EFFECTIVENESS OF SELECTED ANTIMITOTIC AGENTS IN MICROSPORE CULTURE FOR *BRASSICA CARINATA* DOUBLED HAPLOID PRODUCTION

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Abstract

The effects of colchicine, trifluralin and oryzalin on embryo production and chromosome doubling in embryogenic microspore cultures of Brassica carinata genotypes were examined. The application of colchicine stimulated the production of cotyledonary embryos in all genotypes tested when compared to the control variant. Mean rate of doubled haploid plants from the trifluralin treatment was 86.2%, followed by colchicine in vitro (69.0%), oryzalin (63.5%) and finally the in vivo colchicine treatment (49.5%). The level of spontaneous diploidization was only 40.2%. The highest frequency of abnormal plants was observed after oryzalin treatment (11.9%), the lowest from trifluralin (3.5%) and control (4.7%) treatments. The application of 10 µmol/l trifluralin solution during 18 hours of microspore culture was found to be the superior method for doubled haploid production. The use of trifluralin to double chromosomes in microspore culture could be more efficient, cheaper and less toxic substitution of widely used antimitotic agent colchicine for production of doubled haploid lines also in cultivars and breeding materials of Brassica carinata.

Key words: *Brassica carinata*, colchicine, doubled haploid, microspore culture, oryzalin, trifluralin

INTRODUCTION

Brassica is mainly self-pollinating oilseed crop that represents the third most important source of vegetable oil in the world and the third most important oil crop in the highlands of Ethiopia (CSA, 2003). Indeed, it is due to its great demand in the land that it has remained one of the most preferred crops in Ethiopian agricultural sector (Nigussie et al., 1999).

The techniques of isolated microspore culture are being widely used in *Brassica* breeding programmes to generate haploid and doubled haploid plants. The method was modified for Czech cultivars and breeding lines and later applied in the Czech breeding programs of different *Brassica* species.

The doubled haploid methodology is now employed in many *B. napus* and *B. carinata* breeding programs across the world as an alternative/supplement to conventional methods of homozygous line production (Friedt and Zarhloul, 2005). This timesaving method makes it possible to develop completely homozygous genotypes from heterozygous parents in one single generation. Besides, it also allows fixing recombinant gametes directly as fertile homozygous lines. Moreover, scientists are also with the idea that the process can enable a rapid development of homozygous plants and an efficient selection for recessive traits. It could also be used as a versatile genetic manipulation tool for *in vitro* selection and evaluation of desirable genotypes (Barro and Martin, 2001).

Plants regenerated from microspore-derived embryoids can be haploids, dihaploids or polyploids. Treatments with some antimitotic agents such as colchicine, trifluralin, oryzalin have been commonly employed to induce chromosome doubling of several plant species in various *in vitro* systems, e.g. anther culture, microspore culture, ovule culture, flower bud culture, embryo and cell suspension cultures. Additionally, some other compounds such as Polyethylene glykol (PEG), Pronamide, amiprophos-methyl (APM) and brassinosteroids have been used to generate doubled haploid (DH) plants and to improve the microspore embryogenesis in various *Brassica* species as well (Ferrie and Keller, 2007).

Conventional chromosome doubling techniques involves colchicine treatment of whole plants or injecting of a 2 mmol/l colchicine solution into secondary buds. The current methods of doubling the chromosome complements of haploids to produce fertile, homozygous doubled haploids are often inefficient and labour intensive.

Some studies reported that colchicine added directly to the microspore cultures improved embryogenesis and increased diploidization rate up to 80–85% (Weber et al., 2005). Several microtubule depolymerising herbicides also showed to be efficient for *in vitro* chromosome doubling of microspores. Zhao and Simmonds (1995) tested trifluralin and Hansen and Andersen (1996) trifluralin, oryzalin and amiprophos methyl in spring rapeseed (*B. napus*) cultivar Topas.

The main goal of this research work was to develop a simple and rapid procedure to generate fertile doubled haploids in *Brassica carinata* through the application of antimitotic agents (colchicine, trifluralin and oryzalin) to embryogenic microspore culture.

MATERIALS AND METHODS

Three *B. carinata* genotypes provided by the Czech University of Life Sciences, Institute of Tropics and Subtropics and developed by Doc. Ing. Miroslav Be-

chyne and one high productive but high erucic *B. carinata* genotype Dodolla was obtained from Ethiopia´s GeneBank. Donor plants of all *B. carinata* genotypes were grown in the growth chamber under controlled environmental conditions with 16/8 hours photoperiod and at day/night temperature 15/10°C. Mineral fertilizer "NPK" was applied weekly to all donor plants.

Protocol for microspore culture

Microspore isolation was carried out according to Coventy et al. (1988) with some modifications. Microspores were cultivated in NLN liquid medium (Lichter, 1985) supplemented with 13% (w/v) sucrose. The concentration of doubling agents in fresh NLN medium (working solutions) was as follows: 10 µmol/l solution of oryzalin, 10 µmol/l solution of trifluralin and 500 µmol/l solution of colchicine. The density was adjusted to 150 000 microspores per l ml of the solution. Microspore suspensions with individual working solutions were dropped to 9 cm plastic Petri dishes and cultivated for 18 hrs in the thermostat at 30°C in the dark. After 18 hours of treatment the suspensions were centrifuged for 10 minutes at 100 g. After supernatant removal, microspores were resuspended in fresh NLN medium, transferred to fresh Petri dishes and cultivated at the condition described by Klima et al. (2004, 2008). After two weeks of cultivation, Petri dishes were placed on the shaker (60 rpm) with 16/8 hrs. day/night photoperiod and light intensity of 260 µmol/m²/s. Green cotyledonary embryos (at least 3 mm in length) were counted in four-week old cultures.

Green cotyledonary embryos were transferred to differentiation medium (DM) solidified with 0.8% of agar, with 0.2 mg/l BAP, 0.2 mg/l IAA and 2% sucrose and maintained at 20°C with 16/8 hrs. day/night photoperiod and light intensity of 300 µmol/m²/s. After three weeks of cultivation the one third of embryo cotyledons were cut and embryos were transferred to regenerative medium (RM) solidified with 1% of agar. Well established plantlets were transferred to a rooting MS medium without growth regulators. Plantlets with roots were then transferred to the soil.

Doubling of chromosome number

The second method for chromosome doubling was *in vivo* application of aqueous solution, containing 500 µmol/l colchicine and 20 mmol/l dimethyl sulfoxide, to plantlets with 4–6 leaves and well formed roots just before transplanting to greenhouse conditions. The plantlets were washed under running tape water. The elongated roots were trimmed and the plantlets soaked in a colchicine solution. The beaker containing immersed plantlets was covered with poly-sheet and kept at 23°C in the light for 24 hours. The treated plantlets were then washed with distilled water and transferred to pots filled with good soil. To protect from direct light and keep in moisture plants were covered with polyethylene foil for 7 days.

