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ULTRASTRUCTURE OF POLLEN DEVELOPMENT IN EUPHORBIA DULCIS L. 1. DIPLOID PLANTS

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SUMMARY

Ultrastructual development from microspores at the tetrad stage up to the bicellular stage of pollen of diploid *Euphorbia dulcis* L. was investigated.

At the tetrad stage the microspores are enclosed by a thick callose wall. The nucleus occupies a central position in the microspore, is nearly spherical and shows a distinct nucleolus. The thin primexine varies in thickness. Large plastids with starch grains are present.

The generative cell becomes separated from the intine and changes into two short "tails". Many long rough endoplasmic reticulum (RER) cisternae are arranged parallel to the plasmalemma in the tail parts. Plastids are not present. Microtubules are parallel to the plasma membrane of the generative cell.

In the vegetative cell, the plastids undergo two amylogeneses. The first occurs at the tetrad stage and the second after the first pollen mitosis, when the generative cell starts to move to the central position in the vegetative cell. Many lipid droplets surround the generative cell. RER is arranged in aggregations near the generative cell.

After the first mitotic division the intine forms a network of microfibrillar substances around the pores. The cell wall, separating the vegetative and generative cell, is regularly interrupted by plasmodesmata or plasma channels.

1. INTRODUCTION

Embryological studies on *Euphorbia dulcis* mainly concern the female gametophyte development (HEGELMAIER 1901, 1903; CARANO 1925, 1926; CESCA 1961, KAPIL 1961). Little is known about the male gametophytic development (CARANO 1926; CESCA 1961; KAPIL 1961). External pollen morphology of the Euphorbiaceae, including *E. dulcis*, has been studied by PUNT (1962).

CESCA (1961) and CESCA & MUZZI (1972) recorded the presence of three different karyotypes in *E. dulcis* in Tuscany (Central Italy): a diploid ($2n = 12$), a triploid ($3n = 18$) and a tetraploid ($4n = 24$). It is observed that in the diploid karyotype pollen is abundant and the plants have a regular sexual reproduction. In the tetraploid karyotype two types of plants are found: one producing normal, but larger pollen than in the diploid plants and the second producing only abortive pollen.

Our investigation has for aim to elucidate the ultrastructural development

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of the microspore and the pollen grain (up to the bicellular stage) in the diploid, triploid and tetraploid biotype of *Euphorbia dulcis*, starting at the formation of tetrads and ending at the bicellular stage. The pollen grain at anthesis consists of 3 cells, two sperm cells and a vegetative cell.

In the present paper, the results of a study on the cytoplasmic development of the diploid *E. dulcis* pollen are reported and related to other plant species. In following papers the development of the pollen grains in triploid and tetraploid plants will be dealt with.

2. MATERIAL AND METHODS

Plants of the diploid (2n = 12) *Euphorbia dulcis* L., which grew spontaneously in some mountains of Tuscany (Central Italy), were collected and replanted in the Botanical Garden in Siena. Anthers of various sizes were collected from the inflorescences to obtain microspores and developing pollen from the tetrad stage to the bicellular grain stage.

Anthers were fixed for 1 hr at room temperature in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed with the same buffer, post-fixed in 1% OsO_4 for 1 hr, briefly rinsed with water, and dehydrated with a graded ethanol series. The materials were embedded in Spurr's resin. Ultrathin sections were obtained with a LKB Ultratome III, stained with uranyl acetate and lead citrate in an automatic LKB Ultrastainer, and observed in a Zeiss EM 9A, Jeol JEM 100 B, or Philips 301 transmission electron microscope.

The pollen grains were also examined by light microscopy to measure the equatorial (E) and polar axis (P) for calculating P:E (PUNT 1962). This ratio gives an indication about the shape of the pollen grain in the equatorial plane.

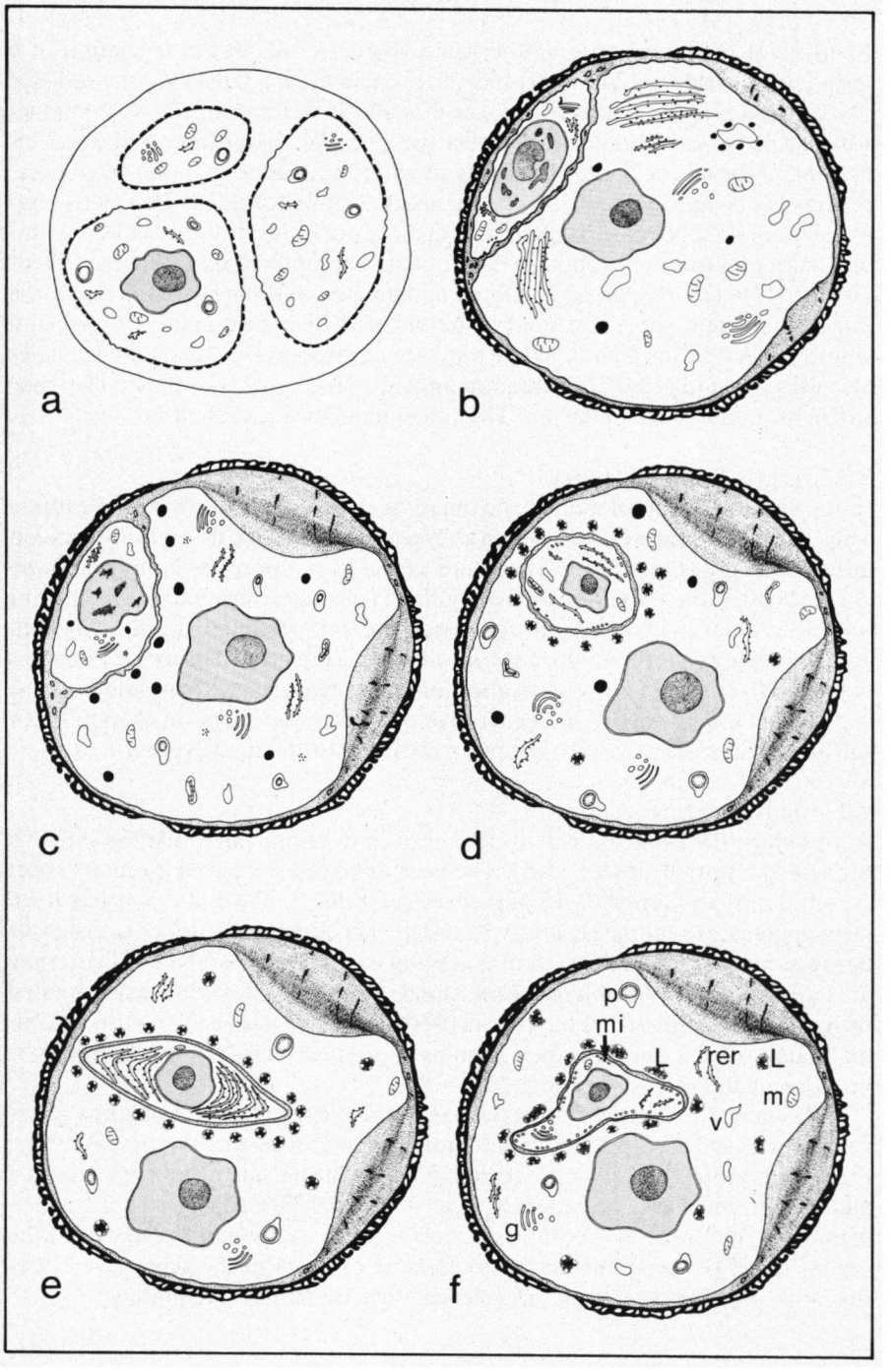
3. RESULTS

Mature pollen grains of *E. dulcis* are spheroidal and triporate. The dimensions are: E = 20.2, P = 21.2. The shape is three-lobate and P/E = 1.05 so the pollen grains of *E. dulcis* belong to the prolate spheroidal class (PUNT 1962)

The development from microspore to bicellular pollen grain has been divided in several stages. The sequence of events is summarized in a scheme (*diagram 1*). The formation of the different cell wall layers as well as the vegetative cytoplasm is schematically included.

Diagram 1. Schematic development of *E. dulcis* pollen from microspore to bicellular pollen grain. a: tetrad stage; b–f: bicellular stage; b: substage 1; c: substage 2; d: substage 3; e: substage 4 and f: substage 5.

