FERTILIZATION IN PLUMBAGO ZEYLANICA: GAMETIC FUSION AND FATE OF THE MALE CYTOPLASM

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ABSTRACT

Developmental phases surrounding the processes of gametic delivery and fusion were examined ultrastructurally in the reduced megagametophyte of Plumbago zeylanica, which lacks synergids. Gametic delivery occurs at the end of pollen tube growth and results in deposition of two male gametes, a vegetative nucleus, and a limited amount of pollen cytoplasm between the egg and central cell. Discharge of these materials from the tube is accompanied by loss of inner and outer pollen tube plasma membranes, loss of sperm-associated cell wall components, and disruption of the formerly continuous cell wall between the egg and central cell. The dispersion of egg cell wall components directly exposes female reproductive cell membranes to the unfused male gametes and pollen tube without disrupting gametic cell plasma membranes. Presence of unfused sperms within the female gametophyte appears to be a transitory phenomenon, lasting less than 5 min at the end of over 8½ hr of pollen tube growth. At the time of gametic deposition, plasma membranes of unfused sperm cells become directly appressed to plasma membranes of both the egg and central cell. Gametic fusion is initiated by a single fusion event between membranes of participating male and female cells, which is rapidly followed by subsequent, secondary fusion events between the same two cells at different locations along their surface. Gametic fusion results in the transmission of male gamete nuclei with co-transmission of nearly the entire sperm cytoplasmic volume and organellar complement, and it is possible to identify heritable male cytoplasmic organelles within both the incipient zygote and endosperm. Paternally originating plastids may be distinguished from maternal plastids by differences in morphology and staining characteristics, whereas paternal mitochondria may be distinguished from maternal mitochondria by populational differences in mitochondrial size which are statistically significant. Such observations further indicate that transmitted paternal mitochondria seem to remain viable, as judged by their ultrastructural appearance, and are transmitted exclusively by sperm cytoplasm rather than discharged pollen cytoplasm. The presence of anucleate, membrane-bound cytoplasmic bodies between the egg and central cell are identifiable on the basis of their enclosed organelles and indicate that fragmentation of a small amount of the sperm cytoplasm associated with the vegetative nucleus commonly occurs. The presence and identification of sperm cytoplasmic organelles and associated membranes within female reproductive cells following gametic transmission represents strong evidence in support of the cellular basis of nuclear and cytoplasmic transmission during sexual reproduction in Plumbago.

IN FLOWERING PLANTS, the concept that male gametes are deposited in the embryo sac through a single receptive synergid is implied by the presence of the pollen tube and its discharged cytoplasm within that synergid (Jensen, 1974). Although the transitory presence of sperm cells in the synergid has been docu-

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tube, or both. Direct structural evidence, which specifically addresses the events of gametic fusion in angiosperms, is almost unavailable. Since the presence of disorganized and densely stained synergid cytoplasm has often interfered with such observations, it has recently been proposed that examination of these processes in flowering plants that lack synergids may play a role in resolving this controversy (Russell, 1980). By studying reduced megagametophytes, such as Plumbago which lack synergids, it may be possible to determine which of the many reproductive functions may be universal in angiosperms during normal reproductive function. Further, the means by which such functions are accomplished in a reduced megagametophyte may also provide insight into the means by which synergid functions are accomplished in more conventionally organized angiosperm megagametophytes.

The mechanism by which gametic nuclei are transmitted to the egg and central cell is still largely unknown; however if transmission occurs by cellular fusion, extra-nuclear components of the male gametes will be transmitted into cells of the female gametophyte and their detection should be possible soon after the arrival of sperm nuclei into the egg and central cell. The problems associated with visualizing these crucial stages and distinguishing between organelles of different cellular origin have largely been resolved in the present study of Plumbago zeylanica. Determining the initial fate of these cytoplasmic organelles provides a technique through which the events of fertilization may be examined and contributes structural information to the process of gametic fusion in flowering plants. The present study outlines the condition of the male gametes after their discharge into the embryo sac and proposes a sequence of events during gametic fusion which terminates in the transmission of male nuclei and heritable cytoplasmic organelles into the egg and central cell.

**Materials and Methods**—Plants of Plumbago zeylanica L. have been maintained at 17 to 23 °C. 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). Unlike many other members of the Plumbaginoideae, P. zeylanica is self-compatible and produces a high proportion of seed containing normal, sexually produced embryos (over 97% seed set). Under these conditions, a chronology of fertilization events was produced to aid in obtaining fertilization stages (Table 1), which otherwise would be difficult to reproduce. This chronology, based on a total of 60 ovules in five different preparative series, is accurate to within ±10 min between different preparations and is accurate to within ±5 min in a given preparative series.

Flowers used in this study were emasculated and artificially pollinated periodically during the morning, and then were collected at 5-min intervals from 8½ to 8¾ hr following pollination. Ovules were immediately dissected from the ovary and immersed in a 3% glutaraldehyde-M/15 phosphate buffer fixing solution (pH 6.8) at room temperature for 6–8 hr. Tissue was subsequently rinsed in the same buffer, fixed for 2 hr in 2% osmium tetroxide-M/15 phosphate buffer at 4 C, dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in low viscosity resin (Spurr, 1969).

Light microscopic preparations were sectioned at 2 to 2.5 μm in thickness, stained with aniline blue-black (Fisher, 1968), and were observed with brightfield or Nomarski interference contrast microscopy using a Zeiss Photomicroscope I. Selected sections were observed unstained in the light microscope and then re-embedded for ultrathin-sectioning according to the method described by Mogensen (1971).

Ultrathin sections were mounted on uncoated nickel or gold grids, stained in a solution of saturated aqueous uranyl acetate and ethanol (1:1, v/v) and in 0.02% lead citrate (Venables and Cogeshall, 1965). Other sections were subjected to the periodic acid-thiocarbohydrazide-silver proteinate reaction (PA-TCH-SP) using a staining regime of 0.5 hr in 1% periodic acid, 6 hr in 0.2% thiocarbohydrazide in 20% acetic acid, and 30 min in newly mixed 1% silver proteinate in the dark (Thiery, 1967). Sections were observed using a Philips 200 or 201 transmission electron microscope.