Determination of ploidy level

Determination of doubled haploid (DH) regenerants was carried out by flow cytometry or by detection of sterile and fertile plants by means of evaluation of the morphological characteristics of inflorescence, production of mature pollen grains and seed set. Plants with different ploidy level than *n* and 2*n* or with abnormal morphological characteristics (e.g. with deformed flowers, low amount of seeds per pod etc.) were considered as abnormal.

Flow cytometry analysis: Flow cytometry analyses were carried out and evaluated at 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, Germany). Simplified two-step (without centrifugation) procedure using Otto I and Otto II buffer (Otto, 1990) and *Lycopersicon esculentum* cv. Stupické polní rané (2C = 1.96 pg) as an internal standard was used for ploidy analysis and genome size estimation.

Data analysis

To prepare the sets of measured values for the analysis of variance, the percentage data was transformed as follows:

The percentage data on abnormal plants was converted via square-root transformation, percentages of regenerants and doubled haploid plants were converted by means of Arc Sine transformation.

Analytic software "Statistica" (StatSoft, Inc., Tulsa, OK, USA) was used both for data analysis and the preparation of graphs.

RESULTS

Embryo frequency

In total, 2909 cotyledonary embryos were derived from all genotypes and treatments (Table 1). In terms of statistical calculations the effect of a genotype, the treatment with antimitotic chemicals and their interactions had significant impact on the embryo frequency.

Genotype BC-1 proved to be less embryogenic than other genotypes derived from control (untreated) variant (Figure 2). The number of cotyledonary embryos in case of genotype BC-1 was only 16.3 while the most productive genotype Dodolla achieved 55.3 embryos per Petri dish, BC-4 48.0 and BC-2 41.7 embryos in untreated (control) variant (Table 1). Significant differences between genotypes were proved within other individual treatments as well (Figure 2). Homogeneous groups on embryo frequency designed from multiple comparisons between means across genotypes and treatments are shown in Table 1.

In general, particular antimitotic agents used did not negatively affect embryogenesis in term of embryo production in any of tested genotypes; conversely, their stimulation effect on embryo production was observed

Tab. 1: The number of cotyledonary embryos per Petri dish and the regeneration ability (%) in individual genotypes and treatments

Genotype	Replication No.	Untreated (Control)			Colchicine			Trifluralin			Oryzalin		
		No. of Embryos*	No. of Regenerants	Regeneration ability [%]**	No. of Embryos*	No. of Regenerants	Regeneration ability [%]**	No. of Embryos*	No. of Regenerants	Regeneration ability [%]**	No. of Embryos*	No. of Regenerants	Regeneration ability [%]**
Dodolla	1	64	45	70.31	147	72	48.98	25	15	60.00	72	48	66.67
	2	49	31	63.27	121	81	66.94	69	47	68.12	79	56	70.89
	3	53	42	79.25	137	90	65.69	86	56	65.12	91	64	70.33
	Mean	efg 55.33	39.33	abc 71.08	a 135.00	81.00	c 60.00	efg 60.00	39.33	bc 65.56	cd 80.67	56.00	abc 69.42
BC-4	1	54	37	68.52	107	63	58.88	61	41	67.21	65	43	66.15
	2	43	29	67.44	110	92	83.64	75	57	76.00	71	56	78.87
	3	47	32	68.09	93	70	75.27	79	54	68.35	81	60	74.07
	Mean	fgh 48.00	32.67	abc 68.06	a 103.33	75.00	ab 72.58	cde 71.67	50.67	abc 70.70	cde 72.33	53.00	ab 73.27
BC-1	1	11	10	72.73	32	21	65.63	16	11	56.25	21	16	57.14
	2	17	12	70.59	27	18	66.67	28	20	71.43	25	17	68.00
	3	21	13	61.90	38	29	76.32	35	27	77.14	30	23	76.67
	Mean	j 16.33	11.67	abc 67.35	hij 32.33	22.67	abc 70.10	ij 26.33	19.33	abc 70.89	ij 25.33	18.67	abc 68.42
BC-2	1	39	27	69.23	79	52	65.82	41	29	70.73	55	40	72.73
	2	45	34	75.56	83	57	68.67	69	50	72.46	70	55	78.57
	3	41	30	73.17	87	61	70.11	56	42	75.00	64	52	81.25
	Mean	ghi 41.67	30.33	ab 72.80	c 83.00	56.67	abc 68.27	efg 55.33	40.33	ab 72.89	def 63.00	49.00	a 77.78
	Total	40.33		69.82	88.42		67.74	53.33		70.01	60.33		72.22

Letters a–j and a–-c in columns and rows designate homogeneous groups (LSD; $P = 0.05$) derived from multiple comparisons between means across genotypes and treatments for the number of embryos per Petri dish (*) or for the regeneration ability from cotyledonary embryos to whole plants (**)

(Figure 3). Treatment with colchicine significantly increased the number of embryos in comparison with control (untreated) variants in all genotypes tested. Furthermore, in case of genotype BC-2 the embryo frequency was demonstrably higher after addition of oryzalin, and from trifluralin and oryzalin treatment in case of genotype BC-4 as well (Figure 4). The most stimulating effect of an antimitotic chemical was observed in genotype Dodolla derived from colchicine treatment, where the number of embryos per Petri dish increased from 55.3 (untreated control) to 135.0 (Table 1, Figure 4).

Embryo development and regeneration ability

Notable differences in embryo development and morphology were detected between particular treatments and in comparison with control variants in all genotypes tested. In general, embryos derived from trifluralin treatment were thinner and with smaller cotyledons than those from control treatment; on the contrary, thicker and smaller embryos were obtained from oryzalin treatment (Figure 1). In addition, trifluralin and oryzalin slowed down embryo development by about one week whereas application of colchicine slightly accelerated embryo development in comparison with control variant. In spite of above mentioned facts, in general, neither the effect of the treatment (nor the genotype, respectively) was significant in terms of regeneration ability (i.e. in the conversion from embryos to regenerants). The percentage of regenerants ranged from 67.7% (colchicine) to 72.2% (oryzalin) between treatments (Table 1)**;** some difference was detected within genotype BC-2, where the value of the regeneration ability from oryzalin treatment (77.8%) was significantly higher than the value from colchicine treatment (68.3%) of the same genotype (Figure 5). Another difference, resulted from multiple comparisons between means of genotypes Dodolla from colchicine (60.0%) and BC-2 from oryzalin treatment (77.8%), is shown in Table 1.

