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ORIGINAL PAPER

The effect of auxin and genotype on the production of *Avena sativa* L. doubled haploid lines

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Abstract Production of doubled haploid (DH) cereals is becoming increasingly important in crop breeding programs, but the methods currently applied still remain inefficient. In this study, we present the procedure for obtaining haploid and DH oat plants by pollination with maize. Thirty-three oat genotypes were used in the experiments. Oat plants (14,543 florets) were pollinated with maize pollen 2 days after emasculation and treated with auxin analogues: 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-2-methoxybenzoic acid (dicamba), at a concentration of 100 mg dm^{-3}. These auxins had no significant influence on the number of haploid embryos developed, but they significantly affected their germination ability, and thus haploid and DH plant production. After application of 2,4-D, 5.06 % of haploid embryos developed per emasculated florets, 1.37 % of haploid plants and 0.54 % of DH lines, whereas after dicamba treatment, 4.3 % of haploid embryos, 0.64 % of haploid plants and 0.25 % of DH lines. Haploid embryos were obtained from all genotypes tested, however, their frequency differed between individual genotypes. The highest number of embryos per emasculated florets (9.0 %) was obtained from the DC09040 genotype after dicamba treatment, and from STH123 × Skorpion (8.9 %) after 2,4-D treatment. The genotype did not significantly affect the development of haploid plants, nevertheless the highest number of DH lines was obtained from the Arab × Typhon genotype. There were 52 DH lines acquired from 28 genotypes, which produced a total of 5227 seeds. The number of seeds varied between the DH lines from 2 to 595. Seeds of all the DH lines produced fertile next generation. DH lines are currently included in breeding programs.

Keywords 2,4-D, dicamba · Oat haploids · Wide crossing · DH lines · F_1 progeny

Introduction

Haploids are plants that contain the gametic number of chromosomes (n). In nature, the spontaneous emergence of haploids in higher plants is very rare (a frequency of 0.001–0.01 %), however, currently there are several techniques available enabling their production. Most commonly, haploids are produced in vivo and in vitro by androgenesis (culture of anthers or microspores), gynogenesis (culture of unfertilized ovules), interspecific or intergeneric crosses, followed by chromosome elimination, or pollination with irradiated pollen (Ferrie et al. 2014). The induction and regeneration of doubled haploids (DHs) allow to produce completely homozygous lines in a single generation. The time required to obtain them using in vitro techniques is very short when compared to traditional breeding schemes, based on multiple self-pollination generations, which take 6–10 years. Haploid plants are very important in various research disciplines, such as plant biotechnology, molecular genetics or traditional plant breeding. They have many applications in basic plant research, such as cytogenetics, crop evolution, mutagenesis induction, genetic transformation and in breeding programs (Philips and Rines 2009; Touraev et al. 2009; Dunwell 2010; Murovec 2013; Hermann et al. 2014).

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Oat (*Avena sativa* L.) haploids have already been produced by in vitro microspore/anther culture (Rines 1983; Sun et al. 1991; Rines et al. 1997; Kiviharju et al. 2005; Tanhuanpää et al. 2008; Kiviharju 2009; Ponitka and Ślusarkiewicz-Jarzina 2009; Sidhu and Davies 2009; Ferrie et al. 2014) and wide hybridizations (chromosome elimination) with maize (Rines and Dahleen 1990; Rines et al. 1991, 1996, 1997; Rines 2003; Sidhu et al. 2006; Nowakowska 2012; Marcińska et al. 2013; Nowakowska et al. 2015). For haploid production, emasculated oat flowers are usually pollinated by maize, seldom by pearl millet (*Pennisetum glaucum* L.), common millet (*Panicum miliaceum* L.), gamagrass (*Tripsacum dactyloides* L.) or sorghum (*Sorghum bicolor* (L.) Moench) (Matzk 1996; Rines et al. 1997; Ishii et al. 2013; Nowakowska et al. 2015). Maize-oat hybrid zygote is formed after fertilization, and subsequently embryo is produced. Although maize chromosomes are preferentially eliminated during early embryo development, it has been found that some of the oat plants retain a single maize chromosome (Liera-Lizarazu et al. 1996; Okagaki et al. 2001; Kynast et al. 2004; Philips and Rines 2009). To enable the formation of cereal haploid embryo, pollinated florets are usually sprayed with different growth regulators: 2,4-D, dicamba, picloram (4-amino-3,5,6-trichloropicolinic acid), IAA (indole-3-acetic acid), PAA (phenylacetic acid), MCPA (4-chloro-*o*-tolyloxyacetic acid), GA_3 (gibberellic acid) or a mixture of 2,4-D and GA_3 (Rines 2003; Sidhu et al. 2006; Kynast et al. 2012; Pratap and Chaudhary 2012; Marcińska et al. 2013; Nowakowska et al. 2015). In rare cases, e.g., in the study of Ishi et al. (2013), pollinated panicles were cut and placed in a solution containing sucrose and 2,4-D.

Endosperms usually fail to develop in the caryopses and isolated embryos must be cultivated on the regenerating medium in order to grow. Haploid embryos of oat have been previously regenerated to haploid plants on the following media: B5 (Gamborg et al. 1968) by Aung (1998) and Sidhu et al. (2006); 190-2 (Wang and Hu 1984) by Marcińska et al. (2013) and Nowakowska et al. (2015); MS (Murashige and Skoog 1962) by Rines and Dahleen (1990), Rines (2003) and Kynast et al. (2012); TL3 (Taira and Larter 1978) by Marcińska et al. (2013), LS (Linsmaier and Skoog 1965) by Ishii et al. (2013). The efficiency of oat haploid plant production per pollinated florets is very low and ranges from 0.5 to 2.0 %, while in wheat it is from 10 to 30 % (Rines et al. 1997). Sidhu et al. (2006) reported the frequency of haploid plant development in the range of 0.8–1.5 %, Marcińska et al. (2013)—0.9 %, and in the study by Nowakowska et al. (2015) it was 0.5–3.1 %.