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**Table 1. Chronology of fertilization events in Plumbago zeylanica based on maximal growth rates following artificial pollination. Times listed are in hours and minutes**

<table>
<thead>
<tr>
<th>Event</th>
<th>Time (hr:min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation of pollen tube growth; penetration of stigma</td>
<td>0:0-0:15</td>
</tr>
<tr>
<td>Rapid pollen tube growth in upper style</td>
<td>0:15-4:30</td>
</tr>
<tr>
<td>Slower pollen tube growth in lower style</td>
<td>4:30-8:10</td>
</tr>
<tr>
<td>Arrival of pollen tube at ovule; penetration of micropyle</td>
<td>8:15-8:20</td>
</tr>
<tr>
<td>Penetration of nucellus and filiform apparatus; entry and discharge of the pollen tube within the embryo sac</td>
<td>8:25</td>
</tr>
<tr>
<td>Gametic fusion</td>
<td>8:30</td>
</tr>
<tr>
<td>Migration of male nuclei; initiation of nuclear fusion; fertilization</td>
<td>8:35-8:40</td>
</tr>
</tbody>
</table>

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Mitochondrial width was determined by measuring median sections of mitochondria using a caliper measuring device (accurate to 0.05 mm) on printed electronmicrographs (final magnification ×7,000 and above) in eight different cell population classes. Cell classes are listed in Table 2. The electron microscope fields were selected randomly with respect to cell organelles. At least five different cells were used in each cell class. Each mitochondrion was measured only once. Whereas other parameters may have been equally useful in discriminating between mitochondria in different cellular populations, mitochondrial width provided three advantages: 1) organelle width is relatively insensitive to orientation during sectioning; 2) mitochondrial shape is typically spherical to roundly ellipsoidal in this material (there were few mitochondria with constrictions in the center, and these were omitted from the counts); 3) mitochondria are small enough that the entire organelle was usually represented in less than five sections. Determining the appropriate organelar section in which to measure mitochondrial width was facilitated by these characteristics. In order to test the reliability of this technique, additional measurements of mitochondria in the unfertilized egg and central cell were taken using electron-micrographs of the unfertilized egg of *P. zeylanica* prepared for a previous study (Cass and Karas, 1974). These measurements did not differ statistically from those made in the present study. Data presented in Table 3 were analyzed using a three-level nested analysis of variance (ANOVA) for unequal population sizes (unless otherwise noted) comparing each population examined (Sokal and Rohlff, 1969).

**Observations**—The entrance and discharge of the pollen tube within the megagametophyte occurs between the egg and central cell in *Plumbago zeylanica*, with pollen tube growth ending 70–80 μm deep within the embryo sac (Russell, 1982). The tube aperture forms at the tip of the pollen tube soon after its arrival within the embryo sac and results in the discharge of the pollen tube and the release of the vegetative nucleus, some pollen tube cytoplasm, and the two male gametes. Regardless of the preparative technique used, the discharged pollen cytoplasm appears to begin degradative changes soon after it is injected into the intercellular space between the egg and central cell (Fig. 3; Russell, 1982, fig. 15). Released pollen tube organelles appear to degenerate rapidly. The vegetative nucleus is situated between the egg and central cell and gradually approaches the

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**Key to Labeling:** CC, central cell; E, egg; EN, egg nucleus; En, endosperm; M, mitochondrion; M₁, mitochondrion—central cell origin; M₂, mitochondrion—sperm cell origin; N, nucleus; Nc, nucellus; P, plastid; P₁, plastid—egg origin; PN, fused polar nuclei; P₂, plastid—sperm origin; PT, pollen tube; S, sperm cell; SN, sperm nucleus; SN₁, sperm nucleus transmitted into the egg; SN₂, sperm nucleus transmitted into the central cell; Sus, suspensor; VN, vegetative nucleus.

Fig. 1–3. 1. Discharged male gamete located between the egg and central cell after release from the pollen tube. The male gamete contains a nucleus with a single prominent nucleolus. Unlabeled arrowheads indicate the edge of the sperm cytoplasm. Figures 1–6 are selected from cross sections of a single megagametophyte. Nomarski interference contrast microscopy; unstained. ×2,600. 2. Second (of two) discharged male gametes between the egg and central cell, proximal to the sperm illustrated above. Edge of sperm cytoplasm, indicated by unlabeled arrowheads. Nomarski interference contrast microscopy; unstained. ×2,600. 3. Transmission electron micrograph of an ultrathin section prepared from the section illustrated in Fig. 2. Sperm cell contains evident cytoplasm and obliquely-sectioned sperm nucleus. Pollen tube and degenerate, discharged pollen cytoplasm are evident between egg and central cell. Uranyl acetate-lead citrate stained (UA-PbCit). ×7,900.

Fig. 4–6. 4. Discharged sperm cell unfused between the egg and central cell from an adjacent section to that in Fig. 3. Sperm nucleus and cytoplasm appear normal and intact prior to gametic fusion. Mitochondria (arrowheads) are present in the cytoplasm surrounding the sperm nucleus. UA-PbCit. ×4,350. 5. Discharged sperm cell unfused between the egg and central cell, prepared from the section illustrated in Fig. 1. Sperm cell is surrounded by a single plasma membrane which is tightly appressed to that of the central cell prior to gametic fusion. Arrowheads indicate microtubules in central cell. Note difference in electron density between ground cytoplasm of sperm and central cell. UA-PbCit. ×25,000. 6. Discharged sperm cell, pollen tube and degenerate pollen cytoplasm from section adjacent to that illustrated in Fig. 5. Note tight apposition of sperm and central cell. Although this tissue was fixed within five minutes of the expected time of pollen tube discharge, pollen cytoplasm and pollen mitochondria (M) are highly degenerate. UA-PbCit. ×22,150.