Abbreviations: C = callosic wall, E = exine, g = Golgi body, gm = generative membrane, gn = generative nucleus, I = intine, IE = intine extension, L = lipid droplets, m = mitochondrion, mi = microtubules, n = nucleus, p = plastid, PE = primexine, rer = rough endoplasmic reticulum, s = starch, sp = sporopollenin, t = tubules, v = vesicle, va = vacuole, vc = vegetative cytoplasm, vm = vegetative membrane.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3.1. Tetrad stage

Meisosis of the microspore mother cell (MMC) results in the formation of a tetrad of microspores. The microspores are enclosed in a thick callosic wall (*fig. 1*). At the sites of the future pore zones the callosic wall remains in close contact with the plasmamembrane of the pollen grain. In *fig. 2*, outside the plasmalemma, the primexine is already present and varies in thickness. In the three poral regions the primexine is very thin. The plasmalemma is folded. The central nucleus is almost spherical and includes a distinct nucleolus. Large plastids containing starch grains are randomly distributed. The mitochondria have different sizes, 0.9–2.6 μm long and 0.2–0.8 μm in diameter, and the largest are near the nucleus. The few, short and small cisternae of RER are irregular in shape and length (about 0.2 μm), and include only some ribosomes. A few small vacuoles are observed. Golgi bodies contain a limited number (2–3) of plated cisternae with a maximal length of 0.6 μm. They seemingly produce vesicles.

3.2. Bicellular pollen grain

In the subsequent development the microspores are released from the callosic wall. The ultrastructure of the pollen after completion of the first mitotic division differs strongly from the ultrastructure of the microspore at the tetrad stage. On the basis of these changes we distinguish 5 substages during the development of the bicellular pollen grain. In substage 1 the first pollen mitosis is completed and two separated cells are formed. In substage 2 the intine bordering the pores thickens. In substage 3 the separation of the generative cell from the intine is completed. In substage 4 the generative cell takes the shape of a spindle. In substage 5 the generative cell occupies a central position in the vegetative cell.

3.2.1. Substage 1

In this stage the exine appears to be ahead in development while the intine is present as a thin layer (*figs. 3–6*). The vegetative cell is marked by many short (about 1 μm) and long (10–15 μm) cisternae in RER. The long cisternae form large aggregates near the generative cell (*fig. 3*). The material inside the cisternae becomes more electron-dense than the cytoplasm. The ends of the RER cisternae are bulbose (*fig. 4*). Vacuoles have increased in number and some are surrounded by lipids. The Goldi bodies have more (4–10) cisternae than before, with a maximal length of 0.8 μm, and they seemingly produce vesicles (*fig. 3*). Starch is not present and plastids are not observable.

The generative cell appears as a flat lengthened cell, separated from the vegetative cell by a relatively thick wall, containing pieces of membranes. At some places the cytoplasmic channels remain (*fig. 5*). The plasma membrane is folded and microtubules can be observed near it (*fig. 5*). The almost round nucleus contains a nucleolus that occupies almost half the volume of the nucleus. The organelles of the generative cell are the same as those in the vegetative cell at this stage (*fig. 6*), however, in the generative cell no plastids are present.

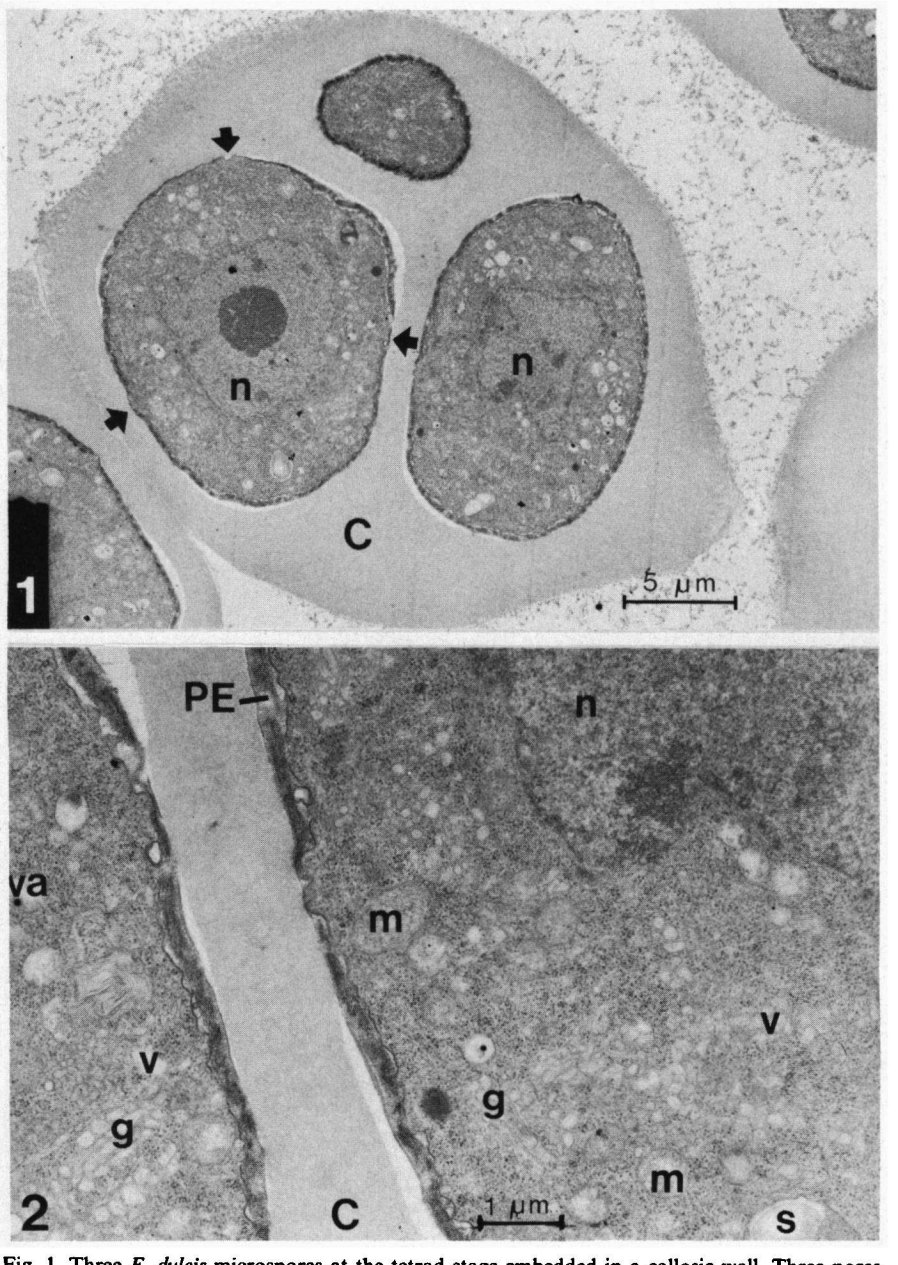

Fig. 1. Three *E. dulcis* microspores at the tetrad stage embedded in a callosic wall. Three pores (arrows) are in development. The central nucleus is preparing for the first pollen mitosis.

Fig. 2. Detail of microspores at tetrad stage. Irregular primexine formation exclude future pores (arrows).