Chromosome doubling is an essential step in the production of oat DH lines. Treatment of haploid plants has been routinely and successfully performed using colchicine (Aung 1998; Rines 2003; Marcińska et al. 2013; Nowakowska et al. 2015). Aung (1998) reported 86 % survival of the developed plants, and 91 % of them produced more than 30 seeds per plant. Similar results were obtained by Sidhu et al. (2006), who induced doubling using colchicine, and approximately 70–80 % of the plants transferred to the soil survived and produced fertile tillers. In the experiments of Marcińska et al. (2013) and Nowakowska et al. (2015), 14 and 15 doubled haploid oat plants, respectively, survived the colchicine treatment and produced seeds.

This report presents the effect of post-pollination treatment with selected auxins on the production of oat haploid embryos by wide hybridization with maize, and the subsequent regeneration of haploid and DH plants that produced seeds. The purpose of our experiments was to determine which auxin should be used after floret emasculation and maize pollination, in order to enlarge the ovaries and stimulate embryo development. The novelty of our research lies in the selection of most effective auxin in the successive steps of the production of oat haploids and DH lines. Thus far, there has been no literature data on the effect of hormones on the further development of embryos into haploid plants and DH lines.

Materials and methods

Plant material

Material consisting of thirty-three oat (*Avena sativa* L.) genotypes (F_1 progeny): Arab × Typhon, Bingo x Zuch, CHD 1193/04 × Bingo, CHD 1430/02 × Typhon, Neklan × Bingo, STH 123 × Ivory, STH 123 × Skorpion, Typhon × Flamingsprofi, Flamingsprofi × Arab, Ivory × Breton, Argon × Husky, Radius × 3DC/DW 2010, Husky × 5DC/DW 2010, DC 1382/05 × Contander, Edelprinz × Celer, Flipper × Radius, Stork × Nekla, Stork × Zolak, Auron × Krezus, Zuch × Krezus, Expander × Skorpion, Chimene × STH85869(b), Chimene × STH85763(b), Chimene × Bingo, Bingo × Bajka, Bingo × SER 20284, Bingo × STH 81957, Bingo × Expander, Bingo × Chimene, Krezus × Expander, DC09012, DC09040 and DC09163 derived from Strzelce Plant Breeding Ltd., Małopolska Plant Breeding Polanowice Ltd. and Danko Plant Breeding Ltd. Maize (*Zea mays* L. var. *saccharata*); a mixture of three genotypes: MPC4, Dobosz and Wania was used as pollen donor. Oat and maize plants were grown under controlled conditions, 16-h photoperiod, in a greenhouse under natural (solar) light during the day and sodium lamps between 6–8 a.m. and additionally 6–10 p.m. on cloudy days. Oat plants were grown at 21/17 °C day/night, whereas maize plants at 21–28/17 °C day/night. All plants were fertilized with a liquid medium once a week (Hoagland and Arnon 1938).

Haploid plant production

Emasculation of oat florets was performed manually using tweezers prior to anthesis. Anthers of the primary florets from the central part of the panicle were discarded. At least 25 panicles were emasculated per genotype. Panicles were covered with transparent fabric bags. Two days after emasculation, oat florets were pollinated with a fresh mixture of maize pollen (collected at 15-min intervals) using a brush. After pollination, panicles were covered with fabric bags. The day after pollination, one drop of 3,6-dichloro-2-methoxybenzoic acid (dicamba) or 2,4-dichlorophenoxyacetic acid (2,4 D) water solution (concentration of 100 mg dm^{-3}) were applied to each oat pistil. Three weeks later, caryopses without endosperms (enlarged ovaries) were isolated, surface-sterilized in 70 % v/v ethanol (1 min), 2.5 % calcium hypochlorite (7 min), 0.1 % mercuric chloride (1 min) and then washed three times with sterile water. Next, isolated haploid embryos were transferred into 60 mm × 15 mm Petri dishes containing 190-2 medium (Wang and Hu 1984) with 9 % (w/v) maltose, solidified with 0.6 % (w/v) agar. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH before autoclaving at 121 °C for 20 min. The haploid embryos germinated at 21 ± 2 °C and light intensity equal to 60 µmol m^{-2} s^{-1} and 16/8 h light/dark cycle. Developed haploid plants were grown on MS medium (Murashige and Skoog 1962). Haploid plants were acclimated to ex vitro conditions by transferring them to a moist perlite and then to the soil. After acclimatization the haploid plants were grown in a greenhouse (conditions as above).

Doubling the number of chromosomes

For chromosome doubling procedure, the developed haploid plants were immersed for 7.5 h in a 0.1 % colchicine solution supplemented with 40 g dm^{-3} dimethyl sulfoxide (DMSO), 0.025 g dm^{-3} gibberellic acid (GA_3) and a drop of Tween. Colchicine treatment was carried out at 25 °C and 80–100 µmol m^{-2} s^{-1} light intensity. Then, the plant roots were washed with running water for 48 h, followed by the transplantation into pots for maturation and seed production.

Ploidy level of plants was evaluated before and after colchicine treatment using a MACS Quant flow cytometer (Miltenyi Biotec GmbH, Germany), equipped with air-cooled laser (488 nm) and MACSQuantify™ software. Approximately 10–15 mg of young leaves was placed in a 60-mm glass Petri dish with 1 ml of the modified PBS buffer (8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g of KH_2PO_4, 2.00 g EDTA, 0.5 % BSA, pH 7.0) (Sambrook et al. 1989). The tissue was chopped with a razor blade to release nuclei from the cells and filtered with a 30-µm nylon mesh filter (Miltenyi Biotec GmbH, Germany) into 5-ml tubes. Suspension of the nuclei was stained with 20 µl of a 2 % propidium iodide (PI) solution. Oat plants derived from the seeds of known diploid DNA content were used as a control.

