

Full Length Research Paper

Establishment of an efficient regeneration protocol of six different varieties of wheat (*Triticum aestivum* L.)

Bo Chen^{1†}, Muhammad Tahir^{2*†}, Fatima³, Sara Zafar⁴, Hui Li⁵, Jia Li¹, Arthur J. Ragauskas^{6,7,8},
Caiming Gou¹, Hayssam M. Ali⁹ and Manzar Abbas¹

¹School of Agriculture, Forestry and Food Engineering, Yibin University, 644000 Yibin, Sichuan, China.

²Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, College of Biological Sciences and Biotechnology, Beijing Forestry University, 100091 Beijing, China.

³Department of Mathematics, University of Karachi, 75270 Karachi, Sindh, Pakistan.

⁴Botany Department, Government College University, 38000 Faisalabad, Punjab, Pakistan.

⁵College of Forestry, Inner Mongolia Agricultural University, 010019 Hohhot, China.

⁶Department of Forestry, Wildlife, and Fisheries, Center for Renewable Carbon, University of Tennessee Institute of Agriculture, Knoxville, TN 37996, USA.

⁷Joint Institute for Biological Science, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA.

⁸Department of Chemical and Biomolecular Engineering, University of Tennessee Knoxville, Knoxville, TN 37996, USA.

⁹Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.

Received 11 December, 2023; Accepted 25 January, 2024

An efficient callogenesis and regeneration protocol was optimized for six different wheat (*Triticum aestivum* L.) varieties including Chakwal-50, Galaxy-01, NARC-09, Pakistan-13, Millat-11, and Borlog-14. For callogenesis, mature seeds of each variety were used as explant, surface sterilization, and culturing were carried out on MS medium enhanced with dissimilar levels (0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D). For regeneration, six different treatments with different combinations of IAA (1.5, 3, and 4.5 mg/L) and Kinetin (1, 2, 3, and 4 mg/L) were applied on each variety. Highest frequency of callogenesis (41.66%) was attained on MS medium in addition with 2.5, 5.5 and 3.5 mg/L 2,4-D in Millet-11, followed by Chakwal-50 (33.33%) and Borlog-14 (29.16%), respectively. Maximum regeneration (41.66%) was observed in Millat-11 on medium enhanced with 1.5 mg/L IAA and 2 mg/L Kinetin. Among all, Millet-11 appeared most responsive genotype towards callogenesis and regeneration, while NARC-09 (16.66%) was least responsive. This study will open new avenues for biofortification of Millet-11 wheat variety and improvement against biotic and abiotic stress.

Key words: Wheat, tissue culture, callogenesis, regeneration, Millet-11, 2,4-D Kinetin.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is mostly growing cereal crop around the world. With the increment of biotic and

abiotic stresses, annual outcome of wheat is less than its potential (Rashid et al., 2012). Wheat is providing energy

*Corresponding author. E-mail: malikuaf2011@gmail.com.

†These authors contributed equally.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

and nutrients to more than two billion people and have essential role in food security as it facilitate with 20% of world calories (Bhatta et al., 2017). Conventional breeding faces two major limitations, that is, restricted gene pool and extended duration. Simultaneously, wheat genome size is ~17000 Mb, which is challenging to improve genetically. As compared to traditional breeding, robust techniques of biotechnology such as CRISPR/Cas9 and other most advanced technology to modify DNA with addition or deletion of nucleotides are more precise and encompass all challenges (El-Sappah et al., 2021), additionally, inter- and intra-species transmission of stress associated traits is also possible with a shortest duration (Singh et al., 2021). To employ aforementioned techniques, a robust and highly efficient regeneration protocol is mandatory (Yu et al., 2008).

