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Optimizing *In Vitro* Regeneration of Wheat via Somatic Embryogenesis Using Endosperm-Supported Mature Embryos

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ABSTRACT: Wheat is a crucial crop for global food security, and effective *in vitro* plant regeneration techniques are considered a precondition for genetic engineering in wheat breeding programs. A practical approach for *in vitro* regeneration of the Kırık bread wheat cultivar via somatic embryogenesis was investigated using endosperm-supported mature embryos. Callus cultures were initiated from mature embryos supported by endosperm, cultured on phytagel-based Murashige and Skoog (MS) basal medium containing dicamba (12 mg/L) and indole-3-acetic acid (IAA) (0.5 mg/L) under dark conditions. This research was designed to examine the impact of putrescine (Put) (0.0 and 1.0 mM) on inducing embryonic callus and the effects of thidiazuron (TDZ) (0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L) on wheat regeneration. Adding 1.0 mM putrescine to MS medium significantly increased ($p < 0.01$) embryogenic callus formation, resulting in a complete (100%) induction rate. Moreover, the highest number of regenerated plants per explant (5.8) was obtained through the synergistic interaction between 1.0 mM putrescine and 0.5 mg/L TDZ. To assess the genetic homogeneity of regenerated plants, 10 different inter-simple sequence repeat (ISSR) primers were utilized, revealing a high level of genetic stability. The results of all the applications of a particular plant tissue culture technique showed a level of somaclonal variation within acceptable limits, indicating that the genetic diversity of the plant populations was protected without compromising the desired traits. These improvements offer a promising tool for wheat biotechnology, especially for genetic transformation.

KEYWORDS: ISSR; plant regeneration; putrescine; somaclonal variation; TDZ

1 Introduction

Wheat (*Triticum aestivum* L.) is a nutritionally and economically significant crop due to its widespread cultivation and use as a staple food worldwide [1]. By 2050, the global population is expected to hit 9.7 billion, leading to a 70% rise in food demand compared to 2005–2007 levels [2]. To address the rapidly increasing demand for food, plant breeders use both traditional and genetically engineered methods to improve wheat crops [3]. Tissue culture is considered a fundamental aspect of genetic engineering, providing an added advantage to crop breeding operations [4,5]. Furthermore, the success of crop genetic engineering and gene transfer research depends on the development of an effective regeneration system [6].

Somatic embryogenesis is a commonly employed regeneration pathway in tissue culture systems for plant regeneration [7–11] and has significant potential for rapid clonal reproduction of different plant



species [12,13]. Additionally, somatic embryos can serve as a dependable source of material required for gene transformation in plants [14]. The main obstacle to genetic engineering programs is poor plant regeneration in various crops, including wheat. Genotype, explant source, culture medium, and their interactions are the primary determinants of wheat plant somatic embryogenesis regeneration frequency [15,16]. Although immature embryos are the most frequently utilized explant type in wheat gene transfer studies due to their high regenerative capacity [5,17,18], different studies have demonstrated that mature embryos can also be utilized [19–21]. Mature embryos can be obtained throughout the year and easily isolated from seeds, thereby maintaining the genetic stability of the regenerated plants [22].

In vitro, plant morphogenesis is governed by chemical agents, primarily plant growth regulators, present in the culture composition. Cytokinin, a cell division stimulant in *in vitro* tissue culture, increases the growth rate of pro-embryogenic mass [23]. In wheat tissue culture, cytokinin was used with or without auxin to stimulate callus regeneration [15,24]. Thidiazuron (TDZ) has gained interest from researchers because of its auxin and cytokinin-like properties on plant tissue culture [25,26]. TDZ also promotes the growth of somatic embryogenesis in plants [27,28].

Polyamines are essential for plant development and somatic embryo formation, just like auxins and cytokinins. Changes in polyamines and their biosynthetic enzyme levels cause hormonal responses [29,30]. Putrescine (Put), one of the polyamines, plays a role in many vital processes in plant cells, including protein synthesis, DNA replication, cell division, root development, tolerance to abiotic stress, and promoting growth and morphogenesis, such as somatic embryogenesis development in *in vitro* plant tissue culture [31–34]. Put has been found to positively influence embryonic development in various plant species, as reported [35–38].

Numerous studies have identified media components as the origins of typical genetic or epigenetic alterations in *in vitro*-grown plants [39]. Genetic changes encompass alterations in DNA sequences and cytogenetic abnormalities, whereas epigenetic changes refer to modifications in gene expression that do not involve changes to the DNA sequences. Furthermore, these are known as somaclonal variations [40]. Somaclonal variation is a crucial factor to consider in selecting an effective regeneration system for genetic engineering and micropropagation [41]. Furthermore, somaclonal variation could make it more challenging to use transgenic plants to determine the function of cloned genes that lack a known purpose [42]. Therefore, somaclonal changes need to be analyzed using molecular markers. Inter Simple Sequence Repeats (ISSRs) are highly effective in assessing the genetic fidelity of regenerated plants across diverse plant species [43–45]. This method, which is inexpensive, simple, and highly discriminatory in determining the variation hidden in plants [46–48], confirmed the genetic stability of regenerated plants across several crops [49–52].