Fig. 7–8. 7. Median longitudinal section of newly fertilized egg, showing relative positions of egg and sperm nuclei soon after gametic fusion. Note apparent partitioning of the electron-dense discharged pollen cytoplasm by regions of modified egg cell wall (indicated by unlabeled arrowheads). Large vacuoles in both the egg and central cell are conspicuous. UA-PbCit. ×1,700. 8. Newly transmitted sperm nucleus (SN₁) within the egg cell at higher magnification. Plastids flanking the sperm nucleus are of male origin. Degenerate, discharged pollen cytoplasm is evident between egg and central cell. Thiocarbohydrazide-silver proteinate staining, without prior periodic acid oxidation (TCH-SP). ×17,500.
chbalazal part of this region. Discharged pollen cytoplasm continues to degenerate following gametic fusion, but undergoes less rapid alteration after fertilization. Even as late as several days after the arrival of the pollen tube it may be possible to distinguish the pollen tube wall and the degenerating remains of the pollen cytoplasm and vegetative nucleus between the suspensor and the endosperm cells.

**Sperm cells in the megagametophyte before gametic fusion**—The transitory nature of developmental stages between gametic discharge and fusion is clearly suggested by the rarity of such reports in the literature (most recently, Cass and Jensen, 1970; Cocucci and diFulvio, 1969; Cass, 1981; Wilms, 1981). In the present study, one megagametophyte observed displayed these stages (Fig. 1–6). In this series, it is evident that the two male gametes remain near the pollen tube aperture, and become tightly appressed against the egg and central cell plasma membranes. They are surrounded by discharged, degenerating pollen cytoplasm on their remaining faces (Fig. 1–3). The male gametes are clearly cellular at this stage and contain a nucleus with a single nucleolus. The two sperms remain closely associated after their expulsion from the pollen tube and are separate cells, each surrounded by a single plasma membrane. At the electron microscopic level, the general cytology of sperms at this stage resembles that of sperms within the pollen grain and tube (Russell and Cass, 1981a). Possibly these two cells remain linked by plasmodesmata as in the pollen grain and growing tube; however, this connection would presumably be disrupted near the time of gametic discharge or before gametic fusion.

The prefusion sperm cell is dominated by a nucleus and contains a number of organelles, which by their appearance, resemble the ultrastructure of viable organelles. Mitochondria are numerous within the sperm cells, spherical to elongate, and approximately 0.2 μm wide and up to 0.5–0.7 μm long (Fig. 4). Plastids, up to 1.5 μm long with dense stromata, are similar to those described within the pollen grain and growing pollen tube (Russell and Cass, 1981a). The ground cytoplasm of sperms appears somewhat less electron dense than that of the surrounding egg and central cell (Fig. 4–6).

The two sperms are each delimited by a single membrane, lacking the surrounding vegetative cell plasma membrane which envelopes the sperms throughout pollen grain maturation and pollen tube growth. Following tube discharge, segments of the sperm plasma membrane appear directly appressed to the plasma membranes of the egg and central cell (Fig. 5, 6). The loose, nonfibrillar cell wall observed at previous stages (Russell and Cass, 1981a) is apparently removed from the sperm cell surface with the removal of the vegetative cell plasma membrane. The junction between the sperms and the egg and central cell varies randomly from 0.02–0.07 μm (Fig. 5) except where pollen cytoplasm is lodged between the cells causing further separation. Adjacent to the discharged sperm cells, longitudinally oriented microtubules may be observed near the plasma membranes of the egg and central cell (Fig. 5). These microtubules frequently appear attached to the egg or central cell plasma membranes (as in Cass and Karas, 1974, fig. 12), and may as in other biological systems, serve a cytoskeletal function.

**Male gametes within egg and central cell subsequent to gametic fusion**—Sperm nuclei pass into the cytoplasm of the egg and central cell soon after the arrival of male gametes within the embryo sac (see Table 1). As the location of the unfused sperm would indicate, the entry of sperm cells likely occurs near the site of their deposition, just beyond the tip of the pollen tube (Fig. 11). At this stage, the two sperm nuclei remain oriented in a linear fashion and the vegetative nucleus is located just beyond the distal sperm nucleus. The sperm nuclei at this stage are typically separated from the egg and central cell nuclei by about 17 μm and 25 μm, respectively (Fig. 7), but this distance is quickly reduced during the ensuing 10 min (see Table 1). The sperm nucleus transmitted into the egg cell is shown in Fig. 7, 8 and 12, whereas the sperm nucleus transmitted into the central cell is seen in Fig. 9–11.
arrows). Vesicles of the associated fusion membranes are indicated by upward-pointing arrow. Plastids evident in adjacent egg cytoplasm represent sperm plastids, located several micrometers from the sperm nucleus transmitted into the egg. PA-TCH-SP. ×37,400. 11. Spatial relationship of transmitted sperm nucleus with the pollen tube aperture (unlabeled arrows) and pollen tube cytoplasm, from a section adjacent to the previous figures but at lower magnification. Membranes associated with the sperm nucleus (unlabeled arrowheads) appear to have arisen during sperm cell fusion with the central cell. PA-TCH-SP. ×11,000. See page 418 for KEY TO LABELING.
The migration of sperm nuclei transmitted into the female reproductive cells appears to occur by the most direct route. Thus subsequent nuclear fusion occurs on the part of the egg nucleus or central cell nucleus nearest the site of gametic deposition and pollen tube discharge (Fig. 20–22). The events of nuclear fusion appear to be identical to those described in other angiosperm taxa (Jensen, 1964; Jensen and Fisher, 1967; Schulz and Jensen, 1977), occurring by the apposition and fusion of nuclear membranes at multiple locations followed by the expansion of these fusion sites in both the incipient zygote and endosperm nuclei (Russell and Cass, 1981b).

**Modifications of egg-central cell wall; sperm membranes following gametic fusion**—Prior to pollen tube arrival, chalazal regions of the egg cell wall are quite different from those in the micropylar and adjacent lateral regions (Russell, 1982). In micropylar parts of the egg, the cell wall contains numerous longitudinally oriented periodate-sensitive fibrils aligned parallel to the pollen tube, which may aid in guiding the pollen tube during its passage into the embryo sac. However, in the lateral egg cell wall, near the region of pollen tube discharge and toward the chalazal end of the egg, the cell wall is homogenous, lacking the linear fibrillar component observed previously (Russell, 1982). Following pollen tube arrival and discharge, the formerly continuous egg cell wall becomes interrupted and is absent over much of the surface of the egg and central cell (Fig. 8, 11; Russell, 1982), thus permitting direct apposition of male and female gamete plasma membranes.

