ANALYSIS OF FACTORS AFFECTING EMBRYOGENESIS IN MICROSPORE CULTURES OF *BRASSICA CARINATA*

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Abstract

Three factors affecting embryogenic processes in microspore culture of Brassica carinata were tested: developmental stage of isolated microspores (late uninucleate to early binucleate), level of the elevated temperature (30; 32 and 25°C) and effect of density 70 000 to 150 000 microspores per 1 ml of the medium. We have developed an efficient and reliable protocol for Brassica carinata microspore embryogenesis. Our results confirmed remarkable effect of all examined factors on the formation and further development of microspore derived embryos, observed in other genotypes from genus Brassica. Optimal values for above mentioned factors were determined in five various genotypes and could largely help to improve the protocol for efficient and rapid production of completely homozygous plants of Brassica carinata.

Key words: *Brassica carinata*, microspore culture, microspore embryogenesis

INTRODUCTION

Brassica carinata B. is mainly self-pollinating oilseed crop that represents the third most important source of vegetable oil in the world (Kidd, 1993) and the third most important oil crop in the highlands of Ethiopia (CSA, 2003).

Growth in *Brassica carinata* production in particular and agricultural production in general in Ethiopia will have to come from increased application of modern agricultural practice. Technological change generated through research will also be vital in the transformation of the health and industry sectors. Biotechnology offers the country an opportunity to solve many of its pressing problems by such means as developing new cultivars of *Brassicas*, increasing food production that support more rapid economic growth or improving the efficiency of agricultural production.

Over the last decades, researchers have made great efforts in developing biotechnology methods to facilitate the breeding of *Brassicas*. Research studies indicated that, with respect to the *Brassica* oilseeds, and indeed any other crop, addressed that modern biotechnology will have a major impact in two areas. Firstly, it provides a new range of techniques enabling the efficient selection of favourable variants in plant breeding programmes. Secondly, it provides the opportunity to improve germplasm by increasing its diversity beyond conventional genetic limitations. Due to the relative ease of genetic transformation, *Brassica* oilseed crops have been amongst the first to be subject to the full range of modern biotechnology methods.

Biotechnology approach towards the production of doubled haploid cultivars through the application of *in vitro* cultures of isolated microspores has been increasingly applied or utilized for development of initial breeding materials with agronomic traits contributing both to a high yield and a desired seed composition. Therefore, high frequency production of doubled haploid microspore embryos is essential for effective and routine utilization in breeding programmes.

The object of this study was to find out proper bud size, level of the elevated temperature and to verify the effect of microspore culture density as important factors affecting embryogenic processes in microspore culture of *Brassica carinata* and thus to optimize the microspore culture protocol in selected genotypes.

MATERIALS AND METHODS

Microspore culture

Plant materials

Five *Brassica carinata* genotypes provided by the Czech University of Life Sciences, Institute of Tropics and Subtropics developed by Doc. Ing. Miroslav Bechyne were utilized. High productive but high erucic *Brassica carinata* genotype Dodolla was obtained from Ethiopia´s Gene bank and attempt for microspore culture was done on it. Donor plants of all *Brassica carinata* genotypes were grown in the growth chamber under controlled environmental conditions with 16/8 hrs photoperiod and at day/night temperatures 15/10°C. Mineral fertilizer “NPK” was applied weekly to all donor plants.

Microspore isolation

Microspore isolation was carried out according to Coventy et al. (1988) with some modifications. Young flower buds with microspores in proper developmental stages were collected from main and lateral branches of the plant. Bud size was optimized for different genotypes of *Brassica carinata* . Buds were surface sterilized with 70% ethanol for one minute and with a 10% solution of

sodium hypochlorite for ten minutes and rinsed three times (with the interval of 5 minutes) with sterile distilled water. Buds were gently macerated with a glass rod in about 2 ml NLN liquid medium (Lichter, 1985) supplemented with 13% (w/v) sucrose. Microspore suspension was filtered through two pieces of sterile nylon mesh (100 μm and 40 μm) gently transferred to a centrifugation tube and the volume adjusted with fresh NLN medium to 10 ml. It was then centrifuged at 100 g for 10 minutes followed by supernatant removal addition of fresh NLN media and centrifugation for 5 minutes. After the second centrifugation the supernatant was removed and microspores were resuspended in fresh NLN medium (cultivation microspores without anti-mitotic agent was directly cultivated on the Petri-dish then taken to the thermostat). Several densities between 60,000 and 164,000 microspores per Petri dish were used. Each microspore suspension was then dropped to 9 cm plastic Petri dish and cultivated for two-weeks in the thermostat at 30°C in the dark or three days in 32°C then after two- three weeks in 25°C in dark. After three weeks of cultivation embryos were counted at torpedo and stage heart (at least 2 mm in length) and the Petri dish were placed on the shaker (70 rpm) under light at 22°C until the embryos turned green.

