Planta (1984) 162:385–391

Planta

Ultrastructure of the sperm of *Plumbago zeylanica*

II. Quantitative cytology and three-dimensional organization*

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Abstract. Pollen grains of *Plumbago zeylanica* L. were serially sectioned and examined using transmission electron microscopy to determine the three-dimensional organization of sperm cells within the microgametophyte and the quantity of membrane-bound organelles occurring within each cell. Sperm cells occur in pairs within each pollen grain, but are dimorphic, differing in size, morphology and organelle content. The larger of the two sperm cells (S_{vn}) is distinguished by the presence of a long (approx. 30 μm) projection, which wraps around and lies within embayments of the vegetative nucleus. This cell contains numerous mitochondria, up to two plastids and, infrequently, microbodies. It is characterized by a larger volume and surface area and contains a larger nucleus than the other sperm cell. The second sperm cell (S_{ua}) is linked by plasmodesmata with the S_{vn}, but is unassociated with the vegetative nucleus. It is smaller and lacks a cellular projection. The S_{ua} contains relatively few mitochondria, but numerous (up to 46) plastids and more microbodies than the other sperm. The degree of dimorphism in their content of heritable cytoplasmic organelles must at fertilization result in nearly unidirectional transmission of sperm plastids into just one of the two female reproductive cells, and preferential transmission of sperm mitochondria into the other.

Key words: Angiosperm reproduction – Mitochondrion (in pollen) – Plastid (in pollen) – *Plumbago* – Pollen – Sperm cell.

* I = Russell and Cass (1981)

Abbreviations: S_{ua} = sperm cell unassociated with the vegetative nucleus; S_{vn} = sperm cell physically associated with the vegetative nucleus

Introduction

Although the sperm cytoplasm of angiosperms is hardly regarded as the expendable "sheath" that it was in the earliest reports of fertilization (Maheshwari 1950) and even into recent times (see review by Jensen 1974), its later influence on the development of the embryo is currently regarded as being, at best, variable and to some extent, taxon-dependent (Birky 1983). If one current model of fertilization is correct, at least some, and perhaps most of the male cytoplasm is transmitted into the female reproductive cells during the process of transmitting the male nucleus (Russell 1983). While some components may have a temporary influence on reproductive development, only the DNA-containing cytoplasmic organelles and the nucleus are expected to have a lasting effect (Gillham 1978; Hagemann 1979).

Genetic evidence shows that the male contribution of plastids and mitochondria in flowering plants can have a great impact on the cytoplasmic characteristics of succeeding generations in a number of different taxa (see review by Gillham 1978). The initial quantity of heritable organelles so transmitted appears to bias the outcome of cytoplasmic assortment (see Birky 1983). Genetic recombination in heritable organelles has also been demonstrated in some angiosperms (Gillham 1978) and when it occurs, paternal characteristics may become fixed within the genome of embryo organelles, even if there are relatively few heritable organelles present in the transmitting sperm cell. The final genetic constitution of organelles within the embryo in either case is sensitive to organellar content in a probabilistic sense (Birky 1983), and the paternal input, as variable as it appears to be in

angiosperms (Hagemann 1979), may determine the extent of paternal influence in the embryo and maturing plant.

Plumbago zeylanica is known through ultrastructural evidence to transmit both mitochondria and plastids into the egg and central cell (Russell 1980, 1983). Since the two sperm cells display recognizable morphological differences (Russell and Cass 1981) and have different fates during double fertilization, the present study was undertaken to determine the numerical content of organelles within each sperm cell and the magnitude of the structural differences.

Material and methods

Specimen preparation. Plants of *Plumbago zeylanica* L. were grown in soil from seed provided by Palmengarten, Frankfurt a.M., FRG, and maintained under 16-h days in a greenhouse. Under these conditions, the plants are fertile with approx. 95% seed set. Pollen grains were collected from newly opened anther sacs and fixed in 3% glutaraldehyde in 0.15 M-phosphate buffer (Jensen 1962) at pH 6.8 at room temperature for the first hour and then transferred to 4° C for an additional 5 h. The grains were rinsed briefly in buffer, fixed for 2 h in cold, buffered 2% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in low-viscosity resin (Spurr 1969).