In chalazal most regions of the egg cell, near the level of the pollen tube aperture to the summit of the egg, modified segments of egg cell wall are evident (Fig. 12, 15, 16; arrowheads) that appear essentially similar to those reported in the unfertilized egg of *P. zeylanica* (Cass and Karas, 1974, fig. 12). In their study, Cass and Karas found electron-lucent regions of cell wall had a constant thickness and were usually associated with straight regions of plasma membrane and “stalked” microtubules. After pollen tube discharge, these segments apparently become electron dense and granular (Fig. 12, 15, 16; arrowheads), although otherwise appearing identical to the egg-central cell junctions observed by Cass and Karas. Microtubules observed in the present study are common near the site of gametic fusion, but are less frequently associated specifically with the modified cell wall regions. The role of these cell wall regions is not directly known; however, their proximity to the site of gametic deposition and their ability to influence the distribution of discharged pollen cytoplasm (arrowheads, Fig. 7, 21), suggests a role in mediating the distance between plasma membranes at the egg-central cell wall interface. Further, such cell wall modifications may limit the movement of discharged sperm cells and the vegetative nucleus prior to gametic fusion. Gametic fusion consistently appears to occur just beyond the tip of the pollen tube amidst these cell wall segments. This location for gametic fusion supports the idea that such modified regions may play a role in controlling the entry of pollen tube ejecta, and stabilizing the position of sperm cells injected into the region between the egg and central cell.

Immediately after gametic fusion, it is possible to observe fragments of plasma membrane surrounding sperm nuclei after entry into the female reproductive cells (arrows, Fig. 9, 10; arrowheads, Fig. 11). Based on staining similarities between these membranes and those of the gametic plasma membranes, it appears that these represent membrane remnants of the fusion process derived from the fusion of the sperm and the respective female reproductive cell with which it fused. These membranes are readily identifiable in PA-TCH-SP-stained material (Fig. 9–11) and are easily discernable from other membrane systems including the endoplasmic reticulum. These fusion membranes are also identifiable in uranyl acetate-lead citrate-stained material (not shown). The presence of such remnant membranes is evidence that although gametic fusion is likely initiated at one point in the cell mem-
branes, that additional fusion points occur. Apparently, some of the sperm cell membrane is not directly incorporated into the two female reproductive cells during cellular fusion. Between the time of gametic fusion and the completion of sperm nucleus migration, the remaining membranes appear to become disorganized by progressive vesiculation (Fig. 9, 10, upward pointing arrow). Later, the only visible remains of the fusion membrane appears to be a small membranous whorl, (Fig. 8). Within 10 min after gametic fusion (as given in Table 1) such membrane systems can no longer be identified.

**Morphological evidence of male cytoplasmic organelle transmission**—Structural evidence for the transmission and incorporation of paternally derived organelles during gametic fusion was observed in both the egg and central cell. The initial fate of two classes of heritable cytoplasmic organelles (mitochondria and plastids) could be determined by the following means: 1) differences in plastid morphology and staining between paternal and maternal plastids permitted individual plastids to be identified with respect to cell of origin; 2) populational differences in average mitochondrial width between paternal and maternally-originating mitochondria permitted populations of mitochondria to be identified on a statistical basis.

Plastids of presumed sperm origin are located next to the sperm nucleus transmitted into the egg cell soon after gamete fusion (Fig. 8, 12) and are noted within adjacent egg cytoplasm in nearby sections (Fig. 10, 11 and 15). Compared to plastids within the unfertilized egg cell (seen in Fig. 14; also Cass and Karas, 1974, Fig. 3, 5, 6) and other plastids of maternal-origin in the megagametophyte (Fig. 16), sperm plastids possess relatively dense stromata in uranium-lead stained material (Fig. 13), are somewhat narrower in width (approx. 1.2 μm or less, instead of 1.5 μm or more), and have a less highly developed internal membrane system (Fig. 13, 15). Plastoglobuli also appear more numerous in these plastids and tend to be aggregated (Fig. 8). Occasional paracrystalline arrays within the stromata are also noticed, but such arrays have been observed in maternally originating plastids, too. At this stage, distribution of sperm plastids within the embryo sac was restricted to within several micrometers of the sperm nucleus and did not extend elsewhere within the large egg cell (Fig. 7). Similarities in structure, morphology, and staining characteristics of these plastids and plastids observed in sperm cells within the pollen grain and tube (Russell and Cass, 1981a) strongly support the co-transmission of male nuclei and organelles during gametic fusion. The approximate number of plastids observed in the egg also agrees with the number counted in sperm cells within the mature pollen grain (Russell, 1981).

**Statistical evidence of male cytoplasmic organelle transmission**—While the presence of dimorphic plastids in the sperm and female gametophyte permits differentiation of maternally and paternally originating plastids on morphological grounds alone, differences in mitochondrial structure, although evident, are better dealt with in a statistical treatment. Both maternally and paternally originating mitochondria are spherical to roundly ellipsoidal and similar in internal structure. Size differences, however, are apparent between mitochondria of male and female origin (Russell, 1980), and suggest that certain mitochondrial dimensions can prove useful in differentiating...
Table 2. Descriptive statistics comparing mitochondrial widths in six different cells of origin and two populations of unknown cellular origin (all measurements given in micrometers)