Statistical analysis

Results were analyzed using two-way ANOVA, Student's *t* test and Duncan's test implemented in the statistical package STATISTICA 10.0 (Stat-Soft, Inc., USA). Significant differences between treatments at $p \leq 0.05$ are marked with different letters.

Results

Two-way ANOVA for production traits of oat doubled haploids indicated no significant differences in the efficiency of haploid embryos. However, the differences were highly significant for haploid plants and DH lines per florets when the type of auxin was the source of variance (Table 1). Furthermore, considerable differences were found between genotypes in the production of embryos, while the differences in the production of haploid plants and DH lines per florets were negligible (Table 1). This indicates the existence of genetic variation only in the induction of haploid embryos, rather than further regeneration steps.

Effect of auxins on oat haploid embryo formation and development of haploid and DH plants

Oat florets from 33 genotypes were pollinated with maize pollen; 6992 of them were treated with dicamba and 7551 with 2,4-D (a total of 14,543 florets). Dicamba treatment resulted in 83.4 % of developed caryopses, and 83.9 % after 2,4-D application (data not shown). Three weeks after pollination, 301 haploid embryos developed in dicamba-treated group (4.3 % haploid embryos per pollinated florets), and 382 in 2,4-D-treated group (5.1 % haploid embryos per pollinated florets) (Table 2; Fig. 1). In total, 683 haploid embryos were obtained from all genotypes. The number of haploid embryos between genotypes varied from 0 to 30. The highest number of haploid embryos (30) was formed by the Arab × Typhon genotype, treated with 2,4-D. Two dicamba-treated genotypes (Argon × Husky, Husky × 5DC/DW2010), and one genotype (Fliper × Radius) treated with 2,4-D, did not form haploid embryos. The highest frequency (haploid embryos per one hundred of emasculated florets) of haploid embryo formation, following dicamba treatment, was observed in the genotypes: DC09040 (9.0 %), DC09012 (7.5 %), while in the 2,4-D experimental group:

Table 1 Analysis of variance in oat doubled haploid production using wide crossing method

Trait	Source of variation	SS	df	MS	F	*p*
Haploid embryos per florets	Auxin	832.430	1	26.01	0.505	0.990^{ns}
Haploid plants per florets		69.639	1	69.63	7.195	0.007^{**}
DH lines per florets		1.395	1	1.39	7.728	0.007^{**}
Haploid embryos per florets	Genotype	2458.160	32	76.82	1.492	0.039^{*}
Haploid plants per florets		310.642	32	9.71	0.997	0.473^{ns}
DH lines per florets		6.971	32	0.22	1.200	0.301^{ns}

SS sum of squares, *df* degrees of freedom, *MS* mean squares, *ns* not significant, *significant at the $p \leq 0.05$, **significant at the $p \leq 0.01$

Table 2 The influence of auxin and oat genotype on the production efficiency of haploid embryos and plants using wide crossing method

Genotype	Pollinated florets		Haploid embryos		Haploid embryos/florets [% ± SE]		Haploid plants		Haploid plants/florets [% ± SE]	
	Dicamba	2,4-D	Dicamba	2,4-D	Dicamba	2,4-D	Dicamba	2,4-D	Dicamba	2,4-D
Arab × Typhon	137	439	8	30	5.8 ± 1.9	6.8 ± 1.4	2	11	1.5 ± 1.5	2.5 ± 0.7
Bingo × Zuch	303	213	16	12	5.3 ± 1.2	5.6 ± 2.9	2	4	0.7 ± 0.6	1.9 ± 1.2
CHD 1193/04 × Bingo	217	329	13	29	6.0 ± 1.6	8.8 ± 1.6	1	7	0.5 ± 0.7	2.1 ± 0.7
CHD 1430/02 × Typhon	187	192	9	12	4.8 ± 1.9	6.2 ± 1.41	2	5	1.1 ± 0.7	2.6 ± 1.1
Neklan × Bingo	197	202	11	8	5.6 ± 1.4	3.9 ± 1.9	1	1	0.5 ± 0.5	0.5 ± 0.6
STH 123 × Ivory	277	276	17	16	6.1 ± 2.0	5.7 ± 1.6	0	3	0	1.1 ± 0.7
STH 123 × Skorpion	225	191	5	17	2.2 ± 1.0	8.9 ± 3.05	0	4	0	2.1 ± 0.8
Typhon × Flamingsprofi	285	211	12	9	4.2 ± 1.3	4.2 ± 1.6	1	2	0.3 ± 0.2	0.9 ± 1.2
Flamingsprofi × Arab	216	197	13	15	6.0 ± 1.6	7.6 ± 1.8	4	4	1.8 ± 0.7	2.0 ± 0.8
Ivory × Breton	264	209	14	15	5.3 ± 2.7	7.1 ± 2.6	6	2	2.3 ± 1.4	0.9 ± 0.4
Argon × Husky	37	25	0	2	0	8.0 ± 3.3	0	0	0	0
Radius × 3DC/DW 2010	45	47	1	2	2.2 ± 3.7	4.3 ± 2.2	1	0	2.2 ± 3.7	0
Husky × 5DC/DW 2010	35	23	0	1	0	4.3 ± 0	0	0	0	0
DC 1382/05 × Contander	85	114	5	2	5.9 ± 2.1	1.7 ± 1.4	0	2	0	1.7 ± 1.5
Edelprinz × Celer	90	79	1	1	1.1 ± 1.8	1.2 ± 0.8	0	1	0	1.3 ± 0.9
Flipper × Radius	129	83	4	0	3.1 ± 1.8	0	1	0	0.8 ± 0.7	0
Stork × Neklan	185	256	4	9	2.2 ± 1.3	3.5 ± 1.2	0	1	0	0.4 ± 0.5
Stork × Zolak	274	367	12	17	4.4 ± 1.1	4.6 ± 1.2	1	4	0.4 ± 0.4	1.1 ± 0.4
Auron × Krezus	269	325	11	16	4.1 ± 1.3	4.9 ± 1.9	4	6	1.5 ± 1.0	1.8 ± 1.2
Zuch × Krezus	264	209	14	18	5.3 ± 3.2	7.1 ± 2.2	0	6	0	2.9 ± 1.1
Expander × Skorpion	267	329	7	16	2.6 ± 1.1	4.8 ± 1.2	0	1	0	0.3 ± 0.4
Chimene × STH85869(b)	186	338	8	12	4.3 ± 1.4	3.5 ± 1.2	1	5	0.5 ± 0.4	1.5 ± 0.5
Chimene × STH85763(b)	305	322	16	16	5.2 ± 1.5	4.9 ± 1.3	2	1	0.6 ± 0.7	0.3 ± 0.5
Chimene × Bingo	384	310	16	13	4.2 ± 0.9	4.1 ± 1.2	1	5	0.3 ± 0.3	1.6 ± 0.8
Bingo × Bajka	367	305	15	14	4.1 ± 1.5	4.5 ± 1.6	4	4	1.1 ± 0.8	1.3 ± 0.6
Bingo × SER 20284	234	275	5	6	2.1 ± 1.0	2.1 ± 1.0	1	2	0.4 ± 0.5	0.7 ± 0.5
Bingo × STH 81957	404	278	12	12	3.0 ± 0.9	4.3 ± 1.1	1	5	0.2 ± 0.3	1.8 ± 1.0
Bingo × Expander	162	301	3	9	1.9 ± 0.7	2.9 ± 0.8	1	1	0.6 ± 0.5	0.3 ± 0.2
Bingo × Chimene	225	188	9	5	4.0 ± 1.6	2.6 ± 0.9	1	2	0.4 ± 0.5	1.0 ± 0.6
Krezus × Expander	275	245	5	9	1.8 ± 0.5	3.6 ± 1.3	1	2	0.4 ± 0.3	0.8 ± 0.6
DC09012	134	163	10	12	7.5 ± 3.8	7.4 ± 3.3	1	4	0.7 ± 0.8	2.4 ± 1.4
DC09040	199	254	18	12	9.0 ± 4.0	4.7 ± 1.4	4	3	2.0 ± 1.2	1.2 ± 0.6
DC09163	129	256	7	15	5.4 ± 1.7	5.8 ± 1.8	1	6	0.8 ± 0.4	2.3 ± 1.3
Total	6992	7551	301	382			45	104		