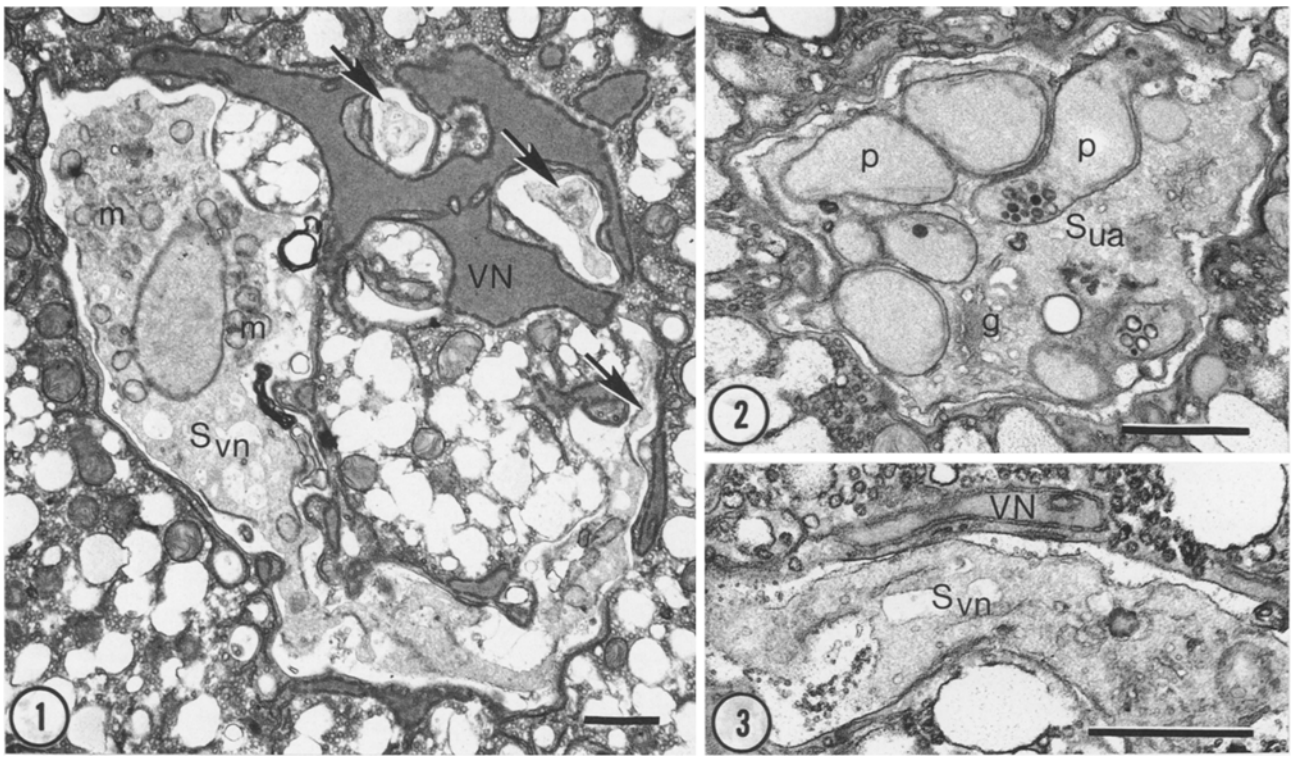
Organelle counts. Serial ultrathin sections were cut at 70 nm using a diamond knife, collected and mounted on Formvar-coated 1·2 mm^2 slot grids (Ted Pella, Tustin, Cal., USA), carbon-coated, and numbered consecutively. Each sperm cell was photographed in a Philips 200 transmission electron microscope (Philips Electronic Instruments, Eindhoven, Netherlands), photographically enlarged and printed at the same magnification. The number of organelles was determined by comparing the position and morphology of mitochondria, plastids and microbodies from section to section and counting each organelle once. Since mitochondria were the smallest organelles present (0.25–0.30 μm in diameter), the maximum number of consecutive sections which could be lost without affecting the accuracy of the total counts, was three. Series in which more than two consecutive sections were missing were omitted from these counts.

