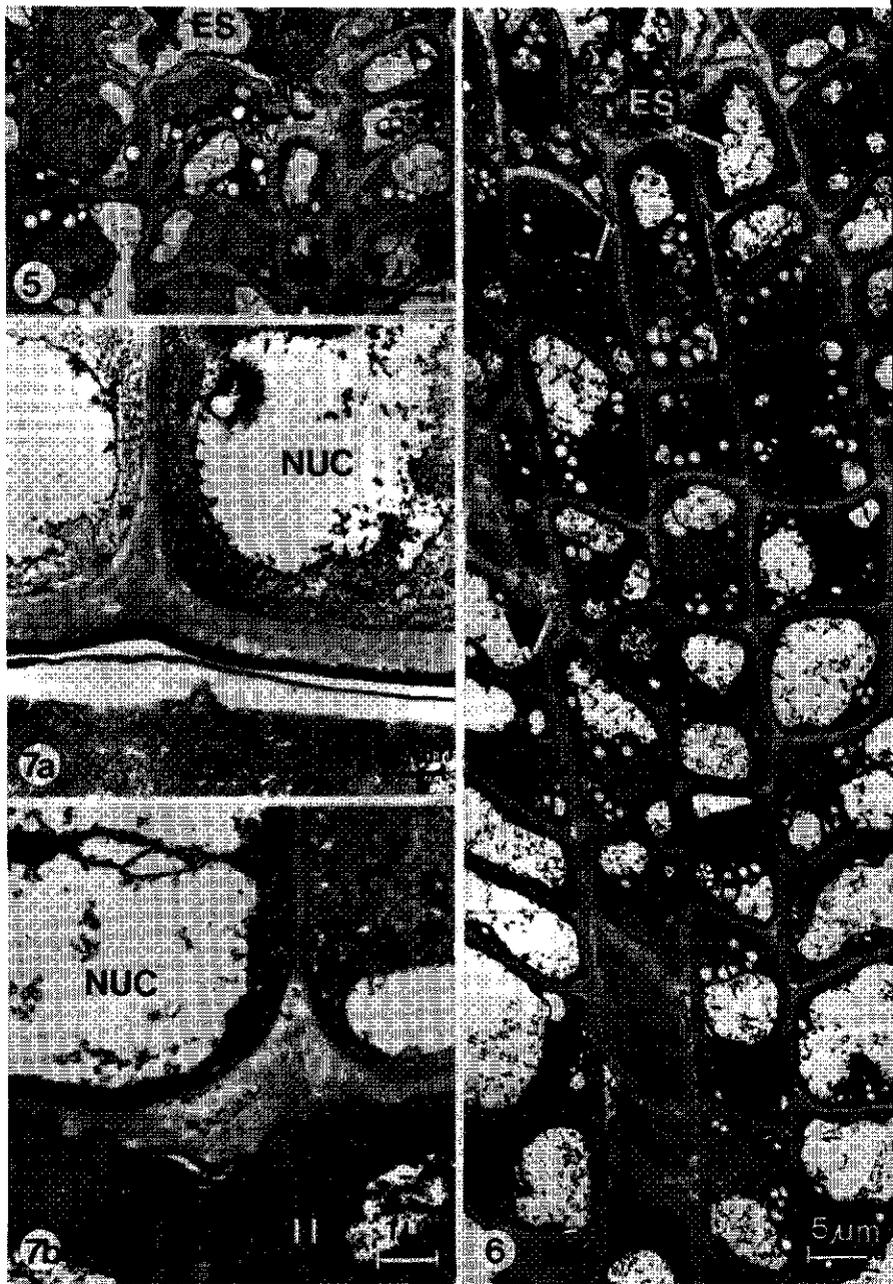


Fig. 5. Young thin-walled nucellar cells against the developing embryo sac. 2000 $\times$ . Fig. 6. Longitudinal section of transmitting nucellar cells near mature embryo sac. Middle lamellae, which are parallel to embryo sac - micropyle axis, are affected (arrows). 2000 $\times$ . Fig. 7. Nucellar cell wall parts at micropyle during two stages of development: a. immature and b. mature. 8000 $\times$ .

