EMPOWERING WHEAT GROWTH: ESTABLISHING A PROTOCOL FOR EFFICIENT PLANT REGENERATION

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Abstract. The recalcitrance of wheat to in vitro culture presents a significant hurdle in its genetic transformation process, directly impacting the ability of explants to regenerate. In light of this challenge, our study aimed to identify the wheat genotype exhibiting the highest responsiveness to callusing and regeneration among elite varieties, with the aim of improving regeneration efficiency within resource and time limitations. We utilized seeds from six diverse wheat varieties as explants. These were cultured on MS media supplemented with different concentrations of 2,4-D to induce callus formation. Following this, we tested six hormonal combinations of IAA and Kinetin to evaluate their impact on regeneration potential from the 2,4-D dichlorophenoxyacetic acid 2,4-D-induced callus. Our results indicate that Millat-11 displayed the most notable regeneration efficiency, reaching 41.66%, particularly when cultured on a regeneration medium containing 1.5 mg/L IAA and 2 mg/L Kinetin. Furthermore, Millet-11 also demonstrated the highest frequency of callus induction, reaching 41.66% on medium supplemented with 2.5 mg/L 2,4-D. In contrast, NARC-09 displayed the lowest responsiveness, with a callus induction frequency of 16.66%. Overall, Millet-11 emerged as the most responsive genotype, suggesting its potential utility in wheat improvement programs. The robust tissue culture response observed in Millet-11 for both callus induction and regeneration underscores its suitability for genetic transformation efforts. These results offer valuable insights for plant breeders seeking to develop new wheat varieties with enhanced adaptability to diverse environmental conditions.
Keywords: *embryogenesis, crop improvement, tissue culture, plant growth regulator, cell division*

Introduction

Triticum aestivum L., the most widely cultivated agronomic crop globally, belongs to the Poaceae family and holds significant importance as a staple food worldwide. However, biotic and abiotic stresses significantly affect wheat yield and production, necessitating the incorporation of resilient genes into the crop genome to mitigate the impact of rapidly changing climatic conditions (Hussain, 2017; Jan et al., 2017). Conventional breeding techniques face limitations due to the hexaploid nature and large genome size of wheat, which restricts access to the gene pool and prolongs the breeding process (El Sayed et al., 2016; Zimin et al., 2017; Singh et al., 2019). Modern biotechnological tools offer a promising solution by overcoming traditional breeding barriers and facilitating the transmission of desirable traits across species and genera (Noor et al., 2009; Aroonluk et al., 2020). Genetic engineering enables the rapid enhancement of wheat characteristics by introducing specific genes into the wheat genome from other species (Sompornpailin and Khunchuay, 2016; Shrawat and Armstrong, 2018). The efficacy of tissue culture methodologies in wheat enhancement

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relies on several critical factors, including the choice of genotype, hormone concentrations in the culture medium, and the regenerative capabilities of the transformed explants (Michard et al., 2019; Chauhan and Khurana, 2017). In wheat tissue culture, a range of explants can be utilized, spanning from seeds to different parts of the plant such as embryos, endosperm, and leaves (Dehghan et al., 2020; Özgen et al., 2017). While immature embryos are favored for their superior regeneration capacity, their availability is limited outside of wheat growing seasons, and their cultivation often demands costly infrastructure. In contrast, mature wheat seeds offer a readily accessible and year-round resource for plant regeneration experiments. Research indicates that optimal callus formation from immature embryo explants can be achieved using Murashige and Skoog medium MS medium supplemented with 3.5 mg/l of 2,4-D 2,4-D dichlorophenoxyacetic acid. Similarly, for regeneration from mature embryo explants, studies have found success with MS medium supplemented with 0.5 mg/l kinetin, 0.5 mg/l BAP, and 25 mg/l tyrosine. To refine tissue culture methodologies for wheat improvement, a thorough investigation into growth media and hormonal influences on various explants is imperative. Thus, this study aimed to identify the most responsive elite wheat genotype for callus induction and regeneration. The overarching goal was to augment regeneration efficiency within the constraints of resources and time, focusing on mature seeds as the primary explant source and considering the multifaceted hormonal effects on diverse wheat varieties.

