Protoplasma 107, 85—107 (1981)

PROTOPLASMA

Ultrastructure of the Sperms of *Plumbago zeylanica*

1. Cytology and Association with the Vegetative Nucleus

S. D. Russell * and D. D. Cass

Department of Botany, University of Alberta, Edmonton, Alberta

Received December 15, 1980
Accepted March 26, 1981

Summary

Male gametes of *Plumbago zeylanica* were examined in pollen grains and tubes using light and electron microscopy of chemically and physically fixed tissues, and Nomarski interference microscopy of isolated, living sperm cells. Male gametes are elongate, spindle-shaped cells containing a nucleus, mitochondria, ER, ribosomes, vesicles, dictyosomes, probable microfilaments, and a variable number of plastids. In mature pollen grains of *P. zeylanica,* the two sperm cells are directly linked; they share a transverse cell wall with plasmodesmata and are enclosed together by the inner vegetative cell plasma membrane. One of these two sperms is also associated with the vegetative nucleus as a consistent feature of pollen grain organization. The basis of this association appears to be a long, narrow projection of the sperm cell (averaging < 1 μm wide and about 30 μm long) which wraps around the periphery of the vegetative nucleus and occupies embayments of that nucleus. This association is maintained throughout pollen tube growth but becomes less extensive near the completion of tube growth and is severed following tube discharge. The consistent occurrence of the sperm-vegetative nucleus association in pollen grains, tubes and isolated pollen cytoplasm suggests that the two structures may be directly connected, but attempts to visualize this type of connection were unsuccessful. Possibly, the entwining nature and extent of complementary interfaces between vegetative nucleus and sperm may have a role in stabilizing their association. Functionally, the two sperms and vegetative nucleus appear to travel as a linked unit within the pollen tube, possibly increasing the effectiveness of gamete delivery and helping to ensure nearly simultaneous transmission of sperms into the receptive megagametophyte.

Keywords: Male cytoplasmic inheritance; *Plumbago;* Pollen grain; Pollen tube; Sperm; Ultrastructure.

1. Introduction

In flowering plants, small nonmotile male gametes fulfill the essential and highly specialized roles of fertilization and endosperm initiation. Apparently passive cells, the sperms are structurally simple; yet their nuclear content

* Correspondence and Reprints: Department of Botany, University of Alberta, Edmonton, Alberta, Canada T6G 2E9.

0033-183X/81/0107/0085/$ 04.60

determines half of the hereditary makeup of the embryo and their cytoplasmic content often plays a significant part in cytoplasmic inheritance in the embryo. The determination of these patterns of cytoplasmic inheritance are clearly influenced by sorting patterns and competition between organelles during generative cell formation (Hagemann 1976) and maturation (Clauhs and Grun 1977), yet few reports specifically concern the descendent sperm cells which actually participate in double fertilization. As our knowledge of male cytoplasmic inheritance in angiosperms expands, it has become evident that a greater understanding of sperm cytology is necessary on both genetic and embryological grounds.

The earliest and most striking evidence for the occurrence of male cytoplasmic inheritance in angiosperms were independent reports in 1909 that plastids, in particular, may follow non-Mendelian patterns of inheritance in *Mirabilis* and *Pelargonium*. In the former plant, Correns (1909) reported exclusively maternal patterns of plastid inheritance; in the latter, Baur (1909) reported biparental patterns of plastid inheritance resulting in variegated patterns during leaf development (see reviews by Grun 1976, Gillham 1978). The complementary information provided by these two reports clearly established the basis for extensive classical and modern research using plastid mutants to follow lines of male inheritance (Hagemann 1976). Although no genetic evidence exists for any zygotic "restriction mechanism" in angiosperms which would systematically eliminate a given class of male (or female) cytoplasmic organelles (Hagemann 1976), to the best of our knowledge this has been directly examined only in the case of plastid inheritance in *Oenothera erythrosepala* (Meyer and Stubbe 1974). Direct observations of sperm organelle structure in pollen grains and during tube growth may provide information on whether specific organelles or classes of organelles undergo alterations in ultrastructure which could affect their viability. It is expected that knowledge of the types and relative proportion of organelles within sperms of *Plumbago zeylanica* may aid in predicting their transmissibility into the zygote and the possibility of their inheritance in the next generation.

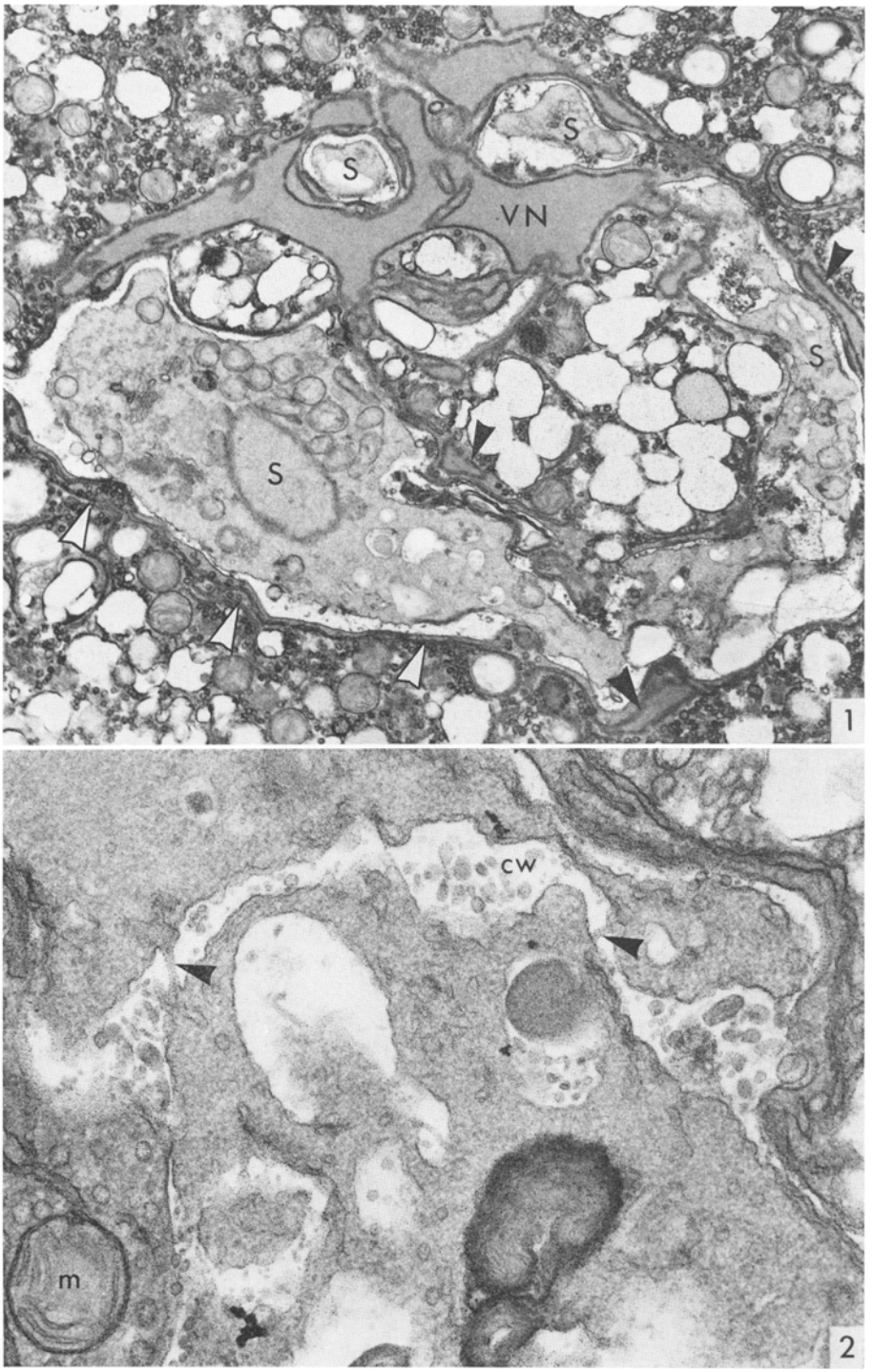
Certain embryological adaptations which may facilitate the passage of sperms within the pollen tube and their subsequent transmission into the embryo and

---

Figs. 1–2. Electron micrographs of chemically fixed mature pollen grains

Fig. 1. Longisection of two sperm cells (*S*) and part of a highly lobed vegetative nucleus (*VN*). Projections of the vegetative nucleus ensheath part of the sperm cell (black arrowheads). Endoplasmic reticulum is found near the outer surface of the sperm (white arrowheads). ×12,300

Fig. 2. Junction between two sperm cells, joined by a common vesiculate cell wall (*cw*) and plasmodesmata (arrowheads). Mitochondrion (*m*) is located in pollen cytoplasm. ×44,600



Figs. 1 and 2

endosperm have remained virtually unstudied, as have the specific structural changes required for gametic transmission. The prevalent assumption that sperms within pollen tubes travel independently of one another and the vegetative nucleus (Maheshwari 1949) does not appear to apply in *P. zeylanica*. In this plant, the association of these three structures may represent an adaptation to promote the passage of the sperms from the stigma to the embryo sac. Only one published work, to our knowledge, has reported detailed observations of sperm ultrastructure during pollen tube passage (Jensen and Fisher 1968). The specific structural changes, if any, required for sperms to fuse with the egg and central cell have been postulated, but are yet to be described from direct ultrastructural observation. Additional embryological issues which may influence the transmission of male cytoplasm, including differences between paired sperm cells and the possible non-transmission of some sperm cytoplasm, are the subjects of continuing research in our laboratory. Contrary to published literature (Maheshwari 1949, Jensen and Fisher 1968, Cass 1973), the two sperms of *Plumbago* appear to differ in cell volume; however, the biological significance of this observation remains unknown.

