biology and life sciences forum

Proceeding Paper

The Effect of Dimethyl Sulfoxide on Embryogenesis and Green Plant Regeneration in Wheat (*Triticum aestivum* L.) Anther Culture †

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† Presented at the 3rd International Electronic Conference on Agronomy, 15–30 October 2023; Available online: https://iecag2023.sciforum.net/.

Abstract: This study aimed to evaluate the effects 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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Citation: Sharafat, A.R.; Ari, E.; Izmirli, A. The Effect of Dimethyl Sulfoxide on Embryogenesis and Green Plant Regeneration in Wheat (*Triticum aestivum* L.) Anther Culture. *Biol. Life Sci. Forum* **2023**, *27*, 46. https://doi.org/10.3390/IECAG2023-16675

Academic Editor: Gianni Bellocchi

Published: 25 December 2023

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most vital grain cereals on the globe and is a key source of calories and protein for humans that provides staple food 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 via additional chromosome doubling treatments. The methods for producing haploids and DHs are remarkably diverse. These techniques offer the possibility of rapidly identifying recessive 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 via wheat anther culture. Nonetheless, despite promising results, anther culture still presents challenges, including genotype dependence, the occurrences of albinism, and suboptimal efficiency in producing healthy green plants.

The genotype, microspore development stage, and culture conditions are the most important factors affecting the microspore's 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 and 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 the 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

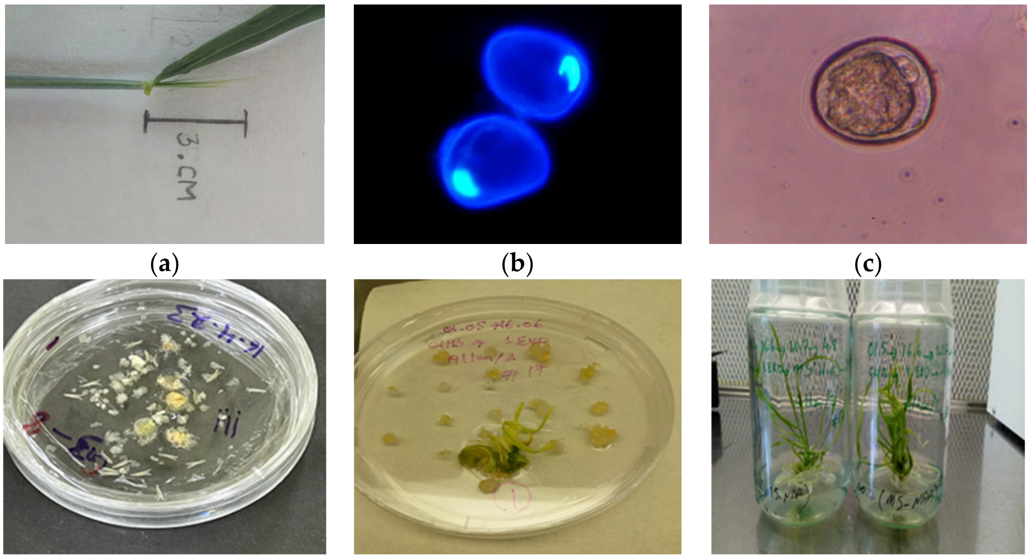
2.1. Plant Materials

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 h photoperiod. After two weeks of seedling growth, they were subjected to a vernalization period at 4 °C, maintaining a 12 h photoperiod for 4 to 6 weeks. Following vernalization, the temperature was adjusted to 15/12 °C with an 18 h photoperiod.

2.2. Spike Collection, Pretreatment and Surface Disinfection

For the anther culture, tillers were carefully collected from donor plants when the anthers contained microspores during 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 to 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 min 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 min 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 S1). The cultured anthers were incubated at 30 °C for 6–7 weeks in darkness. Embryoids derived from the anthers were counted and transferred to 90 mm diameter Petri dishes containing the modified CHB-3 medium (Supplementary Table S1). These Petri dishes were kept in a climate-controlled room at 25 °C with a 16 h 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 h 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 replications, whereas 1 Petri dish was considered a single replication. Fifteen 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–Wallis test in R Studio version 4.3.1 (16 June 2023).

3. Result and Discussion

In the 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. 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 show that the average of the 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] and were reported in the relevant literature.

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 [1]	0.783	0.783	1

[1] Based on the Kruskal–Wallis test, there are no significant differences between variables.

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

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, at 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 a 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 two-fold to four-fold [12]. Still, this approach would require extra practice to develop haploid plants.

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 boosted DH production in the 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 results showed that the inclusion of 1% DMSO in 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 have been no reports 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. Conclusions

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 to 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 to show 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.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/IECAG2023-16675/s1. Table S1: Medium components used in the induction and regeneration medium.

Author Contributions: Conceptualization, E.A. and A.R.S.; methodology, A.R.S.; software, A.R.S.; validation, A.R.S., E.A. and A.I.; formal analysis, A.R.S.; investigation, A.R.S.; data curation, E.A. and A.R.S.; writing—original draft preparation, A.R.S. and A.I.; writing—review and editing, E.A.; supervision, E.A.; project administration, E.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data supporting the reported results are included within the main body of the paper. Additionally, Supplementary Data relevant to the findings can be found in the Supplementary Materials provided, specifically in the Supplementary Table S1.

Acknowledgments: We are very appreciative of Tasaco Tarım Ticaret A.Ş., Antalya, Turkey for providing us with an environment for growing the donor plants.

Conflicts of Interest: The authors declare no conflicts of interest.

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