Plant Physiol. (1987) 83, 464–466
0032-0889/87/83/0464/03/$01.00/0

Communication

Male Germ Unit Isolation from Three Tricellular Pollen Species: *Brassica oleracea, Zea mays,* and *Triticum aestivum*[1]

Received for publication October 12, 1986

ELISABETH MATTHYS-ROCHON*, PHILIPPE VERGNE, SERGE DETCHEPARE, AND CHRISTIAN DUMAS
Reconnaissance Cellulaire et Amélioration des Plantes, Université Claude Bernard, Lyon I, 43 Boulevard du 11 November 1918, 69 622 Villeurbanne Cedex, France

ABSTRACT

A technical procedure is described to follow the *in vitro* release of the 'male germ unit' and the sperm cells from three tricellular pollen species (*Brassica, Zea,* and *Triticum*). The condition of the sperm cell was controlled using light microscopy. In addition, for the first time, the sperm cells viability has been checked by the fluorochromatic reaction test. These preliminary results indicate that this procedure appears to be a prerequisite for the successful preparation of purified viable sperm cells.

In flowering plants, the sexual process of double fertilization is still relatively unknown especially the mechanisms of cell fusions. Two sperm cells (SC1 and SC2)[2] produced by the pollen grain are required: one fuses with the egg to form the embryo and the second fuses with the central cell to give rise to the nutritive endosperm. Today it is a challenge to understand the role played by each sperm cell. With that in mind, attempts have been made to prepare naked pollen gametophytes (sporoplasts) (12) and sperm cell nuclei (11).

On the other hand, Russell (16, 17) first demonstrated in *Plumbago zeytanica* the preprograming of the two dimorphic sperm cells. Similar physical associations between the vegetative nucleus and the sperm cell pair have recently been reported in several species (13, 20) and are termed 'male germ unit' (MGU) by Dumas *et al.* (5) to emphasize the male DNA transmission as a whole in the double fertilization. In addition, Russell (15) pointed out the preferential fusion of the rich plastid sperm cell (SC2) with the egg in *Plumbago.*

All these reports increase the interest in finding ways to isolate living flowering plant sperms. Recently Russell (18) obtained an enriched fraction of sperm cells using a sucrose gradient. Nevertheless the question of their viability remains unresolved. The aim of this paper is to present the technical prerequisites to obtain viable isolated sperm cells from several tricellular pollen grain species: *Brassica zea* and *Triticum.*

[1] Supported by a MRT grant 85 T 0448.

[2] Abbreviations: SC1 and SC2, sperm cells 1 and 2; BK medium, Brewbaker and Kwack medium; DAPI, 4',6-diamidino-2-phenylindole; EB, ethidium bromide; FCR, fluorochromatic reaction; FDA, fluorescein diacetate; MGU, male germ unit.