Mean value ± standard error (SE)

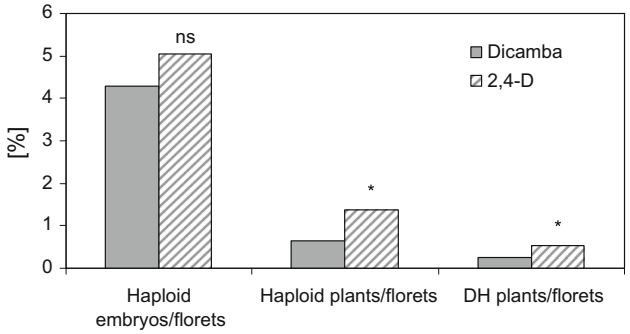

Fig. 1 The influence of dicamba and 2,4-D on the production of haploid embryos, haploid plants and DH plants using wide crossing method; n = 14,543 emasculated oat florets; significant differences according to Student's t-test at the 0.05 probability level are marked with asterisks, *ns* not significant

STH123 × Skorpion (8.9 %), CHD1193/04 × Bingo (8.8 %) and Argon × Husky (8.0 %).

The whole procedure of oat × maize wide crossing in the production of DH lines is presented in Fig. 4. The embryos formed in caryopses without endosperms (Fig. 4a); dicamba solution caused browning of caryopses, whereas after 2,4-D application they were creamy in color. The developed haploid embryos had various shapes and were ca. 1–3-mm-long (Fig. 4b, c).

The average haploid embryo formation induced by 2,4-D was 5.1 %, while in the case of dicamba it was 4.3 % (Fig. 1). Despite the observed difference, the type of auxin used to treat the ovaries did not have statistically significant influence on the induction of haploid embryos in oat (Table 1). As previously mentioned, in contrast to the results related to the effect of auxins on the formation of haploid embryos, these hormones significantly affected the growth of haploids and DH plants.

Forty-eight percent of haploid embryos germinated within 1–3 weeks of culture on 190-2 medium (Fig. 4d), and nearly 22 % of them developed into haploid plants on MS medium (Fig. 4e) (data not shown). The number of haploid plants depended on the auxin applied in ovaries treatments. The application of dicamba resulted in the development of 0.64 % of haploid plants, while 2,4-D stimulated the development of 1.37 % plants (Fig. 1).

After treatment with dicamba, 45 haploid plants were obtained, whereas 2,4-D treatment resulted in 104 haploid plants (Table 2). The highest number of plants (11) was obtained for Arab × Typhon genotype after 2,4-D application. The highest frequency of haploid plants (more than 2 %) was recorded in 7 genotypes after 2,4-D and 2 genotypes after dicamba treatment. Nine genotypes treated with dicamba and 4 treated with 2,4-D did not produce any haploid plants.

As demonstrated in Fig. 1, 0.54 % of DH plants developed after application of 2,4-D, whereas only 0.25 % after dicamba treatment. Colchicine treatment resulted in chromosome doubling in 17 plants of 13 genotypes treated with dicamba, and in 44 plants of 25 genotypes after 2,4-D application (Table 3). The highest number of DH plants formed from the Arab × Typhon genotype, i.e. 1.46 % per emasculated florets after dicamba and 1.37 % after 2,4-D treatment.

