

Generative and sperm cell isolation in *Bauhinia blakeana* (Fabaceae)

Hua Deng¹, Ya-Ying Wang², Xue-Yi Zhu¹ & Hui-Qiao Tian¹

¹⁾ School of Life Science, Xiamen University, Xiamen 361005, China

²⁾ Xiamen Medical College, Xiamen, Fujian 361008, China

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Bauhinia blakeana (Fabaceae) pollen is bicellular and contains only a vegetative and generative cell at anthesis. The pollen grain contents, including the generative cell, can be released *in vitro* following osmotic shock. In this study, when pollen grains were incubated in a 10% sucrose and 5%–15% polyethylene glycol 4500 (PEG 4500) medium, over 50% of the pollen grains germinated and formed a short pollen tube. However, the generative cell did not divide in *in vitro* pollen tubes. *In vivo* pollen tubes were induced to grow from the style using an *in vivo*–*in vitro* method. Sperm cells were subsequently isolated from broken pollen tubes.

Introduction

Sperm cell isolation is the basis for performing *in vitro* fertilization and investigating spermatogenesis in plants (Wang *et al.* 2006). Two types of pollen are characteristic of angiosperms at anthesis: bicellular and tricellular. Sperm isolation is relatively easy in species with tricellular pollen. Typically, tricellular pollen grains are incubated in a medium with some level of osmotic pressure. Under these conditions, the grains readily break releasing the contents, including two sperm cells. The isolation of sperm cells in bicellular pollen species is more difficult due to the formation of two sperm cells in the pollen tube. Therefore, in order to isolate the sperm cells, it is first necessary to induce pollen-tube growth. Most reports of successful sperm-cell isolation have been for herbaceous plants. Chen and Li (1998) isolated sperm cells

from *Ulmus pumila*, a tricellular pollen species. Zhou and Fan (1999) isolated sperm cells from the bicellular pollen species *Liriodendron chinense*, in which the generative cell divided after incubation to form two sperm cells. In the present study, we isolated generative cells from *Bauhinia blakeana* (Fabaceae), which exhibits a bicellular pollen type. The pollen grains were subjected to osmotic shock and sperm cells were removed from the pollen tubes using an *in vivo*–*in vitro* method (Shivanna *et al.* 1988).

Material and methods

Plant specimens

Bauhinia blakeana is a perennial, evergreen tree widely cultivated throughout the Xiamen district in China. The species has large, thin leaves and

from March to May red-purplish flowers. The pollen grains possess a vegetative and generative cell at anthesis, and sperm cells form only in the pollen tube. Therefore, the species is classified as a having a bicellular pollen type. The flowers for the study were obtained from the Xiamen University Campus.

Breaking pollen grains to obtain generative cells

Fresh pollen grains were incubated in solutions containing 5%–20% mannitol (0.273–1.179 mOsmol/kg H₂O) or 5%–20% sucrose (0.149–0.664 mOsmol/kg H₂O) for 5–10 min. It was expected that after incubation some pollen grains would break and release the generative cell from the pollen cytoplasm.

In vitro pollen tube induction

Pollen grains were placed in mediums containing 0.05% (w/v) CaCl₂, 0.01% (w/v) H₃BO₃, 0.01% (w/v) KH₂PO₄, and 10%–20% sucrose at pH 6.5 in room temperature (25 °C). Initial observations indicated that some grains germinated and produced a short pollen tube, but the tube tips enlarged and rapidly broke. Therefore, we amended the medium with 5%–20% polyethylene glycol 4500 (PEG) and achieved longer tubes that exhibited normal morphology.

In vivo–*in vitro* pollen tube induction

The style of *B. blakeana* is approximately 1 cm long and pollen tubes require 10 h to reach the ovary in May temperatures (about 20 °C night and 28 °C day). Therefore, our protocol was as follows: each flower was emasculated at anthesis and the stigma was hand-pollinated with fresh pollen. Pollen tubes were subsequently grown *in vivo* for 6 h. Then, the styles were cut near the ovary and immersed in a medium containing 0.01% (w/v) H₃BO₃, 0.01% (w/v) KH₂PO₄, 0.01% (w/v) CaCl₂ and 20% (w/v) sucrose at pH 5.0 for 3–5 h until pollen tubes emerged from

the cut end of the style. The styles with pollen tubes were transferred into a 6%–8% mannitol solution. Pollen tubes that broke in the mannitol solution released their contents, including two sperm cells.