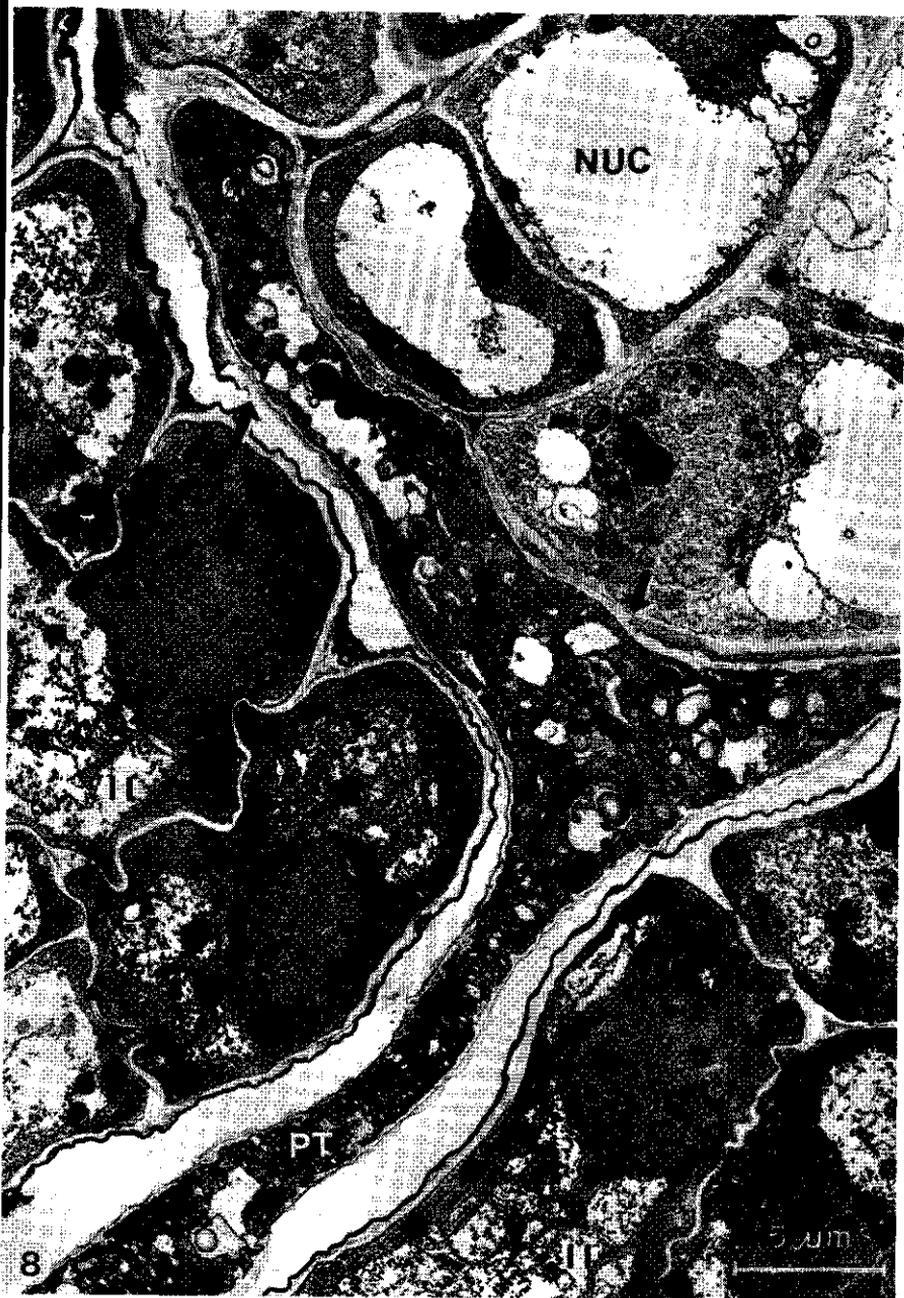


Fig. 8. Longitudinal section through micropylar region. Branching pollen tube penetrates cuticle of nucellus tissue between arrows, whereas middle lamellae region has degenerated (asterisk). 4000  $\times$ .

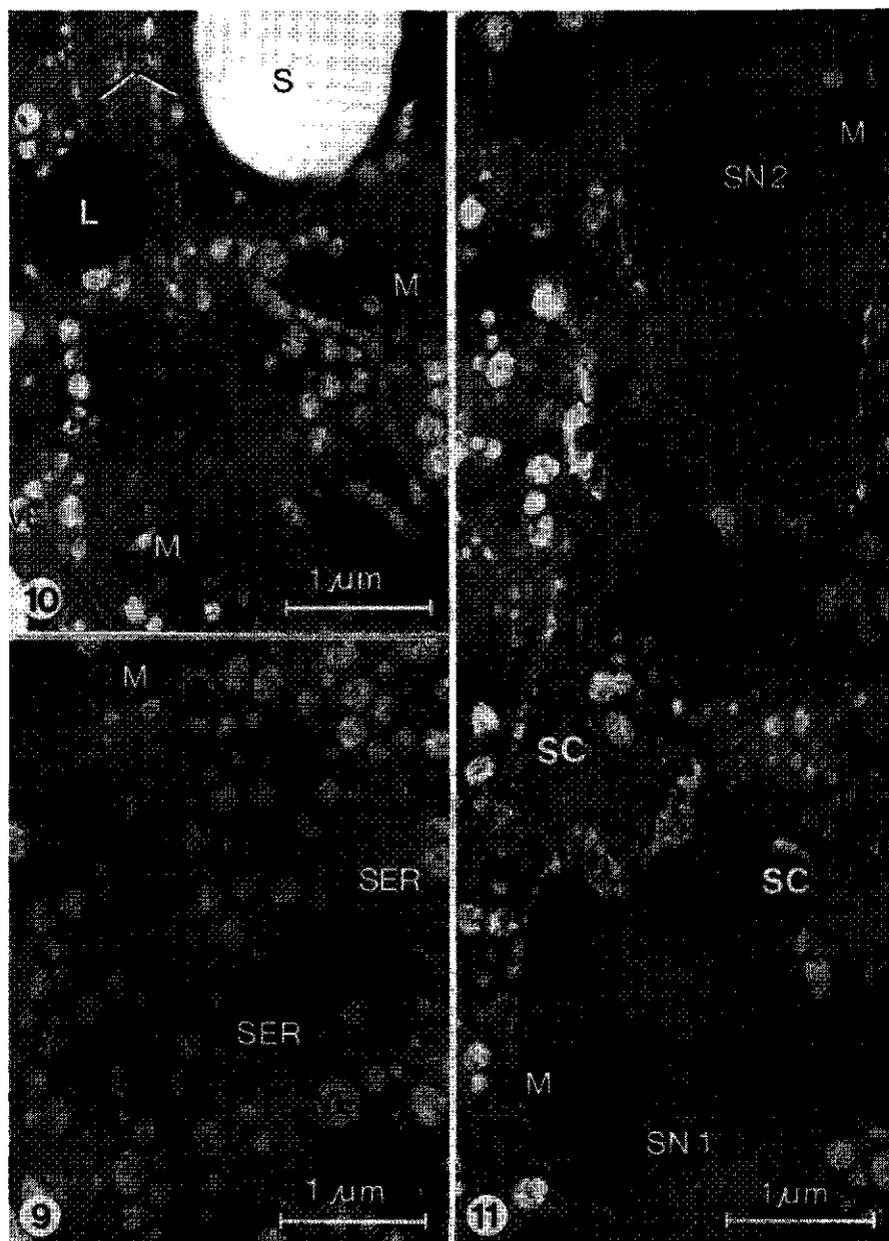


Fig. 9-11. Longitudinal sections of pollen tubes at different places. 20,000  $\times$ . Fig. 9. Tip cytoplasm with vesicles, SER and some mitochondria. Fig. 10. Tube region between tip and sperm cells, containing mitochondria, vesicles with some electron-dense content, an amyloplast and longitudinally oriented strands RER. Fig. 11. Tube region with sperm cells. Connections between sperm cells occur via small cytoplasmic canals (arrow). One common membrane surrounds both sperm cells.