Three-dimensional reconstruction. Computer models of the three-dimensional organization of the sperm cells and their relationship with the vegetative nucleus were made using an Apple II+ microcomputer (Apple, Cupertino, Cal., USA) equipped with a digitizing tablet. Outlines of sperm and vegetative nuclei were converted to digital X,Y locations using a program written by the author. The Z component was assigned based on section number and section thickness using the nearly spherical sperm nucleolus as an internal standard for determining section thickness (Russell and Cass 1981). Objects were then examined using a commercially available computer program for the Apple II ("Apple World", United Software of America, New York, N.Y., USA). This allowed observation of objects from any angle of rotation and relative distance. Images were color-coded and analyzed visually using a color monitor or dumped to a Centronics 739 (Centronics Data Computer Corp., Hudson, N.H., USA) printer using a pin-addressable graphics mode. Additionally, physical models of one sperm-cell assemblage were constructed as the basis for reconstructions illustrated in Figs. 4–6. Volumes and surface areas of sperm cells and nuclei (Table 1) were made by planimetric methods using a microcomputer, and were based on measurements of every third sections using at least 20 sections for each count (Zilles et al. 1982). Graphs of measurements within each sampled cell were made using a digital plotter and the data examined for obvious inconsistencies. Section thickness was estimated as before. Since the degree of error in measuring specimens prepared for electron microscopy may vary based on specimen changes during preparation, section thickness, location in the plastic, inconsistencies in the microscope and other factors, the measurements given here are at best approximate. However, since many of these errors are consistent within a given sample, sperm cells of a specific pair can be compared relatively accurately, but the true cellular dimensions in the living state may differ by as much as 10% or more from the stated figures.

Results

Sperm cell organization. The microgametophyte of *P. zeylanica* consists of two sperm cells and a vegetative cell containing a prominent lobed nucleus. The sperm cells and vegetative nucleus are grouped within the pollen grain and free from the intine. The two sperm cells differ externally in cellular organization and internally in cellular organelles. One of the sperm cells is consistently associated and possibly linked in some manner to the vegetative nucleus; the two sperm cells, in turn, are joined by a common crosswall. For convenience in this paper the sperm are designated as two morphotypes: Sperm S_{vn} and Sperm S_{ua}. Both morphotypes are represented in each pollen grain. The types are distinguished externally by whether one end of the cell is physically associated with the vegetative nucleus ($=S_{vn}$) or whether it lies free in the pollen cytoplasm, physically unassociated with the vegetative nucleus ($=S_{ua}$). The pair of sperm cells arise from a common generative cell and are linked throughout the life of the microgametophyte by the crosswall arising from the division of the generative cell.

The S_{vn} is consistently associated with the vegetative nucleus (Fig. 1) and is morphologically polarized, consisting of a main cell body (approx. 8 μm long, 4 μm wide) at one end and tapering to a narrower cellular projection at the other end (Fig. 1, arrows). This projection forms a long (to 30 μm) 1-μm-thick cellular extension (Figs. 3, 4) which wraps around the vegetative nucleus and lies within embayments on its surface (Figs. 5, 6). The most extensive junctions between the S_{vn} and vegetative nucleus are areas where the sperm projection lies within clasping grooves on the surface of the vegetative nucleus (Figs. 3, 5). Although typically the projection lies near the surface of the vegetative nucleus, at certain points it appears to penetrate



