

In vitro pollen culture and the regeneration of *Brassica campestris* L. plants

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Brassica campestris (*Brassica rapa* L. ssp. *oleifera*) is an important oilseed crop, particularly in Finland. Pollen culture techniques for haploid production have been developed, but *B. campestris* is relatively recalcitrant in pollen culture. Twenty eight genotypes of *B. campestris* were included in this study. The donor plants were grown in the greenhouse and transferred to the growth cabinet before bolting. Buds (2–4 mm long) were selected, macerated in B5 medium, then NLN liquid culture medium was added. The microspores were incubated in the dark at 32°C for 72 h, then at 25°C for a further three weeks. Nineteen genotypes produced microspore-derived embryos. The highest yield was more than 300 embryos per 100 buds. Activated charcoal (150 mg/L) promoted embryogenesis, pollen development was faster and the embryo yield was higher. Plants were regenerated after transferring embryos to a solid B5 medium. Colchicine solution was used to double the chromosome complements. About 100 regenerate plants have been obtained in our laboratory, and these haploids will be useful for the oilcrop breeding.

Key words: oilcrop, genotype, bud size, activated charcoal, donor plant condition, embryogenesis, plant regeneration

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Introduction

Brassica campestris (*Brassica rapa* L. ssp. *oleifera*) is grown in many countries, including Finland, as an oilseed crop. It is mostly self-incompatible and therefore selfed lines are expensive and very difficult to obtain. A haploid breeding technique for the *Brassica* species has been developed during the past two decades. For *B. napus*, pollen culture has become a tool for crop

breeding because the yield of microspore-derived embryos is very high (about 900,000 per 100 buds), (Swanson et al. 1987; Kott et al. 1988; Siebel and Pauls 1989). Haploids are of considerable use in plant breeding and genetic studies. Haploid production is extremely attractive because it not only provides an opportunity to select at the haploid level *in vitro* for desirable agronomic and seed quality characteristics, but it also provides a means of producing genetically stable homozygous lines, fixed by chromo-

some doubling (Kott and Beversdorf 1990). In cross-pollinating species, such as *B. campestris*, doubled haploids are more likely to be used as parents in the production of single- or double-cross hybrids (Dunwell 1985).

A microspore culture technique has been developed for *B. campestris* (Baillie et al. 1992, Burnett et al. 1992) which has a number of specific advantages over anther culture (Pierik 1987). In particular, it is more efficient for haploid production than anther culture (Siebel and Pauls 1989). Androgenesis has become the most important source of haploids produced *in vitro* by far. This is extremely important; as pollen grains are available in considerable quantities and each has the potential to develop into a haploid plant (Pierik 1987). *B. campestris* is relatively recalcitrant in pollen culture, however. The yield of embryos is relatively low and embryos can only be obtained from a few genotypes. Here we report our results on the pollen culture of *B. campestris*, with the aim of establishing a pollen culture system for breeding.

Material and methods

Donor plant and growth conditions

Twenty eight genotypes of *B. campestris* were tested: fourteen cultivars and fourteen breeding lines. The donor plants were grown under controlled-environment greenhouse conditions with 16 h photoperiod (approximately 300 $\mu\text{mol}/\text{m}^2\text{s}$) and a day/night temperature of 25/15°C. The donor plants were grown in individual 12 cm pots, watered daily and fertilized once a week. Before bolting, the donor plants were transferred to a growth cabinet with a 16 h/8h, light/dark regime. The temperature was 10°C during the light period, with a light intensity of 300 $\mu\text{mol}/\text{m}^2\text{s}$, and 5°C during the dark period. The donor plants were watered every two days and fertilized once a week.

Pollen culture

Flower buds (50–75) were removed from donor plants older than 6 weeks. Bud length was 2–4 mm, only those in this range could produce embryos. The optimum timing for microspore culture is during mid-late to very late uninucleate stage, DAPI (4', 6'-diamidino-2-phenylindol) was used to check pollen development. The buds were placed in test tubes and surface sterilized in 4.5 % sodium hypochlorite with one drop of Tween-20 for 15 min, followed by three five-minute washes with sterile, distilled water. The buds were then macerated with a glass rod in cold B5 medium (Gamborg et al. 1968) without iron salt, supplemented with 13% (w/v) sucrose (in B5 medium, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was increased to 750 mg/L), filtered through a 48 μm nylon mesh and pelleted by centrifugation at 1000 rpm for 5 min in the same medium 3 times. The microspores were resuspended in the required amount of NLN medium (Lichter 1982) supplemented with 13 % sucrose and 0.83 mg/L potassium iodide, but without potato extract and hormones. The final density of microspores was 5×10^4 per millilitre. Activated charcoal was added to some of the NLN culture medium at 150 mg/L. Ten millilitres of microspore suspension was dispensed into a 100x15 mm sterile Petri dish. The dishes were then wrapped with double layers of parafilm. Every test was repeated at least three times. The microspores were incubated in the dark for an initial period of three days at 32°C, followed by three weeks of dark culture at 25°C.

