Chromosome Doubling Effects of Selected Antimitotic Agents in *Brassica napus* Microspore Culture

Miroslav KLÍMA, Miroslava VYVADILOVÁ and Vratislav KUČERA

Crop Research Institute, Prague-Ruzyně, Czech Republic

Abstract: Effects of microspore culture treatment with antimitotic agents colchicine, trifluralin and oryzalin on the frequency of embryo formation, embryo development, plant regeneration and diploidization rate in three F_1 hybrids of winter rapeseed cultivars were compared. The ploidy level analysis of 1709 flowering microspore-derived plants showed that *in vitro* applications of all antimitotic drugs increased the rate of doubled haploid (DH) plants significantly. The mean rate of DH plants from the trifluralin treatment was 85.7%, from colchicine 74.1% and 66.5% in the case of oryzalin, while only 42.3% in the untreated control variant whereas *in vivo* additional application of colchicine at the plantlet stage did not significantly increase the mean rate of DH plants (55.6%). Although there were no significant differences in diploidization efficiency between the *in vitro* applications of particular antimitotic agents, trifluralin showed to be the most suitable because of its positive effect on embryo development and conversion into whole plants. In addition, the diploidization rate was sufficient and stable in all genotypes tested. The results indicate that the trifluralin treatment of microspore cultures could provide efficient chromosome doubling for the production of doubled haploid lines from winter oilseed rape breeding materials.

Keywords: antimitotic agents; diploidization; doubled haploid; microspore culture; winter oilseed rape

Microspore culture has been routinely used for doubled haploid line production in oilseed rape breeding programs because of the high frequency of embryogenesis which could recently be achieved in a wide range of genotypes. However, the microspore derived embryo regeneration and frequency of fertile doubled haploids are still unsatisfactory, therefore, the use of effective diploidization techniques such as application of antimitotic agents is essential.

Various chromosome doubling techniques of microspore regenerants by colchicine treatment have been investigated (Mathias & Röbbelen 1991; Chen & Beversdorf 1992; Vyvadilová *et al.* 1993). However, the methods that involve the immersion of roots or whole plantlets in a colchicine solution are laborious, time consuming and require relatively large amounts of an expensive chemical. These procedures may result in ploidy chimeras and poor seed set; in addition, the rate of diploidization rarely exceeds 60%.

It was revealed later that colchicine added directly to microspore cultures improved embryogenesis and increased the diploidization rate up to 80–95% (Chen *et al.* 1994; Möllers *et al.* 1994; Weber *et al.* 2005). Several microtubule depolymerising herbicides were also proved to be efficient for *in vitro* chromosome doubling of microspores. Zhao & Simmonds (1995) tested trifluralin and Hansen & Andersen (1996) trifluralin, oryzalin and amiprophos methyl in the spring rapeseed cultivar Topas. They achieved the mean rate of diploidization from 60% to 65%, which is comparable with the application of colchicine. In addition, herbicides have an advantage because of their lower toxicity than colchicine and because lower concentrations for the treatment are needed.

The purpose of this study was to evaluate the effects of trifluralin and oryzalin application into microspore cultures on the frequency of microspore embryogenesis, embryo development, and thus the efficiency of diploidization in comparison with *in vitro* and *in vivo* colchicine treatment of different winter oilseed rape breeding materials.

MATERIALS AND METHODS

Plant material

Two F_1 hybrids of winter rapeseed cultivars SL-2/04 (Lisek × Stela) and SL-3/04 (Stela × Mohican) from Breeding Station in Slapy near Tábor and one F_1 hybrid of breeding materials OP-41/1(OP-BN-07 × SG-C 23) from Research Institute of Oilseed Crops in Opava were used in the experiments.