Effect of genotype on oat haploid embryo formation and development of haploid and DH plants

The formation of haploid embryos was significantly correlated with genotype (Table 1). All genotypes were able to form haploid embryos, but most of them, based on emasculated florets, developed from the following genotypes: DC09012 (7.5 %), CHD 1193/04 × Bingo (7.4 %), DC09040 (6.9 %) and Flamingsprofi × Arab (6.8 %) (Fig. 2a).

Although the genotype did not have a statistically significant influence on the development of haploid plants, 8 of 33 genotypes (Arab × Typhon, CHD 1430/02 × Typhon, Flamingsprofi × Arab, Ivory × Breton, Auron × Krezus, DC09012, DC09040, DC09163) developed 3.1–4.0 % of haploid plants (Fig. 2b). Only 2 of the 33 genotypes investigated (Argon × Husky and Husky × 5DC/DW 2010) did not develop haploid plants. DH plants were not obtained only from the haploids derived from the genotypes: Radius × 3DC/DW 2010, Expander × Scorpion and Krezus × Expander (Fig. 2c).

Sixty-one haploid plants (41 %) obtained from 28 genotypes in both treatments (Table 3) survived the process of acclimatization and subsequent chromosome doubling (colchicine treatment) (Fig. 4f, g). The ploidy of the plants was confirmed by flow cytometry before and after colchicine treatments (Fig. 3). Although the number of DH plants varied between genotypes, these differences were not statistically significant. Among all genotypes tested, the highest number of DH plants (8) was obtained from the Arab × Typhon genotype (Table 3).

Effectiveness of DH line formation and seed production

Fifty-two fertile plants (DH lines) were obtained that produced seeds (Table 3; Fig. 4h–k). The number of seeds was in the range of 2–595, depending on the genotype and auxin treatment. In total, 5227 seeds were produced (1545 of them were derived from the plants treated with dicamba and 3682 from the plants after 2,4-D treatment). Only nine plants obtained from 8 genotypes (Arab × Typhon, CHD 1193/04 × Bingo, STH 123 × Ivory, STH 123 × Scorpion, Edelprinz × Celer,

Table 3 The influence of auxin and oat genotype on the production efficiency of DH plants and seeds using wide crossing method

Genotype	DH plants		DH plants/florets [% ± SE]		Number of seeds	
	Dicamba	2,4-D	Dicamba	2,4-D	Dicamba	2,4-D
Arab × Typhon	2	6	1.46 ± 1.4	1.37 ± 0.4	0	589
Bingo × Zuch	1	1	0.33 ± 0.5	0.47 ± 0.5	11	137
CHD 1193/04 × Bingo	1	1	0.46 ± 0.7	0.30 ± 0.2	0	182
CHD 1430/02 × Typhon	0	3	0	1.56 ± 1.0	0	84
Neklan × Bingo	1	0	0.51 ± 0.5	0	83	0
STH 123 × Ivory	0	1	0	0.36 ± 0.5	0	0
STH 123 × Skorpion	0	1	0	0.52 ± 0.7	0	0
Typhon × Flamingsprofi	0	1	0	0.47 ± 0.5	0	5
Flamingsprofi × Arab	2	2	0.93 ± 0.4	1.02 ± 0.4	244	595
Ivory × Breton	3	1	1.14 ± 0.9	0.48 ± 0.4	146	7
Argon × Husky	0	0	0	0	0	0
Radius × 3DC/DW 2010	0	0	0	0	0	0
Husky × 5DC/DW 2010	0	0	0	0	0	0
DC 1382/05 × Contander	0	1	0	0.88 ± 1.3	0	176
Edelprinz × Celer	0	1	0	1.27 ± 0.7	0	0
Flipper × Radius	1	0	0.78 ± 0.7	0	143	0
Stork x Neklan	0	1	0	0.39 ± 0.5	0	0
Stork × Zolak	1	2	0.36 ± 0.4	0.54 ± 0.3	172	89
Auron × Krezus	0	2	0	0.62 ± 0.5	0	488
Zuch × Krezus	0	2	0	0.96 ± 0.5	0	62
Expander × Skorpion	0	0	0	0	0	0
Chimene × STH 85869(b)	1	4	0.54 ± 0.4	1.18 ± 0.5	229	317
Chimene × STH 85763(b)	1	1	0.33 ± 0.6	0.31 ± 0.5	108	19
Chimene × Bingo	0	2	0	0.65 ± 0.5	0	38
Bingo × Bajka	1	1	0.27 ± 0.3	0.33 ± 0.4	212	0
Bingo × SER 20284	0	1	0	0.36 ± 0.4	0	2
Bingo × STH 81957	0	1	0	0.36 ± 0.2	0	29
Bingo × Expander	0	1	0	0.33 ± 0.2	0	0
Bingo × Chimene	1	0	0.44 ± 0.5	0	28	0
Krezus × Expander	0	0	0	0	0	0
DC09012	0	2	0	1.23 ± 0.7	0	227
DC09040	0	2	0	0.79 ± 0.5	0	527
DC09163	1	3	0.78 ± 0.4	1.17 ± 0.6	169	109
Total	17	44			1545	3682

Mean value ± standard error (SE)

Stork × Neklan, Bingo × Bajka and Bingo × Expander) did not produce seeds. The highest number of seeds (839 in total, 244 after dicamba treatment and 595 after 2,4-D) was produced by the Flamingsprofi × Arab genotype. In addition, the high efficiency of seed production was recorded in 6 plants from the following 4 genotypes: Auron × Kreazus, Chimene × STH 85869(b) and Bingo × Bajka and DC09040, which produced more than 200 seeds per plant. The seeds were sown on the experimental field to analyze the strength of germination and fertility. Seeds of all DH lines produced fertile plants of the next generation.

