

Isolation of Sperms from the Pollen Tubes of Flowering Plants during Fertilization¹

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K. R. SHIVANNA², H. XU, P. TAYLOR, AND R. B. KNOX*

Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

ABSTRACT

Sperm cells have been isolated from pollen tubes growing in style segments of the dicotyledon *Rhododendron macgregoriae* and the monocotyledon *Gladiolus gandavensis* by the *in vivo/in vitro* method at various stages of fertilization. Pollen tubes emerged from the cut end of the style into agar medium, and more than 95% contained sperm cells. Sperm cells were released from the pollen tubes by osmotic shock or by placing styles in wall-degrading enzymes: 0.5% macerozyme and 1% cellulase. The isolated sperms were ellipsoidal protoplasts of diameter about 2×3 micrometers in *Gladiolus* and about 3×4 micrometers in *Rhododendron*. After isolation, a proportion of the sperm cells occurred in pairs linked at one end by finger-like connections. The pairs of isolated sperms were dimorphic in terms of surface area and volume. By cutting the styles at various positions and times after pollination, the potential exists to detect changes in sperm gene expression associated with fertilization.

MATERIALS AND METHODS

Plant Material. Flowers of *Rhododendron* spp., including *R. arborium*, *R. konori*, *R. macgregoriae*, and *R. nuttallii* were grown in a Melbourne garden. Cut flowers of *Gladiolus gandavensis* were purchased commercially when required.

Pollen Collection. Pollen was collected from freshly dehiscent anthers and was used immediately or stored for 2 to 3 d at 4°C over CaCl₂.

***In Vivo/in Vitro* Technique.** The basic technique developed for *Pyrus* (7) and *Petunia* (16) has been modified for sperm cell isolation. Flower buds were emasculated and bagged 2 to 3 d before anthesis. For *Rhododendron*, flowers were excised on the days of anthesis, and the pistils (together with 1–2 cm of pedicel) were pollinated with fresh compatible pollen and maintained in small vials containing distilled water. The pollen is in tetrads, and usually more than 200 tetrads were applied to each stigma. After 24 h incubation in darkness at $22 \pm 1^\circ\text{C}$, a segment comprising the upper 5 mm of the style (and stigma) or longer was cut transversely with a sharp blade, and the cut end was implanted in Petri plates of an 0.6% agar medium containing 12% sucrose, 100 mg L⁻¹ boric acid, 300 mg L⁻¹ calcium nitrate, 200 mg L⁻¹ magnesium sulfate, and 100 mg L⁻¹ potassium nitrate. While cutting, the style was immersed in this medium (minus agar) with the stigma kept above the liquid. The procedure for *Gladiolus* was similar, except that pollination was performed on the intact flowers, and usually 150 to 200 pollen grains were applied to each stigma. The upper 1 cm segments of the style were excised after 3 h and implanted in the agar medium as described above. Petri plates with implanted styles were examined for emerging pollen tubes. When tubes were visible (*e.g.* after 24 h for *Rhododendron* or 8 h for *Gladiolus*), the styles were gently removed with forceps, the numbers of emergent pollen tubes counted, and the samples used for sperm isolation.

Sperm Isolation and Characterization. To check on the presence of sperm nuclei, emerging tubes were fixed in 3:1 ethanol/ acetic acid for 1 h and were washed and stained in one of the DNA fluorochromes, DAPI³ (3), ethidium bromide, or Hoechst 33258 (8). Preliminary studies to release the sperm cells from the tubes were carried out on microscope slides. Pollen tubes of both genera were incubated in a drop of 5 or 7.5% sucrose medium for osmotic shock or were treated with 0.5% macerozyme R-10 (Serva) and 1% cellulase (Onozuka R-10, Serva) in the culture medium (minus agar). After 1 h of incubation, a drop of a DNA fluorochrome was added and examined by fluorescence microscopy for released sperm nuclei. The number of sperm nuclei released from the tubes was counted. Each sample was replicated ten times. These data enabled the yield of sperms to be calculated on a per style basis. For isolation, the sperm cells were collected directly from enzyme-treated pollen tubes on

The majority of angiosperms possess bicellular pollen grains in which the generative cell is held entirely within the vegetative cell at maturity and division to form the pair of sperm cells occurs within the pollen tube following germination and entry into the style (11). The generative nuclei have been isolated in one system, as a prelude to understanding pollen-specific gene expression (13). In several tricellular pollen types, where sperm cell division occurs in the maturing pollen, the sperm cells have been isolated. Success has been achieved using osmotic shock in *Hordeum* (1), *Plumbago* (17), *Brassica* (14), and *Zea* (6) and using physical grinding in *Brassica* (9), *Gerbera* (18), and *Zea* (2).