MATERIALS AND METHODS

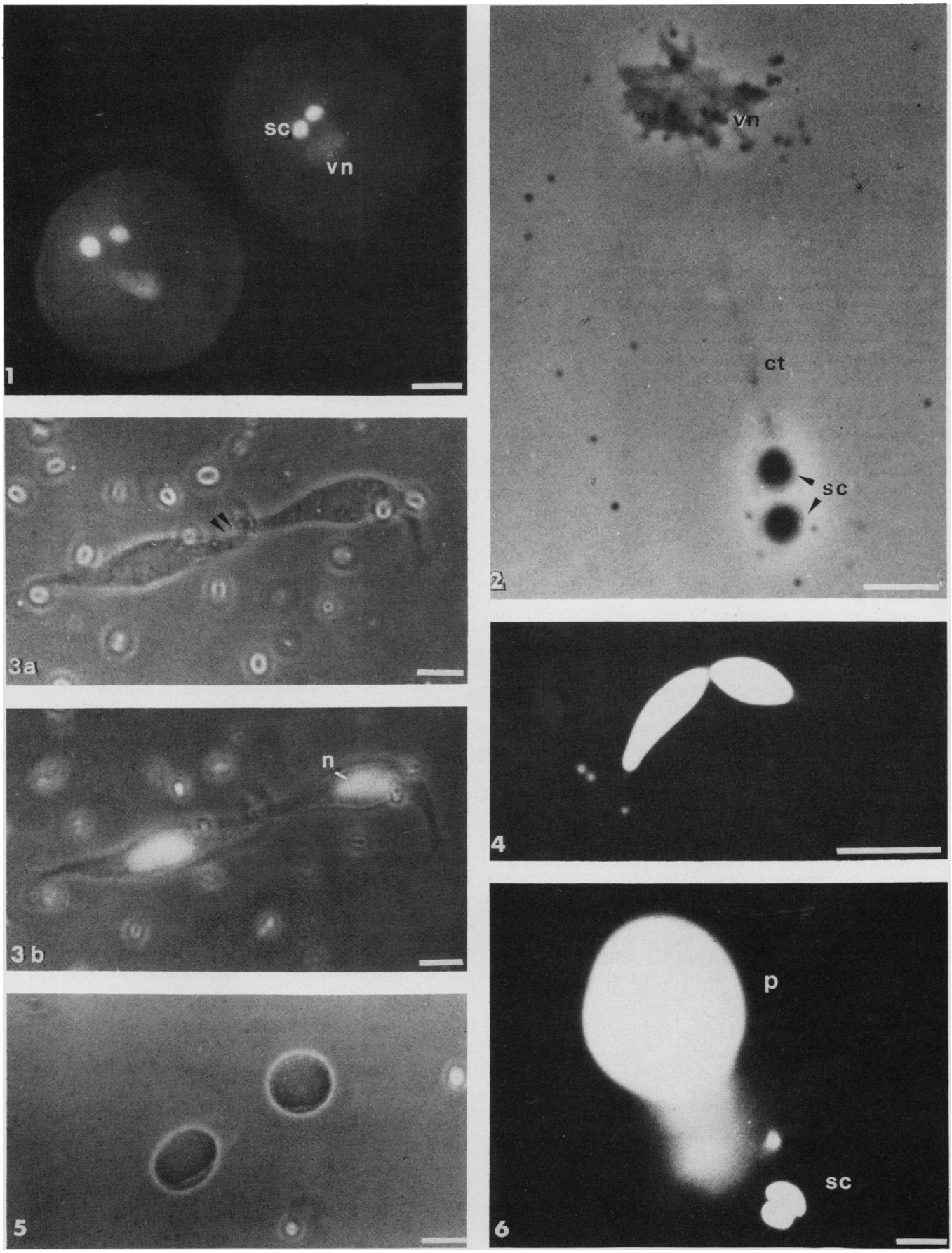
Mature plants of *Brassica oleracea, Zea mays,* and *Triticum aestivum* were grown in a greenhouse with temperatures held above 20°C and 12 to 14 h light. Fresh pollen was collected from dehiscent anthers and pollen viability was tested directly by the usual fluorochromatic FCR test procedure (6, 7). The pollen grains were incubated into the BK medium (1) supplemented with sucrose (15% for *Zea* and *Brassica,* 30% for *Triticum*). Pollen suspensions were observed with a light microscope (Nikon Labophot type 104) equipped with phase contrast and epifluorescence systems. Photographs were taken with a Nikon photomicrographic attachment UFX II loaded with Ilford HP5 film (400 ASA). In order to check nuclear structures, DNA fluorochromes EB (8) and DAPI (3) were used. The details of the technical conditions are reported in Table I.

RESULTS AND DISCUSSION

The pollen species used in this work are tricellular: a vegetative cell containing two sperm cells whose nuclei may be visualized *in situ* by the DNA fluorochrome DAPI (Fig. 1). The isolation of sperm cells first involves the finding of an appropriate medium and technique to break the thick pollen wall. To obtain them free of damage, the release of intact sperm cells must be controlled throughout the isolation procedure. Finally their viability has to be verified and checked.

Thus pollen grains were incubated in a BK sucrose medium to induce a light osmotic shock. For *Zea* and *Brassica* pollen a gentle grinding is coupled with the osmotic shock. In *Zea* pollen congestion of organelles prevents accurate observation of sperm cells which led us to find the following convenient and rapid method: using a light microscope equipped with both fluorescence and phase contrast systems to simultaneously detect the nuclei with a DNA probe (EB or DAPI) and to determine the cellular state by phase contrast microscopy. Under these conditions the release of sperm cells follows a remarkable 'time table.'