This research aims to develop a dependable and efficient *in vitro* regeneration system for wheat using endosperm-supported mature embryos. These embryos provide benefits like year-round access to explants and better culture responsiveness, which can improve the consistency of transformation and breeding processes. Specifically, the research focused on evaluating the effects of TDZ and Put on regeneration efficiency through somatic embryogenesis, and on assessing the genetic stability of regenerated plants, with the ultimate goal of establishing a regeneration protocol suitable for genetic engineering strategies in wheat improvement programs.

2 Materials and Methods

All experimental procedures were carried out in the Tissue Culture and Molecular Analysis Laboratories, which are managed by the Agricultural Biotechnology Laboratories at Atatürk University.

2.1 Plant Material and Preparation of Endosperm-Supported Mature Embryos

The Kırık bread wheat cultivar, sourced from the East Anatolian Agricultural Research Institute in Türkiye, was used as the plant material. The dry seeds were sterilized by first soaking them in 70% ethanol for 5 min, then rinsing with a 5% sodium hypochlorite solution containing 2–3 drops of Tween-20 for 30 min, and finally washed three times with sterile distilled water. The sterilized seeds were stored at +4°C in the dark for 14–16 h. The mature embryos of surface-sterilized seeds were divided into six parts without being separated from the seed, following the methodology described [15].

2.2 Callus Induction, Embryogenic Callus Formation, and Plant Regeneration

Endosperm-supported embryos were cultured in callus induction media (CIM) including MS basal medium [53], 20 g/L sucrose, 1.95 g/L MES Hydrate, 2 g/L of phytigel, 12 mg/L dicamba, and 0.5 mg/L IAA in the darkest conditions at $25 \pm 1^\circ\text{C}$ for 14 days. Afterward, calli were cultured on an embryogenic callus formation medium (ECM), which consisted of hormone-free CIM containing either 0.0 mM putrescine (ECM1) or 1.0 mM putrescine (ECM2), and incubated under dark conditions at 25°C for 14 days. The embryogenic callus formation rate (%) was determined before the calluses were shifted to the plant regeneration medium.

The embryogenic calli were subsequently stimulated to plant regeneration medium (RM), which consisted of the putrescine-free ECM supplemented with different concentrations of TDZ (RM1: 0.0 mg/L, RM2: 0.1 mg/L, RM3: 0.2 mg/L, RM4: 0.3 mg/L, RM5: 0.4 mg/L, and RM6: 0.5 mg/L). Cultures were maintained for 30 days under a 16/8 h light/dark photoperiod (light intensity: $62 \mu\text{mol m}^{-2} \text{s}^{-1}$) at $25 \pm 2^\circ\text{C}$. Then, responsive embryogenic callus rates (%) and the number of regenerated plants per explant (PN) (number) were calculated. When the regenerated plantlets reached a height of 3–4 cm, they were transferred to magenta boxes containing hormone-free regeneration medium and cultured under the same conditions until they grew to a height of 12–14 cm. Later, the plants were transplanted into a peat-soil mixture (1:1) and acclimated in the greenhouse at $24 \pm 2^\circ\text{C}$ with 16 h of light. Before autoclaving at 121°C for 15 min, all media were adjusted to a pH of 5.8 ± 0.1 . Since the autoclave degraded the structure of vitamins and plant growth regulators, they were introduced to the medium ($\sim 50^\circ\text{C}$ – 60°C) through a $0.22 \mu\text{m}$ cellulose nitrate filter (Milipor[®]).

The study was conducted employing a completely randomized design. Each Petri dish was considered an individual replicate, containing 10 endosperm-supported mature embryos. For each concentration of Put, a total of 24 Petri dishes were used. From these, four dishes were randomly selected and assigned to each TDZ concentration for regeneration analysis. The data was analyzed statistically using analysis of variance (ANOVA). A one-way ANOVA was performed to evaluate the effect of Put concentrations on embryogenic callus formation. At the same time, a two-way ANOVA was used to assess the interactive effects of ECM and RM on both regeneration frequency and regeneration efficiency. Mean comparisons were conducted with the Least Significant Difference (LSD) test at a 5% significance level ($p \leq 0.05$). All statistical analyses utilized SAS software (version 9.3).

2.3 Molecular Analysis

The bulk DNA method was used to assess diversity in regenerated plants; ten plants were randomly sampled from each treatment. Genomic DNA (gDNA) was isolated with the CTAB method described by Cota-Sanchez et al. (2006). The DNA amount and purity of the samples were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA; Multiskan Go). DNA samples were stored at -20°C until used.

Twenty-five primers were tested in the control plant DNA, of which the ten primers displaying the highest amplification were used in ISSR analysis (Table 1). The ISSR-PCR process was performed using a thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA, Model 5020). The PCR reaction mix was prepared in a final volume of 25 μ L, containing 50 ng of DNA template, 1x PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, and 0.2 μ M primer. The ISSR-PCR conditions were as follows: 4 min at 94°C, 35 cycles of 30 s at 94°C, 35 s at 41°C–56°C (dependent on primer) (Table 1), 1 min at 72°C, ending with a final extension step of 5 min at 72°C, the program was terminated at 4°C for 10 min.