Embryo induction, development and plant regeneration

Well developed green cotyledonary embryos were transferred to differentiation medium (DM) solidified with 0.8% of agar, with 0.2 mg/l BAP, 0.2 mg/l IAA, growth regulators, and 2% sucrose and maintained at 22°C for 16/8 photoperiod with light intensity of 300 μmol/m²s. After three weeks of cultivation the one third of embryo cotyledons were cut and embryos were transferred to regenerative medium (RM) solidified with 1% of agar.

First method tested in our study was spontaneous chromosome doubling. Well established plantlets were transferred to a rooting MS medium without growth regulators. Plantlets with roots were then transferred to the soil.

The second method for chromosome doubling was in vivo application of 0.05% colchicine and 2% DMSO solution to plantlets with 4-6 leaves and well formed roots just before transplanting to greenhouse conditions. The plantlets were washed under running tape water. The elongated roots were trimmed and the plantlets soaked in a colchicine solution. The beaker containing immersed plantlets was covered with poly-sheet and kept at 23°C in the light for 24 hours. The treated plantlets were then washed with distilled water and then transferred to pots filled with good soil. To protect from direct light and keep in moisture plants were covered with polyethylene foil for 7 days.

RESULTS

Bud length

Torpedo and cotyledonary embryos were derived in microspore cultures established from all bud sizes and genotypes used (Table 1). According to the statistical calculations based on the number of regenerated green embryos per Petri dish, optimal bud size ranged from 2.5 mm to 3.5 mm (Table 1, Figure 1). Significant differences in embryo production between particular lengths of buds were detected in all genotypes tested (Table 1, Figure 2). Statistical analysis of data from optimal variant showed significant differences between some genotypes (Table 1). Dodolla and BC-4 were (in the case of optimal bud size) the most productive genotypes in this experiment with the mean yield of embryos per Petri Dish 56.30 and 56.0, respectively. Genotype BC-1, in the contrary, produced only 24.0 embryos per Petri Dish, while other two genotypes – BC-6 and BC-2 – showed moderate results with the yield 46.3 and 40.7 embryos per Petri Dish (Figure 3). While the range 2.5–3.5 mm was sufficient for all inspected genotypes of *Brassica carinata*, other bud sizes (less than 2.5 mm and more than 3.5 mm) caused poor embryo production and moreover, abnormal embryo development in case of microspores isolated from older buds. Thus, in some replications no green cotyledonary embryos were obtained from buds longer than 4 mm (Table 1).

Temperature treatment

No notable differences in embryo production were detected between continuous and variable temperature treatment (Table 2) after colchicine application. On the contrary, the number of embryos per Petri dish from trifluralin treatment was significantly higher in cultures from variable temperature than from continuous treatment. In case of oryzalin treatment, 95 embryos per Petri dish were derived from variable temperature, while only 72 embryos from continuous temperature treatment.

Positive effect of variable temperature was observed in all genotypes tested (Table 3). Dodolla, the most embryogenic genotype in this experiment, produced 81 cotyledonary embryos per Petri dish from variable temperature, while only 51 from continuous temperature treatment. Distinct increase in the number of derived embryos was observed also in other genotypes tested when variable temperature was applied instead of continuous treatment. For instance, the least responsive genotype BC-1 produced only 15 embryos per Petri dish under continuous temperature treatment; use of variable temperature increased the number up to 30 embryos per Petri dish. Results obtained in other genotypes are shown in Table 3.

