Production and utilization of doubled haploids in *Brassica oleracea* vegetables

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ABSTRACT: A possibility to increase the efficiency of plant regeneration from microspore-derived embryos of selected botanical varieties of *Brassica oleracea* was investigated from 2001 to 2004. More than 400 regenerants of R_1 generation were derived in kohlrabi, cabbage and cauliflower by means of different modifications of microspore culture technique. Distinct genotype differences in embryogenic responsibility and regenerative ability of microspore embryos to whole plants were detected. The highest frequency of embryogenesis and subsequent regeneration of plants were achieved in cauliflower cultivar Siria F1, kohlrabi line P7 and some experimental F1 hybrids of cauliflower. The best production of embryos was obtained when donor plants were grown in the growth chamber under controlled light and temperature conditions. The regeneration of plantlets was considerably improved by repeated subculture of cotyledonary embryos on media with various combinations of phytohormones and excision of the cotyledons from mature embryos. The percentage of plant regeneration from subcultured embryos in kohlrabi ranged from 11.11 to 63.64%, in cauliflower from 23.53 to 46.19% and in cabbage from 5.88 to 52.00%. The utilization of regenerants for doubled haploid line production is often complicated by male sterility also in plants with the normal diploid chromosome number.

Keywords: *Brassica oleracea*; microspore culture; plant regeneration; doubled haploids

Development of doubled haploid lines by means of *in vitro* microspore culture has been increasingly used for homozygous line production in breeding programmes of *Brassica* crops due to a possibility of significant time reduction of cultivar development. Whereas this method is routinely used in oilseed rape (*Brassica napus*), it is still difficult to apply microspore culture techniques to practical breeding of some vegetable brassicas. The main difficulties are the very low embryo yield and insufficient regenerative ability of microspore-derived plants in many of *Brassica oleracea* genotypes (CARLOS, DIAS 1999; RUDOLF et al. 1999). Very often abnormal embryos occur which need to be subcultured many times to induce normal shoots (KUGINUKI et al. 1999). Direct and rapid plant regeneration is very important for eliminating cytogenetic abnormalities and improving the efficiency of doubled haploid system.

Our previous studies (VYVADILOVÁ et al. 1998a,b, 2001) aimed to increase the efficiency of the microspore culture technique, especially by investigating factors affecting pollen embryogenesis and testing the embryogenic responsibility in a broad range of genotypes from *Brassica oleracea* collection and some breeding materials. The previously optimized microspore culture procedure was used in this study, which was directed at increasing the efficiency of whole plant production. Obtained doubled haploid lines will be evaluated for agronomic and quality traits with an emphasis on fungal disease and virus resistance and promising lines will be used in breeding programmes.

MATERIAL AND METHODS

Brassica oleracea genotypes including several botanical varieties such as cabbage, cauliflower and kohlrabi used for experiments were selected according to the previous results regarding the embryogenic responsibility. Eight cultivars and landraces of head cabbage (convar. *capitata* L.) var. *alba* DC. and var. *rubra* DC.; four kohlrabi (var. *gongylodes* L.) open-pollinated cultivars and one self-pollinated line, one commercial F1 hybrid and three experimental hybrids of cauliflower (var. *botrytis* L.) were used for experiments. After vernalization for 4 months at 4°C in a cold room plants of cabbage and kohlrabi were grown in the growth chamber under controlled environmental conditions with a 16 h photoperiod and day/night temperature 18/12°C. Cauliflower plants were grown in isolation cages in the open-air conditions.

The microspore culture technique was modified to make it applicable to a wider spectrum of genotypes (VYVADILOVÁ et al. 2001). Green cotyledonary embryos about 5 mm in size were subcultured (for 5 to 7 days) on differentiation medium with benzylaminopurine, indolyl acetic acid and 2% sucrose, solidified by 0.8% agar (Table 1). Afterwards two thirds of both cotyledons were cut off and embryos were transferred onto regeneration medium without phytohormones, with 1% sucrose and 1% agar (Table 2) and kept at 22°C under 16/8 h photoperiod for three weeks. Well-developed shoots were rooted on MS plant growth medium

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Table 1. Composition of differentiation medium

Component		mg/l
Macroelements	$(NH_4)_2 SO_4$	134
	KNO_3	3,000
	$NaH_2PO_4 \cdot 2 H_2O$	150
	$MgSO_4 7 \cdot H_2O$	500
	$CaCl_2 2 \cdot H_2O$	750
	FeNa-EDTA	40
Microelements	H_3BO_3	3
	$MnSO_4 \cdot 4 H_2O$	10
	$ZnSO_4 \cdot 7 H_2O$	2
	$Na_2MoO_4 \cdot 2 H_2O$	0.25
	$CuSO_4 \cdot 5 H_2O$	0.025
	$CoCl_2 \cdot 6 H_2O$	0.025
Vitamins	thiamine HCl	10
	pyridoxine HCl	1
	nicotinic acid	1
	myo-inositol	100
Amino acids	glutamine	800
	serine	100
Phytohormones	6-BAP	0.2
	IAA	0.2
Sucrose		20 g
Agar		8 g
pH		