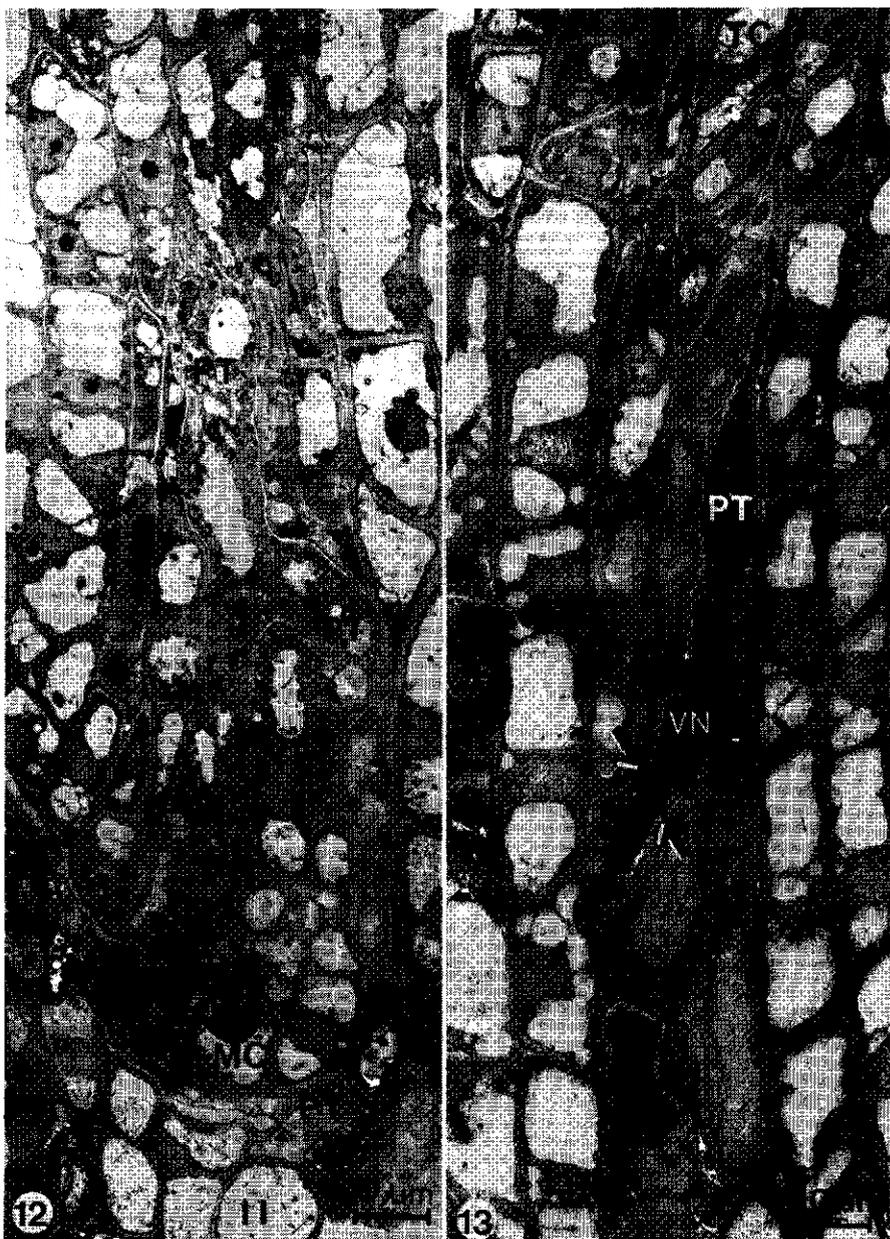


Fig. 12. Branched pollen tube penetrates micropylar nucellus cells and fuses 1000 $\times$ . Fig. 13. Pollen tube growth near transmitting nucellus cells. Cell wall is crossed between arrows. 1000 $\times$ .

