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Enhancing haploid wheat induction efficiency through the wheat × maize cross utilizing silver nitrate and calcium phosphate

Original paper

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Abstract: This study aimed to assess the efficacy of silver nitrate and calcium phosphate in inducing chromosome elimination and promoting haploid wheat generation in the context of wheat-maize crosses. The experiment explored the impact of various treatments, including non-use (control) and the utilization of silver nitrate (3 gr/l) combined with calcium phosphate (0.1 gr/l). Additionally, different durations of treatments post-pollination, spanning 48 and 72 hours, were considered. The study evaluated key characteristics in wheat x maize system, such as the number of florets, number as well as the quantity and percentage of successful seed formations. The findings underscored significant distinctions among the treatments, achieving a level of significance of 1%. The use of silver nitrate in conjunction with calcium phosphate for wheat tillers demonstrated an increased propensity for haploid embryo formation. Specifically, maintaining wheat tillers in a solution of silver nitrate and calcium phosphate for 72 and 48 hours, in comparison to the control group, resulted in haploid embryo formations with proportions of 12.53%, 5.60%, and 3.82%, respectively. The findings suggest that prolonged exposure to the silver nitrate-calcium phosphate treatment increases the development of haploid embryos.

Keywords: haploid, chromosome elimination, interspecific hybridization, embryo rescue, tissue culture.

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Introduction

Haploid plant production serves several crucial purposes, including the acceleration of the breeding process, efficient selection of genotypes, and simplification of genetic analysis (Sharma et al., 2002). This method offers the advantage of achieving complete homozygosity in a single generation, as it results in double haploid plants characterized by pure genetic lines. However, it's important to note that this process can be time-consuming. The application of double haploids is pivotal in the cultivation of many crop varieties, relying on efficient protocols for haploid creation (Niu et al., 2014). Successful production of haploid wheat has been demonstrated through interspecific or intergeneric cross (Sourour et al., 2011). Wheat plants have been induced to become haploid via a chromosomal elimination method, involving crosses with various herbaceous plants such as *Teosinte, Sorghum, Tripsacumpearl, Millet, and Hordeum* Notably, crosses between wheat and maize have shown higher efficiency compared to other crosses, increasing the likelihood of obtaining more haploid plants through this approach (Khan and Ahmad, 2011). However, it's essential to acknowledge that this system comes with its challenges, including slight incompatibility issues, the need for specific conditions to maintain wheat and maize plants, limited embryo differentiation, and early maturity. These challenges must be addressed to optimize the effectiveness of this method.

The application of some growth regulators impresses on the frequency of embryo formation and embryo growth and development (Knox et al., 2000). The most common growth regulators used for this purpose are 2, 4-D, which play an essential role in wheat haploid embryo induction (Suenaga and Nakajima, 1989). Using auxins in the external environment causes to increase in ethylene production (Mensuali - Sodi et al., 1992). Silver nitrate ion controls the external ethylene in the plant and plant parts (Bidmeshkipour et al., 2007). The use of silver nitrate in the culture medium can increase embryogenesis (Sridevi et al., 2010). Calcium phosphate is a practical element in transferring foodstuffs to organs in the plant. Calcium plays a role in response to cell signals and responds to various stimuli; furthermore, it plays a role in metabolism and enzymatic absorption, and hormonal processes. Phosphorus, on the other hand, is an essential element for storing and transferring energy in plants (Upadhyaya et al., 2017). Although the chromosome elimination method in wheat × maize crosses has some disadvantages in this study, we tried to increase the chromosomal elimination method's efficiency to improve the production of wheat haploid embryos in crosses with maize by using silver nitrate with calcium phosphate.

