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Improved approaches in wheat × maize crossing for wheat doubled haploid production

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Wheat × maize crossing is a fast but rather expensive method for doubled haploid production in hexaploid winter wheat. There is an ultimate need for improved efficiency of the method at lower cost. The effect of hormonal treatment and crossing technique on wheat haploid production efficiency were studied. The variation in 2,4-D concentration within the studied range (20–100 mg l^{-1}) had no influence on total efficiency of wheat × maize crosses, while addition of 100 mgl^{-1} $AgNO_3$ to 50 mgl^{-1} 2,4-D solution enhanced embryo formation in wheat × maize crosses from 16.1 to 20.3% (embryos / pollinated florets). The efficiency of wheat × maize crosses was also enhanced by simplifying the crossing technique. Wheat floret emasculation and internode 2,4-D injection was substituted by direct pollination prior to anthesis and subsequent dipping of spikes into 2,4-D solution which resulted in considerable savings of time and labour required.

Key words: wheat × maize crossing, doubled haploid, 2,4-dichlorphenoxyacetic acid (2,4-D)

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is a natural allohexaploid (2n = 6x = 42). Duplication and triplication of the genes in wheat genome leads to complex segregational patterns and epistatic effects [1] which can be difficult to analyze and which complicate wheat breeding. Therefore production of new wheat varieties using the traditionally practiced pedigree system is still a time-consuming process. It requires at least six generations of self-pollination and selection to achieve the levels of homozygosity and hence genotype stability required for a variety to pass the DUS testing, part of which requires that varieties are distinct (D) from others and demonstrate uniformity (U) and stability (S).

The wheat breeding process can be shortened by using doubled haploid (DH) lines. Generally, a minimum of two years can be saved in the release of a new cultivar by the development of recombinant DH populations from inter-varietal F_1s [2]. Laurie and Bennett [3] have been the first to show that a hybrid zygote is formed in the wheat × maize cross, but that the maize chromosomes are rapidly eliminated, usually during the first three embryo divisions. On the other hand, Kynast et al. [4] produced a complete set of maize individual chromosome additions to the oat genome after crossing oat × maize, while Chen et al. [5] had earlier provided molecular evidence to show that a maize-specific DNA fragment was transferred into wheat through wheat × maize hybridization. However, recent studies using AFLP analysis have detected no maize DNA introgression into wheat genome after wheat × maize crossing [6].

Wheat × maize crosses have proved to be more efficient in the production of DH lines than either the 'bulbosum' or the anther culture methods, because of its lower genetic specificity [7]. Despite significant improvements in wheat × maize crossing since the development of the method by Laurie and Bennett [8], the costs of this method remain high. The aim of this work was to study the effect of hormonal treatment and crossing technique in wheat × maize crosses in relation to improving the efficiency of the method for doubled haploid production.

MATERIALS AND METHODS

Plant material

Nine winter wheat F_1 hybrid lines and two maize varieties were used in wheat × maize crossings. F_1 hybrid lines were produced at Lithuanian Institute of Agriculture by intercrossing various winter wheat lines and varieties of local and foreign origin. Two maize varieties, 'Early King' and 'Sundance', were selected as efficient pollinators from the earlier ex-

periments [9]. The selection of maize varieties was based on its availability on the market.

The effect of 2,4-D concentration applied

Five winter wheat F_1 hybrid lines were pollinated with maize variety 'Early King' pollen. The uppermost internode of pollinated tillers was filled with 2,4-D solution, and individual florets were given a drop of the same 2,4-D solution in-between the lemma and the palea 24 hours after pollination. In each of the crossing combinations three different 2,4-D concentrations were applied: (1) 20 mg l^{-1}, (2) 50 mg l^{-1}, and (3) 100 mg l^{-1}. Caryopses and embryo formation was evaluated 17 days after pollination. The number of regenerated haploids was counted 5 weeks after embryo transfer onto 2/3 B5 medium (2/3 of macro and micro salts according to standard B5 composition, 30 g l^{-1} sucrose and 7 g l^{-1} agar). Later, the following efficiency parameters were calculated: caryopses formation frequency (CFF) = number of formed caryopses / pollinated florets, embryo formation frequency (EFF) = number of formed embryos / florets pollinated, haploid regeneration frequency (HRF) = number of regenerated plants / planted embryos, haploid formation efficiency (HFE) = number of regenerated plants / pollinated florets.

