ISOLATION AND ENRICHMENT OF EMBRYOGENIC MICROSPORES IN BRASSICA NAPUS L. BY FRACTIONATION USING PERCOLL DENSITY GRADIENTS

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The technique of isolated microspore culture for producing large quantities of haploid embryos in Brassica napus L. has been improved tremendously in recent years (Lichter, 1982; Keller and Armstrong, 1983; Chuong and Beversdorf, 1985; Pechan and Keller, 1987). This improvement was achieved as a result of studies on donor plant physiology, gametophytic cell development, culture medium composition, and physical culture conditions. Currently, the technique is being employed for production of dihaploid plants in breeding programs, selection of mutants for various agronomic characteristics, and gene transfer via microinjection (Keller et al., 1986; Spangenberg et al., 1987).

In addition to practical applications, isolated microspore culture can serve as a useful system for studies on differentiation from a single gametophytic cell to a sporophytic haploid plant. This type of research usually requires a highly homogeneous and embryogenic microspore population. It has been reported that microspores and suspension cells can be fractionated using a percoll or ficoll density gradient and certain fractions contain microspores or cells having a high frequency of cell division and embryogenesis (Wernicke et al., 1978; Kyo and Harada, 1985; Nomura and Komamine, 1985). In this report, a percoll density gradient system to separate B. napus microspores is outlined and characteristics of microspore populations from the separated fractions are described.

MATERIALS AND METHODS

Plant material. Plants of B. napus cv. 'Topas' were initially grown in a growth room at 20/15°C (day/night) and 6 h illumination. When the plants started to elongate prior to flower bud formation, the temperature was reduced to 10/5°C.

Microspore isolation. The terminal inflorescence and three subsequent branches were harvested when the flowers began to bloom. Buds 2-5 mm in length were selected and separated into two groups. One group had the length of 2-3.5 mm, termed the S buds, and the other with a length of 3.5-5 mm, termed the L buds. The S and L buds were always manipulated separately in the experiments. The buds were surface-sterilized in 7% calcium hypochloride solution for 15 min and rinsed twice in deionized

distilled water (5 min each). The buds were macerated in B_5 medium (Gamborg et al., 1968) free of growth regulators and supplemented with 13% sucrose and then filtrated through a nylon mesh (44 μm). The microspore suspensions obtained were washed three times in B_5 medium by centrifugation (100 g, three min).

Percoll density gradient. A stock percoll solution (80%) was made by mixing 100% percoll with 65% sucrose (8:2). The gradients consisted of 24, 32, 40, 48, and 64% of percoll, which were made by diluting the stock solution with 13% sucrose. The microspore pellets obtained after the last wash were mixed with the 64% percoll and the other concentrations of percoll were layered on in an ascending order. The gradients were centrifuged at 100 g for 6 min. The microspores at each interface of the gradient were collected separately, washed twice in B_5 medium, and plated in a modified Lichter's medium (Lichter, 1982) free of potato extract at a density $0.5X\ 10^5$/ml. The cultures were initially placed in an incubator at 32°C for three days and then transferred to 25°C for two weeks. The embryos formed from each fraction were scored after 20 days of culture.

Cytology. The number of nuclei in the microspores was identified by staining with a fluorochrome, 4; 6-diamidino-2-phenylindole [DAPI (2 ug/ml)]. The microspores were observed on a Zeiss photomicroscope equipped with a 50 W mercury lamp and a filter combination of PB 400-440:LP 470.

RESULTS AND DISCUSSION

Microspores from the S and the L buds separated into four distinct bands in the percoll gradient system. Three major bands were obtained from the microspores of the S buds (Fig. 1). They represented 39.96, 29.73, and 23.14% of the total population, respectively. Microspores from the L buds gave only one major band at 24/32 interface (81.37%). The other three bands had a limited number of microspores and sometimes it was difficult to obtain enough microspores to culture a single plate at the required density.

The microspores of the S buds were predominantly uninucleate, whereas those of the L buds, binucleate. The percentages of uninucleate and binucleate microspores at each interface of the gradient are shown in Table 1. The separation of the uninucleate from the binucleate microspores was achieved by dividing the buds into two groups. Under the growth conditions described above, the first pollen mitosis was observed in microspores from buds 3.5-4 mm in length. Since the development of microspores was not completely synchronized, the microspores from these buds may be uninucleate, binucleate, or at various stages of the mitosis. Therefore, the separation criterion was set up at 3.5 mm. It was also possible to obtain pure uninucleate and binucleate microspore populations by eliminating the 3.5-4 mm buds. However, this would also alter the relative amounts of microspores at different interfaces.

