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Effects of 2, 4-Dichlorophenoxyacetic Acid and Silver Nitrate ($AgNO_3$) on Haploid Embryo Induction in Triticale (× *Triticosecale*) Following Wide Hybridization with Maize (*Zea mays* L.)

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ABSTRACT

Background: Wide hybridization, also known as intergeneric hybridization, has demonstrated encouraging promise for the generation of haploid embryos in triticale when crossed with maize. This strategy makes use of haploid induction, which eliminates the genetic material of maize from the hybrid embryo to produce haploid triticale embryos.

Methods: The study was conducted at the LPU, School of Agriculture, Phagwara, Punjab, during the *Rabi* season of 2023-2024. The experimental materials included selected maize genotype (PMH 10) as pollen parent and Six triticale genotypes were used as female parents (IC 642724, IC 642662, IC 642661, EC 490146, EC 490148 and EC 490150) obtained from the NBPGR (New Delhi). The study investigates the effects of 2, 4-Dichlorophenoxyacetic acid and silver nitrate on Haploid Embryo Induction in Triticale Following Wide Hybridization with Maize. Four treatment combinations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and silver nitrate ($AgNO_3$) were evaluated for their impact on haploid embryo induction.

Result: The results showed that T_4 (2, 4-D + $AgNO_3$, 100 mg/l + 80 mg/l) followed by T_3 (2, 4-D + $AgNO_3$, 100 mg/l + 60 mg/l) was significant in inducing the formation of haploid embryos, suggesting the synergistic effect of 2, 4-D and $AgNO_3$ in promoting haploid induction. However, no significant effect of individual triticale or maize genotypes was observed on the efficiency of haploid embryo induction. The findings contribute to a better understanding of the factors influencing haploid embryo induction in triticale through wide hybridization with maize and provide valuable insights for optimizing this technique.

Key words: 2, 4-D, $AgNO_3$, Auxins, Embryo, Haploid, Triticale.

INTRODUCTION

Triticale (×*Triticosecale wittmack*), a self-pollinating cereal, originated in 1873 when Scottish botanist A.S. Wilson crossed wheat (*Triticum*) and rye (*Secale*) (Yen and Yang 2020). Today, the definition of a “true” allopolyploid triticale dates back to 1888 when German plant breeder W. Rimpau produced a viable hybrid between wheat and rye. Triticale is divided into two main categories: the octoploid type (2n=56) and the hexaploid one (2n=42), (Shkutina and Khvostova 1971). Triticale, a hybrid species created by crossing wheat and rye, combines the good quality, grain yield and stress tolerance from both its parents (Laouar and Hafsi, 2024). The first successful artificial cross between wheat and rye took place in England, but the resulting hybrid was infertile (Wilson, 1873, Wilde and Miedaner 2021). Rimpau (1891) in Germany obtained a fertile type of Triticale. Triticale grains have slightly lower content of fats (2.09%), proteins (13.05%), moisture (10.51%), than those of wheat (2.74; 13.68 and 10.94%) respectively (Abdelaal *et al.,* 2009).

New techniques such as embryo rescue and chromosome doubling using colchicine were developed to make the process of triticale breeding more reliable (Blakeslee and Avery, 1937). Due to its range of uses, triticale has become a crop that is used for a variety of purposes, including as animal feed, human consumption and the generation of biofuel (Oettler, 2005). Wide hybridization, also known as intergeneric hybridization, has demonstrated

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encouraging promise for the generation of haploid embryos in triticale when crossed with maize (*Zea mays*) (Chaudhary *et al*., 2014). This strategy makes use of haploid induction, which eliminates the genetic material of maize from the hybrid embryo to produce haploid triticale embryos (Inagaki and Bohorova, 1995; Laurie and Bennett, 1988). This procedure allows for the production of fully homozygous doubled haploid (DH) lines by doubling the haploid embryos' chromosomal complement, enabling quicker cultivar development and release (Dunwell, 2010; Germana, 2011).

This study aims to determine the key factors affecting the effectiveness of haploid embryo induction in triticale after extensive hybridization with maize. The research examined the effect of different phytohormones and their concentration on haploid embryo induction, embryo culture media and techniques. The research aims to determine the best combinations of phytohormones that maximize the formation and development of haploid embryos and plant regeneration in triticale following wide hybridization with maize.