<table>
<thead>
<tr>
<th>Population*</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>( \bar{x} )</th>
<th>S.D.</th>
<th>0.9500 Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.PG</td>
<td>31</td>
<td>0.215</td>
<td>0.387</td>
<td>0.286</td>
<td>0.042</td>
<td>(0.271, 0.302)</td>
</tr>
<tr>
<td>S.PT</td>
<td>16</td>
<td>0.165</td>
<td>0.291</td>
<td>0.224</td>
<td>0.034</td>
<td>(0.206, 0.242)</td>
</tr>
<tr>
<td>PG</td>
<td>26</td>
<td>0.341</td>
<td>0.517</td>
<td>0.453</td>
<td>0.040</td>
<td>(0.437, 0.469)</td>
</tr>
<tr>
<td>PT</td>
<td>52</td>
<td>0.300</td>
<td>0.554</td>
<td>0.433</td>
<td>0.062</td>
<td>(0.416, 0.451)</td>
</tr>
<tr>
<td>E</td>
<td>79</td>
<td>0.322</td>
<td>0.932</td>
<td>0.623</td>
<td>0.137</td>
<td>(0.592, 0.654)</td>
</tr>
<tr>
<td>CC</td>
<td>37</td>
<td>0.269</td>
<td>0.753</td>
<td>0.489</td>
<td>0.088</td>
<td>(0.460, 0.519)</td>
</tr>
<tr>
<td>S.CC</td>
<td>41</td>
<td>0.132</td>
<td>0.288</td>
<td>0.214</td>
<td>0.037</td>
<td>(0.203, 0.226)</td>
</tr>
<tr>
<td>S.Unfused</td>
<td>8</td>
<td>0.122</td>
<td>0.282</td>
<td>0.203</td>
<td>0.061</td>
<td>(0.155, 0.250)</td>
</tr>
</tbody>
</table>

* S.PG = sperm cell within pollen grain at anthesis; S.PT = sperm cell within the pollen tube during active growth in the lower style; PG = vegetative cell within pollen grain at anthesis; PT = pollen tube during active growth in the lower style; E = unfertilized egg, after artificial pollination but before arrival of pollen tubes; CC = unfertilized central cell, after artificial pollination but before arrival of pollen tubes; S.CC = mitochondrial population within 0.5 \( \mu \)m of the sperm nucleus in the central cell after gametic fusion; S.Unfused = mitochondrial population within unfused cytoplasmic bodies between fertilized egg and central cell.

these organelles on a populational basis. Mitochondrial width is the dimension of choice because it is relatively insensitive to orientation of the organelle during sectioning and is readily measured. The similarity of certain classes of organelles of unknown origin can then be compared with those of known origin and the results statistically compared.

Mitochondrial widths were measured in six different known cell types: 1) sperm in pollen grains at anthesis, S.PG; 2) sperm in growing pollen tubes in the lower style, within 100 \( \mu \)m of the ovule, S.PT; 3) vegetative cell in pollen grains at anthesis, PG; 4) vegetative cell in growing pollen tubes in the lower style, PT; 5) mature, unfertilized central cell, CC; 6) mature, unfertilized egg, E. Average mitochondrial widths, size ranges, standard deviations, and 0.95 confidence intervals are summarized in Table 2. Mitochondrial widths in the sperm cell average 0.29 \( \mu \)m in pollen grains at anthesis (0.95 confidence interval: 0.27–0.30 \( \mu \)m) and in the sperm cells during late pollen tube growth, average 0.22 \( \mu \)m (0.95 confidence interval: 0.21–0.24 \( \mu \)m). Mitochondrial width is greater in the pollen grain (avg. 0.45 \( \mu \)m), pollen tube (avg. 0.43 \( \mu \)m), unfertilized egg (avg. 0.62 \( \mu \)m), and central cell (avg. 0.49 \( \mu \)m).

The 0.95 confidence intervals of mitochondrial width in sperm cells do not overlap with those of the pollen grain, tube, egg, or central cell. Cases in which there is a significant degree of overlap in these confidence intervals include the pollen grain and pollen tube mitochondria and those of the central cell. A minor degree of overlap also occurs between 0.95 confidence intervals of mitochondrial width between the pollen grain and central cell (Table 2). It is interesting to note that 0.95 confidence intervals between sperm in the pollen grain (S.PG) and sperm in the pollen tube (S.PT) do not overlap, probably a consequence of the elongation of sperm mitochondria noted as one of a number of developmental changes occurring during pollen tube growth (Russell and Cass, 1981a).

Differences between populational mean averages, as determined by a two-tailed ANOVA (Table 3) indicate statistically significant differences in mitochondrial width between the various mitochondrial populations. The ANOVA confirms that mitochondrial widths differ significantly between a number of different cell classes (Table 3). Differences in population means are evident in comparing sperm mitochondria with all other classes presented thus far, but are slightly less significant when comparing sperm mitochondria within the pollen grain (S.PG) and those within the pollen tube (S.PT).

While it is inherently interesting that six different classes of gametophytic cells may have consistently different mitochondrial dimensions, the immediate significance of this result is that it permits identifying the origin of certain mitochondria following gametic fusion. Two such test populations were selected: 1) a group composed of mitochondria observed within one-half micrometer of the sperm nucleus within the central cell (S.CC, Table 2) (e.g., Fig. 9, 10); and 2) those within unfused cytoplasmic bodies between the egg and central cell (see following section).

In the first test population (S.CC), the range of mitochondrial widths in this group, varying from 0.132–0.288 \( \mu \)m (avg. 0.21 \( \mu \)m), overlaps closely with the range of mitochondrial widths observed in sperm cells within the growing pollen tube (0.165–0.291 \( \mu \)m, avg. 0.22 \( \mu \)m). The 0.95 confidence intervals corroborate this ob-
servation (Table 2). No such overlap in confidence intervals is observed between this group of sperm nucleus-associated organelles and any other group, including that of the unfertilized central cell in which this group is observed, nor is there significant overlap at the 0.99 confidence interval when this test is repeated (S.CC 0.99 confidence interval is 0.20–0.23 μm; that of the unfertilized central cell, 0.45–0.53 μm).

A two-tailed ANOVA (Table 3) applied to this test class of organelles provides evidence that this population differs significantly from mitochondrial widths in the typical central cell mitochondrion. Furthermore, of the two male gametophytic origins, it is almost equally unlikely that these represent pollen tube mitochondria. This result represents strong evidence that pollen tube mitochondria are entirely excluded during gametic fusion, as in all cases mitochondria fit within the ranges of observed sperm cell mitochondrial dimensions (S.PT), but none overlapped with those of the pollen tube (Table 2).

