Sex Plant Reprod (1993) 6:212–216

Sexual Plant Reproduction

Survival at 20° C and cryopreservation of isolated sperm cells from *Zea mays* pollen grains

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Summary. The survival of isolated sperm cells from maize pollen grains at 20° C and their cryopreservation were studied by means of the fluorochromatic reaction (FCR) test. An osmotic pressure of 500 mOsmol, a pH of 7 adjusted with MOPS buffer, and the ten-fold dilution of Brewbacker and Kwack salts enabled isolated sperm cells to survive for a maximum of 35 h at 20° C. The addition of 3 m*M* of calcium chloride or 0.1% glucose also slightly improved survival. The addition of 1% glutamine gave the best percentage of FCR-positive cells at 20 h. The addition of all of these substances in a unique medium provoked the formation of sperm-cell aggregates that were FCR positive. With respect to cryopreservaton, 70% of the isolated sperm cells remained FCR positive after freezing at −80° C and quick thawing at 37° C in the selected survival medium.

Key words: Corn – Isolated sperm cells – Survival – FCR test

Introduction

Sperm cells have only recently been isolated and available for use in basic and applied science (for reviews see Roeckel et al. 1990; Russell 1991; Theunis et al. 1991), and very few studies on the molecular biology and gene expression of isolated sperm cells have been reported (Haskell and Rogers 1985; Reynolds and Raghavan 1982). In a large extent, this paucity of information is directly related to a lack of knowledge concerning the maintainance and storage of sperm cells. In our opinion, a prerequisite to obtaining any further knowledge on sperm-cell biology is to understand how isolated sperm cells can be maintained.

When we first isolated sperm cells from *Zea mays* pollen grains, we used the BKS15 medium for all of the steps of the procedure (Dupuis et al. 1987); however, after the sperm cells had been incubated for about 5 h at 20° C in this medium, we could only find damaged cells and nuclei in the centrifugate. Thus, it appears necessary to improve the length of their life time at 20° C, which is the maximum temperature at which FCR-positive cells can be detected. We report here our attempts to determine the composition of a medium suitable for corn sperm-cell survival at 20° C.

In addition, we present an easy and reliable procedure for the cryopreservation of isolated sperm cells. The cryopreservation of plant cells has become an important tool for the long-term preservation of plant germ plasm and experimental material without genetic alteration. However, the cryopreservation of isolated sperm cells from corn pollen grains has not yet been established. Simple and reliable techniques would allow the use of frozen sperm cells if they could only exhibit adequate survival.

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Material and methods

Plant material

Zea mays L. hybrid plants from genotype A619 × F1243 were grown in an experimental garden from July to September. Pollen was collected at the time of shedding by shaking the tassels over tin foil.

Aseptic sperm-cell isolation

After collection, corn pollen was immersed in sterile BK*S15 medium (Table 1) (50 mg/ml) and gently homogenized. A maximum of 8 ml of this mixture was placed in an open 10-cm petri dish, which was then gently shaken on a tridimensional shaker under UV light for 5 min. The UV light and shaker were turned off for 1 min to allow the pollen settle; 4 ml of the upper liquid was discarded, and the remaining mixture was transferred to a sterile tube. The following steps were performed under sterile conditions at 4° C according to the procedure described previously (Dupuis et al. 1987). For all of these following steps we used the BK*S15 medium (Table 1) instead of the classical BKS15 medium (Brew-

Table 1. Description of the media used for the survival and cryopreservation experiments