Figs. 1–3. Electron micrographs of mature pollen grains of *Plumbago zeylanica*. **Fig. 1.** The vegetative nucleus (*VN*), associated sperm cell (S_{vn}) and its cellular projection (*unlabeled arrows*). The S_{vn} contains numerous aggregated mitochondria (*m*). ×10500; bar = 1 μm. **Fig. 2.** Sperm cell (S_{ua}) not associated with the vegetative nucleus, containing numerous plastids (*p*) and a Golgi body (*g*). ×17000; bar = 1 μm. **Fig. 3.** Part of the cellular projection of the sperm cell (S_{vn}) associated with the vegetative nucleus (*VN*), illustrating the close physical association of this elongated cell with clasping junctures of the vegetative nucleus. ×21800; bar = 1 μm

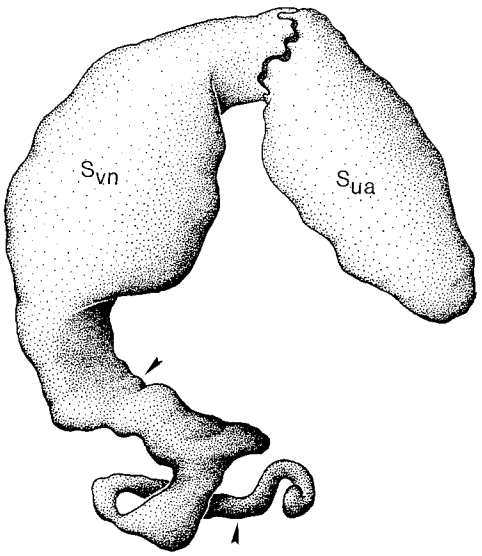


Fig. 4. Reconstruction of the two sperm cells of *Plumbago zeylanica* without the associated vegetative nucleus. The larger of the sperm cells is associated with the vegetative nucleus and is designated S_{vn}. The sperm cell not associated with the vegetative nucleus is labeled S_{ua}. The cellular projection of the S_{vn} is indicated by *arrowheads*

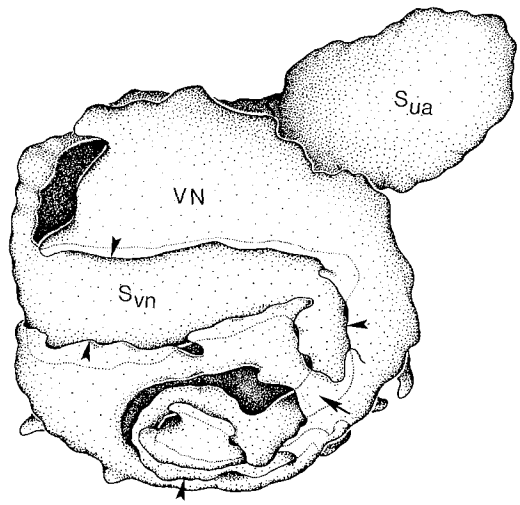


Fig. 5. Reconstruction of the two sperm cells of *Plumbago zeylanica* emphasizing the nature of the physical association between the S_{vn} and the vegetative nucleus (*VN*). The cellular protrusion of the physically associated sperm cell (*arrows*) is found within clasping regions of the lobed VN and extends through a shallow hole in the VN at one location (*arrow*). This view is approx. 120° from that shown in Fig. 4

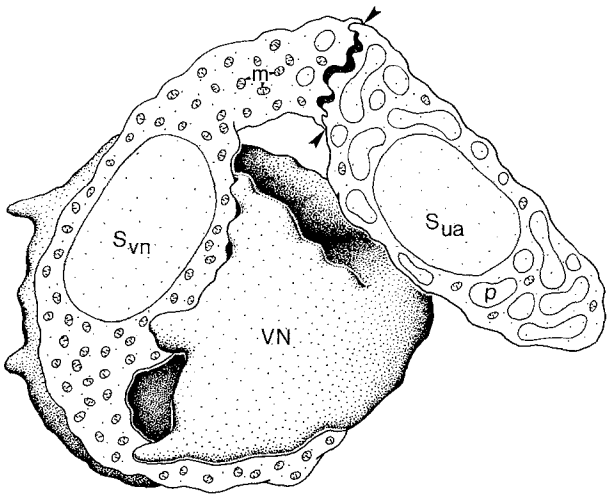


Fig. 6. Reconstruction of the two sperm cells of *Plumbago zeylanica* and the associated vegetative nucleus with superimposed profiles of mitochondria and plastids. The S_{vn} contains a majority of the mitochondria and two plastids near the sperm crosswall (*arrowheads*). The S_{ua} contains most (and usually all) of the plastids and significantly fewer mitochondria. Note the gradation between the main body of the S_{vn} and its cellular projection