Microspore culture treatment with antimitotic agents

Microspore cultures were carried out according to the basic protocol (Klíma *et al.* 2004). Young flower buds with microspores at mid-uninucleate and late-uninucleate developmental stages were collected from the main and lateral branches of donor plants grown under controlled environmental conditions in a culture chamber (light intensity 84 μmol/m^2/s, 22/20°C day/night and photoperiod 16/8 h). Freshly isolated and purified microspores were resuspended in NLN liquid medium (Lichter 1985) supplemented with corresponding amounts of particular doubling agent stock solutions to get the final concentrations of oryzalin 1 μmol/l, trifluralin 10 μmol/l and colchicine 50 mg/l (Vyvadilová *et al.* 1993; Möllers *et al.* 1994; Hansen & Andersen 1996; Zhou *et al.* 2002). The microspore density was adjusted to 10^4 per 1 ml of culture media. Microspores in 60-mm plastic Petri dishes containing 6 ml of suspension were incubated for 18 h at 30°C in the dark. Three chronological replications were carried out for each genotype. Microspore cultures without any treatment were cultivated as a control with each replication to evaluate the frequency of spontaneous diploidization. The microspores were purified after incubation by centrifugation, resuspended in a fresh NLN medium and cultivated in the dark at 30°C. Some control variants were cultivated continuously without media exchange. After three weeks, embryos at torpedo and early cotyledonary stage (at least 2 mm in length) were counted and the Petri dishes were placed on a shaker (70 rpm) under continuous light at 22°C until embryos turned green. Cotyledonary embryos et least 4 mm in length were transferred to a solid differentiation medium with benzylaminopurine (0.2 mg/l), indolyl acetic acid (0.2 mg/l) and 2% sucrose, solidified by 0.8% agar and maintained at 22/20°C, with photoperiod 10/14 h and a light intensity of 300 μmol/m^2/s. After three weeks of cultivation, one third of the cotyledons of each embryo was cut and embryos were then transferred to a solid regeneration medium without growth regulators, with 1% sucrose and 1% agar (Klíma *et al.* 2004). Well-developed plantlets were subcultured onto a rooting MS medium without growth regulators. Regenerants with roots were transferred to pots filled with the peat soil, covered by perforated polyethylene foil for 7 days in order to protect plants from direct light and air-drying.

For *in vitro* application, 50 mg/l of colchicine was dissolved in a liquid NLN medium. The working solution was filter sterilized and stored in the dark at 4°C. For *in vivo* diploidization, colchicine was dissolved in distilled water to 0.05% solution just prior to use. For a stock solution, 33.52 mg of trifluralin and 34.64 mg of oryzalin were transferred to sterile glass beakers in a flowbox and dissolved in a small amount of acetone. After evaporation of acetone, the agents were finally dissolved in dimethyl sulphoxide (DMSO) and concentrations were adjusted to 10 mmol/l. Stock solutions were stored in sterile flasks in the dark at 22°C. The appropriate amount of stock solutions was added to a sterile liquid NLN medium (tempered to room temperature) to prepare a working solution 1 μmol/l of oryzalin and 10 μmol/l of trifluralin just prior to use.

Diploidization *in vivo*

About half of the regenerants, derived from control untreated variants, was used for *in vivo* diploidization with 0.05% colchicine solution for 24 h just prior to transplantation to the soil, according to Vyvadilová *et al.* (1993).

Ploidy level evaluation

Determination of doubled haploid (DH) regenerants was carried out by flow cytometry or by detection of sterile and fertile plants according to the evaluation of morphological characteristics of inflorescence, production of mature pollen grains and seed set.

Flow cytometry analysis

Flow cytometry analyses were carried out and evaluated in the Institute of Botany, Academy of Sciences of the Czech Republic, Laboratory of Flow Cytometry in Průhonice by means of Ploidy Analyser PA-II (Partec GmbH, Münster, Germany), equipped with UV mercury arc lamp and 488 nm argon ion laser. A simplified two-step (without centrifugation) procedure using Otto I and Otto II buffer (Doležel *et al.* 1989; Otto 1990) and *Lycopersicon esculentum* cv. Stupické polní rané (2C = 1.96 pg) as an internal standard was employed for ploidy analysis and genome size estimation. Young intact leaf tissues of a sample and internal standard (typically 1 + 1 cm^2) were cut together with a new razor blade in a Petri dish containing 1 ml of ice-cold Otto I buffer. The suspension was filtered through a 42 μm nylon mesh and then incubated at room temperature for 5–120 min with occasional shaking. The suspension was supplemented with 1 ml of Otto II buffer with fluorochrome DAPI, gently shaken and stored in the dark at room temperature (generally 5–15 min) prior to the determination of relative DNA content of isolated nuclei.

Chimeric plants caused by partial doubling with both sterile and fertile branches were considered as doubled haploids.

Plants with normally developed anthers, which did not set seeds after self-pollination, were interpreted as putative aneuploids.

