

Flow Cytometric Characteristics of Sperm Cells Isolated from Pollen of *Zea mays* L.¹

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ABSTRACT

Sperm cells have been isolated from pollen of maize (*Zea mays* L.) and purified with Percoll density centrifugation. Their flow cytometric characteristics were determined on a FACScan flow cytometer with the fluorescent dyes, fluorescein diacetate and propidium iodide. Freshly isolated sperm cells appeared as a dot cluster on the forward scatter and side scatter dot plot. This dot cluster contained 85 to 95% of the 10 thousand counts collected. More than 98% of cells from the cluster were fluorescein diacetate positive, with no propidium iodide positivity, indicating high cell viability. After 5 hours in 15% (w/v) sucrose at room temperature (23°C), scattering properties, cell number, and percentage of fluorescein diacetate-positive cells remained the same. In contrast, Brewbaker and Kwack salts in 15% sucrose resulted in the emergence of a new cell population, as well as a decrease in cell number at 5 hours. Further investigations with individual components of the Brewbaker and Kwack salts showed that calcium was mainly responsible for the deleterious effects. These results demonstrate the utility of flow cytometry as a tool to determine viability and to monitor morphological changes of plant sperm cells and to challenge current views on the ability of Brewbaker and Kwack salts to maintain viability of isolated sperm cells.

To circumvent prefertilization barriers in interspecific hybridizations, direct fusion of isolated sperm with egg cells has been suggested to create new species. For instance, Jensen *et al.* (10) proposed a protocol for transferring isolated sperm cells to an egg apparatus to increase the taxonomic distance that can be bridged. Recently, electrofusion of sperm cells and egg protoplasts was reported by Kranz *et al.* (12). In these fusion studies, isolated sperm cells may also be considered as gene vectors to produce genetically transformed plants. Therefore, isolation and maintenance of viable sperm cells are necessary.

Sperm cells have been isolated and purified from *Beta vulgaris* (17), *Brassica napus* (14), *Plumbago zeylanica* (23), *Spinacia oleracea* (27), and *Zea mays* (2, 4, 16, 21, 28). Protocols for isolating sperm cells from pollen grains of *Z. mays* were reported by Dupuis *et al.* (4) and Roeckel *et al.*

(21). In their protocols, BKS², which have been documented as essential salts to pollen germination and pollen tube growth (1), were recommended. Roeckel *et al.* (21) suggested that 15% sucrose with BKS as the isolation and suspension medium resulted in adequate viability of isolated sperm cells based on the FCR test.

The FCR test was originally recommended for evaluating pollen viability by Heslop-Harrison and Heslop-Harrison (7). However, recent studies showed that the FCR test reflects neither the vigor nor the germination response of pollen (3, 25). Based on our experience with the FCR test, the intensity of cellular fluorescence decreases with cell aging and becomes too weak for visual detection. Furthermore, when aging cells are stained with both FDA and PI, some cells are not visibly stained by either. This makes it difficult to identify and score FDA and PI positivity of these cells. However, this disadvantage can be overcome by using flow cytometry, which provides high sensitivity to fluorescence. Compared to the limited numbers (usually 100) of cells that are counted with conventional FCR, tens of thousands of cells can be evaluated in a few seconds with a flow cytometer.

To develop a fast and accurate technique for determining viability of sperm cells and evaluating the present protocols for sperm isolation, sperm cells were isolated from pollen grains of *Z. mays*, and flow cytometric characteristics were determined using a FACScan flow cytometer. This is the first report of which we are aware of the use of flow cytometry in the study of sperm cells in plants.

MATERIALS AND METHODS

Preparation of Plant Material

Seeds of maize (*Zea mays* L. cv 129 Crusader, from Stokes Seeds Ltd., St. Catharines, Ontario, Canada) were hydrated overnight in aerated distilled water and planted in clay pots containing growth medium (Metro-Mix 225, W.R. Grace & Co. of Canada Ltd., Ajax, Ontario, Canada). Plants were grown in controlled environment rooms with a 16-h light period (25°C, 60% RH) and 8 h of darkness (20°C, 60% RH). Illumination was provided by five high intensity discharge mercury halide (400 W) and seven high intensity discharge

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² Abbreviations: BKS, Brewbaker and Kwack salts; FCR, fluorescence reaction; FDA, fluorescein diacetate; FL, fluorescence; PI, propidium iodide; FSC, forward scatter; SSC, side scatter; MFS, mean forward scatter channel; MSS, mean side scatter channel.

high-pressure sodium (400 W) lamps. The PPFD was $210 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 1 m above ground level.