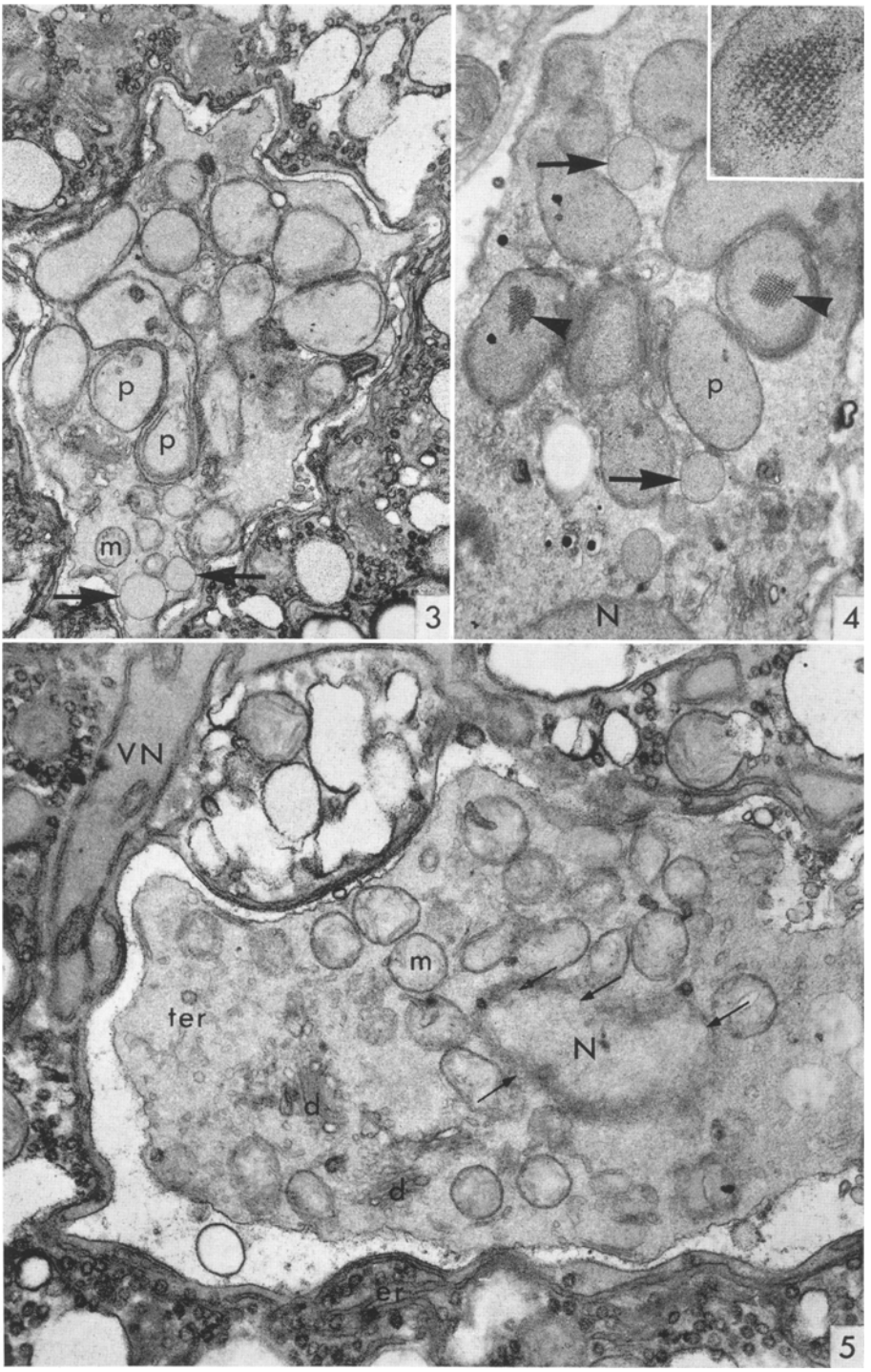
The present study describes sperm cytology and structural relationship in pollen grains and tubes of *P. zeylanica* using light and electron microscopy of chemically and physically fixed tissues, and Nomarski interference microscopy of isolated, living sperm cells. The genus *Plumbago* was originally selected for study in our laboratory because of the structural simplicity of its embryo sac, reported previously in Cass and Karas (1974) and Russell and Cass (in press). The embryo sac of this plant functions without synergids and instead the pollen tube releases male gametes directly between the egg and central cells (Russell 1980). For this reason, *Plumbago* is particularly suitable for studying the structural basis of male cytoplasmic inheritance in angiosperms and determining the fate of the male cytoplasm during the process of double fertilization. The present study represents the first part undertaken in such an investigation.

Figs. 3–5. Electron micrographs of chemically fixed mature pollen grains

Fig. 3. Transection of a sperm cell containing mitochondria (*m*), plastids (*p*), and two unidentified single membrane-bound organelles (arrows). ×16,900

Fig. 4. An aggregation of sperm plastids (*p*), some containing paracrystalline structures (arrowheads). Unidentified single membrane-bound organelles (arrows) are associated with plastids. *N* nucleus. (Fixed with glutaraldehyde-diaminobenzidine and osmium tetroxide.) ×11,400; inset ×64,000

Fig. 5. Longisection of sperm cells containing a nucleus (*N*) with numerous nuclear pores (small arrows), mitochondria (*m*), dictyosomes (*d*), and tubular endoplasmic reticulum (*ter*). *er* endoplasmic reticulum (vegetative cell); *VN* vegetative nucleus. ×23,200



Figs. 3–5

2. Materials and Methods

Plants of *Plumbago zeylanica* L. have been maintained under long-day conditions in University of Alberta greenhouses since 1972 and are the same plants and clones of those used by Cass and Karas (1974).

2.1. Chemical Fixation

Freshly opened anthers (within 1 hour of anthesis) and artificially-pollinated stigmas and styles were fixed 6–12 hours in 3% glutaraldehyde-M/15 phosphate buffer (pH 6.8) at 4 °C. Tissue was briefly rinsed in buffer, postfixed in cold 2% buffered osmium tetroxide, dehydrated in an ethanol series followed by propylene oxide, and embedded in low viscosity resin (Spurr 1969).

In order to compare the effect of different chemical fixatives on sperm cell wall preservation, the following procedures were also employed: 1. 1½ hour fixation in 3% glutaraldehyde with diaminobenzidine (2 mg/5 ml buffer) in 0.05 M propandiol buffer (pH 9.0) (Frederick and Newcomb 1969), rinsed briefly in buffer and osmicated; 2. 3 hour fixation in 3% glutaraldehyde-3% paraformaldehyde (Karnovsky 1965) in phosphate buffer (pH 6.8), rinsed, and osmicated; 3. 2 hour fixation in unbuffered potassium permanganate. All materials were dehydrated and embedded according to procedures outlined above. Among the chemical fixatives used, sperms fixed in standard glutaraldehyde and osmium appeared the least altered by preparation. Except as noted, all chemically fixed material illustrated in this study was prepared according to the first procedure.

2.2. Physical Fixation

Freshly opened anthers and artificially-pollinated gynoecia were immersed in an isopentane 12% methylcyclohexane solution at liquid nitrogen temperatures, transferred to methanol at dry ice temperatures, and dehydrated in several changes of absolute methanol at — 50 °C (Jensen 1962). After several days, material was slowly warmed, transferred to propylene oxide at room temperature, and embedded in low viscocity resin.