When pollen grains discharge their contents, the vegetative nucleus and the two sperm cells appear simultaneously. These three subunits are linked to form a structured set, which has previously been termed the MGU (5), demonstrated from electron microscopic data. We present the first light microscopic observation of the *in vitro* MGU in *Brassica* (Fig. 2). In *Triticum* and *Zea* pollens the abundance of storage products (starch grains) makes the MGU structure more difficult to liberate in good condition. After pollen discharge, the two sperm cells separate from the vegetative nucleus. Then they appear as elongated cells with a reduced 'active' cytoplasm, connected with a membranous tract (Fig. 3, a and b). At that light microscopic level the dimorphic aspect of the two cells cannot always be detected. But



FIGS. 1–6. Male cells of pollen grains. Phase contrast and epifluorescent microscopic observations (scale 10 μm). 1, Tricellular *Brassica* pollen grains: two sperm cell nuclei and vegetative nucleus. DAPI fluorescence (sc, sperm cell; vn, vegetative nucleus). 2, Isolated MGU from *Brassica* pollen grain. Note the vegetative nucleus in connection with the sperm cell pair. Phase contrast (sc, sperm cell; ct, cytoplasmic tract; vn, vegetative nucleus). 3, Two elongated sperm cells from *Zea* pollen: a, phase contrast. The membranous tract between the two sperm cells is clearly visible (arrows). b, EB fluorescence in conjunction with phase contrast in nucleus. 4, A sperm cell pair isolated from *Triticum* pollen. EB fluorescence. 5, Two isolated spherical sperm cells from *Zea* pollen. Phase contrast. 6, FCR test in isolated sperm cells from *Triticum* pollen (p, bursting pollen grain; sc, two sperm cells).

Table I. *Technical Conditions for Using Several Fluorochromes with Different Cytological Targets*

Fluorochromes Fluorescent Products	Concentration in BK Medium	Time of Incubation	Excitation Filter Nikon Fluorescent Equipment	Color of Fluorescence	Cytological Target
	mg/ml	*min*	*nm*		
Ethidium bromide	0.09	1	B_2IF 460–485	Orange	Nucleus
4′,6-Diamidino-2-phenylindole	0.001	10–15	UV 330–380	Blue	Nucleus
Fluorescein as fluorescein diacetate	0.002	5–10	B_2IF 460–485	Green	Cell viability

according to the observations of several authors the SC1 possesses a tail in contiguity with the vegetative nucleus (20). SC1 may correspond to the cell which carries a slender membrane projection with some tiny vesicles (Fig. 4).

Such elongated sperm cell pairs are also found *in vivo* in the narrow tubes of germinating pollen or when these tubes liberate their contents in the BK medium. Tests using variable sucrose solutions have shown that linked *Zea* and *Triticum* sperm cells can be maintained when the sucrose concentration medium is higher than 30%. A few minutes after introduction into the BK medium the physical association between the two sperm cells is ruptured and each of them becomes individualized and shortly thereafter spherical. Nevertheless both cytoplasm and nucleus are still clearly distinguishable (Fig. 5). In *Hordeum* sp. Cass (2) has also noticed the transition from spindle shaped cells to spherical ones which may be facilitated by changes in disposition of sperm microtubules.

The question which arises then is: how can cell viability be assessed? Usually the FCR is employed to test cell viability. This technique has been used since Rotman and Papermaster (1966) (14) have demonstrated the fluorochromasia in living animal cells. Subsequently, it was applied to pollen grains (6, 7), to cell suspensions (9), and cultured plant cells (19). In the last case, the authors correlated the FCR to the cytoplasmic activity and to cell growth. In our laboratory previous observations have shown that FCR^- (negative) pollen grains of the three species (*Zea*, *Triticum*, and *Brassica*) may exhibit two similar green fluorescent spots inside, suggesting that they correspond to sperm cells which respond to the FCR reaction (data not shown). Therefore we checked sperm cell viability—to the best of our knowledge for the first time—by the FCR test. In the presence of FDA the two cells emit bright green fluorescent light (Fig. 6). In addition we demonstrated that isolated sperm cells contain ATP (data not shown). The presence of this nucleotide is known to be an indicator of life (10), and its measurement is used to test the fertility of human semen (4). Moreover the presence of FCR^+ (positive) sperm cells in pollen which is apparently FCR negative implies that sperm cells have a stronger resistance than the pollen grains which contain them. This observation seems to be supported by the fact that isolated sperm cells stored at 4°C for 48 h remained FCR^+ (data not shown).

In conclusion this preliminary work shows that this procedure was an absolute prerequisite for the successful preparation of purified viable sperm cells. Our results have provided the essential technical tools, particularly the feasible assessment of viability by the FCR test.

