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Effect of dimethyl sulfoxide on embryogenesis and green plant regeneration in wheat (*Triticum aestivum* L.) anther culture

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Abstract: This study aimed to evaluate the effect of dimethyl sulfoxide (DMSO) on microspore embryogenesis and green plant regeneration in wheat anther culture. Five culture media, as well as the inclusion of 1% DMSO in the surface-disinfection solution, were investigated in three winter wheat genotypes. Our results showed that the Altındane genotype produced the highest number of embryoids 215 per 100 anthers cultured in CHB-3 medium, whereas the Dariel and Pehlivan genotypes produced 6.6 and 0 embryoids, respectively, from 100 anthers cultured. On the other hand, the addition of 1% DMSO to the same medium adversely affected embryoid production compared to the medium without DMSO. A 70% ethanol solution with 1% DMSO for the surface disinfection of spikes was effective in increasing the embryoids from approximately 0 to 17.8% and from 1 to 48.4% in CHB-3 +1%DMSO and CHB-3 medium, respectively. Furthermore, the Altındane genotype produced 22.2 plantlets/100 anthers (17.7 albino and 4.4 green plants) and 17.7 albino plantlets per 100 anthers in CHB-3 and CHB-3+DMSO, respectively. Our results suggested that the inclusion of 1% DMSO in the disinfection solution increased the number of embryoids without supporting the production of green plants.

Keywords: Dimethyl sulfoxide; doubled haploid; embryogenesis; *Triticum aestivum* L., wheat.

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most vital grain cereals on the globe, being a key source of calories and protein for humans and providing staple foods for 35% of the world's population [1,2]. Among the cereals, wheat ranks first in worldwide production, with 734 million tons. It has been estimated that wheat production should be increased by 38% by 2050 to meet the projected demands of population growth, dietary changes, and increasing biofuel consumption [2]. The biggest threat to food security faced by humanity in the twenty-first century is climate change, which is incredibly unpredictable [3]. With a one-degree Celsius increase in global temperature, global wheat yields would decrease by 6.0 ± 2.9% [4]. Developing more nutritious, resilient, and productive wheat varieties can significantly boost wheat production. Various effective methods can be applied in plant development to expedite the breeding process and enhance the effectiveness of breeding programs. The doubled haploid (DH) plant production technique is a key biotechnological method in contemporary plant breeding [5].

Homogeneity is a fundamental requirement in developing new varieties and hybrids, and one of the main advantages of DH technology is its ability to produce homozygous lines in a single generation [6].

In essence, DH technology relies on the use of haploid cells to develop haploid embryos, which can subsequently become diploid either autonomously or through additional chromosome doubling treatments. The methods for producing haploids and DHs are remarkably diverse. These techniques offer the possibility of rapidly identifying re-

cessive alleles [7], expediting the progression of selected lines toward complete homozygosity, and enhancing selection efficiency [8].

Researchers have employed a range of chemical compounds, including Ionic liquid [9], zearalenone [10], n-butanol [11], DMSO [12], Trichostatin A [13,14] and glutathione [15], in wheat and other cereal androgenesis studies. Furthermore, numerous researchers have dedicated extensive efforts to enhance the overall efficiency of producing viable green plantlets through wheat anther culture. Nonetheless, despite promising results, anther culture still presents challenges, including genotype dependence, occurrences of albinism, and suboptimal efficiency in producing healthy green plants.

Genotype, microspore development stage, and culture conditions are the most important factors affecting the microspores developmental fate. That is how we decided to make some modifications to the cultural conditions for better results. DMSO is a chemical substance and a recognized solvent utilized frequently in cell biology due to its unique physicochemical characteristics, which enhance membrane permeability, reduce lipid bilayer and membrane fluidity [12], as well as its ability to induce alterations at the cellular level of the membrane [16]. In wheat androgenesis, Echávarri and Cistué [12] utilized four different doses of DMSO in a pretreatment medium for the first time. Notably, a concentration of 1% v/v increased the number of green plants in recalcitrant cultivars threefold and reduced the rate of albinism. Additionally, in the case of barley, more green plants resulted in a two- to four-fold increase in all cultivars and F1 crosses [12] So, we conducted this study to ascertain whether adding 1% DMSO to the surface-disinfection solution of explants and the induction medium will increase the efficiency of embryo production and regeneration of green plantlets and will decrease genotype dependency in wheat (*T. aestivum*). To the best of our knowledge, no study on its use as a component of disinfection solution and induction medium in anther cultures has been reported.

