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Impact of selected antimitotic substances on doubled haploid and polyploid regeneration in microspore cultures of swede (*Brassica napus* ssp. *napobrassica* (L.) Hanelt)

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Abstract: Several antimitotic agents were tested in three embryogenic doubled haploid (DH) lines of swede (*Brassica napus* ssp. *napobrassica*). No effect on embryogenesis was observed at the given concentrations of colchicine (5 µmol/L or 50 mg/L) and treatment time of 24 hours. Flow cytometric analysis of microspore embryos revealed a significant increase in the percentage of DH compared to the control for all substances: ethalfluralin, 62.2%; trifluralin, 58.3%; colchicine, 56.1%; amiprophos-methyl, 54.4%; pronamide, 35.0%; control, 13.3%. The occurrence of pure tetraploids was low (0–5%), mixoploids – especially $n+2n$ – were high in all treatments, including the control (29.4–71.1%). Their presence can be explained as a consequence of spontaneous diploidisation and/or endoreduplication during embryogenesis or embryo germination.

Keywords: endopolyploidy; haploids; *in vitro* diploidization; microspore embryos; mixoploids

Swede [*Brassica napus* ssp. *napobrassica* (L.) Hanelt] (AACC, $2n = 38$) is a root vegetable of the Brassicaceae family, used as both food and fodder. Currently, the most important areas in terms of its cultivation and use are the provinces of Eastern Canada, the states of Northern Europe, Scotland, Russia and New Zealand (Spaner 2002; Gowers 2010). Due to its close relationship with oilseed rape (*Brassica napus* L. var. *oleifera*), swede breeding has also undergone a similar route: from population breeding to the production of self-pollinated line varieties and finally hybrids based on self-sterility and cytoplasmic male sterility (see review by Gowers 2010). Bradshaw and Wilson (1993) compared the efficiency of swede hybrid and line breeding and concluded that line breeding was a better choice for the materials and methods studied. Single seed descent (Bradshaw et al. 2009) and the use of the microspore culture approach (Hansen & Svinnset 1993; Hansen & Bratberg 2003) brought further progress in improving the uniformity of swede. *In vitro* chromosome doubling using a variety of antimitotic agents has become an effective technique in breeding programmes for most major crops (see review by Dhooghe et al. 2011). The aim of this study

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was to determine whether the modified microspore culture technique, optimized for winter oilseed rape (*Brassica napus* L. var. *oleifera* subvar. *biennis*), is also applicable for efficient production of microspore embryos in a swede breeding program, and what substance(s) may be suitable for *in vitro* diploidization.