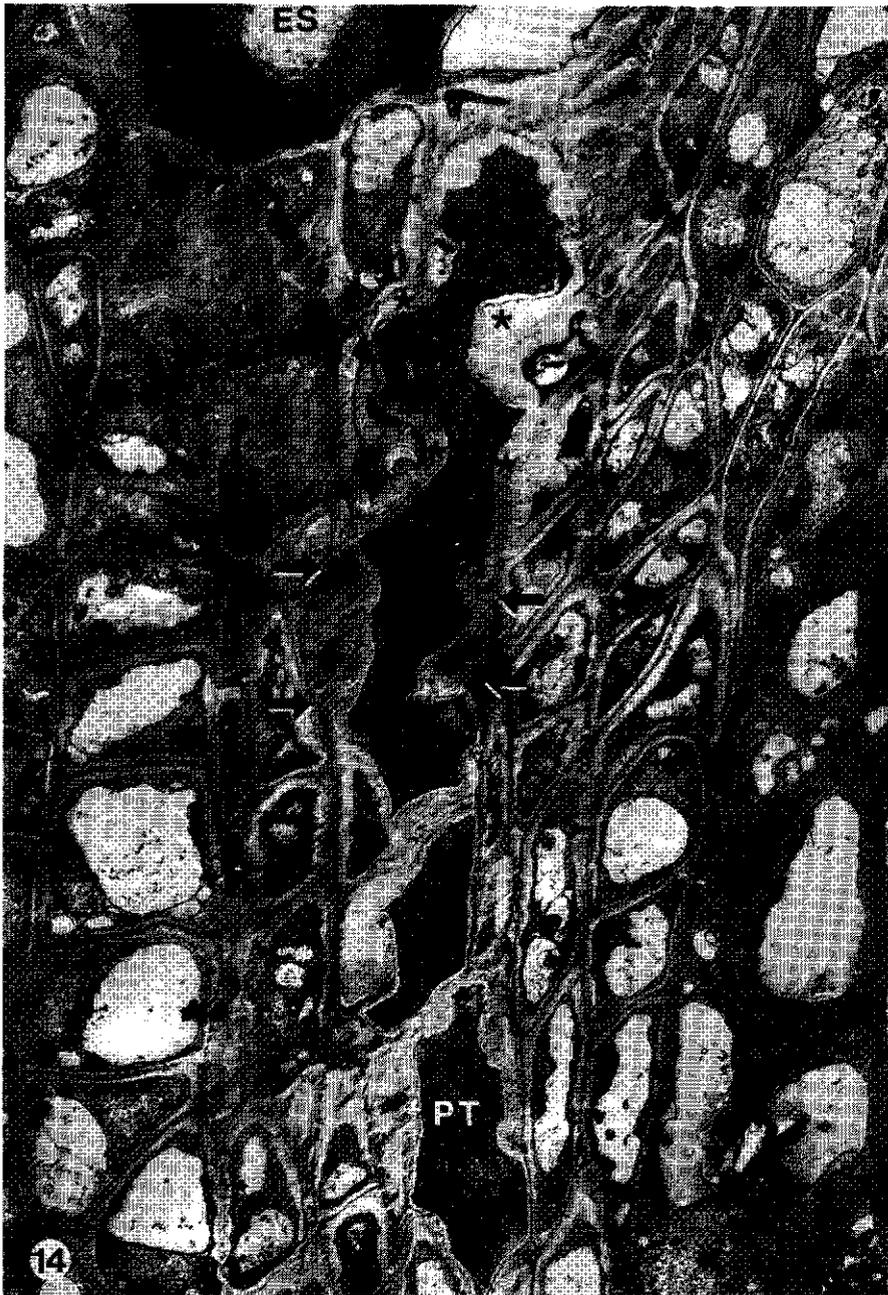


Fig. 14. Pollen tube growth through transmitting nucellus cells. Cell walls are crossed at the arrows; growth is partly through thick cell walls at asterisk. 1700  $\times$ .



Fig. 15. Pollen tube penetration through the filiform apparatus; discharge in degenerated synergid and merging of two cytoplasms, 4000  $\times$ .