Optimization of crossing and 2,4-D application technique

Four winter wheat F_1 hybrid lines were pollinated with maize variety 'Sundance' pollen. The experiment consisted of three treatments. (1) Control: a standard crossing technique was used. Wheat florets were emasculated by removing anthers 1–2 days before anthesis, and maize pollen was applied on the day of anthesis. The uppermost internode of the pollinated tillers was filled with 50 mg l^{-1} 2,4-D solution, and individual florets were given a drop of the same 2,4-D solution in-between the lemma and the palea 24 h after pollination. (2) Application of 2,4-D with $AgNO_3$. Wheat florets were emasculated before pollination as in the control treatment. The uppermost internode of pollinated tillers was filled with 50 mg l^{-1} 2,4-D solution which contained 100 mg l^{-1} $AgNO_3$, and individual florets were given a drop of the same solution in-between the lemma and the palea 24 h after pollination. (3) Dipping without emasculation. Wheat florets were not emasculated but pollinated with maize pollen one day before anthesis. The whole wheat inflorescence was submerged for 10 s into 50 mg l^{-1} 2,4-D solution 24 h after pollination, while no filling of the uppermost internode with 2,4-D solution was performed. Caryopses and embryo formation was evaluated 17 days after pollination. The number of haploid regenerants was counted 5 weeks after embryo transfer to 2/3 B5 medium. Later the following efficiency parameters were calculated: caryopses formation frequency (CFF), embryo formation frequency (EFF), haploid regeneration frequency (HRF), haploid formation efficiency (HFE).

Statistical analysis

The significance of individual factors as well as their interaction were evaluated by performing two-way ANOVA of alternative characters as described in Stelmach [10]. The significance of differences between treatments was evaluated by contigency χ^2 test with software STATISTICA v. 6.0. Correlation coefficients and their significance were calculated with the software STAT v. 1.55 from the package SELEKCIJA (author P. Tarakanovas).

RESULTS AND DISCUSSION

The effect of 2,4-D concentration in wheat × maize crosses

157 to 298 wheat florets were pollinated in each of the experiment treatments, which makes 4258 pollinated florets in total; 3000 (70.5%) of the pollinated florets formed caryopses and 434 (10.2%) developed embryos; 160 (36.9%) of the embryos regenerated wheat plantlets on B5 medium. Thus, the overall haploid formation efficiency (HFE) was 3.8%.

2,4-D concentrations applied in our experiment differed five-fold (20 mg l^{-1} and 100 mg l^{-1}). However, the differences in caryopses formation frequency (CFF) among 2,4-D concentration treatments were not high (on average from 68.7% in 100 mg l^{-1} treatment to 70.3% in 20 mg l^{-1} treatment) (Fig. 1) and were non-significant ($P > 0.05$). Suenaga and Nakajima [11] have shown that 2,4-D treatment is important for successful embryo formation in wheat × maize crosses. 2,4-D induces caryopses swelling and subsequent haploid embryo development up to 14–17 days after pollination. Most probably 2,4-D treatment has a critical concentration for the induction of caryopses formation, and the further increase

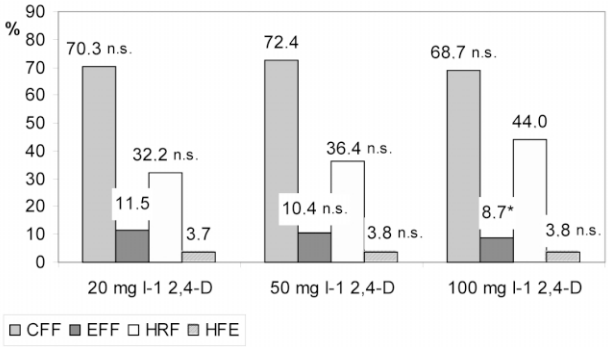

Fig. 1. Effect of 2,4-D concentration on wheat haploid formation efficiency in wheat × maize crosses.
CFF – caryopses formation frequency, EFF – embryo formation frequency, HRF – haploid regeneration frequency, HFE – haploid formation efficiency. *P = 0.01–0.05; n.s. (non-significant) $P > 0.05$ according to contingency χ^2 test

in concentration has less influence on CFF. On the other hand, wheat genotype × 2,4-D concentration interaction had a significant ($P < 0.001$) effect on CFF, implying the genetic specificity of wheat response to 2,4-D treatment applied.