through holes which are occasionally observed in the vegetative nucleus (Fig. 5, arrow). The morphogenetic origin of these holes is not known. Cytologically, the projection is similar to the rest of the cell at maturity, but contains a proportionately smaller number of cytoplasmic organelles.

Cells of the second type (S_{ua}) (Fig. 2) are spindle-shaped, about 8 μm long and 4 μm wide, with one end possessing a common crosswall with the second sperm and the other end tapering to a blunt tip. The second sperm cell is attached to the first sperm cell by a cell wall with plasmodesmata (Russell and Cass 1981). The angle of divergence between the long axis of the two sperm cells is approx. 45–60° as determined from reconstructed cells. The connection of the two sperm cells and their association with the vegetative nucleus is maintained throughout pollen-tube growth (Russell and Cass 1981) and therefore the three bodies travel as an assemblage within the growing pollen tube. During the most rapid phase of pollen-tube growth, the S_{vn} projection alone may extend to 60 μm in length (Russell and Cass 1981); at the completion of pollen-tube growth, the assemblage establishes the order of sperm-cell deposition, usually with the S_{ua} penetrating farthest into the egg. Upon deposition, the common crosswall between the sperm is apparently severed and the S_{vn} projection is torn into one or more pieces, with some of its cytoplasm sequestered into small cytoplasmic bodies which later become isolated between the egg and the central cell (Russell 1983).

Table 1. Cell volumes, surface areas and nuclear volumes of the dimorphic sperm cells of *P. zeylanica* as determined from three-dimensional reconstruction. S_{vn} = sperm physically associated with the vegetative nucleus. S_{ua} = sperm unassociated with the vegetative nucleus. Results of paired *t*-test as applied to organelle types: * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. 0.95 C.I. = 95% confidence interval

Object	Sperm S_{vn}	Sperm S_{ua}
Cell volume (in μm^3)***		
Range	45.6–116.4	32.8–83.9
Mean	69.5	48.9
0.95 C.I.	52.8–86.3	39.2–58.8
Cell surface (in μm^2)***		
Range	100.2–209.9	55.7–119.9
Mean	147.9	84.7
0.95 C.I.	121.1–174.6	72.8–96.6
Nuclear volume (in μm^3)***		
Range	13.8–27.2	9.3–15.4
Mean	19.9	12.1
0.95 C.I.	16.9–22.9	10.9–13.3
Nuclear surface (in μm^2)***		
Range	21.7–43.2	17.3–23.0
Mean	32.4	20.4
0.95 C.I.	27.8–36.9	19.0–21.8
Cytoplasm volume (in μm^3)*		
Range	25.5–90.1	21.1–46.6
Mean	45.2	33.4
0.95 C.I.	31.8–58.6	27.4–39.3

Volume and surface-area measurements. The S_{vn} is the larger of the two cells in surface area and volume, and is also the larger one in cytoplasmic volume exclusive of the nucleus (Table 1); these differences are statistically significant. Cell-surface area and volume differences are statistically highly significant (Table 1). Direct measurements of the S_{vn} projection were not made because the projection grades into the main cell body in a variable manner and therefore the distinction would be arbitrary. Estimates of the S_{vn} projection's volume and surface area, however, appear to be enough to account for most of the differences between the two cell morphotypes. The cytoplasmic volume of the S_{vn} projection is enough to account for all of the difference in cytoplasmic volume exclusive of the nucleus. Presumably, the S_{vn} would still remain the larger of the two cells in volume and surface area after pollen-tube discharge if gametic discharge events occur as proposed (Russell 1983).

