Efficiency of Microspore Culture for Doubled Haploid Production in the Breeding Project "Czech Winter Rape"

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Abstract: A selected set of twenty inter-line F_1 hybrids of *Brassica napus* L. was evaluated for their capacity to produce microspore-derived doubled haploid (DH) plants subsequently utilized in the breeding project "Czech Winter Rape". All tested genotypes were able to produce microspore-derived embryos based on the standardized protocol previously adopted for the Czech winter rape varieties/breeding lines; nevertheless, significant differences were found in the frequency of microspore-derived embryo production as related to particular genotypes. However, the fertile DH regenerants were obtained only in 12 hybrid lines of this set. The other 8 lines failed to produce fertile DH plants for several reasons, mainly due to altered morphology of microspore embryos connected with limited germination/conversion potential and due to unsuccessful colchicine treatment resulting in haploid or mixoploid (chimeric) regenerants exhibiting partial or full sterility. In general, colchicine treatment induced diploid (doubled haploid), polyploid (triploid, tetraploid) and mixoploid (containing both euploid and aneuploid nuclei) plants. The fully aneuploid plants were recorded only sporadically. Some modifications of culture protocol (removal of embryo cotyledons before transfer to a germination medium; production of more plantlets from a single embryo by rooting multiple shoots; repeated colchicine treatment of unsuccessfully doubled plants in a later phase of growth) could enhance the final yield of DH fertile regenerants in our experiments. In total, 590 microspore-derived regenerants were produced in a series of experiments, 236 (40%) of them represented fully fertile DH plants. The R2 seeds (progeny of DH R1-generation) of the most promising hybrid-lines were included in the tests of agronomic performance in field conditions.

Keywords: microspore culture; doubled haploids; winter rape; *Brassica napus* L.; hybrid breeding

Winter rape (*Brassica napus* L. var. *napus*) is the most widespread oil plant and the second most frequently cultivated crop after cereals in the Czech Republic. *Brassica napus* L. is an allotetraploid or amphidiploid species with $n = 19$ chromosomes, derived from 2 diploid species *B. oleracea* ($n = 9$) and *B. rapa* ($n = 10$). The microspore doubled haploid (DH) methodology is now employed in many *B. napus* breeding programmes around the world as an alternative/supplement to conventional methods of homozygous line production (Kučera *et al*. 2002, 2004; Ferrie & Keller 2004; Friedt & Zarhloul 2005; Koprna *et al*. 2005). The doubled haploid method may reduce the time

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needed to develop and release new cultivars by approximately 2 to 4 years in comparison with conventional techniques. In the Czech Republic, the cultivation and breeding of oilseed rapes have become increasingly dominated by hybrid varieties based on seed production from inbreds with cytoplasmic male sterility as a quality donor and a fertility restorer as male line providing pollen. Microspore culture can aid in the selection of restorer lines, and thus increase the efficiency of this programme (Wang *et al.* 1999 in Ferrie & Keller 2004). A biotechnological approach to the production of doubled haploid plants via culture of microspores may be successfully applied to affect a number of morphological /agronomic/ qualitative traits contributing both to high yield and desirable seed composition. An important fact is that efficient selection of superior genotypes as related to substances of interest (e.g. fatty acids) may be realized at an early stage of microspore culture *in vitro* by using one of the two cotyledons for fatty acid analysis while retaining the rest of the embryo (Albrecht *et al.* 1995; Möllers *et al*. 2000). Adamska *et al.* (2004) produced DH lines with a modified fatty acid composition with heritable and environmental stability. According to Ferrie and Keller (2004) there are currently more than fifty oilseed *Brassica* varieties in commercial production derived by a doubled haploid technique.

The breeding programme "Czech Winter Rape" is directed to the development of hybrid cultivars with improved seed yield and quality parameters such as modified fatty acid composition, low glucosinolate content (15–20 μmol/g of seeds) as well as low erucic acid content (0.3–0.8%). In addition, improved resistance to biotic and abiotic stresses and resistance to frost, lodging and fungal diseases (*Phoma lingam*, *Sclerotinia sclerotiorum*) are other significant limiting factors for high yield of oilseed rape (Odstrčilová & Plachká 2005). Thus, the creation of high quality F_1 hybrids used as donor plants for biotechnological approaches is a crucial point which could significantly help conventional methods to produce new oilseed rape varieties.

Lichter (1982) first reported the production of haploid plants from isolated microspores of *B. napus*. In the eighties, mainly spring rape cultivars (e.g. Topas) served as models for the optimization/improvement of the protocol (Coventry *et al*. 1988). The method was adopted and modified for Czech winter rape cultivars and breeding lines by Vyvadilová and Zelenková (1992) and later applied in the Czech programmes of *Brassica* spp. breeding (Kučera *et al*. 1997, 2002, 2004; Vyvadilová *et al.* 1998a, b, 2001; Klíma *et al.* 2004; Koprna *et al*. 2005). Despite the fact that microspore-derived doubled haploid production protocols are well elaborated (and exhibit high efficiency) in model genotypes (e.g. Topas), they cannot usually be simply used for a broad spectrum of breeding materials/lines (Ferrie *et al*. 1995) and should be modified/optimized case by case in order to obtain satisfactory efficiency for practical breeding purposes. Here we present an evaluation of twenty inter-line F_1 hybrids of winter rape as related to microspore-derived embryo induction, embryo conversion into plantlets and final production of doubled haploid plants as revealed by flow-cytometry. The stress was laid mainly on the improvement/optimization of some critical steps of the utilized regeneration protocol in order to improve the efficiency of the whole system.

MATERIALS AND METHODS

Plant material

A set of 20 inter-line F_1 hybrids of winter oilseed rape (*Brassica napus* L.) was prepared in SELGEN, a. s., Plant Breeding Station in Chlumec nad Cidlinou in the framework of the breeding project "Czech Winter Rape" (Table 1). Donor plants (i.e. F_1 hybrids) were grown in the soil in a greenhouse for 4 weeks. Then, the plants with 3 to 4 developed leaves were transferred to a vernalization chamber at 6°C, and 16h photoperiod (irradiance 20.4 μmol/m^2/s) for subsequent 6 weeks. Finally, the vernalized plants were grown in the greenhouse up to the stage of flower bud formation and served as donors for flower bud collection and microspore isolation.