Media	Description
BKS15	Brewbaker and Kwack medium (1963) supplemented with 15% sucrose
BK*	Brewbaker and Kwack salts diluted ten fold with water
BK*S15	BK*, 15% sucrose
A	BK*S15 supplemented with 10 m*M* MOPS, pH 7
B	BK*S15 supplemented with 10 m*M* HEPES, pH 7
C	BK*, 10 m*M* MOPS, 13.5% sucrose, pH 7
D	BK*, 10 m*M* MOPS, 18% sucrose, pH 7
E	BK*, 0.1% glucose, 10 m*M* MOPS, pH 7, osmotic pressure adjusted to 500 mOsmol with sucrose
F	BK*, 1% glutamine, 10 m*M* MOPS, pH 7, osmotic pressure adjusted to 500 mOsmol with sucrose
G	BK*, 3 m*M* $CaCl_2$, 10 m*M* MOPS, pH 7, osmotic pressure adjusted to 500 mOsmol with sucrose
M	BK*, 0.1% glucose, 1% glutamine, 3 m*M* $CaCl_2$, 10 m*M* MOPS, pH 7, osmotic pressure adjusted to 500 mOsmol sucrose

baker and Kwack 1963). The sterility of the isolated sperm cells was checked by culturing aliquots of the sperm-cell fraction on a rich nutritive medium at 24° C for fungal growth, and at 37° C for bacterial growth.

Fluorochromatic test

The viability of the isolated sperm cells was estimated at the end of the isolation procedure, during the survival experiments, and after the thawing of the cells as described by Dupuis et al. (1987). Viability was measured by determining the concentration of cells which fluoresced (FCR^+) per microliter of suspension after exposure to the fluorescein diacetate mixture and observation under fluorescence microscopy. When it was possible 300 sperm cells were counted.

Survival experiments at 20° C and at 4° C

At the end of the isolation procedure, the sperm-cell fraction in BK*S15 medium was collected, and the volume of cell suspension was measured. The number of cells per microliter was determined after microscopical observation as described below. The cell suspension was aliquoted, and each aliquot was diluted to a final cell density adjusted to 500 cells per microliter (t = 0 h). The compositions of the media used for dilution were calculated in accordance to the volume to be added. The final compositions of the liquid media tested are reported in Table 1. With respect to sperm-cell aging, aliquots of the culture were subjected to the FCR test as described above. The results were expressed as a percentage by comparing the concentration of FCR^+ cells obtained at the time t of aging and the initial concentration obtained before aging. At isolation (t = 0 h), the percentages of FCR^+ cells ranged from 90 to 100%.

Light microscopy controls

Throughout the survival studies. sperm cells were examined with phase contrast or fluorescence microscopy using 0.05% ethidium bromide in the survival media. Sperm cells were also observed by phase contrast combined with fluorescence microscopy.

Freezing and thawing procedures

Isolated sperm cells were suspended in BK*S15 or A media (Table 1) at 4° C and frozen either in a −20° C or −80° C freezer or by direct immersion in liquid nitrogen. The cells were held in this state for 1 week before being rewarmed quickly in a water bath held at 37° C; they were then kept at 20° C. In each treatment, 20 tubes containing 50 µl of cell suspension coming from 20 different isolation procedures were used. This represents 60 replicates per treatment.

Three cell clusters from each tube were examined for their viability by means of the FCR test. This was done 3 h after the cells had been thawed out. The survival of the thawed cells was expressed as a percentage of survival over the unfrozen control. Data were analysed using a two-tailed t-test at the 1% significance level.

Results and discussion

Survival at 20° C

Figure 1 A–D shows the aging of four different cell populations in defined media. Some of the media tested were found to increase the life span of the cell populations. Two or three replicates with different cell isolates were carried out to confirm increases in survival rates. Standard variations between replicates were not calculated since the measurements were performed at different cinetics during population agings.

Following experiments in which the BK salts (Brewbaker and Kwack 1963) were diluted, we found that the yield of isolated sperm cell increased ten fold in a medium denoted as BK*S15 (see Table 1) compared to the yield obtained in the classical BKS15 medium (data not shown). In BK*S15 medium 50% of the cells remained FCR^+ for 2 h after isolation at 20° C, and it is after the extended time of 20 h at 20° C that we no longer observed whole sperm cells (Fig. 1 A). After 5 h at 20° C in BK*S15 media, only damaged cells and nuclei were found. We used this BK*S15 medium as a reference medium for the following experiments.