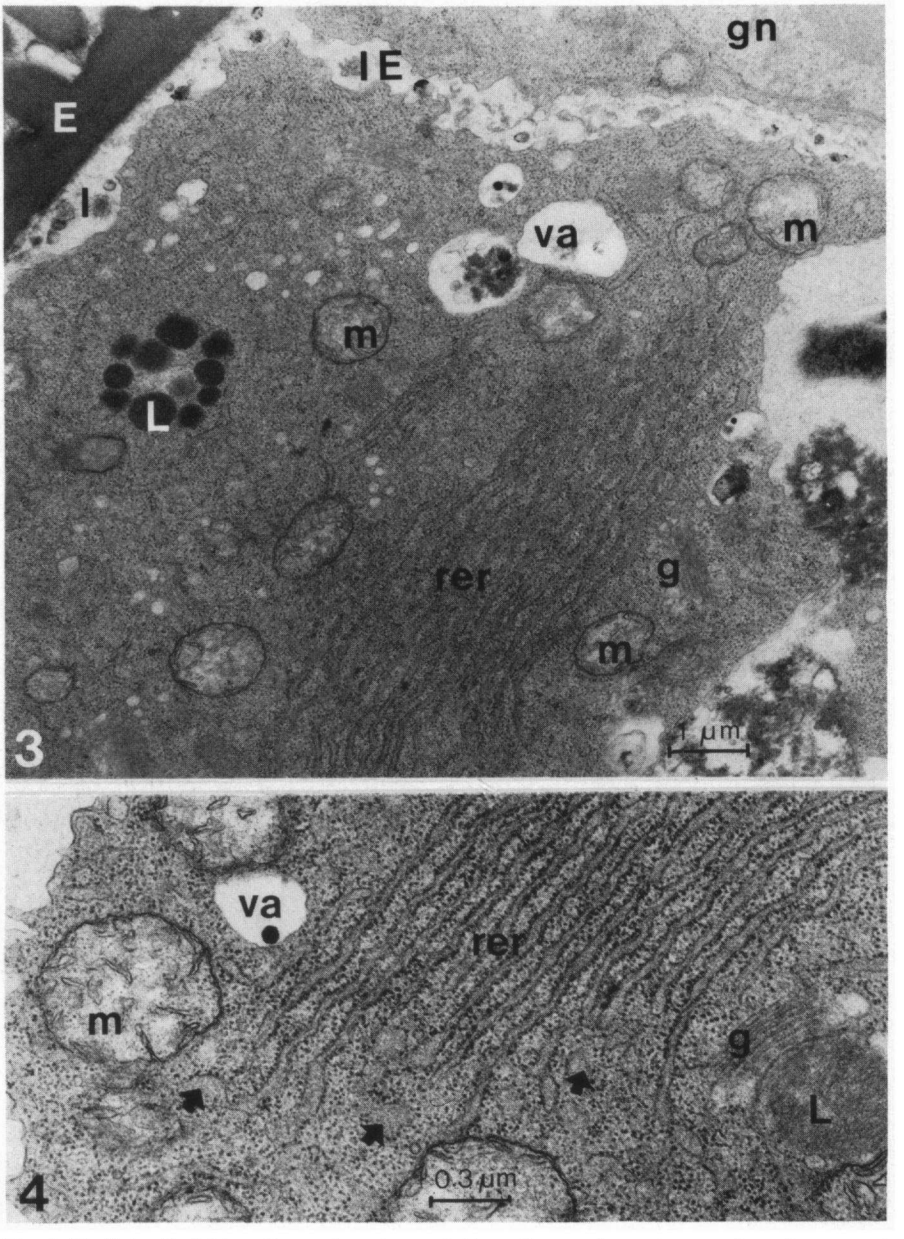

Fig. 3. Bicellular *E. dulcis* pollen grain, substage 1. Part of vegetative and generative cell separated by an extension of the intine.

Fig. 4. Detailed part of the vegetative cytoplasm. Long RER cisternae with bulboseous ends (arrows).

3.2.2. Substage 2

At this stage the thickening of the intine surrounding the pores is completed (*fig. 7*). In both the intine and the wall of the generative cell a network of microfibrillar substances is present. In some parts of the intine this network is electron-dense and different layers can be observed (*fig. 8*). Small parts of cytoplasm or some plasmalemma invaginations forming tubules have been found inside the wall (*fig. 9*). Regularly, a newly formed layer intersects the previous formed tubules (*fig. 10*). In a few cases this new layer is not formed. Lipid drops are concentrating mainly around the generative cell (*fig. 11*). In the plastids starch is formed (*fig. 12*). The cytoplasm contains more mitochondria with clear cristae. The randomly distributed free ribosomes tend to make polysomes. The width of RER cisternae increases, from about 0.2 μm to 0.4 μm. The vacuoles decrease in number and size. The Golgi bodies have 5–7 cisternae and their length is about 0.1–0.2 μm.

The shape of the generative cell becomes more spherical. Its membrane is still folded. The shape of the central nucleus is irregular. The vacuoles are larger and some are fused. Plastids are absent, the other organelles are identical to those in the vegetative cell (*fig. 13*).

3.2.3. Substage 3

In the vegetative cell the lipid droplets lying close to the generative cell as well as those spread within the cytoplasm increase in size by fusion with each other. Within the lipid droplets electron-translucent spots become apparent. The number of plastids increases as the starch increases in size and quantity. The vacuoles disappear. Some of the mitochondria show collapsing membranes and the number of functional mitochondria seems to decrease (*fig. 14*). The cisternae of RER are short (< 3 μm) and sacculated (*fig. 15*). Golgi bodies are present, some being semicircular (about 2 μm long) (*fig. 16*) while others remain straight (about 1 μm). Both seemingly produce vesicles.

The generative cell is spherical and separated from the intine by the vegetative cell. It moves to the central part of the vegetative cytoplasm. Microtubules are found near the plasma membrane. Some mitochondria have collapsed membranes and cristae, also their size is strongly reduced. RER is abundant and their cisternae are almost parallel to the generative membrane (*fig. 17*).

3.2.4. Substage 4

In the vegetative cytoplasm most organelles are the same as in the previous stage. The generative cell stretches and moves to the centre of the vegetative cell. The generative cell has two short "tails" (*figs. 18, 19*). RER is arranged parallel to the plasmalemma in the tail parts. The other organelles do not change morphologically and are sited near the nuccleus.

3.2.5. Substage 5

In the vegetative cytoplasm no morphological changes are observed. The generative cell is irregular in shape (*fig. 20*). The RER is not arranged like in substage

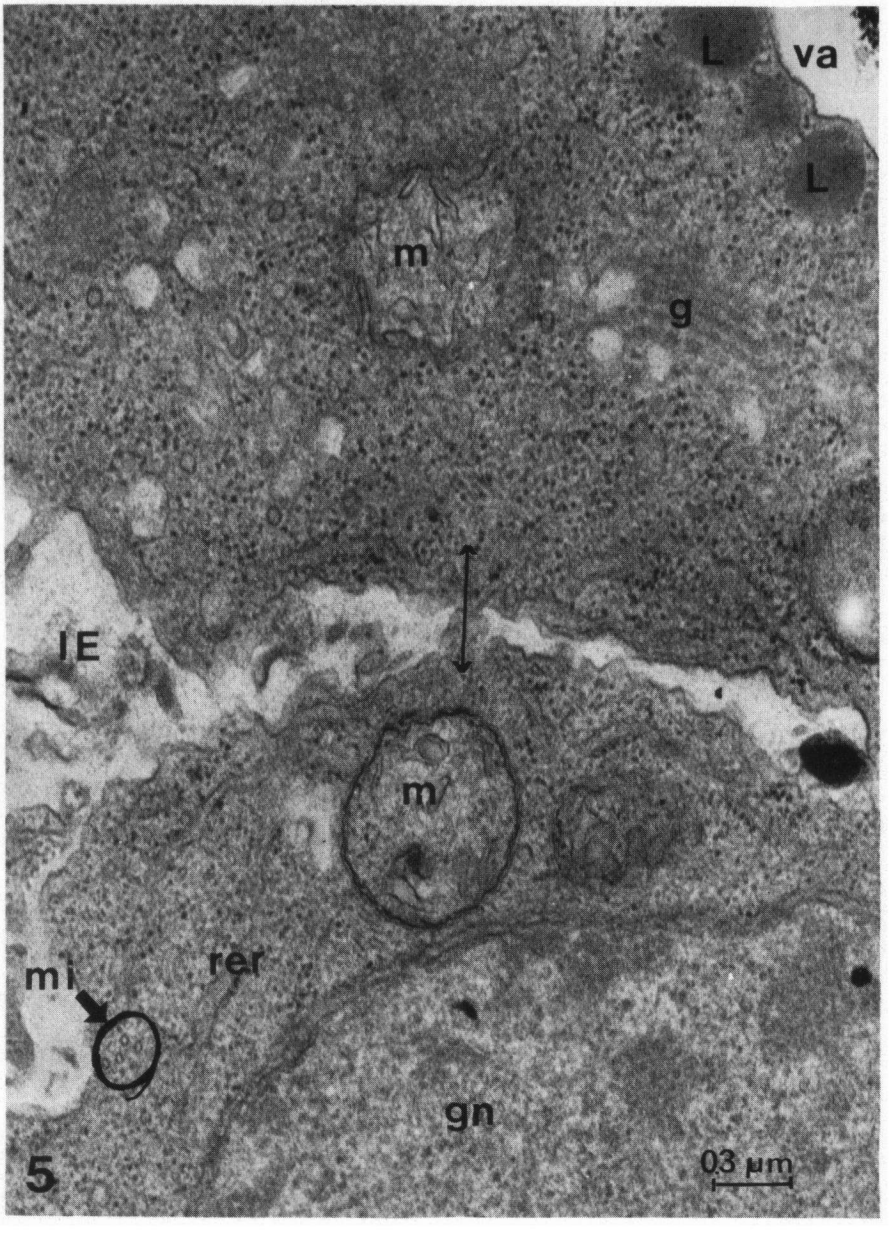

Fig. 5. Bicellular *E. dulcis* pollen grain, substage 1. Detailed part of vegetative cell and part of generative cell. Note encircled microtubules in generative cell and cytoplasmic channel between the cells (arrowed line).