2,4-D concentration had a significant ($P < 0.05$) effect on embryo formation. The highest embryo formation frequency (EFF) was obtained in the 20 mg l^{-1} treatment where 11.5% of florets pollinated formed embryos, while in the 100 mg l^{-1} treatment EFF was significantly ($P < 0.05$) lower reaching only 8.7% (Fig. 1). Treatment with 50 mg l^{-1} 2,4-D yielded an intermediate EFF of 10.4% and the difference was non-significant ($P > 0.05$). As we see, therefore, an increase in 2,4-D concentration has a negative effect on embryo formation, which contradicts the embryo regeneration results. When higher 2,4-D concentrations were applied to pollinated wheat florets, embryos rescued from these florets regenerated more haploid plants (HRF). In a treatment with 100 mg l^{-1} 2,4-D, 44.0% of the planted embryos regenerated haploid plants, while in a treatment with 20 mg l^{-1} 2,4-D regenerants developed only at 32.2% rate (Fig. 1). However, the effect of variation of 2,4-D concentration on HRF could not be proved statistically. The negative correlation between EFF and HRF was also insignificant, showing that variation of 2,4-D concentration in the range of 20–100 mg l^{-1} had no effect on embryo regeneration efficiency. Haploid formation efficiency (HFE) was very similar among the 2,4-D concentrations studied (from 3.7% to 3.8%). A two-way ANOVA revealed that only the wheat genotype had a significant ($P < 0.001$) effect on HFE, while the effect of 2,4-D concentration was not significant ($P > 0.05$).

In conclusion, variation of 2,4-D concentration in the studied range (20–100 mg l^{-1}) had no effect on total efficiency of wheat × maize crosses. However, application of lower 2,4-D concentrations (20 or 50 mg l^{-1}) yielded more embryos, and the total efficiency of the method could be increased by improving *in vitro* regeneration of these embryos.

The effect of 2,4-D application method in wheat × maize crosses

In total, 3164 wheat florets were pollinated by maize variety ‚Sundance' pollen, of them 2710 (85.7%) formed caryopses with 585 embryos (18.5%); 174 embryos regenerated haploid plantlets: 29.7% of the embryos formed or 5.5% of florets pollinated in this experiment.

The caryopses formation frequency (CFF) was significantly ($P < 0.001$) influenced by the 2,4-D treatment method but not by the wheat genotype. The highest CFF 89.9 % of pollinated florets was obtained when 2,4-D was applied with $AgNO_3$ added, and it differed significantly ($P < 0.001$) from the control treatment (Fig. 2). In the treatment 'dipping without emasculation' CFF was also significantly ($P < 0.01$) higher than in the control.

'2,4-D with $AgNO_3$' treatment also yielded a significantly ($P < 0.05$) higher embryo formation frequency (EFF) in comparison to control (20.3% and 16.1% of pollinated florets, respectively), while in the 'dipping without emasculation' treatment EFF was also higher than in the control, but the difference was not significant ($P > 0.05$) (Fig. 2).

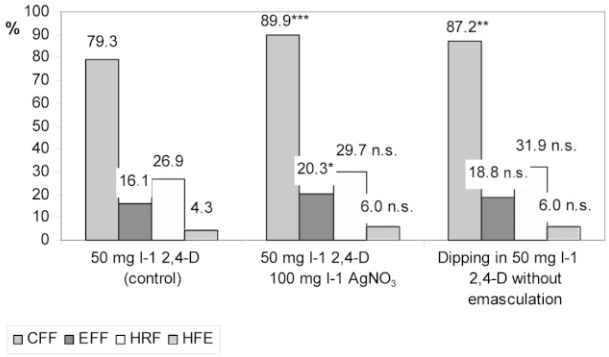

Fig. 2. Effects of 2,4-D treatment method on wheat haploid formation in wheat × maize crosses.
CFF – caryopses formation frequency, EFF – embryo formation frequency, HRF – haploid regeneration frequency, HFE – haploid formation efficiency. ***P < 0.001; **P = 0.001–0.01; *P = 0.01–0.05; n.s. (non-significant) P > 0.05 according to contingency χ^2 test

The haploid regeneration frequency (HRF) was similar in all treatments and the differences were non-significant; 31.9% of the planted embryos regenerated in a 'dipping without emasculation' treatment, 29.7 % in the 2,4-D with $AgNO_3$ treatment, and 26.9% in the control (Fig. 2). Two-way ANOVAs of alternative characters have shown that embryo regeneration in this experiment was influenced neither by wheat genotype nor by the 2,4-D treatment method.