Diploidization frequency

In total, 1 037 flowering regenerants were obtained and analyzed for their ploidy level (Table 2, Figure 10, 11 and 12). All antimitotic chemicals applied *in vitro* and

Tab. 2: Percentage rates of doubled haploid (DH) and abnormal (ABN) plants derived from individual genotypes and treatments [%]

Genotype	Replication No.	Untreated (Control)			Colchicine *in vivo*			Colchicine *in vitro*			Trifluralin			Oryzalin		
		No. of Plants tested	Percentage of DH Plants *	Percentage of ABN Plants **	No. of Plants tested	Percentage of DH Plants *	Percentage of ABN Plants **	No. of Plants tested	Percentage of DH Plants *	Percentage of ABN Plants **	No. of Plants tested	Percentage of DH Plants *	Percentage of ABN Plants **	No. of Plants tested	Percentage of DH Plants *	Percentage of ABN Plants **
Dodolla	1	14	35.71	0.00	20	45.00	5.00	30	73.33	6.67	30	86.67	3.33	30	73.33	13.33
	2	13	38.46	0.00	16	50.00	6.25	24	58.33	4.17	22	90.91	0.00	24	70.83	12.50
	3	15	46.67	6.67	19	57.89	5.26	32	68.75	6.25	20	85.00	0.00	24	75.00	8.33
	Mean		ij 40.28	ab 2.22		fghi 50.96	abcd 5.50		de 66.81	abcd 5.69		ab 87.53	a 1.11		cd 73.06	cd 11.39
BC-4	1	10	40.00	10.00	16	37.50	12.50	32	75.00	9.38	30	93.33	6.67	30	53.33	13.33
	2	14	42.86	0.00	17	64.71	0.00	20	70.00	5.00	20	90.00	5.00	24	66.67	8.33
	3	11	45.45	9.09	15	fghi 53.33	6.67	28	71.43	7.14	14	85.71	0.00	21	71.43	14.29
	Mean		ij 42.77	abcd 6.36		51.85	abcd 6.39		cd 72.14	bcd 7.17		a 89.68	abc 3.89		def 63.81	cd 11.98
BC-1	1	10	30.00	10.00	11	27.27	9.09	10	60.00	10.00	11	81.82	9.09	16	62.50	12.50
	2	6	33.33	0.00	12	58.33	0.00	10	80.00	10.00	12	75.00	0.00	9	55.56	11.11
	3	8	50.00	12.50	9	55.56	11.11	9	66.67	11.11	6	83.33	0.00	11	63.64	18.18
	Mean		j 37.78	abcd 7.50		hij 47.05	abcd 6.73		cde 68.89	cd 10.37		bc 80.05	ab 3.03		defg 60.56	d 13.93
BC-2	1	8	37.50	0.00	26	42.31	11.54	18	66.67	5.56	20	90.00	10.00	20	60.00	15.00
	2	11	36.36	0.00	17	47.06	11.76	20	65.00	5.00	13	84.62	7.69	11	45.45	9.09
	3	26	46.15	7.69	18	55.56	11.11	22	72.73	4.55	8	87.50	0.00	14	64.29	7.14
	Mean		ij 40.01	ab 2.56		ghij 48.31	cd 11.47		de 68.13	abcd 5.03		ab 87.37	abcd 5.90		efgh 56.58	bcd 10.41
	Total	146	40.21	4.66	196	49.54	7.52	255	68.99	7.07	206	86.16	3.48	234	63.50	11.93

Letters a–j and a–d in columns and rows designate homogeneous groups (LSD; P = 0.05) derived from multiple comparisons between means across genotypes and treatments for the percentage of DH plants (*) or for the occurrence of Abnormal plants (**)

colchicine *in vivo* significantly increased the rate of DH plants in comparison with untreated control variant (Figure 6). The most effective antimitotic agent in our experiments proved to be chemical trifluralin (86.2) followed by colchicine *in vitro* (69.0%), oryzalin (63.5%) and finally *in vivo* colchicine treatment (49.5%); the level of spontaneous diploidization was only 40.2% (Table 2, Figure 6). The efficiency of applied chemicals within individual genotypes was similar to the mean from all genotypes tested except genotype BC-2, where the rate of diploidization from *in vitro* colchicine treatment was different from the oryzalin treatment of the same genotype (Figure 7).

While the influence of treatments on the rate of DH plants was important, in general, neither genotype nor interaction between genotype and treatment were relevant. Some differences were observed in case of trifluralin and oryzalin applications, where the genotype BC-1 produced significantly lower rate of DH regenerants than some other genotypes under the same treatment (Figure 8). Differences resulted from multiple comparisons between means across genotypes and treatments, expressed as homogeneous groups, are shown in Table 2.

Occurrence of abnormal plants

Abnormal plants (ABP) were observed in all treatments including control variants and in all genotypes tested (Table 2, Figure 9). The highest frequency of ABP was observed in case of oryzalin treatment (11.9%). On the contrary, the lowest frequencies were obtained in plants derived from trifluralin (3.5%) and control (4.7%) variants. Generally, control and trifluralin treatments generated significantly less ABP than those from oryzalin

and *in vitro* colchicine treatment (Figure 9). Similarly no differences were detected between genotypes in terms of predisposition to particular treatments (data not shown).

DISCUSSION

This is the first study evaluated the efficiency of several *in vitro* and partially *in vivo* applied antimitotic agents on embryo production and induction of doubled haploid plants in selected genotypes of *Brassica carinata.* Our results confirmed the significant impact of the genotype on embryo production in microspore cultures. For instance genotype Dodolla in our experiments produced three-times more cotyledonary embryos than the less productive cultivar BC-1 (55.3 vs. 16.3 embryos per Petri dish). Conformable results were previously published in *B. napus* (Huang et al., 1990) and *B. carinata* (Barro and Martin, 1999).

Reported influence of antimitotic agents on induction of embryogenesis was not observed in all genotypes and treatments tested. Embryo yield in genotypes BC-1 and Dodolla was increased only by application of colchicine. These results may indicate that stimulation of embryogenesis by means of selected antimitotic agents is genotype dependent (Baillie et al., 1992). Generally, colchicine solution had the most stimulating effect on embryo production and rapid development in our experiments. Positive effect of colchicine on embryogenesis was also determined in other genotypes of genus *Brassica* (Zhang et al., 2003). Although the efficiency of other chemicals tested (i.e. oryzalin and trifluralin) was considerably lower in comparison with colchicine, the number of embryos derived from those treatments was in most cases higher than in the control (untreated) variant.