MATERIAL AND METHODS

Three embryogenic doubled haploid (DH) lines of different origins, selected from the swede breeding program (T7, T14 and T15, Table 1), were used for the experiments. Preparation and maintenance of donor plants, collection of buds and establishment of microspore cultures were carried out according to a standard protocol (Smýkalová et al. 2006), modified for use in recent breeding programmes of winter oilseed rape. Donor plants were vernalized at the stage of 5–6 true leaves for 90 days at a temperature of 3–5 °C, a 12-hour light photoperiod and a light intensity of 50 μmol/m^2/s. Vernalized plants were planted individually in a peat substrate in 19 × 19 cm containers and kept in controlled conditions (photoperiod 16/8 h, light intensity 180 μmol/m^2/s and 18/15 °C day/night, Figure 1A). Buds were collected from both main and lateral branches, 40 buds from each genotype. The bud size was determined microscopically to collect buds with the majority of microspores at medium, late uninucleate, and early binucleate stages (Figure 1B). After isolation, microspores were purified by three cycles of centrifugation (10–5–5 min at 100 g) in NLN medium (Lichter 1985). After the third centrifugation, the microspores were resuspended in 6 mL of NLN medium and the suspension was divided into six equal parts. Each part was resuspended in working solutions of antimitotic substances [amiprophos-methyl (O-methyl-O-(2-nitro-p-tolyl)-N-isopropylphosphoramidothionate, AMP), colchicine, ethalfluralin (N-ethyl-N-(2-methyl-2-propenyl)-2,6-dinitro-4-(trifluoromethyl)benzenamine), pronamide (3,5-dichloro-N-(1,1-dimethyl-2-propynyl) benzenamide), trifluralin (2,6-dinitro-N,N-dipropyl-4-trifluoromethylaniline), control], to get the final concentration of 5 μmol/L (or 50 mg/L in the case of colchicine) and 2.5 × 10^5 microspores per 1 mL of cultivation medium. Suspensions were transferred into 60 mm polystyrene (PS) Petri dishes (6 mL each), sealed with a double layer of Parafilm and kept in the dark in a thermostat for 24 h (30.5 °C). Suspensions were then purified in fresh NLN medium in two cycles (10 and 5 min). The microspore concentration was adjusted to 6 × 10^4/mL of cultivation medium, suspensions pipetted to 60 mm fresh Petri dishes and kept in a thermostat, where first divisions and initial formation of globular embryos took place within several days of the culture (Figures 1C and D, respectively). Cultures at the stage of visible globular embryos (usually after 10–14 days, Figure 1E) were then transferred to the orbital shaker (60 rpm) in the cultivation room (25/20 °C, photoperiod 16/8 h and a light intensity of 150 μmol/m^2/s). After 20 days, green, well-developed cotyledonary embryos with a length of at least 4 mm (Figure 1F) were transferred to 90 mm PS Petri dishes on solid differentiation medium with benzylaminopurine (0.2 mg/L), indolyl acetic acid (0.2 mg/L) and 2% sucrose, solidified by 0.8% agar (Klíma et al. 2004). Further cultivation took place at 19 °C, photoperiod 16/8 h, and a light intensity of 300 μmol/m^2/s for 14 days (Figure 1G). For flow cytometric analyses, twenty best developed embryos were taken from each Petri dish of a respective treatment. Microspore cultures and subsequent evaluations were performed in three replications to analyse a total of 60 embryos from each 'genotype × treatment'

Table 1. Doubled haploid genotypes used in experiments

No.	DH line	Components of the initial F_1 cross			
		gene bank accession	accession type/name / country of origin	gene bank accession	accession type/name/ country of origin
T7	T7DH84	BRA 179	cultivar/Patria/unknown	BRA 1001	landrace/unknown/Poland
T14	T14DH4	BRA 1693	cultivar/Angela/Germany	BRA 1685	accession from a collection site/ unknown/Poland
T15	T15DH1	09H2800003	cultivar/Perfection/United States	BRA 1685	accession from a collection site/ unknown/Poland

DH – doubled haploid; BRA – IPK Gatersleben, Germany, https://gbis.ipk-gatersleben.de/gbis2i/faces/index.jsf; 09H2800003 – CRI Prague, Czech Republic, https://grinczech.vurv.cz/gringlobal/search.aspx

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combination. Flow cytometric evaluations were performed from whole embryos without the root part (Figure 1H) according to Doležel et al. (2007). Percentage data subjected to analysis of variance was modified via square root transformation in order to follow normal distribution of errors (Gomez & Gomez 1984). Homogeneity of the variance was accessed via Levene's test, followed by tests of variance (parametric or nonparametric, according to the Levene's test). Statistical evaluations were performed in the Statsoft STATISTICA 12 program.

RESULTS AND DISCUSSION

The production of cotyledonary embryos was sufficient in all genotypes, ranging from 22.9 to 30.8 embryos per ml of medium. Hansen and Svinnset (1993) achieved in the most responsive swede cultivar cv. Gry up to 81.4 embryos per ml of medium, the mean frequency was 27.2 embryos/mL. The average rate of spontaneous diploidization in our experiments reached 13.3% (Figure 2) with a range between genotypes of 11.7–15.0% (Figure 3). A similar result (14.2%) was achieved by Szała et al. (2020) after flow cytometric analyses of oilseed rape leaf segments. In experiments on oilseed rape, a mean of 42.3% and a range of 31.9–55.5% was observed after ploidy evaluations, based on flower morphology (Klíma et al. 2008). Similar rate (44.9%) was obtained by Weber et al. (2005). According to the review by Ren et al. (2017), spontaneous diploidization rates in oilseed rape are mostly between 10–40%.

