IN VITRO CULTURE OF MICROSPORES AND REGENERATION OF PLANTS IN BRASSICA CAMPESTRIS

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INTRODUCTION

Microspore culture techniques have been used to generate haploid and doubled haploid plants in many species (Wei et al., 1986; Cho and Sapata, 1988; Coumans et al., 1989) including the genus Brassica (Lichter, 1982; Keller et al., 1987). This technique has many advantages over anther culture and protoplast culture. The microspore system utilizes a true haploid system. Compared to the protoplast system, it differs in the origin of the tissue and genetic variability. Somaclonal variation associated with protoplast culture is eliminated or at least greatly reduced with the use of microspore culture thereby resulting in more efficient mutation and selection methods. In Brassica napus, microspore culture is ten times more efficient than anther culture for embryo production (Siebel and Pauls, 1989). This single-cell method also eliminates any anther-microspore interaction. Microspore techniques are well established for Brassica napus and have been used for breeding purposes, comparatively limited research has been conducted with the other Brassica species. B. campestris is grown in Canada as a major oilseed crop, however it is recalcitrant in cell and tissue culture. This species is to a large extent self-incompatible and therefore selfed lines are very difficult and expensive to obtain. A microspore culture system would be of benefit in producing homozygous doubled haploids. Here we report preliminary results in our efforts to develop microspore culture methodology for B. campestris.

MATERIALS AND METHODS

Plant Growth Conditions

Four B. campestris genotypes (identified as numbers 2,3,5,6) were evaluated; the seed of these breeding lines was provided out of the Agriculture Canada breeding program. Plants were either grown under greenhouse conditions (16 hour photoperiod with a light intensity of 300 micromoles/m²/sec and a day/night temperature of 25°C/15°C) or in a growth cabinet (16 hour photoperiod with a light intensity of 1500 micromoles/m²/sec and a day/night temperature of 20°C/15°C. Prior to bolting, the temperature in the growth cabinet was lowered to 10°C/5°C, the greenhouse temperatures were unchanged. Plants were fertilized with 14-14-14 Nutricote 100 (slow release fertilizer) and watered biweekly with 0.35 g/l of 15-15-18.

Microspore Culture

Unless specifically indicated, the B. napus protocol was used. Buds were selected on the basis of size. These were placed in lipshaw baskets for sterilization. Buds were surface sterilized in 6% sodium hypochlorite for 15 minutes on a shaker then given three five-minute washes with sterile water. Buds were macerated in 5 ml of B5-13 media (B5 media

supplemented with 13% sucrose) (Gamborg et al., 1968) with a glass rod then filtered through 44 um nylon screencloth into a 50 ml tube. The filter and beaker were rinsed three times with 5 ml of B5-13. The tube containing the microspores and media was centrifuged at 1000-1200 rpm for three minutes, the supernatant was decanted and 5 ml B5-13 was added to the pellet. This procedure was repeated twice (total three washes) then the required amount of NLN-13 pH 6 media (NLN media supplemented with 13% sucrose) (Lichter, 1982 without potato extract and hormones and with the addition of 0.83 mg/L potassium iodide) was added to achieve a density of 10^5 microspores/ml. Ten milliliters of microspore suspension was dispensed into 100 x 15 ml sterile petri plates. Plates were incubated in the dark at 32°C for 24 hours then incubated at 24°C for the remainder of three weeks. The embryos were then counted and put on a shaker (70 rpm) under continuous light at 22°C until the embryos were green. At this stage, the embryos were transferred to solid B5 media free of growth regulators and maintained at 22°C, 14 hour photoperiod with a light intensity of 300 micromoles/m^2/sec. The embryos were subcultured every 3-4 weeks if necessary. The resulting normal plantlets were transferred to a soil-less mix and grown in the greenhouse or growth cabinet. Slow release fertilizer was applied at time of transfer. Plantlets were initially covered with plastic cups to maintain a high humidity. This covering was slowly removed as the plants hardened.