It was observed that the effect of applied chemicals on further regeneration in general was not significant. Even though the embryo morphology could affect shoot tip proliferation and thus the percentage of direct regeneration (Zhao and Simmonds, 1995), this phenomenon was not confirmed in our 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 in general was not discovered as the method of cutting off cotyledons was applied to all cultivated embryos to increase the number of regenerants for further analysis of ploidy level. Thus, conditions for direct regeneration (from apical meristem) were provided even for not properly developed embryos.

The rate of spontaneous diploidization pooled for all genotypes (40.2%) was similar to the results published by Weber et al. (2005) in spring Canadian cultivars of oilseed rape and *B.carinata* (50% of Barro et al. 2001-delete). All *in vitro* tested agents increased significantly the rate of DH plants in comparison with control variants. Positive effect of antimitotic chemicals on the rate of DH plants was observed in spring and winter *B. napus* by Zhou et al. (2002a, b). Likewise in some other experiments with *B. napus* by Zhao and Simmonds (1995) in *B. oleracea*, trifluralin provided the highest rate of DH plants derived (86.2%). The effectiveness of other *in vitro* applied chemicals (colchicine and oryzalin) was slightly lower (69.0% and 63.5%, respectively). Although the rate of DH regenerants obtained from *in vivo* colchicine application (49.5%) was significantly higher when compared to control variants (40.2%), the level of doubled haploids was not sufficient enough to utilize the *in vivo* colchicine application in a large-scale (i.e. for breeding purposes of *Brassica carinata*). Some authors have published similar results on low efficiency (i.e. low embryogenesis and diploidization rate) of *in vivo* colchicine treatment in comparison with *in vitro* colchicine treatment in *B. napus* as well (Hansen and Andersen, 1996, Zhao et al., 1996). Nevertheless, wide differences between successive replications after *in vivo* colchicine application can be explained by some variations in plant material (e.g. in physiological condition, developmental stage etc.) in spite of precious preparation of regenerants in accordance with methodology.

As have been observed, in general, no significant impact of a genotype was detected in the rate of DH plants derived from treatments with antimitotic agents. Conformable results were published by Hansen and Andersen (1996) and Zhao et al. (1996). On the contrary, remarkable differences between genotypes were observed in *Brassica napus*. Furthermore, the highest occurrence of abnormal plants (ABP) was observed in regenerants derived from oryzalin treatment (11.9%), following *in vivo* and *in vitro* application of colchicine. On the contrary, relatively small amount of ABP was derived from trifluralin and control (untreated) treatments. Zhao and Simmonds (1995) demonstrated the highest frequency in the rate of ABP after using continuous treatment with colchicine in *B. napus*.

CONCLUSION

The effect of selected *in vitro* and partially *in vivo* applied antimitotic agents on embryo production, morphology, on the rate of DH and abnormal plants was evaluated in four genotypes of *Brassica carinata.* Traditional method for chromosome doubling – rinsing of plants in aqueous colchicine solution – showed to be not efficient enough and unreliable. The effectiveness of *in vivo* application was nearly identical to spontaneous diploidization. Moreover the appearance of chimeric plants after such application was detected in all genotypes and replications. Finally, for using in breeding stations, huge amount of rather expensive and dangerous colchicine is needed.

Although oryzalin offered better results than *in vivo* colchicine application, the amount of abnormal plants was

relatively high and, additionally, offered smaller and thicker embryos required more precise manipulation.

Colchicine applied *in vitro* appeared to be one of the best chemicals in our experiments for its ability to increase the number of cotyledonary embryos, to speed up the development of maturing embryos and above all for its efficiency in chromosome doubling.

Although the application of trifluralin slightly suppressed embryo development and led to smaller embryos, the percentage of DH plants (86.2%) was the highest and stable among all successive replications and genotypes. In some replications of individual genotypes more than 90% of doubled haploids were achieved. In addition, the frequency of abnormal plants was even lower than in case of the control variant.

With respect to above mentioned facts, trifluralin could be more efficient, cheaper and less toxic substitution of widely used antimitotic agent colchicine for production of doubled haploid lines also in cultivars and breeding materials of *Brassica carinata.*

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Figure 1: Morphological characteristics of 30-day-old microspore embryos in the particular *in vitro* treatments with antimitotic agents (Genotype BC-4)

A - Control, B - Colchicine, C - Oryzalin, D - Trifluralin; bar = 1 millimeter

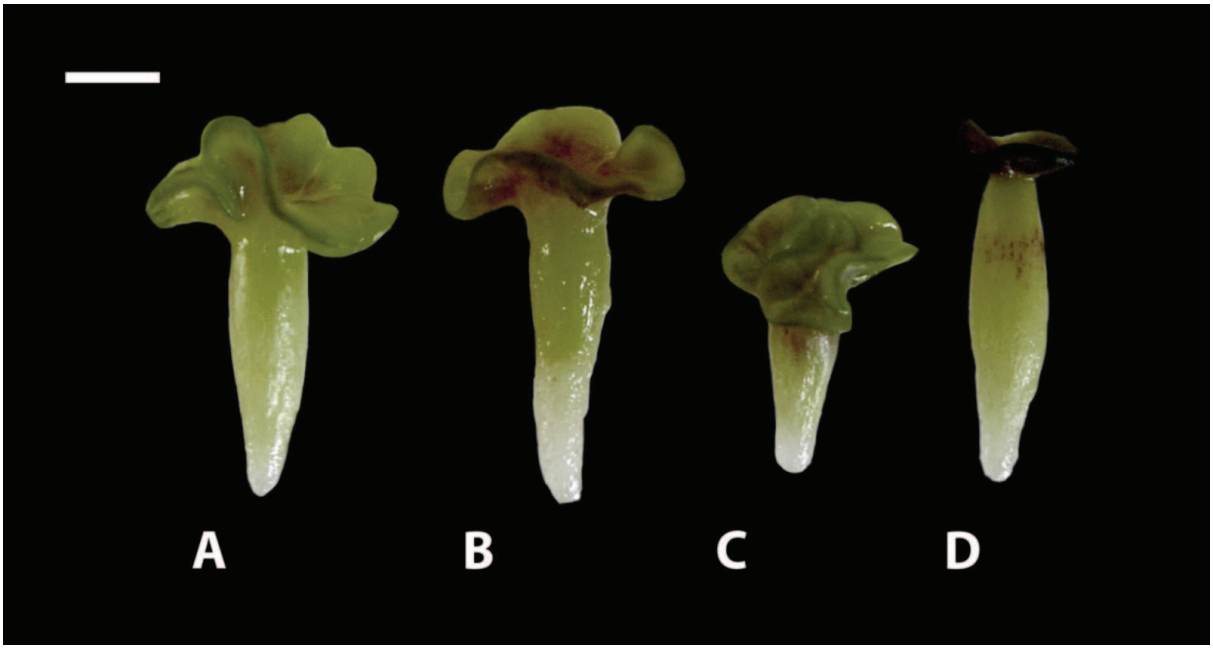


Figure 2: The impact of individual genotypes on the number of cotyledonary embryos per Petri dish in particular *in vitro* treatments; pooled data for three successive replications