MATERIALS AND METHODS

The study was conducted at the Lovely Professional University School of Agriculture in Phagwara, Kapurthala, Punjab, during the *Rabi* season of 2023-24. The experimental materials included selected maize genotype (PMH 10) and six triticale genotypes (IC 642724, IC 642662, IC 642661, EC 490146, EC 490148 and EC 490150) obtained from the National Bureau of Plant Genetic Resources in New Delhi. The triticale lines were sown in a staggered manner at 10-day intervals from the last week of October to November, with 30 cm row-to-row spacing and 2 rows of each line (2 m length). The maize genotypes were sown in a staggered manner at 10-day intervals during the *Rabi* season of 2023-24, with 20 cm plant spacing within rows and 60 cm row spacing between rows.

The haploid embryo induction methodology described by Widzony *et al*. (1998) was followed, involving emasculation of triticale spikes by removing anthers from selected florets using forceps or scissors, followed by pollination with fresh maize pollen collected in sterilized petri plates using a camel toothbrush. Four different phytohormone treatments (100 ppm 2, 4-D, 100 ppm 2, 4-D + 40 ppm $AgNO_3$, 100 ppm 2, 4-D + 60 ppm $AgNO_3$ and 100 ppm 2, 4-D + 80 ppm $AgNO_3$) were applied to the emasculated and pollinated spikes at 24 and 48 hours after pollination using an insulin injection.

The study involved collecting injected spikes from the basal of plants after 13 days and within 16-20 days, haploid plants were preserved when the immature seeds turned transparent. To prevent contamination, 70% ethanol solution was used for washing pseudo seeds. All pseudo seeds were dissected and haploid embryos were identified by cutting them with forceps in an aseptic air flow chamber. The study used Murashige and Skoog (MS) medium to culture haploid embryos from the hybridization of triticale and maize (Niazian and Shariatpanahi 2020). The PT100 packet, a pre-mixed powdered formulation containing essential components of the MS medium, was used to ensure consistent and reproducible media preparation. After identifying haploid embryos, seeds were surface-sterilized with 70% ethanol and dissected to excise immature embryos (Patial *et al*., 2021). The embryo cultures were sub cultured at regular intervals for 2-3 weeks to develop into well-established plantlets with roots and shoots. The culture process was conducted under aseptic conditions, autoclaving the media and culture vessels to maintain sterility. The cultures were incubated at 25°C and subjected to a 16-hour light and 8-hour darkness photoperiod to promote growth and development into green, healthy-looking plants.

The following observations regarding haploid induction parameters were expressed as percentages.

Embryo formation frequency (%) =

$$\frac{\text{Number of pseudo seed carrying embryos}}{\text{Total number of pseudo seed formed}} \times 100$$

Haploid plant regeneration freguency (%)

$$\frac{\text{Number of haploid plant regenerated}}{\text{Total number of pseudo ssed embryo}} \times 100$$

RESULTS AND DISCUSSION

Effect of 2, 4-D and $AgNO_3$ on haploid embryo formation frequency

Out of all four treatments, T_4 and T_3 (100 ppm 2, 4-D + 80 ppm $AgNO_3$) and (100 ppm 2, 4-D + 60 ppm $AgNO_3$) reported to have more haploid embryo formation, 18.71% and 20.13% respectively (Table 1).

The data presented on (Table 2) is the result of a two-way analysis of variance (ANOVA) examining the effects of treatment and genotype for embryo formation frequency. Based on the results, the analysis indicates that there is a significant difference in the data among the effects of various concentrations of the treatment used in the study of haploid embryo formation. This depicts that the treatments were equally good and affect the haploid embryo formation frequency differently with respect to each other. Furthermore, the analysis shows that the genotypes did not demonstrate any notable differences in their impact

Table 1: Mean values of embryo formation frequency.

	T_1	T_2	T_3	T_4
IC 642724	16.67	15.56	18.00	19.59
IC 642662	17.98	17.70	18.89	18.10
IC 642661	14.44	17.35	17.65	20.91
EC 490146	15.05	17.48	18.67	19.13
EC 490148	16.67	16.00	19.05	22.22
EC 490150	16.84	18.45	20.00	20.83
Mean	16.28	17.05	18.71	20.13

on the frequency of embryo formation. This finding underscores the notion that the frequency of embryo formation in triticale is not influenced by the specific genotypes present.