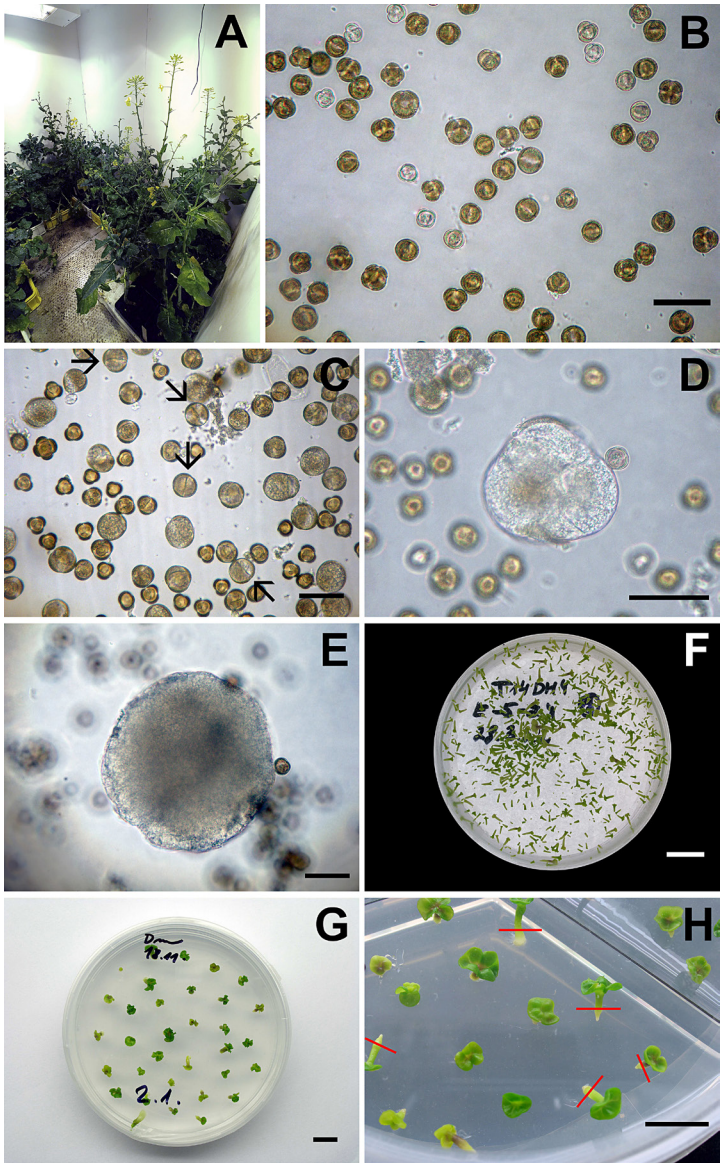

Figure 1. Key stages in the regeneration of swede microspore embryos: donor plants of swede under controlled conditions at the time of bud collection (A); purified suspension in the liquid NLN medium after isolation with a high proportion of microspores at the desired developmental stage (B); the first cell divisions after 48 hours of the culture (arrows indicate cell septa) (C); a multicellular structure (a globular proembryo) on the fourth day after establishment of the culture (D); a globular embryo after 13 days of the culture (E); well developed, green cotyledonary embryos in the 60 mm Petri dish ready for transfer to solid differentiation medium (30 day old culture) (F); cotyledonary embryos after ten days on solid differentiation medium in the 90 mm Petri dish (G); a detail of cotyledonary embryos before their collection for flow cytometric analyses (the red lines indicate the cut site) (H)
Bar = 50 μm (B–E) or 10 mm (F–H)

