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DEVELOPMENT AND COMPOSITION OF THE SPINACH OVULE

H. J. WILMS

Vakgroep Plantencytologie en -morfologie, Botanisch Laboratorium, Landbouwhogeschool, Arboretumlaan 4, 6703 BD Wageningen

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 laterial 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.

1. 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% $KMnO_4$ 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% 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).

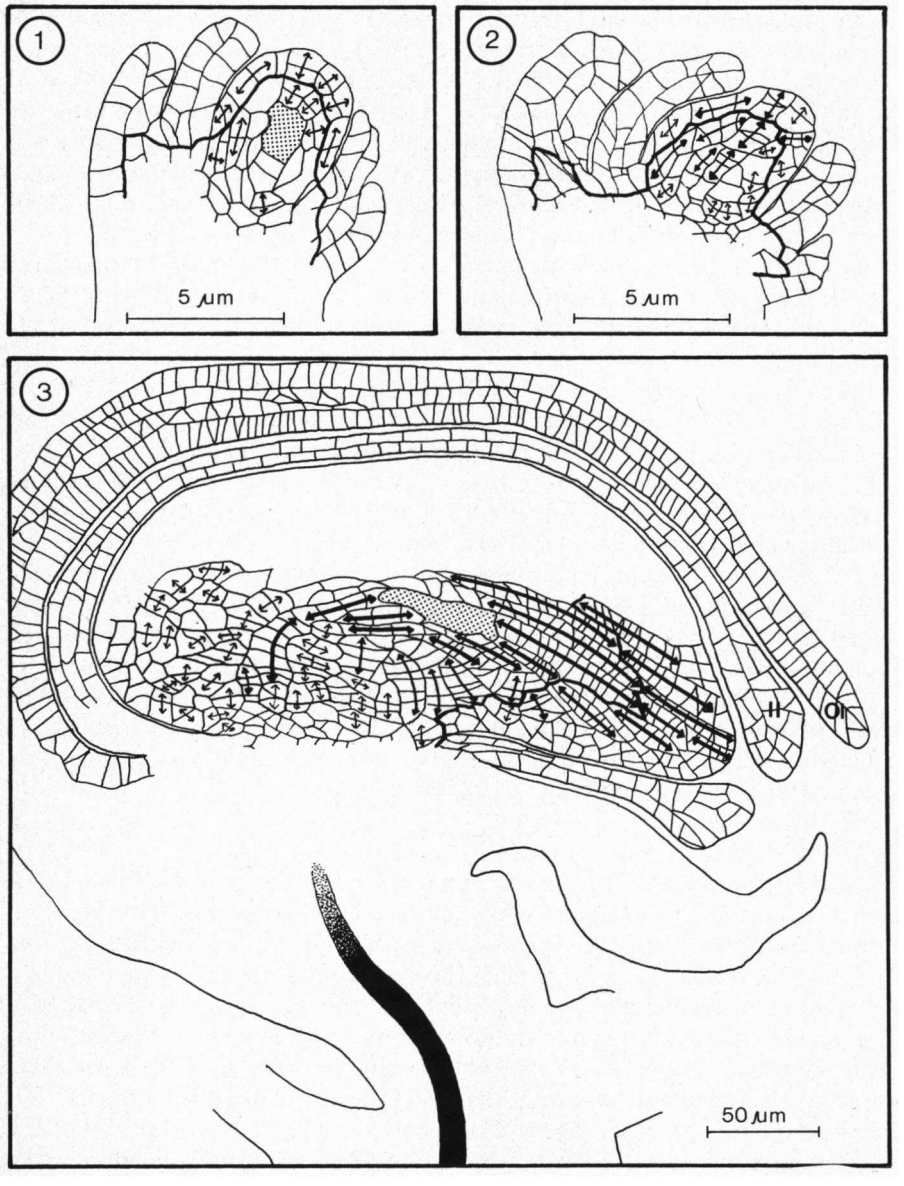
Tissue used for histochemical localization of starch was freeze-fixed in a glycol medium, cut in a IEC CTF cryo-microtome at −10 °C and stained with IKI (JENSEN 1962). Tissue for other histochemical localization procedures was FAA-fixed and sectioned in paraffin wax. For insoluble carbohydrates the periodic 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 μm 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 μm, which is twice the length of the cells of the inner layer. The thickness of the total inner integument remains about 8 μm. 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.

