GENERATION OF DOUBLED HAPLOIDS THROUGH MICROSPORE CULTURE FROM VEGETABLE AND OILSEED *BRASSICA RAPA* CROPS

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Abstract. Microspore culture is a very important and useful tool in plant breeding for doubled haploid production and has been developed for many years. *Brassica rapa* L. is an important species with both vegetable and oilseed crops, but it is relatively recalcitrant in tissue culture including microspore culture. The used microspore culture protocol was based on the extensive manual for *B. napus* microspore culture Custers (2003) and Coventry et al. (1988). Nineteen genotypes of *B. rapa* were included in this study; eleven produced embryos. The highest yield was around 12000 embryos per 100 buds from Chinese cabbage genotype Nejnost, a Russian commercial F, hybrid, which is among the highest yields reported in microspore culture in *B. rapa.* The buds measuring 2.5 mm to 2.8 mm in length responded best to produce embryos, which corresponded to the mid-late to very-late uninucleate stage of pollen development. Activated charcoal (0.02%) added to the liquid NLN-13 medium promoted embryogenesis significantly; embryo development was faster and the embryo yield was significantly higher compared to cultures without activated charcoal. The donor plant condition was considered an important factor influencing embryogenesis; older donor plants (older than five weeks) and a cold treatment during bolting are recommended.

Key words: Brassica rapa, Microspore Culture, Doubled Haploid.

Introduction

B. rapa Diversity Fixed Foundation Set representing a structured sampling of the genetic diversity across the global *B. rapa* genepool is being developed by fixing of accessions from core collections through doubled haploid (DH) production or single seed descent (Zhao et al., 2010). Similarly, for *B. napus* and *B. oleracea* Diversity Fixed Foundation Sets are being developed that represent the genetic diversity present within these crop species (Pink et al., 2008).

Biotechnological DH line production offers various advantages for plant breeders, including the possibility to obtain homozygous lines rapidly, as well as easy selection in segregating populations due to the absence of heterozygosity. Furthermore, die use of DH progeny as mapping population(s) for the development of molecular marker maps is very advantageous since the population is immortal and can be used for repeated phenotyping

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for many traits. It enhances the efficiency of detecting markers, particularly linked to quantitative traits. Furthermore, microspore culture can be easily combined with other biotechnologies and related methods, such as gene transfer or mutagenesis, in order to create novel genetic variation in the starting material.

Materials and methods

Donor plant and growth conditions. Nineteen genotypes representing different subspecies of *B. rapa* were tested (table 1), including some commercial cultivars and some breeding lines. The donor plants were grown in individual 18-cm pots, watered daily and fertilized once a week with NPK liquid fertilizer under controlled environment greenhouse conditions with a 16-h photoperiod and a day/night temperature of 20°C. Thirty days old plants from late flowering accessions were vernalised in a cold room at 4°C for two to eight weeks according to the accession requirements. Plants that initiated bolting and started generative development were transferred to a growth chamber at 10°C under a 16h/8h, light/dark regime. The donor plants were watered as required and fertilized once a week with NPK liquid fertilizer.

Isolation and culture of microspores. Microspore culture was always performed from single plants. Flower buds were harvested from young inflorescences with one or two just opened flowers from plants older then 6 weeks. Per isolation all flower buds of a single plant were used. Buds with length 2.5-2.8 mm were selected. The buds were placed in tea-baskets and surface sterilized in 2% sodium hypochlorite with one drop of Tween-20 for 10 min followed by three 1, 4 and 10 min washes with sterile, distilled water. The buds were then squeezed with a plunger by a turning pressure movement to release free microspores in cold B5 medium (Gamborg et al., 1968), supplemented with 13% sucrose. The solution with squashed buds was filtered through two layers of a 45 цт nylon filter. This microspore suspension was centrifuged 3 times at 800 rpm for 4 min in the same fresh B5 medium. After the last washing step, microspores were resuspended in 1 ml NLN-13 medium (Lichter, 1982) supplemented with 13% sucrose but without potato extract and growth regulators NAA, IAA and BAP since this did not promote embryogenesis (Guo, Pulli, 1996). Microspore density was checked by a Fuchs-Rosenthal counting chamber and resuspended in the required amount of NLN-13 medium for culture at a density of 40,000 microspores/ml. Five milliliters of the microspore suspension was incubated per 60 mm Greiner plastic Petri dish. Sterilized activated charcoal was added to the NLN-13 culture medium at 0.02%.