Culture density
Results obtained from cultures with different density are shown in Table 4. The best embryo yield was achieved in cultures with the density from 97 to 124 × 10 3 microspores per 1 ml of cultivation medium. Higher density (more than 164 or 172 × 10^3 microspores per 1 ml of cultivation medium, respectively) resulted in distinct decrease in the number of derived embryos per Petri Dish. Similarly lower density than optimal value negatively affects embryo production.

Factors affecting embryogenesis in *Brassica carinata* microspore culture

1. Effect of bud size

Table 1: Effect of different bud size on the production of green cotyledonary embryos per Petri dish

Genotype	Replication No.		2–2.5 mm A	2.5–3.5 mm B	3.5–4 mm C	> 4 mm D
	1		15	51	21	0
	2		10	73	16	3
	3		13	45	9	0
Dodolla		**Mean**	**12.67 b**	**A 56.30 a**	**15.33 b**	**1.00 b**
	1		16	55	10	0
	2		8	43	12	0
	3		9	41	15	0
BC-6		**Mean**	**11.00 b**	**A 46.33 a**	**12.33 b**	**0.00 c**
	1		12	67	13	5
	2		7	52	10	3
	3		11	49	9	1
BC-4		**Mean**	**10.00 b**	**A 56.00 a**	**10.66 b**	**3.00 b**
	1		13	35	7	0
	2		9	47	13	2
	3		6	40	13	0
BC-2		**Mean**	**9.33 b**	**AB 40.66 a**	**11.00 b**	**0.67 c**
	1		5	15	2	1
	2		2	30	4	1
	3		7	27	5	2
BC-1		**Mean**	**4.67 b**	**B 24.00 a**	**3.67 b**	**1.33 b**
		Total mean	**9.53**	**44.66**	**16.13**	**1.20**

Letters a, b, c in rows and A, B in the column designate homogeneous groups (LSD; P = 0.05)

2. Effects of temperature

Table 2: Influence of various antimitotic agents on microspore culture of selected genotypes under variable temperature (microspore culture density 100 × 103/ml)

Chemical	No. of embryos per Petri dish	
	temperature treatment	
	variable (32 and 25°C)	continuous (30 °C)
Colchicine	149	149
Trifluralin	83	83
Oryzalin	95	95

Table 3: Embryo yield of selected genotypes under variable temperature

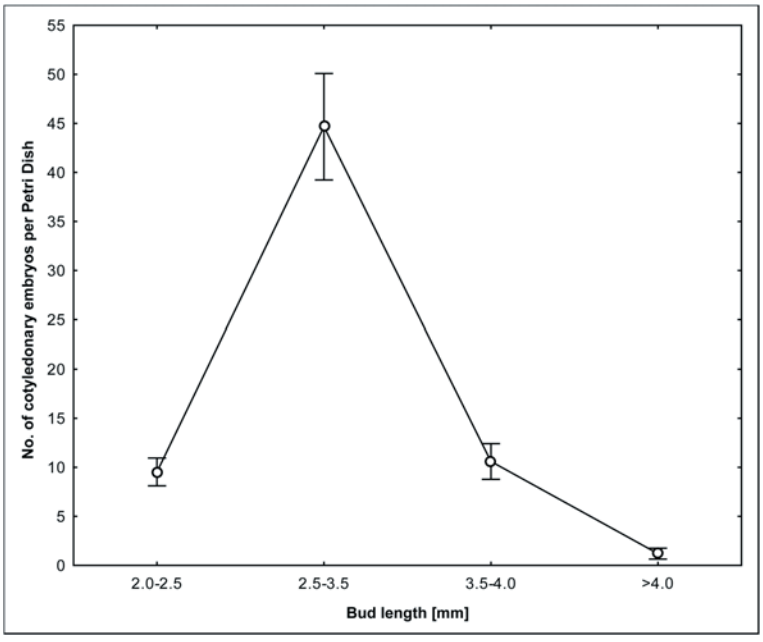
Genotype	No. of embryos per Petri dish	
	temperature treatment	
	variable (32 and 25°C)	continuous (30°C)
Dodolla	81	51
BC-6	75	55
BC-4	79	67
BC-2	53	35
BC-1	31	15

3. Effects of density

Table 4: Embryo yield under different microspore culture density in selected genotypes (haemocytometer counting)

Genotype	Density [× 103/ml]	Average embryo yield
Dodolla	124	++++
BC-1	100	+++
	164	+
BC-2	60	+++
	172	+
BC-4	97	++++
BC-6	100	++++