pH

The optimum pH was taken to be around 7 since sperm cells were destroyed after 3 h at 20° C in BK*S15 with a pH below 6 or above 8 and since they survived 15–20 h in BK*S15 with a pH between 6 and 7.5. In order to modulate pH variations attributed to the use of NH_4^+ and NO_3^- ions by cells (Bligny and Leguay 1987), we investigated a buffer that has the ability to maintain the pH at 7. The presence of the MOPS buffer in BK*S15 medium allowed longer sperm-cell survival than did the BK*S15 medium itself or BK*S15 supplemented with HEPES buffer (Fig. 1 B). As a result, we adjusted the pH of subsequent media to be tested to 7 with 10 m*M* MOPS buffer. Our observation that MOPS is the most suitable buffer is similar to the results obtained by Barrow (1986) for microspores of *Gossypium hirsutum*. For other cell cultures, such as somatic protoplast cultures, the optimum pH is more acidic (Vasil 1987).

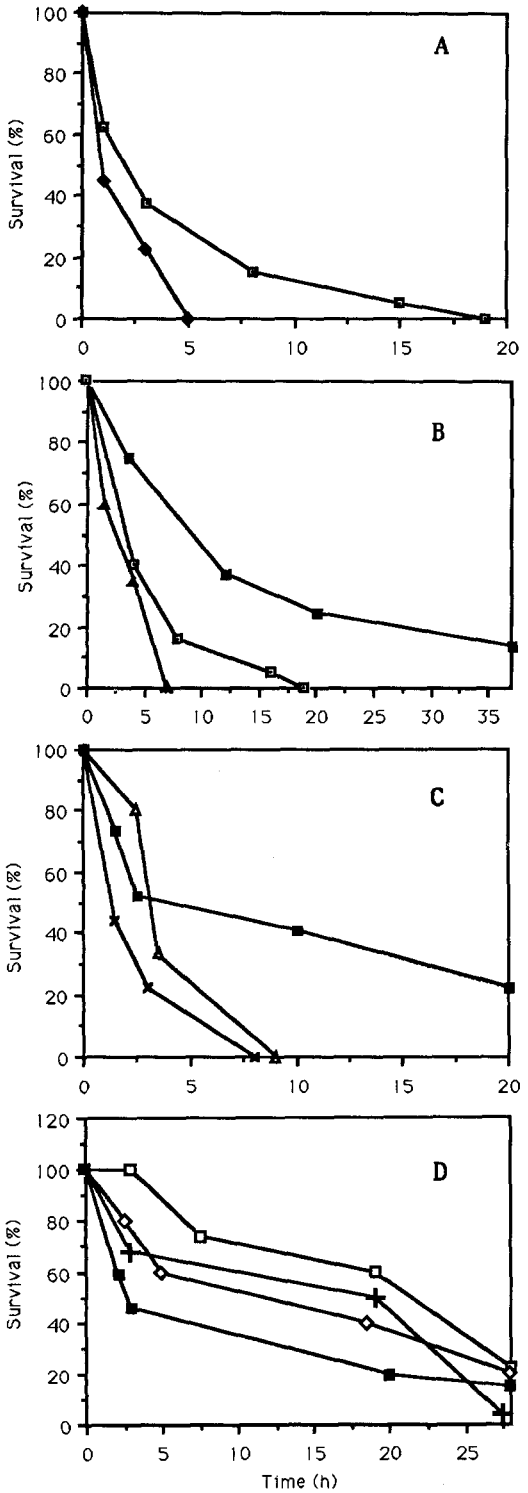

Fig. 1A–D. Sperm cell survival at 20° C in defined media. At the end of the isolation procedure, sperm cells were resuspended in sterile media at a final density of 500 cells per microliter (t=0 h). The percentages of survival, along the Y axis, are represented by the number of FCR^+ sperm cells at t=x divided by the number of FCR^+ sperm cells at t=0, multiplied by 100. Sperm cells were isolated from four different pollen samples. **Figure 1A–D** shows sperm-cell survival of these four isolates in the defined media described in Table 1. **A** Sperm cells were suspended in BK*S15 or in BKS15 (◆); **B** different buffers: sperm cell survival in BK*S15, A (■), B (▲). The pH of these media was adjusted to 7; **C** Different osmotic pressures: media A (■), C (△), D (X). The osmotic pressure of media A, C, D is 500, 430, 532 mOsmol, respectively. **D** Effect of calcium, glucose and glutamine on sperm-cell survival: A (■), E (◇), F (□), G (+)