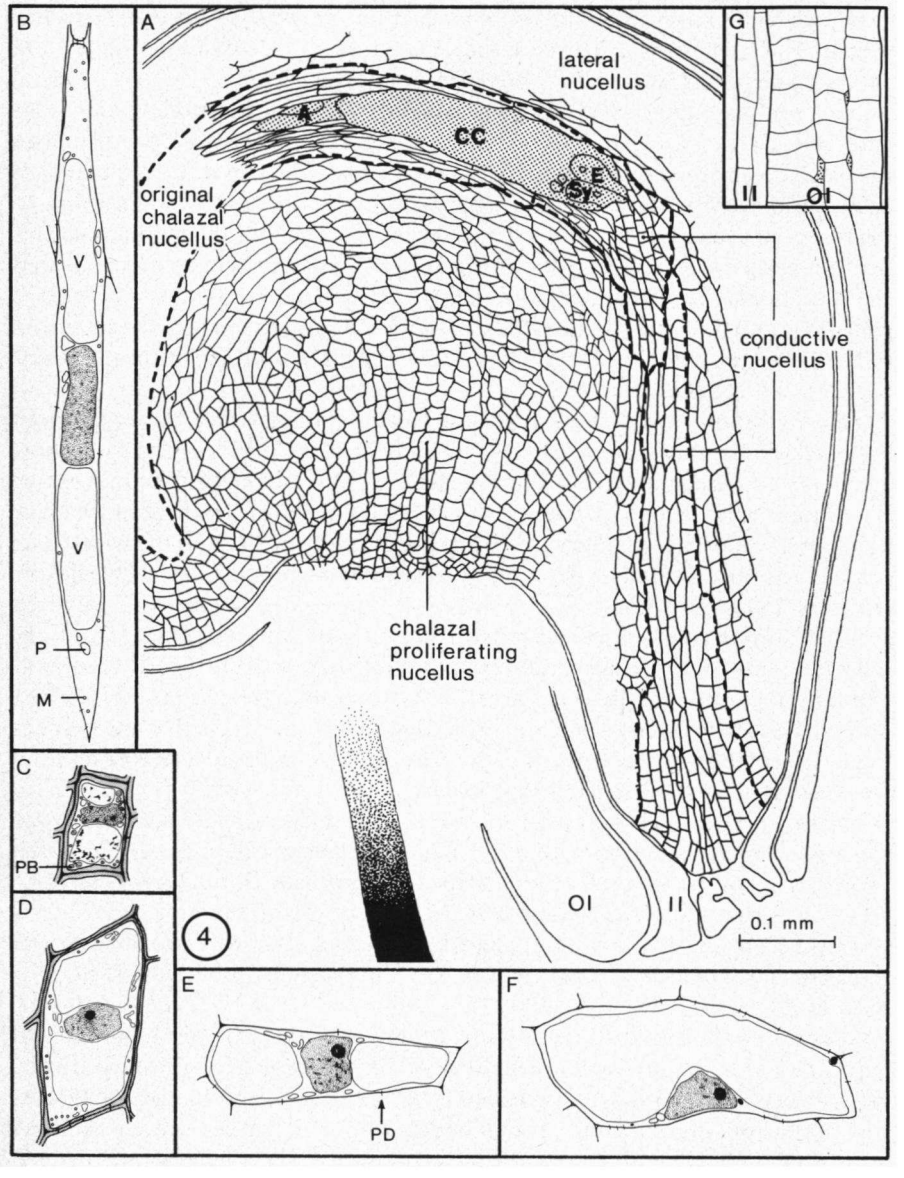


Fig. 4.A. Cell pattern in the mature ovule. The dotted lines mark the various parts of the nucellus. B–G: Cytological drawings of cell types of the nucellus and integuments. B. Original chalazal cell. C. Transmitting cell of the conductive tissue. D. micropylar cell of the conductive tissue. E. Chalazal 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 μm, which is one quarter of the width (12–17 μm). In the mature stage however, the length is as much as 125 μm, 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 μm in length with short cristae, dictyosomes with 4–5 short cisternae (0.3–0.5 μm length) and single strands ER. Also some lipid granules and protein bodies with a diameter of about 1 μm 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 nucellus. 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 loose 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	conductive microp	ant	c c	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 considerating 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.

