

# Isolation and collection of two populations of viable sperm cells from the pollen of *Plumbago zeylanica*

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## Summary

A protocol is described for individually collecting two populations of sperm cells,  $S_{vn}$  and  $S_{ua}$ , from pollen of *Plumbago zeylanica*. Pollen grains were burst in 10 mM MOPS buffer containing 0.8 M mannitol (pH 4.6). Paired sperm cells released from pollen were separated using a microinjector.  $S_{vn}$  and  $S_{ua}$  were then collected individually with a microinjector, based upon known size differences. Collected sperm cells were washed with isolation medium and transferred to liquid nitrogen until use. Fluorochromatic reaction (FCR) test of isolated sperm cells showed a positive reaction, indicating that the isolated sperm cells are viable; most of the sperm cells retain viability for at least 2 h.

Keywords: *Plumbago zeylanica*, Pollen, Preferential fertilisation, Sperm cell isolation

## Introduction

Fertilisation in flowering plants involves two sperm cells: one sperm cell fuses with the egg cell to form the embryo and the other fuses with the central cell to form the endosperm, a nutritive tissue needed for growth of the succeeding generation. Differences in the two sperm cells have been reported in a number of angiosperms (Russell, 1991). In a number of plants, sperm dimorphism has been found that may relate to preferential fertilisation, in which one sperm cell has a greater likelihood of fusing with the egg (Russell, 1992). In *Plumbago zeylanica*, the two sperm cells differ in their content of both mitochondria and plastids. The sperm cell physically associated with the vegetative nucleus ( $S_{vn}$ ) frequently contains no plastids and more than 200 mitochondria, whereas the sperm cell unassociated with vegetative nucleus ( $S_{ua}$ ) contains an average of 24 plastids and fewer than 50 mitochondria (Russell, 1984). Fusion between the egg and the plastid-rich sperm cell ( $S_{ua}$ ) occurs in 94% of the cases

examined (Russell, 1985). The molecular mechanism that causes the two sperm cells to differentiate as well as to participate in preferential fertilisation is unknown. To study potential gene involvement in preferential fertilisation, highly purified viable sperm cells of both the  $S_{vn}$  and  $S_{ua}$ , as separate populations, are needed.

In contrast to readily accessible animal sperm cells, the unique 'cell-within-a-cell' construction of plant sperm cells poses difficult problems for their isolation and further manipulation. In addition, the two sperm cells from a single pollen grain are, in some circumstances, difficult to discriminate and collect separately from one another. Preliminary work has demonstrated that pollen cytoplasmic organelles may represent a major contaminant for mRNA/DNA analysis of sperm cells (Xu, Russell, Zhang and Singh, unpublished data). In this paper, we report a protocol for the individual collection of two populations of viable sperm cells from pollen of *Plumbago zeylanica* ( $S_{ua}$  and  $S_{vn}$ ), which are especially adapted for molecular study.

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## Materials and methods

Plants of *Plumbago zeylanica* L. were grown in a greenhouse at the University of Oklahoma. Fresh pollen collected from flowers was immersed in a solution

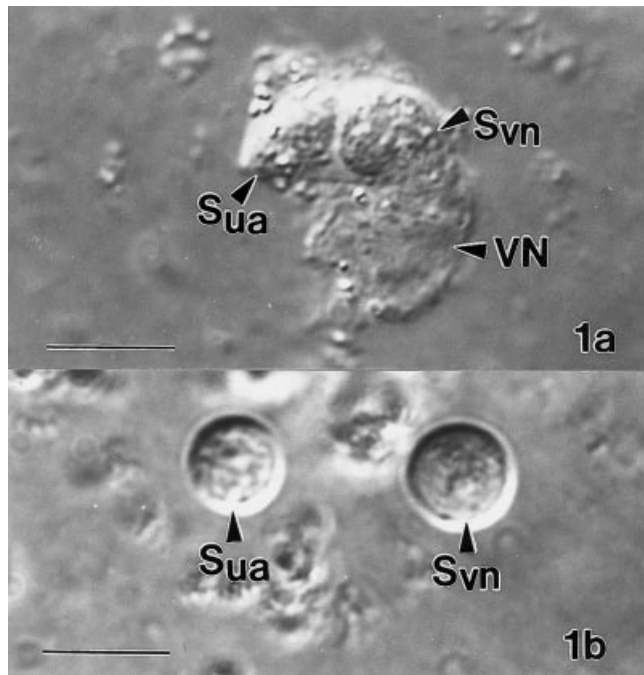
containing 10 mM MOPS and 0.8 M mannitol (pH 4.6) to release the sperm cells. Most of the pollen bursts due to osmotic shock within 5 min, releasing the osmotically more tolerant sperm cells, vegetative nuclei and pollen contents into the solution. Paired sperm cells released from the same pollen grain are separated using a microinjector (Eppendorf, type 5242) using flame-drawn capillaries, trimmed to an aperture size of 15  $\mu\text{m}$ .  $S_{ua}$  and  $S_{vn}$  were then collected in individual groups using the microinjector, based upon their size difference, which known to be consistent from past study (Russell 1984), omitting pairs without a conspicuous size difference. Unpaired cells were omitted because the sperm size ranges partially overlap. Collected sperm cells ( $S_{vn}$  or  $S_{ua}$ ) were then washed two times with the isolation medium to eliminate pollen cytoplasmic organelles. The purified sperm cells were then transferred into an Eppendorf microcentrifuge tube, which was pre-cooled with liquid nitrogen. The sperm cells were stored in liquid nitrogen or dry ice until use.

