AN EFFICIENT METHOD FOR CULTURE OF ISOLATED MICROSPORES OF BRASSICA NAPUS

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INTRODUCTION

Microspores isolated from buds of a number of Brassica napus genotypes can be induced to undergo in vitro embryogenesis and plant regeneration (Lichter 1982; Keller and Armstrong 1983; Chuong and Beversdorf 1985). This process is rapid with fully-differentiated embryos easily detectable after 2-3 weeks. In some instances, frequencies of embryogenesis are satisfactory for the application of the technique to plant breeding programs. However, for application of this technique to genetic engineering and developmental studies, high frequencies of embryogenesis must be consistently obtained. This presentation describes aspects of a major study aimed at refining and optimizing B. napus microspore culture technology for a range of applications in developmental, cell and molecular genetics.

MATERIALS AND METHODS

Plants of B. napus cv. Topas were grown in a growth chamber under a 16-h photoperiod with a light intensity of 300 uE supplied by cool white fluorescent (84% of intensity) and incandescent lights. Day/night temperatures of 20/15, 15/10 and 10/5°C were evaluated in regard to subsequent effect on embryogenesis in the microspore cultures.

Terminal and axillary inflorescences were harvested at the onset of blooming and buds 3-4 mm with minimal pedicel elongation were selected. Cytological evaluation had previously shown that anthers from these buds contained microspores at the uninucleate and early binucleate developmental stage. The selected buds were surface-sterilized by a 15 minute immersion in 7% (w/v) calcium hypochlorite and rinsed three times with sterile, distilled water. Buds, in groups of 10-25, were placed in a 50 ml beaker containing 5-10 ml of "B_5-13" medium (Gamborg et al. 1968) free of growth regulators and supplemented with 13% sucrose; microspores were released as the buds were macerated with the use of a glass pestle. the macerate was filtered through 44 um nylon screen cloth and the filtrate containing the microspores was transferred to 16 x 100 mm glass tubes. The microspores were pelleted by centrifugation at 100 g for three min and resuspended in 10 ml "B_5-13" medium. The washing procedure was repeated three times. After washing the microspores were suspended in culture medium which had the composition described by Lichter (1982) with the exception that potato extract was omitted. Various media modifications were evaluated (see Results and Discussion) in terms of their effect on microspore embryogenesis. All wash and culture media were sterilized by filtration.

Microspore density was adjusted to $2\text{-}5 \times 10^4$ and 1.2 ml of microspore suspension was plated into 60 x 15 mm plates containing 2 ml of culture medium solidified with 0.3% sea plaque agarose (Mandel). The petri plates were sealed with parafilm

and incubated in darkness at 32.5°c for four days followed by transfer to 25°c (dark). All experiments were repeated at least twice.

In experiments in which embryos were cultured, the petri plates were transferred to low intensity fluorescent light (24 uE) for 1-2 days. Embryos 2-3 weeks of age were transferred to B_5 medium free of growth regulators and supplemented with 2% sucrose and 0.8% agar with the pH adjusted to 5.8 prior to autoclaving. Up to 10 embryos were cultured per 20 x 60 mm petri plate. Embryo cultures were maintained under fluorescent light at 25°C and were observed at two-week intervals.

RESULTS AND DISCUSSION

Donor plant growth temperature was a key factor influencing consistency and efficiency of microspore embryogenesis in B. napus (Keller et al. 1987). Plants grown under a 16-h photoperiod and a day/night temperature of 10/5°C gave far higher yields of embryos (in the order of 200 embryos/donor anther) than plants grown at 15/10 (10 embryos/anther) or 20/15 (no response from 18 plants tested). Other investigators (Chuong and Beversdorf 1985; Lichter 1982) reported embryogenesis from microspores isolated from plants grown at temperatures higher than 15°c indicating that genotypic as well as environmental factors may be significant. It should be noted that new buds which develop after harvesting are also suitable for microspore culture. The same donor plants can be harvested at least 5-6 times without diminished yields of embryos.