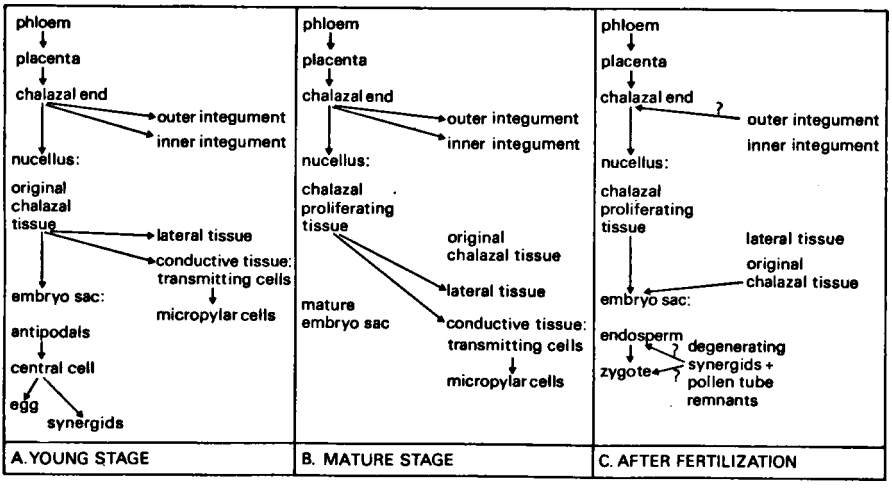


Diagram 1. The proposed pathway of polysaccharides in the spinach ovule during three stages of development, A. young stage; B. mature stage and C. after fertilization has taken place.

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.

