FLOW CYTOMETRIC ISOLATION OF EMBRYOGENIC *BRASSICA NAPUS* MICROSPORES

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Microspore cultures can be used to generate haploid embryos and dihaploid plants (Chuong and Beversdorf, 1984). This technique is a rapid and convenient method for producing homozygous plants and its use in rapeseed breeding programs is being investigated in several countries. However, even with the best cultivars and optimal culture conditions only 1% or less of the microspores that are plated ultimately develop into embryos. In order to follow changes in the viability and cell size of cultured microspores a single laser flow cytometer/cell sorter was used to analyze the light scatter and fluorescence characteristics of fluorescein diacetate-stained microspore cultures over the first seven days.

Microspores were isolated from SV02231 *B. napus* plants grown at 23°C for approximately 7 weeks. Buds, approximately 3 mm large, were harvested and homogenized in B_5 (with 13% sucrose). The suspension was filtered through 44 um and 63 um Nitex screens and centrifuged at 1,000 rpm for 6 minutes. The microspores in the pellet were resuspended and washed 3 times with B_5 and were cultured at a density of 40,000 cells/ml in NLN, which is a modified Nitsch and Nitsch medium (Nitsch and Nitsch, 1967), containing 13% sucrose, 0.05 mg/l BA and 0.5 mg/l NAA at 30°C for three days then at 25°C, in the dark. For flow cytometry the microspores were stained for 10 min. by adding 1.8 ml of a 10 mg/ml solution of fluorescein diacetate to 180 ml of the culture. The cells were washed several times with B_5 and resuspended in NLN for flow cytometric analysis.

Light scatter (related to cell size) and green fluorescence (related to cell viability) were measured using a Coulter EPICS V flow cytometer. Microspores were sorted one day and three days after culture initiation on the basis of their light scatter and fluorescence properties into 96 microwell plates containing 50 ml of NLN. Two thousand live, dead and mixed microspores were sorted separately into 12 microwells each to give, sorted live, sorted dead and sorted mixed populations respectively. The plates also contained a population of control microspores which had been stained but were not sorted (designated manual control). Sorting was better than 90% efficient in selecting live cells. The experiment was repeated three times.

The freshly isolated microspore population had a bell-shaped distribution for light scatter values (Fig. 1A, the values to the left of the graph represent debris). At day one a second population of cells with a larger mean size was evident. This fraction was present in subsequent samples taken throughout the first seven days. Flow cytometry confirmed the visual observation that living cells approximately doubled in size (from 19 um to 38 um) within 1

.ay and then continued to increase at a slower rate. Over time, the proportion of the population falling into the large size category remained consistent (Fig. 1B).

The initial population of microspores (day 0) showed a continuous range of values for fluorescence but within one day the fluorescence profile split into two sub-populations (Fig. 2A). Seventy-five percent of the cells at day one were not fluorescing but the remainder showed stronger fluorescence than had been seen initially. The proportion of cells that fluoresced fell from 40% on day zero to 4% on day seven (Fig. 2B).

Two dimensional plots of fluorescence versus light scatter showed that most highly fluorescent cells were also enlarged (Fig. 3A). Over time the proportion of large, highly fluorescing cells decreased at a rate that was very similar to the decrease in viability estimated visually (Fig. 3B). Only 40 to 50% of the microspores were viable at the time of culture and viability declined to approximately 3% within a week.

The sorting experiments confirmed that flow cytometry is a useful technique for identifying and separating live and dead cells from microspore cultures. No embryo ever developed from the sorted dead population (Table 1). In contrast 0.57 and 4.47 embryos/thousand microspores developed from the live cells sorted at days one and three respectively. This represents approximately a 50 fold increase in embryo formation frequency compared to sorted mixed microspores.

Table 1. Embryo production in unsorted and flow cytometrically sorted microspore cultures. Results are averages of three experiments.

Population Type	Day Sorted	Embryo Produced/ Thousand Microspores Cultured
manual control	1	0.22
	3	0.03
sorted mix	1	0.01
	3	0.11
sorted dead	1	0
	3	0
sorted live	1	0.57
	3	4.47

The results suggest that flow cytometry is a useful method for enriching microspore cultures in embryogenic cells to be used for biochemical and molecular studies.

References

Chuong, P.V. and W. D. Beversdorf, 1985. High frequency embryogenesis through isolated microspore culture in Brassica napus and B. carinata braun. Plant Science 39:219-226.

Nitsch, C. and J. P. Nitsch, 1967. The induction of flowering in vitro in stem segments of Plumbag indica L. I. The production of vegetative buds. Planta 72:355-370.