Dedifferentiation of cells into tissues and altering gene expression pattern within a cell are basic need for regeneration of plants in controlled environment (Bull and Michelmore, 2022). *In-vitro* regeneration of plants happen by three different pathways including repairing of tissues, clonal embryogenesis, and *de novo* organogenesis (Long et al., 2022). First pathway of tissue repairing involves the process of regeneration of tissues after roots, shoots and tips of leaf get wound or cut, and this mostly used in those techniques that utilized non-reproductive plant parts (Xu and Huang, 2014). In cell, tissue and organ cultivation techniques, plants are mostly regrown through non-reproductive cells as well as through *de novo* organogenesis (Hill and Schaller, 2013). *De novo* organogenesis can occur through two ways including direct and indirect *de novo* organogenesis. Direct *de novo* organogenesis takes place naturally while indirect *de novo* organogenesis takes place *in-vitro*. At the time of *de novo* indirect organogenesis, cells of explants pass through dedifferentiation and different plant growth regulators including auxin and cytokinin activate cell division (Sugimoto and Meyerowitz, 2013). Numerous explants of wheat namely seed, immature as well as mature embryo, endosperm, root tips and shoot apical meristem can be employed for *in-vitro* formation of callus and tissues regeneration (Kumar et al., 2017; Mahmood and Razzaq, 2017). Among all, young and immature embryos are best choice (Kumar et al., 2017). For example, immature zygotic embryos of barley (*Hordeum vulgare* L.) displayed significantly high callogenesis and regeneration (Abbas et al., 2023). In order to develop heat stress resistant maize varieties (El-Sappah et al., 2022), immature embryos of maize (*Zea mays*) were employed to induce callogenesis and regeneration, which resulted in significant regeneration rate (Wei et al., 2022). Nonetheless, pre-mature embryos are not accessible consistently throughout the year. Contrastingly, mature seeds are available around the year, therefore, considered best choice (Rahman et al., 2008). In this study, we employed mature seeds in callogenesis and regeneration to investigate their potential.

Murashige and Skoog (MS) media containing specific

concentration of hormones for accelerating growth such as 2,4-D, IAA, and Kinetin is promising media for plant callogenesis and regeneration. MS supplemented with 200 µL/L 1-naphthaleneacetic acid (NAA) and 500 µL/L IBA resulted in significant regeneration of *Populus trichocarpa* (Nisqually-1) (Abbas et al., 2020). Similarly, other researchers propagated *P. trichocarpa* on MS medium in order to perform GUS staining and identified expression of *PtrCs/D5* gene in roots (Peng et al., 2019). Pre-mature embryos of barley (*H. vulgare* L.) grow very well at 2.5 mg/L concentration of 2,4-D on MS medium and displayed excessive callogenesis, while MS medium enhanced with 1 mg/L Kinetin came out in significant regeneration, and supplementation with 1 mg/L IAA resulted in maximum rooting (Abbas et al., 2023). Callus initiation medium enhanced with MS media and dicamba (2 mg/L) showed maximum efficiency of callus induction of 98.22 and 97.33%, respectively, and shoot development media with addition of zeatin at concentration of 5 mg/L 6-benzylaminopurine (BAP) concentration of 5 mg/L 2,4-D concentration of 0.25 mg/L NAA concentration of 0.25 mg/L, and copper sulfate (CuSO₄) concentration of 20 mg/L showed maximum shoot induction in wheat (Phogat et al., 2023). Here, we streamlined MS media enhanced with various concentrations of growth promoting hormones.

Various channels of tissue culture were laid out to upgrade callus formation efficiency in wheat which are extensively including genotypic, culture medium, explant dependent and over expression of callus regenerative gene (Roesler et al., 2018; Chaimae et al., 2021; Yu et al., 2023). For all cultivars, new plants, or species using more than one hormone, tissue culture and growth procedures should be investigated through careful design and testing experiments. Therefore, this study aims to find the most responsive genotypes and most important individuals of wheat having high callus formation and regrowth efficiency, and additionally accelerate the propagation of wheat genotypes that exhibit superior characteristics for genetic advancement based on limited resources and time. The point of our research was to decide reasonable protocol and the selection of most efficient wheat genotype for tissue culture.

MATERIALS AND METHODS

Experimental sites and explant selection

In order to establish tissue culture and regeneration in wheat, we selected common, approved and high yield wheat varieties being extensively cultivated by farmers. We collected mature seeds of wheat in March 2023, packed in clean and microbe free plastic bags, labeled and stored at normal temperature for further use in experiment. Notably, no specific permission was required to collect samples for experimental purpose. Mature seeds of genotypically different six wheat varieties namely Chakwal-50, Galaxy-01, NARC-09, Pakistan-13, Millet-11 and Borlog-14 were employed for callogenesis and regeneration. Laboratory conditions were adjusted as follows: 25±2°C temperature, 60-80% humidity, and white light

Table 1. MS medium enhanced with dissimilar concentrations of IAA and Kinetin for regeneration, while 2,4-D for callogenesis.