Table 2. Composition of regeneration medium

Component		mg/l
Macroelements	NH_4NO_3	1,650
	KNO_3	1,900
	KH_2PO_4	170
	$MgSO_4 \cdot 7 H_2O$	370
	$CaCl_2 \cdot 2 H_2O$	440
	FeNa-EDTA	36.7
Microelements	H_3BO_3	6.2
	$MnSO_4 \cdot 4 H_2O$	22.3
	$ZnSO_4 \cdot 7 H_2O$	8.6
	$Na_2MoO_4 \cdot 2 H_2O$	0.25
	$CuSO_4 \cdot 5 H_2O$	0.025
	$CoCl_2 \cdot 6 H_2O$	0.025
	KI	0.83
Vitamins	thiamine	0.1
	pyridoxine	0.5
	nicotinic acid	0.5
	myo-inositol	100
	glycine	2
Sucrose		10 g
Agar		10 g
pH		

Table 3. Composition of plant growth medium

Component		mg/l
Macroelements	NH_4NO_3	825
	KNO_3	950
	KH_2PO_4	85
	$MgSO_4 \cdot 7\ H_2O$	185
	$CaCl_2 \cdot 2\ H_2O$	220
	FeNa-EDTA	18.35
Microelements	H_3BO_3	3.1
	$MnSO_4 \cdot 4\ H_2O$	11.15
	$ZnSO_4 \cdot 7\ H_2O$	4.3
	$Na_2MoO_4 \cdot 2\ H_2O$	0.125
	$CuSO_4 \cdot 5\ H_2O$	0.0125
	$CoCl_2 \cdot 6\ H_2O$	0.0125
	KI	0.415
Vitamins	thiamine	0.1
	pyridoxine	0.5
	nicotinic acid	0.5
	myo-inositol	100
	glycine	2
Phytohormones	NAA	0.03
Sucrose		30 g
Agar		8 g
pH		

(Table 3) with half-strength of mineral salts and NAA phytohormone under cultivation temperature 20/15°C. Strong well-rooted regenerants with about five leaves were transferred directly onto the soil substrate without any treatment for chromosome doubling and they were grown under greenhouse conditions. Obtained R1 and R2 regenerants were evaluated for fertility and quality parameters in greenhouse and field conditions. Karyological analyses of mature plants of kohlrabi line P7 (parental self-incompatible component of hybrid cultivar Sparta F1) and successive evaluation of flower morphology and seed set per pod were performed. The new regenerants of kohlrabi and cabbage derived in 2004 will be evaluated after vernalization.

RESULTS AND DISCUSSION

Microspore regenerants of selected botanical varieties of *Brassica oleracea* were obtained by means of different modifications of microspore culture technique. In total more than 400 microspore regenerants were derived from all used accessions of kohlrabi, cabbage and cau-

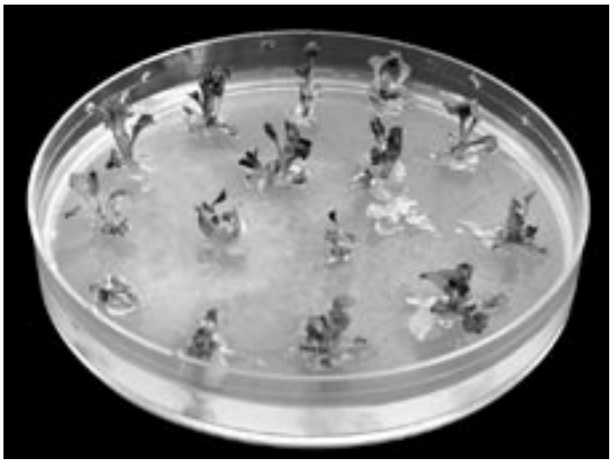

Fig. 1. Embryos of cotyledons on regeneration medium two weeks after dissection (cabbage landrace from Zázrivá)

Fig. 2. Microspore-derived regenerants of various *B. oleracea* genotypes transferred to the soil substrate in the greenhouse

Table 4. Production of embryos and regeneration of whole plants in embryogenic *Brassica oleracea* vegetables

Brassica vegetable	Cultivar	Culture establishment (year)	Number of embryos	Number of whole plants	Regeneration of whole plants (%)
Kohlrabi	Domino	2001	5	3	60.00
		2002	7	3	42.86
	Matouškova modrá	2001	9	1	11.11
		2002	38	10	26.32
		2003	22	14	63.64
	P7	2001	186	98	52.69
	Moravia	2004	9	5	55.56
	Luna	2004	16	6	37.50
Cauliflower	12/B × Br 20/l	2002	132	46	34.85
	12/B × FT 13/1	2002	107	45	42.06
	2/F × Br 20/1	2002	13	7	53.85
	Siria F_1	2002	197	91	46.19
		2003	17	4	23.53
Cabbage	Holt	2002	34	2	5.88
		2003	27	3	11.11
	Křimické	2002	22	4	18.18
		2003	6	1	16.67
	Landrace Zakamenné	2002	17	4	23.53
		2003	61	24	39.34
	Landrace Zázrivá	2002	29	13	44.83
		2003	75	39	52.00
	Landrace Lutiše	2002	23	5	21.74
	Trvanlivé D	2002	7	3	42.86
	Vysocké	2004	27	7	25.93
	Kalibos	2004	39	28	71.79