Table 1: Nucleotide sequences, T_m degrees, marker efficiency indices, bands information, and percentage of polymorphism of ISSR primers

Primer	Nucleotide sequence (5'3')	T _m (°C)	PIC ^a	D ^b	R ^c	TB ^d	MB ^e	PB ^f
UBC-852	TCTCTCTCTCTCTCRA	50	0.12	0.28	5.17	18	3	15
UBC-834	AGAGAGAGAGAGAGAGYT	55	0.14	0.16	2.33	15	6	9
UBC-845	CTCTCTCTCTCTCTCTRG	48	0.14	0.13	1.67	13	4	9
UBC-815	CTCTCTCTCTCTCTCTG	54	0.15	0.10	1.50	16	8	8
UBC-826	ACACACACACACACACC	56	0.14	0.13	2.33	18	4	14
UBC-811	GAGAGAGAGAGAGAGAC	53	0.14	0.13	2.17	17	6	11
HBS-11	GTG TGT GTG TGT CC	44	0.14	0.13	2.50	16	6	10
844-A	CTC TCT CTC TCT CTC TGC	48	0.14	0.12	2.17	17	7	10
17889-A	CAC ACA CAC ACA AC	52	0.14	0.18	3.17	14	2	12
17889-B	CAC ACA CAC ACA GT	41	0.13	0.24	2.00	8	0	8
Average			0.14	0.16	2.50	15.2	10.6	4.6
Total			–	–	–	152	106	46

Note: a: Polymorphic information content [54]; b: Discriminating power [55]; c: Resolving power [56]; d: Total bands; e: Monomorphic bands; f: Polymorphic bands.

PCR products were loaded onto a 1.5% agarose gel containing 2 μ L of ethidium bromide (EtBr), and electrophoresis was conducted at 90 V for 120 min in 0.5 X TBE buffer. Subsequently, the DNA band patterns were visualized and photographed under UV transillumination (Fig. 1). The amplification products (only clear band patterns) were scored as present (1) or absent (0), and the data were constructed as a binary matrix using the computer program TotalLab TL120. Discrimination power (D), polymorphism information content (PIC), marker index (MI), and resolving power (R) were evaluated for each primer based on the treatments using the online iMEC software [57]. A UPGMA dendrogram, generated from the distance matrix using Jaccard's coefficient, was used to visualize genetic similarities.

3 Results and Discussion

The type of explant used is one of the most important factors influencing the success of plant tissue culture, as it directly impacts the plant's growth and response *in vitro* culture conditions. In this study, endosperm-supported developed embryos were used as explants because they are available throughout the year, are structurally stable, and maintain their physiological integrity during the culture initiation process. Adding endosperm tissue creates a favorable microenvironment that facilitates the exchange of nutrients, hormone balance, and signal transmission between the embryo and surrounding tissues [19,21]. This physical and chemical connection may help maintain high natural auxin and cytokinin gradients, which are crucial for controlling dedifferentiation and somatic embryo formation [29]. The endosperm may also act as a biological support, reducing the mechanical and oxidative stress that comes from isolating the explant. This buffering

action helps keep cells alive and maintain the plant's structure, making it easier for calluses to form and the plant to recover [58]. According to [59], endosperm may also help prevent oxidative damage in the early stages of culture by changing the levels of reactive oxygen species (ROS). This will eventually lead to a more efficient conversion of somatic embryos into whole plantlets.

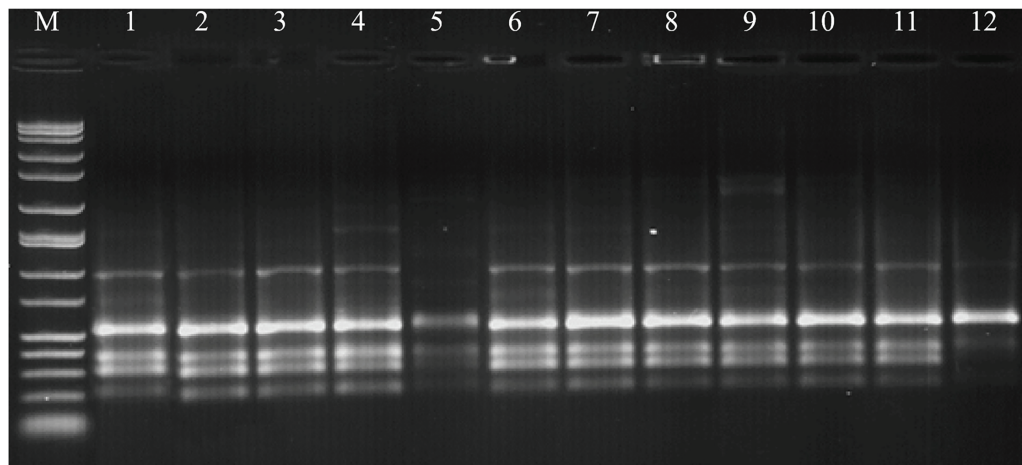


Figure 1: ISSR pattern for primer 844-A based on 12 treatments from combinations of ECM and RM on *Triticum aestivum* L. M: Marker; 1: ECM1 + RM1, 2: ECM1 + RM2, 3: ECM1 + RM3, 4: ECM1 + RM4, 5: ECM1 + RM5, 6: ECM1 + RM6, 7: ECM2 + RM1, 8: ECM2 + RM2, 9: ECM2 + RM3, 10: ECM2 + RM4, 11: ECM1 + RM5, 12: ECM2 + RM6

