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DIVERSITY OF SPERM CELLS OF DIFFERENT SIZE IN PHOTOPERIOD-SENSITIVE GENIC MALE-STERILE RICE

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Abstract

Sperm cells of photoperiod-sensitive genic male-sterile rice (PGMR) were isolated from pollen tubes using two step osmotic shock with BSA and sucrose solution, which were separated and purified using a micromanipulator. The highest ratio of viable sperm cells was obtained when pollen grains cultured in a medium containing 1.5 mM BSA and 10% sucrose solution. Fluorescein diacetate (FDA) stain was used to determine the viable sperm cells. The size of two sperm cells of PGMR nongken 58S were different but the dimorphism of sperm cells in 58S needs to be confirmed by further work.

The sperm cells of about 40 species of flowering plants have been isolated so far, which contain tricellular and bicellular pollen grains. Tricellular pollen grains in which two sperm cells have been formed can be directly isolated (Yu and Russel 1994).

Pollens of *Oryza sativa* L. are tricellular type and consisted of a vegetative cell and two sperm cells. Gou *et al.* (1999) had successfully isolated sperm cells in *indica* variety "K17" and "TeYou 725" by osmatic shock and percoll gradient centrifugation and reported two sperm cells of same size. In this paper a protocol for discovery of sperm cells of different sizes in PGMR is reported.

Plants of PGMR nongken 58S were grown in a glasshouse at 24°C for 8 h in dark and at 27°C for 16 h in light during tiller time, and then under a light/dark cycle of 12/12 ± 1 h at control temperature of 28/22°C during flowering. Unpollinated spikelets of matured flowers were collected for isolation of sperm cells.

Spikelets were surface sterilised in 95% alcohol for 1 min, then in 15% bleach for 5 min and subsequently rinsed with sterilised distilled water three times to obtain aseptic male gametes. The anthers from spikelets, almost ready for anthesis, were collected for isolation. The protocol of sperm cells isolation described by Gou *et al.* (1999) was adopted, with little modification. The pollen grains were collected from about 100 mg of anthers and were suspended in 2 ml of isolation medium at 30°C in a glass Petri dish. To get higher isolate ratio of sperm cells two-step osmotic shock was used. A series of different osmotic concentrations and anther were put into 45, 35 and 20% sucrose and then centrifuged five minutes after grinding the anthers. Upper solution of centrifuged material was thrown out and the remaining was divided into three parts and in these 15, 10 and 5% sucrose was added and kept 14 min at ice condition. After this the separation of sperm cells from pollen grains was carried out using a micromanipulator. Paired sperm cells released from one pollen tube could be operated using a micromanipulator. Two brother sperm cells of PGMR are different in size. Sperm cells viability was tested by a FDA reaction assay described by Heslop-Harrison *et al.* (1984).

Isolation of sperm cells: The sperm cells were isolated by squashing pollen grains and by applying osmotic pressure in the medium. A range of concentrations and incubation periods were used to determine the appropriate concentration(s) of chemicals and incubation period on the

isolation of sperm cells. Media concentration varied (15 - 45%) with different treatment times (0.5 - 2 h). The maximum number of sperm cells (60.9%) was obtained when the medium containing 1.3 mM BSA and 10% sucrose solution was used at a pH 6.0 with a osmolarity of 0.085 mos.mol kg^{-3} H_2O. The size difference of two sperm cells of PGMR nongken 58S is shown in Figs. 1, 2. Eighty per cent of sperm cells showed a bright yellow/green fluorescent staining when stained with FDA, indicating viability.

The BSA effect on storing of sperm cells: It is apparent from the Table 1 that the rate of sperm cells released was different with different BSA, sucrose solution concentration and osmolarity in the medium. The proper BSA concentration could increase the viability of sperm cells. The sperm cells did not show fluorescent staining after 3 h without adding BSA but adding BSA after 3 h show higher fluorescent staining (Fig. 3). BSA concentration of 1.3 mM showed higher number of sperm cells isolated indicating that different concentration of BSA has no effect in isolation of number of pollen grains and number of sperm cells as well. However, increasing the concentration of BSA did not provide any better results and this indicates that optimum concentration of BSA in the media plays a role for higher yield of sperm cells.

