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Isolated microspore culture of Chinese flowering cabbage (*Brassica campestris* ssp. *parachinensis*)

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Abstract Microspores of several genotypes of *Brassica campestris* ssp. *parachinensis* have been cultured *in vitro* and induced to undergo embryogenesis and plant formation. Conditions favourable for embryogenesis in this species include a bud size of 2-2.9 mm, NLN-13 culture medium (Nitsch and Nitsch 1967; Lichter 1981, 1982; Swanson 1990), and an induction through exposure to 32°C for a period of 48 h. Longer periods of an elevated temperature for induction of embryogenesis resulted in embryo abortion at early developmental stages. With the protocol developed here, microspores of 60-80% of donor plants could be induced to produce embryos, although embryo yields were low, i.e. 2-5 embryos per 10 buds. Some genotypes responded to culture conditions with high numbers of embryo formation (100-150 embryos per 10 buds) but most of these subsequently failed to mature. The pattern of cell division and morphological changes of the microspores in culture were studied using various microscopic techniques.

Key words: *Brassica campestris* ssp. *parachinensis* - Microspore - Embryogenesis

Introduction

In *Brassica* ssp. microspore-derived embryogenesis has been achieved *in vitro* with anthers (Keller et al. 1975; Ockendon 1984; Dunwell et al. 1985) and isolated microspores (Lichter 1982; Chuong and Beversdorf 1985). In *Brassica napus* improvements have been made by the use of donor plants grown at low temperature (Keller et al. 1987), the mechanical isolation of microspores from whole buds (Swanson et al. 1987), careful selection of microspores at a specific developmental stage, i.e. prior to and after the first pollen mitosis (Fan et al. 1988, Kott et al. 1988a). These optimizations make *B. napus* microspore embryogenesis one of the most efficient systems for plant regeneration *in vitro*. In contrast, *B. campestris* is comparatively more recalcitrant in terms of embryogenesis and plant regeneration. Successful plant regeneration from isolated microspores has been reported for different Brassicaceae species (Lichter 1989), a variety of Chinese cabbage of *B. campestris* ssp. *pekinensis* (Sato et al. 1989), selected breeding lines of turnip rape ssp. *oleifera* (Burnett et al. 1992; Baillie et al. 1992) and several cultivars of ssp. *chinensis* (Cao et al. 1994).

Chinese flowering cabbage is a subspecies of *B. campestris* which is one of the major vegetable crops in Hong Kong and neighbouring regions known as Choi-sum. There are several varieties available referred to as 50, 60, 70, and 80 days. These varieties are designated according to the number of days from sowing to harvest. Plants from each variety are maintained and selected from an open pollinated population by individual growers. In this paper we report on the

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successful isolation, culture and regeneration of plants from isolated microspores of *B. campestris* ssp. *parachinensis*.

Material and Methods

Microspore culture. Four varieties of *B. campestris* ssp. *parachinensis* (50, 60, 70, and 80 days) were purchased from a local seed supplier. For each experiment, donor plants from each variety were grown in growth chambers (Conviron E15) at 18°C, 16 h light and 15°C, 8 h dark. Lighting was provided by a mixture of incandescent bulbs and cool, white fluorescent tubes (400 $\mu Em^{-2}s^{-1}$). Fertilizer 20-10-20 (N:P:K) at 0.1 g/L was applied with routine watering (4-5 times/week) once plants were past the three-leaf stage. Flower buds (1-5 mm) were excised and surface sterilized in 5% sodium hypochlorite for 15 min then followed by three 5-minute rinses in sterile distilled water. The micro-blender procedure of Swanson et al.(1987) was used. Microspore preparation adjusted to approximately 100,000/ml in NLN-13 medium of pH 6.0, 13% sucrose and BA level of 0.05 mg/L was plated at 2.5 ml/60 x 15 mm Petri dish. Cultures were maintained at 25-30°C for two to three weeks. Effect of induction temperature was studied by culturing the freshly isolated microspores under the temperature range from 25°C to 35°C. Preliminary experiments had indicated that elevated induction temperatures (>30°C) for more than 48 h resulted in increased embryo abortion in all genotypes, therefore an induction temperature of 32°C for 48 h was selected to test the effect of genotype and bud size. The embryo number at torpedo and cotyledonary stages were recorded for all treatments after 21-28 days in culture. Cotyledonary embryos were transferred onto B5 medium with 2% sucrose and 0.8% agarose and cultured at 20-25°C, under fluorescent light. After 4-5 weeks on solid medium, several well-rooted plantlets were produced and subsequently potted in soil after submerging their roots in a 0.34% solution of colchicine for 1.5 h.