In this study, callus formation in endosperm-supported mature embryos was observed after three days of culture initiation in the callus induction medium. All the explants successfully induced well-developed calli (Fig. 2A). Plant growth regulators impact the development of somatic embryos from callus. Furthermore, the presence of plant growth regulators in the media influences each stage of the *in vitro* development procedure [60]. Auxin and cytokinin-based plant growth regulators are commonly employed to promote somatic embryogenesis, though other types of plant growth regulators have also been associated with this process. One of these different plant regulators is polyamines. Polyamines are widely recognized for their many roles in plant physiology, encompassing tasks such as cellular differentiation, induction of totipotency, acceleration of cell division, and facilitation of molecular signaling.

Exogenous polyamine, moreover, usage increased embryogenic structure formation and produced positive results in embryogenesis [61]. Put, one of the polyamines, has a low molecular weight and plays a significant role in cellular mechanisms in plants, such as DNA replication, cell division, and protein synthesis. Furthermore, Put induces over cell division and enlargement, leading to an increase in callus growth, as well as improvements in somatic embryogenesis and plant regeneration, as observed in various studies [62,63]. In this study, 14-day-old calli were transferred to an embryogenic callus formation medium containing 0 (ECM1) or 1 mM (ECM2) Put to evaluate the impact of Put on embryogenic callus development and plant regeneration. Its white colour and watery appearance distinguished the non-embryonic callus. In contrast, the embryonic callus was characterized by its light-yellow colour, friable and compact nature, and the appearance of somatic embryos (Fig. 2B). The embryogenic callus formation rate was determined 85.4% and 92.1 for 0 (ECM1) and 1 mM (ECM2) Put, respectively, and the impact of Put concentration on this parameter was important ($p < 0.05$) (Table 2). Parallel results were shown in *Brassica napus* L., *Cocos nucifera* L., and *Guizotia abyssinica* (Lf) Cass [64,65]. According to a study, 1 mM Put caused a 5-fold increase in embryogenic callus weights and a 2.5-fold rise in embryo formation rate in bitter [66]. Reference [58] found that a 1 mM dose of Put promoted plant formation from responsive embryogenic. The high frequency of

somatic embryos in the indica rice variety was also observed when 1 mM putrescine (Put) was added to the growth medium [29].

