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Application of chosen factors in the wide crossing method for the production of oat doubled haploids

Abstract: Oat (*Avena sativa* L.) has recently gained importance due to the discovery of a variety of health benefits and new opportunities of use. There is no efficient protocol for the production of oat doubled haploid (DH) lines. The aim of this study was to increase the efficiency of obtaining DHs of oat by the wide crossing method. The study was performed on five oat genotypes. We have compared the induction of embryos after pollination with maize, sorghum and millet pollen as well as the development of haploid embryos isolated 2, 3 and 4 weeks after pollination and cultivated on media with different sugar content. Haploid plants were treated with colchicine after or before acclimation to natural conditions. Of the three types of pollen used, the largest number of haploid embryos was obtained using maize pollen. Three weeks after pollination was the most suitable time for the isolation and cultivation of the embryos. The most efficient medium enabling the development of embryos and conversion to plants was 190-2 containing 9% of maltose. Colchicine treatment of acclimated plants provided high survival rate.

Keywords: Doubled haploids, *Avena sativa* L., Pollen donor, Maltose, Embryo development

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1 Introduction

Oat (*Avena sativa* L.) is an important cereal crop from the family Poaceae. The current reduction of world production of oat is associated with a decrease in the population of draft horses and replacement of the grain in fodder with soy beans, corn and other plants. Nevertheless, nowadays oat gets more attention due to its health benefits and new possibilities of application. Moreover, oat can be grown in areas where the prevailing conditions limit the cultivation of other crops [1]. The most characteristic features of oat are high content of protein and valuable fats as well as an amino acid composition favourable for humans when compared to other cereals. Oat grain has a high proportion of shells and contains β -glucans, with high pro-health significance for humans [2]. The potential plethora of applications results in high demand for many different new varieties.

The oat doubled haploids (DHs) are fully homozygous lines, primarily obtained by androgenesis in the anther culture [3] or wide crossing [4,5]. Although several studies have been published demonstrating that the production of oat DHs is possible, fully satisfactory results have not yet been obtained and currently there is no efficient protocol describing the production of DH lines.

Fourteen haploid plants from about 3300 flowers have been obtained in the first experiment involving the pollination of oat by maize (*Zea mays* L.) pollen [4]. Aung [6] stated that oat DH production strongly depended on the genotype of donor plants. The author obtained 1.2% of oat haploid plants by pollination with maize pollen. Not only maize is used as a pollen donor, but also pearl millet (*Pennisetum glaucum* L.) and gamagrass (*Tripsacum dactyloides* L.) [7]. The final efficiency in Matzke experiments [7] was less than 0.1% and the induction of embryos depended on the pollen applied. Partial crosses of oat with pollen donor plants have been observed in the latter study in the population of haploid plants produced [7]. Several maize chromosomes cannot be eliminated during embryogenesis in oat and maize crosses, and retained

maize chromosomes eventually become stabilized. Therefore, these additions of maize chromosomes in the oat lines can be used for physical mapping in maize genomics [8]. Similarly, in the pollination of oat with pearl millet pollen, paternal chromosomes are not eliminated from embryos [9]. Most of the embryo cells derived from the crosses of oat and pearl millet retained all seven chromosomes of pearl millet.

To convert sterile haploids into fertile plants, chromosome duplication is necessary. Chromosome doubling can be performed using treatment with antimicrotubule drugs. Colchicine inhibits microtubule polymerization by binding to tubulin and is the most widely used antimitotic agent. According to Aung [6], a spontaneous doubling of the chromosome number occurred in about 13% of the plants that were not treated with colchicine. Plants treated with colchicine, to induce chromosome doubling, produced seeds efficiently.

The aim of this study was to increase the efficiency of production of oat *Avena sativa* L. doubled haploid by the wide crossing method. The study was performed on five oat genotypes and compared the efficiency of obtaining haploid embryos after pollination with maize, sorghum and millet pollen. Furthermore, the development of haploid embryos isolated at various ages and treated with different levels of colchicine was investigated.