The study compared four treatments (T_1, T_2, T_3 and T_4) using statistical t-tests in (Table 3). The mean values for T_1, T_2, T_3 and T_4 were 16.25, 17.05, 18.70 and 20.12, respectively, with standard deviations ranging from 0.83 to 1.47. Statistical significance was observed in most comparisons, except between T_1 and T_2 ($p = 0.32272$) and between T_3 and T_4 ($p = 0.08029$). Significant differences were found between T_1 and T_3 ($p = 0.00289$), T_1 and T_4 ($p = 0.00805$), T_2 and T_3 ($p = 0.00864$) and T_2 and T_4 ($p = 0.012697$). Correlation coefficients varied from weakly negative to moderately positive across comparisons. These results suggest that treatments T_3 and T_4 generally produced higher values than T_1 and T_2, with T_4 consistently showing the highest mean.

Effect of 2, 4-D and $AgNO_3$ on haploid plant regeneration frequency

Out of all four treatments, T_4 and T_3 (100 ppm 2, 4-D + 80 ppm $AgNO_3$) and (100 ppm 2, 4-D + 60 ppm $AgNO_3$) reported to have more haploid plant regeneration 12.20% and 11.16% respectively (Table 4).

The data presented on (Table 5) is the result of a two-way analysis of variance (ANOVA) examining the effects of treatment and genotype for plant regeneration frequency. Based on the results, the analysis indicates that there is a significant difference in the data among the effects of various concentrations of the treatment used in the study of haploid embryo formation which depicts that the treatments were equally effective and affect the haploid plant regeneration frequency differently with respect to each other. Furthermore, the analysis shows that the genotypes did not demonstrate any notable differences in their impact on the frequency of embryo formation. This finding underscores the notion that the frequency of embryo formation in triticale is not influenced by the specific genotypes present.

The effects of four treatments on an outcome measure compared tabulated in (Table 6), finding an escalating positive effect from T_1 to T_4. The statistical t-test revealed significant differences between the means of T_1 and T_3, T_2 and T_3 and T_2 and T_4, with p-values all below 0.05. T_4 was found to be the most effective treatment for haploid plant regeneration, followed by T_3. The production of wheat haploids through wheat-maize hybridization is summarised on the complete elimination of maize chromosomes from the resultant hybrid embryos (Hussain *et al*., 2013). This phenomenon is mirrored in the generation of triticale haploids *via* analogous intergeneric crossings with maize (Lorenz and Pomeranz 1974). In contrast to androgenic methodologies, these wide hybridization approaches exhibit reduced genotypic dependency, rendering them particularly advantageous in scenarios where the genotype showed resistance to anther or microspore culture techniques. The diminished genotype-specificity of this method confers a significant advantage in breeding programs, as it expands the pool of amenable genotypes and potentially accelerates the development of doubled haploid lines (Chaudhary *et al*., 2014). The present study investigated the effects of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and silver nitrate ($AgNO_3$) on haploid embryo

Table 2: ANOVA for embryo formation frequency.

Source	Df	SS	MSS	F value	P value
Treatment	3	53.399	17.7997	12.8936	0.0001978***
Genotype	5	7.779	1.5557	1.1269	0.3881645
Error	15	20.707	1.3805		

Table 3: Treatment compared for embryo formation frequency.

	Mean	SD	r	T- Stat	p	Significant test
T_1	16.25	1.29	-0.0723	-1.0968	0.32272	Non-significant
T_2	17.05	1.07				
T_1	16.25	1.29	0.53797	-5.42395	0.00289	Significant
T_3	18.70	0.83				
T_1	16.25	1.29	-0.27874	-4.25571	0.00805	Significant
T_4	20.12	1.47				
T_2	17.05	1.07	0.50615	-4.18161	0.00864	Significant
T_3	18.70	0.83				
T_2	17.05	1.07	-0.19097	-3.79545	0.012697	Significant
T_4	20.12	1.47				
T_3	18.70	0.83	0.13862	-2.18804	0.08029	Non-significant
T_4	20.12	1.47				

induction in triticale following wide hybridization with maize. Our findings demonstrate that the addition of these compounds to the induction medium significantly enhanced the frequency of pseudo seed formation and subsequent plant regeneration. These results align with previous research in wheat by Cherkaoui *et al.* (2000) and Laurie and Bennett (1988), who reported similar enhancements in haploid embryo induction using 2, 4-D and $AgNO_3$. The positive effects of these compounds have been observed across various cereal species, including triticale, wheat, maize and durum wheat, highlighting their crucial role in promoting haploid embryo induction through wide hybridization (Sourour *et al*., 2011). Interspecific hybridization is one of the methods of creation of genetic variability and widening of genetic base of a crop species (Mahalingam and Manivannan, 2023).