However, there have been no reports of the isolation of sperm cells in bicellular systems. Here, the sperms must be isolated from the pollen tubes, either during *in vitro* growth, or *in vivo* within the style. Modification of the *in vivo/in vitro* method (7, 16) provides a readily accessible source of pollen tubes containing sperm cells. This paper presents the first isolation and characterization of viable sperm cells from two bicellular pollen systems, the monocotyledon *Gladiolus* and the dicotyledon *Rhododendron*, as part of a study which aims to explore the molecular basis of double fertilization in angiosperms (12).

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² On leave from Department of Botany, University of Delhi, Delhi 110007, India.

³ Abbreviation: DAPI, 4',6'-diamidino-2-phenylindole.

a Nuclepore filter (pore size 1 μm) as described previously for sperms isolated directly from pollen grains (9). Emerging pollen tubes were excised and placed on the filter within a 13 mm Millipore filter unit and incubated in 2 ml of enzyme solution. After 1 h, the enzyme solution was removed, pollen tube debris was removed, and the filter containing the sperms was fixed in 2.5% glutaraldehyde in 0.03 M Pipes buffer (pH 7.2), containing 1.5 mM CaCl_2 and 12% sucrose for 1.5 h at room temperature. Portions of the filter were analyzed for the presence of sperm cells by staining with DAPI or by scanning electron microscopy. For this latter procedure, filters were postfixed in 1% OsO_4 in the same buffer for 30 min and dehydrated in an ethanol series and critical point dried with CO_2 by standard methods and sputter-coated with platinum-palladium before viewing in a JEOL 840 SEM at 5, 10, or 12 kV.

RESULTS

Production of Pollen Tubes. Following incubation of the pollinated styles of both genera, a large number of pollen tubes emerged from the cut end of the style. In *Rhododendron*, style segments cut 5 mm from the stigma (approximately half-style length) and incubated for 24 h showed 100 to 300 tubes per style. In *Gladiolus*, segments 10 mm in length and incubated for 8 h showed 70 to 90 tubes per style, depending on the density of pollination. The length of the emergent tubes, which grew straight and radiating from the cut surface was up to 2 mm. In *Rhododendron*, styles could be cut shorter, and implanted immediately in the agar after pollination, but pollen tubes took 48 h to appear. For longer style segments in which cuts were made closer to the ovary, the tubes were grown *in vivo* for as long as possible in order to prevent microbial contamination of the medium during prolonged *in vitro* culture. DAPI staining of emerging tubes in styles cut at 5 or 10 mm from the stigma showed the presence of sperm nuclei in >95% of tubes in both *Rhododendron* and *Gladiolus*.

Isolation and Characterization of Sperm Cells. Experiments to burst the pollen tubes by osmotic shock in media with sucrose concentration reduced to 5 or 7.5% gave varying success rates in the two genera, as estimated by DAPI staining. A low frequency of success was obtained in *Rhododendron*, with only about 30% of tubes bursting. A higher yield of isolated sperm was obtained in *Gladiolus*, about 93% of expected sperms based on estimated numbers of pollen tubes.

The enzyme treatment gave the opposite results in the two genera. *Rhododendron* pollen tubes showed over 90% dissolution of the tube tips within 1 h, with many nuclei closely associated in pairs (Fig. 1). With *Gladiolus* pollen tubes, however, the recovery of sperm cells was only about 59%.

Scanning electron microscopy of the enzyme-treated sperm cells showed that the sperms are ellipsoidal, about 3 to 4 μm in diameter in *Rhododendron* (Figs. 2 and 3) and 2 to 3 μm in *Gladiolus* (Figs. 4 and 5). A notable feature of *Rhododendron* sperms is that the nucleus of the cells is frequently observed apparently attached to the plasma membrane, which has a raised sculptured appearance at such sites (Fig. 3). Analysis of scanning electron micrographs showed that in *Rhododendron* about 75%

of the isolated sperms occurred singly, while 25% remained in pairs following the enzyme treatment. In *Gladiolus*, only 9% of the sperms remained associated in pairs following the same isolation technique. In both genera, the cells are linked by a single circular connection at one end with finger-like processes (Figs. 2–5) and broken connections are evident on all the single sperms.

Sperm cell volumes are significantly different between the larger and smaller sperm cells in each pair. In *Rhododendron*, the largest sperm had a cell volume approximately half as large again as the smaller sperm of each pair (Table I). The smaller of the pair had a more granular surface structure than the larger smoother sperm as seen by scanning electron microscopy (Fig. 3). In *Gladiolus*, both the mean surface area and volume of the larger sperm were approximately double those of its smaller partner (Table I).