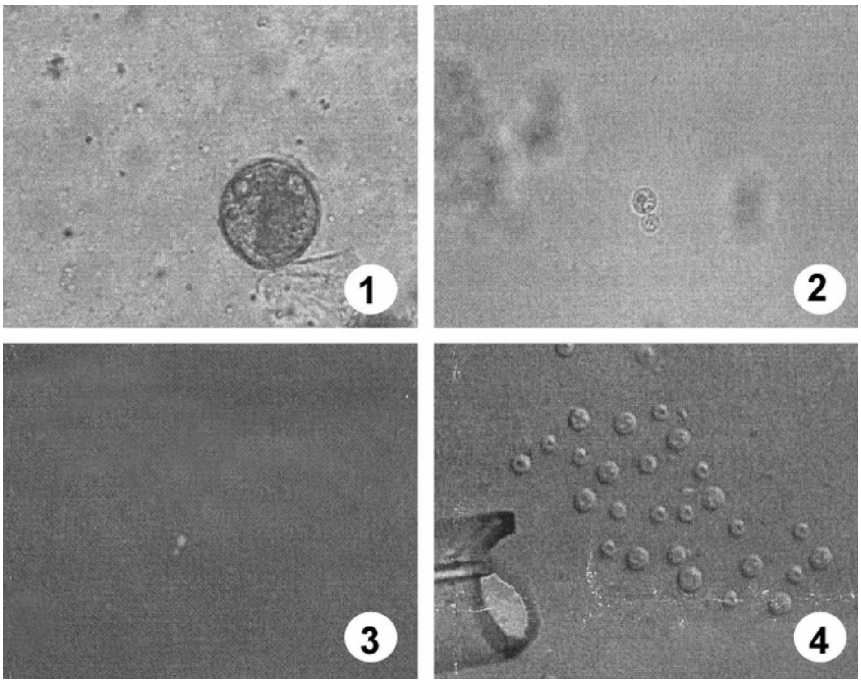
Table 1. Effect of media composition and concentration on sperm cell isolation.

BSA (mM)	Sucrose (%)	No. of pollen grains	No. of sperm cells isolated	Ratio of sperm cells isolated
0.50	15	390	39	10.0
0.50	10	376	55	14.6
0.50	5	387	96	24.8
1.30	15	407	144	35.3
1.30	10	396	241	60.9
1.30	5	404	153	37.8
2.10	15	395	111	28.1
2.10	10	400	94	23.5
2.10	5	386	85	22.0
3.60	15	377	75	19.9
3.60	10	410	124	30.2
3.60	5	393	148	37.7

Purification of sperm cells: Pollen contains one vegetive and two sperm cells and some impurity. Gou *et al.* (1999) obtained many sperm cells using percoll gradient centrifugation. In the present study pure sperm cell population was obtained using micromanipulator (Fig. 4).

In this study, the effect of different concentrations, of BSA and sucrose, has been observed (Table 1). The rice sperm cells were small in size (Fig. 1). The isolation of a large number of sperm cells from tricellular pollen species has been reported in maize (Dupuis *et al.* 1987); spinach (Theunis and Went 1989, Theunis (1992) and perennial ryegrass (Van der Maas *et al.* 1993, 1994). Since the phenomenon of sperm dimorphism was found in bicellulr pollen species *Plumbago zeylanica* in 1985, two sperm cells have been found dimorphic in pollen tube of

tobacco (Yu and Russell 1994, Tian *et al.* 2001); and two populations of sperm cells of tobacco have also been isolated (Yi-Lan *et al.* 2004). It appears from these reports that all sperm cells are small in size, after isolation, they become spherical and contain a very large nucleus and a small volume of cytoplasm. Gou *et al.* (1999) had successfully isolated the sperm cells of two *indica* variety of rice, namely (K17 and TeYou 725) by osmatic shock and percoll gradient centrifugation and in which the two sperm cells were same in size. In this paper, the authors report that there are different sizes of sperm cells in PGMR and got distinct isolated sperm cells using micromanupulation. The possibility of dimorphism in rice sperm cells needs further confirmation in other varieties. The present finding will provide an insight for preferential fertilization of rice.



Figs. 1-4: 1. The two different sperm cells of pollen grain in "nongken 58S" (× 630). 2. Released sperm cells of "nongken 58S (× 510). 3. Two different sperm cells of "nongken 58S" FDA stain (× 510). 4. Sperm cell population of "nongken 58S" (× 630).

Viability of sperm cells decreases in natural condition. It was found during this study that the viability of isolated sperm cells decreases with the increased storage time. FDA tests revealed that about 50% viability was lost within 3 h of storage. A longer viability of sperm cells was observed when BSA was added to the isolation media. The purity of sperm cells was important to carry out molecular work in rice sexual production. Collection of sperm cells by density gradient percoll, results in some impurity in the sperm cells population. Collection of sperm cells by micromanipulation and two step shock, could avoid impurity in sperm cells and could be used for further work.

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