The isolated sperm cells were tested for their viability with fluorescein diacetate (FDA, Sigma, F7378). After testing the sperm viability, the sperm cells were washed twice in a breaking solution and the pollen tube organelles were removed. The purified generative cells and sperm cells were transferred to an Eppendorf microcentrifuge tube in preparation for making a sperm cell cDNA library assay.

The osmolality of all solutions was measured using an osmometer OSMOMAT 030.

Results

Generative cell isolation

Following incubation in a mannitol or sucrose osmotic regulator solution for 5–10 min, some *B. blakeana* pollen grains ruptured and released their cytoplasmic contents including the generative cell (Fig. 1A and B). The ratio and timing of ruptured pollen grains was related to the type and concentration of the osmotic regulator. The lower the osmotic regulator concentration was, the greater the number of broken pollen grains. In a 5% mannitol or sucrose solution, the percentage of broken pollen grains was 98% and 99%, respectively. Increases in solution concentration, resulted in a decrease in the percentage of broken pollen grains. In a 20% mannitol or sucrose solution, the percentage of broken pollen grains decreased to 6% and 23%, respectively (Table 1).

The generative cell was of an elongated type, and became essentially spindle-shaped soon after release from the pollen grain; it finally assumed a round shape. The shape of the generative cells was related to the two osmotic regulators and the osmotic pressure of the isolation solution. In a 5% mannitol or sucrose solution, although the percentage of ruptured pollen grains was highest, the generative cells were inflated and broken soon after release. In the 10% mannitol solution, the generative cells took on a round shape within