S/N	Treatment	IAA + Kinetin (mg/L)	2,4 -D (mg/L)
1	H1	1.5 + 1	0.5
2	H2	3 + 1	1.5
3	H3	4.5 + 1	2.5
4	H4	1.5 + 2	3.5
5	H5	1.5 + 3	4.5
6	H6	1.5 + 4	5.5

intensity was 7000 lux. Prior to kick-off experimental work, laminar airflow cabinet was sterilized with UV lamp for 20 min.

Surface sterilization and inoculation of seeds

Mature seeds of all six wheat varieties were picked out as an explant material and disinfected three times with 50% Clorox for 20 min with constant agitation in a laminar airflow hood. Washing of seeds were performed thrice for 10 min with ddH₂O to remove Clorox and placed at sterilized filter paper to become dry. Disinfected seeds were then shifted on an autoclaved Whatmann filter papers to absorb excessive water. After that, disinfected seeds were cultured on MS media supplemented with regeneration and callus initiation media with the help of sanitized forceps. All experimental procedure was performed inside 6-inch vicinity of spirit lamp.

Regeneration and callogenesis

In order to initiate regeneration, surface sterilized dry seeds of wheat were cultured on MS media supplemented with different concentrations of IAA and Kinetin (Table 1). Each variety of wheat was tested at six different combinations of hormones in order to observe their regeneration potential, and response to various combinations of hormones, on the base of different genetic makeup. Each treatment was performed thrice, data was collected, and average was calculated to avoid any error. Inoculated seeds were incubated in growth room at temperature at $25 \pm 1^\circ\text{C}$, 60-80% humidity, and 7000 lux intense white light.

Germination initiated after 4 days of culturing and day-by-day germination data was collected and saved on excel sheet. For the purpose of callus production, inoculation of surface sterilized seeds was performed on MS media enhanced with various levels of 2,4-D (Table 1), and cultures were placed in dark condition for prompt callus induction. Notably, after 2 weeks of incubation in dark condition significant callus formation was observed in almost all wheat varieties. All reading were obtained in next 3 to 4 weeks, then separation of calli from seeds was performed with the help of sterilized forceps under sterilized conditions in order to identify best wheat variety with maximum potential of callogenesis.

Statistical analysis

Collected data were statistically analyzed by using slide writer software and ANOVA to check out the differences between treatments and within the treatments, in concern of percentage of callus formation and tissues regeneration. To get optimum result, 20 explants of every variety used for each treatment in three

replications. The mean was processed from each treatment.

RESULTS

Study aim

The aim of this study was to foster a strategy for compelling crop improvement via genetic modification. For this purpose, an established and reproducible callus induction and regeneration protocol was optimized using mature seeds of six genetically different wheat genotypes. Although, fully mature seeds of wheat are available around the year, but we collected seeds of six different varieties of wheat in March 2023, labelled name of each wheat variety, and stored in sterile bags in order to avoid exposure to microbes such as fungal spores. Notably, it is very hard to induce callus formation in mature seeds as compared to immature embryo, cotyledons or leaves, but mature seeds are available around the year. This is why we employed mature seeds for callogenesis and regeneration. This optimized callus induction and regeneration protocol will be further employed in development of biofortified wheat varieties with biotic and abiotic stress resistance. For the purpose to check the potency of six wheat cultivars for callus development and regeneration, six unique combinations of hormones (IAA, Kinetin and 2,4-D) for six treatments were applied.

Regeneration

Diverse concentrations and combinations of IAA (1.5, 3, and 4.5 mg/L) and Kinetin (1, 2, 3, and 4 mg/L) were considered to advance the regeneration combination (Table 1). To obtain high regeneration activity, various amount of Kinetin was mixed in MS media enhanced with multiple levels of IAA. The outcomes showed significant contrast among wheat varieties against growth regulators (IAA and Kinetin) of various levels.

Regeneration of tissues is genotypes dependent; therefore, behavior of different wheat genotypes was different at various concentrations of growth regulators.



Figure 1. 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.