Culture media

A liquid washing medium for microspore isolation/preparation contained basal major and minor salts and Fe-EDTA solution of B5 medium (Gamborg *et al*. 1968), 100 mg/l myo-inositol and 10% sucrose. Liquid NLN-13 medium (Lichter 1982) with 13% sucrose was used for microspore culture, subsequent embryo induction and initial development. In the initial series of experiments,

Table 1. The efficiency of production of microspore-derived morphologically normal embryos and subsequent plant regeneration as related to the genotype and sequence of culture media (NLN-13 → MD → MR → MS); number of embryos is expressed as the mean ± standard error, related to 20 initially used flower buds; MD – medium for embryo differentiation; MR – medium for further culture of embryos after cotyledon removal; MS – medium for rooting and plantlet formation; pooled data of several independent experiments

Genotype (F_1-sample designation)	Origin	Total replications of experiment/responsive replications	No. of responsive buds	Culture medium				
				NLN-13/MD	MR		MS	
				initial mean No. of embryos (= 100%)	mean No. of embryos	frequency (%)	mean No. of plantlets	frequency (%)
8/03	HSH 1023 × LBN 249/58	6/1	23	191.0*	178.0*	93.2	124.0*	65.0
9/03	HSH 1023 × NSL 01/80	8/5	113	229.6 ± 39.6	209.2 ± 35.7	91.1	124.0 ± 13.3	54.0
11/03	HSH 1023 × NSL 01/84	4/2	59	14.0 ± 7.0	11.0 ± 4.0	78.6	2.0 ± 2.0	14.3
12/03	HSH 1023 × NSL 01/85	5/4	100	377.3 ± 223.3	312.5 ± 185.5	82.8	195.3 ± 135.6	51.8
13/02	Rasmus × Magnum	5/3	72	26.7 ± 12.5	26.3 ± 12.2	98.5	12.3 ± 11.4	46.1
15/03	HSH × Capitol	4/3	66	365.0 ± 199.3	308.0 ± 163.8	84.4	112.7 ± 70.5	30.9
16/03	GPR 456 × Odila	5/2	47	78.0 ± 57.0	77.0 ± 56.0	98.7	27.0*	34.6
20/03	HRH 934 × LBN 249/58	3/1	20	278.0*	201.0*	72.3	25.0*	9.0
24/03	HRH 934 × NSL 01/85	3/3	56	71.0 ± 21.1	65.7 ± 17.2	92.5	0.0	0.0
37/02	Sl. Stela × Rasmus	8/8	220	60.6 ± 20.9	54.8 ± 20.2	90.4	25.4 ± 8.9	41.9
40/02	Sl. Stela × Magnum	9/5	130	139.8 ± 47.7	137.6 ± 48.0	98.4	38.8 ± 18.9	27.8
51/02	Magnum × (Bristol × Falcon)	9/8	217	71.1 ± 26.6	70.7 ± 26.7	99.4	26.0 ± 7.6	36.6
54/02	Orkan × FPR 226	9/7	187	106.4 ± 52.5	99.9 ± 47.9	93.9	46.3 ± 30.3	43.5
62/02	Mohican × Rasmus	11/2	49	6.0 ± 2.0	6.0 ± 2.0	100.0	0.0	0.0
63/02	Mohican × FPR 226	9/7	187	166.7 ± 54.5	145.4 ± 43.5	87.2	37.9 ± 10.8	22.7
65/02	Mohican × Magnum	11/4	93	23.0 ± 14.8	5.5 ± 2.2	23.9	3.5 ± 2.4	15.2
73/02	(Lirajet × Wotan) × Jesper	10/3	90	3.7 ± 1.8	3.7 ± 1.8	100.0	1.3 ± 1.3	35.1
75/02	(Lirajet × Wotan) × Orkan	10/6	208	46.8 ± 17.3	46.3 ± 17.3	98.9	24.2 ± 8.8	51.7
76/02	(Lirajet × Wotan) × Mohican	3/1	20	25.0*	20.0*	80.0	0.0	0.0
81/02	(Accord × Prestol) × FPR 226	3/3	49	53.0 ± 13.5	46.0 ± 15.0	86.8	0.0	0.0
Mean		7/4	100	116.6	101.5	87.6	41.3	29.0

*Data from one replication only; not evaluated statistically

the germination/conversion of microspore-derived embryos was tested on B5 solid medium (Gamborg *et al*. 1968) with 0.1 mg/l GA_3 for 2 weeks, followed by MS medium (Murashige & Skoog 1962) supplemented with 1 mg/l kinetin (KIN) and 1 mg/l BAP for another 2 weeks, and finally on MS medium without growth regulators. Later, another sequence of media was used to support optimum embryo development and plant regeneration. Media for embryo development (MD) and for plantlet regeneration (MR) were prepared as modified B5 media (Gamborg *et al*. 1968). MD medium contained increased KNO_3 up to 3000 mg/l and $CaCl_2.6H_2O$ up to 1100 mg/l with addition of amino acids (800 mg/l glutamine, 100 mg/l serine), 20 g/l of sucrose and growth regulators: 0.2 mg/l BAP and 0.2 mg/l IAA. In MR medium, the composition of macroelements was modified as follows: 1650 mg/l NH_4NO_3, 1900 mg/l KNO_3, 170 mg/l $KH_2PO_4.2H_2O$, 370 mg/l $MgSO_4.7H_2O$; the medium was supplemented with 2 mg/l of glycine and 10 g/l of sucrose. MS rooting solid medium (Murashige & Skoog 1962) with addition of 0.03 mg/l NAA contained 10 g/l of sucrose. pH of all media was adjusted with KOH to 5.8–6.0. NLN-13 medium was filter sterilised (0.22 µm filter, Millipore) under sterile conditions in air laminar flow, B5 liquid (washing) and solid B5, MS, MD and MR media were sterilised by autoclaving (121°C, 15 min).