Collection and Bursting of Pollen Grains

Pollen was collected by shaking tassels over a tray, sieved through 80- μm nylon mesh, weighed, and hydrated in a glass chamber with a moisture-saturated atmosphere at room temperature (23°C) for 20 min. After hydration, the pollen was immersed in unbuffered sucrose solution (15% [w/v], pH 6.7) in a ratio of 1 g pollen per 10 mL solution and shaken at 130 rpm at room temperature for 20 min. The bursting mixture was sieved through 40- μm nylon mesh to remove pollen exines, and the resulting filtrate containing sperm cells was saved for density gradient centrifugation as described below.

Preparation of Percoll Density Gradients and Isolation of Sperm Cells

Discontinuous Percoll density gradients were prepared in 15-mL Corex centrifuge tubes by layering 1.5 mL each of 30, 15, and 10% Percoll (Sigma) solutions in 15% sucrose (pH 6.7). The filtered sperm cell suspension (5–10 mL) was layered on top of the 10% Percoll and centrifuged at 3000g for 60 min at 4°C. Sperm cells located at the interface of 15 and 30% Percoll were collected with a glass pipette, washed with 15% sucrose, and centrifuged at 3000g for 7 min. The supernatant was largely removed, leaving 0.2 mL to prevent disturbing the pellet. Cells were resuspended and divided into two tubes. Control cells were diluted to 1 mL with 15% sucrose. Cells for BKS treatments were diluted by adding 0.4 mL 15% sucrose and 0.5 mL of 2 \times concentrated BKS in 15% sucrose, with a final concentration (mM) of 1.27 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.99 KNO_3 , 0.81 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.62 H_3BO_3 as described by Brewbaker and Kwack (1). The same dilution procedure was used with individual salts replacing total BKS. Isolation and suspension were carried out under sterile conditions when prolonged suspension was needed. Addition of the BKS or individual salts to 15% sucrose did not change the osmolarity significantly (data not shown). These solutions were therefore considered osmotically equivalent.

Flow Cytometric Analysis of Isolated Sperm Cells

Cells were stained with 0.5 $\mu\text{g}/\text{mL}$ FDA and/or 50 $\mu\text{g}/\text{mL}$ PI according to the method of Ross *et al.* (22). Viability of sperm cells was estimated 1 h after isolation by intracellular green (FDA) and/or red (PI) fluorescence using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Viable cells are FDA positive and PI negative, and nonviable cells are FDA negative and PI positive. Fifteen percent sucrose, filtered through a 0.22- μm filter (Millipore Filter Co., Bedford, MA) was used as the sheath fluid. A 530-nm filter with a bandwidth of 30 nm and a 585-nm filter with a bandwidth of 42 nm were used to collect FL1 (green) and FL2 (red) signals, respectively. When both FDA and PI were used to stain cells, compensation levels of 10 and 48% were set for FL1–%FL2 and FL2–%FL1 to subtract the spectral overlap between the FL1 and FL2 signals. Because freshly isolated sperm cells are not permeable to PI,

cell preparations were heated over a burner for a few seconds to obtain PI positivity to calibrate the compensation. The time course of staining with FDA and PI showed that maximal intensity of fluorescence was obtained between 5 and 10 min (data not shown); this was chosen as the routine staining period. Ten thousand counts were collected for each sample. Data was analyzed using ReproMan Flow Cytometry Analysis Software for Personal Computers (1990) (True Facts Software Inc., Seattle, WA). FL1 and FL2 histograms and quadrant statistics were used to evaluate the viability of sperm cells, and FSC and SSC were used to evaluate changes in cell volume and shape. All parameters were expressed in channel numbers on linear scales. Average rates (number of cells collected per second) were used to estimate percentages of cells remaining relative to the control samples.