Discussion

The objective of this study was to investigate the influence of auxins on the production of haploid oat plants and further development into the fertile DH lines. Various growth regulators have been examined for induction of caryopses and embryos to produce haploid plants using wide crossing method (Rines et al. 1997, 2009; Rines 2003; Sidhu et al. 2006; Aung 1998; Kynast et al. 2012; Ishii et al. 2013; Marcińska et al. 2013; Nowakowska et al. 2015). However, there is no data available on the effects of hormones on the

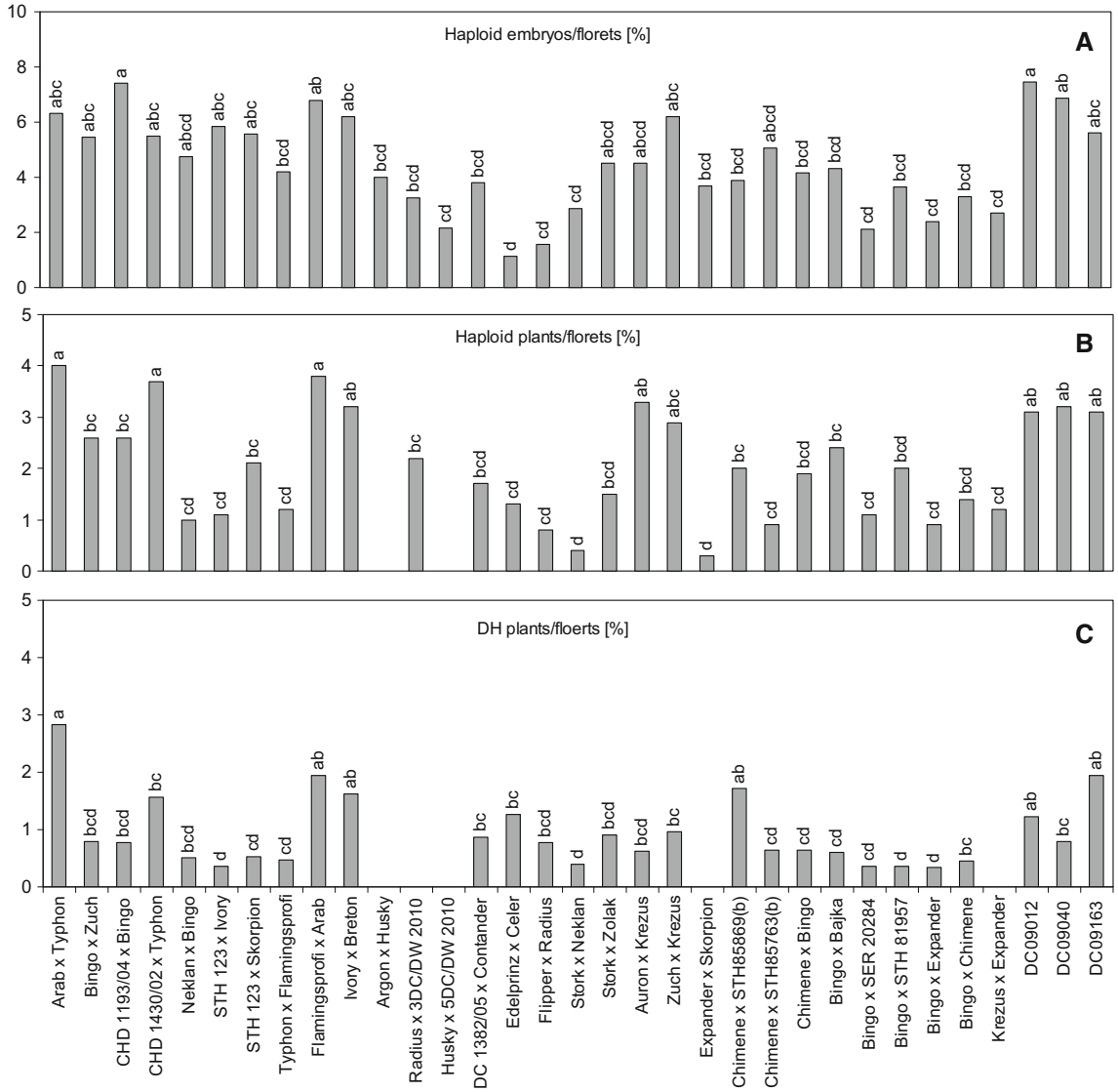

Fig. 2 The influence of genotype, independent of the auxin type, on the production of: **a** haploid embryos, **b** haploid plants and **c** DH plants using wide crossing method; n = 14,543 emasculated oat florets; significant differences according to Duncan's test at the 0.05 probability level are marked with different letters. a, b, c, d—significant differences according to Duncan's test, $p \leq 0.05$

following stages of development of haploid embryos, haploid plants and DH lines. The most commonly used compound for these purposes is 2,4-D administered at a concentration of 100 mg dm^{-3}. It is applied alone or in combination with 50 or 100 mg dm^{-3} of GA_3. Sidhu et al. (2006) tested three auxin analogues, i.e., 2,4-D, picloram and dicamba; Marcińska et al. (2013) used two auxins: 2,4-D and dicamba, while Nowakowska et al. (2015) studied dicamba alone. All of the hormones were applied at a concentration of 100 mg dm^{-3}. Therefore, in our study, the oat plants were treated with the same concentration of dicamba and 2,4-D as used by the above listed authors.

Rines (2003) reported that the percentage of embryo recovery usually ranged between 2 and 10 % of maize-pollinated oat florets. The rate of germination of these embryos into vigorous plants is typically low and falls below 20 %. This low efficiency makes it difficult to conduct experiments of an adequate scope to statistically compare the factors affecting the frequency of plant recovery. Genotypic variation in the response of different oat lines and maize pollen have been reported, but they may be obscured by the reaction of lines to growth conditions applied. In general, the more vigorous the plants, the higher frequency and quality of embryos recovered.