Materials and Methods

Plant materials and Plant growth conditions

Plant material used in this study comprised six genotypes of the F_2 generation resulting from hybrid crosses between hexaploid wheat "Mortared" and "Man" as well as"Mortared" × "Pishgam". These genotypes served as the maternal parent, while three commercial maize cultivars, namely SC400, SC260 and SC301, were utilized as the pollinator parent. This research was conducted at the Seed and Plant Improvement Institute of Karaj, Iran. Maize seeds were cultivated 45 days earlier than wheat seeds to synchronize their flowering for pollination. The seeds were treated with carbon thiram 40% and then were sowed in pots with a diameter of 8 centimeters and a height of 10 centimeters with a bed of sand in a greenhouse (250c with 16h/8 light/dark).

The sowing of maize seeds was done continuously until the end of pollination time. After germination and growth sufficiently, the seedlings (2-3 leaf stage) were transferred in the pots with a diameter of 23 cm and a height of 18 cm with a bed of farm soil, sand, and peat moss, respectively, with ratios of 5: 1: 1. Because the wheat seeds were elite, twenty seeds of each genotype were cultivated in laboratory conditions in Petri dishes with 10 centimeters span diameter on filter paper after sterilization with 2% sodium hypochlorite for 5 minutes and 70% ethanol for 1 minute and three times washing with distilled water. The Petri dishes were then exposed to a temperature of 4 degrees Celsius; if they had seed dormancy, they would be broken, and the other growth condition was the same. After 24 hours, they were kept in the

germinator at 22C for 2-3 weeks. After that, they were transferred in pots with a diameter of 13 centimeters and a height of 13 centimeters supplemented with farm soil, sand, and peat moss with ratios of 5: 1: 1, respectively, at 200C and 16h/8h light/dark. Wheat and maize plants were fed with commercial fertilizers containing all the elements needed for plant growth.

Pollination, treatment, and maintenance tillers

The crosses were randomized regarding the availability of spikes from each genotype and fresh pollen from maize plants. When two-thirds of the spikes emerged from their boot leaf, and the middle-floral spikelet had two branches and a fluffy stigma, it was considered the best condition for accepting pollen. The appropriate spikes were harvested from the stem's last part and transferred to the laboratory. Then, the upper and lower florets on the spikes were removed, and two-thirds of the florets in each spike were cut with scissors (without emasculation and removing central florets). Immediately afterward, fresh pollen from each maize variety was collected in the greenhouse on an aluminum foil and brought to the laboratory. Pollination was conducted using a very soft brush.

The pollinated spikes on the stems were then transferred to an MS medium supplemented with 0.1 g/l 2, 4-D, 0.1 g/l silver nitrate, three g/l calcium phosphate, 40 g/l of sucrose, and 8 ml sulfurous acid. After 48 and 72 hours, the ends of the stems were shortened to size 2 to 3 centimeters. This medium was replaced with another medium containing 40 g/l sucrose and 8 ml sulfurous acid until the harvesting of the seeds, cutting the end of stems was done every 2 to 3 days, and a fresh medium was replaced. The seeds were harvested after 20 to 22 days, and the self-pollinated seeds were removed. Embryo rescue was done for seeds in a laminar airflow after sterilization of seeds with 2% sodium hypochlorite for 5 minutes and 70% ethanol for 1 minute. The seeds were washed three times with distilled water to remove the effects of sodium hypochlorite and ethanol. Then, they were placed on binoculars so that the line on the surface of the seeds was downwards. Due to the absence of endosperm, the formed embryos were definitely haploid, so they were cultured in an MS medium.

Sampling and statistical analysis

The number of pollinated florets, the number of seeds, and the number of haploid embryos were recorded. The following formula calculated the percentage of seed and embryo formation.

$$\text{Frequency of seed formation} = \frac{\textit{Number of seed formed}}{\textit{Number of pollinated florets}} \times 100$$

$$\text{Frequency of embryoformation} = \frac{\textit{Number of embryo formed}}{\textit{Number of seed formed}} \times 100$$

The data were compared using the Chi-square x^2 test.

Results

Seed formation

According to Table 1 seed formation was significantly greater in the treated florets than in the control. Furthermore, the highest seed formation (71%) was observed in the silver nitrate treatment containing calcium phosphate for 48 hours (see Figure 1).