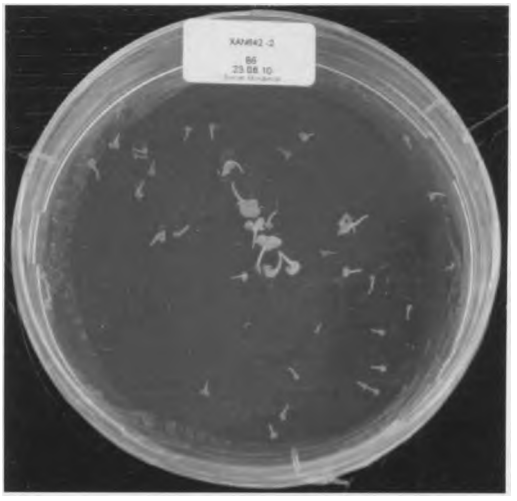


Fig. 1. *B.rapa* ssp. *pekinensis* (acc. Xan 642) embryos in cotyledon stage after 20 days in NLN-13 medium dark culture at 25°C

The microspores were incubated for an initial period at 33°C for 48 hours (heat shock) in the dark followed by three weeks of dark culture at 25°C.

Plant regeneration. After three weeks of microspore culture, the embryos in cotyledon stage were scored (fig. 1). Mature embryos were transferred to solid B5 medium

containing 2.5% sucrose and 1.1% phytoblend agar without growth regulators (Gamborg et al., 1968) and cultured at 25°C in a continuous light (fig. 2).

Obtained plantlets were individually potted and placed in a greenhouse under plastic cups to maintain a high humidity (fig- 3).

Cytological analyses. Development of the microspores was analyzed with the DNA-specific 4 ' ,6-diamidino-2-phenylindole (DAPI) fluorescent stain to visualize the microspore nuclei. Initial samples taken from microspore suspension left over in the centrifugation tubes were spined down for 2-4 min at 4000 rpm, supernatant was removed and the pellet were resuspended in 10 pi of DAPI solution. 10 pi of prepared mixture were mounted on a microscope slide and covered with a cover slip. After about 5-10 min the samples could be used for observation under a Carl Zeiss microscope equipped for light fluorescence microscopy.

The developmental stages represented in the initial microspore populations were scored as early uninucleate (EU), mid uninucleate (MU), late uninucleate (LU), early binucleate (EB), mid binucleate (MB), late binucleate (LB) and trinucleate (T), according to similar stages described by Custers (2003).

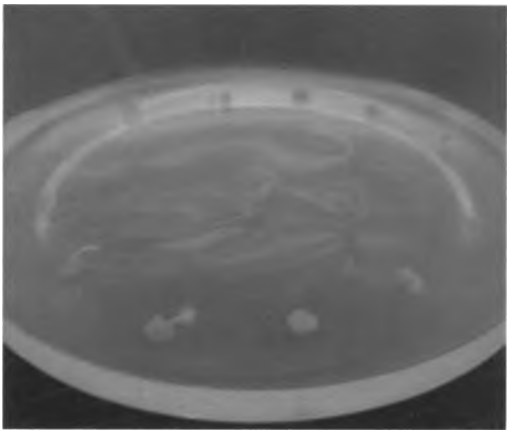


Fig. 2. *B.rapa* ssp. *pekinensis* (acc. Xan 642) embryos after two days of culturing on solid B5 medium at 25°C in a continuous light

Fig. 3. S. rapa ssp. *pekinensis* (acc. Xan 642) plantlet after two weeks of growing on substrate at 20°C in a continuous light

Results and discussion

Influence of genotype. The *B. rapa* genotype is considered to be one of the key factors controlling the embryogenic response of the microspores (Baillie et al., 1992; Burnett et al., 1992) and much research is needed in developing and optimizing tissue culture conditions for a broad range of genotypes. For *B. napus,* doubled haploid production also genotype dependent (Chuong et al., 1988). *B. rapa* is mostly self-incompatible and open-pollinated, and therefore many accessions, especially open pollinated cultivars and landraces, are in fact heterogeneous populations. There is plant to plant variation for microspore culture response between but possibly also within accessions (Ferrie and Keller, 1995). From a total of nineteen tested *B. rapa* accessions including Chinese cabbage, pakchoi, broccoletto, turnip and others, eleven produced embryos (table 1).