2 Experimental Procedures

2.1 Plant material and growth conditions

Five oat genotypes (F_1 progeny) derived from Strzelce Plant Breeding Ltd., selected from the previous experiment as the most responsive to haploidization [10], were used in the present experiment. The genotypes originated from: 80031 - Deresz x Szakal, 81350 - Krezus x STH 454, 82072 - Bajka x STH 454, 82091 - Bajka x STH 7706, 83213 - Flamingstern x Chwat. Maize (*Zea mays* L. var. *saccharata*), sorghum (*Sorghum bicolor* (L.) Moench) and common millet (*Panicum miliaceum* L.) were used as pollen donors.

Oat plants were grown in controlled conditions in a growth chamber [temperature: 21/17°C day/night, with 16/8 h light/dark photoperiod and photosynthetic photon flux density (PPFD) of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Maize, sorghum and common millet plants were grown in a greenhouse [temperature: 21-28/17°C day/night, natural (solar) light during the day and sodium lamps (6-8 a.m., 6-10 p.m. on cloudy days), 16 h photoperiod]. All plants were fertilized once a week with a Hoagland

liquid medium [11]. Haploid embryos were germinated in an *in vitro* chamber (temperature: 21°C, with 16/8 h light/dark photoperiod and photosynthetic photon flux density (PPFD) of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Haploid plants after acclimatization were grown in a greenhouse (conditions as above). Colchicine treatment was conducted at 25°C with 24 h light and photosynthetic photon flux density (PPFD) of 80-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

2.2 Methods

Oat florets from the central part of the panicle were emasculated before anthesis, when the panicles were growing out from the leaf sheath. Emasculation was performed manually using tweezers. Anthers from the primary and secondary florets were discarded. Panicles were protected with fabric bags. Emasculated oat florets were pollinated (two days after emasculation using a brush) with a fresh pollen mixture of sweet corn cultivars (Ambrosia, Dobosz, Wania and Waza) and pollen of sorghum or millet (collected at 15 minute intervals). The day after pollination, one drop of 3,6-dichloro-2-methoxybenzoic acid (dicamba) water solution (concentration 100 mg dm^{-3}) was applied on each oat pistil.

Enlarged ovaries were collected at 2, 3 and 4 weeks after pollination. They were surface-sterilized in 70% ethanol (1 min), 2.5% calcium hypochlorite (20 min), 0.1% mercuric chloride (1 min) and then washed five times with sterile water. The embryos were immediately isolated from enlarged ovaries and placed individually in 6 mm Petri dishes with the 190-2 regenerating medium [12] containing 0.5 mg dm^{-3} kinetin, 0.5 mg dm^{-3} 1-naphtaleneacetic acid (NAA) and 6% or 9% maltose. Regenerated plants were transferred separately to Magenta vessels (Sigma-Aldrich, USA) with MS medium [13]. Then the plants were acclimated to natural conditions by transfer to a wet perlite and subsequently to the soil. Ploidy level of plants was evaluated using flow cytometry. Approximately 10-15 mg of young leaves was placed in 60 mm glass Petri dish. Ice-cold lysis buffer (45 mM MgCl_2 , 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonic acid – MOPS, 0.1% Triton X-100 in distilled water, pH 7.0) [14] was added to the plant material. The tissue was chopped with a razor blade to release nuclei from cells. Individual samples were then filtered to remove debris with a pre-separation filter 30 μm nylon mesh (Miltenyi Biotec GmbH, Germany) into 5 ml tubes. The nuclei suspension (1.5 ml) was stained with 30 μl of a 2% propidium iodide (PI) solution. Each sample was gently mixed and incubated for 10 min. Aliquots (50 μl) of stained nuclei were mixed and ploidy was analyzed using a flow cytometer MACS Quant (Miltenyi

Biotec GmbH, Germany) equipped with air-cooled laser (488 nm, fluorescence channel 4), and MACSQuantify™ software (Miltenyi Biotec GmbH, Germany). In total, the fluorescence of at least 10,000 nuclei was analyzed in each sample. Nuclei were gated on the basis of logarithmic plots of PI fluorescence and side scatter area (PI-hlog vs. SSC-A). Doublets were excluded using a forward height/forward area scatter plot (FSC-H vs. FSC-A). The control sample from the seed-derived oat plant of known diploid DNA content and potentially haploid/diploid plants were used to set the diploid and haploid gates.