Embryo formation

Table 2 shows that treatments with silver nitrate containing calcium phosphate for 48 and 72 hours were effective significantly in enhancing wheat embryo formation in the wheat x maize hybridization. The formation of wheat embryos increased in alignment with longer with longer treatment time when a chemical combination of silver nitrate and calcium phosphate was applied. The highest frequency of embryos (12.53%) was obtained from 72 hours of treatment which showed a significant difference compared to both 48 hours (5.61%) and control (3.82 %) treatments (refer to Figure 2). Therefore, maintaining tillers for 72 hours immediately after pollination in the MS medium supplemented with 0.1 g/l silver nitrate, 3 g/l calcium phosphate was the best. Since the frequency of haploid embryos obtained from seeds reached 12.53%, which was 2.2 times more than the haploid production in the 48-hour treatment and 3.3 times more than in the control treatment. Figure 3 illustrates the various steps of haploid embryo induction in wheat, starting from the emasculation of the florets to seed formation.

Table 1. The effect of treatments in seed formation (caryopsis) in the wheat x maize crosses.

Treatments	Number of pollinated florets	Seed formation (no)	df	χ^2
Control (without $AgNO_3$+$Ca_3(PO_4)_2$)	22,561	16,143		17.95
48 hours use of $AgNO_3$+$Ca_3(PO_4)_2$	14,286	10,698		46.83
72 hours use of $AgNO_3$+$Ca_3(PO_4)_2$	8,533	5,960		175.80
Total	45,380	32,801	**10**	240.58**

**, ns: significant at 1% level and nonsignificant, respectively

Table 2. The effect of treatments in embryo formation in the wheat x maize crosses.

Treatment	Number of seeds	Embryo formation (no)	df	χ^2
Control (without $AgNO_3$+$Ca_3(PO_4)_2$	16,143	617		26.18
48 hours use of $AgNO_3$+$Ca_3(PO_4)_2$	10,698	600		45.18
72 hours use of $AgNO_3$+$Ca_3(PO_4)_2$	5,960	747		41.15
Totals	32,801	1,964		10
			10	112.50**

**,ns: significant at 1% leveland nonsignificant, respectively

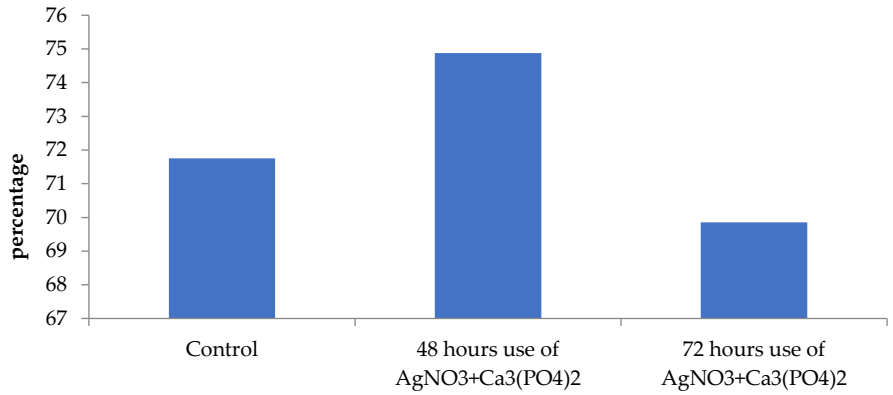

Figure 1. Percentage of seed formation with and without (control) exogenous application of $AgNO_3$+$Ca_3(PO_4)$.