2. Methods and materials

2.1. Plant materials

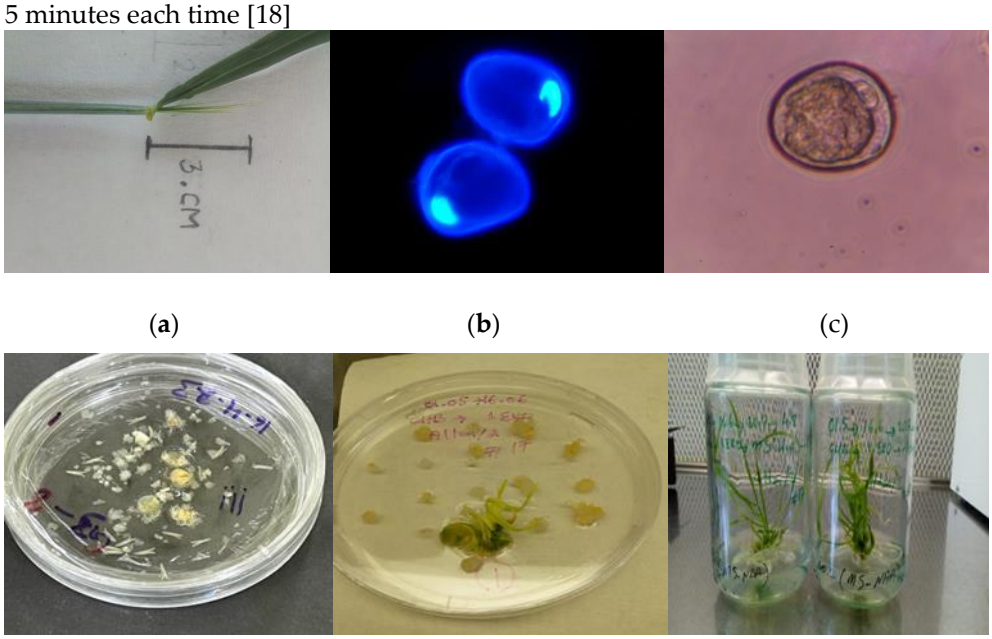
In this research, we employed three winter bread wheat genotypes: Altındane, Dariel, and Pehlivan. These plant materials and growth conditions were provided by Tasaco Tarım Ticaret A.Ş., Antalya, Turkey. The seeds were initially grown in a controlled environment with a day/night temperature of 20/18 °C and an 18-hour photoperiod. After two weeks of seedling growth, they were subjected to a vernalization period at 4 °C, maintaining a 12-hour photoperiod for 4 to 6 weeks. Following vernalization, the temperature was adjusted to 15/12 °C with an 18-hour photoperiod.

2.2. Spike collection, pretreatment and surface-disinfection

For anther culture, tillers were carefully collected from donor plants when the anthers contained microspores at the mid-to-late-uninucleate developmental stage. To evaluate and discern the microspore population, a staining technique was employed using 4,6-diamidino-2-phenylindole (DAPI), following the methodology outlined by Ari et al [6]. Following that, microspores were isolated from various spikes with emergent lengths ranging from 0-9 cm. A careful examination revealed that the majority of microspores within the emergent lengths of 1-3 cm had reached the mid-to-late uninucleate stage[14]. (Figure 1).

Afterward, selected spikes were placed in a 250-ml jar containing 50 ml of cold water, covered with a plastic bag, and kept at 4 °C for 4-6 days. Following this, all leaves and awns were trimmed. Care was taken not to excessively trim the awns to avoid exposing the top of the floret, which could allow bleach to penetrate, potentially harming the microspores [17]. For surface disinfection, the spikes were immersed in a 70% ethanol solution in a 250-ml jar for one minute in a laminar flow cabinet. Simultaneously, for

the Pehlivan genotype, we mixed 70% ethanol with 1% DMSO. Subsequently, the spikes were rinsed with cold, sterile ddH_2O for three minutes. Afterward, the spikes were subjected to an additional disinfection step for 15 minutes using a 30% bleach solution (containing 5% sodium hypochlorite) mixed with 4 drops of tween-20 in a 250-ml jar. The spikes were thoroughly rinsed three times with cold, sterile ddH_2O for approximately 3-5 minutes each time [18]



(**a**) (**b**) (c)

(d) (e) (f)

Figure 1. (**a**) suitable spike for androgenesis; (**b**) mid to late uninucleate stage (c) released microspore development in anther culture (d) embryoid production in CHB-3 induction medium (e) regeneration of green and albino plants (f) green regenerated plantlet.