Hemacytometric Quantification of Isolated Sperm Cells

Hemacytometry was used to monitor changes in the number of isolated sperm cells. Cell numbers were counted with a Zeiss microscope using a $\times 25$ phase-contrast objective after 1, 2, 4, 8, and 12 h of suspension in 15% sucrose with or without BKS. Cell numbers were expressed as a percentage of cells remaining relative to the number at time 0 (100%).

RESULTS

Flow Cytometric Characteristics of Isolated Sperm Cells

Microscopic examinations of isolated sperm cells showed a clean isolation with few starch grains (Fig. 1A). The cells were dark spheres under phase-contrast microscopy. Micrometer measurements gave cell diameters of $7.3 \pm 0.6 \mu\text{m}$. Flow cytometric analysis showed that the cell preparation contained four populations as observed on the FSC and SSC dot plot

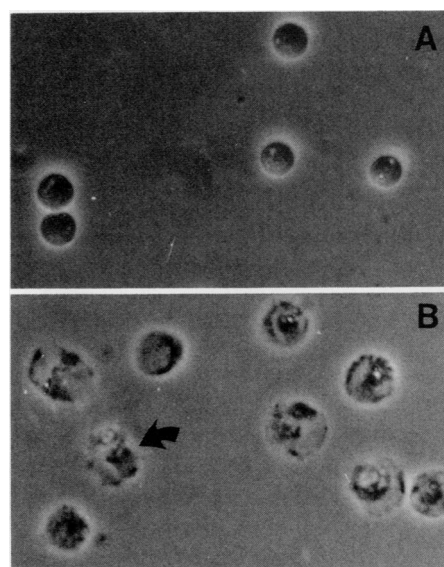


Figure 1. Light microscopy of isolated sperm cells. A, Phase-contrast micrograph of freshly isolated sperm cells; B, phase-contrast micrograph of heat-damaged sperm cells and cell debris after cell lysis (arrow). $\times 650$.

(Fig. 2A, gates 1–4). The dot cluster in gate 1 accounted for 85 to 95% of total counts, with a MFS of 78 and a MSS of 43. This population appeared as a major peak on the three-dimensional histogram (Fig. 2B). Gate 2 accounted for about 10% of the total counts, with a MFS of 16 and a MSS of 45 (Fig. 2A). One to 2% of the total counts had high SSC and spread on the top of the plot (Fig. 2A, gates 3 and 4). These were derivatives from the last two populations.

FDA and PI response showed that sperm cells isolated using 15% sucrose as the bursting and isolation medium were viable. There was no FL1 signal before FDA staining. After FDA staining, there was a fluorescence peak accounting for 85% of the total counts between channels 100 and 200, with a mean channel of 152 and a peak channel of 157 (Fig. 3A). Gating analyses show that this fluorescent peak resulted from the dot cluster in gate 1 of Fig. 2A. Ninety-five to 98% of counts in the dot cluster were FDA positive. Furthermore, 99% of derivatives from the dot cluster as observed in gate 3 were FDA positive. In contrast, 85 and 90% of the counts in gate 2 were FDA negative, indicating that most counts in these populations were cell debris (Fig. 3A). No FDA positivity was found in gate 4, the derivatives of gate 2 (data not shown). PI staining did not increase FL2 signal in the dot cluster compared with that of unstained cells (Fig. 3B). When double