Presence and identification of unfused cytoplasmic bodies between the egg and central cell—The second test population was derived from unfused, anucleate, membrane-bounded cytoplasmic bodies observed between the egg and central cell after the time of gametic fusion (Fig. 17, 18). These bodies are restricted in distribution to within approximately 10–12 μm beyond the aperture of the pollen tube, usually within micrometers of the vegetative nucleus and may persist well after fertilization (Fig. 19) without undergoing gross degenerative changes. They contain ribosomes, endoplasmic reticulum, and have a less electron-dense ground cytoplasm than either the egg or central cell; the density of the ground cytoplasm is similar to that in unfused sperm cells prior to gamete fusion (Fig. 4–6).

The mitochondria observed in these bodies range from 0.19–0.29 μm in width (mean average, 0.25 μm) and appear similar in size and structure to those observed within the sperm cell in the pollen tube (S.PT). The 0.95 confidence interval of the distribution overlaps with that of sperm mitochondria in the pollen grain, tube, and central cell, but does not overlap with that of mitochondria in the pollen grain or tube, egg, and central cell (Table 2), even when the confidence interval is raised to the 0.99 level. A two-tailed ANOVA of the population means (Table 3) showed a very significant difference in mitochondrial widths within cytoplasmic bodies from those of the pollen (grain and tube) and female gametophyte. There are insignificant differences in comparing mitochondrial widths within cytoplasmic bodies with those of the sperm (S.PG, S.PT, S.CC).

These statistical tests support an interpretation that these cytoplasmic bodies represent isolated segments of the sperm cell. This is corroborated by the location of these cytoplasmic bodies near the vegetative nucleus and the relative numbers of mitochondria observed within these bodies. Further, the small proportion of mitochondria located within these bodies suggests more specifically that these segments originate in the sperm projection associated with the vegetative nucleus (Russell and Cass, 1981a; Russell, 1981). This slender sperm cell projection (approximately 1 μm wide and up to 30 μm long during rapid phases of pollen tube growth; Russell and Cass, 1981a) is possibly severed during expulsion of the vegetative nucleus and gametes from the pollen tube. The ratio of mitochondria observed flanking the sperm nucleus in the central cell relative to that in cytoplasmic bodies (roughly 8:1) is smaller than numerical comparisons of mitochondria in the main cell body and projection made in sperm within pollen grains at anthesis (range, approx. 1.4:1 to 4.9:1; avg.

### Table 3. Results of statistical tests comparing mitochondrial widths in eight cellular populations. Except as noted, the following are the results of three-level nested ANOVA calculations

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* Key to abbreviations given in Table 2: * = $P = 0.05$; ** = $P = 0.01$; n.s. = not significant.

* Paired data (same preparation and electron micrographs). Analyzed by single classification ANOVA for populations with unequal variances.
2.45:1) (Russell, 1981, and unpubl. data). However, if one assumes that the sperm projection becomes less elongated during pollen tube growth, as reported previously (Russell and Cass, 1981a), and that the probability of segmentation likely increases near the ends of the projection, the 8:1 ratio observed may be an accurate estimate of the proportion of mitochondria in the main cell body transmitted into the female gametophyte versus those untransmitted during gamete fusion in cytoplasmic bodies between the egg and central cell.

**Discussion**—The paucity of information presently available concerning the events of gametic deposition, gametic fusion, and events accompanying nuclear fusion in angiosperms is largely the result of difficulties in analyzing the extent of cellular participation in gametic fusion and determining the initial fate of male and female cell contents. In Plumbago zeylanica, such an examination is favored by the absence of synergids, whose densely staining contents commonly obscure these processes in conventionally organized angiosperm megagametophytes. Despite years of such studies, the structure, condition, and ultrastructure of the sperm cytoplasm upon deposition in the female gametophyte is poorly understood. Although genetic studies have demonstrated that numerous angiosperms transmit certain characteristics through genes located within male cytoplasmic organelles (Kirk and Tilney-Bassett, 1978), in the majority of angiosperms the influence of these organelles during later embryogenesis and maturation is controversial. For the most part, embryologists have assumed that the issue is unproven or unprovable (Kapil and Bhatnagar, 1975), and regard the cytoplasmic contribution as unimportant to the fertilization process. However, a myriad of genetic phenomena owe their explanation to the occurrence of cytoplasmic transmission of heritable cytoplasmic organelles and certain cytoplasmic organelles may serve as useful intracellular markers for transmitted sperm cytoplasm. Moreover, these organelles possess inherent genetic importance as the mitochondria and plastids contain DNA and some independent hereditary characteristics (Grun, 1976; Gillham, 1978; Kirk and Tilney-Bassett, 1978).

From previous studies, it is evident that the sperms represent true cells, invested with a cytoplasm and organelles which seem viable as evidenced by their ultrastructural appearance (Russell and Cass, 1981a). Among the four recent embryological studies which report the condition of male gametes deposited within the degenerate synergid, all are in agreement that the sperm remain cellular up to the time of gametic fusion (Cass, 1981; Cass and Jensen, 1970; Cocucci and diFulvio, 1969; Wilms, 1981). Similarly, the male gametes of Plumbago also remain cellular prior to gametic fusion. The fate of these organelles during fusion, however, has been disputed. Presently, the only widely accepted basis for non-transmission of male organelles in angiosperms is the exclusion or elimination of organelles within the generative cell (Hagemann, 1979). However, the potential exists for exclusion of some sperm cytoplasmic organelles during gametic fusion. Although a number of embryological accounts have reported the non-transmission of sperm cytoplasm, only Jensen and Fisher have provided convincing evidence in a conventionally-organized female gametophyte which would suggest that sperm cytoplasm is shed prior to gametic fusion (Jensen and Fisher, 1968, fig. 19, 20; Fisher and Jensen, 1969). In Plumbago, there is also evidence that some of the sperm cytoplasm is not transmitted during gametic fusion, but the amount is minor. Less than 15% of the sperm organelles observed within the female gametophyte were seen in unfused cytoplasmic bodies.