For the chromosome doubling, the roots of haploid plants were treated for 7.5 h with a 0.1% colchicine solution supplemented with 40 g dm⁻³ dimethyl sulfoxide (DMSO), a drop of Tween and 0.025 g dm⁻³ gibberellic acid (GA₃). Then the plant roots were washed in running water for 48 h. The effectiveness of chromosome doubling was evaluated by cytometrical analysis of plant ploidy two weeks after colchicine treatment. All plants were grown in the greenhouse until maturation.

2.3 Statistical analysis

Results concerning the influence of pollen type, maltose concentration and age of the embryos were analyzed using Duncan's test incorporated 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. The influence of pollen donor on embryo formation was tested in 979 oat florets pollinated by maize, 434 pollinated by sorghum and 265 pollinated by millet. The number of replications depended on the availability of pollen from each pollen donor. The effect of maltose concentration and the age of embryos on their germination and development was tested in 26 replications for each treatment.

3 Results

Pollen type had a significant influence on the induction of embryos in oat (Table 1). Maize pollen caused the induction of embryos in all oat genotypes, whereas sorghum did so in two oat genotypes and millet in one. The number of embryos depended on the pollen donor. Forty-three embryos were obtained after pollination with maize, 2 with sorghum and 1 with millet. As shown in Figure 1, the percentage of haploid embryos for genotypes pollinated with maize was 2.5–6.9%, sorghum 1.3% and millet 1.2%.

To recover the haploid plants, 2, 3 and 4-week-old embryos were isolated from enlarged ovaries and cultured on 190-2 medium with 6% or 9% maltose. Embryos obtained by maize pollination germinated within 3 weeks of culture. After this period, the embryos were transferred

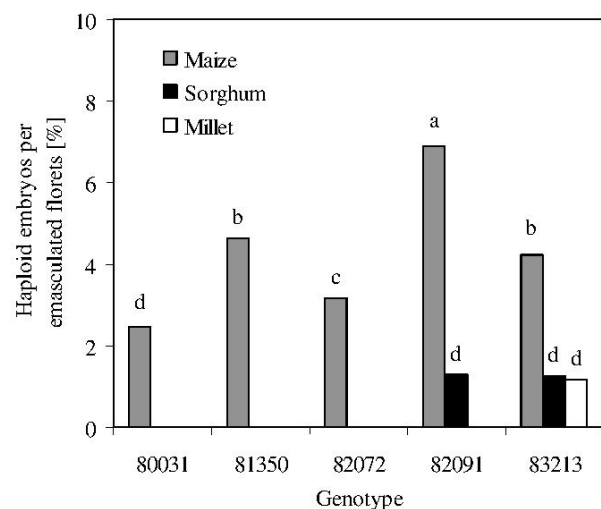


Figure 1: The percentage of haploid embryos obtained after pollination by maize, sorghum and millet in relation to oat genotype. Bars represent mean values ($n = 25 - 236$); significant differences according to Duncan's test at 0.05 level of probability are marked with different letters.

Table 1: Number of oat embryos obtained after pollination by maize, sorghum and common millet.

Genotype	Maize		Sorghum		Millet	
	Emasculated florets	No of embryos	Emasculated florets	No of embryos	Emasculated florets	No of embryos
80031	162	4	97	0	33	0
81350	173	8	63	0	53	0
82072	190	6	117	0	69	0
82091	218	15	78	1	25	0
83213	236	10	79	1	85	1
Total	979	43	434	2	265	1