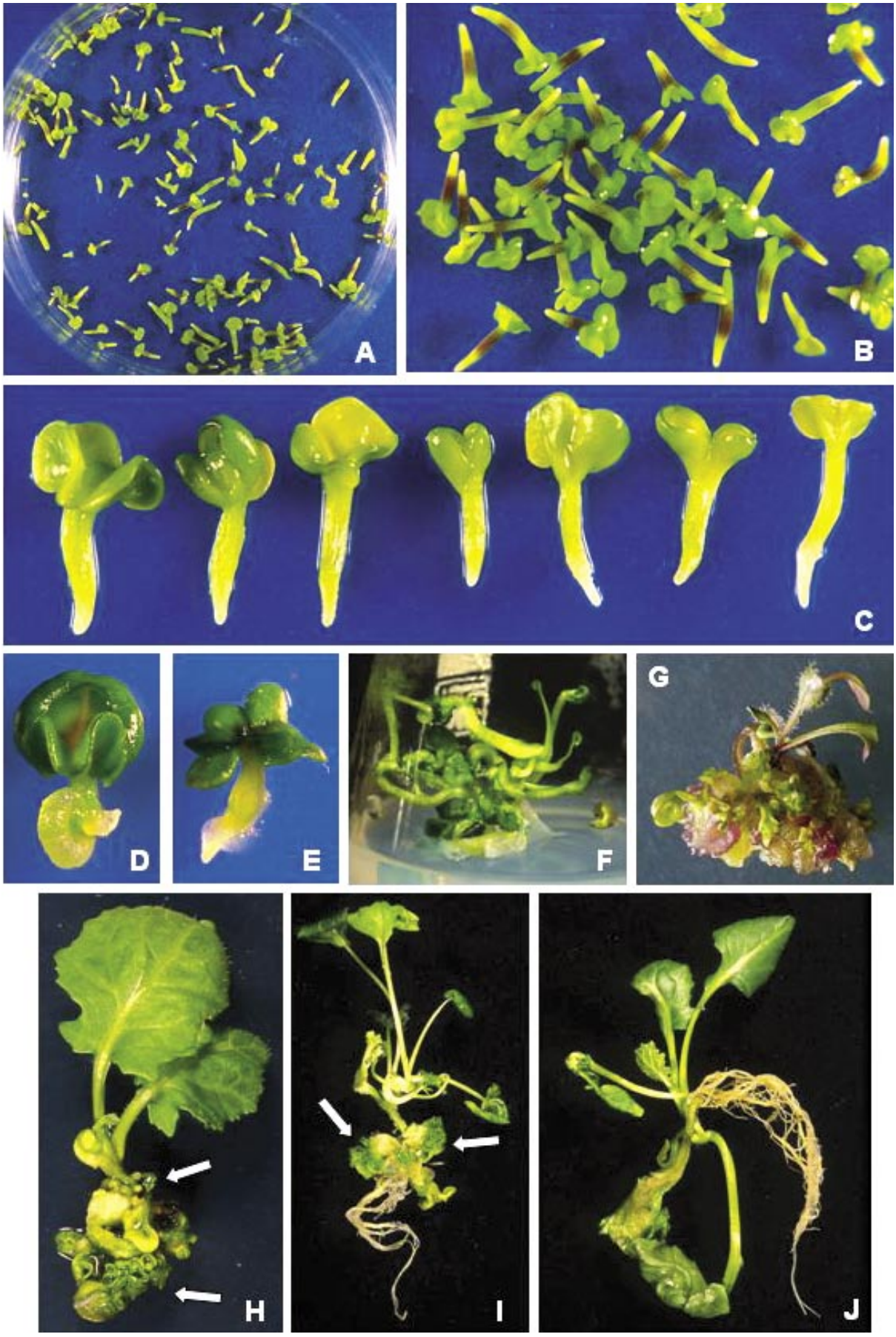
Microspore isolation

The procedure of microspore isolation was done according to Coventry *et al*. (1988) and Vyvadilová and Zelenková (1992). Unopened flower buds 2.5–3 mm in length at the mid-uninucleate stage of pollen development were surface sterilised with 70% ethanol for 2 min, first 10 min and then washed three times with cold sterile deionized water. The flower buds were carefully crushed using a glass stick in a small volume of the washing medium. The crushed flower buds were filtered through 44 µm and 70 µm nylon screen meshes, diluted with washing medium to the final volume of about 10 ml and centrifuged (1000 g) for 10 min. The supernatants were decanted and the pellets were washed twice with washing medium by centrifugation for 5 min. The obtained pellets containing microspores were diluted with NLN-13 medium (Lichter 1982) up to a density of about 75 000 to 100 000 microspores per 1 ml of media. The microspore culture was sampled each 5 ml per 60 mm Petri dish.

Embryo induction, its development and plant regeneration

The isolated microspores were incubated in NLN-13 medium at 30°C, in darkness for 10 to 14 days. After visual detection of small embryoids about 1–1.5 mm in size, the Petri dishes were transferred onto a gyratory shaker (60 rpm) and cultured on the same medium at 26 ± 1°C, 16 h photoperiod, irradiance 20.4 µmol/m^2/s for further 10 to 14 days. Thus, after 3 to 4 weeks from microspore isolation, fully morphologically developed embryos were obtained with distinct root pole, hypocotyl and cotyledons. The first signs of root elongation and root hair formation were recorded at the end of this culture period.