2.3. In vitro culture conditions

After surface disinfection, anthers were aseptically dissected from spikes and cultured in sterile 60-mm-diameter petri dishes containing 8 ml of induction medium (Supplementary Table 1). The cultured anthers were incubated at 30 °C for 6-7 weeks in darkness. Embryoids derived from anthers were counted and transferred to 90-mm-diameter petri dishes containing modified CHB-3 medium (Supplementary Table 1). These petri dishes were kept in a climate-controlled room at 25 °C with a 16-hour photoperiod. After 2 weeks, the number of green and albino regenerants was counted. The green plantlets were transferred to a jar containing 30 ml of hormone-free medium consisting of MS salts [19] and NN vitamins [20]. After 15 days in this medium, they were moved to the same medium supplemented with 2 mg l^{-1} NAA to encourage root development. Following an additional 15 days in this medium, the green plantlets were then transferred to small pots filled with a mixture of 1:2 perlite and peat mass. These pots were kept in a controlled environment at 18 °C with a 16-hour photoperiod for a duration of 2 weeks. Finally, the regenerated plantlets were ready for their last transfer, this time to larger pots with sizes ranging from 12 to 18 inches. In these pots, they continued to grow and mature.

2.4. Research design and data analysis

This investigative study employed a simple randomized design approach that included the examination of three genotypes along with the use of five culture media. Additionally, two spike disinfection methods were employed. Each treatment had 3 replica-

tions, whereas 1 petri dish was considered a single replication, 15 anthers were aseptically removed from the middle of the spike for a single petri dish. Variables such as the number of embryoids, green plantlets, and albino plantlets were recorded and expressed as averages per 100 anthers.

Statistical analysis was performed using Kruskal-Walli's test in R Studio version 4.3.1 (2023-06-16).

Table 1. The average number of embryoids, albino plants, and green plants per 100 anthers.

Treatments	Embryoids / 100 anthers	Albino plants / 100 anthers	Green Plantlets / 100 anthers
Genotypes			
Altındane	46.66	8.00	0.88
Dariel	1.33	0.44	0.00
Pehlivan	0.00	0.00	0.00
F- probability	<0.05	<0.05	<0.05
Medium			
MS1	0.00	0.00	0.00
MS2	0.00	0.00	0.00
CHB-3	74.07	8.15	1.48
CHB-3+MS2	0.00	0.00	0.00
CHB-3+1%DMSO	5.92	5.92	0.00
F- probability	<0.05	<0.05	<0.05
Kruskal-Wallis	0.783	0.783	1

[1] Based on the Kruskal- Walli's test there is not significantly different between variables.

3. Result and discussion

In androgenesis studies of wheat and other cereal crops, many factors influenced the process of embryogenesis. One of the most important factors is culturing anthers at the mid-to-late uninucleate development stage of microspores [10]. This is because the typical pollen-formation pathway changes at this point. Additionally, callus and androgenic structures are generated as microspores undergo frequent mitotic divisions. Furthermore, genotype dependency is another important factor for haploid studies, which could significantly affect the process of embryogenesis [17,18].

Tables 1 and 2 present the responses of anthers from all three genotypes. Observable differences emerged: Dariel produced 1.3 embryoids, while Altındane yielded 215 embryoids per 100 anthers, respectively. These differences are believed to be influenced by the tested genotypes. The highest number of embryoids achieved in our study surpassed figures reported by Echávarri and Cistué [12] in wheat (190.4 embryoids per 100 anthers) and by Lantos et al [21] in split wheat (173.3 embryo-like structures per 100 anthers). Our data shows that the average of obtained embryoids from 100 anthers is above 16, which is higher than 10 androgenic structures per 100 anthers [10], 7.8 calli per 100 anthers [22]and 6 embryo-like structures per 100 anthers [9] were reported in the relevant literature.

Another challenge to using anther culture in some wheat cultivars is albinism. Some genotypes have 100% albinism. In our study, the number of albino plants and green plants was also affected by genotypes. The highest production of albino plants was in genotype Altındane (22.2/100 anthers), while the lowest was in genotype Pehlivan (2.2/100 anthers). Green plant production was only produced from genotype Altındane, about 0.8 green plants from 100 anthers. Other researchers enhanced the productivity of a different culture by utilizing responsive genotypes (11.4% of 100 anthers[23], and various pretreatment techniques. For example, n-butanol mixed with Ca

in macronutrient pretreatment enhanced the green plant from 0 to 27 per 100 anthers [11]. Additionally, adding 1% DMSO to the pretreatment media enhanced the number of green plants twofold to fourfold [12]. Still, this approach would require extra practice to develop haploid plants.