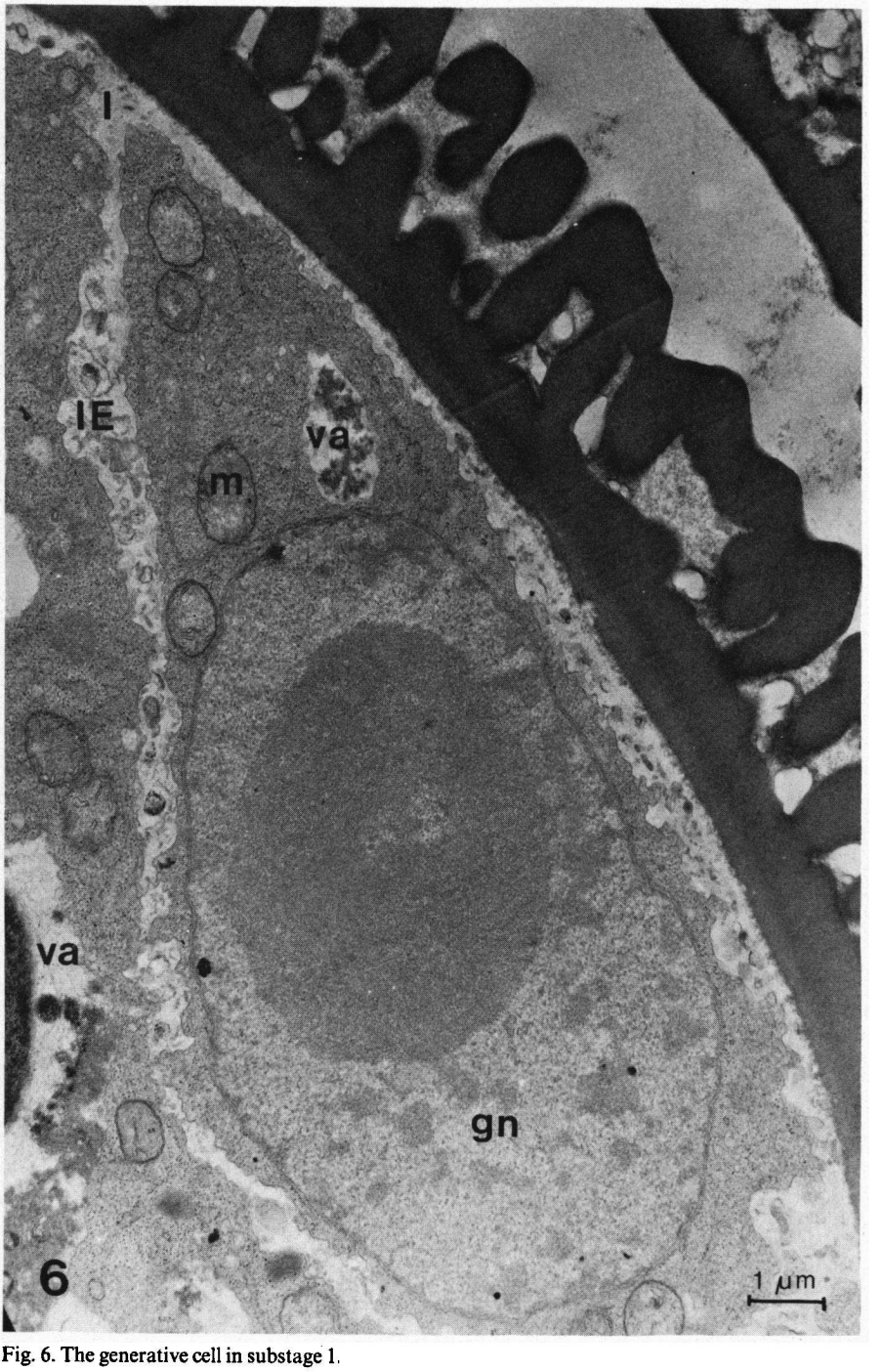

Fig. 6. The generative cell in substage 1.

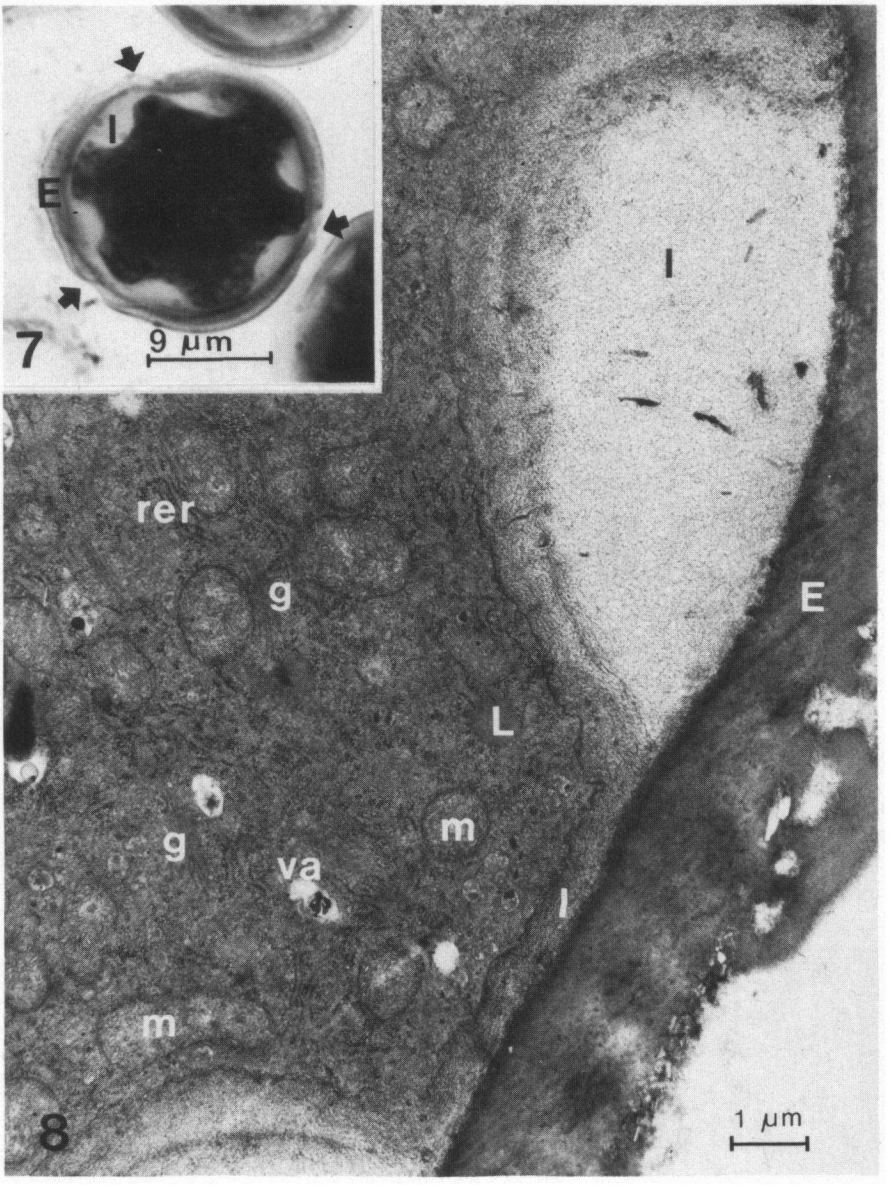

Fig. 7. Light microscopical view of bicellular *E. dulcis* pollen grain, substage 2. Note the particular intine layer. Arrows indicate pores.
Fig. 8. Detail of the vegetative cell, substage 2 near one germination pore.