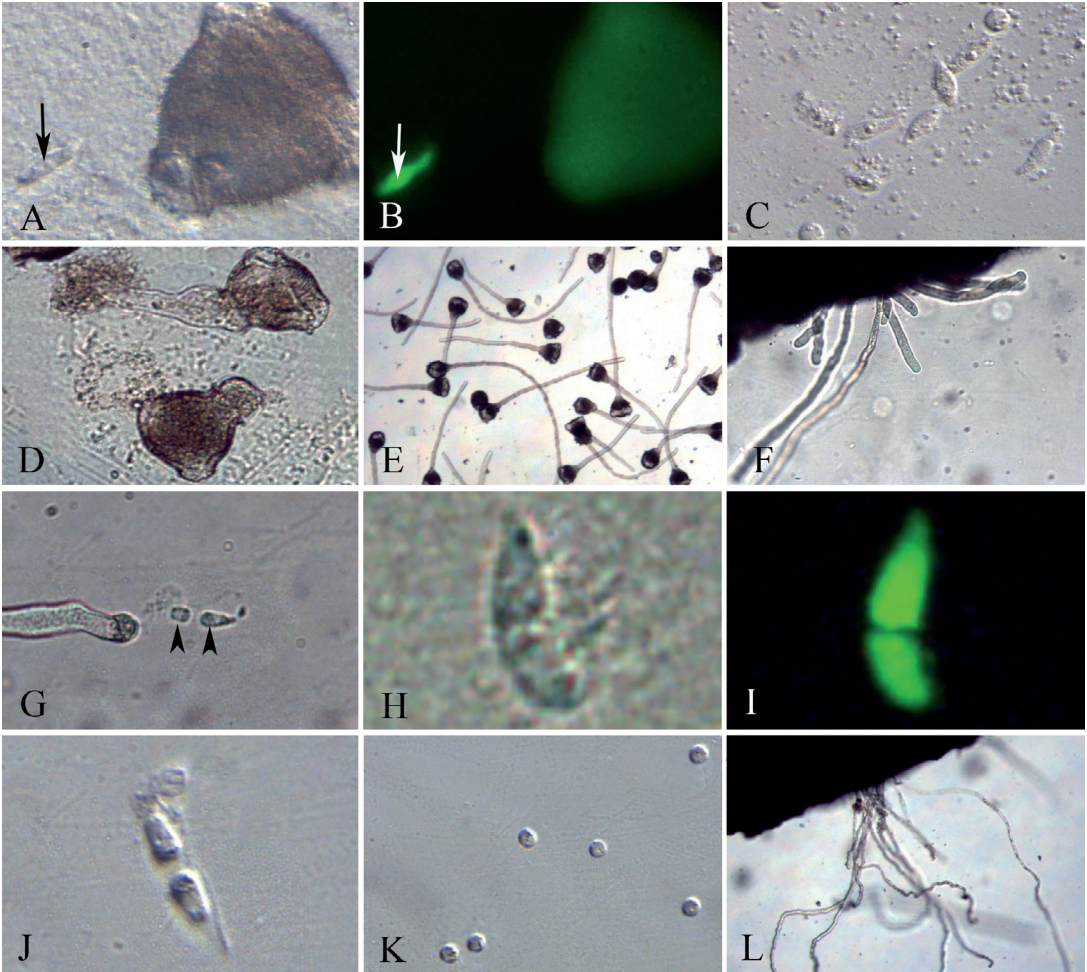


Fig. 1. Generative and sperm-cell isolation in *Bauhinia blakeana*. — **A:** A just released generative cell (arrow) from a pollen grain ($\times 1500$). — **B:** Elongated generative-cell (arrow) viability after the FDA reaction ($\times 1500$). — **C:** Collected generative cells ($\times 1500$). — **D:** Pollen grains germinating irregular pollen tubes ($\times 800$). — **E:** Pollen tubes in a PEG-amended medium ($\times 400$). — **F:** Pollen tubes growing from the cut end of a style ($\times 600$). — **G:** A pair of sperm cells (arrowheads) just released from a pollen tube ($\times 800$). — **H:** A pair of associated sperm cells ($\times 1500$). — **I:** Sperm-cell viability after the FDA reaction ($\times 1500$). — **J:** A pair of ellipsoidal sperm cells ($\times 1500$). — **K:** Collected sperm cells ($\times 400$). — **L:** Long and curly pollen tubes when the styles were cultured for over 5 h ($\times 400$).

Table 1. The effect of mannitol and sucrose concentrations on pollen breaking and generative-cell shape within 2 min after release.

Osmotic regulators	Osmotic pressure (mOsmol/kg)	No. of pollen grains	Broken pollen grains (%)	Ratio of spindle generative cells (%)
5% mannitol	0.273	115	98.26 $\pm$ 2.76	0 $\pm$ 0
10% mannitol	0.566	128	78.87 $\pm$ 3.57	39.2 $\pm$ 0.52
15% mannitol	0.887	109	46.83 $\pm$ 2.23	65.72 $\pm$ 2.16
20% mannitol	1.179	117	5.42 $\pm$ 0.42	95.82 $\pm$ 5.82
5% sucrose	0.149	112	96.94 $\pm$ 2.08	0 $\pm$ 0
10% sucrose	0.305	103	73.52 $\pm$ 2.69	35.84 $\pm$ 1.18
15% sucrose	0.474	125	67.79 $\pm$ 12.76	95.88 $\pm$ 3.96
20% sucrose	0.664	106	23.53 $\pm$ 1.01	100

2 min after release, however in 10% sucrose, the generative cells remained spindle-shaped for 10 min. In 15% sucrose, the spindle generative cells maintained their shape for 20–30 min (Table 1). The spindle generative cells were easily collected and purified in large numbers (Fig. 1C).

Pollen tube *in vitro* induction

When 0.01% H₃BO₃, 0.01% KH₂PO₄, or 0.01%–0.05% CaCl₂ was added to the 10% sucrose solution, many pollen grains germinated and produced short pollen tubes. Among the three chemicals, CaCl₂ had the greatest effect on preventing pollen breakage and inducing pollen germination (Table 2).

In the calcium-enriched medium, pollen tubes grew for only a short time and the tube length did not exceed 100 μm, which was quickly followed by inflation and breakage of the tube tips (Fig. 1D). However, tube growth was markedly improved after amending the medium with 5%–20% PEG. In the lower PEG concentration, the tube tip still broke; and in the higher PEG concentration, pollen germination decreased. The addition of 15% PEG resulted in normal pollen tube morphology and the tube

length reached 230 μm after 20 min and 410 μm after 1 h (see Fig. 1E and Table 3).

Sperm cell isolation

In the above-mentioned medium, the generative cell did not divide in the *in vitro* pollen tubes, even following 3 h of culture. An *in vivo*–*in vitro* method was used to induce generative cell division, in which pollen tubes were permitted to grow in style *in vivo* and then styles were cut and immersed in a medium. The osmolality of the medium was 0.758 mOsmol/kg H₂O. Following approximately 2–3 h of incubation, some pollen tubes emerged from the cut end of the style (Fig. 1F) and exhibited conspicuous cytoplasmic streaming. To release the sperm cells, the cut end of the style was transferred and immersed in a breaking solution of 6%–8% mannitol (0.342–0.458 mOsmol/kg H₂O). Some pollen tubes ruptured and pairs of long sperm-cells were released (Fig. 1G). These sperm cells were generally elongated, but rapidly assumed an ellipsoid shape and finally a round appearance in the 6% mannitol solution. In the 8% mannitol solution, sperm ellipsoid shape could be retained for a longer time than in the 6% mannitol solution. The two sperm cells remained connected and

Table 2. The effect of different concentrations of CaCl₂ on pollen germination in 10% sucrose solution with 0.01% H₃BO₃ and 0.01% KH₂PO₄.