The fully morphologically developed embryos about 5 mm in length were transferred onto MS solid medium for direct germination and plant regeneration. As this simple procedure did not result in a satisfactory embryo germination frequency, a sequence of media was later applied in order to improve the efficiency of plant regeneration. Embryos from liquid NLN-13 medium were placed onto MD medium in a culture room at 20–22°C, 16 h photoperiod (cool-white fluorescent tubes, irradiance 20.4 µmol/m^2/s) for 7 to10 days. After removing cotyledonary leaves (Kott & Beversdorf 1990), embryos were transferred onto MR medium at the same culture conditions as mentioned above for further 3 to 4 weeks. The germinating embryos were transferred onto the rooting medium MS for 4 to 6 weeks to support root growth. Colchicine treatment for the production of doubled haploid plants was performed with rooted plantlets containing 2 to 3 leaves. The plantlets were submersed in a solution of 0.5% colchicine + 0.1% DMSO in a culture room at 20–22°C and 16 h photoperiod for 24 h. After colchicine treatment, the plantlets were placed onto sterile perlite saturated with half-strength MS medium for 2 weeks. Then the regenerated plants were transferred to the soil and grown in a greenhouse up to the stage of at least 4 leaves, when they were subjected to vernalization in a vernalization room (6°C, 16 h photoperiod, irradiance 56 µmol/m^2/s) for 6 weeks. For the flow-cytometric analysis only those leaves were sampled that newly differentiated after colchicine treatment. Vernalized plants were grown up to flowering and seed setting in a greenhouse.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Determination of ploidy level

The ploidy status/DNA content of regenerated plants was determined using flow-cytometry (PA, Partec, GmbH, Münster, Germany). Measurements were calibrated with two standards: (1) Partec DNA standard kit and (2) seed-derived winter rape plants with diploid status ($2n$ = 38) as a diploid control. 25 mg FW sample of young leaf was cut in extraction buffer OTTO I (Otto 1990) for the isolation of nuclei. 180 ml OTTO II solution containing DNA-specific fluorochrome DAPI (4,6-diamino-2-phenylindole) was added to the obtained suspension of isolated nuclei to visualize them (Hause *et al*. 1992), and then the solution was filtered through a nylon filter mesh (30 μm). After short-term incubation in the extraction solution, the filtrate was immediately analysed with flow-cytometer. After measurements of about 2–5 thousand nuclei, the relative content of DNA (corresponding to $1n$, $2n$, $3n$, $4n$ or mixoploid/aneuploid status) was determined. The measurements were done in triplicate.

RESULTS

Embryo induction, its development and plant regeneration

Evident differences in capacity to produce microspore-derived embryos were found between the twenty tested inter-line winter rape hybrids (Table 1). The mean number of embryos per 20 flower buds produced in the liquid induction medium varied from 3.7 (line 73/02) to 377.3 (line 12/03); the mean number of induced embryos per tested set of hybrid lines was 116.6. There was no hybrid line with zero response in a series of experiments; however, the differences/variation in embryo production in subsequently repeated experiments (2002, 2003, 2004) with the same line were high (see standard errors in Table 1). As related to the parental effect on embryogenic competence in hybrid combinations, HSH genotype (e.g. sample 8/03, 9/03, 12/03, 15/03) seems to exhibit a good embryogenic capacity and may serve as a potential donor/parent of regeneration competence in hybrid combination designs (Table 1). The hybrid lines were classified on the basis of their embryogenic capacity into three categories:

(1) very good regeneration – 100 to 500 embryos per 20 flower buds (fb);
(2) good regeneration – 50 to 100 embryos per 20 fb;
(3) weak regeneration – less 50 embryos/20 fb (Table 2).

Once induced, originally globular/spherical microspore-derived proembryos developed very quickly and within 2 weeks they reached the size of ca. 5 mm in length and exhibited typical bipolar organization with distinct white-yellowish root pole, green or violet-brown hypocotyl (according to the genotype) and green cotyledons (Figure 1 A, B, C). There was practically no morphological variation as related to the root and hypocotyl organization (Figure 1 A, B, C), while cotyledons – on the other side – exhibited a spectrum of morphological changes (as compared to regular dicotyledonous embryos), namely horn-shaped or leafy single cotyledon, 3 cotyledons or fused cotyledons (trumpet-shaped and jar-like structures) (Figure 1 A, B, C). The microscopic analysis of some morphologically abnormal embryos showed the improperly formed shoot apical meristem. This fact negatively influenced direct embryo germination and plantlet formation (see the text below). Three- to four-weeks-old embryos usually started to spontaneously germinate via the hypocotyl and root elongation and root hair formation already in MD medium (Figure 1 C). However, the transfer of embryos onto the first tested sequence of media (B5 + GA_3, MS + KIN + BAP, B5) did not result in continuous germination, but all parts of embryo gradually swelled (Figure 1 D, E), sometimes with the signs of vitrification, or turned into the callus

Figure 1. Microspore culture of winter rape; A, B – Fully developed embryos in liquid NLN-13 medium 3 weeks after microspore isolation; C – Detail of microspore-derived embryos with distinct root pole and cotyledons exhibiting variable morphology; the shoot apical meristem is not visible macroscopically; D, E – Swelling of embryos cultured on the sequence of media (B5 + GA_3, MS + KIN + BAP); F – Direct leaf/shoot organogenesis on swollen microspore-derived embryos on the sequence of media (B5 + GA_3, MS + KIN + BAP, MS); G – Formation of organogenic callus from swollen embryos on the sequence of media (B5 + GA_3, MS + KIN + BAP, MS); H, I – Plantlets developed from swollen embryos; arrows indicate abundant multiple buds on swollen hypocotyl and cotyledons of embryos; J – Rooted shoot (= complete plantlet) isolated from organogenic embryo-derived callus; MS medium + NAA