to MS medium. Embryos derived from sorghum and millet pollination did not germinate. Observation of embryo germinations after three weeks of culture showed that the process was related to the age of isolated embryos and maltose concentration in the medium (Figure 2A). Embryos isolated 2 or 3 weeks after pollination germinated more effectively than the 4-week-old embryos. Embryos isolated at 2, 3 and 4 weeks had the following germination rates: 88%, 93.3% and 66.7% on 190-2 medium with 9% maltose and 78.6%, 70.0% and 77.8% on 190-2 medium with 6% maltose, respectively. Thus, the medium containing 9% maltose was more effective than that with 6% maltose. Number of haploid plants developed was also dependent on the age of isolated embryos and the percentage of maltose in the medium (Figure 2B). The highest percentage of haploid plants (60%) was recorded on medium with 9% maltose. The majority of the haploid plants were obtained from embryos isolated at the age of 3 weeks. Of 43 embryos developed after pollination with maize, 33 haploid plants were obtained (Table 2). All plants were green. The efficiency of haploid plant production varied from 0.5% for genotype 83213 to 3.1% for genotype 82072. Fifteen plants were acclimated to natural conditions and treated with colchicine for chromosome doubling. Number of DH plants was also associated with the genotype and varied from 0.8% to 2.3% per emasculated floret. As demonstrated in the cytograms, all haploid plants had double chromosome number (Figure 3). Sixty-four percent of haploid plants survived treatment with colchicine. DH plants were fertile and produced seeds.

4 Discussion

4.1 Effect of the pollen donor on haploid embryo formation

Reports on the use of the wide crossing method indicated the possibility of using several species of the

monocotyledonous family as pollinators in cereals, e.g., barley (*Hordeum bulbosum*), teosinte (*Zea mays* ssp. *Mexicana*), sweet maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) or millet (*Pennisetum glaucum* L. = *P. americana*) [15]. Rines and Dahleen [4] used pollen originating from 4 different genotypes of maize (A188, B73, Honeycomb, A619 x W 64A) to pollinate oat. The authors did not observe any differences in the ovary enlargement and the formation of embryos using different genotypes of maize in any of the four genotypes of pollinated oat. The study of Matzk [7]

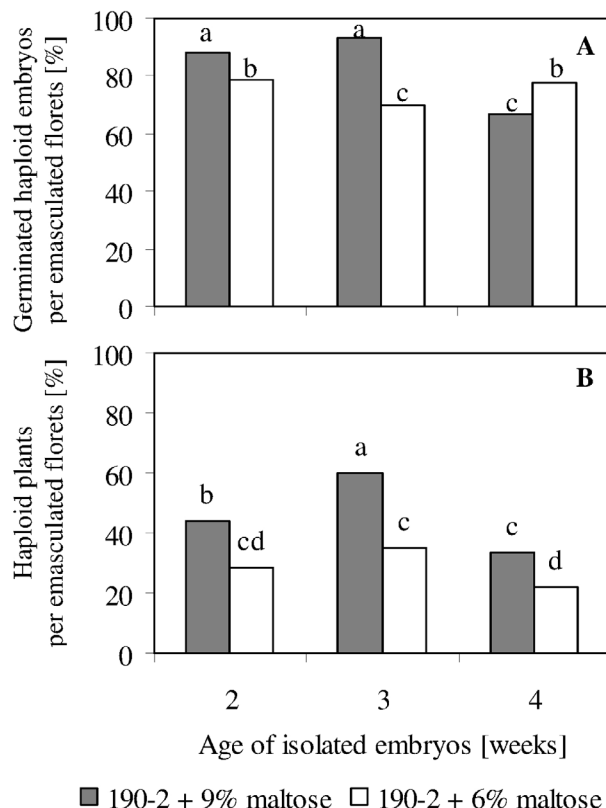


Figure 2: The percentage of germinated haploid embryos (A) and plants (B) after three weeks of culture on 190-2 medium with 6% and 9% maltose. Bars represent mean values ($n = 26$); significant differences according to Duncan's test at 0.05 level of probability are marked with different letters.

Table 2: The efficiency of plant production in five oat genotypes pollinated with maize.

Genotype	Haploid plants	Plants/ Emasculated florets (%)	DH plants	DH plants/ Emasculated florets (%)
80031	6	2.3	6	2.3
81350	1	0.6	0	0
82072	9	3.1	3	1.0
82091	6	2.7	4	1.8
83213	11	0.5	2	0.8
Total	33	1.84	15	1.18

used five cultivars of oat and pollinated them with pollen of 2x and 4x maize, pearl millet [*Pennisetum americanum* (L.) Leeke] and eastern gamagrass (*Tripsacum dactyloides*