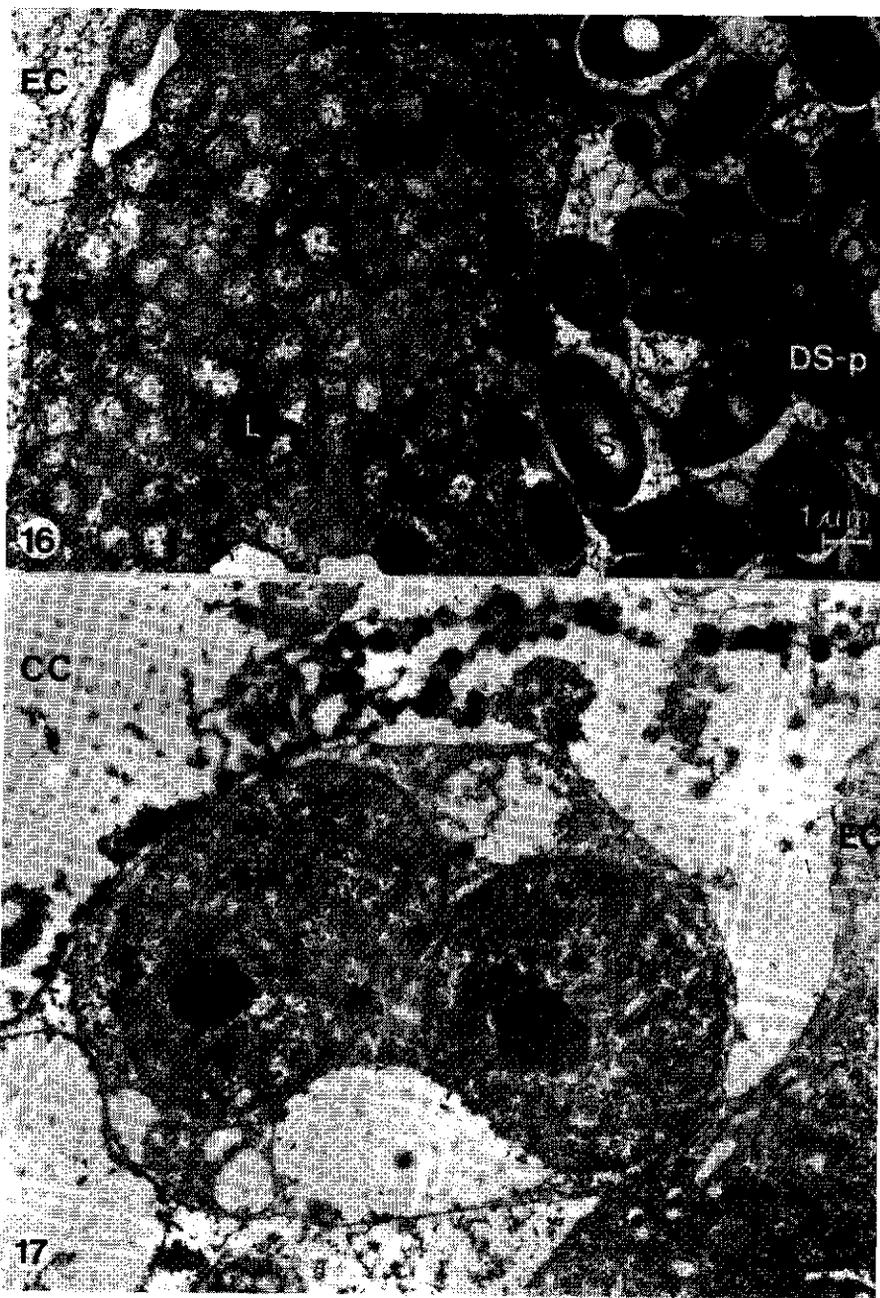


Fig. 16. Enlarged view of egg cell and degenerated synergid after discharging of pollen tube contents. 6500 $\times$ . Fig. 17. Single membraned, two nucleate sperm cell in chalazal part of degenerated synergid with two sperm nuclei. 14,000 $\times$ .

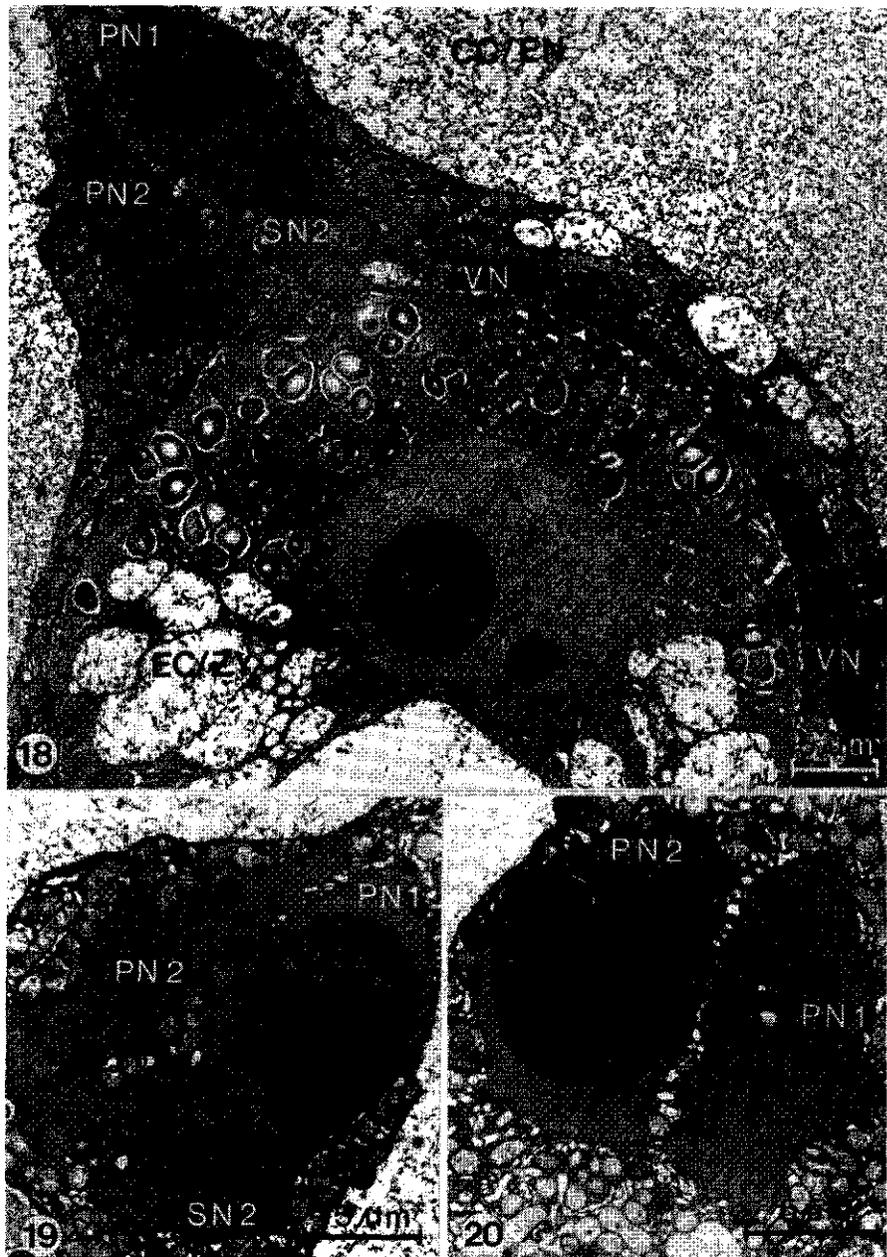


Fig. 18. Fertilization in sensu stricto; fusion of sperm nuclei with egg and polar nuclei. Vegetative nucleus is pierced between egg and central cells. 2400 $\times$ . Fig. 19, 20. Enlarged parts from serial sections of fusing sperm nucleus with polar nuclei. 3500 $\times$ .



Fig. 21. After successful pollen tube discharge in degenerated synergid a second pollen tube arrives at persistent synergid without penetration.  $1900\times$ . Fig. 22. Part of two-nucleate endosperm with starch accumulation in plastids.  $5500\times$ .

### 3. PISTIL DEVELOPMENT, POLLINATION AND FERTILIZATION AS PROCESS

The knowledge on reproduction has increased with the ultrastructural methods and histochemical techniques. Many descriptions are the result of studies of mature structures alone without a sufficient knowledge of the complete sequence of development. Although the ontogenetic approach has almost become a matter of routine in investigations of pollen wall structure, type of embryo sac, endosperm and embryo, etc., hardly any attention has been paid to the total process of development from initiation, differentiation of the generative organs and fertilization in *sensu lato*, to zygote and endosperm formation. The analysis of ontogenetic processes must form a more important part of embryological research than was hitherto the case, the mode of ovule initiation and the differentiation of the nucellus being rather neglected subjects. An ultrastructural investigation of the embryology of a given species must include the integral study of the complete development sequence from the first stages of ovule initiation to complete seed maturity. Also practical breeders and growers should be more interested in the time sequence of flower development and its flowering, the duration of the development of the various cells and tissues of the nucellus and embryo sac, the duration of the pollen tube growth and its pathway and the possible obstructions.