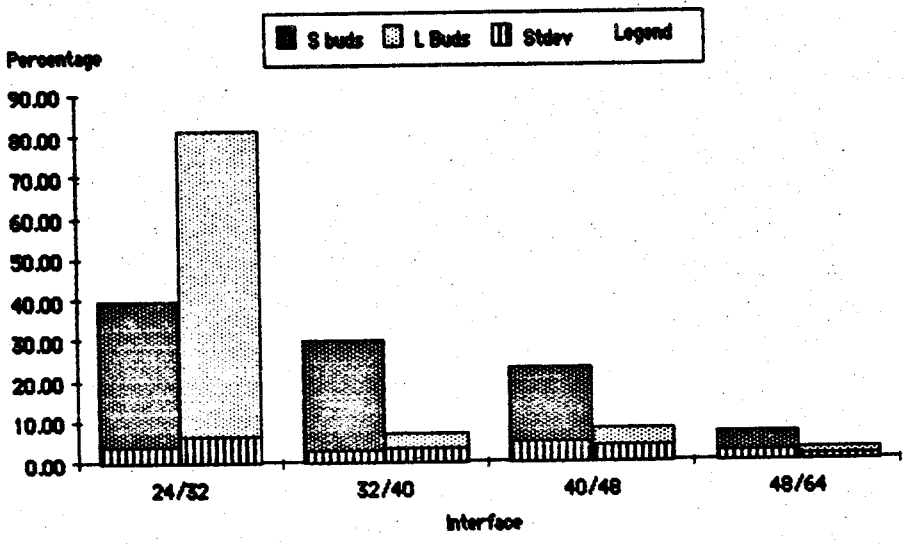

FIG. 1. Percentage of microspores from the S and L buds at each interface of the gradient.

TABLE 1. Percentage of uninucleate and binucleate microspores at each interface of the percoll density gradient

Bud type	Spore type	Interface 24/32	32/40	40/48	48/64
S	Uninucleate	89.25±3.00	95.31±3.24	96.47±1.98	98.43±0.69
	Binucleate	10.73±3.00	4.69+3.24	3.53±1.98	1.57±0.69
L	Uninucleate	6.42±2.68	4.57±1.57	17.40±4.49	1.57±1.17
	Binucleate	93.58±2.68	95.43±1.57	82.60±4.49	94.66±1.17

The microspores from the S buds at the 24/32, 32/40, and 40/48 interfaces were very uniform and were significantly different in size (Table 2). This was also the same for their nuclei. Those at the 48/64 interface varied much in size, suggesting that they may be damaged microspores. The size differences were also observed with the microspores from the L buds at the 24/32 and 32/40 interfaces. The size of those at the 40/48 and 48/64 interfaces was not significantly different due to their large size variances. From Table 2, it appears that the size of microspores is negatively correlated with their density; the larger microspores had a lower density with the smaller type having a higher density. The

developmental stages of the microspores also appears to be correlated with the density since the larger microspores were usually at a more advanced developmental stage.

TABLE 2. Diameters of microspores and their nuclei at each interface of the percoll density gradient.

Bud type	Interface			
	24/32	32/40	40/48	48/64
S	22.28a(11.23a)*	18.87b(7.68b)	17.68c(5.84c)	20.69abc(6.60abc)
L	22.59a(5.76a,9.44a)	20.20b(4.02b,6.18b)	20.93ab(4.76ab,7.37ab)	22.37a**

*The format is microspore diameter (nucleus diameter). The values followed by different letters are significantly different at 0.01 level using the student t test.
**The nuclei were very lightly stained, an accurate measure could not be obtained.

The microspores from the S buds at the 24/32 interface gave the highest frequency of embryo formation [14.05%; Table 3)], indicating that the later uninucleate microspores were highly embryogenic. The corresponding microspores from the L buds produced 8.15% embryos, indicating that some of the early binucleate microspores are also embryogenic. However, the embryos derived from these two fractions were morphologically different. The embryos of the S buds had a normal root, cotyledon, and hypocotyl, and could be easily regenerated into plants, while those of the L buds had abnormal cotyledon which proliferated into callus-like growth, and only grew a root or a malformed shoot.

TABLE 3. Percentage of embryos formed from microspores at each interface of the percoll density gradient.

Bud Type	Interface			
	24/32	32/40	40/48	48/64
S	14.05±5.06	6.81±1.76	1.25±1.28	0.00
L	8.15±4.11	2.19±0.51	0.90±1.41	0.00

In summary, a highly uniform and embryogenic microspore population was obtained by fractionation using a percoll density gradient. This would be very useful for studies of cytological, biochemical, and physiological aspects of microspore embryogenesis.

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