2.3. Specimen Preparation

Ultrathin sections were routinely cut using a Reichert OM U2 ultramicrotome and collected on uncoated nickel or gold grids. Selected stages in pollen tube growth were first thick-sectioned, photographed using Nomarski interference optics, and then reembedded for ultrathin

---

Figs. 6–10. Frozen-substituted pollen grains stained with uranium and lead (Figs. 6 and 7) and by PA-TCH-SP reaction (Figs. 8, 9, and 10)

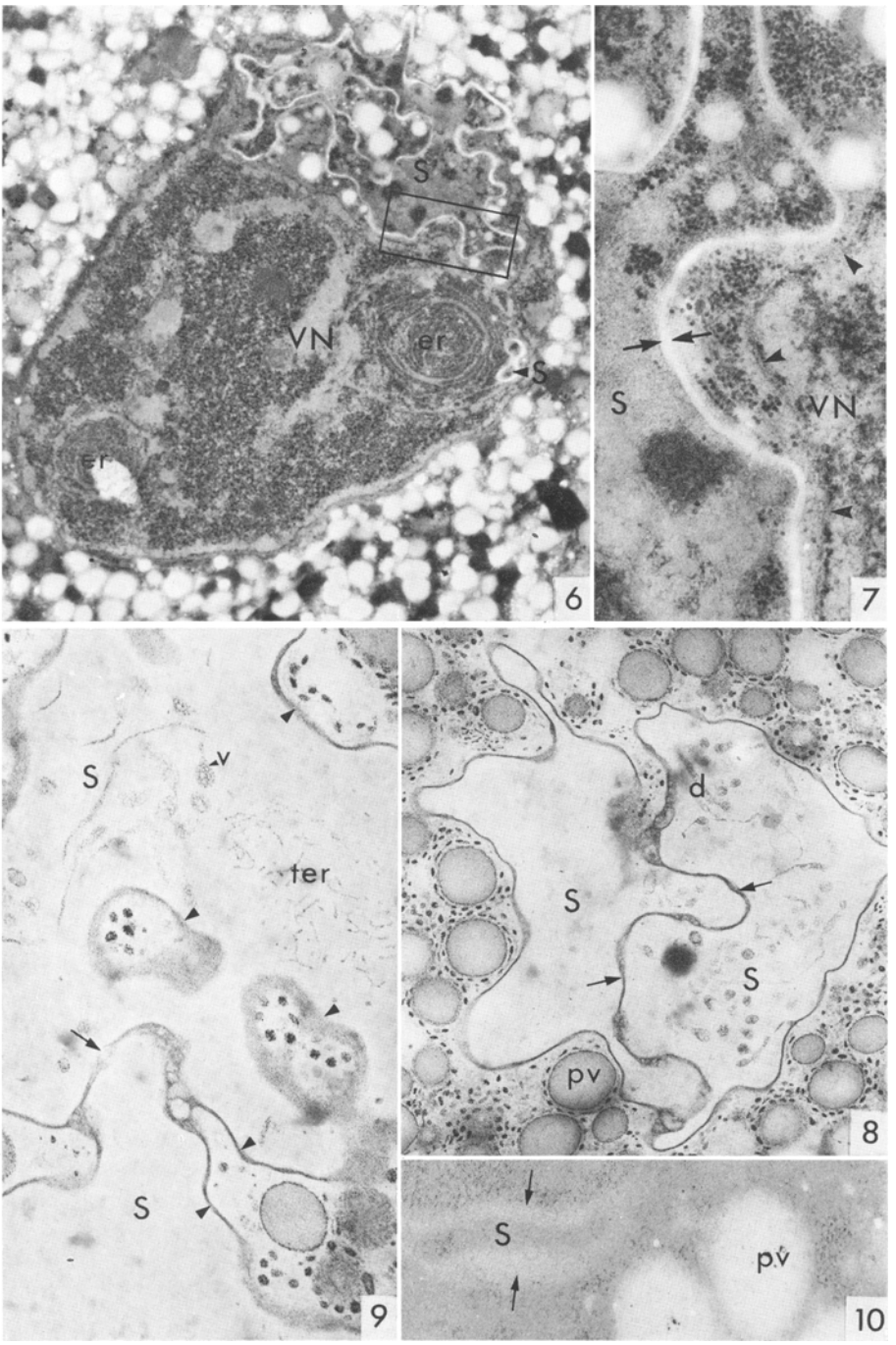
Fig. 6. Near-median section of vegetative nucleus (*VN*) showing a sperm cell (*S*) inserted within uniformly gray perinuclear cytoplasm. Two aggregations of rough endoplasmic reticulum (*er*) are present near sperm projection (labeled arrowhead). Region enclosed in box is shown in Fig. 7. ×7,400

Fig. 7. Sperm cell (*S*), cell wall (small arrows) and vegetative nucleus (*VN*). Unlabeled arrowheads indicate nuclear envelope. ×35,200

Fig. 8. Junction between two sperm cells (*S*) with an extensive segment of shared cell wall (arrows). *d* dictyosome; *pv* polysaccharide vesicle. ×13,700

Fig. 9. Sperm cells (*S*) delimited by PA-TCH-SP reactive cell walls (small arrowheads). Possible plasmodesma at the sperm cell junction is indicated by an arrow. *ter* tubular endoplasmic reticulum; *v* vesicle. ×30,600

Fig. 10. Sperm (*S*) and cell wall (arrows) after PA-TCH-SP reaction without periodic acid oxidation. (This figure was printed with the same contrast photographic paper as in Figs. 8 and 9, but exposed three times longer.) *pv* polysaccharide vesicle. ×35,000



Figs. 6–10

sectioning according to the technique described by Mogensen (1971). Sections were stained using 2% uranyl acetate in 45% ethanol followed by 0.2% lead citrate (Venable and Coggeshall 1965), or by subjecting material mounted on gold grids to the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) reaction (Thiéry 1967), with 30 minutes oxidation in 1% periodic acid, 6 hours in 0.2% thiocarbohydrazide in 20% acetic acid, and 30 minutes in 1% silver proteinate solution.

2.4. Serial Reconstruction of Sperm Cells

Serial ultrathin sections were collected using naked slot grids, transferred on the surface of a drop of water, and mounted on Formvar-coated slot grids (Galey and Nilsson 1966). Serial sections containing sperm cells were numbered consecutively, photographed in the electron microscope, and printed at the same magnification. A two-dimensional line was plotted over the center of the sperm projection using a plastic overlay. At bends in the line, points were plotted and the section number containing the center of the sperm projection at that point was recorded. Average section thickness was determined by measuring the diameter of the sperm nucleolus (assumed to be spherical) and dividing by the number of sections in which the nucleolus penetrates the section. The distance and height between two adjacent points on the line was converted into micrometers and recorded. The total length of the sperm projection was then calculated as the sum of the distances between adjacent points using the Pythagorean theorem.

2.5. Observation of Isolated, Living Sperm Cells

Isolated, living sperm cells were examined by releasing the contents of mature pollen grains in a pollen tube growth medium and observing them in Nomarski interference optics. The sucrose content of the medium was adjusted so that sperm cell volume remained constant throughout the 30 minutes observation period (Cass 1973). Sperm cells which displayed cytoplasmic activity were photographed with a Zeiss Photomicroscope I at 2- to 5-minute intervals during the observation period.

3. Results

3.1. Ultrastructure Within the Mature Pollen Grain

3.1.1. Sperm Cell Organization

Mature pollen grains of *P. zeylanica* each contain two roughly spindle-shaped male gametes which are delimited by the sperm plasma membrane, a rudimentary cell wall, and enclosed together within an internal pollen plasma membrane (Figs. 1–3, 5, and 12). These sperms are derived from the same generative cell and share a common transverse cell wall, but differ significantly with respect to their morphology. The male gamete immediately adjacent to the vegetative nucleus has a long, slender (usually < 1 μm wide) projection which wraps around the edge and occupies embayments of the vegetative nucleus (Figs. 1, 11, and 12); the other male gamete has a short free end projecting into vegetative cytoplasm. In one sperm cell reconstructed from serial ultrathin sections, this projection reached a length of 30 μm (Fig. 11), while the other sperm cell (not shown) was, in its entirely, only 8 μm long. While the two sperm appear to differ with respect to cell volume, no other cytological differences related to their differing morphologies were observed (see section 3.2.2.).

Both sperm cells contain a normal complement of organelles, the majority of which are clustered near the nucleus in the widest part of the cell (Figs. 1, 4, 5, and 12). The sperm cells observed contain numerous mitochondria, plastids, endoplasmic reticulum (ER), dictyosomes, vesicles, and small vacuoles. Nuclei are elongate (about 3 by 6 μm) with numerous pores in the nuclear envelope (Fig. 5). A nucleolus is typically present in the nucleus and although small (approx. 1 μm), it is readily identifiable in light microscopic preparations (Figs. 15 and 18). Chromatin, visible in the nucleus, is only slightly condensed (Fig. 12).