liflower in 2001–2004. Significant genotype differences in embryogenic responsibility and regenerative ability of microspore embryos were detected. The highest frequency of embryogenesis was achieved in cauliflower cultivar Siria F1, kohlrabi line P7 and two experimental F1 hybrids of cauliflower. High frequency was obtained in one commercial open pollinated cabbage cultivar Holt and two cabbage landraces from Zakamenné and Zázrivá.

Numerous published experimental results indicate that direct haploid plant production from microspore derived embryos could be enhanced after the exposition of embryos to specific types of environmental stresses such as high dosages of exogenously applied abscisic acid (WAKUI et al. 1994), exposure of embryos to short cold period (CEGIELSKA-TARAS et al. 2002) or desiccation of microspore-derived embryos (HANSEN 2000). The excision of cotyledons from mature embryos also showed to increase regeneration (KOTT, BEVERSDORF 1990). The method of cutting cotyledons of approximately 3 weeks old embryos, which has been successfully applied in *Brassica napus*, enhanced the efficiency of regeneration in our experiments with investigated genotypes of *Brassica oleracea*. The rate of regeneration was significantly improved by repeated subculture of embryos at the cotyledonary stage on media with special combinations of phytohormones and excision of both cotyledons from mature embryos after five days of cultivation on a solid medium. The reduction of both cotyledons had a positive effect on stimulation of shoot development from apical meristems of microspore-derived embryos (Fig. 1). The percentage of regenerated whole plants from subcultured embryos in kohlrabi ranged from 11.11 to 63.64%, in cauliflower from 23.53 to 53.85% and in cabbage from 5.88 to 52.00% (Table 4, Fig. 2).

The majority of regenerants in kohlrabi line P7 has a normal ploidy level according to the results of karyological analyses. Out of the total number of 91 regenerants 82 plants were grown up to flowering and self-pollinated in buds. Fifteen plants with diploid chromosome number and the best seed set were selected for further reproduction of the line. These sublines will be used to improve the self-incompatibility degree of the original self-pollinated line P7 and to increase the percentage of hybridity of the cultivar Sparta F1.

The regenerants of kohlrabi from the cultivars Domino and Matouškova modrá showed to be completely

self-sterile even after self-pollination in buds in spite of normal developed anthers. Most of the cabbage and cauliflower regenerants were also completely sterile with abortive anthers or self-sterile despite normal morphology of flowers. This problem was described previously by STIPIC and CAMPION (1997). Despite the high number of diploid regenerants, the frequency of fertile regenerants was very low in their experiments with cauliflower. In addition, KUGINUKI et al. (1999) reported that less than a half of DH lines of cabbage had high seed production.

These results indicate that although the efficiency of embryogenesis and plant regeneration from microspore-derived embryos has been distinctly improved, it is still difficult to apply the microspore culture technique to practical breeding of *Brassica oleracea* vegetables. To overcome this problem it is necessary to produce a large amount of regenerants from various *Brassica oleracea* genotypes by means of both improved microspore culture and whole plant regeneration technique.

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Produkce a využití dihaploidů u zelenin druhu *Brassica oleracea*

ABSTRAKT: Byla zjišťována možnost zvýšení účinnosti produkce celistvých rostlin z mikrosporových embryí vybraných botanických variet *Brassica oleracea* v letech 2001 až 2004. Různými modifikacemi techniky mikrosporových kultur bylo odvozeno více než 400 regenerantů R1 generace u kedlubnu, zelí a květáku. Byly zjištěny výrazné genotypové rozdíly v embryogenní schopnosti a regeneraci celistvých rostlin z mikrosporových embryí. Nejvyšší frekvence embryogeneze a následná regenerace celistvých rostlin byla dosažena u květáku Siria F1, u linie kedlubnu P7 a u několika experimentálních hybridů květáku. Nejvíce embryí bylo získáno při pěstování donorových rostlin v růstové komoře v kontrolovaných světelných a teplotních podmínkách. Regenerace celistvých rostlin se značně zlepšila opakovaným pasážováním plně vyvinutých embryí na média s různými kombinacemi fytohormonů a odřezáváním částí děloh z plně vyvinutých embryí. Procento regenerace celistvých rostlin z pasážovaných embryí se pohybovalo u kedlubnu od 11,11 do 63,64 %, u květáku od 23,53 do 46,19 % a u zelí od 5,88 do 52,00 %. Využití regenerantů pro odvozování dihaploidních linií je často komplikováno výskytem samčí sterility i u rostlin s normálním počtem chromozomů.

Klíčová slova: *Brassica oleracea*; mikrosporové kultury; regenerace celistvých rostlin; dihaploidy

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