DISCUSSION

The *in vivo/in vitro* technique (7, 16), combined with enzymic digestion of the pollen tube walls, has enabled the isolation of sperm cells from bicellular pollen systems. The sperms are produced at second pollen mitosis within the pollen tubes *in vivo* and are accessible for experimental analysis after brief *in vitro* growth. This method overcomes a major limitation in studying the physiology and biochemistry of sperm cells in the majority of angiosperms that possess bicellular pollen grains. The technique is simple and can be extended to other genera after suitable optimization of culture media and time of implantation.

This is the first report of the use of cell wall-degrading enzymes to isolate sperm cells, which has previously been achieved by osmotic shock (14, 16) or by physical grinding (2, 9). The variation between monocot and dicot pollen tube systems is shown in this study, where enzyme treatments were most effective in isolating *Rhododendron* tubes, while osmotic shock was the more effective method for *Gladiolus*. We have found for the first time in a bicellular system that a proportion of the sperm cells are linked together in pairs following isolation from pollen tubes. The sperms in each pair differ significantly in surface area and cell volume. Dimorphism of sperm cells within pollen grains is known in several systems (15), but there are no previous data from pollen tubes. The single sperms isolated in this study possess surface membrane outgrowths suggesting that the paired associations have become detached during isolation. In tricellular systems, a special structural organization, the male germ unit (4, 5) comprises an association of the pair of sperm cells in which one is linked with the vegetative nucleus, *i.e.* the linking of all the DNA of male heredity. The significance of such structural organization is that the sperm cells may be preprogrammed as a transmitting unit for double fertilization. Complete units have been isolated from mature pollen grains in a tricellular system (14).

In *Gladiolus* and *Rhododendron* pollen tubes, there is no evidence from the present study that the vegetative nucleus remains associated with the sperm cells following isolation. This may be due to the change in shape, the rounding off of the sperm cells when isolated. Cytological evidence from three-dimensional

Table I. Comparison of Physical Characteristics of the Pairs of Isolated Sperm Cells from Pollen Tubes of *Gladiolus* and *Rhododendron*, as Determined by Image Analysis of Scanning Electron Micrographs

Species	Larger Sperm	Smaller Sperm	Significance
A. Sperm surface area (μm^2)			
<i>Gladiolus</i>	4.1	2.4	$t = 5.18$ $df = 5$ $P = 0.003$
<i>Rhododendron</i>	7.4	5.3	$t = 5.22$ $df = 6$ $P = 0.002$
B. Sperm cell volume (μm^3)			
<i>Gladiolus</i>	5.8	2.7	$t = 5.62$ $df = 5$ $P = 0.003$
<i>Rhododendron</i>	12.8	8.8	$t = 4.75$ $df = 6$ $P = 0.003$