Materials and methods

Seeds from six distinct wheat varieties were examined for experimentation. Laboratory conditions were meticulously calibrated, maintaining a temperature of $25 \pm 2°C$, with a white light intensity of 7000 lux, and humidity levels set between 60-80%. To prepare the seeds for experimentation, a rigorous sterilization process was employed. This involved immersing the mature seeds in a 50% Clorox solution for 20 min, followed by three thorough rinses with autoclaved distilled water. Once sterilized, the seeds were carefully dried on autoclaved filter papers, and the culture media were subsequently autoclaved to ensure sterility. The sterilized seeds were then inoculated onto Murashige and Skoog (MS) media for growth and induction of callus under aseptic conditions. For regeneration studies, the explants were cultured on MS media supplemented with varying concentrations of Indole-3-acetic acid (IAA) and Kinetin (Kn) to evaluate their potential for regeneration. Each wheat variety underwent six different treatments comprising different hormonal combinations, with three replications for each treatment. The cultures were maintained at ambient room temperature, and germination data were meticulously recorded after a 4-day incubation period. Simultaneously, callus induction was initiated by placing sterilized seeds on MS media supplemented with varying levels of 2,4-D, and the cultures were kept in darkness to stimulate callus formation. After two weeks, the induction of callus was carefully observed. Following this, the callus was meticulously separated from the seeds, and after an additional 3-4 weeks, the weight of the callus was recorded to determine the optimal callus production.

Statical analysis

The data collected underwent rigorous statistical analysis using slide writer software. This analysis adhered to the method outlined by Steel et al. (1997), ensuring robustness and reliability in the results. A total of 24 explants were utilized for the analysis, with

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triplicate samples meticulously maintained for each treatment to ensure consistency and accuracy. Mean values were calculated for each treatment, allowing for a comprehensive assessment of the overall response and facilitating comparisons between different experimental conditions.

Results

The choice of mature seeds as explant material stemmed from their consistent availability throughout the year. The ultimate goal was to establish a protocol that could pave the way for the development of novel wheat varieties harboring desirable traits. To evaluate the multi-hormonal effects on regeneration and callus induction, six treatments incorporating various hormone combinations (IAA, Kinetin, and 2,4-D) were meticulously applied to each of the six wheat varieties.

Regeneration

The experiment explored a range of concentrations and combinations of indole acetic acid (IAA) and kinetin to fine-tune the regeneration process (see *Table 1*). Varying concentrations of kinetin were introduced alongside optimized levels of IAA in MS media to bolster regeneration frequency. Results, as shown in *Table 2,* unveiled significant disparities among wheat varieties in their reactions to different growth regulator levels (IAA and kinetin). It became evident that the efficiency of regeneration varied across wheat genotypes, with each genotype exhibiting distinct responses to varying growth regulator concentrations. The most notable finding was the remarkable regeneration rate of 41.66% achieved with the Millet-11 variety on medium containing 1.5 mg/l IAA and 2 mg/l kinetin. Conversely, all varieties exhibited diverse responses to changes in indole acetic acid and kinetin concentrations. Millet-11 showcased the highest germination rate at 31.24%, closely followed by Borlog-14 at 29.16%, with an average highest germination percentage of 27.77% recorded on media containing 1.5 mg/l IAA and 2 mg/l kinetin. Particularly, NARC-09 displayed the least responsiveness to tissue culture among the selected wheat varieties (refer to *Table 2; Figs. 1* and *2*). As a result, the findings indicate that Millet-11 and Borlog-14 emerge as the most responsive genotypes for tissue culture among the studied varieties. The process of plant regeneration from wheat explants in response to various concentrations of IAA and kinetin is visually depicted in *Figures 3* and *4.*

Callus induction

Callus formation serves as a crucial precursor in various tissue culture processes, delineated into embryogenic and non-embryogenic types based on their distinct characteristics. Among the growth regulators explored, 2,4-D emerged as the most efficacious in inducing callus formation, aligning with prior research findings (Tanida and Shiota, 2019). A spectrum of 2,4-D concentrations, ranging from 0.5 mg/L to 5.5 mg/L, was applied to instigate callus formation in the six wheat varieties. Response to callus induction varied among the varieties, with some exhibiting notably higher induction rates than others. The onset of callus formation manifested as white spongy tissue on the seed surface, with marked discrepancies observed in the timing and texture of callus development across genotypes and media formulations (Weckx et al., 2019). During observations, two discernible types of callus were identified: embryogenic

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callus, characterized by its compact, spongy texture, and fibrous appearance, and non-embryogenic callus, which displayed a softer, watery texture with a faint white hue. Analysis of callus induction percentages unveiled Millet-11 as the most responsive variety, particularly notable at a concentration of 2.5 mg/l 2,4-D. Furthermore, this variety exhibited superior callus weight production, particularly evident at a concentration of 3.5 mg/l 2,4-D (refer to *Tables 3* and *4*).