Bars represent individual 95% confidence intervals
Letters a-d, A-C, α-β and A-Γ designate homogeneous groups (LSD; $P = 0.05$)

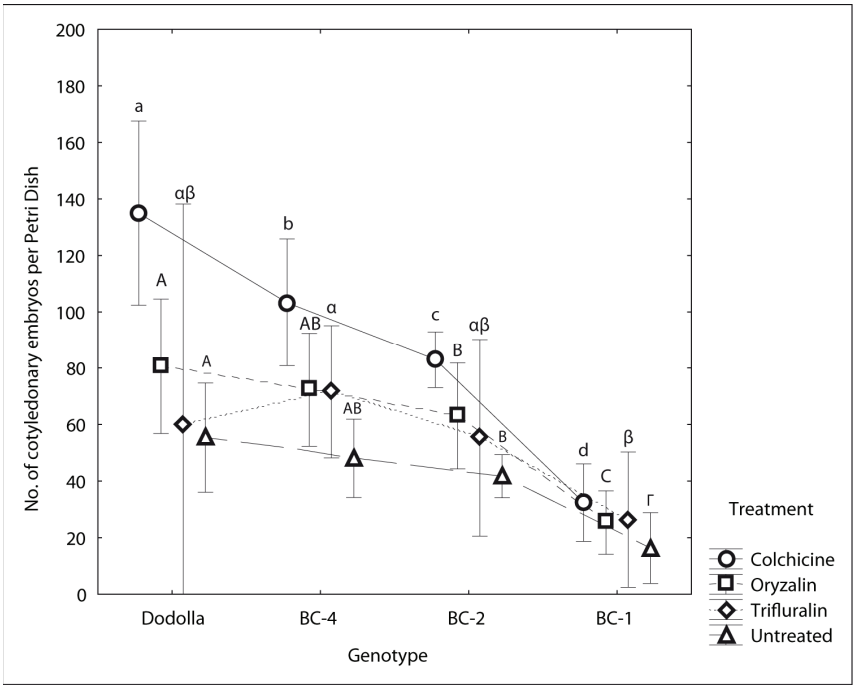


Figure 3: The impact of particular *in vitro* treatments on the number of cotyledonary embryos per Petri dish; pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals
Letters a-c designate homogeneous groups (LSD; $P = 0.05$)

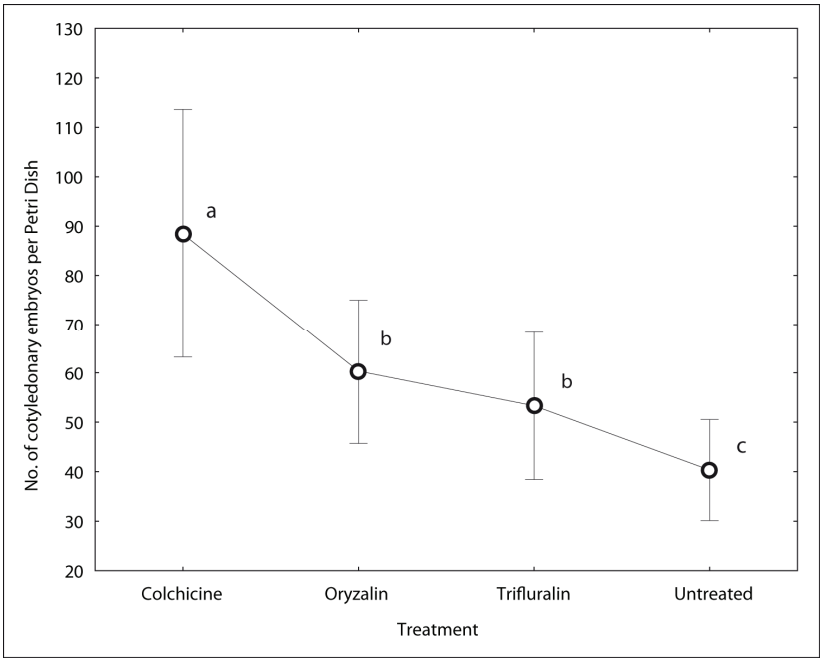


Figure 4: The impact of particular *in vitro* treatments on the number of cotyledonary embryos per Petri dish in individual genotypes; pooled data for three successive replications

Bars represent individual 95% confidence intervals
Letters a-b, A-C, α-β and A-Γ designate homogeneous groups (LSD; $P = 0.05$)

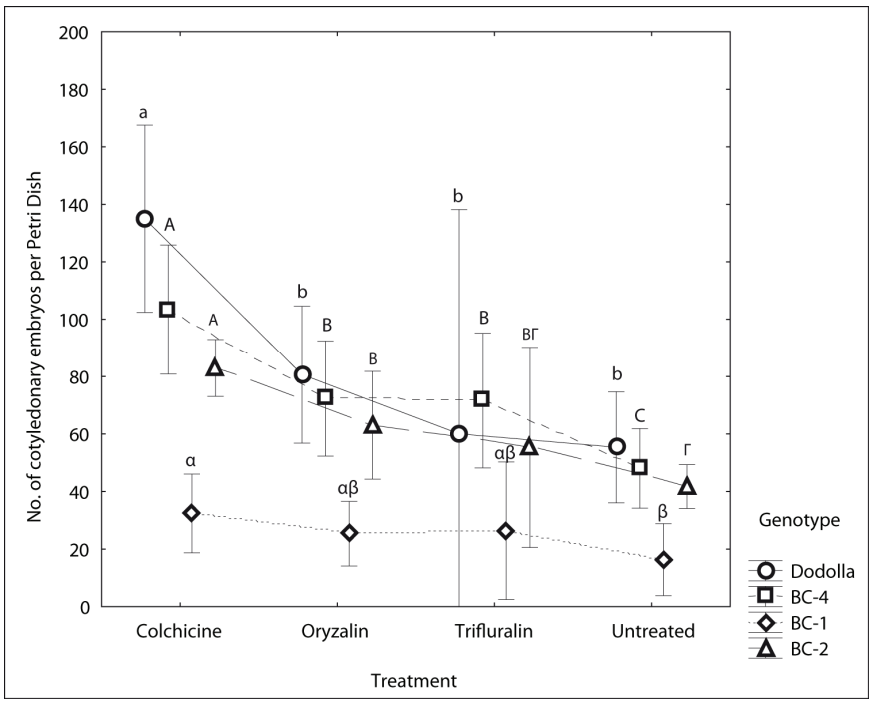


Figure 5: The impact of particular *in vitro* treatments on the regeneration ability [%] in individual genotypes; pooled data for three successive replications