Plating of microspores onto an agarose underlayer improved consistency of the embryogenic process and also enhanced embryo development with a higher proportion of embryos in advanced stages of differentiation in comparison to embryos developing in liquid layers in petri plates. Embryos in liquid layers could be induced to undergo further development if they were slowly agitated (30 rpm) after 7 days in culture (Pechan unpublished) indicating that aeration may be a major factor influencing embryo development.

The omission of plant growth regulators from the culture medium generally had a positive effect on frequency of embryogenesis. The presence of naa at the level originally recommended by Lichter (1982) tended to reduce embryo yield. Brassica napus cv. Topas embryos which differentiated in growth regulator-free medium appeared to be superior to those developing in medium with growth regulators in regard to shoot regeneration capacity in subsequent embryo culture. Higher frequencies of embryos from growth regulator free media developed normal shoots during the first four weeks of culture than those obtained from medium containing growth regulators (Table 1).

Reduction of macronutrients to one-half strength enhanced frequencies of embryogenesis (Table 2). Embryos were also more fully-developed on half-strength medium in comparison to control medium. Microspores are apparently sensitive to media salts as even the original Lichter medium is relatively low in macronutrient content in comparison to other tissue culture media in common use. Attempts to induce embryogenesis in B_5 (Gamborg et al. 1968) or MS (Murashige and Skoog 1962) basal salts media were unsuccessful. The composition of the culture medium currently employed for B. napus cv. Topas microspore culture is given in Table 3.

When optimal conditions with regard to donor plant growth temperature and culture medium composition are employed, 1-5% of the microspores will undergo embryogenesis. percoll gradient fractionation can be used to obtain microspore fractions which undergo embryogenesis at frequencies of 15% (Fan et al. 1987). by working with individual buds, it is possible to observe frequencies of embryogenesis in the order of 50% (Pechan and Keller 1987). Frequencies of embryogenesis of 1% or less will lead to the production of sufficient numbers of plants for breeding purposes providing that selection of specific genotypes is not occurring. Frequencies of 5% are sufficient for mutant selection as 10^6 microspores can be obtained per plant and 20 donor plants can be easily grown under controlled climate conditions. Studies involving the selection of herbicide-resistant embryos in mutagenized microspore cultures are currently in progress in our laboratory.

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TABLE 1. Effect of growth regulators in microspore culture medium on subsequent embryo development

Growth regulator concentration (mg/l) BA	NAA	No.embryos cultured	% survival	% survivors with normal shoot development
0	0	272	99.6	30.2
0.05	0	200	99.5	12.6
0.5	0	84	100	29.8
5.0	0	100	97	18.6
0	0.05	172	98.8	22.9
0	0.5	121	90.9	5.4
0.05	0.05	100	97	8.2
0.5	0.5	100	100	9.0
0.05*	0.5*	372	99.5	8.6

*Concentrations used in original medium published by Lichter (1982)

TABLE 2. Effect of macronutrient concentration on embryogenesis in isolated microspores of *Brassica napus* cv. Topas

Macronutrient* concentration factor	Embryos/donor anther
1/4	119
1/2	126
1	83
2X	17
4X	1

*Macronutrients defined by Lichter (1982)

TABLE 3. Composition of the medium for isolated microspore culture in Brassica napus

Component	Concentration (mg/l)
KNO_3	62.5
$MgSO_4.7H_2O$	62.5
$Ca(NO_3).4H_2O$	250.0
KH_2PO_4	62.5
FE.EDTA (Na SALT)	40
$MnSO_4.4H_2O$	25
H_3BO_3	10
$ZnSO_4.4H_2O$	10
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
myo-inositol	100
nicotinic acid	5
glycine	2
pridoxine	0.5
thiamine hcl	0.5
folic acid	0.5
biotin	0.05
glutathione	30
l-glutamine	300.0
l-serine	100
sucrose	130,000

pH adjusted to 6.0; sterilize by filtration