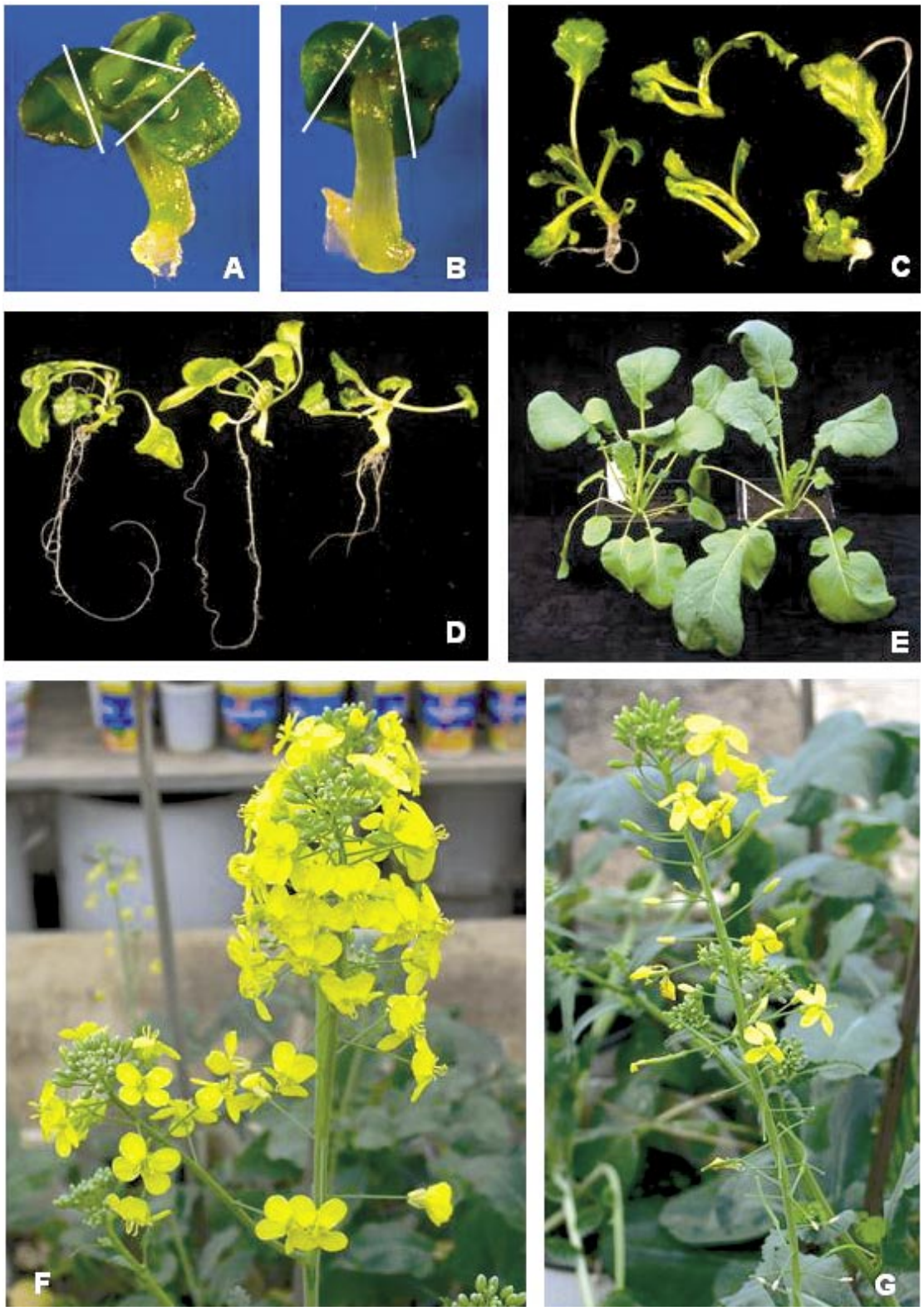

Figure 2. Microspore culture of winter rape; A, B – Adjustment of swollen embryos (= partial removal of cotyledons; MD medium) for better germination/conversion; C – Leaf (and root) development from embryos with partially removed cotyledons on MR medium; D – Regular root induction and development on embryos/shoots on MS medium; E –Regenerated plants in the soil 3 weeks after colchicine treatment; F, G – Flowering plants regenerated from microspore-derived embryos after colchicine treatment; Inflorescence of doubled haploid plant with fertile flowers (F), inflorescence of chimeric plant containing both sterile and fertile flowers and exhibiting a haploid habitus (G)

Table 2. Classification of winter rape genotypes (F_1 hybrids) into categories (I, II, III) based on the regeneration ability, i.e. production of microspore-derived morphologically normal embryos on liquid induction medium NLN-13 (mean number of embryos per 20 flower buds; pooled data of several experiments)

Regeneration category (mean No. of embryos per 20 flower buds)		
I (500–100)	II (100–50)	III (< 50)
12/03 > 15/03 > 20/03 > 9/03 > 8/03 > 63/02 > 40/2 > 54/02	16/03 > 51/02 > 24/03 > 37/02 > 81/02	75/02 > 13/02 > 76/02 > 65/02 > 11/03 > 62/02 > 73/02

(Figure 1 G). Nevertheless, in a part of cultures (ca. 15%) the culture on the above-mentioned sequence of media resulted in multiple shoot bud and leaf formation on swollen embryos (Figure 1 F, H, I) or in the formation of organogenic callus (Figure 1G). In the course of further development, usually a single shoot dominated above the others and a single plantlet was formed (Figure 1 H), sometimes with parallel root formation (Figure 1 I). In other cases, the non-vitrified shoots/leaf clumps formed on swollen embryos or organogenic calli could be cut off and rooted on an auxin-supplemented medium (0.03 mg/l NAA; Figure 1 J). The direct conversion of embryos into plantlets was in general difficult and low – only regular dicotyledonous embryos with the properly developed shoot apical meristem germinated/converted more easily. Thus the initially promising yield of microspore-derived embryos was in fact drastically reduced by their poor germination/conversion ability. This low efficiency of plantlet formation from microspore embryos could be partially compensated by producing more than 1 regenerated plant from a single embryo (possibility of isolating several shoots/leaves able to root per embryo in some cases; Figure 1F).

The unsatisfactory direct germination/regeneration efficiency made us modify the regeneration protocol: in order to stimulate shoot proliferation, the leafy structures developed from/on cotyledons of elongated embryos (MD-medium with BAP and IAA) were cut off (Figure 2 A, B) and such adjusted embryo-explants were transferred into MR-medium with specifically changed macroelement composition. These explants formed clumps of well-developed leaves (Figure 2 C, D) which were relatively easily rooted in sterile or semi-sterile conditions and subsequently transferred and acclimatized *ex vitro*. The mentioned modifications increased the efficiency of the whole protocol up to the mean final frequency 29% (i.e. the number of obtained viable

Table 3. Proportion of haploid, doubled haploid and polyploid regenerants after colchicine treatment; pooled data of several experiments

Genotype*	No. of evaluated regenerants	Haploids	Doubled haploids	Polyploids**	Doubling efficiency (%)
9/03	92	55	28	9	40.2
12/03	150	59	67	24	60.7
15/03	19	10	4	5	47.4
28/03	4	0	4	0	100.0
37/03	39	34	5	0	12.8
40/02	44	39	5	0	11.4
51/02	8	7	1	0	12.5
54/02	11	7	4	0	36.4
63/02	33	22	8	3	30.3
75/02	14	13	1	0	7.1
Σ	414	246	127	41	40.6

*Selected F_1 inter-line hybrids; for the hybrid origin of tested genotypes see Table 1

**Polyploids include triploids and tetraploids

plantlets related to the initial mean number of fully developed embryos; range 0 to 65% according to the particular hybrid lines; Table 1).