Bars represent individual 95% confidence intervals
Letters a-b designate homogeneous groups (LSD; $P = 0.05$); individual genotypes, not marked with letters, are members of one group only

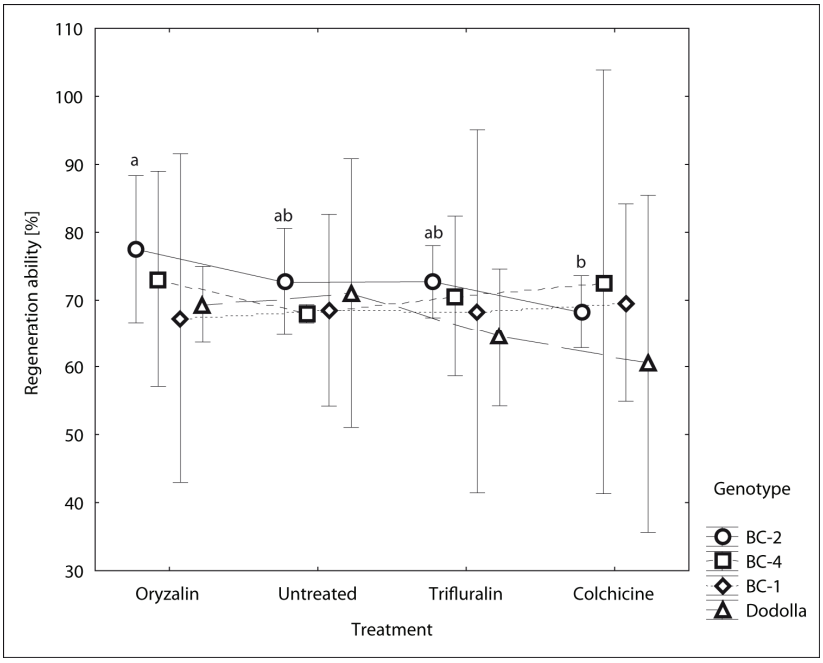


Figure 6: The impact of particular treatments on the rate of DH plants [%]; pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals
Letters a-d designate homogeneous groups (LSD; $P = 0.05$)

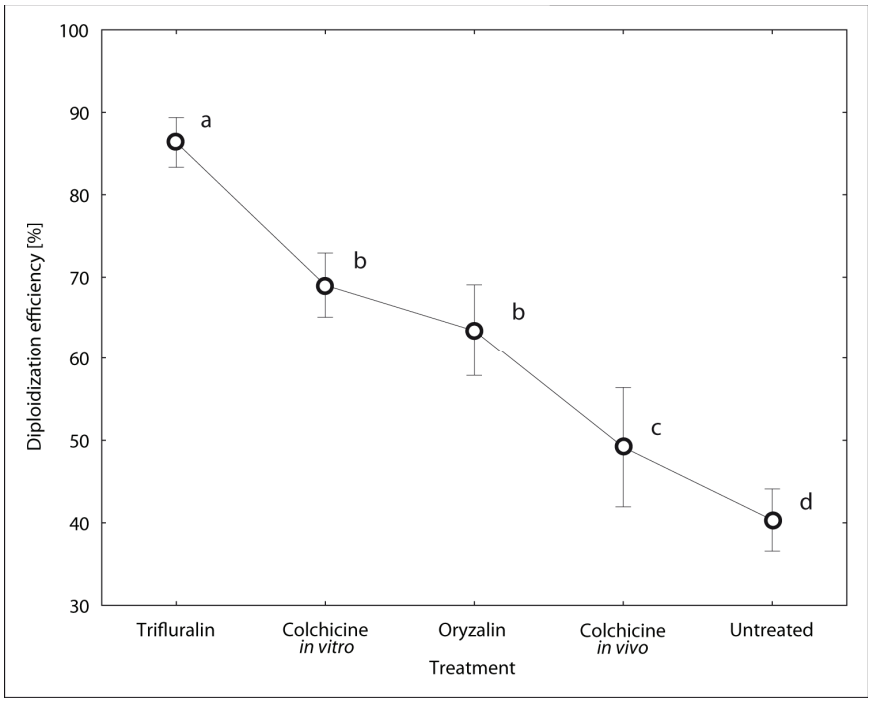


Figure 7: The impact of particular treatments on the rate of DH plants [%] in individual genotypes; pooled data for three successive replications

Bars represent individual 95% confidence intervals
Letters a-d, A-D, α-δ and A-Δ designate homogeneous groups (LSD; $P = 0.05$)

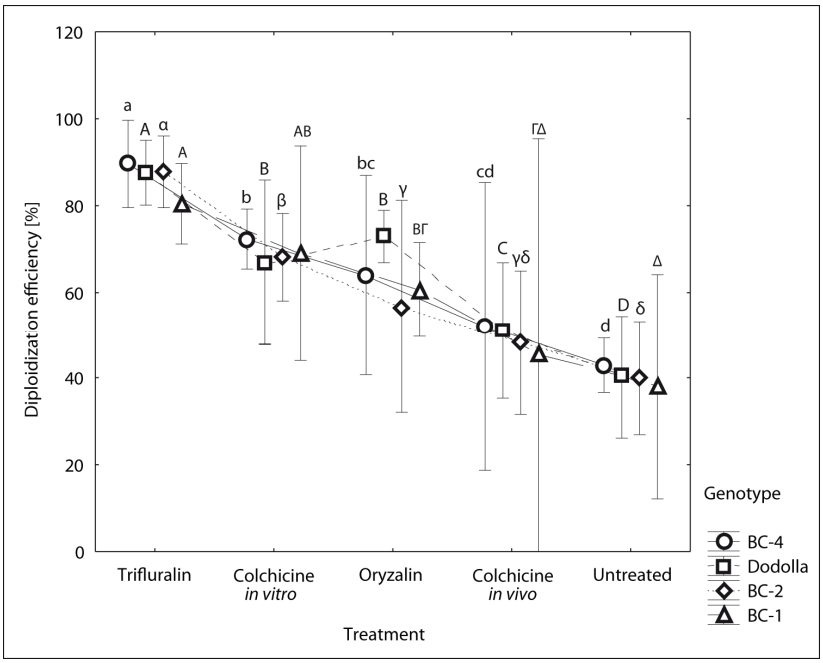


Figure 8: The impact of individual genotypes on the rate of DH plants [%] in particular treatments; pooled data for three successive replications

Bars represent individual 95% confidence intervals
Letters a-b and A-B designate homogeneous groups (LSD; $P = 0.05$); particular treatments, not marked with letters, are members of one group only