Differences in the volume and surface area of the sperm nuclei in these two morphotypes are also

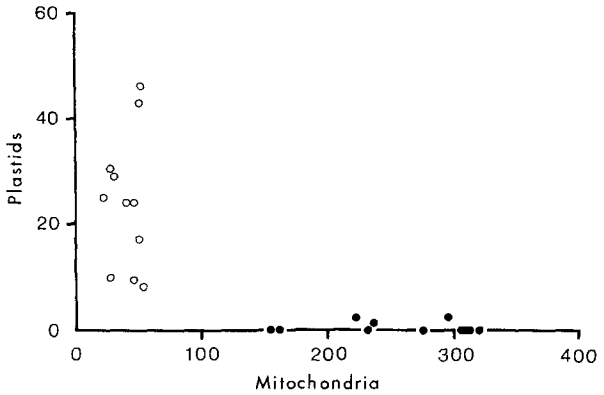


Fig. 7. A scatter diagram illustrating the content of mitochondria and plastids within individual sperm cells in pollen grains of *Plumbago zeylanica*. Solid circles (●) indicate data from sperm cells associated with the vegetative nucleus (S_{vn}); open circles (○) represent data from the unassociated sperm cell (S_{ua})

statistically very highly significant, with the size of the S_{vn} nucleus greater than that of the S_{ua} nucleus in all cases examined (Table 1). These differences are also visible in the light microscope when sperm cells are isolated using the techniques described by Russell and Cass (1981). The ratio of nuclear volume to cell volume is nearly identical in the two morphotypes: in the S_{vn}, it is 0.29 and in the S_{ua}, 0.25. Nuclear size and cell size appear to be related in the sperm cells. The greater nuclear surface area of the S_{vn} (Table 1) reflects both its greater volume and its tendency toward a more ellipsoidal shape.

Organelle content. The individual content of each of 22 paired sperm cells is indicated in the scatter diagram in Fig. 7, and their average content in Table 2. The S_{vn} possesses numerous mitochondria (range observed: 154–311) and relatively few plastids (range: 0–2). No plastids were present in eight of 11 S_{vn} cells analysed in this study (Fig. 7). When present in this sperm, however, plastids are located within 1 μm of the crosswall between the sperm cells (Fig. 6). Mitochondria appear to be randomly distributed between the main body and projection (Table 3), but appear locally aggregated (Fig. 1). The number of microbodies in these cells also varied from 0 to 2 (Table 2). Microbodies appear to be randomly distributed (Table 3).

The other sperm cell, S_{ua}, has fewer mitochondria (range observed: 22–52), many more plastids (range: 8–46), and may be regarded as a plastid-rich cell (Table 2, Fig. 2). The membrane-bound organelles present in this sperm cell are distributed throughout the cell and are not visibly polarized. Plastids are tightly packed in this morphotype (Fig. 2) with few representatives of other cytoplasmic organelle types present (Table 2, Fig. 6). Microbodies are more common in this morphotype than in S_{vn}, with 0–8 microbodies observed in the eleven sperm cells analyzed in this study (Table 2).

Differences in sperm-cell volume (Table 1), although related to morphotype, do not account for the differences observed in mitochondrial numbers. When the main bodies of the sperm cells

Table 2. Heritable organelles and microbody content in two sperm morphotypes of *P. zeylanica*. S_{vn}, S_{ua} as in Table 1. Results of paired *t*-test as applied to organelle types: * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. 0.95 C.I. = 95% confidence interval

Organelle type	Sperm S_{vn}	Sperm S_{ua}
Mitochondria***		
Range	154–311	22–52
Mean	256.18	39.81
0.95 C.I.	216.04–296.32	32.46–47.18
Plastids***		
Range:	0–2	8–46
Mean	0.45	24.3
0.95 C.I.	−0.91–1.00	15.56–32.80
Microbodies***		
Range:	0–2	0–8
Mean	0.36	3.18
0.95 C.I.	−0.08–0.82	1.43–4.93