Colchicine treatment, doubled haploid plant production

As the spontaneous diploidization of microspore-derived plants in *Brassica napus* is low, the *in vivo* colchicine treatment was necessary in our experiments in order to increase the number of doubled haploids. The doubling rate varied between hybrid genotypes and ranged from 7 to 100% (Table 3). However, the colchicine treatment also induced a certain portion of polyploid (triploid, tetraploid) and mixoploid plants – the analysed sample usually consisted of 2 different euploid cell populations (e.g. haploid and diploid cell population of comparable size) or was composed of euploid and aneuploid cell population, as revealed by flow-cytometry (Figure 3). Fully aneuploid plants were recorded only sporadically (Figure 4 B). Our results suggest about 10% spontaneous diploidization in the totally obtained regenerated plants. On the other hand, the diploidization by colchicine treatment was not completely effective and a relatively high portion (depending on the genotype) of the treated plantlets remained in a haploid status (Table 3). Nevertheless, some of the regenerated plantlets with recorded haploid status also produced a few seeds, because the young leaves used for flow-cytometric determination were often mixoploid, i.e. the measured sample represented probably chimeric tissues. After vernalization of haploid plants we tested a possibility of repeated diploidization using colchicine treatment on axillary bud meristems. Within repeatedly colchicine-treated haploid plants, the diploid status was additionally obtained in 11 hybrid lines (Table 4). The total time needed for the whole procedure, i.e. from seed germination of donor plants (inter-line hybrids) to seed production (R1) on DH

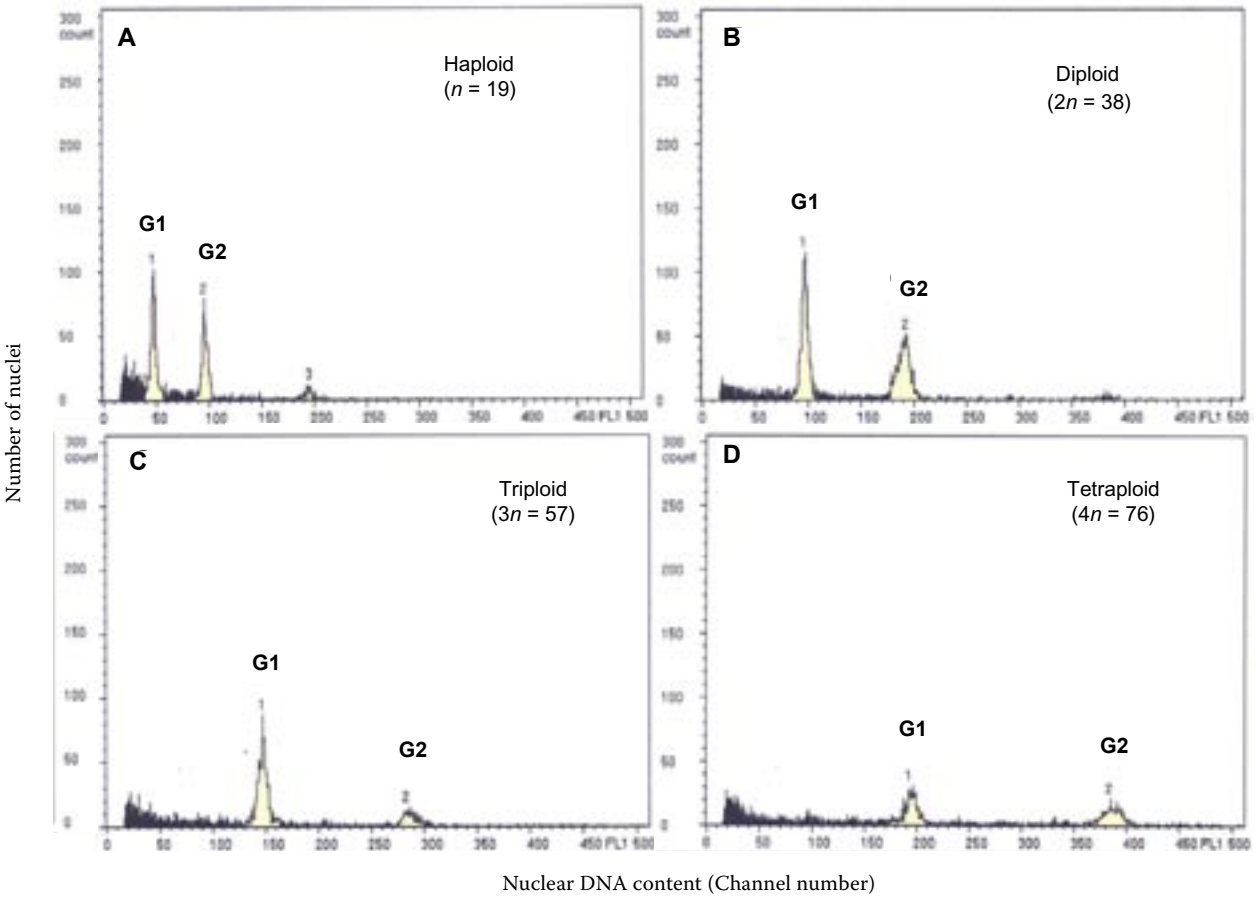

Figure 3. Flow-cytometric determination of DNA content/ploidy level of microspore-derived plants of winter rape; A – haploid plant (1*n* = 19); in fact a mixoploid with a small population of diploid cells (peak 3); B – diploid /doubled haploid plant (2*n* = 38); C – triploid plant (3*n* = 57); D – tetraploid plant (4*n* = 76)

Table 4. Proportions of fertile/sterile plants within the microspore regenerants obtained from selected F_1 inter-line hybrids; pooled data of several experiments