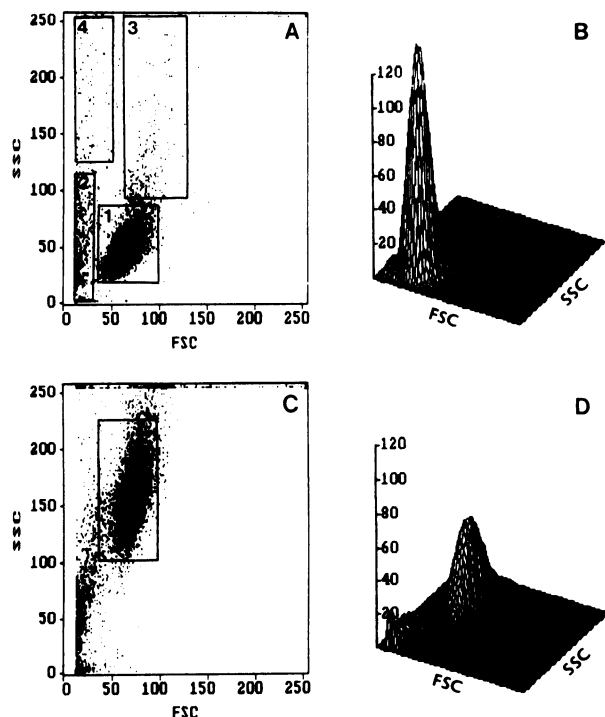


Figure 2. FSC versus SSC dot plots and three-dimensional histograms of isolated sperm cells. A, FSC versus SSC dot plot of freshly isolated sperm cells. Gate 1, Major cell population accounting for 85 to 95% of total counts; gate 2, cell debris accounting for about 10% of total counts; gates 3 and 4, derivatives from gates 1 and 2 accounting for <2% of total counts. B, Three-dimensional histogram of A. C, FSC versus SSC dot plot of heat-damaged sperm cells. D, Three-dimensional histogram of C. Z axes in B and D, Numbers of cells.

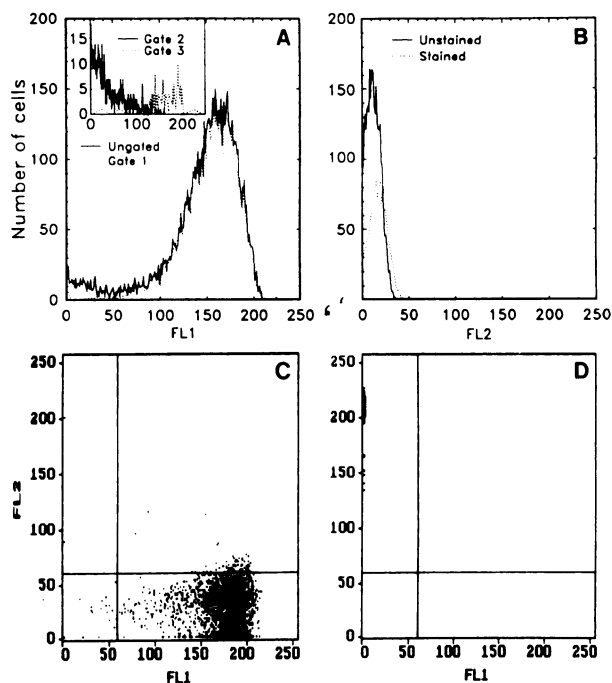


Figure 3. FL1 and FL2 histograms and quadrant statistics of freshly isolated (A, B, C) or heat-damaged (D) cells. A, FL1 histograms from whole cell preparation and gate 1. Inset, FL1 histograms of gates 2 and 3 (see Fig. 2 for gates). B, FL2 histogram from the dot cluster (Fig. 2A, gate 1) of PI-stained and unstained cell preparations. C, Quadrant statistics of FL1 and FL2 from the dot cluster of FDA- and PI-stained cell preparations. D, Quadrant statistics of FL1 and FL2 from dot cluster of heat-damaged cell preparations.

stained with both FDA and PI, more than 96% of the counts from the dot cluster appeared in quadrant 4, the FDA-positive region of the quadrant statistics. Only 0.1% were in quadrant 1, the PI-positive region (Fig. 3C). These results suggest that the dot cluster on FSC and SSC dot plots represents viable cells. Further analyses focused on this population.