Immediately prior to gametic fusion, several structural changes are evident in both the female and male gametes of P. zeylanica. The major change occurring in the female gametophyte, as discussed previously, relates to the disruption of the formerly continuous egg cell wall near the tip of the discharged pollen tube. This is evident in both chemically fixed (Fig. 3–6, 11) and physically fixed, freeze-substituted ovules (Russell, 1982, fig. 15). In the male gametophyte, possibly accompanying gametic deposition, sperm structure is modified in a manner possibly analogous to the concept of sperm capacitation in other biological systems. In Plumbago, gametic deposition entails the breakdown of both the external and internal pollen tube plasma membranes and releases the sperms as intact cells. The released sperms each possess a plasma membrane, but lack the additional layers of membranes and soluble cell wall components which normally envelope these cells during their descent in the pollen tube. This modification results in the direct exposure of sperm plasma membranes to the immediate environment and permits direct contact to occur between sperm plasma membranes and both the egg and central cell plasma membranes. A similar modification of sperm-associated membranes has also been noted in plants with conventionally organized megagametophytes (Wilms, 1981). Other modifi-
cations of sperm structure observed prior to gametic delivery in *Plumbago* include shape changes in the cell and mitochondria, and progressive decreases in the thickness of the sperm cell wall during passage in the pollen tube (Russell and Cass, 1981a).

As discussed previously (Russell, 1980; present study), the participation of male cytoplasm during gametic fusion is evidenced by the presence of transmitted sperm organelles in both the egg and central cell. The events of gametic fusion in *P. zeylanica* are also evidenced in the presence of membrane segments associated with the transmitted sperm nucleus in the central cell. Based on staining similarities between these membranes and those of the plasma membrane it appears that the membranes are remnants of the fusion process, derived from the fusion of the sperm and the respective female reproductive cell with which it fused. These membranes are readily identifiable in PA-TCH-SP-stained material (Fig. 9–11), and are easily discernable from other membrane systems including the endoplasmic reticulum. The distribution of these membrane fragments flanking the sperm nucleus and its associated organelles supports the idea that these membranes originated from the fusion of the sperm and central cell. On a populational basis, the small mitochondria seen associated with the sperm nucleus have been statistically identified as paternally originating organelles. The presence of such remnant membranes is strong evidence that although gametic fusion is likely to occur initially at one point in the cell membranes, that additional fusion points occur. Thus, similar to the study of membrane fusion events during exocytosis (da Silva and Kachar, 1980; Ornberg and Reese, 1981), and studies of cell fusion (Fumagalli et al., 1981), some of the sperm cell membrane in *P. zeylanica* does not appear to be directly incorporated by the two female reproductive cells during fusion. As in myoblast fusion studies (Fumagalli et al., 1981) cytoplasmic continuity may be established at several points nearly simultaneously, causing several fusion points to form.

Although non-participation of small membrane segments during cellular fusion may be a common feature in biological systems, one must assume that the location of fusion inside the megagametophyte may impose additional spatial constraints. Presumably, such constraints may originate in structural features which stabilize the egg-central cell boundary, in particular 1) the presence of microtubules associated with the plasma membrane (presumably functioning as a cytoskeletal element), 2) frequent specialized junctions of modified cell wall between the egg and central cell (Cass and Karas, 1974; present study), and 3) a continuous cell wall elsewhere between these two cells (Russell, 1982). Following gametic fusion, the remnant membranes appear to become disorganized by progressive vesiculation (Fig. 9–11). Later, the only visible remains of the fusion membrane appear to be a small membrane whorl as is evident in Fig. 8. (In this particular example, the lesser extent of such supernumerary membranes in the egg suggests that gametic fusion with the egg occurred prior to the fusion of the other sperm cell with the central cell.)

What is the sequence of events during gametic fusion? Based on the observations presented herein, it appears that the process of gametic fusion in *Plumbago* begins with the delivery of two single membrane-bounded male gametes between the plasma membranes of the egg and central cell. The geometrical location of the sperm cells between the egg and central cell results in the sperm cells becoming tightly appressed to the plasma membranes of both of the female reproductive cells (Fig. 23). Gametic fusion would logically be expected to begin at a single location on each sperm plasma membrane, as shown in Fig. 24 (arrowhead near SN1); however, there is evidence for subsequent instances of membrane fusion at multiple locations, as shown in Fig. 24 (arrowheads near SN1) and Fig. 25 (arrowheads near SN2). Fusion membranes disorganize by progressive vesiculation of remaining segments of membrane (Fig. 9, 10, 25). Isolated membrane segments (like those in Fig. 9–11) are likely composed of elements of both the sperm and the female cell involved. Vesiculation of fusion membranes may be completed in less than five minutes following gametic fusion. In Fig. 25, only a small remnant of the fusion membrane may be visible near the sperm nucleus in the egg (arrowheads near SN2).

What are the apparent prerequisites for gametic fusion? Gametic fusion in angiosperms as in other biological systems appears to be primarily a membrane-based event, and as such the condition, exposure, and apposition of gametic plasma membranes prior to fusion may be critical. Membrane apposition in *Plumbago* is facilitated by the disappearance of pollen tube plasma membranes surrounding the sperms, and the dispersion of cell wall components at the surface of the egg and central cell. Each sperm cell in *Plumbago* is thus directly apposed to both the egg and central cell prior to gametic fusion. In more conventionally organized female gametophytes with synergids, such conditions are present only upon
entry of the pollen tube into the degenerating synergid. Synergid degeneration appears to entail loss of the synergid plasma membrane and dispersion of any remaining cell wall materials near the egg and central cell, and therefore presents exposed cell surfaces similar to those seen in the megagametophyte of Plumbago. Further evidence that such modifications in synergid structure are necessary for normal reproductive function include infrequently reported cases in the literature in which gametic fusion has not occurred in normally appearing embryo sacs, despite the presence of pollen tubes within the ovule (Mogensen, 1978). In the rare cases in which the pollen tube has entered or approached the intact (persistent) synergid, successful pollen tube-megagametophyte interactions have not resulted. In these instances, the pollen tube may 1) continue growth through the persistent synergid, then enter and discharge its contents into the degenerated synergid (Jensen and Fisher, 1968); 2) discharge sperm cells into the persistent synergid which are not subsequently transmitted into female reproductive cells (Cass and Jensen, 1970; Cocucci and diFulvio, 1969); or 3) cease further tube growth prior to penetration of the persistent synergid (Mogensen, 1978). Apparently, there are yet no reports of normal fertilization resulting from the transmission of sperm cells into the persistent and presumably intact synergid. Even when ovules are removed from their normal milieu and cultured in vitro, the ensuing events of synergid degeneration and