The study of Rines et al. (1997) showed that the post-pollination treatment with 2,4-D appeared to be beneficial for the embryo and plant recovery. The authors reported that after application of 10 mg dm^{-3} 2,4-D, embryos

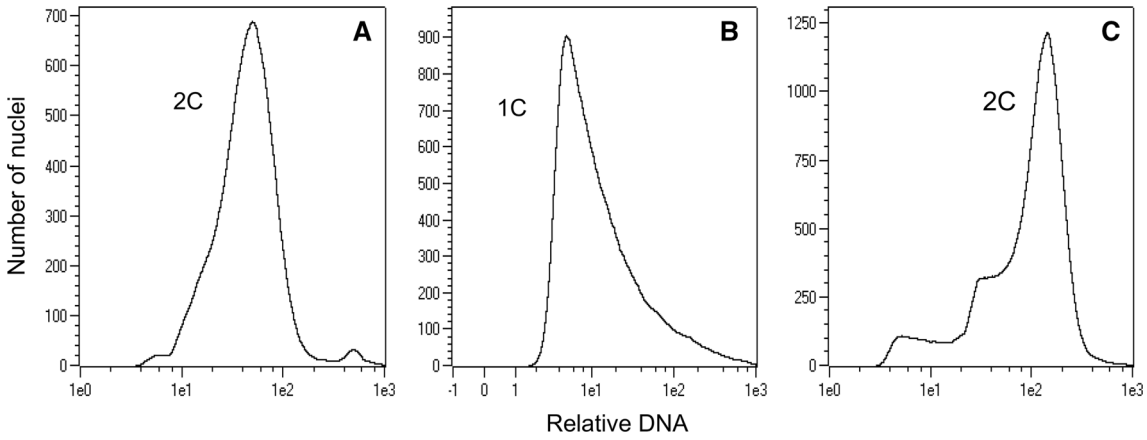

Fig. 3 Flow cytometry histograms of oat plants (genotype Arab × Typhon); **a** control 2n, **b** haploid 1n and **c** doubled haploid 2n

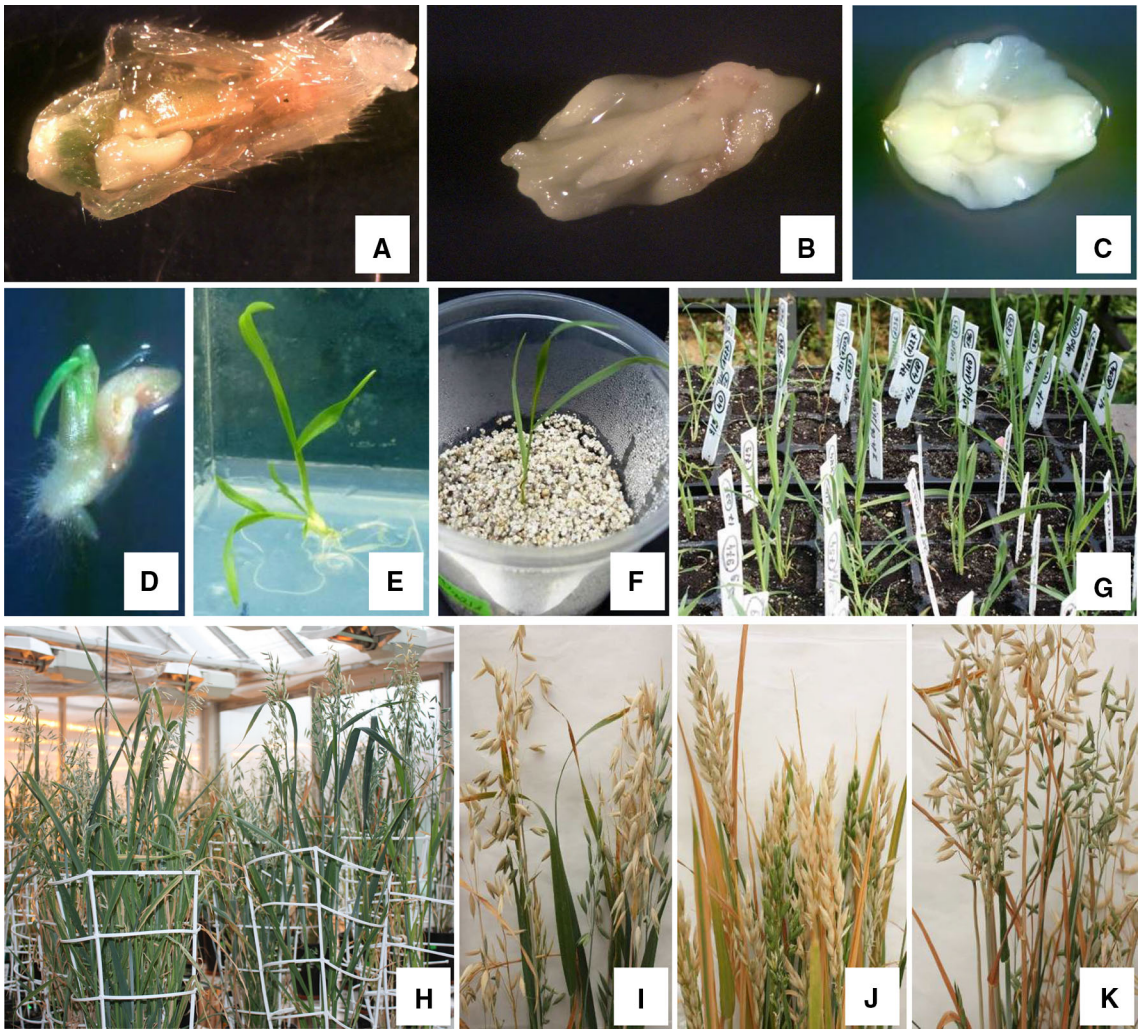

Fig. 4 Procedure of oat × maize wide crossing for DH oat production: **a** haploid embryo formed in caryopsis without endosperm (magnification ×10); **b**, **c** haploid embryos isolated from caryopses (magnification ×15); **d** germinated haploid embryo on 190-2 medium (magnification ×5); **e** developed haploid plant on MS medium; **f** acclimatization of haploid plant in perlite; **g** plants after colchicine treatment; **h** DH plants in the greenhouse; **i–k** maturing panicles of DH lines Chimene × STH 85763 (**b**), Bingo × SER 20284 and Neklan × Bingo, respectively

seemed more normal in shape and less browning of the caryopses occurred than at higher 2,4-D concentrations. Moreover, in the same experiment, these authors used additional concentration of 2,4-D (100 mg dm^{-3}), because the development of the endosperms and whole embryos appeared to be enhanced by increased concentrations of this growth regulator. In our work, endosperm development was not observed and embryos developed normally, despite the fact that dicamba caused browning of caryopses.