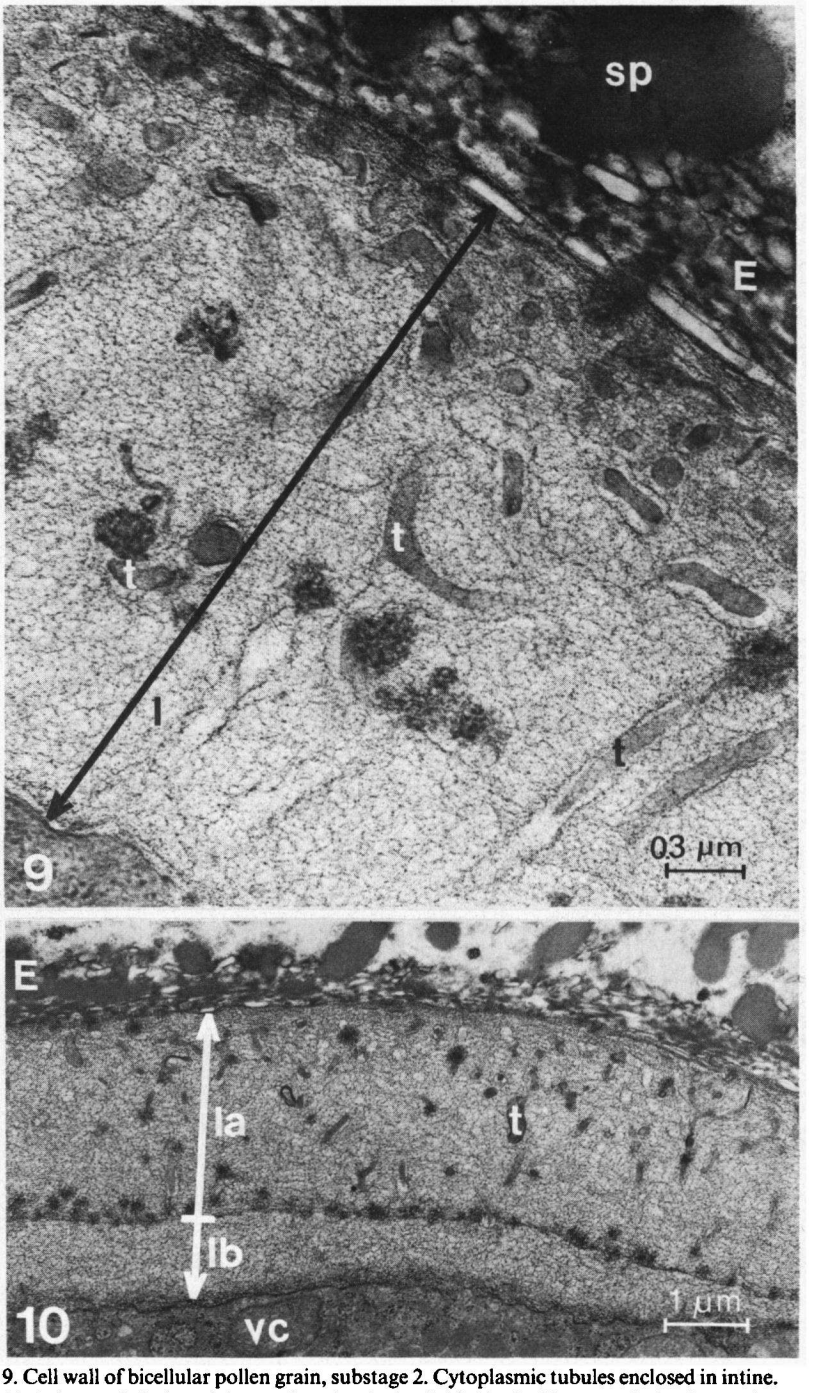

Fig. 9. Cell wall of bicellular pollen grain, substage 2. Cytoplasmic tubules enclosed in intine.
Fig. 10. Substage 2. Intine with cytoplasmic tubules (Ia) is blocked by a new intine layer (Ib).

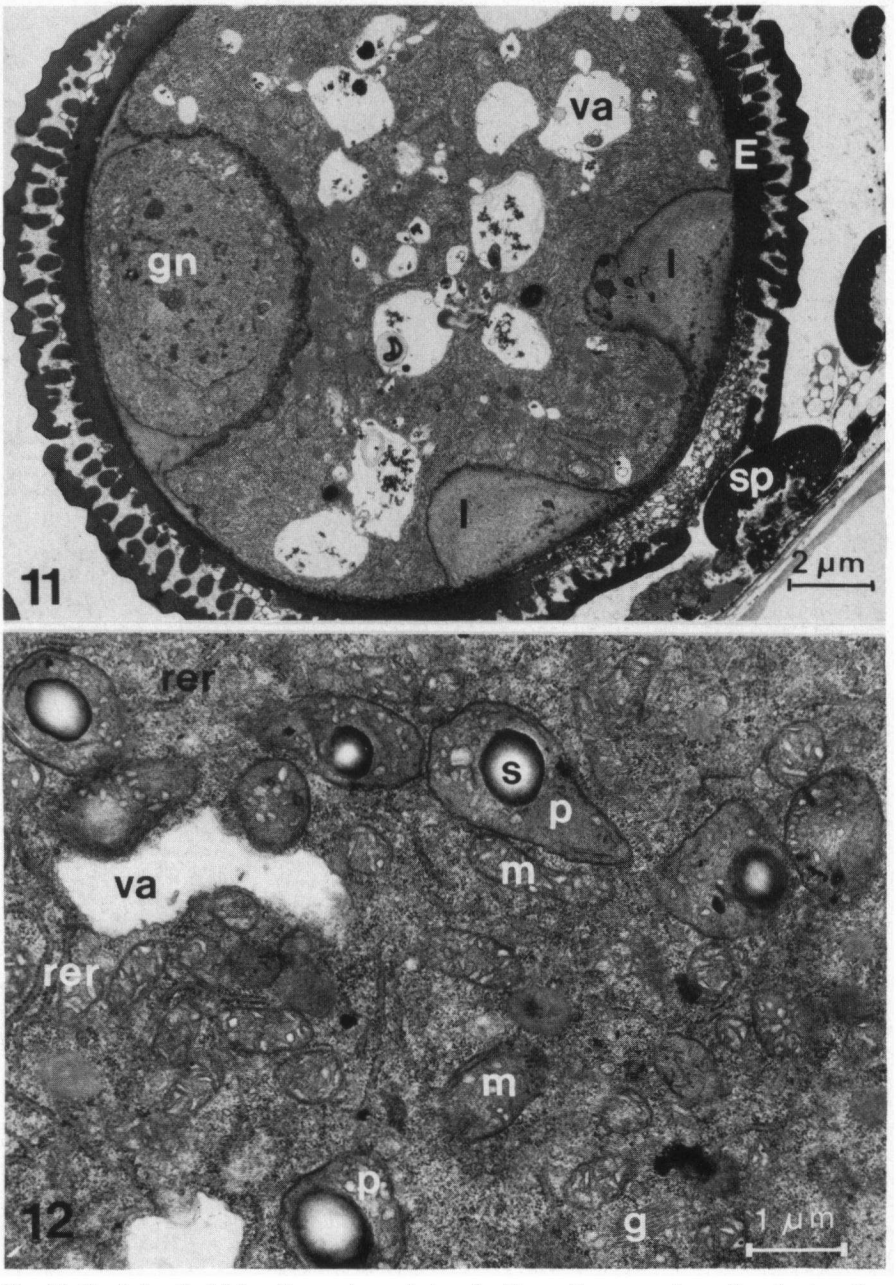

Fig. 11. Bicellular *E. dulcis* pollen grains, substage 3 with median generative cell and submedian vegetative cell. In vegetative cell vacuoles and one pore with thick intine layers are present.
Fig. 12. Cytoplasm of vegetative cell in substage 3.

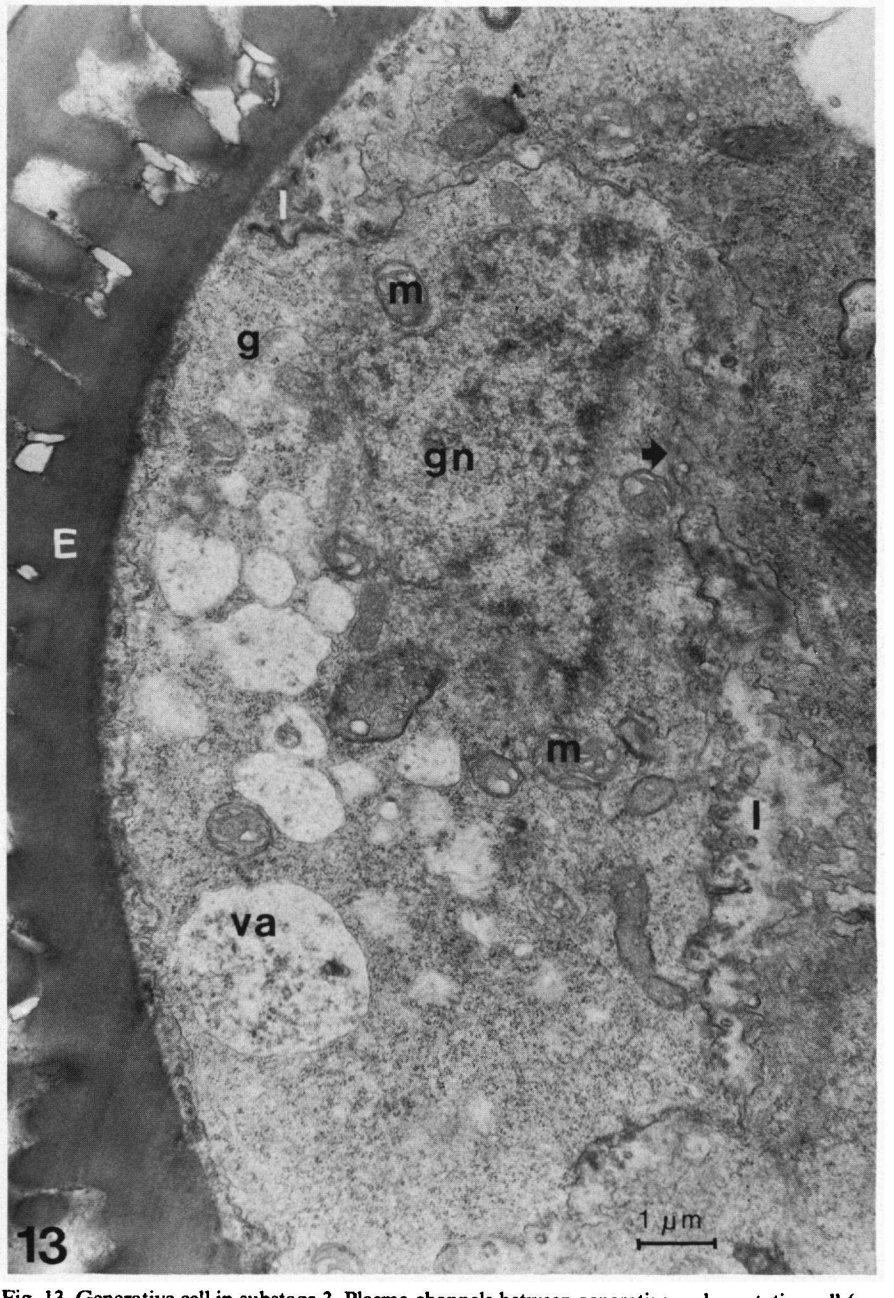

Fig. 13. Generative cell in substage 3. Plasma channels between generative and vegetative cell (arrow).