The process of reproduction mainly is an interaction between the development and differentiation of the pistil parts and the ingrowth of the pollen tube. The ovules, i.e. the nucelli and the embryo sacs, need a certain stage of development before pollen tubes are able to penetrate. The embryo sac has to reach the fertilizable stage. In the species studied this means a functional egg cell and central cell, and a varying stage in development of the synergids and the antipodals.

The synergid, which will receive the pollen tube, starts degeneration at variable times. In some plant species this synergid degenerates already before the pollen tube entry, in other species synergid degeneration starts after pollen tube entry. In all cases the other synergid persists till after fertilization.

The size, condition and number of antipodals in the angiosperms are highly variable. It can be emphasized that there are two general classifications, one for the monocotyledons and one for the dicotyledons. In both, the initial development of the antipodals is similar, generally resulting in three functional antipodals when maturity of the embryo sac is almost reached. From the ultrastructure and histochemistry a high cellular activity is attributed to these cells. In the phase from just before maturity to the fertilizable stage the subsequent development is different in both classes.

In the monocotyledons the number of antipodals increases strongly by cell division. In *Triticum* the number of cells increases before fertilization to 15-18 and can be some time after

fertilization as high as 28 (KALTSIKES et al. 1974). In *Stipa elmeri* even a cell number of 140 is reached (MAZE & BOHM 1973). The active developing period of the antipodals continues until endosperm cell wall formation starts, which occurs in *Triticum* 96-120 hrs after fertilization. Ultrastructurally these cells remain in an active state; they possess many mitochondria and ribosomes, abundant ER and dictyosomes (MAZE & LIN 1975). After the endosperm becomes cellular the degeneration of the antipodals occurs rather rapid.

In the dicotyledons the functional antipodals are ephemeral (KAPIL & BHATNAGAR 1979). Just before maturity of the embryo sac they reach their complex structural development and subsequently degeneration commences. Generally the antipodals have disappeared before fertilization (D'ALASCIO-DESCHAMPS 1972; GODINEAU 1973; NEWCOMB 1973; RIFOT 1973). In one case, *Capsella*, the antipodals persist till the formation of the endosperm, but remain small inactive appearing cells (SCHULZ & JENSEN 1971).

The functional antipodals have nuclei which are rich in DNA and cytoplasm, usually with a high concentration of RNA and proteins, characteristic profiles of RER, abundant dictyosomes, ribosomes and mitochondria, indicating a metabolically active state. The antipodal walls possess papillate projections and are traversed by plasmodesmata. This indicates a possible function for the antipodals as a pathway for metabolites from the nucellus to the central cell and egg apparatus. Based on the structural development the antipodals of spinach become functional before the mature embryo sac stage is reached. In this period the antipodals indeed function as a pathway for metabolites from the original chalazal nucellus to the embryo sac. Up to maturity the chalazal proliferating tissue develops and causes a structural cut off of the direct pathway of metabolic transport to the original chalazal tissue. The antipodal function diminishes and ends at or even before the embryo sac reaches its fertilizable stage. Nutrition of embryo and endosperm therefore occurs by a different pathway.

The mature ovule of spinach consists of various nucellus tissues, of which one is the conductive tissue between the embryo sac and the micropyle. The micropylar and transmitting cells of this tissue play a role in relation to the growth of the pollen tube. A gradual dissolution of the longitudinal middle lamellae of these cells has started at the embryo sac and extends to the micropylar region. Ultimately the nucellar cuticle is attached and disintegrated. The changing morphology of the nucellus during ovule development appears necessary to allow pollen tube growth into the nucellus, since no pollen tube is able to penetrate young ovules.

In discussions concerning the ovule classification of the flowering plants mostly only the shape of the ovule, the number of integuments, the type of nucellus, and the presence or absence of an "aril" have been included or applied (see MAHESHWARI 1963).

The crassinucellate and bitegmic condition is generally considered to represent the basic type among angiospermous groups. The crassinucellate condition appears to be highly correlated with the presence of two integuments: 92% of the crassinucellate taxa studied are also bitegmic, as against only 3% of the tenuinucellate ones (BOUMAN 1974). SPORNE (1969) emphasized the occurrence of crassinucellate ovules as a highly significant characteristic marker among 21 characters to state the primitivity of such dicotyledonous families. It is a striking thing that the Chenopodiaceae with crassinucellate ovules get an advancement index of 80%, which is as high as the Balsaminaceae and the Compositae with their tenuinucellar ovules. The specific differentiation into the various tissues of the multicellular nucellus in spinach may be an indication of a much greater phylogenetic advance than in the homogeneous crassinucellate ovules of plants like the Malvaceae.

Germination and tube growth in spinach is rapid. The sticking of pollen grains to the papillae does not depend of the developmental stage of the stigmata and the pollen grains need only a short time to realize germination and subsequent tube growth. The growth of the pollen tube starts quickly, whereas penetration of the cuticle takes more time. After formation of the pollen tube, the sticking force between the pellicle and Pollenkitt decreases probably by desiccation and when it becomes less than the tube tension the grain comes off the stigma papilla.

In mature pistils the tube growth in a particular tissue is always faster than in the corresponding tissue of the young pistil. Penetration into the nucellus occurs immediately in the mature pistils, whereas in the immature pistils there is no ingrowth in the nucellus. This means that the pollen tube growth through the stigma and the style and into the ovule is dependable of the developmental stage of the pistil tissues.