Osmotic pressure

As shown in Fig. 1C, longer sperm-cell survival was observed in a media having an osmotic pressure of 500 mOsmol than in those adjusted to 430 or 570 mOsmol. Indeed, 50% of the cells were still FCR^+ after 10 h in A medium (500 mOsmol) whereas we did not observe FCR^+ cells in the two other assays. We therefore adjusted the osmotic pressure of the media to be tested to 500 mOsmol with sucrose. For long-term cell culture (somatic protoplast) mannitol is usually preferred over sucrose as the means to maintain osmotic pressure because cells do not seem to metabolize it (Vasil 1987). Mannitol can be used in a survival medium for sperm cells (data not shown), however we preferred to use sucrose in this preliminary work because it can provide both an osmotic pressure stabilization and a carbon source for a short period of time.

Miscellaneous

We also tested the effect of glucose as a carbon source, glutamine as a nitrogen source, and calcium. The addition of any one of these substances in the A medium either slightly improved or did not modify sperm-cell survival. The survival curves are shown in Fig. 1D.

Sucrose and glucose are the most common carbon sources employed in plant cell culture (Bligny and Leguay 1987). While sucrose alone enhanced sperm-cell survival, the addition of 0.1% glucose did not greatly improve the cell's fate. For example, after the end of the isolation procedure, about 10% of the sperm cells remained FCR^+ in A medium while only 20% FCR^+ cells were observed in the E medium (Table 1).

Most cell cultures are able to grow on media containing only a mineral nitrogen source; however, the addition of organic nitrogen may improve cell survival (Jullien and Guern 1979). Our media contained mineral nitrogen derived from the diluted BK salts (Brewbaker and Kwack 1963). We checked the effect of glutamine on sperm-cell survival because it is one of the "principal" free amino acids found in maize pollen grains (Gay 1986). Sperm cells are embedded within the vegetative cell, which constitutes almost the total volume of the pollen grain. Thus, we can suppose that assays on free amino acids from pollen grains are representative of the natural medium surrounding the sperm cells in situ which would explain the positive effect of 1% glutamine on sperm-cell survival. For example, 35% of the FCR^+ cells were still found after 20 h at 20° C in F medium, while only 10% of the FCR^+ cells were observed in A medium (Fig. 1D). The effect of other amino acids has still to be tested.

We also noticed that sperm-cell death occurred by membrane rupture. Since calcium is known to rigidify

membranes, we tested the effect of calcium chloride at different concentrations (1 m*M*, 3 m*M*, 5 m*M*) on sperm-cell survival (data not shown). The survival curve obtained with 3 m*M* $CaCl_2$ appeared to be the best among those tested and is shown in Fig. 1D. The addition of 3 m*M* $CaCl_2$ seems to improve the percentage of survival during the first 20 hours of sperm-cell culture compared to the percentage of survival observed in the A medium.

Mixture of substances improving sperm-cell survival

On the basis of the preceding results, we designed a new medium, M, that contains all of the substances that appeared to improve sperm-cell survival. Its composition is described in Table 1. Surprisingly, after sperm cells had been incubated for 2 h in M medium at 20° C, the formation of cell doublets was observed; these were subsequently joined by more cells (Fig. 2a). One hour later, some multinuclear structures were also visible with ethidium bromide staining (Fig. 2c). Under phase contrast microscopy, these nuclei seemed to be enclosed in a large cell (Fig. 2d). Most of these structures were found to be FCR^+ (Fig. 2b). We can suppose that these aggregates arose from bonds created between substances of the medium and membrane residues. Based on these results, we studied sperm-cell survival in modified M media in which either $CaCl_2$, glucose, or glutamine were excluded. The elimination of glucose decreased the frequency of aggregate occurrence (data not shown). From our frequent observation of doublets, we can suppose that certain specific molecules are present on the sperm-cell surface that makes isolated sperm cells particularly susceptible to fusion with each other. Also, the facility of sperm cells to bind each other could be related to the existence of links existing between sperm cells within the pollen grain (for review, see Matthys-Rochon and Dumas 1988).