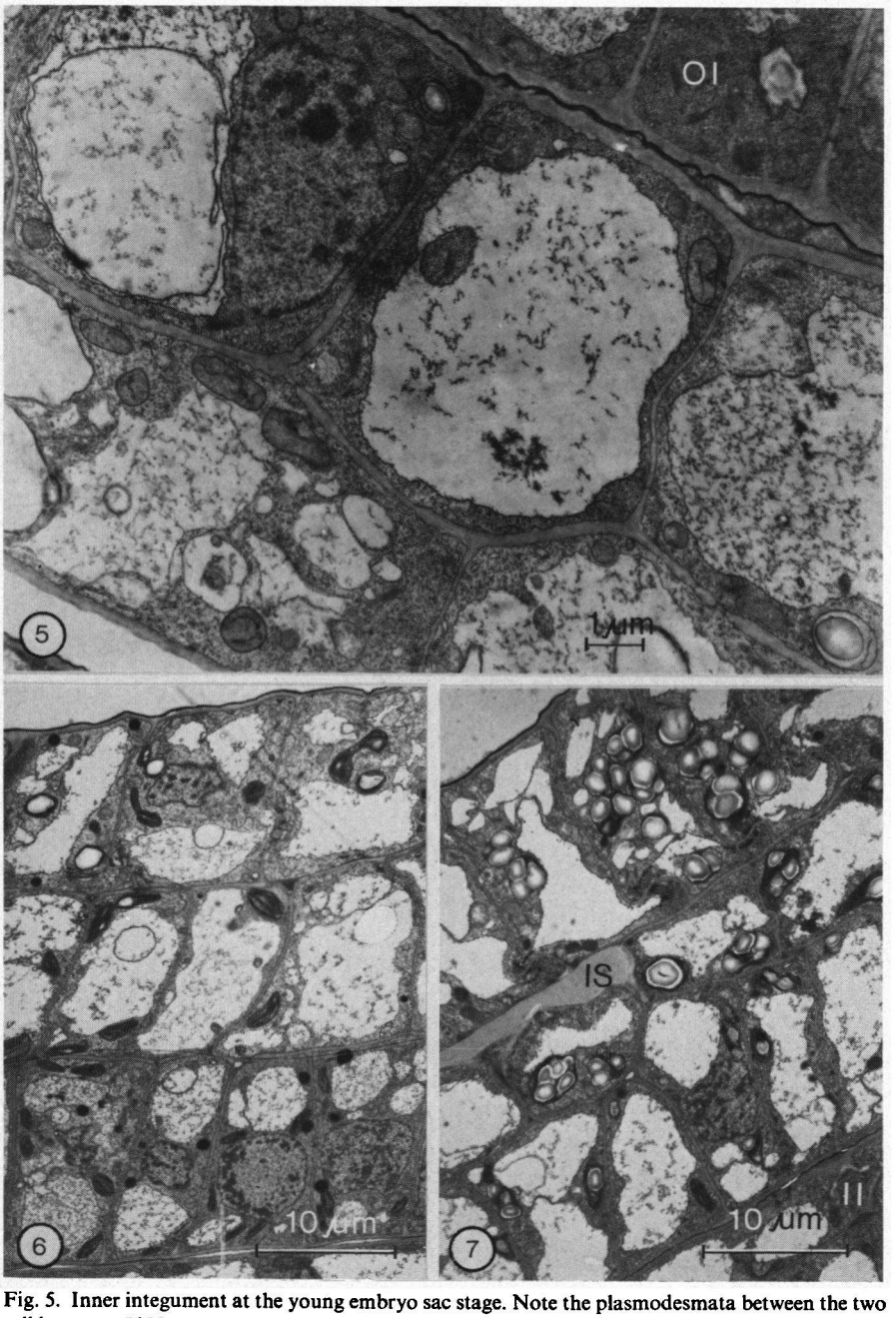

Fig. 5. Inner integument at the young embryo sac stage. Note the plasmodesmata between the two cell layers. × 8000.
Fig. 6. Outer integument at young embryo sac stage. × 2000.
Fig. 7. Outer integument at mature embryo sac stage. × 2000.