L.). Efficiency of embryo induction varied depending on the pollen donor. Most embryos formed when the millet pollen was used (9.8%), and least in the case of tetraploid maize (0.4%). The final efficiency of oat haploid production was lower than 0.1%, because some of the germinated embryos died. Matzk [7] obtained four plants, including oat haploids and a cross with millet and gamagrass. Ishii *et al.* [9] reported pollination of oat, wheat, triticale, rye and other Triticeae by pearl millet (*Pennisetum glaucum* L.) pollen. Complete elimination of millet chromosomes occurred in the majority of wheat embryos. However, in other species, fragments of millet chromosomes were inserted in part of the cells. Oat was a unique species, as in these embryos the presence of fragments of all seven chromosomes derived from pearl millet was observed. In our experiments, maize pollen induced a significantly higher number of embryos compared to sorghum and millet. Sorghum pollen showed no effect in three of the oat genotypes, while only two embryos were obtained in the remaining two genotypes. Millet pollen did not induce embryo formation in four oat genotypes, whereas only two embryos were formed in the fifth genotype. Our results clearly demonstrated that from the three pollen types used in wide crossing, i.e., maize, sorghum and millet, the best effect (expressed as the largest number of haploid embryos formed) could be obtained using maize pollen.

4.2 Effect of culture conditions on the development of haploid plants

Rines and Dahleen [4] cultivated whole oat ovaries after pollination with maize pollen, which potentially contained the embryos. The authors applied MS medium, containing 7% sucrose, supplemented with amino acids according to the protocol described by Norstog [16]. They have also tested the medium without amino acids and medium supplemented with filtered coconut milk. The percentage of oat embryos obtained in a study of Sidhu *et al.* [5] regenerated on B5 medium ranged from 34.6 to 49.3%, depending on the genotype. The experiments of Marcińska *et al.* [10] on oat haploid embryos, derived by the wide crossing method, involved 190-2 medium containing 9% maltose and TL3 medium [17] containing 5% sucrose. On the TL3 medium, 5.6% of plants were obtained, while 190-2 with maltose generated almost 4 times more plants (19.6%). The present work used embryos at different developmental stages (2, 3 and 4-week-old) that were placed on 190-2 medium containing 6% or 9% maltose. More plants formed from the embryos isolated at 3 weeks, which were cultivated on the medium containing 9% maltose in comparison to other media. These results