The influence of these factors on haploid formation efficiency (HFE) was also non-significant. However, treatments with both '2,4-D with $AgNO_3$' and 'dipping without emasculation' yielded slightly higher HFE than the control, respectively 6.0% for each experimental treatment and 4.3% for control. As we see, the efficiency of '2,4-D with $AgNO_3$' and 'dipping without emasculation' methods is similar to that of the standard Laurie and O'Donoughue [12] method (control treatment).

On the other hand, the efficiency of the method could be estimated not only by the yield of haploids, but also by the labour required to produce a certain number of haploid plants. Following the standard wheat × maize crossing method [12], wheat florets are emasculated 1–2 days before anthesis by removing anthers, and the uppermost internode of wheat

stem is filled with 2,4-D solution with a hypodermic syringe one day after pollination. These procedures both require skills and occupy most of the time required for crossing. On the contrary, the 'dipping without emasculation' method requires no emasculation of wheat florets but pollination with maize pollen 1–2 days before anthesis to prevent wheat selfing, and the 2,4-D solution injections are substituted by simply dipping the whole wheat ear into 2,4-D solution one day after pollination. Therefore, 'dipping without emasculation' is more efficient than the standard method, despite the same haploid yield obtained, as it requires less labour and a higher number of crosses can be performed at the same time.

$AgNO_3$ showed a positive effect on both caryopses and embryo formation in the wheat × maize crosses studied. More caryopses and embryos were formed in the '2,4-D with $AgNO_3$' treatment as compared to the control. 2,4-D with $AgNO_3$ was also used in durum wheat [13] and proved to be more efficient in embryo formation than 2,4-D without $AgNO_3$. Almousalem et al. [13] have raised a hypothesis that silver nitrate added to 2,4-D solution inhibits the production of ethylene which is induced as a side effect by the application of 2,4-D. The application of $AgNO_3$ is likely to delay the abscission process, and hence facilitatation of embryo development is ensured.

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PATOBULINTAS KVIEÈIØ DVIGUBØ HAPLOIDØ GAVIMO BÛDAS SUKRYÞMINUS KVIEÈIUS IR KUKURÛZUS

Santrauka

Kvieèiø ir kukurûzø kryþminimas yra efektyvus, taèiau sàlyginai brangus kvieèiø homozigotiniø linijø gavimo metodas. Ðio darbo tikslas – padidinti metodo efektyvumà modifikuojant kryþminimo bûdà bei veikimà hormonais. Kukurûzø þiedadulkëmis apdulkintus kvieèiø þiedus paveikus ávairios 2,4-D koncentracijos tirpalu, nustatytas panaðus bendras kvieèiø ir kukurûzø kryþminimo metodo efektyvumas mûsø tirtos 2,4-D koncentracijos ribose (20 mg l^{-1} – 100 mg l^{-1}). Vis tik, naudojant maþesnæ 2,4-D koncentracijà (20 mg l^{-1} ar 50 mg l^{-1}), kvieèiai suformuoja daugiau gemalø, taigi ir bendra haploidø iðeiga galëtø bûti didesnë optimizavus gemalø regeneracijos *in vitro* sàlygas. Sidabro nitratas kryþminant kvieèius ir kukurûzus skatina haploidiniø gemalø formavimàsi, todël, papildþius 2,4-D tirpalà sidabro nitratu, galima padidinti kvieèiø haploidø iðeigà. Kvieèiø ir kukurûzø kryþminimo metodo efektyvumas buvo padidintas supaprastinus kryþminimo metodikà. Atsisakius kvieèiø þiedø kastravimo, o 2,4-D tirpalo injekcijà virðutinio kvieèiø stiebo tarpubambliui pakeitus trumpalaikiu (10 sek.) kvieèiø varpos pamerkimu á 2,4-D tirpalà, bendra haploidø iðeiga lieka panaði, taèiau sutaupomas laikas bei darbo sànaudos – taigi sumaþëja ir metodo savikaina.