CaCl ₂ (%)	No. of pollen grains	Breaking pollen grains (%)	Germination grains (%)
0	78	86.95 ± 2.30	13.3 ± 1.59
0.01	96	15.55 ± 1.29	80.47 ± 2.56
0.03	103	17.63 ± 0.94	77.04 ± 2.24
0.05	89	13.63 ± 1.87	87.59 ± 0.23

Table 3. The effect of polyethylene glycol (PEG) on pollen germination and tube growth in 10% sucrose solution with 0.01% H₃BO₃, 0.01% KH₂PO₄ and 0.05% CaCl₂ after 1 h in the culture medium.

PEG (%)	No. of pollen grains	Germinating grains (%)	Length of pollen tubes (μm) (min–max)	Appearance of pollen tubes
5	88	77.19 ± 0.14	465 (460–470 μm)	Tube tip not inflated but still broken
10	78	62.11 ± 2.61	480 (475–485 μm)	Tube curly
15	89	56.31 ± 0.50	410 (406–414 μm)	Tube straight
20	59	25.50 ± 1.23	535 (248–822 μm)	Tube length strongly variable

fluorescein diacetate (FDA) staining indicated the cells were viable (Fig. 1H and I). It was also observed that in some cases, the two sperm cells appeared to be associated with the vegetative nucleus as a male germ unit (MGU) (Fig. 1J). The vegetative nucleus swelled and deteriorated quickly, but the sperm cells remained associated with each other for at least 30 min. During this 30 min, the two sperm cells were easily collected using a micromanipulator (Fig. 1K).

The quality of pollen tubes is vital for successful release of the pollen tube contents, including the two joined sperm cells. Generally, the pollen tubes growing from the cut end of style were of good quality within 2–3 h of the style placement in the *in vitro* culture solution. However, if the styles were retained in the solution and cultured over 5 h, the pollen tubes grew too long and curved irregularly (Fig. 1L). These pollen tubes hardly burst during transfer to the breaking solution.

Discussion

Sperm cells of bicellular pollen species are formed in the pollen tube. Therefore, pollen germination and tube induction occur before sperm cells can be isolated. However, in some species, Read *et al.* (1993) reported that the generative cell did not divide to form two sperm cells in *in vitro* pollen tubes. *Bauhinia blakeana* is an arboreal plant, its pollen is bicellular, and a mature pollen grain is comprised of only a vegetative and a generative cell. Generative- and sperm-cell isolation in *B. blakeana* had not been achieved prior to the present study. We used a protocol in which pollen grains were ruptured and large numbers of generative cells were collected. We applied an *in vivo*–*in vitro* technique to culture the entire style that had been grown 6 h *in vivo*. After pollen tubes emerged from the cut end of the style following 2–3 h of *in vitro* growth, two nearly mature sperm-cells were released from the broken tubes. At this point, a “population” of sperm cells can be collected with a micromanipulator and studied using molecular methods. In this way, our sperm isolation method provides the basis for identifying unique genes and proteins that may be specifically involved in sper-

matogenesis in *B. blakeana* and other species.

During *B. blakeana* sperm cell isolation, two factors affect the results. One is the effect of environment on pollen tube growth, which is highly sensitive to changes particularly in temperature and humidity. Pollen tube growth in the style is optimal under conditions of continuous sunny days, and in culture the tubes grow readily from the cut end of the style. Cloudy or rainy conditions result in unstable tube growth and a limited number of tubes grow from the cut end of the style when cultured. Another important factor is *in vivo*–*in vitro* timing for style culture. If the pollen tube growth period *in vivo* is short, pollen tube growth is limited during the *in vitro* phase. However, if the culture time is too long, the pollen tubes will be severed when the styles are prepared for culture. It is most suitable for the style to be given 6 h *in vivo*, then cut and cultured for 3 h. The pollen tubes just emerging from the style are straight, of similar diameter and exhibit conspicuous cytoplasmic streaming. Pollen tubes with these attributes are easily burst when transferred to the breaking solution. If the style is cultured for too long, i.e. over 5 h, the pollen tube will be longer, but curly and with slow cytoplasmic streaming. Pollen tubes with these characteristics are difficult to rupture after transfer to the breaking solution.

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