Table 2. The average numbers of embryoids, albino plants, and green plants per 100 anthers in genotype Pehlivan.

Medium	Normal surface disinfection			+1%DMSO surface disinfection		
	Embryoids / 100 anthers	Albino plants / 100 anthers	Green Plantlets / 100 anthers	Embryoids / 100 anthers	Albino plants / 100 anthers	Green Plantlets / 100 anthers
MS1	0.00	0.00	0.00	0.00	0.00	0.00
MS2	0.00	0.00	0.00	0.00	0.00	0.00
CHB-3	0.00	0.00	0.00	41.6	2.2	0.00
CHB-3+MS2	0.00	0.00	0.00	0.00	0.00	0.00
CHB-3+1%DMSO	0.00	0.00	0.00	4.4	0.00	0.00

As we mentioned before, there are many chemical compounds available that can be used in induction media for the process of wheat androgenesis [9, 10, 13, 14]. The use of DMSO in pretreatment media has been reported by Echávarri and Cistué [12]. They found that low concentrations of DMSO and mannitol as stressors, boosting DH production in anther culture of barley and wheat, but the use of DMSO in induction medium has not yet been reported. The utilization of DMSO in induction media is reported in this study, our result showed that the inclusion of 1% DMSO to the induction medium yielded a lower rate of embryoid production. Specifically, Medium CHB-3 demonstrated the highest embryo production, with an average of 74 embryoids per 100 anthers. This result suggests that CHB-3 provides optimal conditions for embryo induction in wheat anther culture. In contrast, medium CHB-3+1%DMSO yielded a lower rate of embryoid production, and MS1, MS2, and CHB-3+MS2 did not produce any embryos. We observed that only the CHB-3 medium elicited responses for green plants in the Altındane genotype. Overall, the average for green plants across all genotypes was 1.48 per 100 anthers. Notably, the Altındane genotype exhibited a substantially higher average of 4.34 green plants per 100 anthers.

To date, approximately the same surface disinfection methods have been applied, and there is no report regarding the uses of DMSO in surface disinfection for wheat androgenesis. That is why we decided to add 1% of DMSO to the surface disinfection solution, because cell culture experiments reported that the rise in permeability and a reduction in lipid bilayer thickness caused by DMSO could promote the permeation of substances, which could facilitate the uptake of nutrients in the initial phases of embryogenesis and lead to faster embryo development [12]. The preference for the disinfection method had an extensive impact on embryoid production. Surface disinfection of the spikes with 1% DMSO increased embryoid production from 0 to 46 embryos per 100 anthers (Table 2). Furthermore, when 1% DMSO was used in the induction medium, there was a noticeable decrease in embryoid production across all genotypes and media. As an important result, the presence of DMSO at some point during induction can also inhibit embryoid formation. A similar effect was observed in a study by Echávarri and Cistué [12] in recalcitrant genotypes in both barley and wheat plants. When they used different concentrations of DSMO in a pretreatment medium, DMSO also contributed to an increase in the number of green plant productions only in responsive genotypes.

4. Conclusion

In conclusion, this study emphasizes the complex nature of wheat anther culture by showing that anther development stage, genotype, culture medium, and surface-disinfection procedures, with a particular emphasis on the function of DMSO, are all necessary conditions for effective embryogenesis. While challenges such as albinism persist, the findings open avenues for further research and advancements in the field. The use of 1% DMSO in the surface disinfection solution allowed us to increase the numbers from 0–46 embryoids per 100 anthers, but the inclusion of 1% DMSO in the induction medium decreased the number of embryoids across all genotypes and media. To our knowledge, this is the first report showing that 1% DMSO could be used as a successful enhancer for the process of wheat androgenesis when added to the solution of surface disinfection. The findings of the investigation of DMSO's dual function as an inhibitor and facilitator of embryoid formation show the complexity of additional research. These findings showed considerable advancements in crop improvement and agricultural innovation and provided helpful recommendations, like the high production of embryoids for the development of haploid studies and wheat breeding initiatives.

4. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Medium components used in the induction and regeneration medium.

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