Sidhu et al. (2006) compared the capacity of picloram, dicamba and GA_3 with 2,4-D for induction of caryopsis and embryo formation. The authors found significant differences between the study genotypes and growth regulators stimulating the development of caryopses and embryos. The number of caryopses in the first experiment involving 2,4-D treatment varied depending on the genotype from as low as 35.7 to 83.3 %. In the second experiment, dicamba treatment resulted in the highest percentage (about 94.0 %) of developed caryopses, picloram produced the second largest number of caryopses (about 70.0 %), followed by 2,4-D (about 40.0 %) and GA_3 (about 10.0 %). There was no significant difference observed between the individual genotypes, likewise no important correlations were found between the genotypes and growth regulators. The AK-1 genotype produced more embryos per floret than 01,095 regardless of the growth regulator applied. However, the differences between particular growth regulators or interactions of growth regulators and genotypes were not statistically significant.

Recent experiments of Kynast et al. (2012) showed that the mixture of 2,4-D with GA_3 was repeatedly proved more effective in haploid embryo stimulation than the previous more concentrated 2,4-D solution without GA_3. Rines et al. (1997) reported in turn that the size of the embryos varied greatly in the range of about 0.5–4.0 mm in length, and this trait had no apparent relation to any of the 2,4-D treatments used. In the present study, haploid embryos excised from caryopses also varied in size. Similarly to the results obtained by Marcińska et al. (2013), treatment with dicamba and 2,4-D, generated a comparable number of enlarged ovaries and embryos in the present work. However, in our experiments, the ovaries following dicamba treatment were smaller than those after the 2,4-D treatment, which have reached a size even two times larger than normal.

According to Rines et al. (1997), the recovery of plants from oat × maize crosses is often sporadic without the consistent presence or absence of the endosperm. Successful plant development most often occurs in the case of medium to large size embryos, but instances of successful recovery from small (<1 mm) embryos were also reported. Overall, the frequency of haploid plant recovery from oat × maize crosses described in the literature ranged from approximately 0.5–2.0 % of pollinated florets. Sidhu et al. (2006) examined 5 oat genotypes for haploid production via pollination by maize and obtained 0.8–6.7 % of haploid embryos and 0.8–1.5 % of haploid plants per emasculated florets. In the study of Rines et al. (2009), four genotypes tested produced on average 7.2 % of haploid embryos. Similar rate of haploid embryo formation (7.8 %) was recorded by Marcińska et al. (2013). Kynast et al. (2012) reported that up to 5.0 % of rescued haploid embryos germinated and developed vigorous plantlets. The efficiency of haploid embryo formation in the experiments of Nowakowska et al. (2015) varied between 2.5 and 6.9 %. All the authors noted a considerable variation in the frequency of haploid embryo formation among the oat genotypes studied. Our results are consistent with the data of the authors cited above, which indicates that the efficiency of haploid embryo formation also depends on the genotype and ranges from 1.1 to 9.0 %.

Rines and Dahleen (1990), in their first study, concerning the production of oat haploid plants by wide crossing, obtained 14 haploid plants (0.42 % of haploids per emasculated florets), while Matzk (1996) produced 4 haploid plants (0.018 % of haploids per emasculated florets). Those authors did not provide the results with respect to the production of DH lines. However, Rines et al. (1996) reported spontaneous chromosome doubling in oat haploid plants and seed production by these plants. The study of Sidhu et al. (2006) demonstrated a considerably higher efficiency of haploid plant production, when compared to the former authors, which ranged from 0.8 to 1.5 %, depending on the genotype. Aung (1998) described the preparation of oat DH lines and the efficiency of this production was on average 1.2 %. Individual genotypes of oat exhibited different response to DH production mediated by maize pollen, which ranged from 0.2 to 2.6 %. Most of the oat plants were essentially sterile and the number of seeds produced was very low. Thirty-three percent of plants produced less than 30 seeds per plant, while 54.0 % did not produce any seeds. Eighty-six percent of haploid plants survived colchicine treatment. Sidhu et al. (2006) also induced chromosome doubling using colchicine and showed that approximately 70–80 % of plants transferred to the soil survived and produced fertile tillers. Marcińska et al. (2013) obtained 0.9 % of haploid plants, and after colchicine treatment, 14 plants acclimated and produced seeds. Nowakowska et al. (2015) reported that the number of DH plants depended on the genotype and varied from 0.8 to 2.3 % per emasculated florets. After colchicine treatment, 64 % of haploid plants survived, and 15 DH plants produced seeds.

In conclusion, the efficiency of haploid plant production in our investigations was primarily dependent on the auxin treatment. In total, we have obtained 149 haploid plants from all genotypes tested. Sixty-one plants survived

chromosome doubling procedure, whereas 52 of them (85 %) were fertile and successfully produced seeds (5227 in total). To our knowledge, this is the first report showing that treatment with auxin after pollination exerted an effect not only on embryo development, but also on the production of haploid and DH oat plants. All the DH lines have been incorporated into breeding programs of Polish breeding companies.

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Conflict of interest The authors declare no conflict of interest.

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