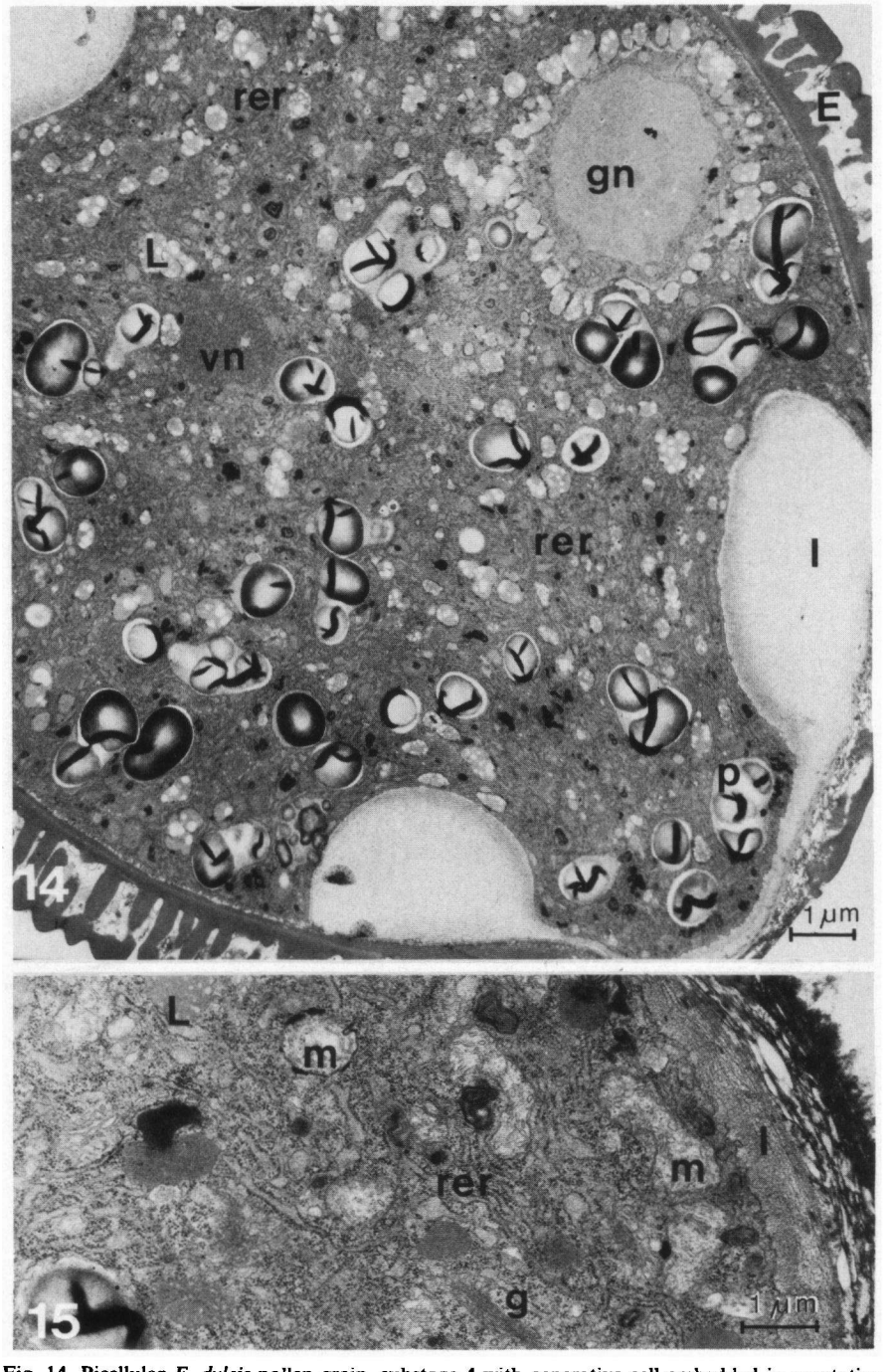

Fig. 14. Bicellular *E. dulcis* pollen grain, substage 4 with generative cell embedded in vegetative cytoplasm. Thick intine around the pores. Lipid droplets surround the generative cell.
Fig. 15. Detail of vegetative cytoplasm near the pore of *fig. 14.*

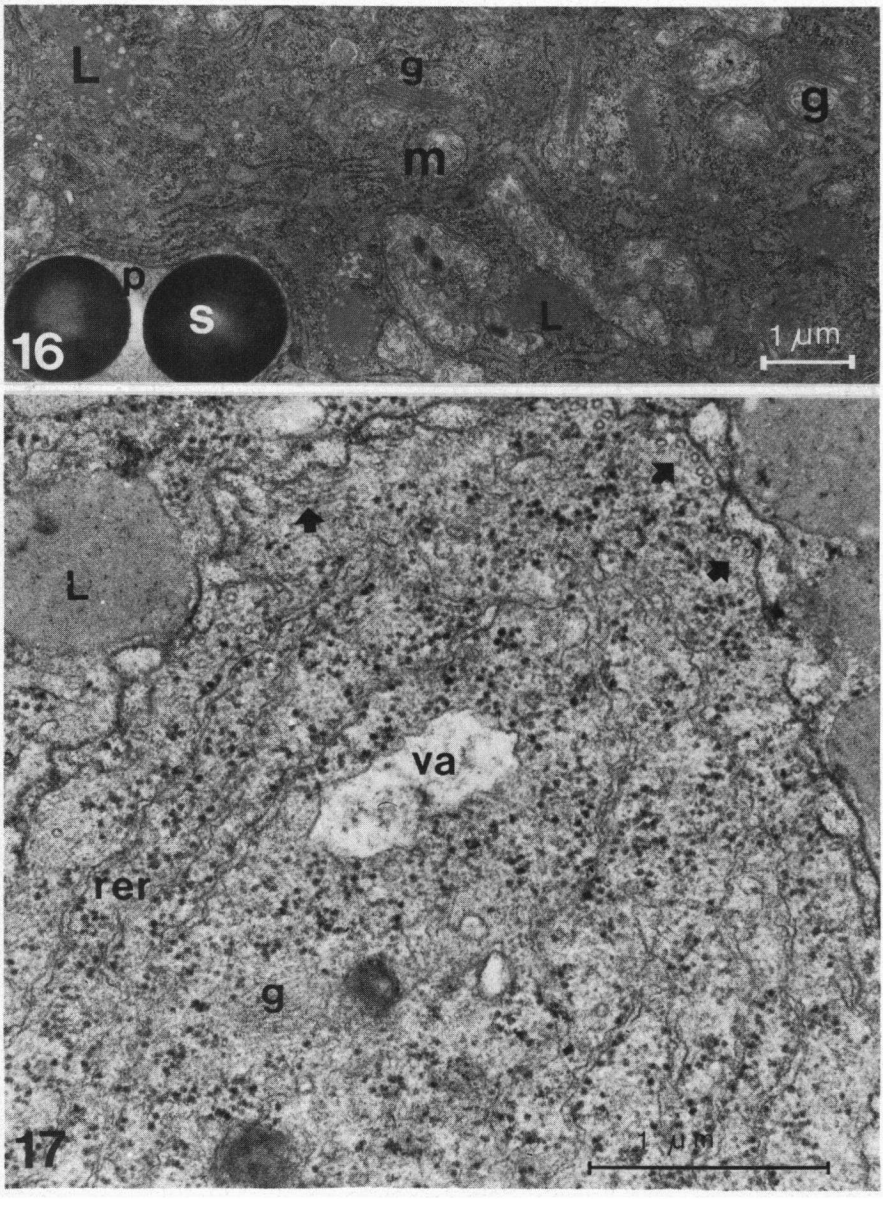

Fig. 16. Detail of vegetative cytoplasm in the centre of *fig. 14.*
Fig. 17. Detail of tail part of generative cell of substage 4. Microtubules (arrows) parallel to plasma membrane.