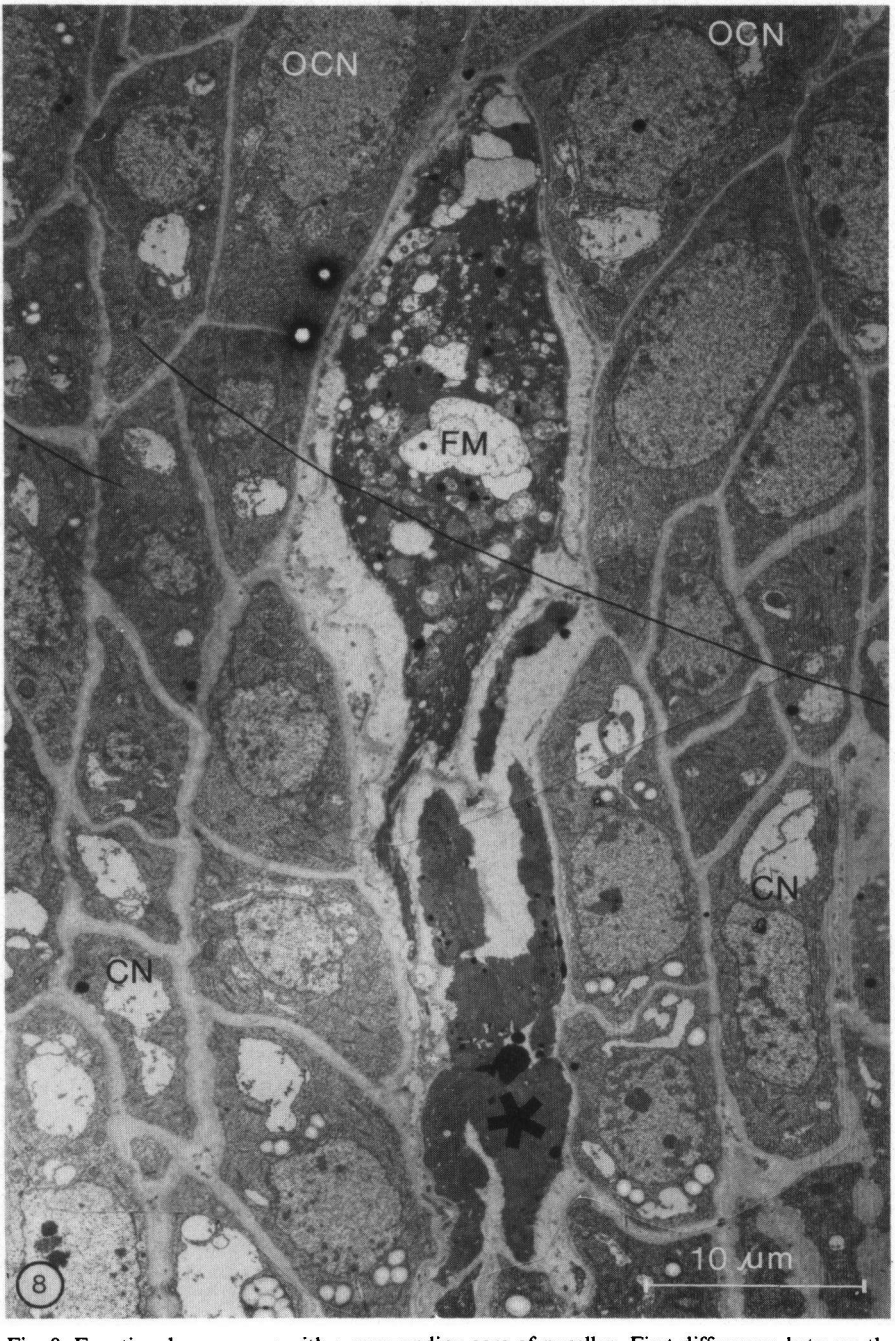

Fig. 8. Functional megaspore with a surrounding core of nucellus. First differences between the chalazal and micropylar nucellar cells can be observed. Note the degenerating megaspore (asterisk). × 3000.