Table 1

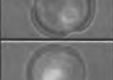
B. rapa **L. accessions tested for microspore culture system**

No.	Number of accession	Genotype name	Collection resource*	Gene bank accession No.	Subtaxa in originating gene bank	Cultivar name	Country of origin
1	N123	BRO-030	WUR	CGN06829	*broccoletto*	Sessantina	Italy
2	N47	PC-214V	VIR	214	*chinensis*	Nicanme Jukijiro Taisai	Japan
3	N46	PC-106V	VIR	106	*chinensis*	Yantsai	China
4	V77	O-135V	VIR	135	*dichotoma*	Ds 17	India
5	V82	O-205V	VIR	205	*dichotoma*	Sarson	Pakistan
6	V75	O-106V	VIR	106	*oleifera*	Lotni mustard	India
7	N155	MIZ-128	WUR	CGN17279	*mizuna*	Round Leaved Mibuna	Japan
8	V49	O-108V	VIR	108	*oleifera*	Arlo	Sweden
9	V55	O-302V	VIR	302	-	NILS	Nepal
10	N31	CC-48V	VIR	48	*pekinensis*	Wong-Bok	Netherlands
11	N72	CC-53V	VIR	53	*pekinensis*	Local	Kazakhstan
12	N39	CC-56V	VIR	56	*pekinensis*	Da-tsin-kou	China
13	Xa-642	—	RSAU	—	*pekinensis*	Xa-642	Russia
14	Mal.Chudo	—	RSAU	—	*pekinensis*	Mal.Chudo	Russia
15	Nejnost	—	RSAU	—	*pekinensis*	Nejnost	Russia
16	N1	NG-372V	VIR	372	*Japanese green*	Bansei Mana	Japan
17	W-vt-008	VT-008	WUR	CGN06711	*vegetable turnip*	Pusa Chandrina	India
18	V96	O-218V	VIR	218	*sylvestris*	Nabo silvestre	Peru
19	V95	O-176V	VIR	176	*sylvestris*	v. sylvestris	Italy

* WUR: accessions from the core collection as described in Zhao et al, 2010; VIR: accessions from the Vavilov Research Institute of Plant Industry, St. Petersbourg, Russia; RSAU: accessions from the Russian State Agrarian University, Moscow, Russia

Table 2

Comparison of different accessions microspore nucleate stage and yield of embryos for flower bud length 2.5 and 2.8 mm

Accession number	Flower bud length, mm	Microspore development stage		Number of well formed embryos per 100 buds
N46	2.5	early-middle uninucleate		9
	2.8	middle-late uninucleate		139
N47	2.5	early-middle uninucleate		200
	2.8	middle-late uninucleate		328
N39	2.5	middle-late uninucleate		250
	2.8	late uni & binucleate		64
V75	2.5	early-middle uninucleate		184
	2.8	middle-late uninucleate		395
Mal. Chudo	2.5	middle-late uninucleate + binucleate		845
Nejnost	2.5	middle-late uninucleate		11859
V77	2.5	late uninucleate		0
	2.8	binucleate		0
V82	2.5	late uninucleate		0
	2.8	binucleate		0
V49	2.5	binucleate		0
	2.8	trinucleate		0
N31	2.5	late uninucleate		0
	2.8	binucleate		0

Influence of bud size on embryo yield in microspore culture. Bud size plays an important role during pollen culture; microspores had embryogenic potential only during a short and specific period of development. A range of bud sizes was tested, smaller than 2.5 mm, 2.5-2.8 mm, 2.9-3.2 mm and larger than 3.3 mm. For most of genotypes only buds between 2.5 mm and 2.8 mm in length responded well to produce embryos. The optimum timing for *B. napus* microspore culture is the late uninucleate stage accompanied with 10-40% early binucleate pollen, slightly younger or older microspores have a drastically reduced embryogenic performance (Custers, 2003). Postculture observations (6-8 h after culture) indicated that the embryogenic spores appeared spherically swollen, distinctly vacuolate, and with a clear cytoplasm (Guo, Pulli, 1996), similar to microspore culture in *B. napus* (Kott et al., 1988). In table 2 the microspore developmental stages are listed for different bud sizes of selected accessions. The embryo yield was highest in all accessions for the middle-late uninucleate stage. However, this developmental stage corresponds to different bud sizes in the accessions tested. For example, the

Table 3

Influence of activated charcoal (AC) on embryo yield. Accession numbers are listed (see table 1) with in parenthesis the bud size