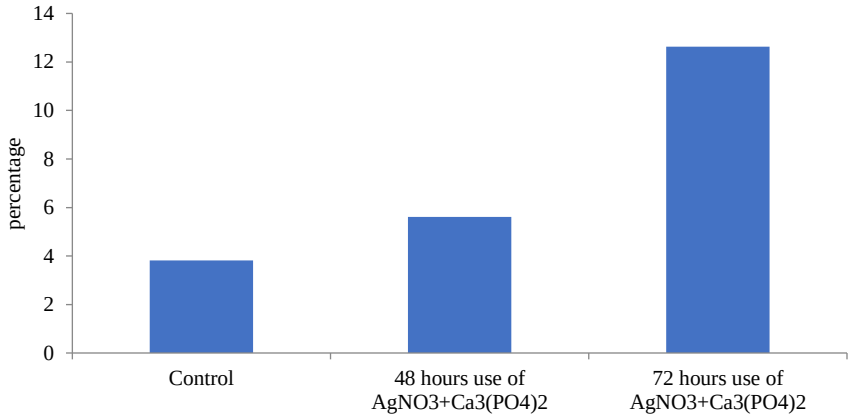

Figure 2. Percentage of embryo formation with and without (control) exogenous application of AgNO3+Ca3(PO4).

Figure 3. Different steps of haploid induction in wheat embryos, A: wheat spike in the optimal growth stage for pollination, B: emasculated spike, C: Spikes ready to receive pollen D: Collection of maize pollen E: Pollination of wheat spike with a soft brush F: pollinated spike G: Maintenance of pollinated spikes in a liquid medium in germinator H: seed formed after 20-22 days I: Haploid embryo.

Discussion

The highest seed formation was seen in silver nitrate treatment with calcium phosphate for 48 hours. Upadhyaya et al. (2017) reported that calcium phosphate is an influential factor in transferring foodstuffs to plant organs. Also,

calcium plays a role in response to cell signals and various stimuli in metabolic processes and absorbing enzymes and growth regulators (Upadhyaya et al., 2017). Nevertheless, phosphorus is an essential element that needs to store and transfer energy in plants. Therefore, using these two substances results in an improved percentage of seed formation. However, according to the present study's results, the treatment time influenced the frequency of seeds significantly. So, with increasing treatment time, the result was reversed, and the seed frequency decreased.

Although 72 hours treatment resulted in the lowest hybrid seed formation in our experiment, it is important to note that the primary goal of the present experiment was to improve production of wheat haploid embryos capable of generating haploid plants via *in vitro* culture. The production ability to form haploid embryos might depend on the application of silver nitrate and growth regulators treatment, alone or in combination (Slama-Ayed et al., 2019). It was reported that treatment with silver nitrate and 2,4-D is an efficient method in wheat and maize cross-system, and also, keeping crossed tillers in 2,4-D solution saves time and reduces problems associated with injection of this substance into the plant (Brazauskas et al., 2005). Bidmeshkipour et al. (2007) used silver nitrate along with 2,4-D in their research, and they expressed that using these substances in optimum concentrations can increase the production of the embryo and haploid plant (Bidmeshkipour et al., 2007). Applying silver nitrate in the culture medium can increase embryogenesis (Sridevi et al., 2010). Increased haploid embryo production through silver nitrate with 2,4-D treatment obtained in the study was conducted by Khan et al. (2012) They reported that treating wheat crossed tillers with 100 milligrams of silver nitrate in maintenance media resulted in a frequency of 52.53% and 28.95% seed and embryo formation, respectively. Also, increased silver nitrate in 150 mg causes more production of seed (54.5%) and embryo (26.96%) (Khan et al., 2012). Khan and Ahmad (2011) declared that the optimum concentration of silver nitrate for seed and embryo formation is 100 milligrams per liter (Khan and Ahmad, 2011). In another study, the optimal concentration of silver nitrate and 2,4-D for this aim was 100 mg/l 2,4-D combined with 75 mg/l silver nitrates (Sourour et al., 2011). In another study, Patial et al. reported in 2020, according to their research, that the response to silver nitrate as an ethylene inhibitor of silver nitrate in the medium does not affect the production of haploid embryos.