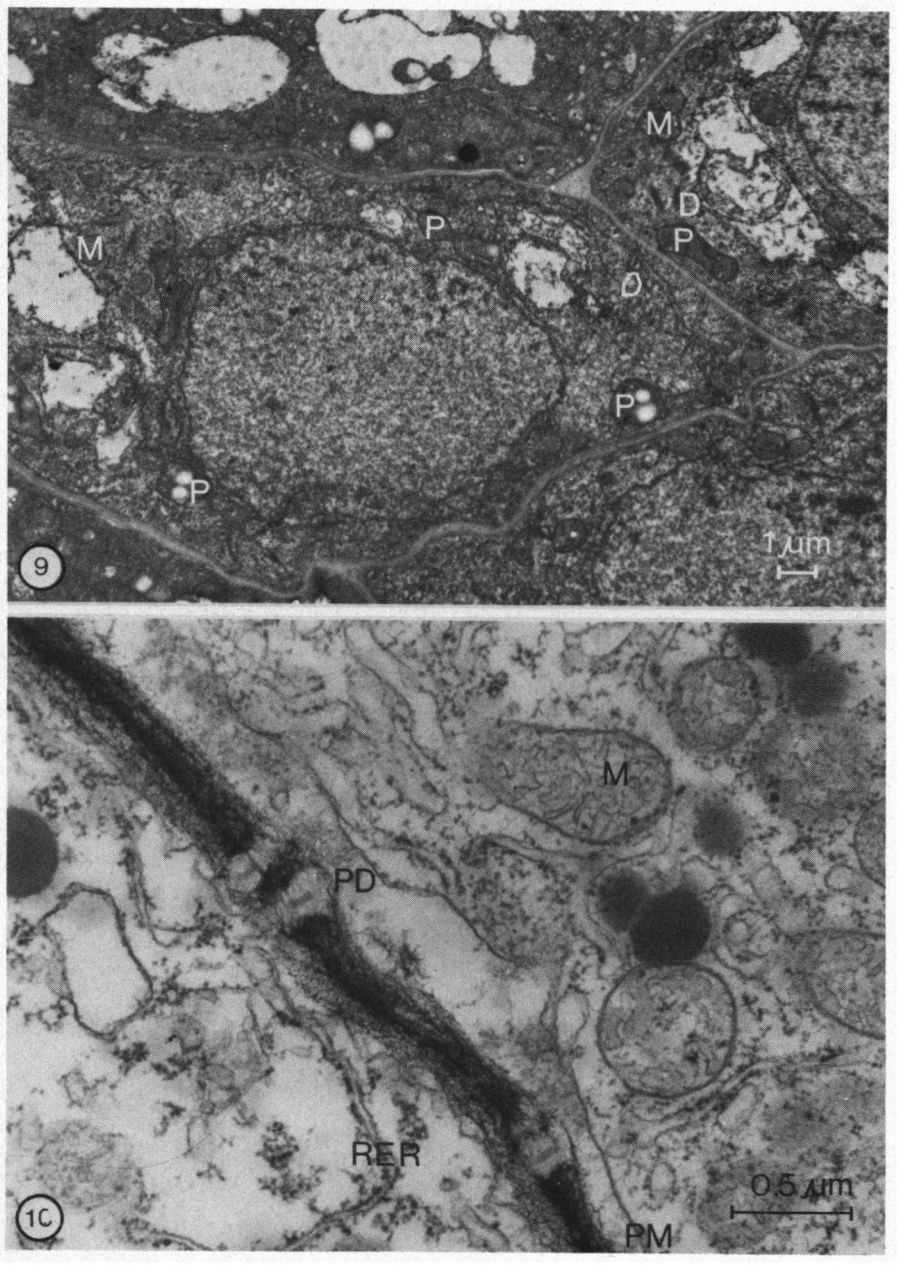

Fig. 9. Young original chalazal tissue. × 5000.
Fig. 10. Mature original chalazal tissue. Cytoplasm near the end wall between two cells. × 32,000.