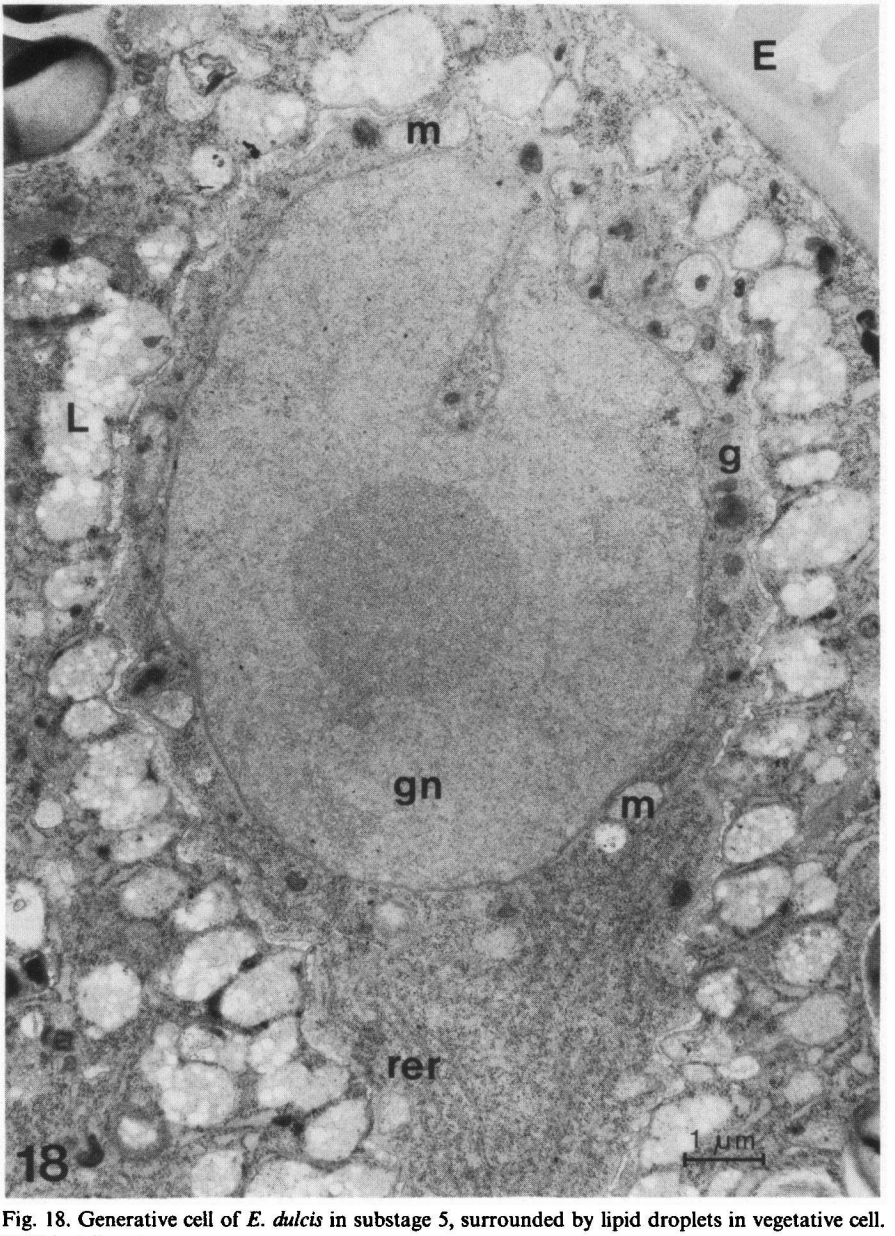

Fig. 18. Generative cell of *E. dulcis* in substage 5, surrounded by lipid droplets in vegetative cell. RER in tail part.

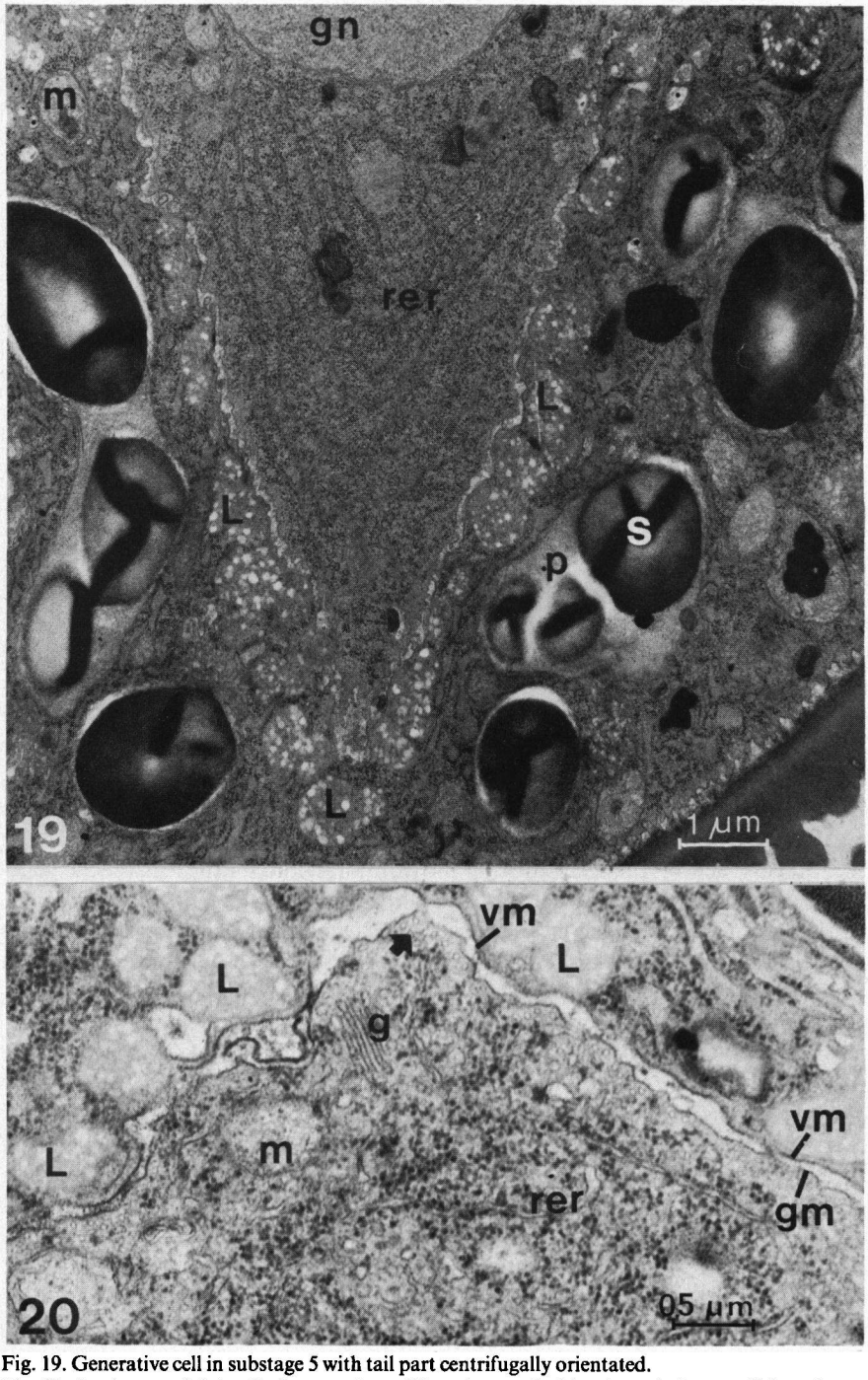

Fig. 19. Generative cell in substage 5 with tail part centrifugally orientated.
Fig. 20. Semitangential detail of generative cell in substage 5 with microtubules parallel to plasma membrane.

4 in the tails, but the individual cisternae become randomly spread. Connections between the two membranes surrounding the generative cell are observed. Microtubules are distributed parallel to the generative membrane, Golgi bodies consist of 4–5 cisternae with a length of about 0.5 μm. Mitochondria, Golgi bodies and ribosomes remain randomly distributed.

4. DISCUSSION

Since the formation of the pollen exine starts at the tetrad stage, the cells within the tetrad must be regarded as young pollen rather than as microspores. The intine is formed while the callosic wall dissolves. In *Euphorbia dulcis* plants the plasma membrane and the intine remain longer in contact with the callosic wall in the areas, where the three pores are formed. DICKINSON (1976) suggested that such event is related to the abscence of exine formation in the poral zones.

The plastids in the vegetative cell are present during the pollen development, and they undergo two amylogeneses. The first occurs during the tetrad stage, the second after the first pollen mitosis when the generative cell starts to move to the central part of the vegetative cell. It is found that amylogenesis is correlated with vacuolation. When one increases the other decreases, and vice versa, which comfirms the statement of SHIVANNA & JOHRI (1985).

In *Euphorbia dulcis* pollen the generative cell does not contain plastids. The plastid distribution, present in the vegetative cell and absent in the generative cell, is according to the *Lycopersicon* type (HAGEMANN 1981, 1983). Also in *Impatiens walleriana* and *I. glandulifera*, the plastids are only present in the vegetative cell (VAN WENT 1984). They are clustered in the cell opposite to the region where the generative cell is formed. In *Secale cereale* the disposition of plastids is only near the pore in the vegetative cell (WEBER, pers. communication). This situation is different from the one that is observed in *Lilium martagon*, (SCHRÖDER 1984). By contrast, he found plastids during the formation and maturation of the generative cell. In the vegetative cell, however, he observed degeneration of plastids.