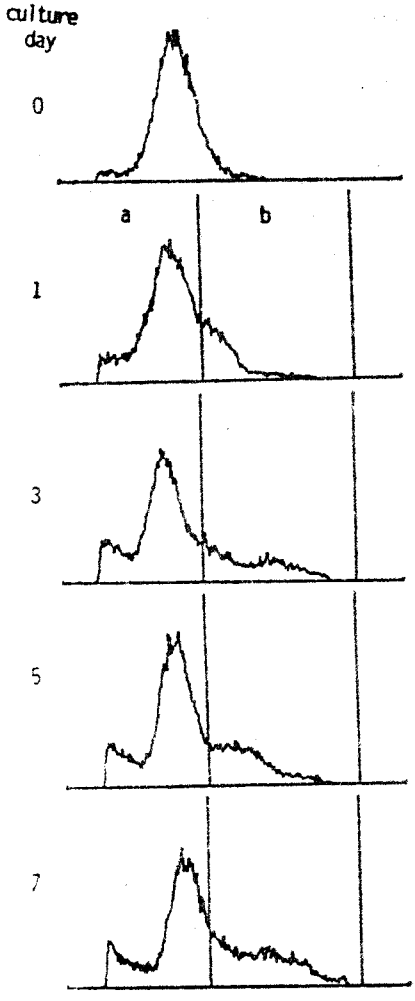

Fig. 1A. Flow cytometric light scatter profiles for B. napus microspore cultures at day 0, 1, 3, 5 and 7. Profiles were divided into two intervals (a and b) for analysis.
x = log light scatter intensity
y = number of cells/channel

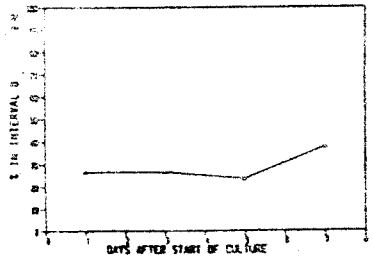

Fig. 1B. Percentage of enlarged microspores (interval b in Fig. 1A) over time in culture.

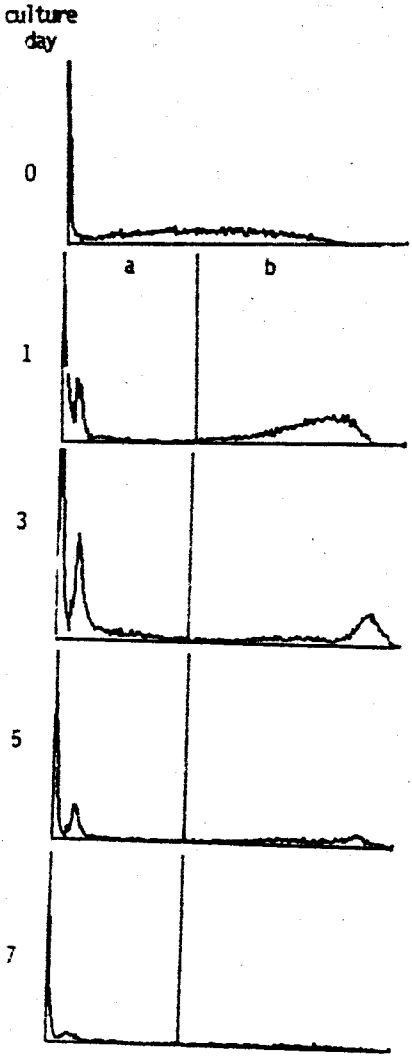

Fig. 2A. Flow cytometric fluorescence profiles for B. napus cultures at day 0, 1, 3, 5 and 7. Profiles were divided into two intervals (a and b) for analysis.
x = log integrated fluorescence intensity
y = number of cells/channel

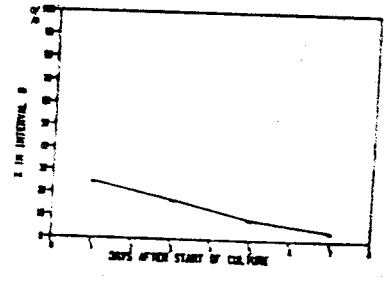

Fig. 2B. Percentage of highly fluorescent microspores (interval b in Fig. 2A.) over time in culture.

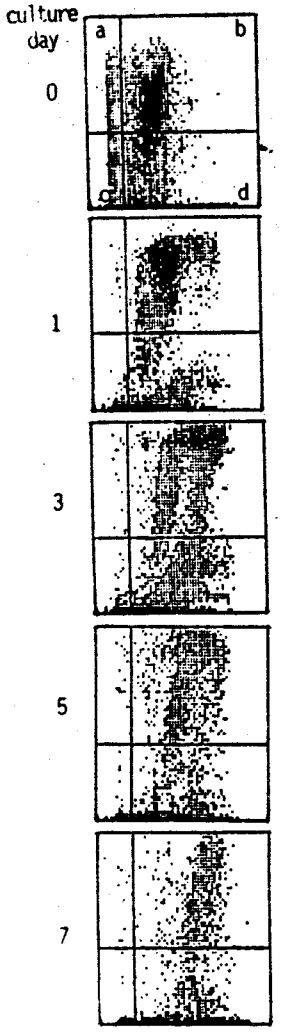

Fig. 3A. Flow cytometric scatter plots of light scatter vs. fluorescence for B. napus microspore cultures at day 0, 1, 3, 5 and 7. Plots were divided into 4 quadrants (a, b, c, and d) for analysis.
x = log integrated fluorescence intensity
y = log light scatter

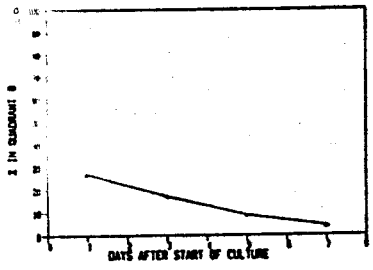

Fig. 3B. Percentage of highly fluorescent enlarged microspores (quadrant b in Fig. 3A.) over time in culture.