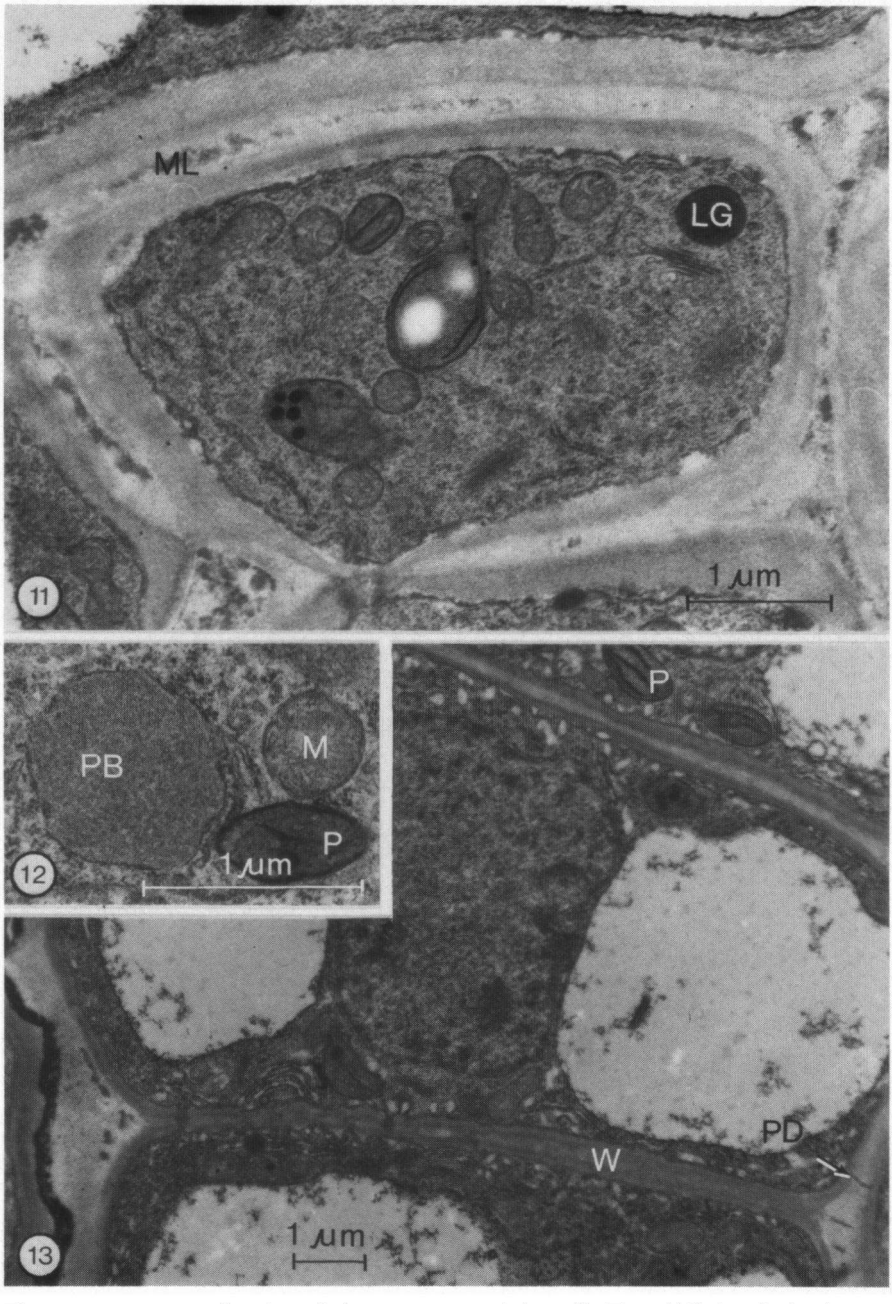

Fig. 11. Transverse section through the mature transmitting cells. The middle lamella has become disintegrated. × 20,000.

Fig. 12. Detail of the cytoplasm of the mature transmitting cell, in which protein bodies are accumulated in the cisternal phase of the ER. × 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. × 10,000.

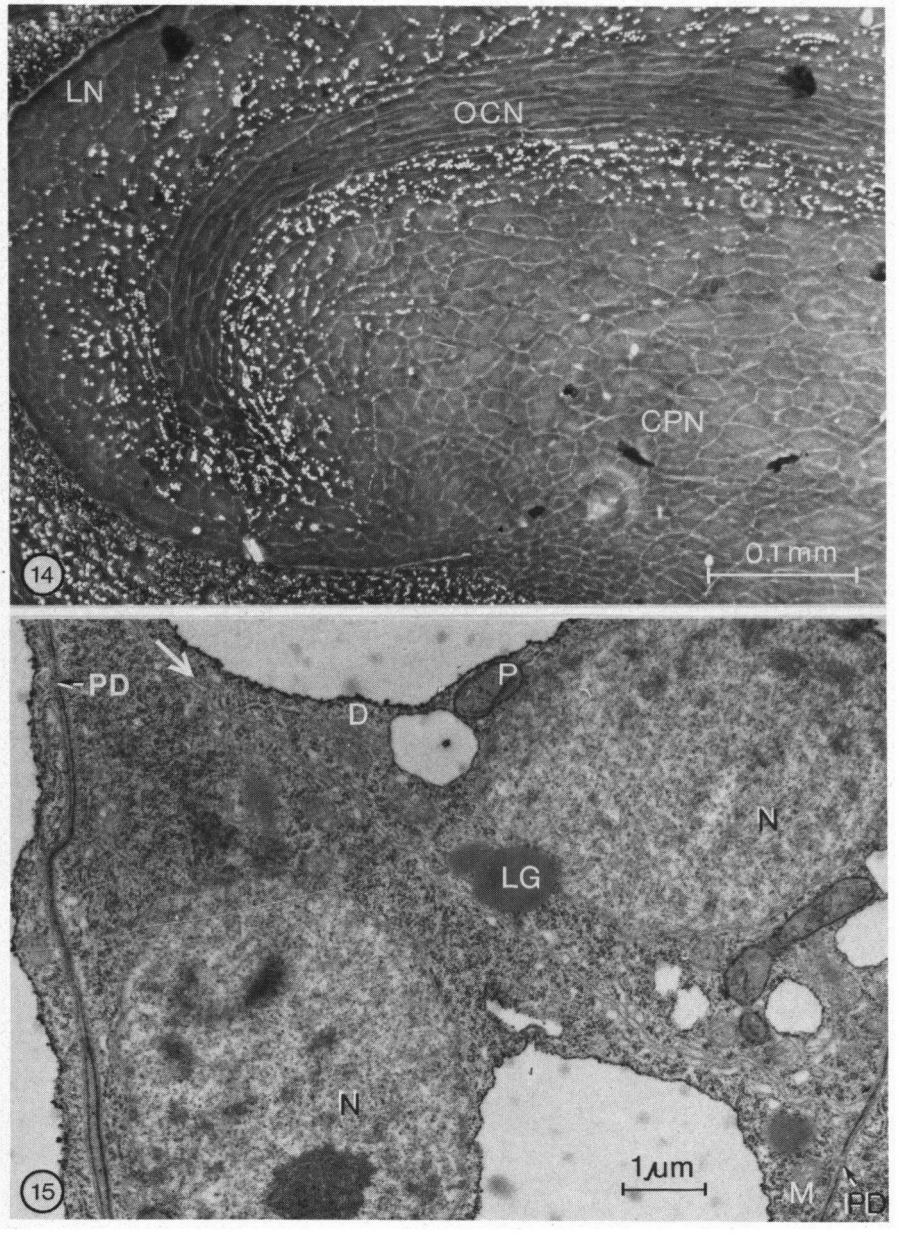

Fig. 14. Starch accumulation around the cells of the original chalazal tissue. Phase-contrast photograph. × 220.
Fig. 15. Part of the chalazal proliferating tissue. Note the newly formed cell wall (arrows). × 11,000.