RESULTS

Microspore embryogenesis and plant regeneration

Applied concentrations of all tested antimitotic drugs did not markedly affect the frequency of embryogenesis in comparison with untreated control (Figure 1 and 3). However, some differences in the microspore embryo development and morphology were observed (Figure 2). The presence of colchicine in the microspore culture medium accelerated embryo development and embryos with not deformed apical part, thinner hypocotyls and bigger cotyledons were derived as compared to the control variant. The treatment with oryzalin delayed embryo development, resulted in smaller embryos with thicker and shorter hypocotyls, and slightly deformed cotyledons. Results of the trifluralin treatment were similar to oryzalin, but hypocotyls were thinner and longer. There were no significant differences in the conversion of embryos into whole plants between individual treatments and genotypes as the method of cutting off cotyledons was applied to all cultivated embryos; however, vitrification in some regenerants occurred in the control variants and oryzalin application, and repeated subcultures to improve regeneration were necessary.

Ploidy level and diploidization efficiency

In total, 1709 flowering regenerants were derived and investigated for their ploidy level (Table 1).

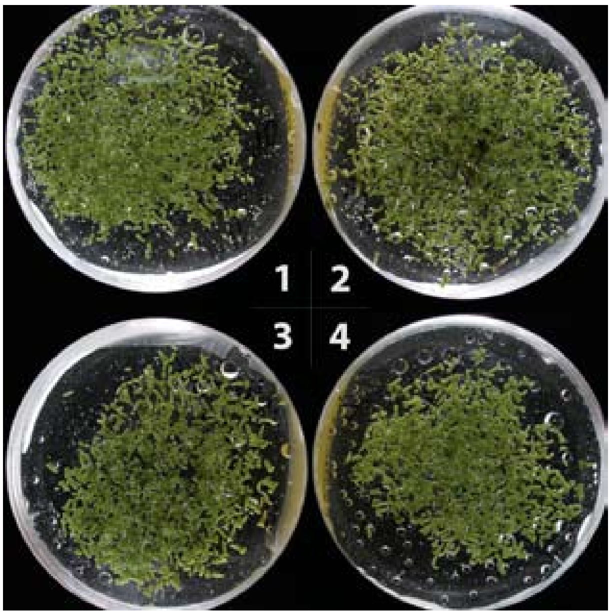

Figure 1. Comparison of microspore-derived embryo frequency between individual variants in genotype OP-41/1, replication No.1 after 23 days of culture

1 – Trifluralin, 2 – Colchicine, 3 – Oryzalin, 4 – Control; Petri dish 60 mm in diameter

Figure 2. Morphological characteristics of 30-days-old microspore embryos derived from individual treatments (genotype SL- 3/04)

A – Control, B – Colchicine, C – Oryzalin, D – Trifluralin; Bar = 1 millimetre

The statistical analysis (Figure 4) showed that *in vitro* applications of all antimitotic drugs significantly increased the rate of doubled haploid plants in comparison with the control. The mean rate of DH plants from the trifluralin treatment was 85.7%, from colchicine 74.1% and oryzalin 66.5%, while only 42.3% in the untreated control variant. Whereas, the *in vivo* additional application of colchicine at the plantlet stage did not significantly increase the mean rate of DH plants (55.6%) even on a 95% confidence level (Figure 4).

However, some significant differences in the rate of DH plants derived from individual treatments were detected between genotypes. Namely, the *in vivo* application of colchicine significantly increased the rate of DH plants in genotype OP-41/1, and the application of oryzalin in genotype SL-2/04 did not increase the percentage rate of DH plants in comparison with the untreated variant. Significant differences were detected between some genotypes in the rate of DH plants in control variants, *in vivo* colchicine and in oryzalin treatment, while no significant differences between genotypes were detected after *in vitro* colchicine and trifluralin treatment (Table 1). DH chimeras were observed only in regenerants from *in vivo* col-

Table 1. Percentage rates of doubled haploid (DH) plants derived from individual treatments