According to Russell (15), the preferential fusion of the SC2 with the egg and SC1 with the central cell indicates a peculiar recognition at the gamate level, which may reflect specific differences in the cell surface. Thus viable sperm cell isolation may give the opportunity to analyze plasma membranes of these highly specialized cells with appropriate markers. In our laboratory we are trying to get an enriched fraction of sperm cells by separating them from pollen organelles and debris through a Percoll gradient centrifugation step. All these studies may contribute to a better understanding of double fertilization.

Acknowledgment—We thank Miss Anne-Marie Thierry for typing this article.

LITERATURE CITED

1. Brewbaker JL, BH Kwack 1963 The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50: 859–865
2. Cass DD 1973 An ultrastructural and Nomarski interference study of the sperms of barley. Can J Bot 51: 601–605
3. Coleman AW, LJ Goff 1985 Applications of fluorochromes to pollen biology. I. Mithramycin and 4′,6-diamidino-2-phenylindole (DAPI) as vital stains and for quantitation of nuclear DNA. Stain Technol 60: 145–154
4. Comhaire F, L Vermeulen, K Ghedirak, J Mas, S Irvine, G Callipolitis 1983 Adenosine triphosphate in human semen: a quantitative estimate of fertilizing potential. Fertil Steril 40: 500–504
5. Dumas C, RB Knox, T Gaude 1985 The spatial association of the sperm cell and vegetative nucleus in the pollen grain of *Brassica*. Protoplasma 124: 168–174
6. Heslop-Harrison J, Y Heslop-Harrison 1970 Evaluation of pollen viability by enzymatically induced fluorescence intracellular hydrolysis of fluorescein diacetate. Stain Technol 45: 115–120
7. Heslop-Harrison J, Y Heslop-Harrison, KR Shivanna 1984 The evaluation of pollen quality and a further appraisal of the fluorochromatic (FCR) test procedure. Theor Appl Genet 67: 367–375
8. Hough T, P Bernhardt, RB Knox, EG Williams 1985 Applications of fluorochromes to pollen biology. II. The DNA probes ethidium bromide and Hoechst 33258 in conjunction with the callose-specific aniline blue fluorochrome. Stain Technol 60: 155–162
9. Jones KH, JA Senft JA 1985 An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J Histochem Cytochem 33: 77–79
10. Karl DM 1980 Cellular nucleotide measurements and applications in microbial ecology. Microbiol Rev 44: 739–796
11. LaFountain KL, JP Mascarenhas 1972 Isolation of vegetative and generative nuclei from pollen tubes. Expt Cell Res 73: 233–236
12. Loewus FA, BG Baldi, VR Franceschi, LD Meinert, JJ McCollum 1985 Plant sporoplasts: dissolution of pollen walls. Plant Physiol 78: 652–654
13. McConchie CA, S Jobson, RB Knox 1985 Computer assisted reconstruction of the male germ unit in pollen of *Brassica campestris*. Protoplasma 127: 57–63
14. Rotman B, BW Papermaster 1966 Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA 55: 134–141
15. Russell SD 1985 Preferential fertilization in *Plumbago zeytanica*: ultrastructural evidence for gamete level recognition in an angiosperm. Proc Natl Acad Sci USA 82: 6129–6132
16. Russell SD, DD Cass 1981 Ultrastructure of the sperms of *Plumbago zeytanica*. I. Cytology and association with the vegetative nucleus. Protoplasma 107: 85–107
17. Russell SD, DD Cass 1983 Unequal distribution of plastids and mitochondria during sperm cell formation in *Plumbago zeytanica*. *In* DL Mulcahy, E Ottaviano, eds, Pollen Biology and Implications for Plant Breeding. Elsevier Biomedical, New York, pp 135–140
18. Russell SD 1986 Isolation of sperm cells from the pollen of *Plumbago zeytanica*. Plant Physiol 81: 317–319
19. Widholm JM 1972 The use of fluorescein and phenosafranin for determining viability of cultured plant cells. Stain Technol 47: 189–194
20. Wilms HT, AC Van Aelst 1983 Ultrastructure of spinach sperm cells in mature pollen. *In* O Erdelska, ed, Fertilization and Embryogenesis in Ovulated Plants. Center of Biological and Ecological Science, Slovak Academy of Science, Czechoslovakia, pp 105–112