Table 3. Distribution of organelles in the sperm physically associated with the vegetative nucleus. Main body was defined as the region within 4 μm of the sperm nucleus which was wider than 1.5 μm in sectional view. The protrusion was defined as the region less than 1.5 μm in sectional view. Results of paired *t*-test as applied to organelle types: n.s. = not significant; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. 0.95 C.I. = 95% confidence interval

Organelle type	Sperm S_{vn} main body	Sperm S_{vn} projection
Mitochondria***		
Range	107–257	36–122
Mean	176.0	80.2
0.95 C.I.	144.8–207.2	59.8–100.5
Plastids n.s.[a]		
Range	0–2	0
Mean	0.45	0
0.95 C.I.	−0.10–1.01	
Microbodies n.s.[b]		
Range	0–1	0–1
Mean	0.27	0.09
0.95 C.I.	−0.04–0.58	−1.11–0.29

[a] Insignificant differences in main body and projection are artifacts of small sample size; all plastids observed were within 1 μm of the sperm crosswall

[b] No polarization of microbodies was observed

were compared, the number of mitochondria in the S_{vn} (range observed: 107–257) (Table 3) was much larger than the number of mitochondria in the S_{ua} (range: 22–52) (Table 2). A paired *t*-test indicates that this is very highly significant ($P < 0.0001$). Differences in plastid content are equally highly significant between the two morphotypes. The number of microbodies is highly significant but to a somewhat lesser degree ($P < 0.001$). The number of plastids was positively correlated with the number of microbodies in the 22 sperm cells counted ($P < 0.01$).

The strong relationship between sperm morphology and organellar content is illustrated in Table 2 and Fig. 7, and indicates the ease with which the two sperm types may be distinguished. This pattern is consistent in the over 200 pollen grains examined closely during this study. When, in infrequent cases, the plastid-rich sperm appeared to be associated with the vegetative nucleus, closer examination consistently showed it to be an artifact of orientation and that it indeed lacked a cellular projection.

Discussion

At maturity, each pollen grain of *P. zeylanica* contains two dimorphic sperm cells, each differing externally in both morphology and dimensions, and internally in organelle content and distribution of organelles. These differences are consistent from pollen grain to pollen grain and are statistically very highly significant. One sperm cell (S_{vn}) is physically associated with the vegetative nucleus at one end and is directly linked by plasmodesmata (Russell and Cass 1981) with the other sperm cell (S_{ua}). In *Plumbago,* the two sperm cells and vegetative nucleus thus form an assemblage that is present in the pollen grain and maintained throughout pollen-tube growth. This has the biological implication of ordering the nuclei within the pollen grain and developing tube (Russell and Cass 1981). Superimposed on the organization of this assemblage are differences in cellular volume, nuclear volume and surface area in the two sperm-cell types, with the S_{vn} larger than the S_{ua} in all respects.

Perhaps the most dramatic of the sperm differences is the relative content of heritable organelles. The S_{vn} is mitochondrion-rich and typically lacks plastids, whereas the S_{ua} is relatively mitochondrion-poor and has numerous plastids. If plastids and mitochondria are transmitted and expressed in the offspring of this plant, the identity of the sperm cell which fused with the egg would be crucial to the pattern of cytoplasmic inheritance in the embryo. The influx of plastids and mitochondria from the other sperm cell that is introduced into the central cell (Russell 1983) might also have an important impact on the developing endosperm, particularly since the relatively brief lifespan of the endosperm might limit genetic sorting-out (see Birky 1983).