In all angiosperm plant species studied the pollen tubes always follow one specific pattern of growth through stigma and style and into the ovule. In spinach, however, pollen tubes can follow various pathways as intercellular, through the outer part of the cell wall and between the plasm membrane and the cell wall. Already from the first moment of growth, pollen tubes are able to grow through wall layers. The pollen tubes of some species appear to have pectinases (see VASIL 1974) and in the case of spinach these are probably able to affect the pectic substances in wall layers of the stigma papillae, and the transmitting cells in the style and in the nucellus. Besides, in spinach pollen tubes are able to pass completely through the cell walls of the transmitting cells in the style and in the nucellus. It is not clear if this is caused only by mechanical pressure or the involvement of enzymes or the preference of nutrition on cellular level as well.

After successful fertilization pollen tube growth into the nucellus stops and the following pollen tubes continue their growth in the micropyle around the nucellus tissue and between

the integuments. It seems that fertilization causes a trigger mechanism, probably originating in the embryo sac, that regulates the growth of the pollen tubes. This can be seen in a similar way as the earlier initiated dissolving or affecting of the middle lamellae of the longitudinal cell walls of the nucellar cells between the embryo sac and the micropyle. The feature of tube branching does occur more frequently from now on. This branching and the continuing growth of tubes in the micropylar region does not stop before the tubes reach a certain length. This length seems related to the distance from the stigma to the embryo sac. During the passage of the tubes no adjacent ovarian cells appear affected. In the male gametophyte most substrates, except water, are present sufficiently. Mainly the internal organization of the male gametophyte seems responsible for the final tube growth.

Much detailed information has been gained on the processes of pistil development, pollination and fertilization in spinach, ending up with the impression that no male cytoplasm has been involved in embryo and endosperm formation. More ultrastructural evidence has to prove this. The spinach pistil appears a good example to obtain information on regulated pollen-pistil interaction experiments. Plant conditions, development and differentiation of cells and tissues, nourishment and attraction are components for the team-work, which contribute to a successful reproduction. All have to be in the right stage and each individual component can influence the process specifically.

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## 4. SAMENVATTING

### Ontwikkeling en samenstelling van de zaadknop

De ontwikkeling en differentiatie van de integumenten, nucellus en de megagametofyt resulteert in spinazie in een ortho-amphitrope zaadknop. In het nucellus worden vier verschillende weefsels onderscheiden: het geleidend weefsel, het oorspronkelijke chalazale weefsel, het uiteindelijk chalazaal "proliferating" weefsel en het laterale weefsel. De cellen van de onderscheiden weefsels hebben overeenkomstige als ook sterk verschillende eigenschappen, die gerelateerd zijn aan de positie binnen het geheel en aan hun functie.

De integumenten vertonen verschillen in hun ontwikkeling. Het buiten integument omvat 3-5 cellagen met daartussen intercellulaire holten van variabele grootte. Het binnen integument wordt gevormd door twee zich verschillend ontwikkelende cellagen. Het plasmodesmaal contact tussen deze twee cellagen vermindert en is verdwenen bij volgroeiheid.

De megagametofyt heeft vlak na de coenocytische periode plasmodesmata in de chalazale celwand tussen de chalazale antipode en de aangrenzende nucelluscellen.

De localisering van reservemateriaal, zoals zetmeel, andere polysacchariden, proteïnen en lipiden, is bestudeerd in de zich ontwikkelende zaadknop om de voedingsbaan naar de embryozak en het embryo vast te stellen. In de jonge zaadknop gaat de aanvoer van voedingsstoffen via het oorspronkelijk chalazale weefsel naar de zich ontwikkelende vrouwelijke gametofyt en het geleidend weefsel, waarbij opslag vooral in dit laatste plaatsvindt.

Tijdens de groei van de zaadknop ontwikkelt het uiteindelijk chalazaal "proliferating" weefsel zich waarbij dit het oorspronkelijk chalazale weefsel wegdrukt en de transportfunctie ervan overneemt. Hiermee gaan veranderingen gepaard in de waargenomen hoeveelheden reservematerialen. De opslag van zetmeel neemt sterk toe in het buiten integument en in de cellen, die de baan van oorspronkelijke chalazale cellen - embryozak omgeven. Na de bevruchting wordt dit zetmeel geleidelijk afgebroken.

Proteïnen worden vooral aangetroffen in de weefsels die betrokken zijn bij de groei en penetratie van de pollenbuis naar de embryozak.

### Ontwikkeling van de embryozak

Vanaf de vorming van de coenocyt toont de ontwikkeling van de embryozak twee fasen: de eerste start met de vorming van de cellen en gaat tot het bereiken van de uiteindelijke grootte van de cellen. Nu begint de tweede fase, die loopt tot het stadium waarop de embryozak bevruchtingsrijp is. Gedurende de eerste fase zijn de dimensies, oppervlakten en volumens van de verschillende cellen en celonderdelen gemeten en met elkaar vergeleken. De celvergroting

is in hoofdzaak het gevolg van vacuolisatie. Het protoplasma van de antipoden blijkt nauwelijks toe te nemen, terwijl dat van de synergide verzesvoudigd en dat van de eicel en de centrale cel ongeveer vertienvoudigd. Gedurende de tweede fase ontwikkelen de cellen tot hun uiteindelijke ultrastructuur.

Bij de vorming van de cellen blijkt kwalitatief de samenstelling van iedere cel ongeveer gelijk: een kern met een onregelmatige vorm, veel ER en ribosomen, de nodige mitochondriën en dictyosomen, weinig plastiden en geen lipid. De ontwikkeling en differentiatie van de onderscheiden celtypen geschiedt verschillend voor zowel de wanden als hun plasmatische onderdelen.