The auxin-like activity of 2, 4-D plays a key role in stimulating cell division and embryo development (Warchoł *et al*., 2016). $AgNO_3$, on the other hand, regulate ethylene biosynthesis, which may influence embryo survival and development (Kumar *et al.* 2009). Recent research by Testillano (2019) suggests that epigenetic modifications, particularly changes in DNA methylation patterns, may also contribute to the enhanced haploid induction efficiency observed with these compounds. However, the study did not find significant effects of individual triticale or maize genotypes on haploid embryo induction efficiency. This contrasts with previous reports, such as those by Cherkaoui *et al.* (2000) and Wędzony *et al.* (2015), which emphasized the influence of genotypic differences on haploid induction rates. The lack of genotypic effect in our study suggests that the optimized concentrations of 2, 4-D and $AgNO_3$ may have overridden potential genotypic variations, leading to consistent haploid embryo formation across the evaluated triticale and maize genotypes. This finding is particularly promising for breeding programs, as it indicates the potential for developing a more universally applicable protocol for haploid production in triticale and mung bean by Ujianto *et al.* (2019). Recent advancements in understanding the molecular mechanisms of haploid induction have opened new avenues for improving the efficiency of the process. For instance, Kelliher *et al.* (2017) identified a pollen-specific phospholipase, MATRILINEAL (MTL), as a key factor in haploid induction in maize. The discovery of similar genes in other cereal crops could lead to the development of more efficient haploid inducers for triticale. Additionally, Ren *et al.* (2017) reported that the manipulation of centromere histone H3 (CENH3) could enhance haploid induction rates in wheat, suggesting a potential target for genetic improvement of haploid induction efficiency in triticale.

Table 4: Mean value of haploid plant regeneration frequency.

	T_1	T_2	T_3	T_4
IC 642724	6.67	7.14	11.11	15.79
IC 642662	6.25	7.14	11.76	10.53
IC 642661	7.69	5.88	13.33	13.04
EC 490146	7.14	5.56	7.14	9.09
EC 490148	6.67	6.25	10.00	13.33
EC 490150	6.25	10.53	13.64	12.00
Mean	6.78	7.08	11.16	12.20

Table 5: ANOVA for plant regeneration frequency.

Source	Df	SS	MSS	F value	P value
Treatment	3	142.384	47.461	16.0745	5.941e-05***
Genotype	5	29.633	5.927	2.0072	0.1359
Error	15	44.289	2.953		

Table 6: Treatment compared for plant regeneration frequency.

	Mean	SD	r	T-Stat	p	Significant test
T_1	6.83	0.56	-0.67126	-0.27517	0.794192	Non-significant
T_2	7.08	1.80				
T_1	6.83	0.56	-0.14728	-4.17703	0.00868	Significant
T_3	11.16	2.39				
T_1	6.83	0.56	0.193027	-5.8273	0.002103	Significant
T_4	12.29	2.33				
T_2	7.08	1.80	0.602834	-5.137631	0.003653	Significant
T_3	11.16	2.39				
T_2	7.08	1.80	0.104982	-4.55966	0.006059	Significant
T_4	12.29	2.33				
T_3	11.16	2.39	0.404225	-1.07345	0.332122	Non-significant
T_4	12.29	2.33				

CONCLUSION

In conclusion, phytohormone treatments significantly influenced the efficiency of haploid embryo induction in triticale. Treatment 4 (T_4), which combines 100 mg/l (2, 4-D) and 80 mg/l ($AgNO_3$), was found to be the most effective combination, resulting in the highest mean embryo formation frequency (20.13%) and haploid plant regeneration frequency (12.30%) across all genotypes evaluated. Treatment 3 (T_3) also showed promising results, with mean embryo formation and haploid plant regeneration frequencies of 18.71% and 11.16%, respectively. However, the study did not detect significant effects of individual triticale or maize genotypes on the efficiency of haploid embryo induction. The findings highlight that as the concentration of $AgNO_3$ increases, the efficiency of haploid embryo production also increases up to the level tested. Hence, optimizing phytohormone concentrations can enhance the efficiency of haploid embryo induction, leading to the rapid generation of homozygous lines and exploration of genetic variability for crop improvement in triticale.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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