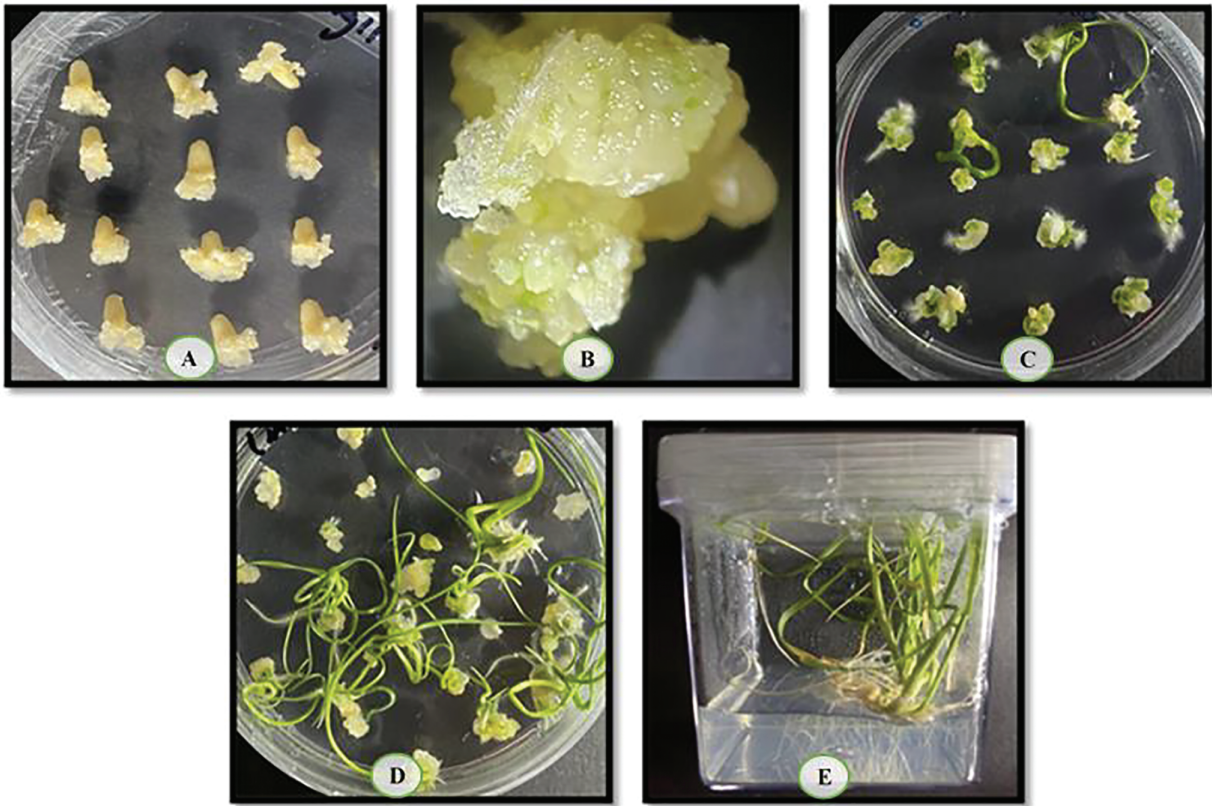


Figure 2: (A) Callus induction; (B) embryogenic callus; (C) responded embryogenic callus; (D) plant regeneration; and (E) regenerated plants in magenta boxes

Table 2: Analysis of variance (ANOVA) results for the variables studied in the research

Parameters	Source of variation	df	MS	F-Value	<i>p</i> -Value
ECF (%)	ECM (E)	1	533.3	5.12	0.028
	Error	46	104.2		
	Total	47			
REC (%)	ECM	1	3887.0	33.38	0.000
	RM (R)	5	1091.3	9.37	0.000
	ExR	5	497.5	4.27	0.004
	Error	36	116.5		
	Total	47			
PN (Number)	ECM (E)	1	19.0008	58.61	0.000
	RM (R)	5	13.7975	42.56	0.000
	ExR	5	2.4748	7.63	0.000
	Error	36	0.3242		
	Total	47			

Not only is regenerative plant formation not observed in all somatic embryos in embryogenic calli, but also plant regeneration may not occur in the entire embryogenic calli. Therefore, in this study, the responsive embryonic callus was defined as calli that form plants with roots and shoots (Fig. 2C,D). The main impacts of ECM and RM on the REC rate were significant ($p < 0.01$) (Table 2). The REC rate (85.1%) in 1 mM Put (ECM2) was higher than 0 mM Put (ECM1) (67.1%). On the other hand, the highest REC rate of 96.1% was determined at a 0.5 mg/L TDZ concentration (RM6). In contrast, the lowest REC rate (62.8%) was observed in 0.2 mg/L TDZ concentration (RM3) (Fig. 3). Because the effects of the plant regeneration medium differed based on the embryogenic callus formation medium, the ECM $\times$ RM interaction was significant ($p < 0.01$) (Table 2). The highest REC ratio (97.5%) was observed using 1 mM Put as the embryogenic callus formation medium (ECM2) and 0 mg/L TDZ as the plant regeneration medium (RM1). The lowest rate (47.1%), however, was determined using a medium containing 0 mM Put for embryogenic callus formation (ECM1) and 0.2 mg/L TDZ for the regeneration medium (RM3) (Fig. 4).

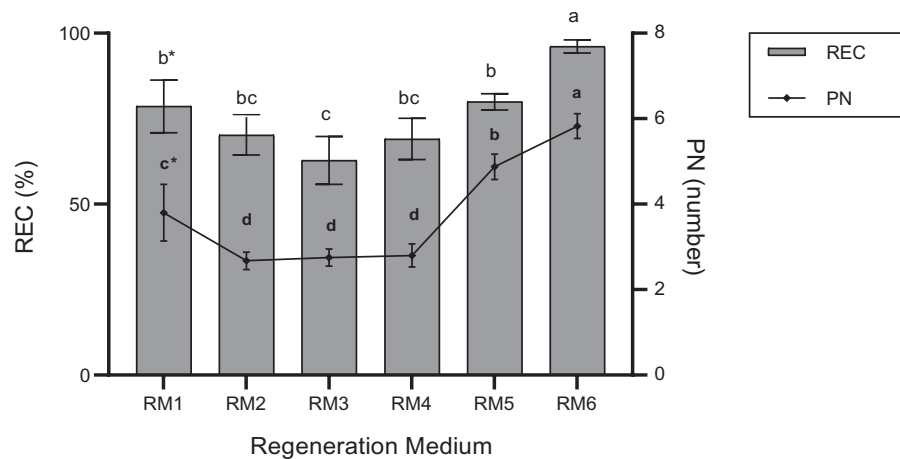


Figure 3: The main impact of regeneration medium on REC (%) and PN (number). The bars represent the mean values, and standard error (mean + SE). *: Means with different letters are significantly different from each other at the 5% significance level, based on the Least Significant Difference (LSD) test