De antipoden bereiken hun functionele structuur snel, leven korstondig en degenereren volgens een vast patroon.

De eicel groeit eerst zeer snel, vervolgens langzaam en ondergaat in het volgroeide stadium niet lang voor de bevruchting een hernieuwde vermeerderingsactiviteit van mitochondriën.

De ontwikkeling van synergiden en centrale cel is geleidelijk. Voor de bevruchting degenerereert een van de synergiden, terwijl in de centrale cel de poolkernen talrijke kernuitlopers vormen, die gedeeltelijk fuseren.

De structurele veranderingen in ieder celtypen zijn gerelateerd aan hun mogelijke functies. Opslagen afbraak van reservemateriaal wordt verklaard. In de bijna rijpe embryozak is het aanbod groter dan nodig; dit leidt tot opslag in de vorm van zetmeel. Dit geschiedt achtereenvolgend in de eicel, centrale cel en synergiden en tenslotte ook nog iets in de antipoden. Wanneer de zaadknop volgroeit is stopt de aanvoer wegens het niet meer functioneren van de aanvoerweg. Het zetmeel wordt geleidelijk afgebroken, beginnende in de meest chalazale cel. Ten tijde van de bevruchting is het vooral de eicel die nog zetmeel bezit.

### Stempel en stijl

Het receptieve gedeelte van de stamper bestaat uit 4 lange stempels. Het bovenste gedeelte van een stempel bevat uitsluitend papilcellen met een breed centraal deel en een smal spiraliserend staartgedeelte. Het onderste gedeelte bezit bovendien nog cilindrische parenchymcellen. In iedere stempel wordt een centrale zone gevormd, allereerst door de staartgedeelten, later samen met de kleine parenchymatische cellen. In de stijl komen deze centrale zones bijeen en fuseren tot één zone van geleidend weefsel. Aan de basis buigt dit geleidend weefsel af in de richting van de micropyle. De intercellulairtussen de verschillende cellen van de stempelen van de stijl verschillen sterk in grootte en in electronen-dichtheid van hun matrix.

### Pollenbuisgroei in stempel en stijl.

De kieming geschiedt binnen 10-20 minuten na bestuiving, terwijl de Pollenkitt reeds binnen 7-10 minuten met de pellicula van de stempelpapil fuseert. De pollenbuis dringt door de pellicula en

de aangetaste cuticula en groeit via de buitenste wandlaag naar de basis van de papil en vervolgt zijn weg via intercellulair. De pollenbuisgroeï door stempel en stijl veroorzaakt geen structurele veranderingen in de aangrenzende cellen. In de stijl kan de pollenbuisgroeï ook via celwanden en/of tussen celwand en plasmamembraan door. In alle gevallen bereikt de pollenbuis de ruimte tussen buiten integument en vruchtwand. Soms groeit een pollenbuis eerst nog een periode via de cellen van de vruchtwand. Een afsluitende cuticula ontbreekt op deze plaats aan de binnenzijde van de vruchtwand. Ongeveer 6 uur na bestuiving bereiken de eerste pollenbuizen de micropyle.

### Penetratie in de zaadknop

Indien de zaadbeginsels nog niet volgroeïd zijn, kunnen pollenbuizen dit nucellus niet penetreren en klusteren dientengevolge in en om de micropyle tesamen. Wanneer de zaadknop volgroeïd is, blijkt bestuiving uitscheiding van "substanties" vanuit de synergiden te stimuleren. Deze uitscheiding leidt tot aantasting van de longitudinale middenlamellen van het nucellus dat tussen de embryozak en de micropyle gelegen is. Uiteindelijk wordt ook de nucellaire cuticula nabij de micropyle aangetast. Vanaf dit tijdstip kunnen pollenbuizen het nucellusweefsel penetreren. De pollenbuisgroeï geschiedt aanvankelijk uitsluitend intercellulair en vervolgens via wegen, overeenkomstig aan die in de stijl. Meerdere pollenbuizen kunnen de embryozak bereiken, maar slechts één pollenbuis penetreert de degenererende synergide via het faden-apparaat. Spermacellen, vegetatieve kern en pollenbuis cytoplasma met vele karakteristieke amyloplasten worden via een terminale opening geloosd in de degenererende synergide.

### Bevruchting

De fusie van de gameten vindt 7-9 uur na de bestuiving plaats, waarbij blijkbaar geen of zeer weinig organellen van de spermacellen zijn betrokken. Celfusie vindt plaats tussen spermacel en eicel, respectievelijk centrale cel, waarbij de desbetreffende membranen fuseren. De fusie tot zygote kern geschiedt snel, de fusie tot endosperm kern is veel geleidelijker. De eerste mitotische deling is in de endosperm kern 16-17 uur na de feitelijke bevruchting voltooid, terwijl dan de zygote kern nog in de aanloopfase verkeerd.

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## CURRICULUM VITAE

His Jack Wilms werd op 5 juni 1944 geboren te Anna-Paulowna. Hij bezocht de H.B.S. te Den Helder, Schagen en Alkmaar en behaalde in 1963 het eindexamen b. In hetzelfde jaar ging hij studeren aan de Landbouwhogeschool te Wageningen. Na in 1969 kandidaatsexamen richting Plantenveredeling te hebben gedaan, was hij gedurende zeven maanden werkzaam bij het Department of Agronomy, Agricultural University, te Gainesville, Florida. In 1971 behaalde hij het Ingenieursdiploma Plantenveredeling met als verdere specialisaties Erfelijkheidslcer (verzwaard) en Plantenfysiologie.

Sinds oktober 1971 is hij als wetenschappelijk medewerker werkzaam bij de afdeling Plantkunde van de Landbouwhogeschool, welke tegenwoordig de vakgroep Plantencytologie en -morfologie heet.