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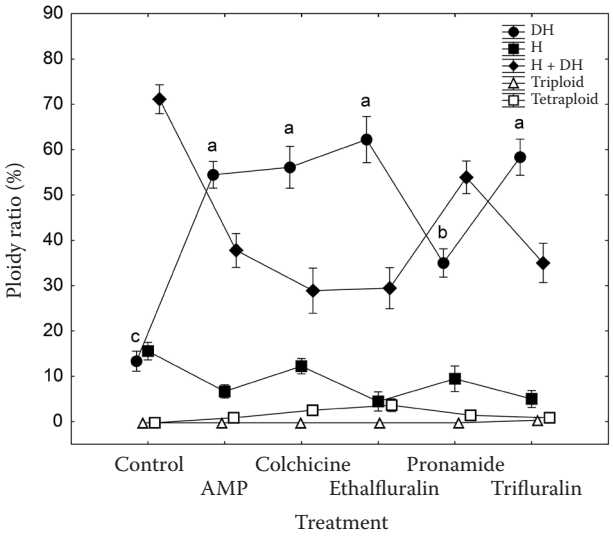

Figure 2. Percentage rates of microspore embryos with selected ploidy levels derived from individual treatments
Pooled data for three genotypes and three successive replications; letters a–c denote homogeneous groups for doubled haploid ratio (Tukey HSD test; $P = 0.05$)
DH – doubled haploid; H – haploid; AMP – amiprophos-methyl; bars denote standard errors

In our experiments with swede, no significant effect of antimitotic substances on the frequency of embryogenesis was noted. The same result was achieved by Klíma et al. (2008) in oilseed rape for colchicine and trifluralin treatments. In contrast, Zhao and Simmonds (1995) recorded a decrease in embryogenesis after the application of both of these substances in oilseed rape, Hansen and Andersen (1996) obtained a similar result in terms of a decrease in embryogenesis with trifluralin from a concentration of 3 µmol/L. Braasch (2008) noted a significant decrease in embryogenesis in the case of pronamide when used at a concentration of 3 µmol/L. On the contrary, Klutschewski (2012) did not note a negative effect at the same concentration, but reported a decrease after the use of AMP, likewise Hansen and Andersen (1996) for AMP from a concentration of 3 µmol/L. Surprisingly, Zhou et al. (2002) reported the stimulating effect of colchicine on embryogenesis.

We recorded a significant increase in the ratio of DH compared to the control for all antimitotics tested (ethalfluralin 62.2%, the range between genotypes 56.7–70.0%; trifluralin 58.3, 55.0–60.0; colchicine 56.1, 53.3–58.3; AMP 54.4, 51.7–56.7; pronamide 35.0, 33.3–36.7; control 13.3%, 11.7–15.0%) according to Tukey HSD test ($P = 0.05$) (see Figures 2 and 3). In experiments on oilseed rape (Klíma et al. 2008), a higher proportion of 2*n* plants after treatment with antimitotics (trifluralin 85.7%, colchicine 74.1%, control 42.3%) was obtained as well. Trifluralin was tested in oilseed rape by Zhao and Simmonds (1995) and proved to be better compared to colchicine (58% *vs* 22%). Pronamide was tested by Braasch (2008) along with colchicine and trifluralin in two oilseed rape genotypes; colchicine indicated better results, with a value of 75% (trifluralin 68%, pronamide 48%, control 29.5%). Klutschewski (2012) examined the