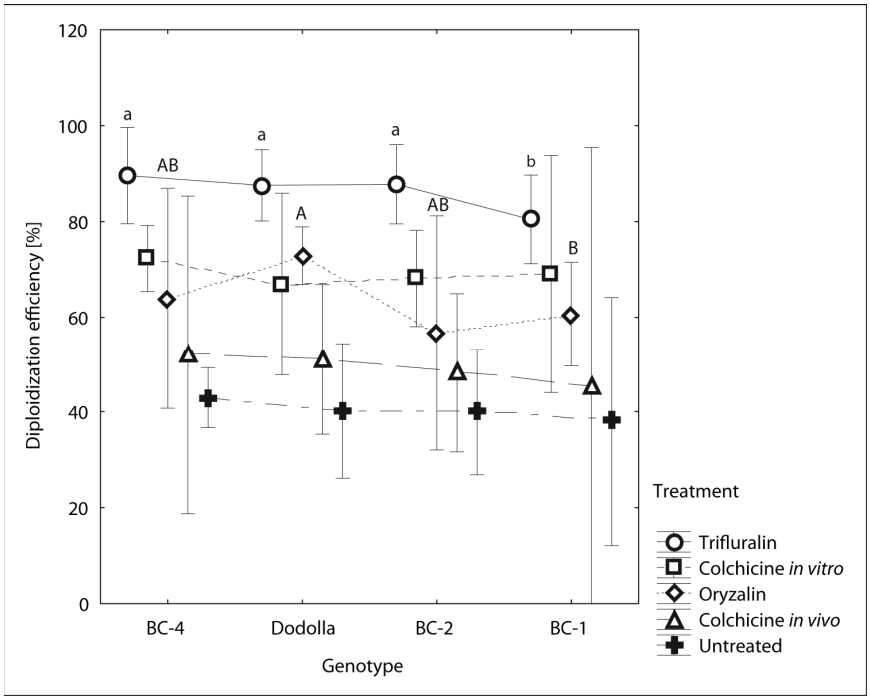


Figure 9: The impact of particular treatments on the rate of abnormal plants [%]; pooled data for three genotypes and three successive replications

Bars represent individual 95% confidence intervals
Letters a-c designate homogeneous groups (LSD; $P = 0.05$)