The viability of collected sperm cells was evaluated by FCR (fluorochromatic reaction) (Heslop-Harrison & Heslop-Harrison, 1970), using 0.0002% fluorescein diacetate (FDA). The FDA was mixed as 0.1% (w/v) FDA in acetone to form a stock solution, which was then diluted 1:50 with water; 10  $\mu\text{l}$  of the diluted stock was used for each 100  $\mu\text{l}$  of isolation solution.

## Results

The newly released sperm cells from pollen are generally paired and normally spindle-shaped (Fig. 1a). The sperm cells rapidly become ellipsoidal to nearly spherical, but are still connected by membranous surrounding materials, often including the inner pollen plasma membrane. The distance between the two sperm cells increases with time after isolation. The average diameters of the  $S_{vn}$  and  $S_{ua}$  are  $7.98 \pm 0.50 \mu\text{m}$  and  $6.91 \pm 0.44 \mu\text{m}$ , respectively (Fig. 1b). The vegetative nucleus is frequently observed within a few minutes of isolation associated with the sperm cells, forming the so-called male germ unit (Dumas *et al.*, 1984) (Fig. 1a). Vegetative nuclei were excluded from harvest, however, because of their fragility and large size, which matched that of the microinjector pipette tip (15  $\mu\text{m}$  diameter).

Newly released sperm cells initially aggregate with pollen cytoplasm forming clusters that are difficult to separate with a microinjector (Fig. 2a–c). As the sperm cells are transferred from the isolation medium to the first wash, sperm cells readily separate from much of the surrounding pollen material (Fig. 2d), although some cells still have pollen organelles associated with them. With the second wash, most of the organelles