Patterns of cytoplasmic organelle inheritance in angiosperms have been clearly elucidated only for plastids. Both uniparental (maternal) and biparental patterns of plastid inheritance have been described in angiosperms (for review, see Kirk and Tilney-Bassett 1978) and these relate closely to three general patterns of plastid content in the generative cell (for discussion and review, see Hagemann 1979). In the first, typified by *Lycopersicon* and *Solanum,* plastids are either initially excluded from the generative cell or selectively eliminated, respectively. In either case, plastids are absent as an organelle type within the mature generative cell and plastid inheritance is strictly maternal. In the second major type, represented by *Pelargonium,* intact plastids continue to be present and apparently remain functional within the mature generative cell; in some plants, they are numerous. These plants display strong biparental inheritance. *Antirrhinum* typifies an intermediate case in which plastids are infrequently observed and the pattern of plastid inheritance may be either maternal or weakly biparental. The correlation between plastid content of the generative cell and pattern of plastid inheritance appears to be consistent enough to predict whether plastid inheritance will be uni- or biparental (Hagemann 1979). In *Plumbago,* plastids are numerous within the generative cell, but are transmitted largely into just one of the two sperm cells. One sperm cell appears to resemble *Pelargonium* in its plastid content, while the other more closely resembles *Antirrhinum.* Therefore, the two sperm cells of *Plumbago* may be expected to differ greatly in their ability to transmit plastids into the offspring.

Although the generative cell of *Plumbago* contains numerous plastids, only one of its descendent sperm cells may be regarded as a consistent source of transmitted male plastids. This dimorphism in content of DNA-containing organelles may be of particular genetical importance if the sperm cytoplasm is the principal vehicle for transmitting male organelles, as is widely assumed. Ultrastructural evidence from the fertilized female gametophytes of *Plumbago* indicates that transmission via the sperm cytoplasm is the sole source of transmitted paternal DNA-containing organelles (Russell

1983). If this is true, the degree of asymmetry in plastid content found in the plastid-poor S_{ua} is extreme enough that it could result in functional exclusion of plastids in the female reproductive cell with which it fuses.

The origin of dissimilarities in organelle content between the two sperm cells probably results from the polarized cytoplasmic conditions observable within the immature generative cell (Russell and Cass 1983). Initially, following detachment from the intine the immature generative cell is nearly spheroidal and is unpolarized. However, as the generative cell elongates at one end to form the characteristic cellular projection of the S_{vn}, mitochondria appear to aggregate toward that half and plastids toward the opposite end. The distribution of organelles present in each half of the late generative cell is fixed at the time of cytokinesis (Russell and Cass 1983). The content of organelles in each half of the generative cell thus determines the initial concentration of organelles in the sperm cells. Any further differences in the content of plastids and mitochondria between the two sperm cells would therefore be the result of different rates in organellar synthesis or senescence.

Sperm dimorphism has been reported in a small number of seed-plant taxa. In gymnosperms, several taxa differ notably in size and nuclear volume between the two sperm cells (for review, see Chamberlain 1935; Singh 1978), and this may be a fairly common phenomenon (Chamberlain 1935). In angiosperms, reports of sperm dimorphism have been slower to gain acceptance. To my knowledge, this report is the first to present clear evidence for such differences in an angiosperm and to relate these to differences in heritable cytoplasmic organelles. In *Plumbago,* these differences are related to the physical association of one sperm cell with the vegetative nucleus. Such associations of one sperm cell with the vegetative nucleus have also been noted in *Spinacia oleracea* (Wilms and van der Aelst 1983), *Brassica oleracea* (Dumas et al. 1984), and *Brassica campestris* (McConchie et al. 1984). Differences in organelle content are indicated by preliminary results in both *B. oleracea* (Dumas et al. 1984) and *B. campestris* (McConchie et al. 1984). In *Alopecurus pratensis*, the association between the sperm and vegetative nucleus may be lacking (Heslop-Harrison and Heslop-Harrison 1984). In plants lacking such an association, it may be difficult to distinguish naturally occurring variation from genetically programmed dimorphism. Such dimorphism could be genetically important if it determines the fate of a particular type of sperm cell during double fertilization.

This research was supported in part by National Science Foundation grant PCM-8208466. I wish to thank Susan M. Heinrichs, Steven Eckroat and Anabella Opazo for technical assistance.

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Received 17 February; accepted 12 June 1984