Experiment 1: Genotype. Four genotypes were evaluated using the B. napus protocol.

Experiment 2: Bud size. Buds were selected and catergorized according to length, less than 2.0 mm, 2.0-2.9 mm, 3.0-3.9 mm, and 4 and greater than 4 mm. The B. napus protocol was used.

Experiment 3: Media components. NLN media was used with varying levels of sucrose and pH. The standard media was NLN-13 pH 6 which was compared to NLN-10 pH 6, 6.2, 6.8, and 7.2.

Experiment 4: Media change. Initially microspores were cultured in NLN-17 pH 6.2 media. After 48 hours, the media was changed to NLN-10 pH 6.2 for half of the plates. The other half of the plates were kept at NLN-17 pH 6.2. The media and microspores were pipetted into a 50 ml tube and centrifuged at 1000-1200 rpm. The supernatent was decanted and media with a lower sucrose level (NLN-10 pH 6.2) was added.

Experiment 5: Incubation temperatures. Initial incubation temperatures were evaluated. These were 35°C for 24 hours, 35°C for 24 hours followed by 32°C for 24 hours, 32°C for 24 hours, and 32°C for 48 hours. The experiment used NLN-10 pH 6.2 media.

RESULTS AND DISCUSSION

Genotypic differences for embryogenic capability in B. campestris were evident. Genotype 3 was the most embryogenic line of the four lines tested (Table 1).

Table 1. The influence of genotype on B. campestris microspore culture.

Genotype	Total embryos	Embryos/plate	Embryos/100 buds
2	232	1.3	16.0
3	875	2.2	22.4
5	273	0.8	6.5
6	71	0.4	3.9

Preliminary experiments determined the optimum bud size for the production of embryogenic microspores. A range of bud sizes were

examined, less than 2 mm, 2.0-2.9 mm, 3.0-3.9 mm, and 4 mm and greater. The range of 2.0-2.9 mm produced embryos whereas the other bud sizes did not. This is in contrast to B. napus where a larger bud size yields embryogenic microspores.

The environment in which the donor plants are grown is extremely important. An experiment compared growth cabinet conditions versus greenhouse conditions. Embryos were produced from the plants grown in the growth cabinet whereas no embryos were produced from plants grown in the greenhouse. The lower temperature (10°C/5°C) causes slower plant growth and allows for a longer period for sampling buds. Better embryo development also results from the lower growing temperature.

The sucrose level and pH of the media is a key factor in influencing microspore embryogenesis. The standard media used for B. napus is NLN-13 pH 6. This media yielded embryos when used for B. campestris microspore cultures, however several sucrose levels and pH levels were evaluated. Ten percent sucrose was used as previous work has indicated that the optimal sucrose concentration for B. campestris anther culture is 10% (Keller et al., 1975). Microspore research on B. campestris spp pekinensis has also used 10% sucrose (Sato et al., 1989). The media NLN-10 pH 6.2 was the best media yielding 9.8 embryos/100 buds (Table 2). The B. napus standard media yielded 6.1 embryos/100 buds.

Table 2. The effect of sucrose and pH on B. campestris microspore capability*.

Media	Total embryos	Embryos/plate	Embryos/100 buds
NLN-13 pH 6	105	0.4	6.1
NLN-10 pH 6	2	0	0.3
NLN-10 pH 6.2	501	1.2	9.8
NLN-10 pH 6.8	29	0.3	2.9
NLN-10 pH 7.2	1	0	0.1

* Genotypes were pooled.