Nonviable cells showed distinctive patterns of light scattering. When damaged by brief heating, cells showed an increase in cell size, coagulation of cytoplasm, and cell lysis (Fig. 1B). Flow cytometry showed that the dot cluster shifted to a higher SSC region (Fig. 2, C and D) compared with undamaged cell preparations (Fig. 2, A and B). The new cluster had a MFS of 69 with a peak channel of 70 and MSS of 161 with a peak channel of 144, indicating changes in light-scattering properties. When these damaged cells were stained with FDA and PI, >99% of the cells were found in quadrant 1 of the quadrant statistics (Fig. 3D). These results show that flow cytometry with FDA and PI staining provides an accurate means of evaluating viability of plant sperm cells.

Evaluation of the Effect of BKS on Viability of Isolated Sperm Cells

Previous studies showed that BKS were essential to pollen germination and pollen tube growth (1), as well as maintenance of viability of isolated sperm cells (21). To evaluate the effects of BKS on viability of *Z. mays* sperm cells, freshly

isolated cells were placed in 15% sucrose with or without BKS, and light-scattering properties were determined after 1 and 5 h. Compared with cells in 15% sucrose (Fig. 4A), addition of BKS did not affect FSC and SSC of these cells in 1 h (Fig. 4C). After 5 h, the FSC and SSC of cells from 15% sucrose remained unchanged (Fig. 4B). However, a new cell population with a higher FSC (165 in MFS) and higher SSC (92 in MSS) appeared in the BKS treatment (Fig. 4D, gate a), suggesting that BKS induced changes in cell morphology. The new population accounted for 11% of the total cells. Determination of the effects of individual salts from the BKS showed that $\text{Ca}(\text{NO}_3)_2$ was primarily responsible for the new cell population found with BKS (Fig. 4E, gate a). Some of the cells from the dot cluster shifted toward the higher SSC region when cells were treated with MgSO_4 after 5 h in suspension (Fig. 4F). No noticeable differences were found with KNO_3 or H_3BO_3 (Fig. 4, G and H). There was no difference in the intensity of FDA fluorescence of cells with or without BKS after 1 or 5 h of suspension. Further gating analysis showed that the new cell population in either BKS or $\text{Ca}(\text{NO}_3)_2$ was FDA positive (data not shown).

Analysis of cell numbers remaining after 5 h of suspension distinguished between treatments with or without BKS. Whereas the cell number in the dot cluster remained the same after 5 h suspension in 15% sucrose, the cell number decreased by 40% in the presence of BKS (Fig. 5A). Further investigations with individual salts showed that the cell number decreased by 23% in the presence of $\text{Ca}(\text{NO}_3)_2$; no statistical differences were found with other salts compared with 15% sucrose (Fig. 5A). Effects of BKS were confirmed by hema-

cytometric studies. When cells were suspended in 15% sucrose, cell numbers remained unchanged for 8 h. However, the percentage of remaining cells decreased by 28% after 12 h. In contrast, decreases of 24, 38, and 62% were observed after 4, 8, and 12 h, respectively, in the presence of BKS (Fig. 5B). These results challenge the present views on the adequacy of the BKS for maintenance of viability of isolated cereal sperm cells.

DISCUSSION

During flow cytometric analyses, sperm cells isolated from *Z. mays* pollen appeared as a condensed dot cluster on the FSC and SSC dot plots. FDA positivity and PI negativity indicate that cells prepared using 15% sucrose alone as the bursting, isolation, and suspension media are viable cells. Furthermore, the FDA positivity and PI negativity of these live cells and PI positivity and FDA negativity of heat-damaged cells collectively showed the utility of flow cytometry as a means of evaluating viability of isolated plant sperm cells. Information concerning morphological changes can also be obtained with flow cytometry, as reflected in changes in FSC and SSC. These morphological parameters are more sensitive than differences in the intensity of fluorescence between experimental treatments. Furthermore, changes in the number of counts in the dot clusters can also provide useful information concerning changes in numbers of intact cells.

