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 amiprophen methyl in the spring rapeseed cultivar Tospas. 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 F₁ 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 F₁ 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/m²/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 (Vvadilová et al. 1993; Möllers et al. 1994; Hansen & Andersen 1996; Zhou et al. 2002). The microspore density was adjusted to 10⁴ 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 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 (Vvadilová et al. 1993; Möllers et al. 1994; Hansen & Andersen 1996; Zhou et al. 2002). The microspore density was adjusted to 10⁴ 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 resuspended in NLN liquid 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 Vvadilová 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 cm²) 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).
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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Replication No.</th>
<th>No. of plants tested</th>
<th>Control</th>
<th>Colchicine <em>in vivo</em></th>
<th>Colchicine <em>in vitro</em></th>
<th>Oryzalin <em>in vitro</em></th>
<th>Trifluralin <em>in vitro</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-3/04</td>
<td>1</td>
<td>174</td>
<td>61.76</td>
<td>66.67</td>
<td>77.78</td>
<td>72.73</td>
<td>89.29</td>
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<td></td>
<td>2</td>
<td>166</td>
<td>50.00</td>
<td>70.83</td>
<td>70.45</td>
<td>91.67</td>
<td>80.00</td>
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<tr>
<td></td>
<td>3</td>
<td>220</td>
<td>54.84</td>
<td>75.76</td>
<td>81.63</td>
<td>93.67</td>
<td>71.43</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>560</td>
<td>mean 55.53 a</td>
<td>71.09 a</td>
<td>76.62 a</td>
<td>86.02 a</td>
<td>80.24 a</td>
</tr>
<tr>
<td>OP-41/1</td>
<td>1</td>
<td>177</td>
<td>36.36</td>
<td>51.11</td>
<td>86.96</td>
<td>75.76</td>
<td>87.50</td>
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<td></td>
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<td>136</td>
<td>34.48</td>
<td>47.83</td>
<td>68.00</td>
<td>70.00</td>
<td>93.10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>302</td>
<td>47.17</td>
<td>58.02</td>
<td>76.92</td>
<td>63.64</td>
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<td>mean 39.34 ab</td>
<td>55.65 ab</td>
<td>77.29 a</td>
<td>69.80 a</td>
<td>88.07 a</td>
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<tr>
<td>SL-2/04</td>
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<td>19.05</td>
<td>53.33</td>
<td>56.25</td>
<td>32.69</td>
<td>90.70</td>
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<td>187</td>
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<td>88.89</td>
<td>43.75</td>
<td>93.48</td>
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<tr>
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<td>154</td>
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<td>60.00</td>
<td>54.90</td>
<td>81.82</td>
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<tr>
<td></td>
<td>total</td>
<td>534</td>
<td>mean 31.88 b</td>
<td>39.94 b</td>
<td>68.38 a</td>
<td>43.78 b</td>
<td>88.67 a</td>
</tr>
<tr>
<td>Mean</td>
<td>total</td>
<td>1709</td>
<td>total mean 42.25</td>
<td>55.56</td>
<td>74.10</td>
<td>66.53</td>
<td>85.66</td>
</tr>
</tbody>
</table>

Letters a and b in columns designate homogeneous groups (LSD; *P* = 0.05)
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 with antimitotic agents.
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.

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