Fig. 23–25. Semi-diagrammatic reconstruction of gametic fusion events in Plumbago zeylanica based on longitudinal sections illustrated in Fig. 8, 11. Pollen tube aperture evident on the left in each drawing; egg and central cell nuclei (not shown) are located to the right of the drawing. Duration of these events (based on Table 1) is probably less than 5 min. Approx. ×3,500. 23. Deposition of male gametes (S₁ & S₂) and their associated cytoplasmic organelles. Plasma membranes of egg and central cell indicated by unlabeled arrowheads. 24. Gametic fusion is initiated at one location as illustrated in the central cell (arrowhead). This is followed by several fusion events at other sites in the appressed membranes (arrowheads in the egg) and formation of numerous small vesicles by breakdown of remaining fusion membranes. 25. Vesicles generated by breakdown of remnant fusion membranes are indicated by unlabeled arrowheads in central cell. At late stages of gametic fusion, only membrane whorls are evident (arrowhead in the egg). Unlabeled arrow indicates membrane-bound cytoplasmic bodies presumably arising from fragmentation of a sperm cellular projection associated with the vegetative nucleus. See page 418 for KEY TO LABELING.
physical mediation of gametic fusion appear to follow similar developmental patterns (Olson and Cass, 1981) to those previously described.

Detailed fertilization studies to date indicate that the discharge of the successful tube occurs exclusively into the degenerated synergid in normally fertilized ovules (Jensen, 1974; Mogensen, 1978); however, this does not imply that the degenerated synergid is an intact cell at the time of gametic deposition and fusion. Moreover, the majority of electron microscopic studies to date indicate that the penetrated synergid lacks a plasma membrane and cellular integrity at the time of gametic deposition (Cocucci and Jensen, 1969; Jensen and Fisher, 1968; Maze and Lin, 1975; Mogensen, 1972; Mogensen and Suthar, 1979; Schulz and Jensen, 1968; van der Pluijm, 1964; van Went, 1970; Vazart, 1969; Wilms, 1981). In conventionally organized megagametophytes, it also seems a frequent observation that the vegetative nucleus and discharged pollen cytoplasm are located directly between the egg and central cell and not within the synergid at all (cotton, Fisher and Jensen, 1969; Petunia, van Went, 1970; Linum, Vazart, 1969; Spinacia, Wilms, 1981). In fact, Vazart (1969) suggested that the region occupied by these degenerating products, rather than the synergid proper, may normally constitute the site of male gamete deposition and transmission. Another parallel which seems apparent in comparing fertilization events in Plumbago with more typically constructed female gametophytes is the presence of simultaneous membrane contacts between sperm cells and both the egg and central cell. Of the three accounts describing the condition of the sperm within the synergid in detail (barley, Cass, 1981; Cass and Jensen, 1970; Spinacia, Wilms, 1981), each of these accounts showed simultaneous sperm cell contacts with both the egg and central cells. At least functionally, gametic deposition and fusion in other angiosperms may be similar to that in the Plumbago reproductive system. Whether the region of gamete fusion is originally derived from an intracellular region (the synergid) or intercellular region (outside of the synergid) may not greatly alter the function of the gametes during fertilization.

Since fertilization events in flowering plants are so highly dependent on the geometrical organization of the cells composing the egg apparatus, any study which proposes a relationship between reduced megagametophytes such as that of Plumbago must also consider the salient features of the conventionally organized megagametophyte. Particular significance must be given to cellular interfaces, as their location, condition, and intervening cell wall characteristics together play an important role in determining the potential fate of sperms deposited within the embryo sac. In light of the present findings, how does the egg apparatus of Plumbago compare with those of more typical angiosperms? The typical, conventionally organized female gametophyte possesses an egg apparatus composed of three roughly pyriform cells: a single egg cell and two flanking synergids. The synergids are placed nearest the micropyle and are thus well situated to receive the pollen tube. Each of the synergids possesses cell wall interfaces with the egg and central cell in addition to an interface with the other synergid. Prior to gametic fusion, the synergid cell typically possesses a poorly developed cell wall at its chalazal end, or one that is disrupted during gametic deposition. At this interface both the egg and central cells have exposed plasma membrane surfaces next to the synergids. In contrast, the micropylar ends of the two synergids possess a substantial cell wall which may physically block cellular fusion of sperms with these cells. The typical organization of the egg apparatus in angiosperms includes the presence of a thin, discontinuous cell wall within the degenerate synergid prior to gametic deposition (Jensen, 1974). Both Plumbago (Russell, 1982) and Capsella (Schulz and Jensen, 1968) represent exceptions, as the female gamete of these plants is invested by a continuous cell wall; however, the disruption of this cell wall during normal gametic deposition testifies to a unique consistency of the cell wall, apparently adapted to the transmission of the male gamete. Whether the discontinuity of the egg cell wall occurs prior to gametic deposition or shortly thereafter, the female reproductive cells consistently lack a continuous cell wall at the time of gametic fusion.

In Plumbago, the absence of synergids superficially appears to be a major departure from the more conventionally organized embryo sacs which possess synergids. Functionally, however, the egg of Plumbago appears to represent a reduced egg apparatus in which typical synergid structures and responsibilities are transferred to the egg cell (Russell and Cass, 1981b). The intercellular location of gametic deposition, which appears to distinguish Plumbago from the majority of the angiosperms, similarly may not represent a significant functional difference: The possibility of simultaneous contact of each sperm cell membrane with both of the female reproductive cells appears to be a more significant issue. If other angiosperms
also possess simultaneous membrane contacts between male and both female gametic cells prior to gametic fusion, this may represent a fundamental similarity between the conventionally organized egg apparatus and that of Plumbago. More importantly, the occurrence of such contacts between male gametes and both female reproductive cells constitutes the minimum structural requirement for possible gametic recognition in angiosperms.

LITERATURE CITED