Genotype	No. of plants				
	total	fertile	%	sterile	%
8/03	8	2	25.0	6	75.0
9/03	28	11	39.3	17	60.7
12/03	77	55	71.4	22	28.6
15/03	10	7	70.0	3	30.0
37/02	13	11	85.0	2	15.0
40/02	13	5	38.0	8	62.0
51/02	4	2	50.0	2	50.0
54/02	9	4	44.4	5	55.6
63/02	11	4	36.0	7	64.0
65/02	1	1	100.0	0	100.0
75/02	11	10	91.0	1	9.0
Σ	185	112	60.5	73	39.5

plants, was approximately 17 months. The seed set of repeatedly colchicine-treated plants was very variable; some of them produced a sufficient amount of seeds, some only a few seeds and others were completely sterile (Table 4). Nevertheless, the repeated colchicine treatment increased the total number of DH plants per genotype and thus the final efficiency of the protocol. The seed progenies of produced DH lines were included in the current breeding programme "Czech Winter Rape" directed to desirable modifications of seed quality (data will be presented elsewhere).

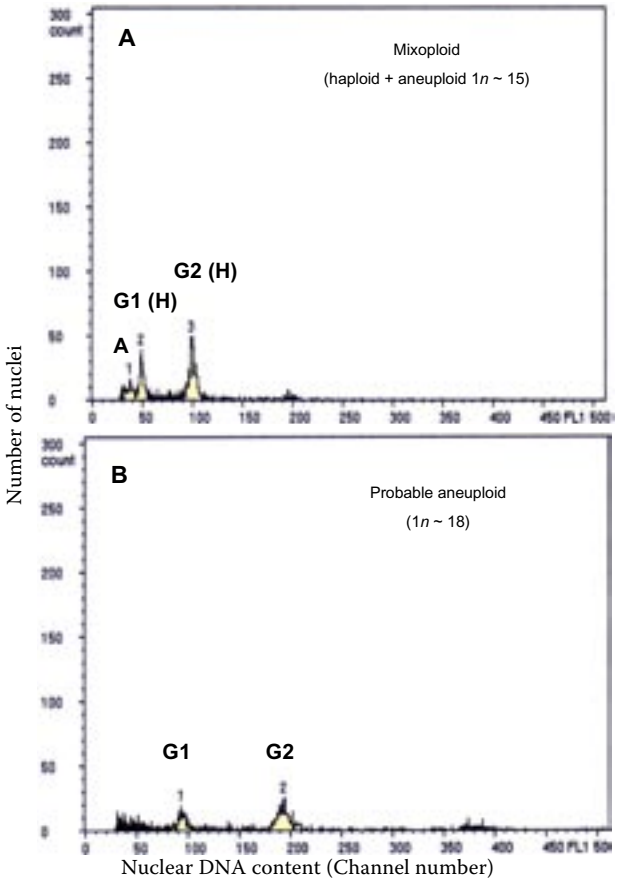

Figure 4. Flow-cytometric determination of DNA content/ploidy level of microspore derived plants of winter rape; A – mixoploid plant, i.e. predominantly haploid tissue (H; 1*n* = 19; peak 2 and 3) with a population of aneuploid cells (A; 1*n* ≅ 15 (19–4); peak 1); hybrid line 12/03; B – probable aneuploid plant (1*n* = 18 (19–1), 2*n* = 36); hybrid line 9/03

DISCUSSION

A series of the experiments was carried out in the course of four years (2002–2005) with the aim to adapt the published protocols of winter rape (*B. napus*) microspore embryogenesis and to optimize them into the form of routine methodology which would be applicable in breeders' practice. The genotype responsiveness was the most important factor affecting the efficiency of direct microspore-derived embryo production in our experiments (Table 1). It is not surprising

because even in responsive genotypes there may be differences between individual plants, particularly in self-incompatible species (Ferrie *et al.* 1995). It was evident that the presence of a definite parent (e.g. HSH, Table 1) in the hybrid combination played an important role in the competence for microspore-derived embryogenesis within the tested hybrid lines. As the knowledge of genes responsible for microspore embryogenesis in *B. napus* (two multiple gene loci with additive effects; Zhang & Takahata 2001) is currently available, it should be easily possible to cross highly embryogenic lines with recalcitrant ones in order to transfer the embryogenic potential (Ferrie & Keller 2004; Weber *et al*. 2005). In general, spring rape varieties (e.g. the frequently used model variety Topas; Pechan & Keller 1988; Huang *et al.* 1990; Custers 2003) have better responsiveness to microsporogenesis *in vitro* than winter forms (Gu *et al*. 2004). Thus, the protocols formulated for spring oilseed rape cannot be easily applied to winter rape germplasm.

In addition to genotype competence, embryogenesis was affected by the year as the same genotype behaved differently in particular years (data not shown) although standardized *in vitro* methodology (nutritional as well as physical conditions of *in vitro* culture) was used, thus reflecting mainly the actual physiological state of donor plants grown in the non-strictly controlled environment (greenhouse – vernalization room – greenhouse). It is well known that donor plants grown in a growth chamber in controlled conditions usually have higher frequencies of embryogenesis than plants grown in the field or in a greenhouse (Ferrie & Keller 2004). An optimum flower bud length giving the highest unicellular microspore yield with best embryogenic response was 2.5 to 3 mm in our experiments, which is a little different from other reports (e.g. 4–4.2 mm in Pechan & Keller 1988; 3.2–3.3 mm in Custers 2003); this difference may be explained mainly by specific growth conditions of donor plants and by the used genotypes (spring versus winter *B. napus*).