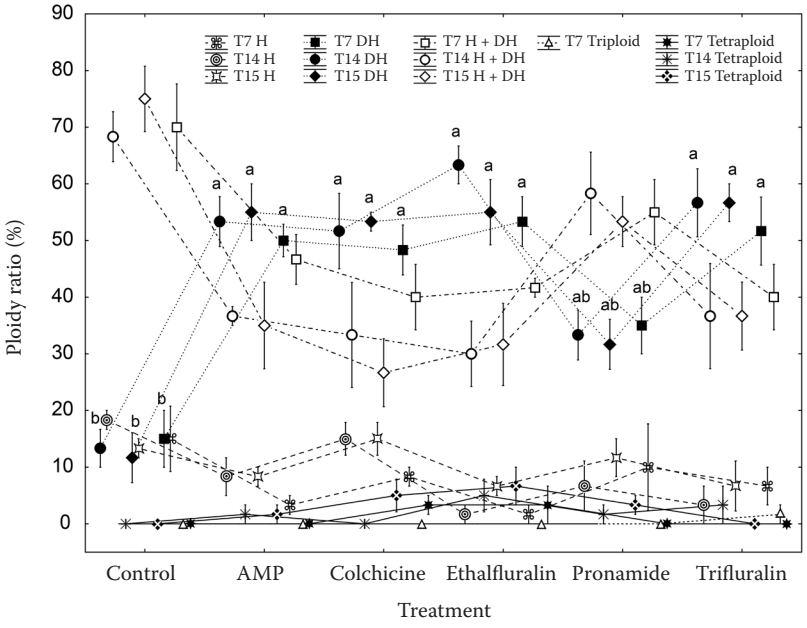

Figure 3. Percentage rates of microspore embryos with selected ploidy levels derived from individual treatments and genotypes
DH – doubled haploid; H – haploid; AMP – amiprophos-methyl; pooled data for individual genotypes T7, T14 and T15; letters a–b denote homogeneous groups for doubled haploid ratio (Tukey HSD test; $P = 0.05$); bars denote standard errors

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efficiency of AMP and pronamide (both at 3 µmol/L) in 8 oilseed rape genotypes and achieved 52% in the case of pronamide and 33.4% in AMP. Hansen and Andersen (1996) recorded comparable efficiency for both AMP and trifluralin (around 60% and 63%, respectively, control 18%). Ethalfluralin was used to successfully induce tetraploids in watermelon (*Citrullus lanatus*) (Li et al. 1999; Nasr et al. 2004).

A significant impact of the genotype on the DH ratio was not detected (Figure 3). Opposite results were reported previously for the application of colchicine in oilseed rape; for example, Kampouridis et al. (2016) evaluated the percentage of DH in microspore embryo cotyledons and recorded a range of 23.9–58.7% for 10 genotypes. Even more pronounced differences were observed by Szała et al. (2020), where a range of 15.8–94% was observed for 24 genotypes. Flow cytometric analyses detected triploids and tetraploids (Figure 4D, E) in addition to haploids and doubled haploids (Figure 4A–C, respectively). Nevertheless, the share for the first two listed was low; a triploid genotype was recorded in only one case, and the percentage of tetraploids varied between 0–5% (Figure 2), which also corresponds to the results and conclusions