Microscopy Microspores at various stages of development *in vitro* were fixed in glutaraldehyde (3%) in phosphate buffer for 2-4 h at room temperature. Samples were washed in buffer and then fixed in osmium tetraoxide (1%) for 1-2 h. After post-fixation the samples were washed in buffer before being dehydrated in alcohol. Samples for transmission electron microscopy were embedded in epon for sectioning and stained with uranyl acetate and lead citrate (Lewis and Knight 1992). Samples for scanning electron microscopy were dried in the CO_2 critical-point drying system (Reid and Beesley 1991). Microspore staging of samples from various bud length was stained with Hoechst 33342 according to the procedure by Swanson et al. (1990).

Results and Discussion

1. *Effect of genotype*. The four varieties (50, 60, 70, and 80 days) tested were not pure lines. They were bred and selected based on different dates of maturity. Twenty plants from each variety were used to test the embryogenic response of their isolated microspores from forty buds of the same size (2-2.9 mm) from each plant. There were responsive and non-responsive isolated microspores obtained from plants within each variety. The overall results (not shown) indicated about 60 to 80 % of the plants from each variety were able to produce microspores responsive to culture conditions. Results are presented in Table 1. Microspores from the two late maturing varieties were more embryogenic than the early ones, although the embryogenic frequency was very poor in comparison to *B. napus*. With a few donor plants, very high levels of embryo formation of 100 to 150 per 10 buds have been achieved from their microspores. However, most of these early embryos never survived more than 7-10 days in culture. These donor plants may reflect the genetic variation for their embryogenic potential. Attempts to subculture the small globular and heart shaped embryos on fresh medium also failed. This may be the result of some negative growth factors produced which are inhibiting embryo formation as reported by Kott et al. (1988b).

Table 1. Effect of genotype on embryogenesis in *B. campestris* ssp. *parachinensis* microspore culture.

Variety	Total embryos	Embryo/10 buds[1)]
50 days	116	1.5±0.3
60 days	104	1.3±0.3
70 days	280	3.5±0.7
80 days	368	4.6±1.6

[1)] Numbers represent average of 20 x 40 buds/plant (N=20,±S.E.).

2. *Effect of bud size.* Donor plants from the four varieties were grown under the controlled conditions as indicated in the Material and Methods section. Various bud sizes from 1-1.9 mm, 2-2.9 mm, 3-3.9 mm, and 4-4.9 mm were examined for the developmental stage of microspores at the time of excision. As examined by light microscope with nuclear staining, 60% of the microspores from bud size of 2 to 2.9 mm were at the late uninucleate stage (Table 2). Generally, the microspores isolated at late uninucleate stage produced the highest number of embryos (Table 3). This is in agreement with results for *B. napus* microspores (Kott et al. 1988a; Pechan and Keller 1988) and *B. campestris* ssp *oleifera* (Burnett et al. 1992). Flower buds ranging from 2 to 2.9 mm were selected for use in all subsequent experiments.

Table. 2 Comparison of bud size with microspore nuclear stages from *B. campestris* ssp. *parachinensis*.

	% Nuclear stages[1)]			
Bud size (mm)	1-1.9	2-2.9	3-3.9	4-4.9
EU	26.7±0.1	2.1±0.6	1.0±0.7	0
MU	64.3±3.3	33.1±1.2	25.5±1.9	4.5±1.5
LU	8.9±1.6	60.7±1.6	56.1±1.8	18.5±5.9
BN	0	4.1±1.5	16.8±2.1	71.8±5.5
TN	0	0	0	5.3±2.6

[1)] Numbers represent average of the % of nucleate stages determined from the isolated microspores from each of the four varieties (N=4, ±S.E.). 800 microspores were examined from each variety. EU Early-uninucleate; MU Mid-uninucleate; LU Late-uninucleate; BN Binucleate; TN Trinucleate.

Table 3. Effect of bud size in *B. campestris* ssp. *parachinensis* microspore culture.

	Number of embryos per 10 buds[1)]			
Bud Size (mm)	1-1.9	2-2.9	3-3.9	4-4.9
Variety				
50 days	1.0±0.1	1.8±0.4	0	0
60 days	0	1.5±0.1	0	0
70 days	0.8±0.1	3.8±0.4	1.1±0.2	0.2±0.1
80 days	0.6±0.3	4.3±0.8	1.8±0.2	0.1±0.1

[1)] Numbers represent average of 3 x 40 buds/variety (N=3,± S.D.).

3. *Effect of induction temperature.* Preliminary testing indicated that prolonged elevated induction temperatures above 30°C for more than two days resulted in arrested embryo development. Therefore, the duration of 48 h was used to evaluate the induction temperature range of 25°C, 28°C, 30°C, 32°C, and 35°C for the freshly isolated microspores. The results of temperature effects during embryo induction were pooled from the cultures of four varieties (Table 4). The optimal induction temperature of 32°C is very similar to the result observed for *B. campestris* ssp. *chinensis* (Cao et al. 1994). They also indicated that the effective temperature treatment was 33°C for two days then changed to 25°C for embryo yield.