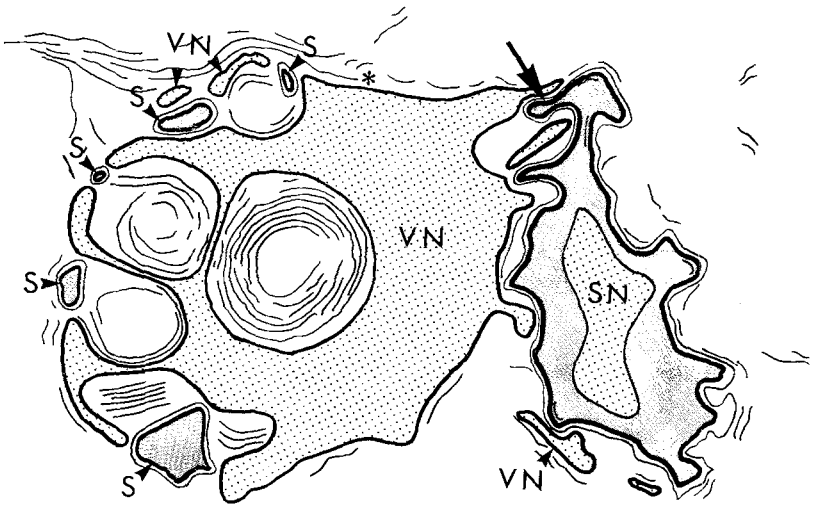


Fig. 11. Schematic representation of the association between one sperm (*S*) and the vegetative nucleus (*VN*) in a pollen grain as traced from an electron micrograph. A reconstruction of this sperm prepared using serial sections revealed that this sperm's projection (labeled arrowheads) was about 30 μm long, extending from the arrow, wrapping clockwise around the periphery of the vegetative nucleus, and terminating near the asterisk. Note aggregations of ER (represented by thin lines). Embayments and ensheathing lobes (labeled arrowheads) of the vegetative nucleus are evident. *SN* sperm nucleus. ×10,400

Sperm mitochondria are usually smaller than those in vegetative cytoplasm and are spheroidal to ellipsoidal in shape, possessing narrow but well-developed cristae (Fig. 5). Plastids (Figs. 3 and 4) are larger than mitochondria, and their stromata are electron dense in uranium and lead stained material. Plastids contain a number of rudimentary lamellae, internal vesicles, ribosomes, and occasionally, paracrystalline structures in their stromata (Fig. 4 and inset). Paracrystalline structures like these have been found within immature and senescent plastids in somatic tissues and are believed to be composed of accumulated enzymes or ferritin (Toyama 1980). Mitochondria and plastids are often found in clusters composed almost exclusively of either

mitochondria (Fig. 5) or plastids (Fig. 4), and the two are rarely seen together in a given thin section of sperm cell. (An exception can be seen in Fig. 3.) An as yet unidentified class of single-membrane bound organelles was occasionally seen in association with plastids (Figs. 3 and 4, unlabelled arrows). These bodies could represent either electron-dense vesicles or microbodies. Microbodies, although rare in angiosperm gametophytes, have been reported in the female gametophyte of *P. zeylanica* (Cass and Karas 1974).

Sperm cell dictyosomes are abundant, well-developed, and possess numerous cisternae, with clear vesicles present near the maturing face of the dictyosome. Occasionally, an apparently anastomosing network of tubular ER is seen near dictyosomes (Fig. 5). Lamellar cisternae of ER occur as isolated segments at the periphery of the cell but are rarely well developed (Figs. 5 and 11). In frozen-substituted material (Figs. 8–10), dictyosomes, some vesicles, ER, and the sperm cell wall are all stained by the PA-TCH-SP reaction.

A rudimentary cell wall appears to surround the sperms in chemically (Fig. 2) and physically fixed pollen grains (Figs. 6 and 7, unlabelled arrows). In chemically fixed tissues, the cell wall varies from 0.1 to 0.2 μm in thickness and contains numerous vesicles. Polysaccharides were poorly stained by the PA-TCH-SP reaction in chemically fixed tissues (Fig. 3; compare with Fig. 2, prepared without PA). In contrast, frozen-substituted sperms have narrower cell walls, 0.05 to 0.1 μm wide, with vesicles restricted to the segment of wall shared by the two sperms. Physically-fixed cell walls stain intensely after the PA-TCH-SP reaction (Figs. 8–10). Comparing the speed of fixation, the uniformity of wall thickness, and the density of PA-TCH-SP staining after freeze-substitution, it appears that physically fixed sperm cell walls are less altered by preparation than those fixed by chemical means.

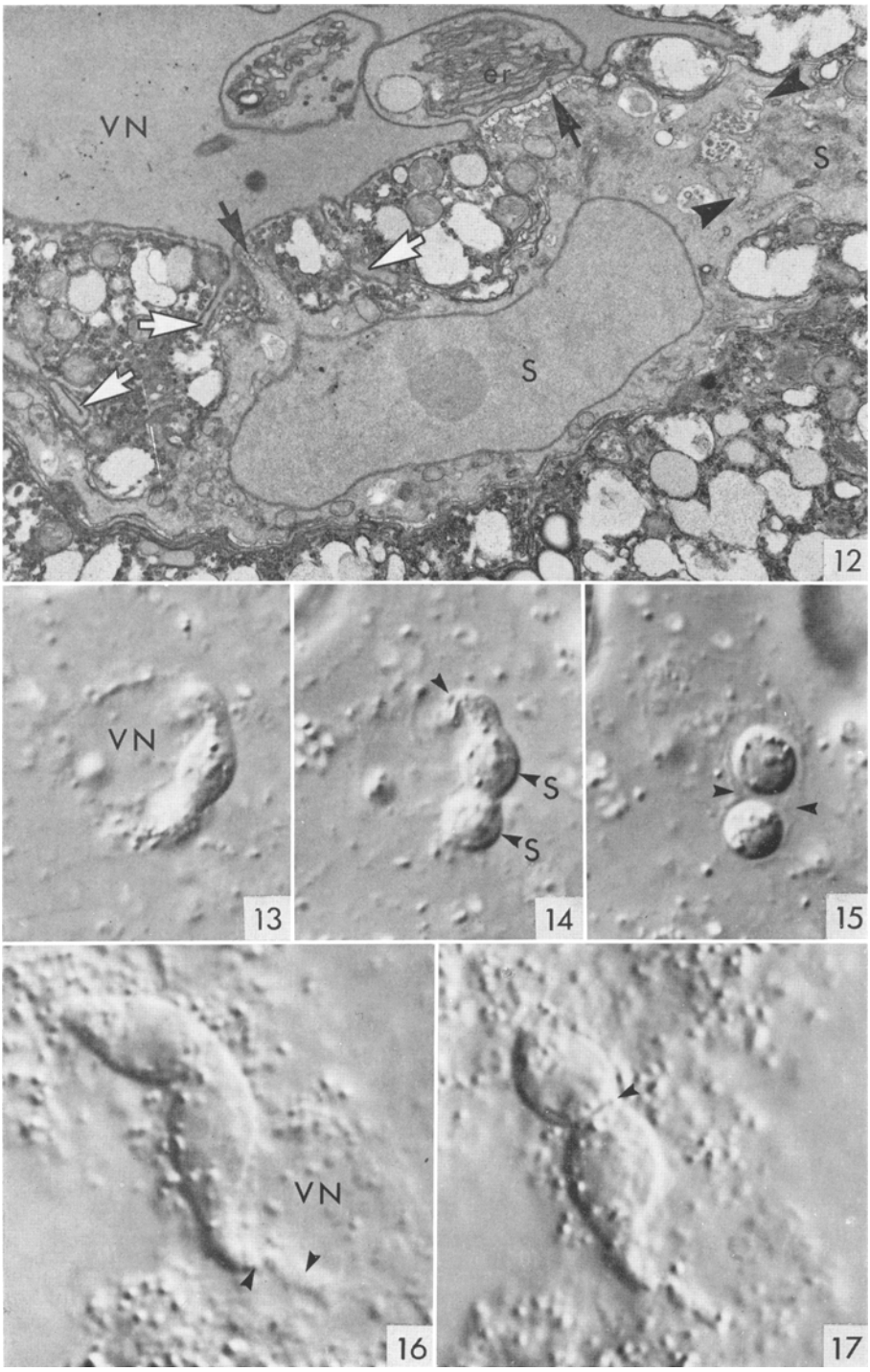
Microtubules were not observed in sperm cells during the course of this study, although identical techniques reveal numerous microtubules in generative cells of *P. zeylanica* (unpublished observations). At anthesis and throughout pollen tube growth, microtubules are rare or possibly absent. Probable micro-