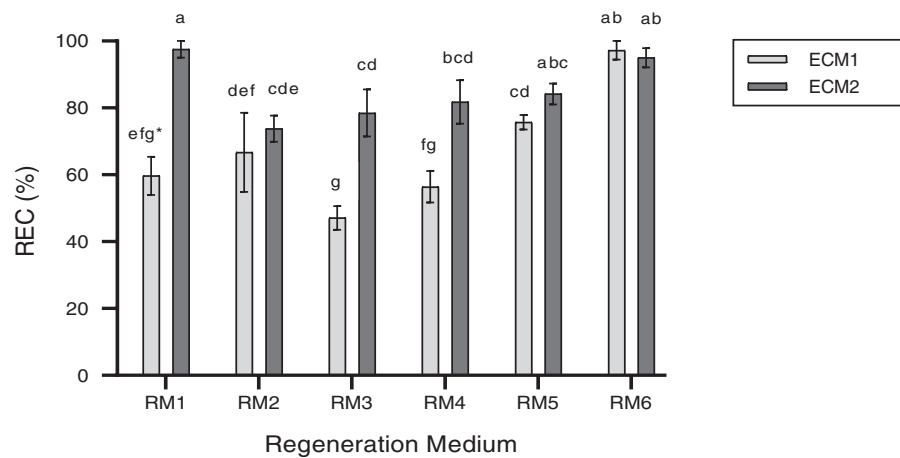


Figure 4: REC rate (%) in the regeneration media according to embryogenic callus formation media. The bars represent the mean values, and standard error (mean + SE). *: Means with different letters are significantly different from each other at the 5% significance level, based on the Least Significant Difference (LSD) test

Furthermore, the results indicated that the number of regenerated per explant was remarkably affected by the ECM and RM and their interactions (Table 2). When the main impact of the regeneration medium was evaluated, the number of regenerative plants per explant in ECM2 (1 mM Put) was higher than in ECM1 (0 mM Put). Also, when the main effect of the regeneration media was considered, the RM6 medium gave the highest number of regenerated plants per explant, which was 5.8 (Fig. 5). The average number of regenerated plants per explant (regeneration efficiency) ranged from 2.1 to 6.5 based on ECM and RM (Fig. 5). The highest number of regenerating plants (PN) (6.5) per explant was observed using ECM2 (1 mM Put) as embryogenic callus formation medium and RM6 (0.5 mg/L TDZ) as plant regeneration medium (Fig. 2E). The lowest PN (2.1) was determined in the use of ECM1 and RM1 as embryogenic callus formation and regeneration media, respectively.

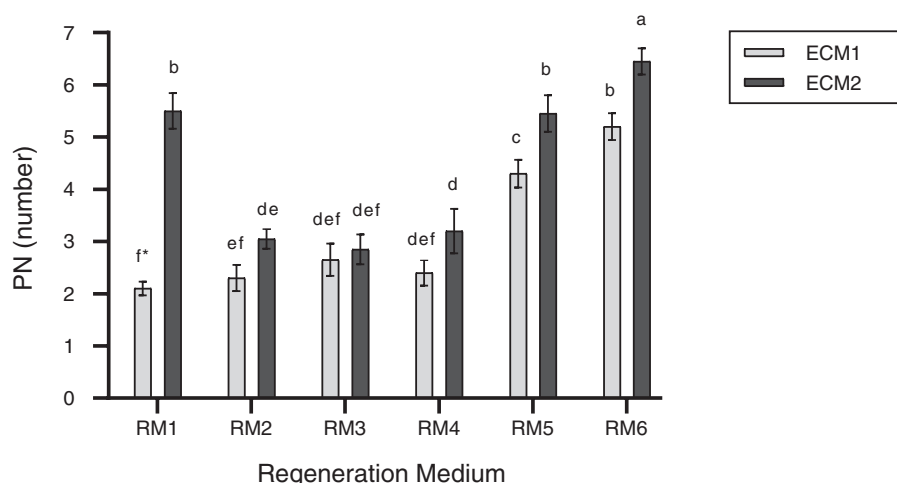


Figure 5: PN (number) in the regeneration media according to embryogenic callus formation media. The bars represent the mean values, and standard error (mean + SE). *: Means with different letters are significantly different from each other at the 5% significance level, based on the Least Significant Difference (LSD) test

Previous research has demonstrated that TDZ plays a functional role in initiating somatic embryogenesis [67–71] and is also a highly effective inducer of morphogenesis, causing cell dedifferentiation and reprogramming of the cell cycle before the formation of meristematic centers, which lead to *de novo* morphogenesis, such as random shoot growth or somatic embryogenesis [72,73]