Table 1. *Media used for regeneration and callus induction of wheat varieties*

Regeneration Treatments (IAA with kinetin)	Callus induction Treatments (2, 4 -D)
H1 = 1.5 mg/l IAA with 1 mg/l kinetin	H1 = 0.5 mg/L with MS media
H2 = 3 mg/l IAA with 1 mg/l kinetin	H2 = 1.5 mg/L with MS media
H3 = 4.5 mg/l IAA with 1 mg/l kinetin	H3 = 2.5 mg/L with MS media
H4 = 1.5 mg/l IAA with 2 mg/l kinetin	H4 = 3.5 mg/L with MS media
H5 = 1.5 mg/l IAA with 3 mg/l kinetin	H5 = 4.5 mg/L with MS media
H6 = 1.5 mg/l IAA with 4 mg/l kinetin	H6 = 5.5 mg/L with MS media

Table 2. *Effect of IAA & KN on regeneration (%) of wheat*

	H1	H2	H3	H4	H5	H6	Mean
IAA	1.5 mg/l	3 mg/l	4.5 mg/l	1.5 mg/l	1.5 mg/l	1.5 mg/l	
Kn	1 mg/l	1 mg/l	1 mg/l	2 mg/l	3 mg/l	4 mg/l	
Chakwal-50	16.66	20.83	16.66	25	20.83	26	20.83
Galaxy-01	29.16	20.83	16.66	25	25	16.66	22.22
NARC-09	20.83	8.33	20.83	12.5	16.66	20.83	16.66
Pakistan-13	20.83	16.66	12.5	29.16	20.83	16.66	19.44
Millet-11	29.16	25	25	41.66	37.5	29.16	31.24
Borlog-14	37.5	20.83	20.83	33.33	37.5	25	29.16
Mean	25.69	18.74	18.74	27.77	26.38	22.2	