---

Fig. 12. The structural association of sperm cell (*S;* black arrows) and vegetative nucleus (*VN;* white arrows) in a mature pollen grain. The other sperm cell is visible in the upper right corner, immediately adjacent (arrowheads) to the first sperm cell. ×10,700

Figs. 13–15. Sperm cells (*S*) and vegetative nucleus (*VN*) immediately after isolation in a pollen tube growth medium. Photographed with Nomarski differential interference optics at 5 minute intervals. Unlabeled arrowheads indicate a sperm cell projection in Fig. 14 and the internal pollen plasma membrane in Fig. 15. ×1,000

Figs. 16 and 17. Same as Figs. 13–15, except isolated in pollen cytoplasm and photographed over a 20 minute interval. Arrowheads indicate a sperm cell projection in Fig. 16 and a modified sperm-to-sperm junction in Fig. 17. ×1,550



Figs. 12–17

filaments were seen in *P. zeylanica* (Fig. 24, unlabelled arrow), but difficulties in preserving microfilaments may exist since they were observed infrequently.

3.1.2. Vegetative Cell Organization

The vegetative cell contains a large polymorphic nucleus, many mitochondria, plastids, occasional starch grains, ER, ribosomes, and numerous dictyosomes and polysaccharide vesicles. The nucleus, up to 13 μm long, is a highly lobed, irregular structure with deep embayments (Figs. 1, 6, 11, and 12) and numerous nuclear pores (Figs. 1 and 5). Within embayments of the nuclear envelope and immediately surrounding the nucleus, rough endoplasmic reticulum often forms intricate membrane arrays (Figs. 11 and 12). Elsewhere, lamellar ER is often found adjacent to sperm cells becoming especially pronounced on the surface opposite to the vegetative nucleus (Figs. 1, 5, 11, and 12). In frozen-substituted preparations, the perinuclear cytoplasm contains few vesicles or other organelles and sometimes appears to surround cellular projections of the sperm (Figs. 6 and 7).

The vegetative pollen cytoplasm contains numerous, small vesicles with PA-TCH-SP stained polysaccharides (Figs. 8–10). These vesicles can be divided into two classes on the basis of size and staining characteristics, and can be identified in both chemically and physically fixed tissues. Following periodic acid oxidation the smallest vesicles, 0.05 to 0.1 μm in size, stained densely with the PA-TCH-SP reaction (Figs. 8 and 9, compare Fig. 10 without PA oxidation). The remaining vesicles, from 0.3 to 0.6 μm in median section, have an electron-dense PA-TCH-SP reactive perimeter and less densely stained contents (Figs. 8 and 10). In frozen-substituted preparations of vegetative cytoplasm, PA-TCH-SP reaction products were also detected in dictyosomes, starch grains, and the pollen intine.

Figs. 18–22. Chemically-fixed style with growing pollen tube.

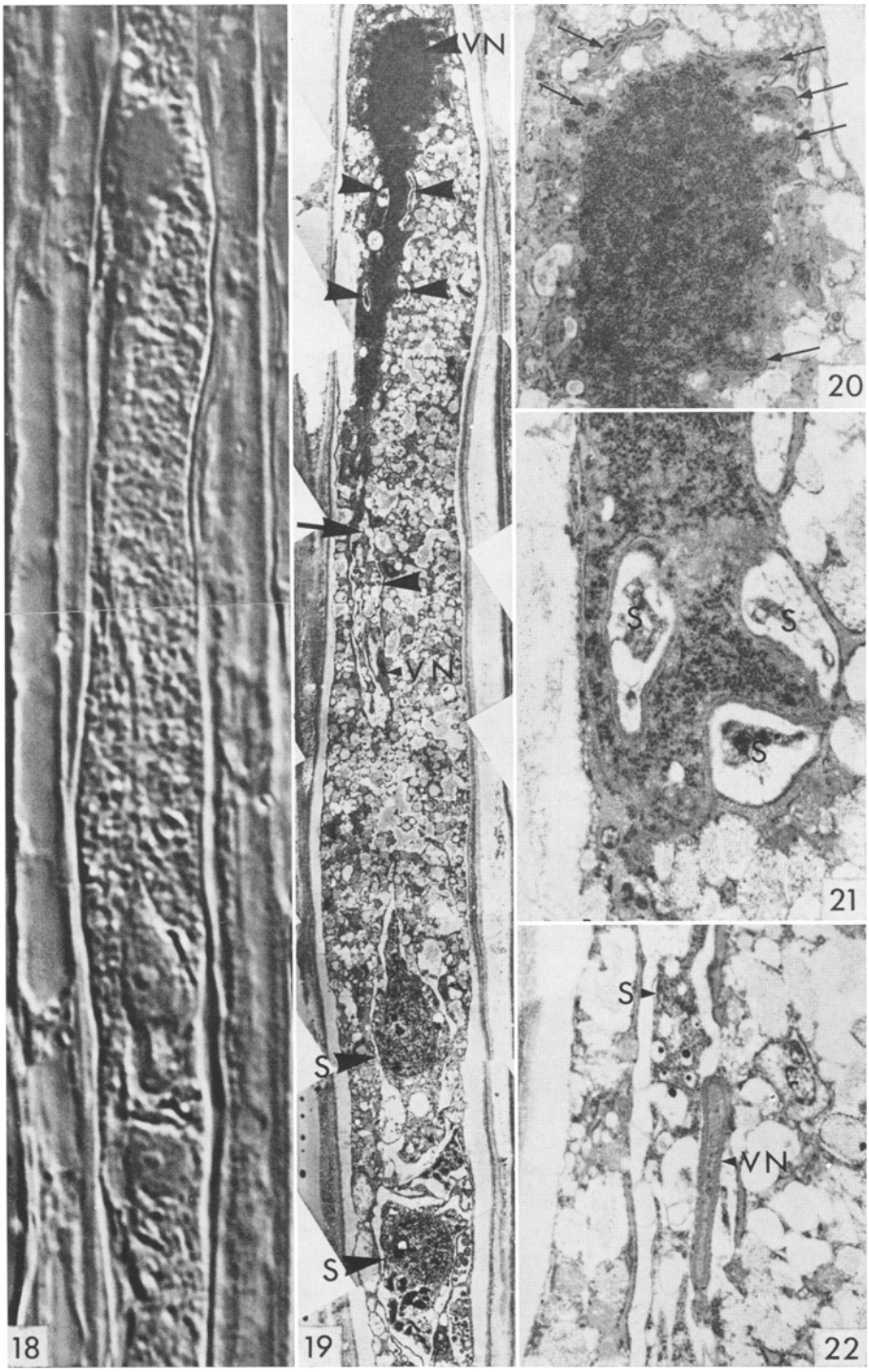
Fig. 18. Nomarski interference micrograph of the vegetative nucleus and two sperms during the rapid elongation phase in a pollen tube growing through the style. The pollen tube apex is oriented toward the top of the page. Arrowheads indicate a strand of vegetative nucleus and sperm linking these two bodies. ×2,000

Fig. 19. Correlative electron micrograph obtained by reembedding and ultrathin-sectioning the pollen tube in Fig. 18. Unlabeled arrowheads indicate strands of sperm cell cytoplasm associated with elongate vegetative nucleus (*VN*). Two sperms (*S*) remain linked by a shared cell wall. ×2,300

Fig. 20. The leading face of the vegetative nucleus with numerous lobes at its periphery (small arrows). ×7,750

Fig. 21. Segments of sperm cell (*S*) visible near the thin perinuclear cytoplasm at the trailing edge of the same vegetative nucleus. ×14,500

Fig. 22. The most proximal portion of vegetative nucleus (*VN*) is also associated with sperm cell. The same segment of VN is indicated by a labeled arrowhead in Fig. 19. ×10,150



Figs. 18–22

3.2. Structural Interrelationships of Sperms and Vegetative Nucleus

3.2.1. Associations Within Mature Pollen Grains

In mature pollen grains of *P. zeylanica*, the two sperm cells are directly linked; they share a transverse cell wall with plasmodesmata and are enclosed together by the inner vegetative cell plasma membrane (Figs. 2, 8, and 9). One of these two sperms is also associated with the vegetative nucleus as a consistent feature of pollen grain organization (Figs. 1, 5, 6, 11, and 12). The basis of this association appears to be a long, narrow projection of the sperm cell (averaging < 1 μm wide and about 30 μm long; Fig. 11) which wraps around the periphery of the vegetative nucleus and occupies embayments of that nucleus. Analysis of serial ultrathin sections also reveals that nearly 70% of the sperm projection and 10% or more of the main cell body may be ensheathed by lobes of the vegetative nucleus. While sperms never directly contact the vegetative nucleus, sometimes sperm projections are separated from the nuclear envelope by as little as 0.1 μm; projections are typically embedded in a morphologically distinct perinuclear cytoplasm (Figs. 1, 6, 7, 11, and 12). Although we looked specifically for cellular structures which might directly link one sperm or its surrounding membranes with the vegetative nucleus, we were unable to find any.