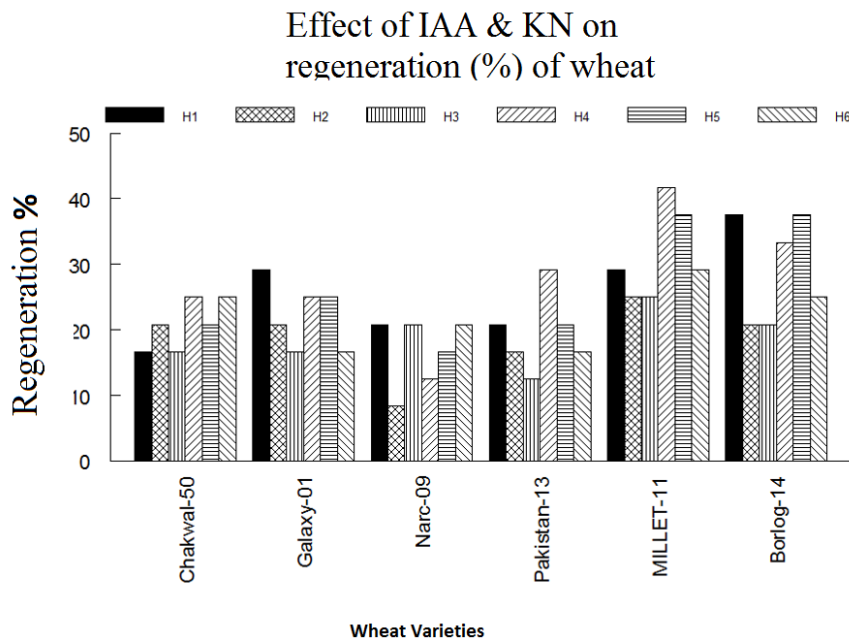


Figure 2. Effect of IAA & Kinetin (KN) on regeneration of wheat.

Millet-11 showed the highest regeneration potency on IAA concentration of 1.5 mg/L and on Kinetin concentration of 5 mg/L on tissue regeneration media (Figure 1). Behavior and regeneration ability of each genotype altered with alteration of IAA and Kinetin levels (Figure 2). However, maximum average regeneration among varieties at all combination of growth regulators

was examined in Millet-11 (31.24%), next to 29.16% in Borlog-14, and 27.7% mean maximum regeneration was acquired among all varieties at combination of IAA and Kinetin 1.5 + 2 mg/L, respectively. Wheat cultivar NARC-09 was considered a poor genotype in terms of regeneration as it produced minimum regeneration percentage of 16.6% declared as least active genotype to

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

Variety	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	25	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	22.22	

**Figure 3.** Initiation of callus started as a white spongy tissue.

among all wheat cultivars for the purpose of tissue culturing (Table 2). However, outcomes of this research presented Millet-11 cultivars as best wheat cultivars for use of tissue culture on the base of giving maximum percentage of regeneration and Borlog-14 was considered as second-best genotypes having potency of regeneration.

Callus induction

Callus formation is the essential step in different processes involved in applied and analytical tissue culture. The callus formation was divided into two different calli. Calli that were dense in look, nodules like shape, creamy-whitish to light greenish in color and embryo-like structures were named as “embryogenic calli” (Figure 4a). Calli that showed color of dirty-whit and soft-watery texture were named as “non-embryogenic calli” (Figure 4b). For callus induction, hormones play important role and, in our research, we found that only 2,4-D was prime for callus development and it was elaborated that 2,4-D is the mostly utilized growth regulators for the purpose of callus formation and its maintenance (Aadel et al., 2016; Eshagi et al., 2021;

Phogat et al., 2023).

For the purpose of callus development, various concentrations of hormone 2,4-D (0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mg/L) were evaluated. In order to find out excellent wheat genotype in terms of callus induction, six wheat genotypes were treated with six different levels of 2,4-D. Callus formation examined as a white spongy tissue on upper side of seed during the periods of 10 to 12 days and it was also genotype and medium dependent (Figure 3). Almost same types of results were disclosed by Mehmood et al. (2013). Culturing was carried out in 2,4-D hormones for up to three weeks and after that data was calculated.”

The data indicated that behavior of each variety was significantly different under unchangeable cultural environment and medium concentration because of their potency to develop callus. However, callus induction media ought to be well standardized to study the potency of a genotype to make maximum callus on the base of its genetic makeup. Results showed that in the light of different levels of 2,4-D, Millet-11 (41.66%) recovered maximum number of calli percentage at 2,4-D concentration of 2.5 mg/L next to 33.33% by variety Chakwal-50 and 29.16% by variety Borlog-14 (Figure 5).