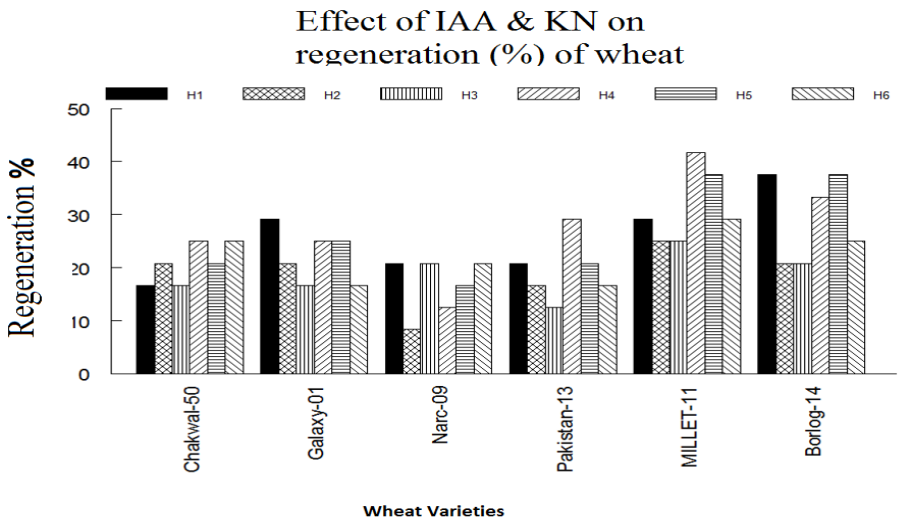

Figure 1. *Effect of IAA & KN on regeneration of wheat*

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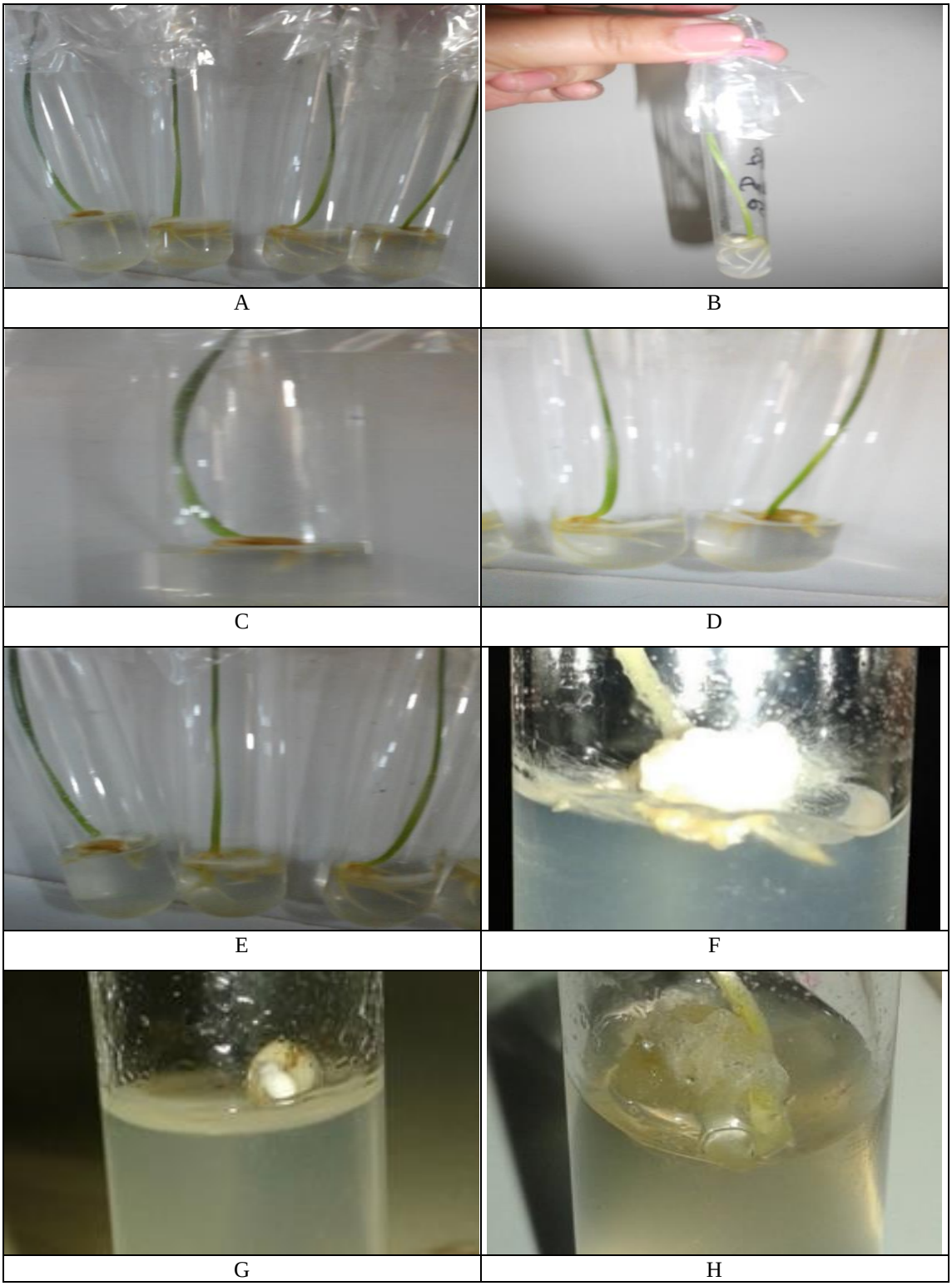

Figure 2. *(A) The maximum regeneration obtained (41.66%) on regeneration medium having 1.5 mg/l IAA and 2 mg/l kinetin by the var. Millet-11. (B) Plant regeneration from explant of wheat at 1.5 mg/L IAA with 2 mg/L KN. (C) Plant regeneration from explant of wheat at 1.5 mg/L IAA with 2 mg/L KN. (D) Plant regeneration from explant of wheat at 1.5 mg/L IAA with 1 mg/L KN. (E) Plant regeneration from explant of wheat at 1.5 mg/L IAA with 4 mg/L KN. (F) Initiation of callus started as a white spongy tissue. (G) Embryonic wheat callus induction. (H) Non-embryonic wheat callus induction*