This action of TDZ is connected to a series of metabolic pathways, including a signaling event, the buildup and transmission of endogenous plant signals, a system of secondary messengers, and concurrent stress response mechanisms [74]. The reason for the positive impact of TDZ on plant regeneration may be due to its involvement in modulating plant endogenous growth regulators, particularly cytokinins and auxins [75,76]. The cytokinin-like activity of TDZ is implicated in the gathering of ions and enzymes in plant tissues and induces the expression of somatic embryogenesis genes [77]. Additionally, TDZ facilitates the accumulation and production of endogenous growth hormones in the plant [78]. The possible metabolism of TDZ by plant cells, oligomerization of proteins in solutions by TDZ, and improved utilization and sugar catabolism (5C and 6C) by TDZ in the medium are only a few of the numerous plausible explanations for TDZ's excellent role [79]. The study findings indicated that the application of Put and TDZ elicited a significant enhancement in plant regeneration. Many studies have revealed the beneficial impacts of Put on somatic embryogenesis formation and plant regeneration in various plants, containing bitter melon [66], *Momordica charantia* [80], wheat [58], palm [81], sweet pepper [82], sugar cane [83], rice [29] and *Litchi*

chinensis Sonn [84]. The main reason for the enhancing effect of Put on plant regeneration may be that it increases the development and formation of somatic embryos. One of the primary challenges in somatic embryogenesis is the low rate of transformation of these structures into plantlets. Reference [85] demonstrated that low conversion rates have been caused by low somatic embryo quality and a lack of maturation and desiccation tolerance in various species. Treatment is known to promote various osmotic substances, such as amino acids, soluble sugars, and proline, which contribute to drought stress tolerance and reduced reactive oxygen species (ROS) overgeneration [86]. Study [59] indicated that the amount of Put increased during the maturation stages of somatic embryos in grapevine and reported that somatic embryogenesis is linked to the accumulation of large amounts of polyamines. Furthermore, Study [58] reported that polyamines are primarily involved in the maturation of somatic embryos and may have positive effects on plant regeneration by protecting DNA from the detrimental impacts of ROSs.

A simple medium composition, minimal manufacturing phases, a predictable degree of propagation, and genetically stable products are all necessary components of an efficient regeneration system [87]. *In vitro* tissue culture conditions can be mutagenic, and plants regenerated from calli, organ cultures, somatic embryos, and protoplasts can exhibit phenotypic and genotypic diversity [88]. Meanwhile, although cellular organization is essential for plant growth, the loss of cellular regulation *in vitro*, resulting in disorderly growth, may generate somaclonal variation [89]. Moreover, somaclonal variation in tissue cultures is induced, in part, by variations in nutritional conditions, culture duration, phytohormone concentrations, and the auxin–cytokinin ratio [87]. Somaclonal variation is a serious problem when cultivating plant tissue for genetic engineering and micropropagation. Thus, regeneration systems need to be evaluated in terms of genetic fidelity. Previous research has employed PCR-based techniques utilizing various molecular markers to investigate the genetic stability and somaclonal diversity of diverse regenerated plant specimens [90–93]. The regenerated plants in the study were screened for genetic differences using the ISSR approach, which was chosen because of its cost-effectiveness, simplicity, widespread distribution throughout the genomic DNA, and more precise genetic conclusions [94].

Ten ISSR primers were utilized to evaluate the genetic stability of the regenerated plants obtained from a total of 12 different treatments consisting of different embryogenic callus formation and regeneration media. A total of 152 bands were acquired, with 106 of them being polymorphic bands. The number of polymorphic bands varied between 8 and 15, and all bands of primer 17889 B were polymorphic bands (Table 1). According to the results of marker efficiency, although the highest and lowest PIC values were determined in the UBC-815 (0.15) and UBC-852 (0.12) primers, respectively, the primers were found to have similar values in general. On the other hand, regarding the resolving power (R), the UBC-852 primer showed the highest value (5.17) while primer UBC-815 showed the lowest (1.50), and the mean of the primers was determined as 2.5. In addition, the D ranged between 0.10 (primer UBC-815) and 0.28 (primer UBC-852), with a mean value of 0.16. The ISSR band patterns generally displayed an acceptable level of polymorphism induction. A dendrogram constructed using Jaccard's similarity coefficient and the UPGMA algorithm illustrates the genetic relationships between treatments (Fig. 6).

According to the results, similarity coefficients among 12 treatments ranged from 0.776 to 0.940 with a mean of 0.845 (Table 3). Maximum similarity was observed between ECM1 + RM1 and ECM2 + RM6 (Table 3).