Figure 1: Effect of different bud size on the production of cotyledonary embryos per Petri dish; pooled data for three genotypes and three successive replications

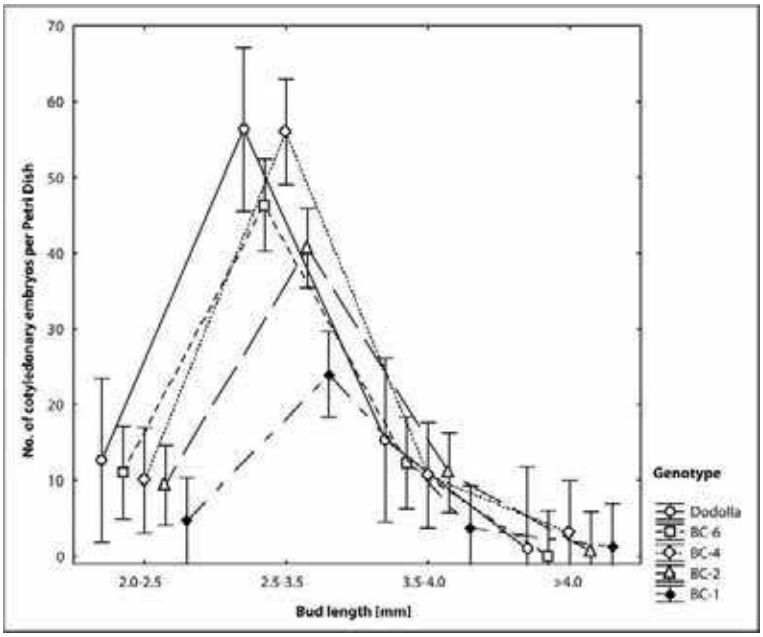


Bars represent individual 95% confidence intervals

DISCUSSION

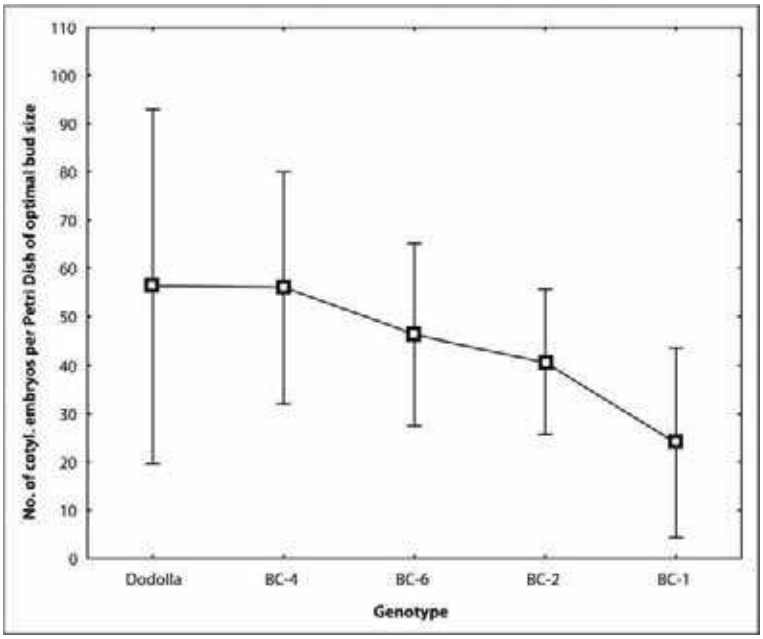
Although viable and green cotyledonary embryos were observed in microspore cultures from all individual bud lengths, sufficient number of embryos was obtained only in cultures established from buds 2.5–3.5 mm long. Moreover, the abnormal morphological characteristics of derived embryos from other than optimal bud sizes often complicated further regeneration into whole plants. These results are in accordance with previously published experiments on *B. carinata* and other genotypes of the genus *Brassica*, where the size of buds has also been considered as an important factor affecting microspore embryogenesis, (Telmer et al., 1992, Vincente and Dias, 1996). This could be explained by tight correlation between bud size (i.e. bud length) and developmental stage of immature pollen grains, where only microspores at middle or late uninucleate stage can undergo embryogenesis (Pechan and Keller 1988, Hansen and Svinnset 1993). Deformed embryos often derived from non-optimal bud sizes (especially with the large percentage of binucleate microspores) might be a result of the presence of various substances in culture medium, indigenous from shattered older microspores and mature pollen grains (Kott et al., 1988b).) Barro and Martin (1998), and Thruling and Chay (1984) illustrated significant effect of a genotype on optimal bud size in *Brassica carinata* and *Brassica napus*. For instance, Barro and Martin (1998) in B. carinata found in ten lines out of 16 tested genotypes good responsibility to the optimal bud size between 2.5 and 3.5 mm, whereas Chuong and Beversdorf (1985) recommended for one breeding material (R-2128) of *B. carinata* bud size 2.5 to 3 mm. Thurling and Chay (1984) observed the corresponding bud size from 2 to 3 mm for tested genotypes of *Brassica napus* ssp. *oleifera* but they also reported