Cryopreservation

We observed an important difference between sperm cells isolated from summer pollen obtained from our experimental garden versus those from greenhouse pollen. The isolation yield and the sperm-cell quality (in terms of response to the FCR test) were 10–100 times higher when pollen from the experimental plot was used (data not shown). As a result, the ability to cryopreserve isolated sperm cells would enable us to work all year long with good quality cells isolated during the summer.

We tried simple methods of freezing, such as direct freezing in a −20° C or −80° C freezer, or immersion into liquid freezer nitrogen. As shown in Fig. 3, the imposition of an ultra-rapid cooling rate by direct immersion in liquid nitrogen and quick rewarming, in order to avoid intracellular ice formation, was not adequate. As well, no success was obtained after cooling at −20° C. However, following cooling to −80° C and quick rewarming at 37° C, we were able to recover 70% of the FCR^+ cells (Fig. 3). Sucrose was present in the media at a high concentration of 15% and can be considered to be a cryoprotective agent. We can assume that

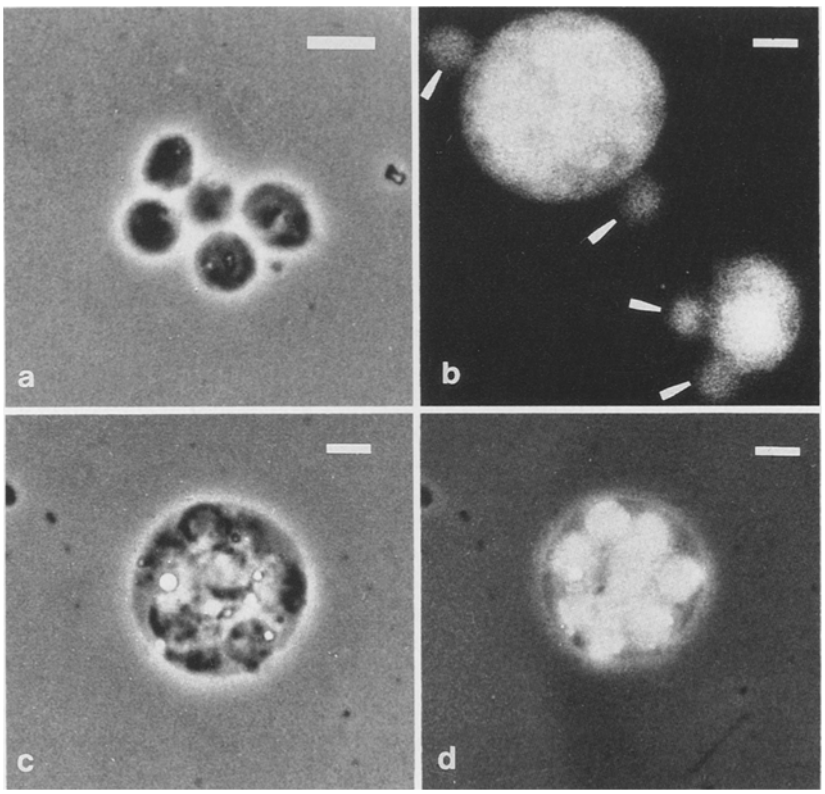

Fig. 2A–D. Observation of isolated sperm cells in M media (see Table 1 for the composition of this media). **A** Isolated sperm cells were observed with phase contrast microscopy after 2 h in M media. **B** After 3 h in M media FCR^+ aggregates were observed by fluorescence microscopy following the fluorochromatic reaction (FCR) test. *Arrows* indicate single sperm cells that join the aggregate already constituted. After 3 h in M media the presence of multi-nuclear-like structures within the same surrounding membrane was observed with phase contrast microscopy (**C**) coupled with fluorescence microscopy after staining with ethidium bromid (**D**). *Bar:* 10 μm