3.2.2. Associations of Sperms and Vegetative Nucleus Within Isolated Pollen Grain Contents

Reconstructing the sperm-vegetative nucleus association in the living state is simplified by releasing pollen grain contents into an isotonic medium and briefly observing them with Nomarski interference optics. Newly isolated sperm cells appear roughly spindle-shaped and are closely associated with the vegetative nucleus and with one another (Figs. 13 and 16). While both sperms are similar in shape, the one adjacent to the vegetative nucleus has a longer projection and greater cellular volume than the other sperm. Part of

---

Fig. 23. Frozen-substituted pollen tube fixed during the slow phase of tube growth near the ovule. Somewhat rounded sperm cells (*S*) remain surrounded by a clear zone (arrowheads) which represents cell wall. Vegetative nucleus (*VN*) remains closely associated with sperms. The pollen tube apex is shown in the upper right corner of this figure. ×2,060

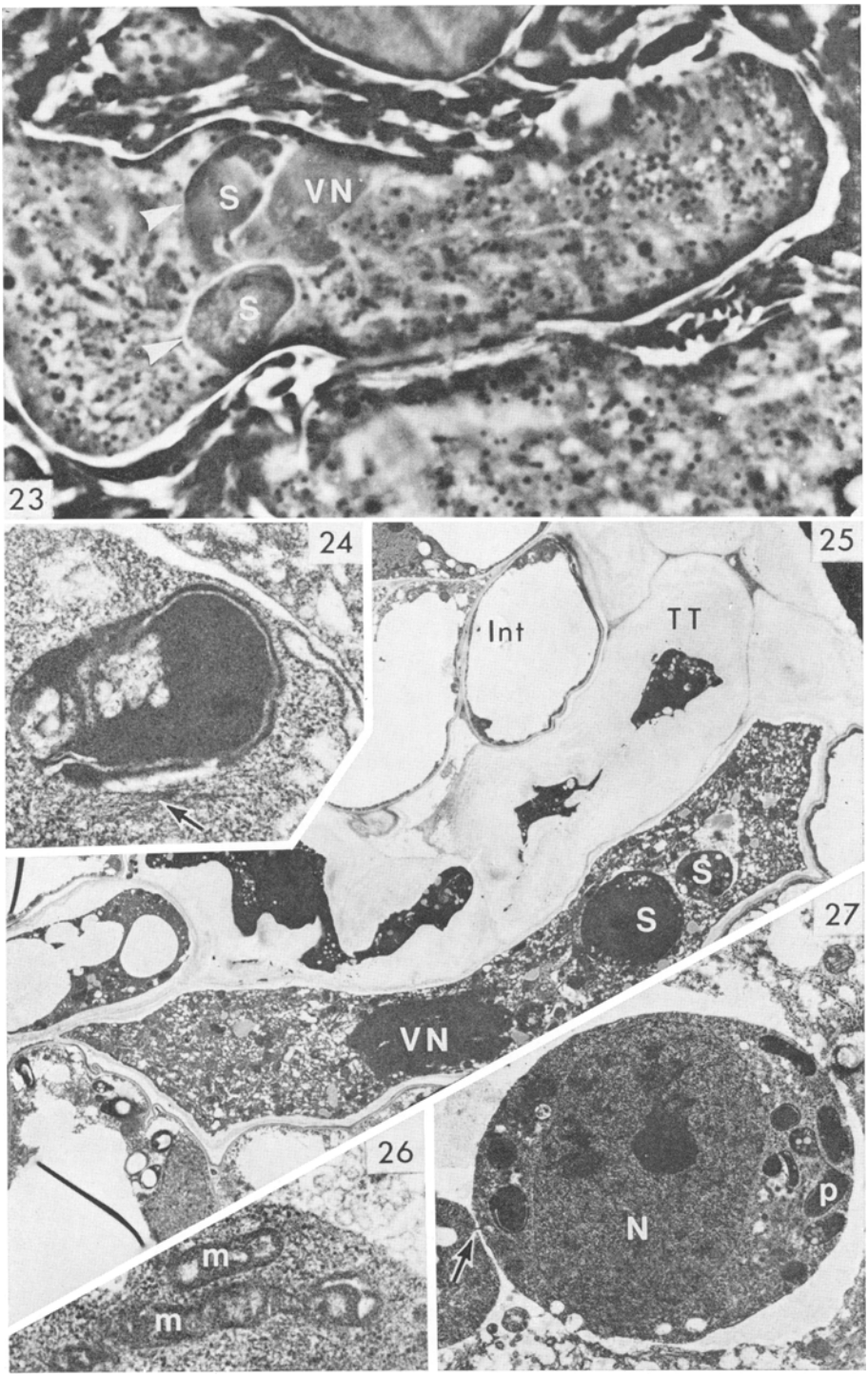
Figs. 24–27. Electron micrographs of chemically fixed pollen tubes in the style

Fig. 24. A sperm plastid during late pollen tube growth near the ovule; unlabeled arrow in pollen cytoplasm indicates probable microfilaments. ×44,000

Fig. 25. The vegetative nucleus (*VN*) leading two rounded sperm cells (*S*) as pollen tube leaves the stylar transmitting tissue (*TT*) and contacts an integument (*Int*). ×2,900

Fig. 26. Sperm mitochondria (*m*) become elongate, and cristae expand during late phases of pollen tube growth. ×41,000

Fig. 27. Two rounded sperm cells remain linked (arrow) as pollen tube approaches the ovule. *N* nucleus; *p* plastid. ×9,400



Figs. 23–27

this sperm appears to extend just within the edge of the vegetative nucleus membrane, presumably in embayments of the nuclear envelope (Figs. 14 and 16).

Observation of living sperm cells in isolation also provides information on the regulation of sperm cell shape and the possibility of independent cell movement. Sperms remaining surrounded by their native pollen cytoplasm do not change greatly in cell shape or structural association over the observation interval (Figs. 16 and 17). However, sperms which were separated from surrounding pollen cytoplasm appeared to undergo rapid degenerative changes in both cell shape and structural association within 10 minutes (Figs. 13–15). Cytoplasmic activity was observed in sperm cells throughout the observation period, but in none of the sperms observed were conformational changes reversed. Sperms did not appear to display any shape changes related to motility or directional movement.

3.2.3. Associations of Sperm and Vegetative Nucleus Within the Pollen Tube

The nature of the sperm-vegetative nucleus association remains unchanged throughout pollen tube growth, but the distance between these two structures is greatly altered during rapid tube elongation. Early pollen tube growth is rapid (up to 5 mm/hour or more, *in vivo*) and pollen tube diameter is relatively narrow. During this phase, the main body of the vegetative nucleus precedes the sperm cells by up to 50 μm (Figs. 18 and 19) and is nearly as wide as the bore of the tube. The vegetative nucleus is highly elongated, with numerous lobed extensions near its apex (Fig. 20, arrows). Sperm cells travel within the tube as a linked pair, and continue to share a common cell wall.

In light microscopy, a strand passing between the vegetative nucleus and the leading sperm cell can be distinguished (Fig. 18). Electron microscopic examination reveals that this strand is composed of vegetative nucleus, internal pollen plasma membrane, and projections of the leading sperm cell (Figs. 19 to 22). Projections of sperm cytoplasm are relatively narrow and sometimes appear to be isolated within a thin layer of perinuclear cytoplasm (Fig. 21). The leading sperm cell is demonstrably contiguous for over 30 μm from the center of the strand (Fig. 19, unlabelled arrow) to the sperm cell common wall; it may become more than 60 μm long, since sperm cytoplasm can also be recognized near the main body of the vegetative nucleus (Fig. 19, unlabelled arrow-heads). The vegetative nucleus, itself, stretches to > 40 μm at this stage (Figs. 19 and 22). The region of sperm-vegetative nucleus overlap may extend for a distance of > 35 μm (a length consistent with that of the sperm projection in pollen grains; see Fig. 11).