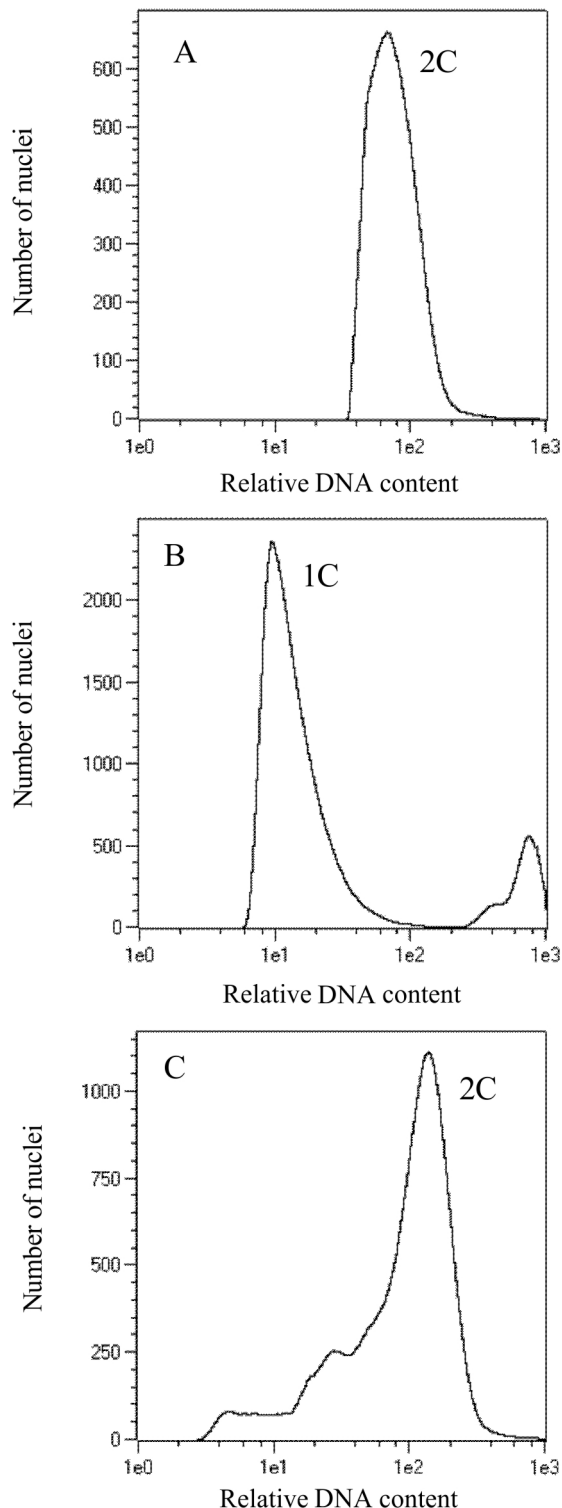


Figure 3: Flow cytometry histograms of control (A), haploid (B) and doubled haploid (C) oat plants (genotype 82072).

are consistent with the study of Marcińska *et al.* [10], where haploid plants were also obtained from embryos cultivated on medium with maltose. A unique part of our experiment was the isolation of haploid embryos at different ages. The results indicate that embryos are the most suitable for isolation and cultivation on the rescue medium 3 weeks after pollination. In summary, the conclusion of the above-described experiments is that maltose is a more accessible source of carbohydrates for oat than sucrose. 190-2 medium containing 9% of that sugar enables the development of haploid embryos and their conversion to plants.

4.3 Effect of colchicine on DH production

In the study of Aung [6], 86% of haploid plants survived colchicine treatment. Of these, 91% produced more than 30 seeds. In the group of untreated plants, only 13% had more than 30 seeds per plant, as a result of spontaneous doubling of the chromosome number. Kiviharju *et al.* [18] obtained 13 haploid oat plants by androgenesis, of which one plant spontaneously doubled the number of chromosomes. Eleven plants were treated with colchicine and doubled the number of chromosomes, resulting in doubled haploid lines capable of producing seeds. Panicles producing seeds ranged from 10 to 100% per plant, and the number of fertile florets ranged from 11 to 76%. Haploid oat plants obtained by Sidhu *et al.* [5] were acclimated to the soil. The plants were washed, trimmed and treated with a solution containing 0.2% colchicine for 3 hours with aeration in the tillering phase. Then they were rinsed with tap water for one hour and transplanted to pots. The results for the two genotypes revealed that about 70-80% of the plants survived and formed seeds. The experiments of Marcińska *et al.* [10] were based on treating 14 haploid oat plants with 0.1% colchicine solution for 7 hours and subsequent rinsing for 48 hours. All of them survived and produced seeds. In the present study, not all of the plants survived the treatment with 0.1% colchicine for 7.5 hours probably due to the prolonged time of colchicine action. The study of Marcińska *et al.* [10] and the current work treated haploid plants with colchicine after acclimatization to the natural condition. This method was more effective in comparison to colchicine treatment prior to the acclimatization [19]. All DH lines obtained in this study produced seeds in different quantities.

In conclusion, the type of pollen donor had the influence on the effectiveness of oat haploid embryos and plant production. Maize was the most effective pollen donor among plants tested. The highest frequency of

haploid embryo germination and number of plants were obtained for embryos isolated 3 weeks after pollination. Higher concentration (9%) of maltose in the medium when compared to 6% concentration was more effective for embryo formation, germination and the development of haploid plants. This study had an innovative character, as it demonstrated that factors applied exerted reproducible effects and significantly enhanced the process of haploidization in such a difficult cereal as oat.

Conflict of interest: Agata Nowakowska at the time of the experiments was a scholarship fellow of the “Doctus – Małopolska scholarship for PhD students” project funded by EU under the European Social Fund and the study was partially supported by the Ministry of Science and Higher Education, PhD grant No. N N130 452138, National Centre for Research and Development, grant No. NR 12002904/2008 and the Ministry of Agriculture and Rural Development, grant No. HORhn-801-1/13. The rest of the authors has nothing to disclose.

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