After inductive treatment with a high sucrose concentration (13%) and elevated temperature (30°C) followed by a drop to 26°C a portion of cultured microspores switched from their gametophytic pathway to start symmetric divisions (Smýkal 2000). Proembryos appeared and embryos became macroscopically visible within 10–14 days. However, the other part of microspores did not respond to this inductive treatment – probably a high portion of dead pollen cells in the liquid medium negatively affected cultured microspores, which led to the loss of their embryogenic competence (Binarová *et al*. 1997). The optimum microspore density used in our experiments was higher ($7.5–10 \times 10^4$/ml) than published elsewhere – in general 4×10^4/ml (Huang *et al*. 1990; Custers 2003; Friedt & Zarhloul 2005). In fact, the culture density higher than 10×10^4/ml is considered as inhibitory to embryogenesis (Huang *et al*. 1990; Weber *et al*. 2005). Nevertheless, a plating density 10 to 15×10^4/ml was necessary to achieve the highest embryogenesis frequency in *B. carinata* (Barro & Martin 1999). Thus, it is not possible to draw a general conclusion on optimum density not only for all *Brassica* species but also for the cultivars/lines/genotypes within this species (Friedt & Zarhloul 2005).

The induced microspore-derived embryos looked morphologically normal (Figure 1 A,B,C), but the rate of their direct conversion was very low. Similarly like other authors (Swanson *et al.* 1987; Cao *et al.* 1994), we mostly regenerated plants "indirectly" via adventitious shoot bud organogenesis from swollen embryos or callus (exactly as described by Tian *et al.* 2004: "...the majority of embryos developed into irregular masses of tissue with some leaf-like structures"). In contrast, we did not observe the typical secondary embryogenesis as a way of regenerating plants from original microspore embryos (Swanson *et al.* 1987; Tian *et al.* 2004). Detailed microscopic observations revealed the alterations connected with a shoot pole, namely cotyledon fusion accompanied by the absence of shoot apical meristem or by the presence of non-functional shoot apical meristem. Various abnormalities during embryo development, such as absence of meristems, early necrosis and polycotyly were reported in *B. rapa* (Cao *et al.* 1994). In our experiments germination of such abnormal embryos usually resulted only in root growth, while the shoot pole developed irregularly and embryos failed to produce normal complete plantlets. A similar situation was observed in pea somatic embryos (Griga 1998); these morphological defects could be overcome by the application of thidiazuron, a substance with strong cytokinin-like effect, which induced abundant multiple shoot formation on morphologically abnormal embryos. The removal of the cotyledons (Kott & Beversdorf 1990; Klíma *et al*. 2004)

improved the proliferation of the shoot pole of the embryos and resulted either in complete plantlets or in shoots/leaves which were subsequently easily rooted. Changes in nutritional conditions also led to some improvements in embryo development and germination: an increased level of Ca^{2+} and a higher supply of vitamins in MD medium (Tian *et al*. 2004) resulted in the increased frequency of regenerated plants (up to 65%; mean 29%). A more synchronous population of healthy-looking embryos could also be obtained by replacement of the high level of sucrose for polyethylene glycol with only 0.08–0.1% sucrose (Ilić-Grubor *et al*. 1998) or cold pretreatments of microspores (Gu *et al*. 2004). Cegielska-Taras *et al*. (2002) reported a very high germination/conversion rate (above 85%) in *B. napus* when a specific temperature regime was used for microspore-derived embryo culture, i.e. 1°C for 14 days, and then 24°C for the next 21 days. Embryos directly converting into plantlets may minimize possible genetic variation caused by callus formation and secondary embryogenesis or organogenesis (Tian *et al*. 2004).

The flow-cytometric determination of ploidy status revealed 10% frequency of spontaneous diploidization. It is a lower value than observed by Weber *et al*. (2005) in *B. napus* F_1 hybrids (ca. 23%). Spontaneous diploidization is also genotype-dependent (Möllers *et al.* 1994) and generally lower in hybrids than in parental genotypes (Weber *et al*. 2005). The induced diploidization using the whole plant body immersion in a colchicine solution was not completely effective. The other method, when the apical meristems, secondary buds, tillers or roots were treated, led to an increase in the diploid status by about 40%. This low frequency of diploidization can be increased either by the replacement of doubling agents or by another method of colchicine treatment. The recommended efficient direct application of colchicine to the microspore culture (Zhou *et al*. 2002) decreased the embryo yield in our experiments (Klíma, unpublished data). Another doubling agent trifluralin can be used to increase the frequency of non-chimeric fertile plants close to 60% in *Brassica napus* cv. Topas (Zhao & Simmonds 1995) or in *Brassica oleracea* (Rudolf *et al*. 1999). In our experiments the colchicine treatment resulted not only in doubled haploid but also triploid and tetraploid plants. Mixoploidy (aneusomaty) was also a frequently observed situation when a single sample contained cell populations of different ploidy (including the combination of euploid and aneuploid nuclei). Pure aneuploids were recorded only sporadically (Figure 4 B). Mixoploid plants usually produced some branches/flower parts with haploid or doubled haploid cells (Figure 2 G); the latter produced viable seeds. The repeated colchicine treatment of axillary flower bud meristems of plants originally determined as haploid also frequently led to chimeric (mixoploid) inflorescence formation with both fertile (doubled haploid) and sterile (haploid, mixoploid, aneuploid) flowers exhibiting altered flower morphology and flowering time. In resynthesized species of *B. napus* the allopolyploid phenotypes have many intermediate characters, e.g. different leaf and flower morphology, flower colour or flowering time (Himelblau *et al*. 2004; Osborn 2004) in consequence of instabilities in the genome structure and gene expression (Osborn 2004). Of course, from the breeding point of view, the other ploidy levels than doubled haploid ones are of little use – very few tetraploid plants set seed and no seeds were obtained from haploid, triploid, octoploid or aneuploid plants in *B. oleracea* (Farnham 1998).

In conclusion, we were able to produce fertile doubled haploid plants in twelve F_1 hybrids out of the total of twenty F_1 hybrid combinations requested by a breeder. The R2 generation of these twelve DH lines is now under field trials to evaluate their agronomic characters. Two steps of the used protocol seem to be the most critical: (1) correct development and high rate of direct germination of microspore-derived embryos, and (2) more efficient approach to diploidization (a higher proportion of doubled haploids as compared to haploids, polyploids and mixoploids in the treated population). The latest literature data show that a microspore colchicine treatment may improve both these parameters. In addition, the temperature changes as well as supplemental calcium and vitamins improve regular development of microspore-derived embryos and stimulate their high frequency of direct conversion. These methodological improvements will now be used for DH production within the "Czech Winter Rape" hybrid breeding programme.

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