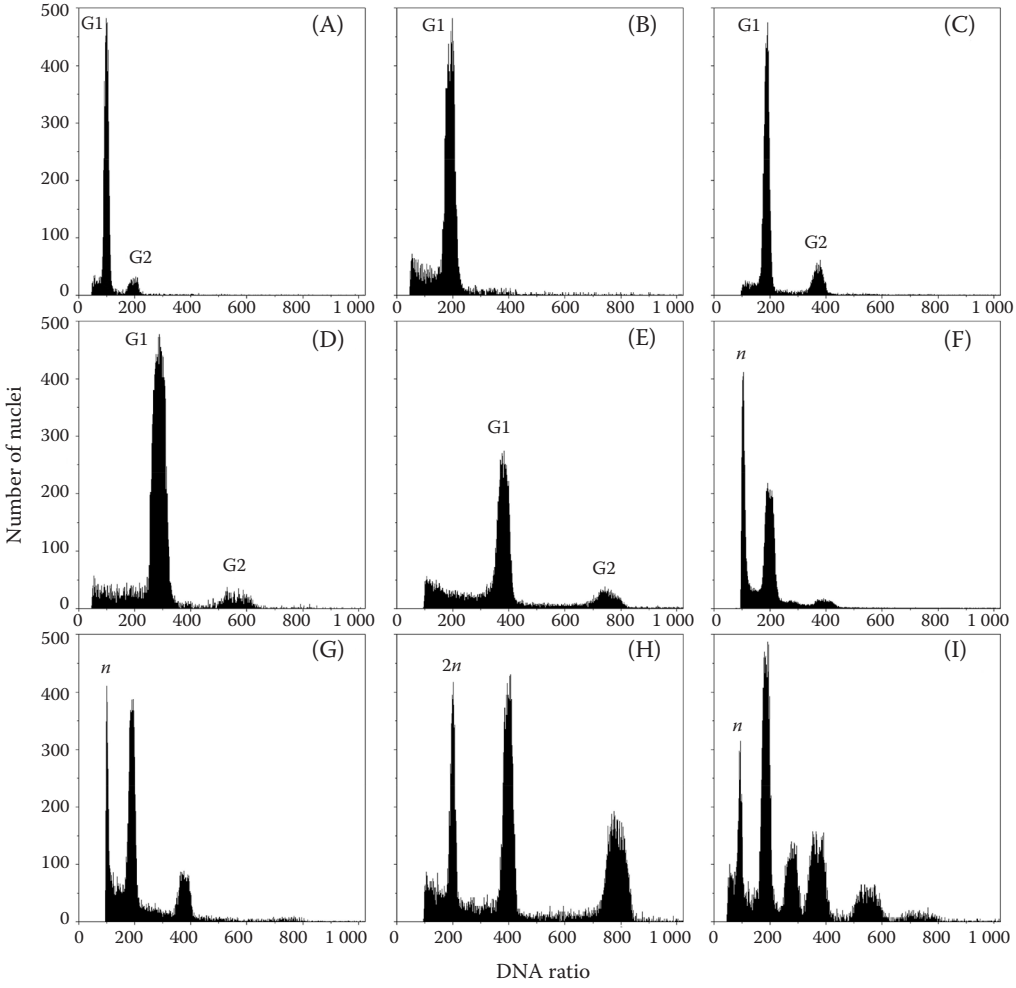

Figure 4. Assessment of ploidy levels via flow cytometry; representative flow cytometric histograms of microspore embryos: haploid (A); doubled haploid (B, C); triploid (D); tetraploid (E); mixoploids with peaks, denoting haploid and doubled haploid nuclei (and possible overlapping peaks between haploid, spontaneous doubled haploid or endopolyploid nuclei) (F, G); mixoploid with peaks, denoting doubled haploid and tetraploid nuclei (and possible overlapping peaks between doubled haploid, tetraploid or endopolyploid nuclei) (H); mixoploid with peaks, denoting haploid, doubled haploid, triploid and tetraploid nuclei (and possible overlapping peaks between haploid, spontaneous doubled haploid, tetraploid or endopolyploid nuclei) (I)

G1 – nuclei in G1 phase; G2 – nuclei in G2 phase; *n* – haploid nuclei in G1 phase; 2*n* – doubled haploid nuclei in G1 phase

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of Szała et al. (2020) in oilseed rape. However, the occurrence of mixoploids, especially *n*+2*n* (Figure 4F, G) was high in all treatments, including the control (29.4–71.1%, Figure 2). The presence of other types (Figure 4H, I) was less frequent and was included in the calculations among doubled haploids or haploids, according to their lowest degree of ploidy. The appearance of mixoploids can be considered as a consequence of endoreduplication (so-called endopolyploidy), a commonly occurring phenomenon in germinating embryos, their cotyledons, and first true leaves in the genus *Brassica* (see Kudo & Kimura 2001), and/or spontaneous di- or polyploidization during embryogenesis. Therefore, possible overlapping peaks (see Figures 4F, G, I) may complicate the identification of spontaneous or induced doubled haploids (Hilgert et al. 2015).

CONCLUSION

It can be concluded that the modified technique, currently used in winter oilseed rape breeding programmes, may also be applicable to swede, including the *in vitro* diploidization approach. Despite the aforementioned issues mostly related to the phenomenon of endopolyploidy, it was also possible to compare the effectiveness of individual antimitotic agents. From this point of view, there was an interesting positive result for ethalfluralin, which has not yet been tested for *in vitro* diploidization in the genus *Brassica* and showed at least the same effectiveness as other substances for all three genotypes tested.

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