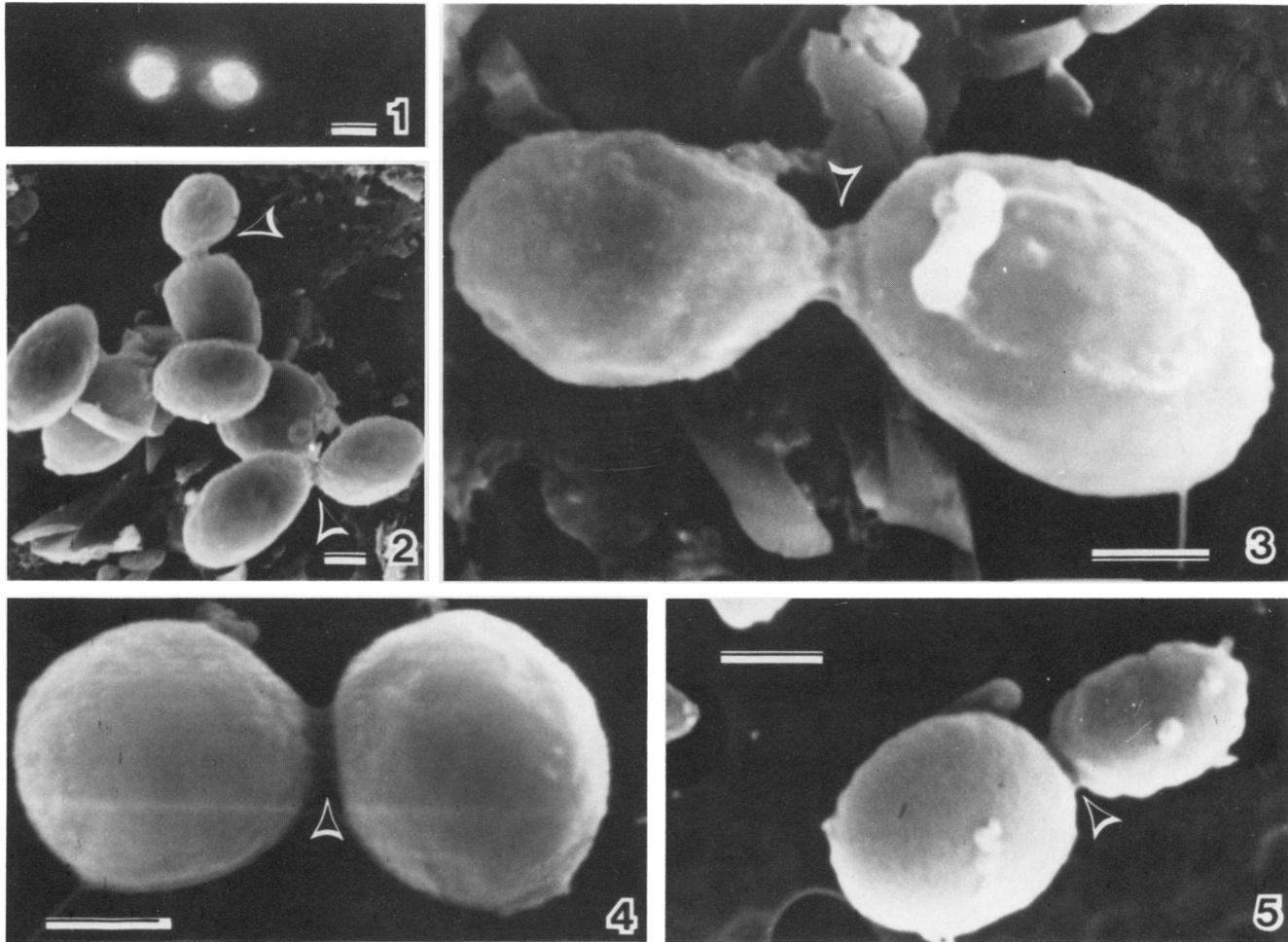


FIG. 1 to 5. Isolated sperm from pollen tubes of *R. macgregoriae* (1–3) and *G. gandavensis* (4–5); magnification bars, 1 μm . 1, Pair of sperms released from tube after enzymic digestion, visualized by DAPI fluorescence. 2 and 3, Scanning electron micrographs of isolated *Rhododendron* sperm cells. Note some sperms remain associated in pairs, connected by finger-like processes (arrows); in 3, note the granular surface pattern of the smaller sperm (LHS), and the smooth surface pattern of the larger sperm (RHS) except for the raised pattern of the underlying nucleus. 4 and 5, Scanning electron micrographs of pairs of sperm cells of *Gladiolus*; in 4, the sperms are almost isomorphic, while the pair in 5 is markedly dimorphic.

reconstructions demonstrates that male germ units are formed within the pollen tube in two bicellular systems, *Petunia* (19) and *Rhododendron* (10). The ellipsoidal shape of the sperms after isolation contrasts with the markedly elongate shape of *Rhododendron* sperms in intact pollen tubes viewed by three-dimensional reconstruction of serial thin sections of pollen tubes (RB Knox, V Kaul, unpublished data). The function of the vegetative nucleus in such associations remains unknown.

The physiological significance of this study lies in the provision of structural evidence favoring the specific recognition hypothesis of fertilization (12). The linkage of the sperms in pairs just prior to fertilization in the pollen tube, combined with the significant dimorphism, suggests that each of the pair of sperm cells has its own pathway of differentiation and functional specialization for double fertilization.

Which of the pair of sperms is the true male gamete that fertilizes the egg? This new technique offers us an experimental approach to this question. By cutting the style at different lengths or times after pollination, samples of sperm cells at various stages can be produced from the second pollen mitosis which occurs soon after entry into the style, right up until entry of pollen tubes into the ovule. Since the sperm cells from tricellular types that have been previously isolated are from mature dormant pollen

(2, 6, 7, 9, 14, 17, 18), it is likely that these sperms are physiologically inactive. The *in vivo/in vitro* technique, however, provides populations of sperm cells from pollen tubes that are approaching fertilization, when these cells are about to perform their biological role. The number of tubes in replicated styles is sufficient for molecular analysis of the differences in sperm surface macromolecules, particularly for screening of monoclonal antibodies (9). Given further improvements in techniques, it should be possible in future to isolate polyadenylated RNA for studies of the expression of fertilization-specific sperm genes.

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