During a slower phase of pollen tube elongation (about 250 μm/hour, *in vivo*) which occurs near the ovule, tube diameter increases until the two sperms can fit beside each other in the tube (Fig. 23). Sperms remain associated with the vegetative nucleus even after entering the micropyle (unpublished data), but

more loosely than before as the sperms become more rounded (Figs. 24 and 27). In these last two figures, sperm projections do not occur, but lobes of vegetative nucleus are located as closely as 0.1 μm from the leading sperm. Sperm cells appear to remain connected by plasmodesmata as the pollen tube nears the micropyle (Fig. 27, arrow), and remain closely associated even after their discharge from the pollen tube within the embryo sac (RUSSELL 1980).

3.3. Sperm Cell Modifications During Pollen Tube Growth

During the course of pollen tube growth, sperms become more rounded and less irregularly shaped in both chemically and physically fixed stylar tissues (Figs. 18, 23, and 25). In frozen-substituted material, sperm cells become rounded and more regularly shaped, but not spherical. Sperms continue to possess a periodic acid-Schiff's (PAS) reactive cell wall, visible in unstained sections as a clear region (Fig. 23); however, the stainability and extent of this wall decreases during pollen tube growth. Sperm cell projections are usually observed but are less extensive than in pollen grains.

Sperm organelles are not greatly modified during pollen tube growth, but changes in the condition of mitochondria appear noteworthy. Sperm mitochondria, which are round at anthesis and have narrow cristae (Fig. 5), elongate during pollen tube growth and their cristae expand considerably (Fig. 26). These alterations were also observed in the pollen tube and were not observed in surrounding stylar tissue (unpublished data). The sperm nucleus becomes more nearly spherical near the completion of tube growth, presumably as a result of changes in cell shape. Nuclear contents appear to become slightly more condensed (Figs. 23 and 25). No significant changes were observed in the nucleolus or in plastids.

4. Discussion

4.1. Sperm Cell Structure

Sperm and generative cells examined to date share a number of common cytological features. These include the presence of a nucleus, free and bound ribosomes, endoplasmic reticulum, vesicles, dictyosomes, and mitochondria [1]. Additionally, generative and sperm cells are delimited by their own plasma membrane and are surrounded by the inner vegetative cell membrane. However, the cytology of flowering plant sperms appears to vary with regard to the inclusion of plastids, microtubules, microfilaments, and the presence and extent of a surrounding cell wall (CASS 1973, CASS and KARAS 1975, CHENG, SHIYI, LIYUN, XIURU, and JIAHENG 1980, HOEFERT 1969, JENSEN and FISHER 1968, KARAS and CASS 1976).

[1] In a single exception, *Epidendrum,* the generative cell appears to lack mitochondria (COCUCCI and JENSEN 1969).

The presence of generative cell plastids appears to be a prerequisite for the transmission and inheritance of male plastome genes (Hagemann 1976). Angiosperms which display uniparental (maternal) patterns of plastome inheritance, as do the majority of flowering plants (Gillham 1978), typically exclude plastids during generative cell cytokinesis (Hagemann 1976) or eliminate plastids by means of a "lethality factor" during generative cell maturation (Clauhs and Grun 1977). However, the presence of plastids in the generative cell, while permitting transmission of paternal plastids into the sperm, does not necessitate their genetic expression in the embryo. In some varieties of *Oenothera,* a well-described but apparently little understood process of nucleocytoplasmic incompatibility appears to decrease the replication rate of male plastids, alters their genetic expression, and eventually may result in the elimination of paternal plastids within the mature plant (Grun 1976, Gillham 1978). A number of other possibilities in which generative cell plastids may be transmitted but uninherited include: 1. spontaneous or nuclear-induced failure of sperm plastids to replicate or to express certain genetic characteristics; 2. differential plastid apportionment during generative cell division; 3. transmission of sperm plastids into a cell lineage which is unrepresented in the mature plant (*e.g.*, the suspensor or basal cell); 4. failure of sperm cytoplasm to enter the egg during gamete fusion. Although cytological evidence contradicts the fourth alternative in *P. zeylanica* (Russell 1980), an evaluation of the remaining options will require a thorough knowledge of plastome genetics and ultrastructure in this plant. In considering the transmission of other heritable structures in the generative or sperm cell cytoplasm, including mitochondria or virions, similar arguments may be made. Presuming that conditions of non-inheritance like those described above are not in effect, one would expect, based on the present study of sperm cytology, that *P. zeylanica* will display biparental inheritance patterns of plastid and mitochondrial genes, and transmit other heritable structures present in male cytoplasm.

The nature of the cell walls surrounding male gametes may be expected to influence sperm cell physiology and gametic transmission, yet there is no clear, general understanding of their construction and extent. In *P. zeylanica,* plasmodesmata and vesicles are present in sperm cell walls regardless of preparative techniques used; fibrillar substructure is not visible. In four different chemical fixation regimes, variable distances were observed between sperm and vegetative cell membranes and attempts to stain the sperm cell wall with the PAS and PA-TCH-SP reactions produced negligible results. Thus, sperm cell walls which were chemically fixed appeared to be altered during preparation. Physically-fixed, frozen-substituted mature pollen grains produced a far more consistent image of sperm cell wall structure and thickness, staining intensely after PAS and PA-TCH-SP reaction. In these preparations, the sperm cell wall is 0.05 to 0.1 μm wide and decreases after several hours

of pollen tube growth to less than 0.03 μm. Near the completion of pollen tube growth, the sperm boundary appears to consist of only a thin layer of polysaccharides rather than a cell wall in the conventional sense. Following discharge from the pollen tube, sperm cell wall appears to disperse almost completely (unpublished data), and therefore would not be expected to interfere with the completion of gamete fusion.

The regulation of mature sperm cell shape in *P. zeylanica* is an intriguing question in light of the structural relationships reported between sperms and the vegetative nucleus. In this regard, the presence and extent of microtubules and microfilaments should receive special consideration, as these two structures are generally believed to have major cytoskeletal roles. The establishment of the spindle shape typical of mature sperm cells occurs in the progenitor (generative) cell in *P. zeylanica.* At this stage the generative cell has numerous, longitudinally-oriented microtubules located adjacent to the plasma membrane. Presumably, these microtubules serve, at least in part, a cytoskeletal role and may as in *Endymion* and *Haemanthus* generative cells aid in the transformation of the immature, ellipsoidal generative cell into its mature spindle shape (BURGESS 1970, SANGER and JACKSON 1971). Apparently, in *Endymion,* some cytoskeletal microtubules may become incorporated in forming the mitotic spindle during generative cell division (BURGESS 1970). In *P. zeylanica,* a similar phase of microtubular depletion seems to occur during sperm cell formation; however, in sperms of this plant, unlike sperms of other trinucleate pollen reported to date (*Beta,* HOEFERT 1969, *Hordeum,* CASS 1973, CASS and KARAS 1975; *Secale,* KARAS and CASS 1976; *Triticum,* CHENG *et al.* 1980), cytoskeletal microtubules apparently are not reestablished after generative cell division. In *Plumbago* unlike the latter plants, cellular morphogenesis appears to precede sperm cell formation (unpublished data), whereas in *Hordeum, Secale,* and *Triticum,* the onset and completion of morphogenesis apparently follows sperm formation. *Beta* appears to represent an intermediate case, in which the onset of morphogenesis precedes generative cell division, but is not completed until after the two sperms are formed (HOEFERT 1969, 1971).

The presence of infrequently observed microfilaments may contribute to the regulation of sperm cell shape but the extent of their influence is unknown. Correlated light microscopic examination of living, isolated sperm cells reveals that immediately upon release from the pollen grain, local irregularities in sperm shape decrease and that sperms initially retain their spindle shape and structural associations. Sperms also display extensive cell plasticity *in vivo* during passage in normally growing pollen tubes (Figs. 18 and 19). Such observations of sperm cytology and behavior suggest that the external environment of sperm cells plays an important role in maintaining cell shape. Observed shape changes *in vivo* may result largely from changing cytoplasmic conditions in the pollen grain and growing pollen tube. Based on the apparent

absence of highly organized cellular components known to influence cell movement in other biological systems, and our own observations of living, isolated sperms, we feel that the possibility of directional motility in sperm cells of *P. zeylanica* is highly unlikely.