Results from analyses of light-scattering properties as well as hemacytometry suggest that BKS may not be appropriate for maintaining maximal viability of isolated sperm cells from

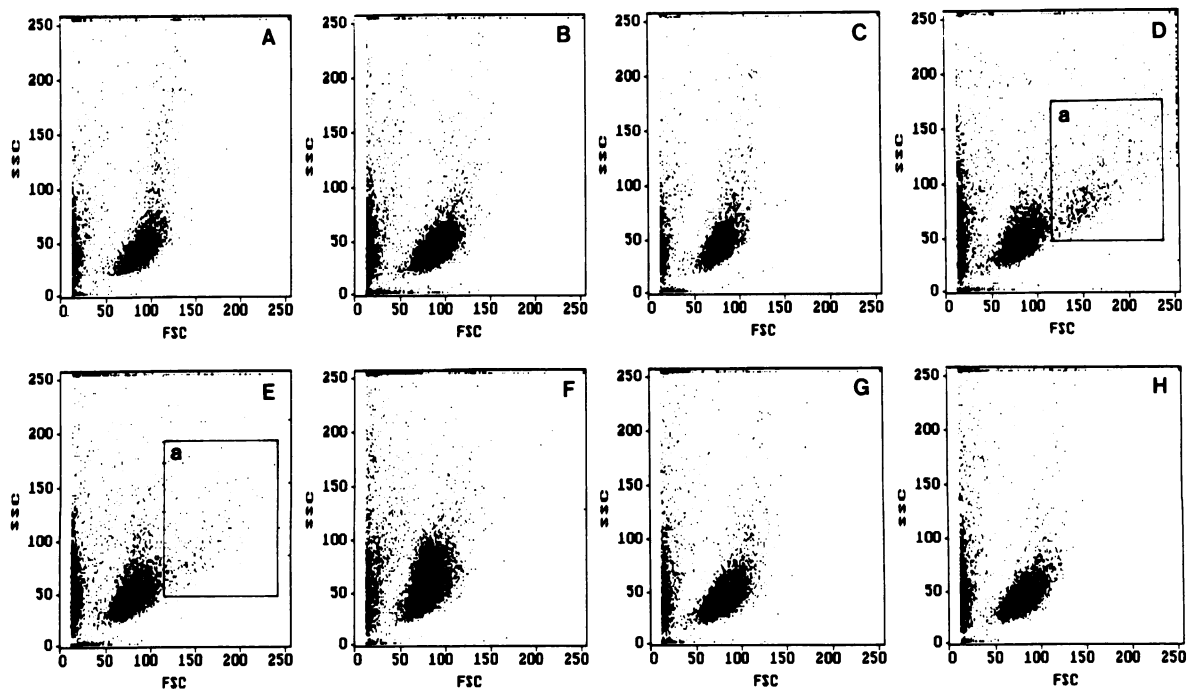


Figure 4. FSC versus SSC dot plots of sperm cells after 1 or 5 h in 15% sucrose (A, B), 1 or 5 h in 15% sucrose with BKS (C, D), and 5 h in 15% sucrose in the presence of $\text{Ca}(\text{NO}_3)_2$ (E), MgSO_4 (F), KNO_3 (G), and H_3BO_3 (H). Dots in gate a in D and E, New populations in presence of the BKS or $\text{Ca}(\text{NO}_3)_2$.