Genotype	Replication No.	No. of plants tested		Control	Colchicine *in vivo*	Colchicine *in vitro*	Oryzalin *in vitro*	Trifluralin *in vitro*
SL-3/04	1	174		61.76	66.67	77.78	72.73	89.29
	2	166		50.00	70.83	70.45	91.67	80.00
	3	220		54.84	75.76	81.63	93.67	71.43
	total	560	mean	55.53 a	71.09 a	76.62 a	86.02 a	80.24 a
OP-41/1	1	177		36.36	61.11	86.96	75.76	87.50
	2	136		34.48	47.83	68.00	70.00	93.10
	3	302		47.17	58.02	76.92	63.64	83.61
	total	615	mean	39.34 ab	55.65 ab	77.29 a	69.80 a	88.07 a
SL-2/04	1	193		19.05	53.33	56.25	32.69	90.70
	2	187		36.59	43.90	88.89	43.75	93.48
	3	154		40.00	22.58	60.00	54.90	81.82
	total	534	mean	31.88 b	39.94 b	68.38 a	43.78 b	88.67 a
Mean	total	1709	total mean	42.25	55.56	74.10	66.53	85.66

Letters a and b in columns designate homogeneous groups (LSD; $P = 0.05$)

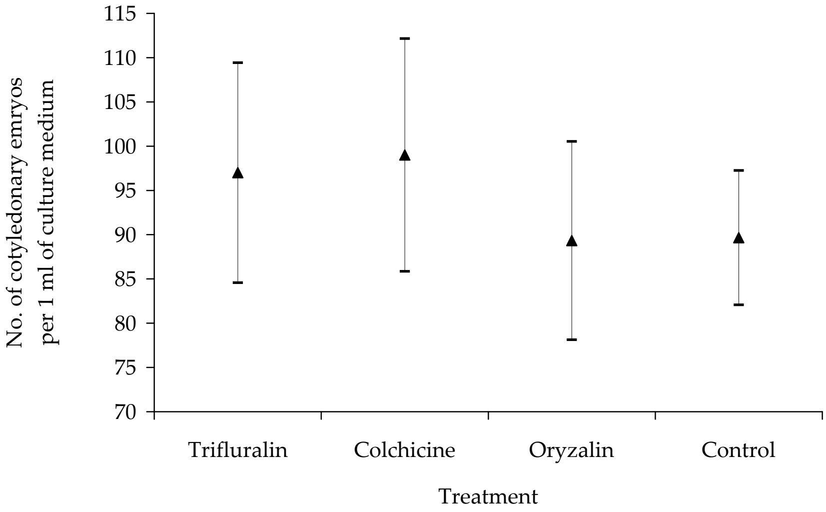

Figure 3. The number of cotyledonary embryos per 1 ml of culture medium in the particular *in vitro* treatments with antimitotic agents; pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals

chicine treatment. The mean rate of DH chimeras was 22.4% from all DH regenerants of this treatment. The occurrence of putative aneuploid plants (Figure 5) was significantly higher after oryzalin treatment (13.6%) when compared with untreated control (3.0%). However, in genotype SL-3, the rate of aneuploid plants increased significantly after the application of trifluralin.

DISCUSSION

The results demonstrate that the applied concentration of all tested antimitotic agents did not markedly affect the frequency of embryogenesis. Oryzalin and trifluralin slightly delayed embryo development but more direct and rapid regeneration of embryos to whole plants from cultures treated

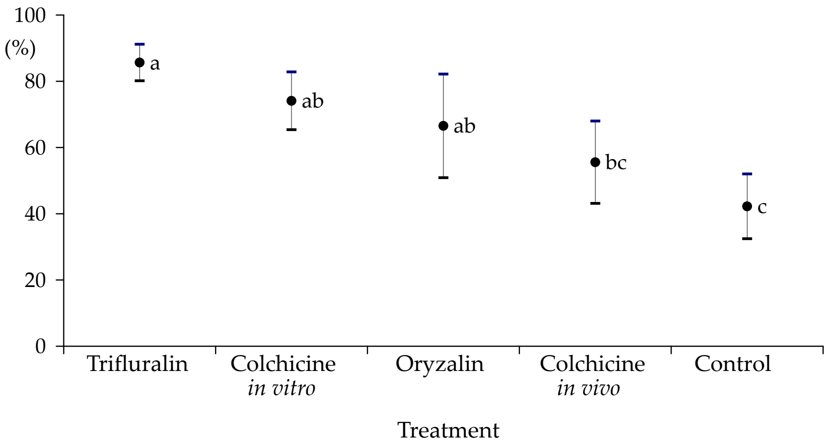

Figure 4. Diploidization frequency of individual treatments and occurrence of aneuploid plants (%); pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals. Letters a, b and c designate homogeneous groups