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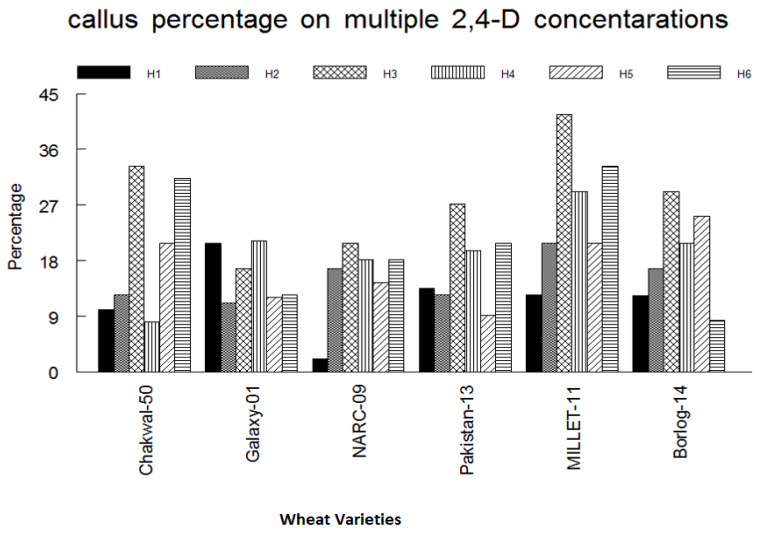

Figure 3. *Callus formation percentage of various wheat varieties in response to multiple 2, 4-D concentrations*

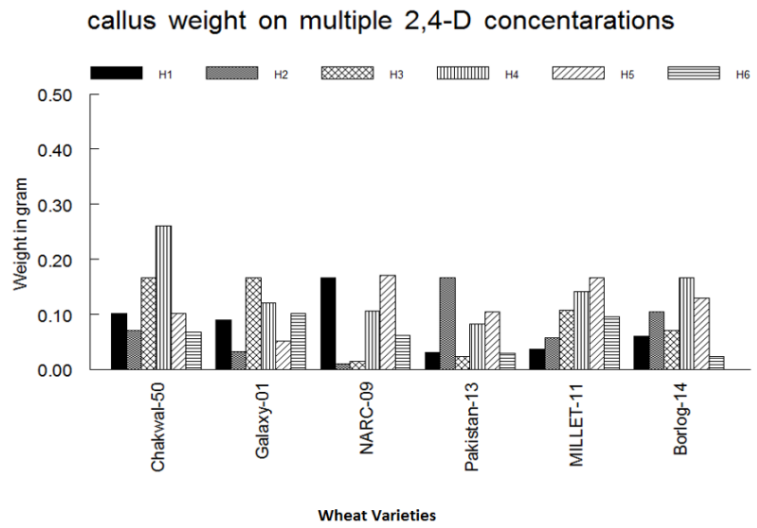

Figure 4. *Callus weight of various wheat varieties in response to multiple 2, 4-D concentrations*

Table 3. *Callus percentage (%) on multiple 2, 4-D concentrations*

	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**Mean**
2, 4-D	0.5 mg/l	1.5 mg/l	2.5 mg/l	3.5 mg/l	4.5 mg/l	5.5 mg/l	(g)
CH-50	10.12	12.50	33.33	8.01	20.83	31.22	19.34
GA-01	20.83	11.08	16.66	21.20	12.05	12.50	15.72
NARC-09	2.08	16.66	20.83	18.16	14.50	18.16	15.07
Pak-13	13.50	12.50	27.16	19.66	9.08	20.83	17.12
Millet-11	12.50	20.83	41.66	29.17	20.83	33.33	26.39
Borlog-14	12.38	16.66	29.16	20.83	25.16	8.33	18.75
Mean (%)	11.9	15.04	28.13	19.5	17.08	0.07	

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Table 4. *Callus weight (g) on multiple 2, 4-D concentration*