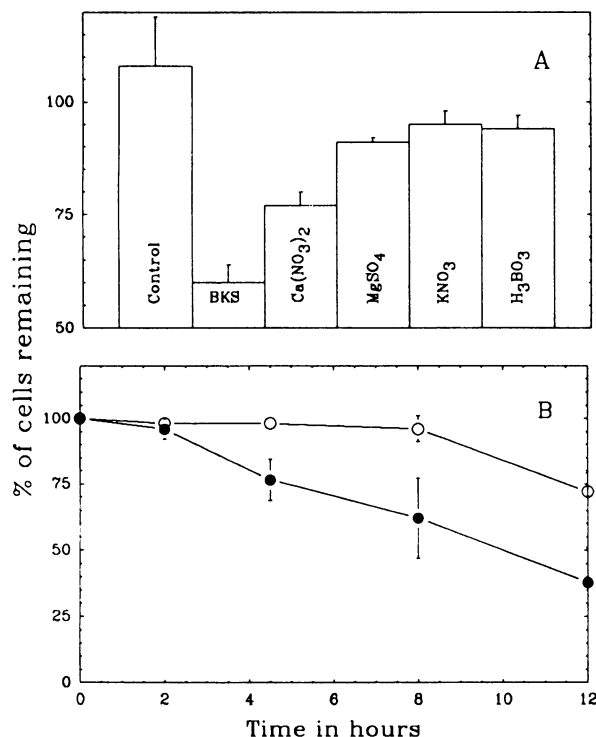


Figure 5. A, Percentage of cells remaining after 5 h of suspension in 15% sucrose by itself (control) or with BKS, Ca(NO₃)₂, MgSO₄, KNO₃, and H₃BO₃ as determined by flow cytometry. Percentage of cells remaining was calculated based on number of cells in the dot clusters on the FSC and SSC dot plots in Figure 4. Columns, Means of two individual experiments; bars, \pm sd. B, Percentage of cells remaining after 5 h of suspension in 15% sucrose with or without BKS as determined by hemacytometry. Points, Means of three to six experiments; bars, \pm sd.

Z. mays as suggested by Roeckel *et al.* (21). Emergence of a new cell population with different light-scattering properties in the presence of BKS suggests changes in morphology. Although the exact nature of these changes in light scattering is unknown, the new population may result from changes in cell size or shape, vesicle formation, or other morphological changes concomitantly with the demise of cells as confirmed by decreases in cell number. Such a conclusion needs confirmation by morphological studies. Salzman *et al.* (24) suggested that under some conditions the intensity of light scattering from cells is a nonlinear function of cell volume. However, this presumption has been questioned recently by McGann *et al.* (15) who determined the relationship between light scattering and cell volumes in anisotonic solutions and after a freeze-thaw stress. Their results showed that light scattering and cell volumes can vary independently (15). Furthermore, changes in light scattering may result from changes in properties of the plasma membrane and/or internal structure of cells (11). Although the mechanism is unknown, the BKS-induced decline in cell number confirmed the deleterious effects of these salts on the viability of isolated sperm cells. Results from the individual salts of the BKS suggest that Ca(NO₃)₂ is the primary salt responsible for the

changes in light-scattering properties and decreased cell number. Further studies with the same concentration of CaSO₄ indicated that calcium, not nitrate, is responsible for these effects.

Although the deleterious effect of calcium on *Z. mays* sperm cells conflicts with the requirement for calcium in pollen germination (1, 19), our results clearly showed the detrimental effects of calcium on the longevity of isolated sperm cells. It is probable that growing pollen tubes provide the sperm cells they contain with a microenvironment different from the medium in which the tubes grow. Previous studies showed that the calcium concentration in the first 10 or 20 μ m of pollen tubes of *Lilium longiflorum* is 10 to 100 times higher than in the rest of the tube (9, 20). In an effort to determine the location of sperm cells in pollen tubes, we observed >200 growing pollen tubes in *Z. mays*; no sperm cells were found in the first 60 μ m, suggesting that sperm cells are proximal to the region of elevated calcium concentration.

Data documenting the effect of calcium on the metabolism of plant sperm cells are not available. Although calcium is usually included in the isolation of protoplasts (6), calcium was not recommended for isolation of mitochondria from pollen of *Typha latifolia* (8). Calcium ions may inhibit some enzymes necessary for sperm function (26) or may activate undesirable functions during isolation and suspension. Recent reports of the effect of calcium on microsomal membranes indicate that physiological levels of calcium promote membrane deterioration by inducing lipid degradation and proteolysis (5, 13, 18). Physiological as well as biochemical effects of BKS, especially the calcium ions, on the sperm cells need to be investigated.

In conclusion, our results suggest: (a) flow cytometry using analyses of FDA and PI, FSC, and SSC provides a fast and accurate means of evaluating viability of isolated sperm cells of *Z. mays*, (b) sperm cells prepared with 15% sucrose without the addition of any salts or cations were viable, with the cell numbers and flow cytometric properties remaining unchanged for 8 h after isolation, and (c) the BKS, which have been widely used in pollen germination, are not appropriate for isolation and suspension of sperm cells from *Z. mays*. Experiments to further improve the isolation and maintenance conditions and the effect of calcium ions on metabolism of these cells are underway.

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