Microtubules are observed in the generative cell of *E. dulcis*. Some authors hypothesize that they are involved in various processes and help to maintain the shape of the cell (SANGER & JACKSON 1971; CRESTI et al. 1984; LLOYD 1982).

In substage 2 in *E. dulcis* the intine is completed with particular wall formation. A new layer is present against the vegetative membrane that breaks the preceding formed tubules. This was also observed in Malvaceae and in *Olea europea* (HESLOP-HARRISON 1973; PACINI & CRESTI 1977; PACINI & JUNIPER 1979). In *E. dulcis* the intine is thick near the pores and forms a circular wall round the pores. It grows thicker during the development of the bicellular pollen grain. According to KNOX et al. (1976) the tubules are commonly considered as storage sites of gametophytic proteins involved in the pollen stigma recognition process. A similar intine is found in *Parietaria officinalis* L. (FRANCHI & PACINI 1980) under the pores. The wall between the generative cell and the vegetative cell is initially intercepted by plasmodesmata or wider plasma channels. Also in *Lilium longiflorum* the generative cell is connected to the vegetative cell

by many plasmodesmata (NAKAMURA & MIKI-HIROSIGE 1985). On the contrary the generative cell in *Tillandsia* tends to become isolated from the vegetative cell, and plasmodesmata disappear (BRIGHIGNA et al. 1981). Also KARAS & CASS (1976) in *Secale cereale* did not find plasmodesmata between generative and vegetative cell.

In *E. dulcis* the cisternae of RER are arranged in aggregations in the vegetative cytoplasm at the moment when the generative cell is in peripheric position. Later on, when the generative cell moves to the centre, cisternae are not stacked anymore. Other authors have also observed this, but only in mature pollen (KROH 1967; JENSEN et al. 1974; CRESTI et al. 1975. 1985). The last authors indicated that these cisternae are places for storage of proteins for later tube growth. Also JENSEN et al. (1974) found in some Scrophulariaceae similar ER cisternae surrounding the plastids and lipids. Such ER can be an energy source. In *E. dulcis* no RER was found fused with the generative or vegetative cell wall as reported for *Lilium longiflorum* (NAKAMURA & MIKI-HIROSIGE 1985).

At substage 4 in *E. dulcis* the generative cell is ellipsoidal with sharp ends. This is similar to the generative cell in *Rhododendrón* pollen, when the long coiled tails are not considered (THEUNIS et al. 1985).

Drops of lipid surround the generative cell. While the generative cell goes to the central position the lipid drops become electron-translucent spots. The lipid droplets never fuse with the generative cell wall as in *Lilium longiflorum* (NAKAMURA & MIKI-HIROSIGE 1985), where lipid droplets are present in the inner surface of the generative cell.

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REFERENCES

BRIGHIGNA, L., A. CECCHI FIORDI & M. R. PALANDRI (1981): Ultrastructural investigation on the two-nucleate pollen grain of Tillandsia caput-medusae Morr. (Bromeliaceae). *Amer. J. Bot.* **68**(8): 1033–1041.

CARANO, E. (1926): Ulteriori osservazioni su Euphorbia dulcis L. in rapporto col suo comportamento apomittico. *Annali Bot.* **17**: 50–79.

CESCA, G. (1961): Ricerche cariologiche ed embriologiche sulle Euphorbiaceae. I. Su alcuni biotipi di Euphorbia dulcis L. della Toscana. *Caryologia* **14**(1): 79–96.

— & D. MUZZI (1972): Ricerche citotassonomiche nelle Euphorbiaceae: Euphorbia angulata Jacq. ed E. dulcis L. *Giorn. Bot. Ital.* **106**(2): 89–99.

CRESTI, M., E. PACINI, G. SARFATTI & C. SIMONCIOLI (1975): Ultrastructural features and storage function of Lycopersicum peruvianum pollen. In: D. L. MULCAHY (ed.): *Gamete competition in plants and animals*. pp. 19–28. (2x) North Holland Publ. Co., Amsterdam.

—, F. CIAMPOLINI & R. N. KAPIL (1984): Generative cells of some angiosperms with particular emphasis on their microtubules. *J. Submicrosc. Cytol.* **16**: 317–326.

—, —, D. L. M. MULCAHY & G. MULCAHY (1985): Ultrastructure of Nicotiana alata pollen its germination and early tube formation. *Amer. J. Bot.* **72**(5): 719–727.

DICKINSON, H. G. (1976): Common factors in exine deposition. In: I. K. FERGUSON & J. MULLER (eds.): *The evolutionary significance of the exine,* Linnean Soc. Symp. Series 1: 67–90.

FRANCHI, G. G. & E. PACINI (1980): Wall projections in the vegetative cell of Parietaria officinalis L. pollen. *Protoplasma* **104**: 67–74.

HAGEMANN, R. (1981): Unequal plastid distribution during development of the male gametophyte of angiosperms. *Acta Soc. Bot. Poloniae* **50**(1–2): 321–327.

— (1983): The formation of generative and sperm cells without plastids in angiosperms and the underlying mechanisms. In: O. ERDELSKÁ (ed.): *Fertilization and embryogenesis in ovulated plants,* pp. 97–99, Veda, Bratislava, Czechoslovakia.

HEGELMAIER, F. (1901): Ueber einen neuen Fall von habitueller Polyembryonie. *Ber. deutsch. bot. Gesell.* **19**: 488–499.

— (1903):Zur Kenntnis der Polyembryonie von Euphorbia dulcis Jacq. (purpurata Thuill). *Ber. deutsch. bot. Gesell.* **21**: 6–19.

HESLOP-HARRISON, Y., R. B. KNOX & B. HOWLETT (1973): Pollen-wall proteins: "gametophytic" and "sporophytic" fractions in the pollen walls of the Malvaceae. *Ann. Bot.* **37**: 403–412

JENSEN, W. A., M. ASHTON & L. R. HECKARD (1974): Ultrastructural studies of the pollen of subtribe Castilleanae, family Scrophulariaceae. *Bot. Gaz.* **135**(3): 210–218.

KAPIL, R. N. (1961): Some embryological aspects of Euphorbia dulcis L. *Phytomorphology* **11**(1–2): 24–36.

KARAS, I. & D. D. CASS (1976): Ultrastructural aspects of sperm cell formation in rye: Evidence for cell plate involvement in generative cell division. *Phytomorphology* **26**: 34–45.

KNOX, R. B., A. CLARKE, S. HARRISON, P. SMITH & J. J. MARCHALONIS (1976): Cell recognition in plants: Determinants of the stigma surface and their pollen interactions. *Proc. Natl. Acad. Sci. USA* **73**(8): 2788–2792.

KROH, M. (1967): Fine structure of Petunia pollen germinated in vivo. *Rev. Paleobot. Palynol.* **3**: 197–203.

LLOYD, C. W. (1982): *The cytoskeleton in plant growth and development.* Academic Press, London.

NAKAMURA, S. & H. MIKI-HIROSIGE (1985): Fine-structural study on the formation of the generative cell wall and intine 3-layer in a growing pollen grain of Lilium longiflorum. *Amer. J. Bot.* **72**: 365–375.

PACINI, E. & M. CRESTI (1977): Viral particles in developing pollen grain of Olea europea. *Planta* **137**: 1–4.

—, B. E. JUNIPER (1979): The ultrastructure of pollen grain development in the olive (Olea europea). 1. Proteins in the pore. *New Phytol.* **83**: 157–163.

PUNT, W. (1962): Pollen morphology of the Euphorbiaceae with special reference to taxonomy. *Wentia* **7**: 1–129.

SANGER, J. M. & W. T. JACKSON (1971): Fine structure study of pollen development in Haemanthus katherinae Baker. II. Microtubules and elongation of the generative cells. *J. Cell Sci.* **8**: 303–315.

SCHRÖDER, M. B. (1984): Ultrastructural studies on the plastids of generative and vegetative cells in the family Liliaceae. 1. Lilium martagon L. *Biol. Zbl.* **103**: 547–555.

SHIVANNA, K. R. & B. M. JOHRI (1985): *The angiosperm pollen – structure and function,* Wiley Easter Ltd., New Delhi.

THEUNIS, C. H., C. A. MCCONCHIE & R. B. KNOX (1985): Three-dimensional reconstruction of the generative cell and its wall connection in mature bicellular pollen of Rhododendron. *Micron and Microscopia Acta* **16**: 225–231.

WENT, J. L. VAN (1984): Unequal distribution of plastids during generative cell formation in Impatiens. *Theor. Appl. Genet.* **68**: 305–309.