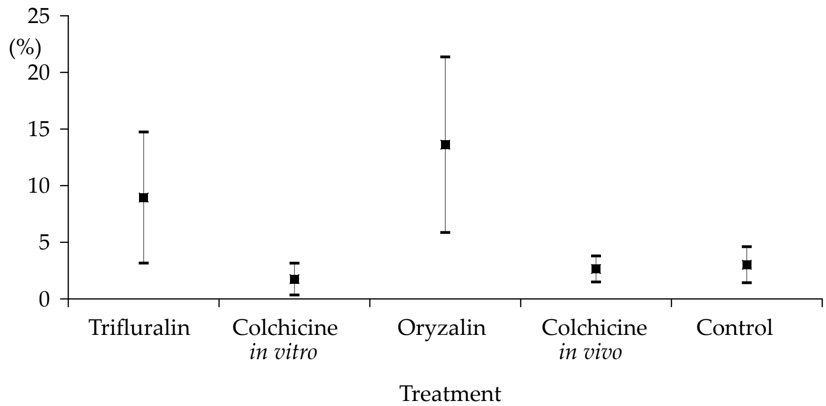

Figure 5. Occurence of aneuploid plants (%); pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals with these agents was observed. The results are consistent with previous reports on the positive effect of colchicine on embryo development (Zaki & Dickinson 1995; Zhou *et al.* 2002), direct plant regeneration (Weber *et al.* 2005) and improvement of embryo germination by trifluralin (Eikenberry 1994). The lower embryo frequency in the untreated control in comparison with *in vitro* colchicine-treated variants described by Zhou *et al.* (2002) in spring rape was not proved. However, some differences in the number of embryos and embryo morphology appeared between control variants with and without change of the media. Therefore, embryogenesis and further embryo development could be stimulated by the media exchange after 18 h of cultivation. Significant differences in embryo frequency were also detected between replications within genotypes, which could be explained by various portions of microspores in the optimal developmental stage and different physiological conditions of donor plants between successive replications of experiments. Although the divergence in embryo morphology between particular treatments was observed and embryos with deformed cotyledons occurred (namely in embryos from oryzalin and trifluralin treatments), differences in the conversion into whole plants between treatments was not discovered as the method of cutting off cotyledons was applied to all cultivated embryos. Thus, conditions for direct regeneration (from apical meristem) were provided even for not properly developed embryos. Our experiments revealed that a high frequency of doubled haploids could be obtained using the *in vitro* treatment of microspores with trifluralin (85.7%) and colchicine (74.1%) while the mean frequency of doubled haploids after oryzalin treatment was 66.5%. Significant differences in the percentage rate of doubled haploid plants were detected between some genotypes after the *in vivo* application of colchicine from 39.9% to 71.1% and in oryzalin treatment from 43.8% to 86.0%, whereas the percentage rate between control variants ranged from 31.9% to 55.5%. Previous results (Smýkalová *et al.* 2006) with *in vivo* colchicine treatment showed even larger differences between genotypes (7.1%–100%), which could be explained according to Hansen and Andersen (1996) as a result of different growing conditions for donor plants, affecting the capacity for microspore embryogenesis, chromosome doubling and plant regeneration.

The mean rate of spontaneous doubled haploids from all tested genotypes was 42.3%. Similar results were reported by Weber *et al.* (2005) in spring Canadian cultivars. They further revealed that spontaneous diploidization showed a large variation depending on the genotype. This corresponds to the results of Rudolf *et al.* (1999), who also observed differences in spontaneous genome doubling between closely related cabbage genotypes. Möllers *et al.* (1994) considered the different stages of microspores in cultures as a determining parameter for spontaneous diploidization. Thus, it could be improved by a more precise preparation of donor material.

DH chimeras were observed only after the *in vivo* colchicine treatment in our experiments. *In vitro* diploidization avoided the occurrence of chimeric plants in comparison with the colchicine treatment of plantlets. According to Zhao and Simmonds (1995), it is so because chromosomes are doubled very early in a microspore culture.

Although there were no significant differences in chromosome doubling between trifluralin, oryzalin and *in vitro* application of colchicine, trifluralin in our experiments showed to be the most suitable agent. These results are consistent with Zhao and Simmonds (1995), who demonstrated the efficiency of trifluralin for chromosome doubling in spring oilseed rape, cultivar Topas. The higher efficiency of trifluralin in relation to *in vitro* chromosome number doubling in comparison with oryzalin was also described by Rudolf *et al.* (1999) in *Brassica oleracea*. According to Zhao and Simmonds (1995), a great advantage of trifluralin use is that embryogenesis is normal and proceeds to direct embryo germination and vigorous plant growth.