Producing haploid embryos is a significant factor in chromosomal elimination techniques. This method's frequency of haploid embryo production in bread wheat is shallow compared to the number of pollinated florets and seeds formed. While the efficiency of haploid plant production from these embryos is high, and the resulting plants are often routine and stable. In most reports, the frequency of haploid embryos obtained from seeds forming by crossing between maize and wheat is less than 5%, and it causes to increase in crosses and embryo rescue for seed production. This will laborious work whit improving the process for embryo formation in crossing between wheat and maize that some treatments for wheat-crossed tillers can reduce. In this study, the frequency of embryo formation in the control treatment was 3.82, but using silver nitrate plus calcium phosphate in tillers maintenance media during the initial 48 hours after pollination improved embryo formation by a frequency of 5.61%. The maintenance of these tillers over 48 hours in this media led to a significant increase in embryo production. So, by maintaining tillers for 72 hours immediately after pollination in this media, the frequency of haploid embryos obtained from seeds reached 12.53%, 2.2, and 3.3 times more than haploid production in 48 hours and control treatments, respectively.

Conclusion

The efficiency of haploid embryo induction is critical to implementing chromosomal elimination techniques aiming at haploid plant production in wheat. However, the frequency of haploid embryo production in bread wheat is low to considering the ratio of the number of pollinated florets and the number of seeds set. While the efficiency of haploid plant production from the induced embryos is high, and the resulting plants are often normal and stable. In most reports, the frequency of haploid embryos obtained from seeds forming by crossing between maize and wheat is less than 5%. It translates the requirement for a high in number of crosses and

embryo rescue for seed production. To reduce this laborious work we implement a treatment of wheat florets following their pollination with maize pollens . In this study, the frequency of embryo formation in comparison with the control was increased 1.5 fold when tillers were kept in medium containing silver nitrate plus calcium phosphate for 48 hours after pollination. Nonetheless, by maintaining tillers for 72 hours immediately after pollination in this treatment, the frequency of haploid embryos obtained from seeds reached 12.53%. This was 2.2, and 3.3 times more than haploid embryo production in 48 hours and control treatments, respectively.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization R.B, R.K; methodology, H.M, R.K, R.B; software, H.M; validation, H.M, R.K, R.B; formal analysis, R.B; investigation, H.M, R.K, R.B; data curation, H.M; writing—original draft preparation, H.M; writing—review and editing, H.M, R.K, R.B; supervision, R.B; project administration, H.M, R.B; funding acquisition, R.K, R.B; Authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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مقاله پژوهشی

بهبود کارایی القای گندم هاپلوئید از طریق روش تلاقی گندم × ذرت با استفاده از نیترات نقره و فسفات کلسیم

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چکیده: این مطالعه با هدف بررسی اثر نیترات نقره و فسفات کلسیم در بهبود تولید گندم هاپلوئید با روش حذف کروموزوم در تلاقی گندم با ذرت انجام شد. در این مطالعه تأثیر تیمارهای مختلف، از جمله عدم استفاده (شاهد) و استفاده از نیترات نقره (۳ گرم در لیتر) همراه با فسفات کلسیم (۰.۱ گرم در لیتر) مورد بررسی قرار گرفتند، همچنین تیمارهای زمانی مختلف پس از گرده افشانی، شامل ۴۸ و ۷۲ ساعت، در نظر گرفته شد. در این مطالعه ویژگی‌های کلیدی مانند تعداد گلچه‌ها، همچنین تعداد و درصد تشکیل دانه مورد ارزیابی قرار گرفت. بین تیمارها تفاوت‌های قابل ملاحظه در سطح معنی‌دار ۱ درصد مشاهده شد. استفاده از نیترات نقره همراه با فسفات کلسیم در محیط نگهداری ساقه‌های گندم تولید بیشتر جنین هاپلوئید را به دنبال داشت. نگهداری ساقه‌های گندم در محلول نیترات نقره و فسفات کلسیم به مدت ۷۲ و ۴۸ ساعت تشکیل جنین هاپلوئید را با ۱۲.۵۳، ۵.۶۰ درصد نسبت به شاهد با ۳.۸۲ درصد بهبود بخشید.

کلمات کلیدی: هاپلوئید، حذف کروموزوم، هیبریداسیون بین‌گونه‌ای، نجات جنین، کشت بافت.

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