Plant regeneration

After three weeks of microspore culture, the cotyledonous embryos were counted and placed on a slow shaker (50 rpm) under continuous light at 25°C until they were green. At this time, the embryos were transferred to a solid B5 medium containing 2% sucrose and 0.3% phytigel without growth regulators (Gamborg et al. 1968) and cultured at 25°C, 16 h light period. Plantlets were obtained and then labeled individually. Prior to

transfer of plantlets to soil, the roots of the plantlets were immersed in a 0.2% colchicine solution for 3 h followed by several water washes. The plantlets were then potted and placed in a greenhouse under plastic cups to maintain a high humidity level.

Results and discussion

Influence of genotype

B. campestris genotype is considered to be a key factor in obtaining microspore-derived embryos; only a few genotypes can produce microspore-derived embryos (Baillie et al. 1992, Burnett et al. 1992). Sorvari (1985) indicated that the highest embryo yield was from cross type "dihaploid x dihaploid" and high-glucosinolate genotypes. For *B. napus*, haploid production was also genotype dependent (Chuong et al. 1988). *B. campestris* is mostly self-incompatible and open-pollinated, genotypes are mixed to various degrees in the population. There is plant to plant variation in microspore culture response within genotypes (Ferrie and Keller 1995). In our research, from a total of 28 genotypes tested, 19 produced embryos.

Influence of bud size

Bud size plays an important role during pollen culture; microspores had embryogenic potential only during a short and specific period during pollen development. A range of bud sizes was tested: smaller than 2.0 mm, 2.0-2.9 mm, 3.0-3.9 mm and bigger than 4.0 mm. Only the buds measuring 2.0 mm to 3.9 mm in length were easy to produce embryos. The optimum timing for microspore culture is during the mid-late to very late uninucleate stage. Optimum bud lengths were different for different genotypes; for example, the optimum bud length for Emma (approx. 3.2 mm) was slightly higher than for Candle (approx. 2.8 mm).

Table 1. Response of genotypes in pollen culture. Activated charcoal was contained in the culture medium. Mean is from at least 8 replicates.

Genotype	Embryos/100 buds	SD
Bc3059	326	64
Bc3158	283	77
Kulta	120	34
Candle	78	29
Bc3211	76	21
Bc3063	54	17
Bc3161	49	15
Bc3230	36	9
Emma	32	9
Bc3234	30	11
Kova	27	4
Bc3066	23	9
Bc3139	15	9
Bc3131	13	7
Sisu	13	6
Tobin	11	8
Tyko	8	6
Hian Vankka	4	5
Bc3134	3	2
Bc3129	0	0
Bc3163	0	0
Bc3141	0	0
Turkel	0	0
Hanne	0	0
Nopsa	0	0
Ww1774	0	0
Palle	0	0
Vallti	0	0

SD: standard deviation.

Effect of activated charcoal

In some of our tests, we found that the activated charcoal was beneficial for embryogenesis. A series amount of activated charcoal was added to the liquid NLN media and 150 mg/L of activated charcoal was found the best for the microspore culture (data not presented); pollen development was faster and the yield of embryos was higher in the culture medium with activated charcoal than those in the culture medium without activated charcoal. It has been reported that endogenously produced toxins within cultures

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Table 2. Microspore embryogenesis for various bud lengths of genotype Bc3158. Activated charcoal was contained in the culture medium. Mean is from at least 6 replicates.

Bud length	Embryos/100 buds	SD
< 2.0 mm	0	0
2.0-2.9 mm	203	79
3.0-3.9 mm	77	18
> 4.0 mm	2	2

SD: standard deviation.

play a negative role in the initiation and development of embryos in *B. napus* (Kott et al. 1988). High levels of phenylacetic acid and *p*-OH-benzoic acid have been found in *Daucus* cultures; 2,6-OH-enzoic acid and benzoic acid have been found in *Allium* cultures; benzoic acid, pelargononic acid and caprylic acid have been found in *Haplopappus* cultures (Fridborg et al. 1978). It was also shown that *p*-OH-benzoic acid had inhibitory effects on the embryogenesis in *Daucus* cultures (Fridborg et al. 1978), whereas the media with activated charcoal did not. This evidence suggests that activated charcoal adsorbs such toxins. We noted that, in the absence of activated charcoal, cell division, embryo formation and development were inhibited in the culture. Often there was no growth or development *in vitro*; or initial growth was followed by the abortion of embryos. When activated charcoal was added to the media, such effects were no longer apparent, the reason is perhaps due to interference with polyphenolic compounds produced by the explants and partial adsorption of the exogenous auxin by the activated charcoal (Dumas and Monteuis 1995). In our work, the embryos obtained from microspore culture with activated charcoal and microspore culture without activated charcoal are normal developed, and both of them have the potential to develop to the plantlets. For the microspore culture of *Brassica* species, media change was considered a good method to enhance embryo yield (Baillie et al. 1992, Burnett et al. 1992, Hansen and Svinnet 1993). There is no toxin in the fresh medium, so the pollen can develop well in the new medium.