Accession number	Number of embryos per plate			Mean
	1	2	3	
N46 (2.8)	0	0	0	0,0
N46 (2.8)+AC	19	15	5	13,0
N47 (2.5)	2	0	0	0,7
N47 (2.5)+AC	17	15	19	17,0
N39 (2.5)	2	1	1	1,3
N39 (2.5)+AC	49	26	22	32,3
V75 (2.5)	0	0	0	0,0
V75 (2.5)+AC	17	23	20	20,0
V95 (2.5)	20	23	19	20,7
V95 (2.5)+AC	52	57	67	58,7
Nejnost (2.5)	172	108	144	141,3
Nejnost (2.5)+AC	478	324	376	392,7

optimum bud length for accession N39 (Chinese cabbage) was 2.5 mm, which was lower than for accessions N46 (pakchoi), N47 (pakchoi) and V75 (sarson) with optimum bud length 2.8 mm. For accession V49 (oiltype) bud length 2.5 and 2.8 correspond to binucleate and trinucleate stages of microspore development, respectively that is too late for microspore culture. For the non responding accessions smaller bud sizes need to be selected for microspore culture.

Effect of activated charcoal on embryo yield in microspore culture. In some of our tests, we found that the activated charcoal (AC) was beneficial for embryogenesis. Embryo development was faster and the yield of embryos was higher in the culture medium with activated charcoal than in culture medium without activated charcoal (table 3). It has been reported that endogenously produced toxins within cultures play a negative role in initiation and development of embryos in *B. napus* (Kott et al., 1988).

We noted that cell division, embryo formation and development were inhibited in the culture in the absence of activated charcoal; often there was no growth and development *in vitro* (the embryo development often stopped after several divisions or during transition from the globular embryo stage to the heart-shaped embryo stage, after which embryos died). When activated charcoal was added to the media such effects were not apparent, possibly due to interference with polyphenolic compounds produced by the explants and partial adsorption of the auxin (endogenous) by the activated charcoal (Dumas and Monteuus, 1995). For microspore culture of Brassica species, refreshment of culture media was considered a good method to enhance embryo yield (Baillie et al., 1992; Burnett et al., 1992; Hansen and Svinnset, 1993). There is no toxin in the fresh medium, so the microspores can develop well in the new medium. However, changing the medium is time consuming, easily leads to contamination and loss of a percentage of the microspores.

Growth conditions of donor plants. Donor plants have to be grown in an environmentally controlled growth chamber which minimizes stress. Optimal growth conditions will produce healthy plants and enhance embryogenic responses. Factors such as temperature, light, water and nutrients are important in order to obtain healthy plants.

For microspore culture of Brassica species, donor plant conditions were an important factor in embryo production. Baillie et al. (1992) recommended a low temperature pre-treatment for donor plants in the pollen culture of *B. rapa.* In the anther culture of *B. oleracea* var. *capitata,* more embryos were produced after cold pretreatment of flower buds (Osolnik et al., 1993).

We also established that embryos could be produced from plants of at least 6 weeks old more easily. The younger donor plants did not respond well 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 buds of older plants had a higher embryo yield than those of younger ones (Takahata et al., 1991).

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Аннотация. Технология культуры микроспор, разрабатываемая в течение многих лет, в настоящее время является очень важным и полезным инструментом в производстве дигаплоидов и селекции растений. Вид *Brassica rapa* L., включающий ряд важнейших овощных и масличных культур, по-прежнему остается относительно малоизученным в области культуры

ткани и культуры микроспор в частности. Использованная в данном исследовании методика основана на руководстве по культуре микроспор для *B. napus* Custers (2003) и Coventry et al. (1988). Исследовано девятнадцать генотипов *B. rapa,* для одиннадцати из них успешно получены эмбриоиды. Наибольший выход эмбриоидов около 12000 на 100 бутонов был получен на образце капусты пекинской F, Нежность селекции Селекционной станции им. Н.Н.Тимофеева (Москва); данный показатель является одним из наиболее высоких среди опубликованных выходов эмбриоидов в культуре микроспор *B. rapa.* Установленный оптимальный размер бутонов для изолирования микроспор 2,5-2,8 мм соответствовал среднепоздней и очень поздней одноядерной стадии развития пыльцы. Добавляемый в жидкую питательную среду NLN-13 активированный уголь в концентрации 0,02% существенно стимулировал эмбриогенез, при этом отмечено более быстрое развитие эмбриоидов и наблюдался более высокий их выход. Отмечено значительное влияние состояния растения-донора на эмбриогенез: более отзывчивыми являются взрослые растения (старше 5 недель), рекомендовано содержание цветущих растений при пониженной положительной температуре.