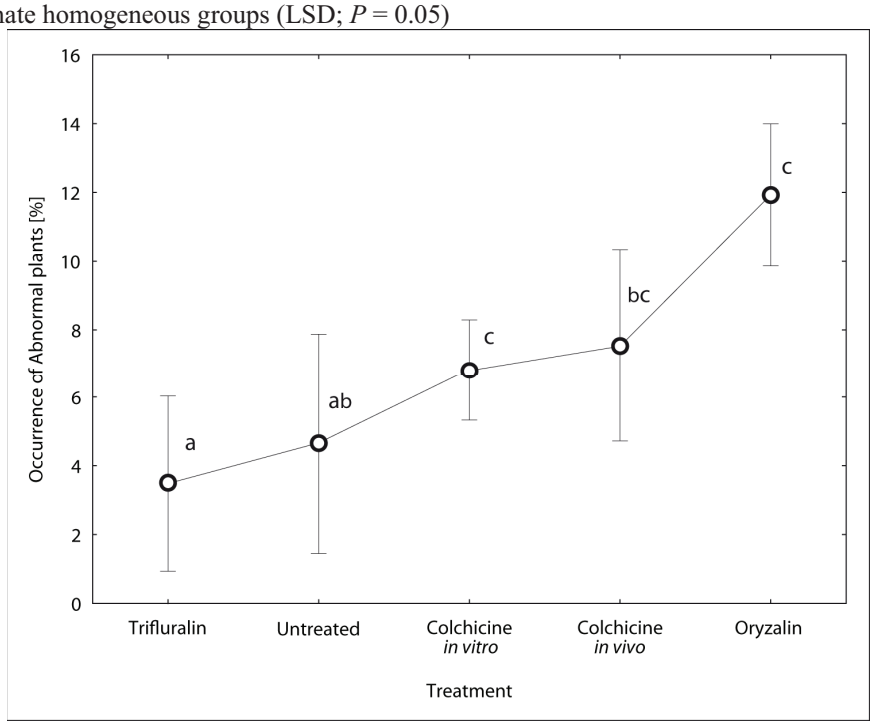


Figure 10: Flow cytometry histograms of microspore derived haploid plant

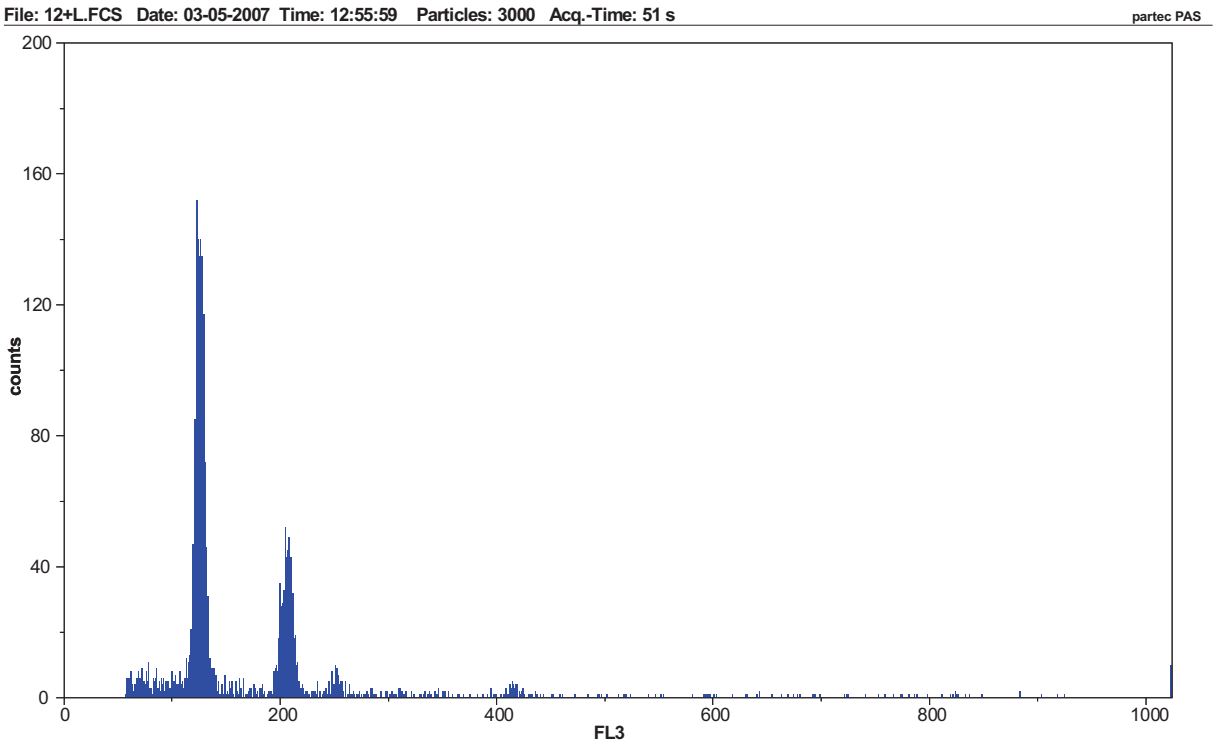


Figure 11: Flow cytometry histograms of microspore derived doubled haploid plant

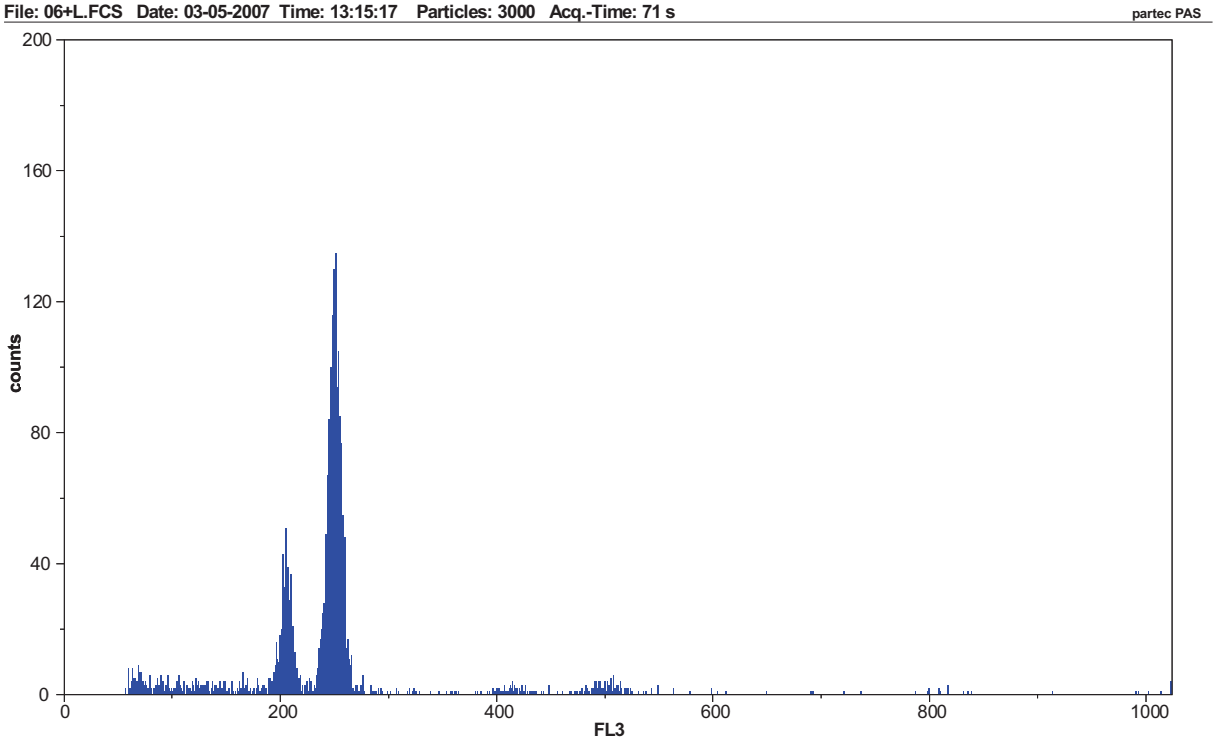
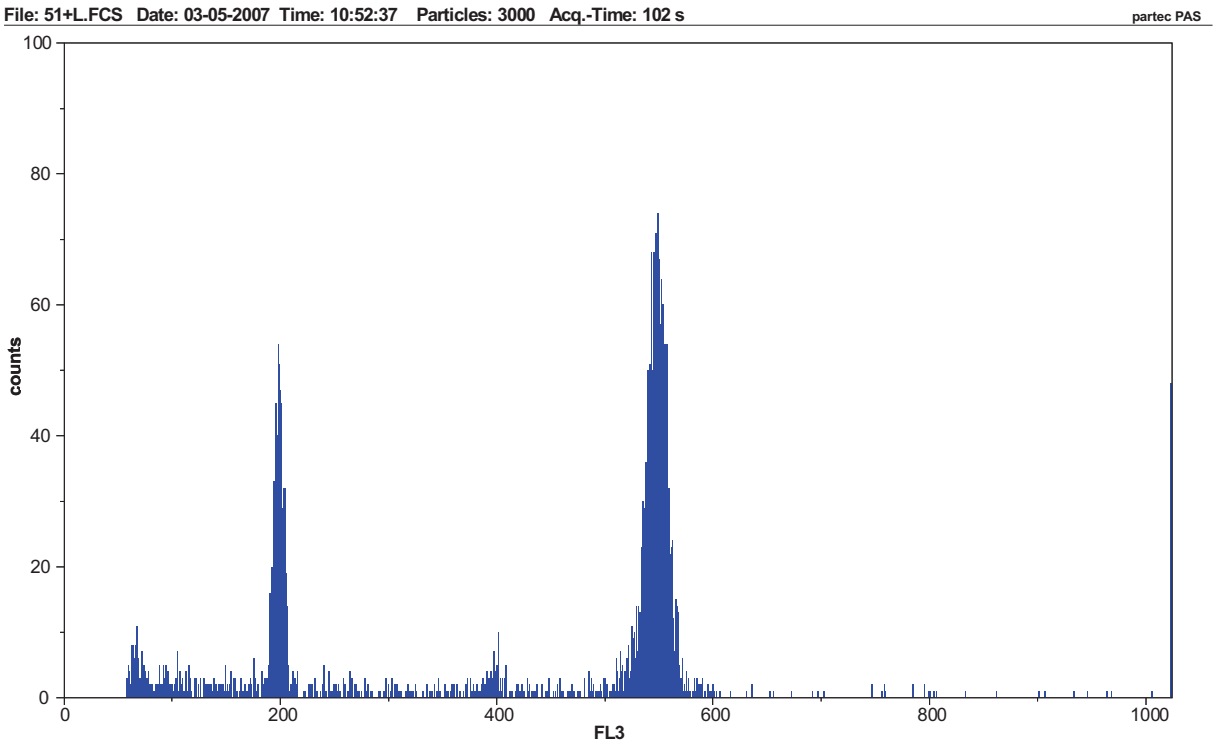


Figure 12: Flow cytometry histograms of microspore derived nonaploid plant



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