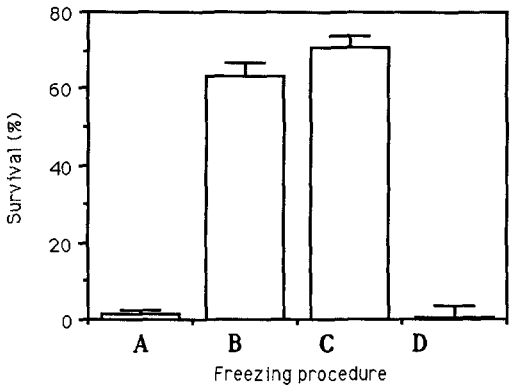

Fig. 3. Cryopreservation of isolated sperm cells. Isolated sperm cells were resuspended in BK*S15 or in BK*S15 MOPS media at a final concentration of 500 cells per microliter. They were frozen quickly at −20° C in BK*S15 (**A**), −80° C in BK*S15 (**B**) or BK*S15 MOPS (**C**), or in liquid nitrogen in BK*S15 media (**D**). After 1 week, the sperm cells were thawed out, placed at 20° C for 3 hours, and then assessed for their viability by the FCR test. The percentage of survival, along the Y axis, is represented by the number of FCR^+ cells recovered after thawing divided by the number of FCR^+ cells before freezing, multiplied by 100. *Error bars* indicate standard deviations

putting the cells at −80° C set up a cooling rate at which the media, highly concentrated in sucrose, were able to solidify into a metastable ice state. This process is known as "vitrification" (McGann and Walterson 1987). Following this treatment, no significant difference was observed between survival percentages in BK*S15 or A media after 3 h at 20° C. Furthermore, we observed that the survival rate of thawed cells in medium A is similar to the survival rate of fresh cells in the same medium (data not shown). In addition, we must point out the importance of the cell suspension volume of 50 µl. With such small volumes, temperature exchanges between the environment (−80° C or 37° C water bath) and the cells are facilitated. Thus, sperm-cell populations can be homogeneously frozen and thawed.

A similar technique of freezing at −80° C has been used previously to stock wheat nuclei (Luthe and Quatrano 1980). According to Wagner and Dumas (1989), the sperm-cell nucleus represents 38.5% of the total cell volume. The resulting nucleoplasmic ratio of 0.63 is particularly high. In these conditions isolated sperm cells could have behaved as nuclei during the freezing process, which would explain the success encountered with the freezing method of −80° C.

In conclusion, the results presented in this paper constitute the basis for further in vitro manipulation of isolated sperm cells. First, we have determined a medium in which sperm-cell survival is extended to up to 35 h at 20° C. This constitutes a significant improvement since the initial survival time was less than 5 h at the same temperature. The definition of such a survival medium will allow researchers to perform studies on sperm-cell physiology. Moreover, the study of isolated sperm-cell gene expression can then be initiated. Second, we have also shown an easy and reproducible method for the long-term storage of isolated sperm cells, which presents the opportunity to work all year round with good quality sperm cells isolated during the summer season.

The hereby demonstrated improvement in the long-term viability of sperm cells is a pre-requisite for immunogenic characterization via monoclonal antibodies, cell sorters, etc. Moreover, this improvement will enable researchers to transform isolated sperm cells; for example, by electroporation. Transformed sperm cells could become the optimum vectors for plant transformation through intergametic fusion (Kranz et al. 1991).

Acknowledgements. We thank Dr. J. Drevet and R. Meidinger for proof reading the present manuscript. This work was supported in part by the company Sanofi Elf Bio-recherches.

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