Table 4. Effect of induction temperature in *B. campestris* ssp. *parachinensis* microspore culture.

Temperature	Total Torpedo Embryo	Embryos/ 10 buds[1)]
25°C	12	0.2±0.1
28°C	49	0.6±0.2
30°C	112	1.4±0.5
32°C	192	2.4±0.8
35°C	86	1.1±0.5

[1)] Number represent average of 4 x 200 buds/variety (N=4,± S.D.).

4. *Morphological changes.* Morphological studies using light and scanning electron microscope showed that after 1-2 days of culture the microspores increased in size from 5-8 μm

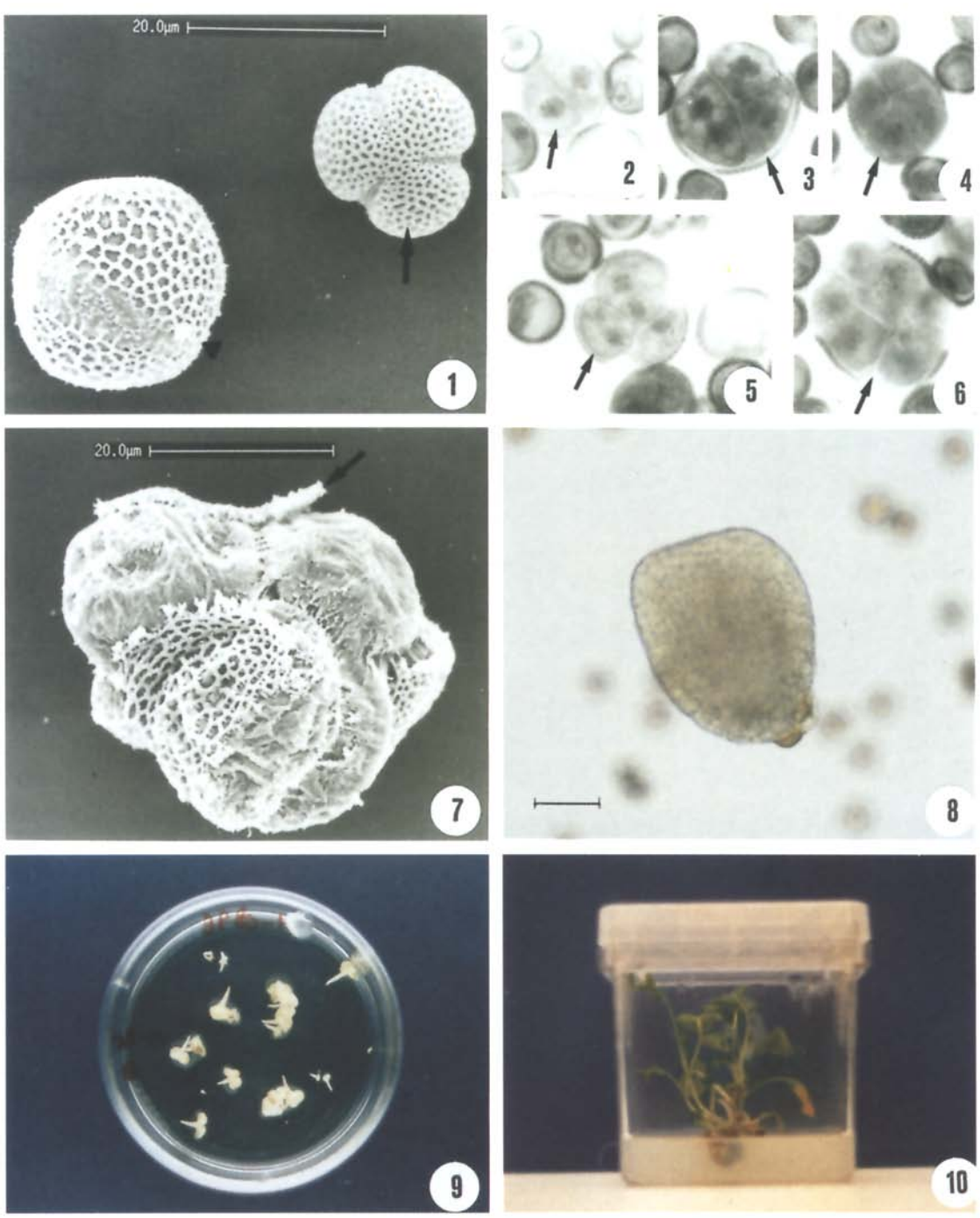

Fig. 1 Scanning electron micrograph of a microspore before culturing (arrow) and a microspore after culturing for about 2 days (arrow head). **Fig. 2** A microspore at 2-cell stage (arrow) x460. **Fig. 3** A microspore at 3-cell stage (arrow) x460. **Fig. 4** A 4-cell stage microspore (arrow) x460. Note that the cells of the microspore are arranged in a tetrad-like pattern. **Fig. 5** Microspore at 4-cell stage of development (arrow) x460. The pattern of cell division in this microspore different from that seen in Fig. 4. **Fig. 6** A pro-embryo at 8(or more)-cell stage. Showing the point of rupture at arrow x460. **Fig. 7** Scanning electron micrograph of a microspore at 8-(or more) cell stage with arrow showing portion of the ruptured exine. **Fig. 8** A heart shaped embryo developed from microspore after 14 days in culture. Bar=100μm. **Fig. 9** Embryos developed from microspores in a 60 mm petri dish after 28 days in culture. **Fig. 10** Regenerated plantlet from a microspore derived embryo cultured in solid medium.

to 15-20 μm in diameter (Fig.1). Those microspores that failed to divide would shrink during culture. Microspores capable of division would further increase in size. The first cell division took place inside the microspore without rupturing the exine and intine (Fig.2). When the microspore divided into three cells the exine invariably ruptured (Fig.3). The ruptured exine could not be easily detected at the light microscope level, but with the scanning electron microscope the ruptured exine could be easily detected (Fig.7). The intine of the microspore did not appear to rupture and became continuous with the walls of the newly formed cells of the developing embryo. At the light microscope level the pattern of division of the microspore was also followed. After the 3-cell stage the cells further divided to give variable patterns including 4-cell stage (Fig.4), a 1+3 pattern (Fig.5) and a uniseriate pattern (not shown) which is most likely a dead end in further development. Both the 1+3 or 4-cell stage are likely to contribute pro-embryos of 8-26 cells (Fig.6,7), within a period of 7 days and subsequent haploid plants.