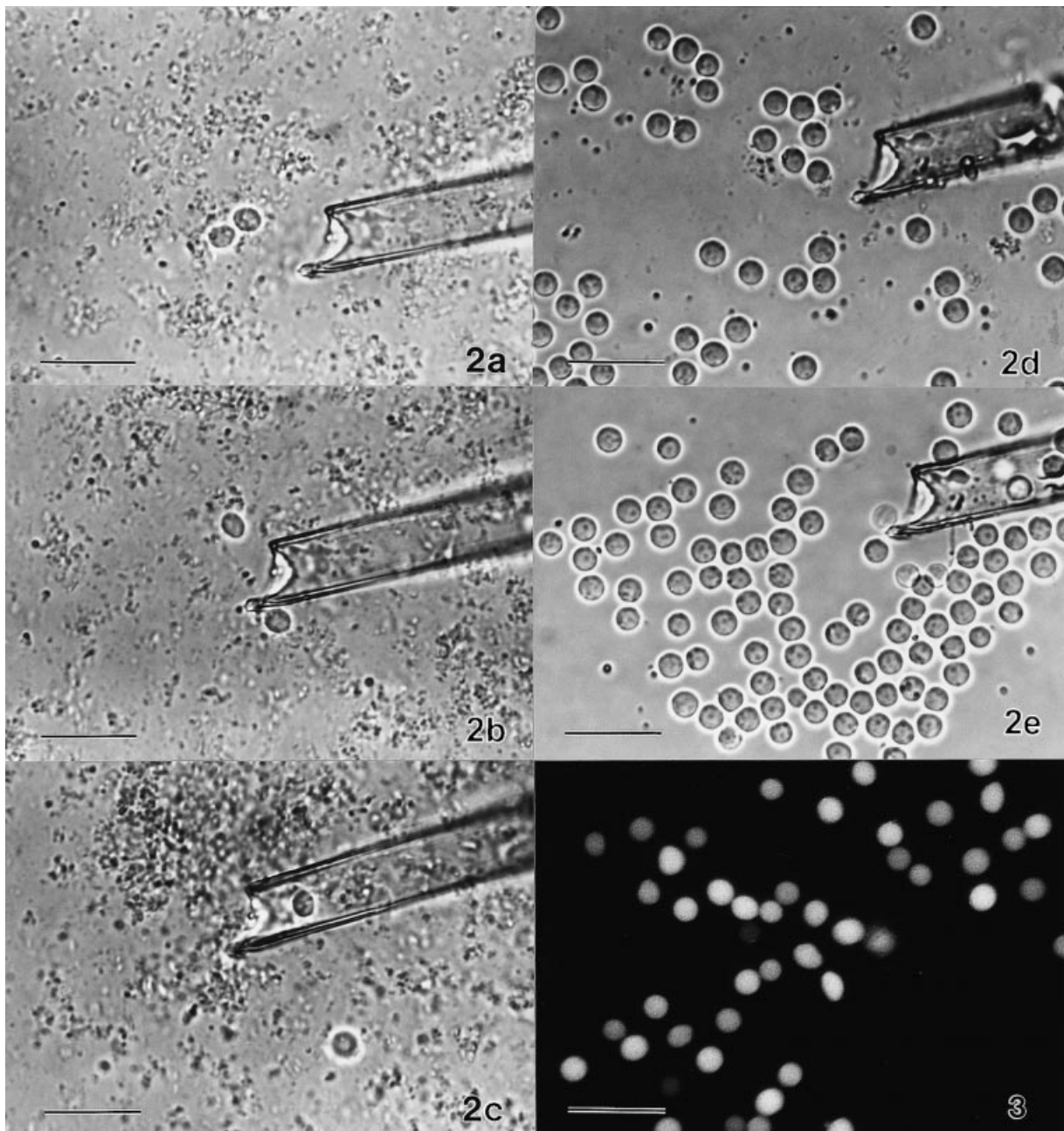


**Figure 1** Interference contrast micrograph of a pair of sperm cells isolated from pollen of *Plumbago zeylanica*. (a) Newly released sperm cells ( $S_{ua}$  and  $S_{vn}$ ) associated with the vegetative nucleus (VN). (b) Five minutes later, the sperm cells have separated from each other and become spherical. The  $S_{vn}$  is the larger of the two cells and is initially physically associated with the vegetative nucleus. Scale bars represent 10  $\mu\text{m}$ .

have been eliminated (Fig. 2e). As the sperm cells are purified further, they tend to aggregate in solution, separated by a 200–500 nm perimeter (Fig. 2e). At this stage, the contamination is reduced to less than one organelle per sperm cell. Collection efficiency can routinely reach nearly 100 sperm cells per hour with excellent purity. The sperm cells display a positive FCR indicating that isolated sperm cells are viable (Fig. 3). Sperm cells retain viability for at least 2 h.

## Discussion

The first attempt to isolate sperm cells from angiosperms was reported by Russell (1986), who used 20% sucrose as bursting solution and obtained an enriched fraction of sperm cells from the pollen of *Plumbago zeylanica*. Viability, as measured using the FCR test, was less than 5 min in this solution, although Evans' blue continued to be excluded from sperm cells for up to 24 h; this suggests that cell membranes are largely intact but that cell integrity is in some ways compromised. The protocol reported here results in FCR-positivity for more than 2 h after isolation of sperm cells. This is indicative of a significant improve-



**Figure 2** Collection of two populations of sperm cells with a microinjector. (a) Two sperm cells released from the same pollen grain are found in pairs. (b) Sperm cells are separated with the microinjector. (c) One sperm cell ( $S_{ua}$ ) is drawn into the microinjector. (d) First wash of collected sperm cells ( $S_{ua}$ ). (e) Second wash of collected sperm cells ( $S_{ua}$ ). Fewer than one organelle per sperm cell is left in solution. Scale bars represent 30  $\mu\text{m}$ .

**Figure 3** FCR test of isolated sperm cells ( $S_{ua}$ ), showing a positive reaction with epifluorescence microscopy. Scale bar represents 30  $\mu\text{m}$ .

ment in the quality of the sperm cells of *Plumbago*, since FCR-positivity appears to be required for *in vitro* fertilisation (Kranz & Lörz, 1993; Faure *et al.*, 1994) and is highly desirable for other methods of experimental manipulation.

Numerous protocols have emerged for isolation of plant sperm cells *en masse* (Shivanna *et al.*, 1988; South-

worth & Knox, 1988; Russell, 1991; Theunis *et al.*, 1991). *En masse* isolation of sperm cells involves either centrifugation or a discontinuous gradient or filtration using a polycarbonate filter. In none of these protocols, however, are sperm cells so highly purified from contaminating pollen organelles as with the present method, and nor could individual types of sperm cells

( $S_{vn}$  and  $S_{ua}$ ) be collected into separate populations. Using the highly purified populations of sperm cells from each morphotype ( $S_{vn}$  and  $S_{ua}$ ), as described here will improve the utility of sensitive techniques such as immunoblotting, elicitation of monoclonal antibodies or the preparation of PCR-amplified cDNA libraries. The use of these techniques in sperm cell biology will enhance our understanding of the unique properties that the male gametes of angiosperms may possess and contribute to an understanding of the nature of preferential fertilisation.

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