	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**Mean**
2, 4-D	0.5 mg/l	1.5 mg/l	2.5 mg/l	3.5 mg/L	4.5 mg/l	5.5 mg/l	(g)
CH-50	0.10	0.07	0.16	0.26	0.10	0.06	0.12
GA-01	0.09	0.03	0.16	0.12	0.05	0.10	0.09
NARC-09	0.16	0.01	0.01	0.10	0.17	0.06	0.08
Pak-13	0.03	0.16	0.02	0.08	0.10	0.03	0.07
Millet-11	0.03	0.05	0.10	0.14	0.16	0.09	0.10
Borlog-14	0.06	0.10	0.07	0.16	0.13	0.02	0.09
Mean (g)	0.08	0.07	0.09	0.14	0.12	0.06	

Discussion

Tissue culture techniques offer a valuable avenue for rapidly propagating plants under controlled conditions, facilitating the development of desirable traits. In the context of wheat improvement, establishing a genotype-independent transformation system is crucial for transferring beneficial genes across different wheat varieties. This study aimed to optimize tissue culture conditions using various hormonal combinations to identify wheat genotypes responsive to callus induction and regeneration, thereby enhancing the potential for genetic enhancement of wheat varieties. The role of hormones such as IAA, kinetin, and 2, 4-D in callus induction and regeneration was investigated across six wheat varieties. While IAA alone may not be pivotal for whole plant regeneration, its inclusion was found to enhance regeneration rates significantly, as evidenced by previous studies (Zhang et al., 2019; Afzal et al., 2010;). The results revealed varying responses among the wheat varieties to different concentrations of IAA and kinetin, with Millet-11 exhibiting the highest responsiveness to tissue culture conditions. The study identified optimal concentrations of 2,4-D crucial for maximizing callus production. Specifically, 2.5 mg/l 2,4-D was deemed optimal for inducing efficient callus formation, while a concentration of 3.5 mg/l 2,4-D was found to be ideal for promoting the development of high-weight callus. The stability of 2, 4-D as an auxin and its resistance to enzymatic degradation make it a key component in regeneration media, particularly when combined with cytokinins to induce shoot and root formation. The significance of high-weight callus in genetic studies, such as gene transformation, underscores the importance of optimizing tissue culture conditions for efficient regeneration. The findings of this study align with previous research demonstrating successful regeneration and callus induction in wheat using IAA, kinetin, and 2, 4-D combinations (Ma et al., 2016; Gholami and Tarinejad, 2017; Jasdeep et al., 2019; Roesler et al., 2018). Furthermore, standardized concentrations of 2, 4-D have been established for various wheat genotypes, emphasizing the genotype-dependent nature of callus induction and regeneration responses (Sarkar and Biswas, 2002; Yasmin et al., 2009; Afzal et al., 2010). Notably, the use of mature embryos as explants proved highly efficient, offering a practical advantage over immature embryos, which are not readily available year-round and are more challenging to handle (Mahmood and Razzaq, 2017; Mahmood et al., 2012). The robust protocol developed in this study for mature wheat embryos demonstrates its potential applicability across different wheat species for breeding programs, facilitating the efficient transfer of beneficial traits.

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Overall, these findings provide valuable insights into optimizing tissue culture techniques for wheat improvement and genetic enhancement.

Conclusion

Optimizing the growth regulators is pivotal for maximizing plantlet regeneration in tissue culture-based studies. Among the six wheat varieties evaluated, Millet-11 exhibited high potential for both regeneration and callus induction. The results suggest that combining 1.5 mg/l IAA with 2 mg/l KN can effectively enhance the regeneration frequency of wheat varieties. Additionally, the importance of 2,4-D in wheat tissue culture is underscored, as it plays a critical role in regulating callus induction and maintenance. Notably, a concentration of 3.5 mg/l 2,4-D was found to be optimal for inducing high-weight callus in wheat. These results hold promise for future gene transformation efforts aimed at improving agronomic traits. The notable tissue culture responsiveness of Millet-11 underscores its potential utility in wheat improvement programs. These findings offer valuable insights for plant breeders, providing a basis for the development of new wheat varieties with enhanced adaptability to diverse environmental conditions. Ultimately, leveraging these results could contribute to the creation of more resilient and productive wheat cultivars.

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Conflict of interest. The authors declare that they do not have any conflict of interest.

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