It can be concluded that the microspore culture treatment with trifluralin in micro-molar concentrations is inexpensive, less toxic in comparison with colchicine and, unlike the use of oryzalin, resulting in well-developed embryos, better conversion into whole plantlets and lower percentage of aneuploid regenerants. In addition, the effect of trifluralin treatment on diploidization was sufficient and stable in all genotypes tested. This implies that the procedure could provide efficient chromosome doubling for the production of doubled haploid lines from winter oilseed rape breeding materials.

Acknowledgements. Supported by the Ministry of Agriculture of the Czech Republic, Project No. 0002700602.

References

Chen J.L., Beversdorf W.D. (1992): Production of spontaneous diploid lines from isolated microspores following cryopreservation in spring rapeseed (*Brassica napus* L.). Plant Breeding, **108**: 324–327.

Chen Z.Z., Snyder S., Fan Z.G., Loh W.H. (1994): Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breeding, **113**: 217–221.

Doležel J., Binarová P., Lucretti S. (1989): Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum, **31**: 113–120.

Eikenberry E. (1994): Chromosome doubling of microspore-derived canola using trifluralin. *Cruciferae* Newsletter, **16**: 51–52.

Hansen N.J.P., Andersen S.B. (1996): *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. Euphytica, **88**: 156–164.

Klíma M., Vyvadilová M., Kučera V. (2004): Production and utilization of doubled haploids in *Brassica oleracea* vegetables. Horticultural Science (Prague), **31**: 119–123.

Lichter R. (1985): From microspores to rape plants. A tentative way to low glucosinolate strains. In: Sorensen H. (ed.): Advance in the Production and Utilisation of Cruciferous Crops. Martinus Dordecht, Boston, Lancaster, Nijhoff M., Junk W. Publishers, 268–277.

Mathias R., Röbbelen G. (1991): Effective diploidization of microspore-derived haploids of rape (*Brassica napus* L.) by *in vitro* colchicine treatment. Plant Breeding, **106**: 82–84.

Möllers C., Iqbal M.C.M., Röbbelen (1994): Efficient production of doubled haploid *Brassica napus* plants by colchicine treatment of microspores. Euphytica, **75**: 95–104.

Otto F. (1990): DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Crissman H.A., Darzynkiewicz Z. (eds): Methods in Cell Biology, Vol. 33, Crissman Academic Press, San Diego,105–110.

Rudolf K., Bohanec B., Hansen M. (1999): Microspore culture of white cabbage, *Brassica oleracea* var. *capitata* L.: Genetic improvement of non-responsive cultivars and effect of genome doubling agents. Plant Breeding, **118**: 237–241.

Smýkalová I., Větrovcová M., Klíma M., Macháčková I., Griga M. (2006): Efficiency of microspore culture for doubled haploid production in the breeding project "Czech Winter Rape". Czech Journal of Genetics and Plant Breeding, **42**: 58–71.

Vyvadilová M., Zelenková S., Tomášková D., Košner J. (1993): Diploidization and cytological control of *Brassica napus* L. haploids. Rostlinná výroba, **39**: 129–137.

Weber S., Ünker F., Friedt W. (2005): Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment *in vitro* and ploidy determination by flow cytometry. Plant Breeding, **124**: 511–513.

Zaki M., Dickinson H. (1995): Modification of cell development *in vitro*: The effect of colchicine on anther and isolated microspore culture in *Brassica napus*. Plant Cell, Tissue and Organ Culture, **40**: 255–270.

Zhao J., Simmonds D.H. (1995): Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. Physiologia Plantarum, **95**: 304–309.

Zhou W.J., Tang G.X., Hagberg P. (2002): Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regulation, **37**: 185–192.

Received for publication August 21, 2007
Accepted for publication February 27, 2008

Corresponding author:

Ing. Miroslav Klíma, Výzkumný ústav rostlinné výroby, v.v.i., Drnovská 507, 161 06 Praha 6-Ruzyně, Česká republika
tel.: + 420 233 022 369, fax: + 420 233 310 636, e-mail: klima@vurv.cz