It has been demonstrated that a high level of sucrose is beneficial for initial microspore survival and division but a lower level is important for continuation of microspore division (Dunwell and Thurling, 1985). Experiment three included the culture of microspores at NLN-17 pH 6.2 continuously and NLN-17 pH 6.2 for 48 hours followed by a media change to NLN-10 pH 6.2. The results indicate that the media change did improve the microspore capability to produce embryos (Table 3). This was evident for all genotypes evaluated. For genotype 2, there was 1.4 times as many embryos/100 buds produced after media change when compared to continuous NLN-17. For genotypes 3 and 5 this figure was 6.9 and 3.5, respectively. For genotype 6, no embryos were produced from media with the continuous high sucrose level. With a media change, embryo production increased to 7.3 embryos/100 buds.

Table 3. The effect of media change B. campestris microspore capability.

Genotype	Media	Total embryos	Embryos/plate	Embryos/100 buds
2	NLN-17	64	5.8	61.5
2	NLN-17--> NLN-10	122	8.7	85.3
3	NLN-17	72	0.7	7.3
3	NLN-17--> NLN-10	467	4.5	50.3
5	NLN-17	3	0.1	1.6
5	NLN-17--> NLN-10	10	0.5	5.6
6	NLN-17	0	0	0
6	NLN-17--> NLN-10	4	0.7	7.3
All	NLN-17	139	1.0	9.5
All	NLN-17--> NLN-10	603	4.1	46.3

The incubation temperature and time period is an important factor in microspore culture. The microspores are incubated initially at a high temperature then maintained at 24°C. For B. napus, the initial incubation period is 32°C for 72 hours. Various times and temperatures were evaluated. From the results, the higher temperature (35°C) is of no benefit. Microspores incubated at a temperature of 32°C for 48 hours yielded the most embryos (Table 4). This was evident for genotype 3. Results were similar for 24 hours and 48 hours incubation for genotype 5. There was no response for genotype 6 at three of the temperature regimes (35°C, 35°C-32°C, and 32°C), 32°C for 48 hours was not evaluated. For genotype 2, 32°C for 24 hours was the best treatment, however 32°C for 48 hours was not evaluated.

Table 4. The effect of incubation temperature on B. campestris microspore capability*.

Incub.°C(hr)	Total embryos	Embryos/plate	Embryos/100 buds
35(24)	9	0.1	0.9
35(24),32(24)	117	1.0	12.3
32(24)	383	3.6	45.3
32(48)	169	7.0	114.2

* Genotypes were pooled.

Normal plantlets were regenerated from microspore-derived embryos, however less that 20% of the embryos produced developed directly into plantlets. The remaining embryos elongated and produced a thickened hypocotyl. These were subcultured several times which resulted in some shoot formation. Over 250 plants have been regenerated either directly from embryos or through repeated subcultures. The majority of the plants were sterile and were considered to be haploid, however approximately 20% of the plants produced pollen and were considered to be spontaneous diploids. Bud pollination resulted in some selfed seed from these plants.

It has been noted that there is a genotypic effect on microspore capability to produce embryos however, there also appears to be a plant effect. In the above mentioned experiments a number of plants were used. Several produced many embryos whereas others did not. These embryogenic plants were maintained and using those plants, the efficiency of microspore embryogenesis would increase.

An effective microspore culture technique could be most invaluable for development of homozygous diploid self-incompatible B. campestris. Such a protocol could also be used in genetic studies, and as it is a

single cell system could be used for direct DNA uptake and recipient of foreign genes through Agrobacterium-mediated gene transfer or microinjection. It also represents a monoploid cell culture system that could be useful in mutant selection.

CONCLUSIONS

The development of a microspore culture protocol for B. campestris has been developed. This protocol is similar to B. napus with several modifications. The plants are grown in a growth cabinet with a day/night temperature of 10°C/5°C. The buds selected for microspore culture are between 2-2.9 mm in length. The media used for culture is NLN-17 pH 6.2 with a media change to NLN-10 pH 6.2 48 hours after culture. These plates are incubated in the dark at 32°C for 48 hours then maintained at 24°C for 19 days. The regeneration media was B5 without hormones. Plants have been regenerated from microspore-derived embryos using this protocol. The majority of plants were not normal and require several subcultures before normal shoot and roots develop.

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