Donor plant conditions

For pollen cultures of the *Brassica* species, donor plant conditions are also an important factor in embryo production. In our work, the donor plants were tested with different lengths of exposure to low temperatures. The plants were grown in a controlled-environment greenhouse (25/15°C, 16 h lightperiod), before bolting (about 6 weeks), they were then transferred to the growth cabinet (10/5 °C, 16 h lightperiod). We established that embryo yield was directly proportional to the length of the low temperature pre-treatment, not to the age of donor plants. The yield of embryo was similar, not significantly different if buds were moved from 6th week to 9th or 10th week (data not presented). Baillie et al. (1992) recommended a low temperature pre-treatment for donor plants. The temperature in the growth cabinet was lower and more stable than it was in the greenhouse, and this benefited the plants. Low temperature pre-treatment has become usual in the haploid breeding of cereal crops (Nitsch, 1972; Wang et al. 1974).

We also established that embryos could only be produced from older plants. The donor plants were transferred into a growth cabinet (10/5°C, 16 h lightperiod) after six weeks growth in the greenhouse. The too young donor plants failed to produce embryos (data not presented). Burnett et al. (1992) also showed that four to five week old plants did not produce embryos. For *B. napus*, microspores isolated from the buds of older plants had a higher embryo yield than those of the younger ones (Takahata et al. 1991).

Table 3. Effect of activated charcoal (AC). Mean is from at least 8 replicates.

Genotype	Em / 100 buds ±SD with AC	Em/100 buds ±SD without AC
Bc3158	196 ±65	69 ±32 *
Kulta	101 ±16	52 ±12 *

SD: standard deviation.

* Significant at 1% level.

Table 4. Effect of cold treatment. Activated charcoal was contained in the culture medium. Mean is from at least 6 replicates.

Genotype	Cold treatment week(s)	Em/100 buds \pm SD
Bc3059	0 (control)	61 \pm 23 ^c
	1	172 \pm 47 ^{ab}
	2	194 \pm 35 ^{ab}
	3	264 \pm 53 ^a
	#	
Kulta	0	40 \pm 17 ^f
	1	77 \pm 43 ^d
	2	62 \pm 21 ^{de}
	3	63 \pm 29 ^{de}
	4	74 \pm 27 ^d
	#	

Plants ceased to produce buds.

SD: standard deviation.

Means followed by the same letter are not significantly different according to the Duncan multiple range test at the 5% level.

The microspore culture technique for *Brassica* species has existed for some years, and currently the microspore culture technique for *B. napus* is very successful as a breeding tool. The yield of embryos from *B. campestris* is relatively poor, however. To improve the microspore cultures for *B. campestris* breeding, it is important to enhance the yield of embryos. The effect of several factors including optimal media, donor plant conditions and culture temperature will be tested in the future.

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SELOSTUS

Kevättrypsin mikrosporiviljely *in vitro* ja vihreiden kasvien tuottaminen alkiosta

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Maatalouden tutkimuskeskus

Tutkimus sisälsi 28 perimältään erilaista kevättrypsinlinjaa ja -lajiketta. Tutkimusmateriaali kasvatettiin alkuvaiheessa kasvihuoneessa ja siirrettiin ennen kukintaa kasvatuskammioon. Mikrosporiviljelyssä parhaan tuloksen tuottivat mikrosporit eli epäkypsät siipölyhiukkaset, joiden kehitysvaihe oli myöhäinen 1-tumavaihe, mikä edellyttää, että kukkasilmun pituus on 2-4 mm. Oikeassa kehitysvaiheessa olevat kukkasilmut murskattiin B5-nestealustalla ja mikrosporit viljeltiin NLN-nestealustalla, ensin 72 h ajan

+32°C:ssa ja seuraavaksi kolme viikkoa +25°C:ssa. Viljellyistä mikrosoreista alkiota tuotti yhdeksäntoista kevättrypsigenotyyppeä. Parhaalla genotyypillä saatiin 100 mikrosporista 300 alkiota. Aktiivihäili 150 mg/l paransi mikrosporien kehitystä ja lakionmuodostusta sekä lisäsi alkioiden lukumäärää. Alkiot kasvatettiin vihreiksi kasveiksi kiinteällä B5-alustalla. Tutkimuksen ensimmäinen vaihe tuotti noin 100 haploidista kevättrypsikasvia.