4.2. Structural Associations Involving Sperm Cells

The two sperms and the vegetative nucleus are consistently associated in pollen grains and tubes of *P. zeylanica* independent of the mode of fixation or other preparative procedures (see section 3.2.). The two sperms are joined by a transverse cell wall with plasmodesmata (Fig. 2, arrowheads) and are surrounded by the inner vegetative cell membrane. The transverse cell wall probably originates during generative cell cytokinesis. One of the two sperm cells is consistently associated with the vegetative nucleus, sharing complex complementary boundaries over much of its surface. The basis of this association appears to be a long, narrow projection of the associated sperm cell which wraps around and occupies embayments of the vegetative nucleus; only a thin sheath of perinuclear cytoplasm appears to separate these two structures. Lobes of the vegetative nucleus, in turn, partially ensheath this associated sperm cell. Two important features of the sperm-vegetative nucleus association, namely: 1. the extensive surface area shared by sperm and vegetative perinuclear cytoplasm, and 2. the elaborate morphological relationship between one sperm and the vegetative nucleus, suggest that the conformational relationship of these two structures may help stabilize their association. The only recent report which presents information comparable to ours is that of Jensen and Fisher (1970), in which the sperms of cotton are apparently associated with the vegetative nucleus, but this association lacks the morphological complexity observed in *Plumbago*. Since it seems unlikely that two large structures like these could be stabilized exclusively by their conformation, we looked specifically for cellular structures which might directly link one sperm or its surrounding membranes with the vegetative nucleus. We were, however, unable to observe any such structures.

The occurrence of associations between sperm cells and the vegetative nucleus may facilitate the passage of sperms within the pollen tube and assure their nearly simultaneous delivery into the embryo sac. Rapid pollen tube growth, while being an obvious advantage in gametophytic competition, would be counterproductive if it resulted in the inadvertent exclusion of male gametes from the growing pollen tube. Possibly, especially in light of rapid pollen tube elongation rates in *P. zeylanica* (which reach 5 mm/hour ($>$ 80 μm/minute) in artificially pollinated styles; unpublished observations), such a linkage may represent a competitive advantage in delivering sperms to the lower style and ovule more effectively. As pollen tube growth slows, the sperms and vegetative nucleus approach the growing tip and seem less tightly associated: they share less surface area and the sperm projection becomes less

conspicuous. Sperm-to-sperm connections are retained throughout pollen tube growth and may also aid in delivering both male gametes into the region between the egg and central cell where gamete fusion occurs (RUSSELL 1980, RUSSELL and CASS, in press). Successful gamete transmission in the *Plumbago* system, as well as in the more common system in angiosperms where gamete transmission is mediated by a synergid, probably requires some form of gamete recognition with specific conditions of membrane receptivity. Since the conditions favorable for gamete fusion are likely to be transient following pollen tube discharge, the close association of sperm cells could promote their efficient co-transmission at a time when the receptivity of target cell membranes is being rapidly altered.

Sexual reproduction in *P. zeylanica,* and apparently all members of the tribe *Plumbagineae* (RUSSELL 1980), occurs in the absence of synergids, the cell type in angiosperm megagametophytes which normally receives the pollen tube and physically mediates sperm transmission. Using previous studies of male gamete structure as a basis for comparison (CASS 1973, CASS and KARAS 1975, CHENG *et al.* 1980, HOEFERT 1969, JENSEN and FISHER 1968, and KARAS and CASS 1976), we have been unable to find any unique modifications in *P. zeylanica* sperm structure which may facilitate the process of gamete delivery in a synergidless angiosperm, with the possible exception of sperm-to-sperm connections and associations with the vegetative nucleus. Theoretically, simultaneous delivery of both male gametes should be favored in angiosperm reproductive systems where sperms must each be delivered to different female cells to effect double fertilization; however, the generality of the structural features described in *Plumbago* is yet unknown and may prove to be relatively common as other flowering plant sperms are critically examined.

Acknowledgements

We gratefully acknowledge research support provided by Natural Sciences and Engineering Research Council of Canada operating grant A6103 to Dr. D. CASS and facilities of the Biological Sciences Electron Microscope Laboratory.

Portions of this research were conducted during the tenure of a University of Alberta Dissertation Fellowship awarded to S. RUSSELL. We thank Drs. R. A. STOCKEY and D. J. PETEYA for critically reviewing the manuscript.

References

BAUR, E., 1909: Das Wesen und die Erblichkeitsverhältnisse der „varietates albomarginatae hort" von *Pelargonium zonale*. Z. Vererbungs. 1, 330—351.

BURGESS, J., 1970: Cell shape and mitotic spindle formation in the generative cell of *Endymion non-scriptus*. Planta **95**, 72—85.

CASS, D. D., 1973: An ultrastructural and Nomarski-interference study of the sperms of barley. Can. J. Bot. **51**, 601—605.

— KARAS, I., 1974: Ultrastructural organization of the egg of *Plumbago zeylanica*. Protoplasma **81**, 49—62.

— — 1975: Development of sperm cells in barley. Can. J. Bot. **53**, 1051—1062.

Cheng, Z., Shiyi, H., Liyun, X., Xiuru, L., Jiaheng, S., 1980: Ultrastructure of sperm cell in mature pollen grain of wheat. Sci. Sinica **23**, 371—379.

Clauhs, R. P., Grun, P., 1977: Changes in plastid and mitochondrion content during maturation of generative cells of *Solanum* (*Solanaceae*). Amer. J. Bot. **64**, 377—383.

Cocucci, A., Jensen, W. A., 1969: Orchid embryology: Pollen tetrads of *Epidendrum scutella* in the anther and on the stigma. Planta **84**, 215—229.

Correns, C., 1909: Vererbungsversuche mit blaß (gelb) grünen und buntblättrigen Sippen bei *Mirabilis jalapa*, *Urtica pilulifera* und *Lunaria annua*. Z. Vererbungs. **1**, 291—329.

Frederick, S. E., Newcomb, E. H., 1969: Cytochemical localization of catalase in leaf microbodies (peroxisomes). J. Cell Biol. **43**, 343—353.

Galey, F. R., Nilsson, S. E. G., 1966: A new method for transferring sections from the liquid surface of the trough through staining solutions to the supporting film of a grid. J. Ultrastruct. Res. **14**, 405—410.

Gillham, N. W., 1978: Organelle heredity. New York: Raven Press.

Grun, P., 1976: Cytoplasmic genetics and evolution. New York: Columbia University Press.

Hagemann, R., 1976: Plastid distribution and plastid competition in higher plants and the induction of plastom mutations by nitroso-urea compounds. In: Genetics and biogenesis of chloroplasts and mitochondria (Bücher, T., Neupert, W., Sebald, W., Werner, S., eds.), pp. 331—338. Amsterdam: North-Holland.

Hoefert, L. L., 1969: Fine structure of sperm cells in pollen grains of *Beta*. Protoplasma **68**, 237—240.

— 1971: Pollen grain and sperm cell ultrastructure in *Beta*. In: Pollen: development and physiology (Heslop-Harrison, J., ed.), pp. 68—69. New York: Appleton-Century-Crofts.

Jensen, W. A., 1962: Botanical histochemistry. San Francisco: W. H. Freeman and Co.

— Fisher, D. B., 1968: Cotton embryogenesis: The sperm. Protoplasma **65**, 277—286.

— — 1970: Cotton embryogenesis: The pollen tube in the stigma and style. Protoplasma **69**, 215—235.

Karas, I., Cass, D. D., 1976: Ultrastructural aspects of sperm cell formation in rye: Evidence for cell plate involvement in generative cell division. Phytomorph. **26**, 36—45.

Karnovsky, M. J., 1965: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. **27**, 137 A.

Maheshwari, P., 1949: The male gametophyte in angiosperms. Bot. Rev. **15**, 1—75.

Meyer, B., Stubbe, W., 1974: Das Zahlenverhältnis von mütterlichen und väterlichen Plastiden in den Zygoten von *Oenothera erythrosepala* Borbas (syn. *Oe. lamarckiana*). Ber. dtsch. bot. Ges. **87**, 29—38.

Mogensen, H. L., 1971: A modified method for re-embedding thick epoxy sections for ultratomy. J. Ariz. Acad. Sci. **6**, 249—250.

Russell, S. D., 1980: Participation of male cytoplasm during gamete fusion in an angiosperm, *Plumbago zeylanica*. Science **210**, 200—201.

— Cass, D. D., In Press. Ultrastructure of fertilization in *Plumbago zeylanica*. Acta Soc. Bot. Pol.

Sanger, J. M., Jackson, W. T., 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.

Spurr, A. R., 1969: A new low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**, 31—43.

THIÉRY, J. P., 1967: Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J. Microscopie **6**, 987—1018.

TOYAMA, S., 1980: Electron microscopic studies on the morphogenesis of plastids. X. Ultrastructural changes of chloroplasts in morning glory leaves exposed to ethylene. Amer. J. Bot. **67**, 625—635.

VENABLE, J. H., COGGESHALL, R., 1965: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**, 407.