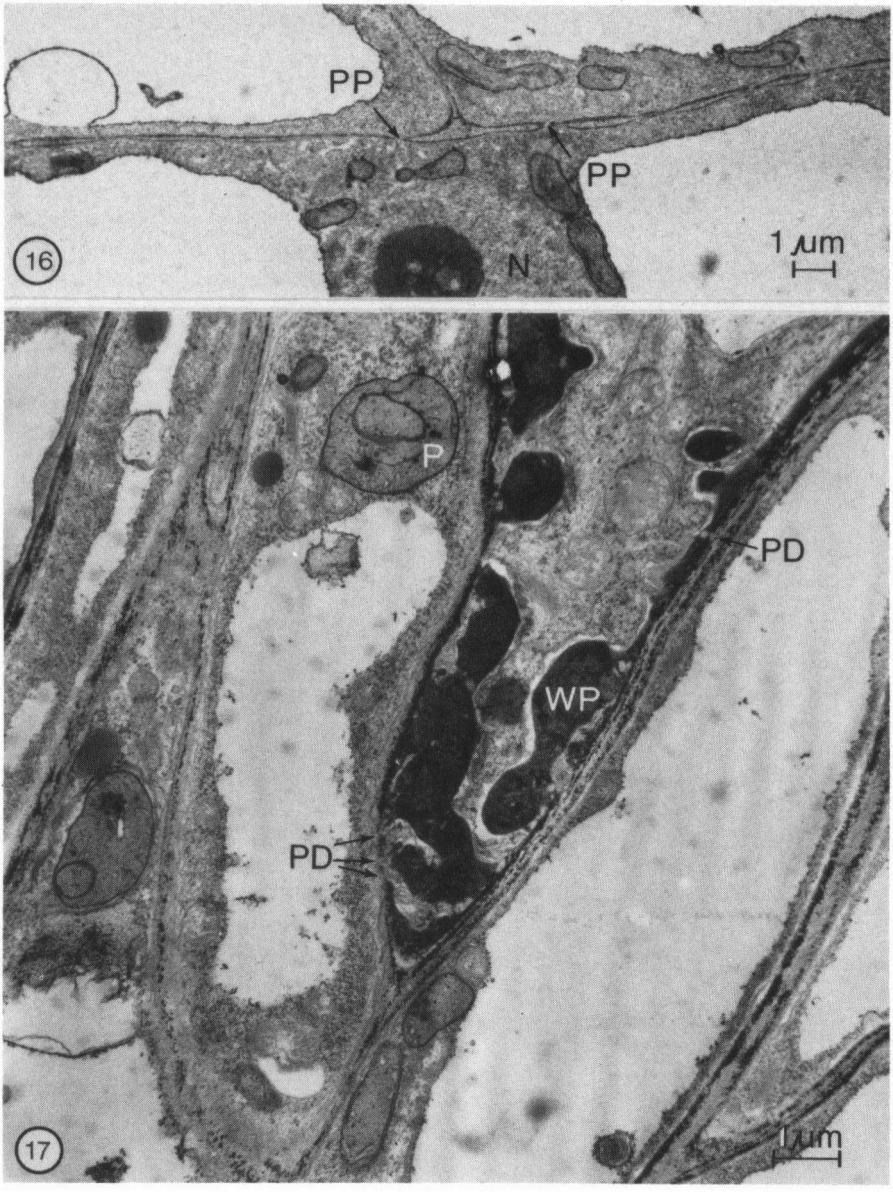

Fig. 16. Part of the lateral tissue. × 5000.
Fig. 17. Chalazal part of the chalazal antipode surrounded by original chalazal cells. In the antipodal wall plasmodesmata are present. × 12,000.