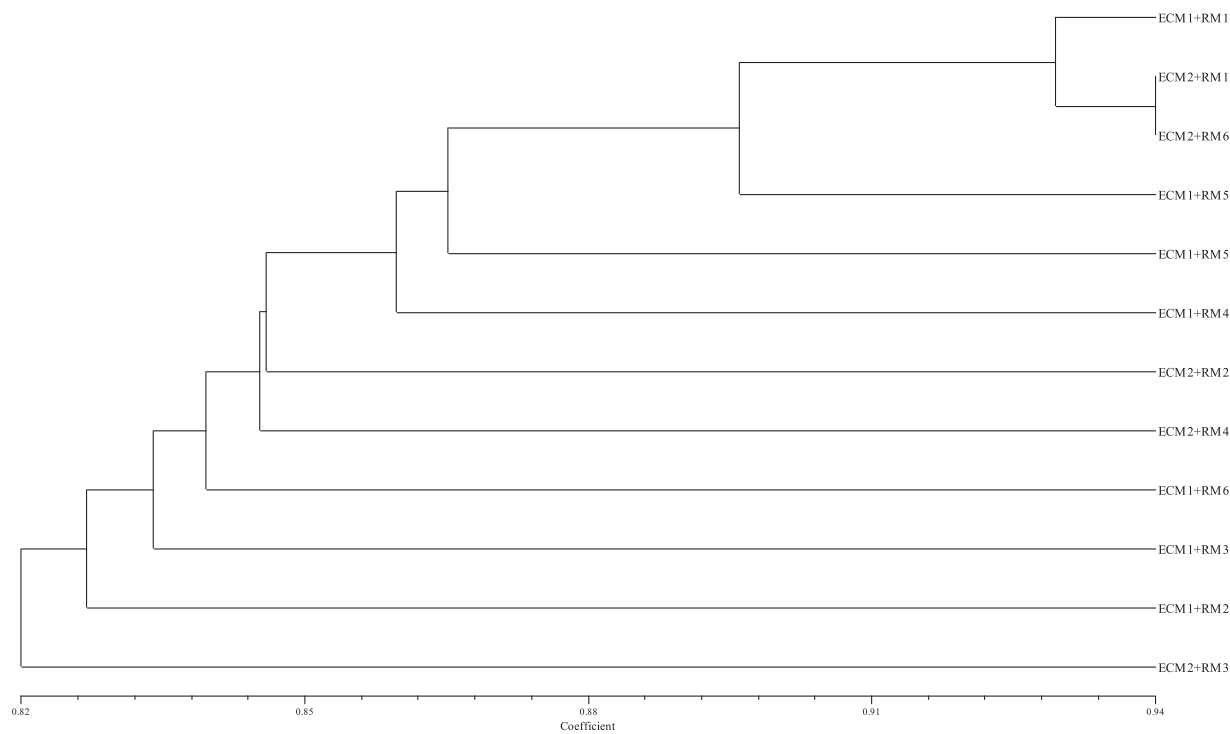


Figure 6: Dendrogram of genetic relationship between treatments

Table 3: Jaccard's similarity coefficient

Treatment	1	2	3	4	5	6	7	8	9	10	11	12	
ECM1 + RM1	1	1	0.829	0.854	0.861	0.861	0.842	0.926	0.849	0.866	0.854	0.882	0.934
ECM1 + RM2	2		1	0.813	0.820	0.820	0.813	0.836	0.820	0.776	0.826	0.829	0.881
ECM1 + RM3	3			1	0.809	0.809	0.803	0.873	0.821	0.801	0.815	0.854	0.882
ECM1 + RM4	4				1	0.840	0.833	0.855	0.816	0.808	0.846	0.861	0.888
ECM1 + RM5	5					1	0.821	0.855	0.828	0.808	0.809	0.849	0.901
ECM1 + RM6	6						1	0.849	0.833	0.826	0.827	0.842	0.882
ECM2 + RM1	7							1	0.855	0.860	0.849	0.888	0.940
ECM2 + RM2	8								1	0.796	0.846	0.849	0.888
ECM2 + RM3	9									1	0.789	0.841	0.868
ECM2 + RM4	10										1	0.842	0.882
ECM1 + RM5	11											1	0.921
ECM2 + RM6	12												1

The potential cause of this polymorphism observed in this study may be ROS. *In vitro* culture conditions are stressful [95]. They also are thought to be subjected to elevated levels of oxidative stress [96]. ROSs caused genomic instability [97–99]. Moreover, genomic instability occurring *in vitro* has been associated with chromosomal irregularities, gene amplification, point mutation, and DNA methylation alteration [41,100–102]. The probable cause of ISSR polymorphism may have resulted from the retrotransposons’ movement. DNA methylation is essential for regulating gene expression and silencing transposons in plants [103].

Reference [104] indicated that Put gives rise to the retrotransposon's movement. Furthermore, alterations in cellular polyamine levels have been shown to impact the level of DNA methylation [105]. No morphological changes were detected in all the regenerated plants in this study. These findings support our thinking that it should be verified in the next generation obtained from regenerated plants to wholly illuminate the cause of polymorphism determined in ISSR.

4 Conclusion

This study successfully established an efficient and reproducible *in vitro* regeneration system for wheat (*Triticum aestivum* L., cv. Kırık) through somatic embryogenesis using endosperm-supported mature embryos. The incorporation of 1.0 mM Put significantly enhanced embryogenic callus induction, while the application of TDZ, particularly at 0.5 mg/L, markedly improved regeneration efficiency. The combined application of 1.0 mM Put and 0.5 mg/L TDZ resulted in the highest regeneration rate and the greatest number of plantlets per explant, highlighting the synergistic effect of polyamines and cytokinin-like regulators in enhancing somatic embryogenesis and plant regeneration. Genetic fidelity assessment using ISSR markers confirmed a high level of genetic stability among the regenerated plants, with polymorphism remaining within acceptable limits and no morphological abnormalities observed. However, observed ISSR polymorphisms in some treatments suggest potential underlying epigenetic or retrotransposon-related changes, likely triggered by *in vitro* stress conditions, warranting further investigation in subsequent generations. Overall, the regeneration protocol established here offers a practical and scalable platform for wheat improvement programs, especially in the context of modern breeding, genetic engineering, and biotechnological applications.

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