In Hong Kong and neighbouring regions, Chinese flowering cabbage is one of the staple vegetables for the local diet. No serious breeding efforts or genetic improvement have been done or reported for this crop. It is the intent of this study to develop the microspore system for the genetic improvement and breeding of this vegetable. We have observed that *B. campestris* ssp *parachinensis* microspores can be induced to form embryos and plants. These results enable the further development of a culturing procedure for use in the breeding program based on double haploid technology. Further, it was noted that early embryo development was high in some genotypes and it may be possible to determine methods or conditions for overcoming the factors which are precluding the complete development of embryos and plants in these lines.

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References

Baillie AMR, Epp DJ, Hutcheson D, Keller WA (1992) Plant Cell Reports 11:234-237

Burnett L, Yarrow S, Huang B (1992) Plant Cell Reports 11:215-218

Cao MQ, Li Y, Liu F, Dore C (1994) Plant Cell Reports 13:447-450

Chuong PV, Beversdorf WD (1985) Plant Science 39:219-226

Dunwell JM,Cornish M, De Courcel AGL (1985) J. Exp. Bot. 36:679-689

Fan Z, Armstrong KC, Keller WA (1988) Protoplasma 147:191-199

Keller WA, Rajhathy T, Lacapra J (1975) Can. J. Genet. Cytol. 17:655-666

Keller WA, Arnsion PG, Cardy BJ (1987) In *Plant Tissue and Cell Culture* Alan R. Liss Inc. 223-241

Kott LS, Polsoni L, Beversdorf WD (1988a) Can. J. Bot. 66:1658-1664

Kott LS, Polsoni L, Ellis B, Beversdorf WD (1988b) Can. J. Bot. 66:1665-1670

Lewis PR, Knight DP (1992) In: A.M. Glauert (ed.) *Practical Methods in Electron Microscopy vol 14* Elsevier, Amsterdam

Lichter R (1981) Z. Pflanzenphysiol. Bd 103:229-237

Lichter R (1982) Z. Pflanzenphysiol. Bd 105:427-434

Lichter R (1989) Plant Breeding 103:119-123

Nitsch C, Nitsch JP (1967) Planta 72:355-370

Ockendon DJ (1984) Ann. Appl. Biol. 105:285-291

Pechan PM, Keller WA (1988) Physiologia Plantarum 74:377-384

Reid N, Beesley JE (1991) In: A.M. Glauert (ed.) *Practical Methods in Electron Microscopy vol 13* Elsevier, Amsterdam

Sato T, Nishio T, Hirai M (1989) Plant Cell Reports 8:486-488

Swanson EB, Coumans MP, Wu SC, Barsby TL, Beversdorf WD (1987) Plant Cell Reports 6:94-97

Swanson EB, Yarrow SA, Coumans M, Erickson L (1990) Stain Technology 65:251-257