Figure 2: Effect of different bud size on the production of cotyledonary embryos per Petri dish in selected genotypes; pooled data for three successive replications



Bars represent individual 95% confidence intervals

Figure 3: The number of cotyledonary embryos obtained from optimal bud size (2.5–3.5 mm) culture; pooled data for three successive replications



Bars represent individual 95% confidence intervals

significant difference in proper bud size between genotypes. Lichter (1989) observed that the bud size with microspores in optimal stage for *Brassica napus* genotypes was between 3 to 4 mm. On the contrary, no genotypic effect affecting bud size was detected in our experiments; the size 2.5–3.5 mm was optimal for all tested genotypes. Identical bud size suitable for different genotypes was presented also by George and Rao (1982) 2mm for *Brassica juncea* and by Baillie et al., (1992) for *Brassica rapa.*

The importance of heat shock pre-treatment for the initialization of embryogenesis was described in various experiments concerning microspore cultures of Brassicas (Binarova et al., 1997, Baillie et al., 1992, Ferrie et al., 1995). Comparison of two types of temperature treatment in our experiments (i.e. variable and continuous pre-treatment) showed higher efficiency of variable temperature pre-treatment in all variants with antimitotic agents. Even in less responsible variants with trifluralin variable temperature increased the number of generated embryos more than three times. Positive effect of higher initial temperature followed decreased cultivation temperature of 25ºC as compared with continuous moderate temperature of 30ºC was presented by Keller and Armstrong (1979) in *Brassica rapa* and by Chuong and Beversdorf (1985) on *B. napus* and on one genotype of *B. carinata*. Effects of heat shock exposure to microspore culture were sufficient in all tested genotypes of *Brassica carinata* when compared to continuous temperature treatment, preferred in *Brassica napus* (Chuong and Beversdorf, 1985).

Culture density has significant effect on embryogenesis, embryo morphology and thus the capability of direct regeneration into whole plants (Huang et al., 1990; Kott et al., 1988). In our experiments, the best embryo yield and good embryo morphology was achieved with the density from 97 to 124 × 10^3 microspores per 1 ml of cultivation medium. Barro and Martin (1999) reported optimal density in *Brassica carinata* 100 000–150 000 microspores/ml and Lichter (1982) and Zhou et al. (2002b) reported the optimal density for *Brassica napus* 2 × 10^4 per ml. Higher and lower density caused remarkable decrease in number of derived embryos per Petri Dish and in the case of high density often protracted development and incomplete embryos. This issue could be the cause of nutrient competition among developing embryos and/or some toxic substances when older microspores are presented in the cultivation medium.

CONCLUSION

The influence of different bud size, temperature treatment and microspore culture density on embryo production was evaluated in selected genotypes of *Brassica carinata*. Our results confirmed remarkable effect of all examined factors on the formation and further development of microspore derived embryos,

observed in other genotypes from genus *Brassica*. Optimal values for above mentioned factors were determined in five various genotypes and could largely help to improve the protocol for efficient and rapid production of completely homozygous plants of *Brassica carinata*.

Acknowledgement

This work was supported by the Ministry of Agriculture of the Czech Republic, Project No. 0002700602 and we extend our deep appreciation to Dr. V. Kucera, J. Sramek, P. Bartosova and V. Streskova from CRI for their interest and help in this work.

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Received for publication on April 3, 2008
Accepted for publication on April 28, 2008

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