Other than this, variety Chakwal-50 gave the best

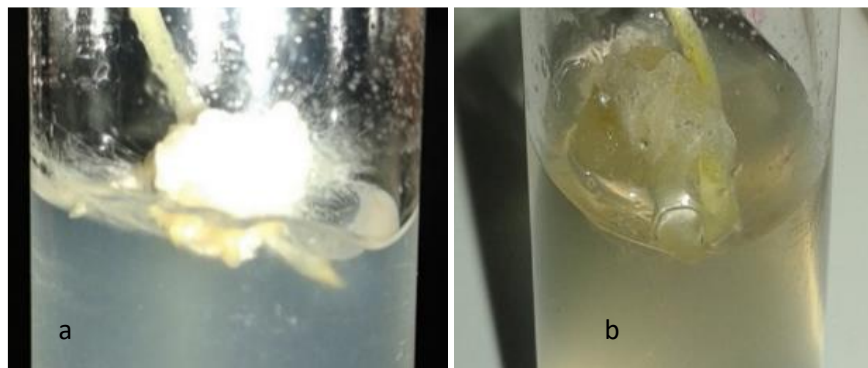


Figure 4. Different types of callus induction in wheat: (a) embryogenic callus; (b) non-embryogenic callus.

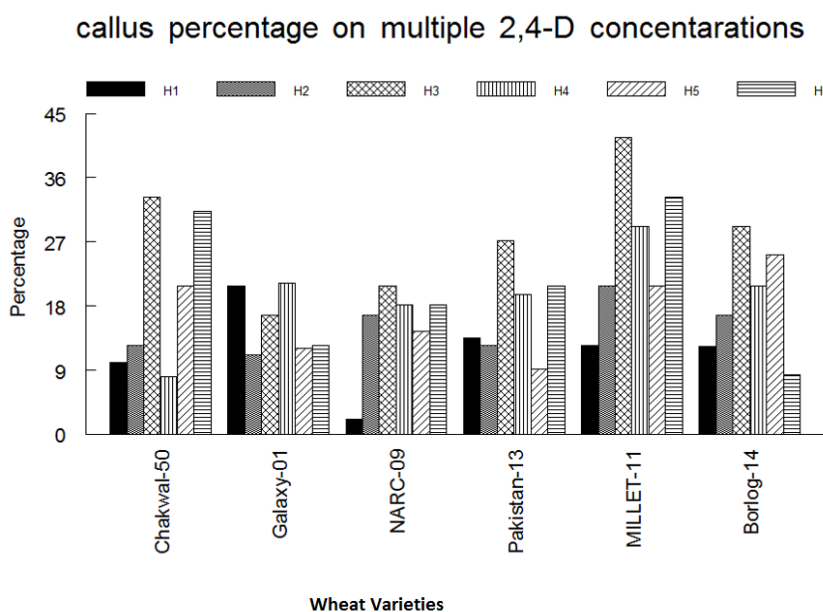


Figure 5. Callus formation percentage of various wheat varieties in response to multiple 2,4-D concentrations.

outcomes by producing maximum callus weight (0.26 g) at MS media holding 3.5 mg/L 2,4-D followed by Borlog-14 (0.16 g) and Millet-11 (0.14 g) (Figure 6). In general examination of multiple varieties at every giving level of 2,4-D media disclosed that Millet-11 (26.39%) is a genotype exhibited superior characteristics for callus development and 2.5 mg/L concentration of 2,4-D was viewed as best concentration for production of maximum callus (Table 3), and 3.5 mg/L 2,4-D was viewed best for wheat crop to produce highest weight of callus (Table 4).

DISCUSSION

Tissue culture is one of the latest techniques that involves exposure of plant tissue to a specific type of nutrients,

different kinds of hormones and artificial light under *in-vitro* circumstances to develop many plants, all of them are clones of explants, over a very small interval of time. Plant tissue culture is a crucial technique for making disease-free, stress resistant planting material and in short time production of uniform plants in developing countries. In botany, callus cells are that kinds of cells that heal plant wound after injury. Callus formation is stimulated from any type of explants materials after being sterilized and *in-vitro* planting on culture medium contains different hormones and growth regulators. Plants growth controlling hormones such as IAA, Kinetin, and 2,4-D are added in plating medium for the purpose to commence callus production or somatic embryogenesis.

In the present research, six wheat genotypes were utilized to observed *in-vitro* effect of different hormones

callus weight on multiple 2,4-D concentrations

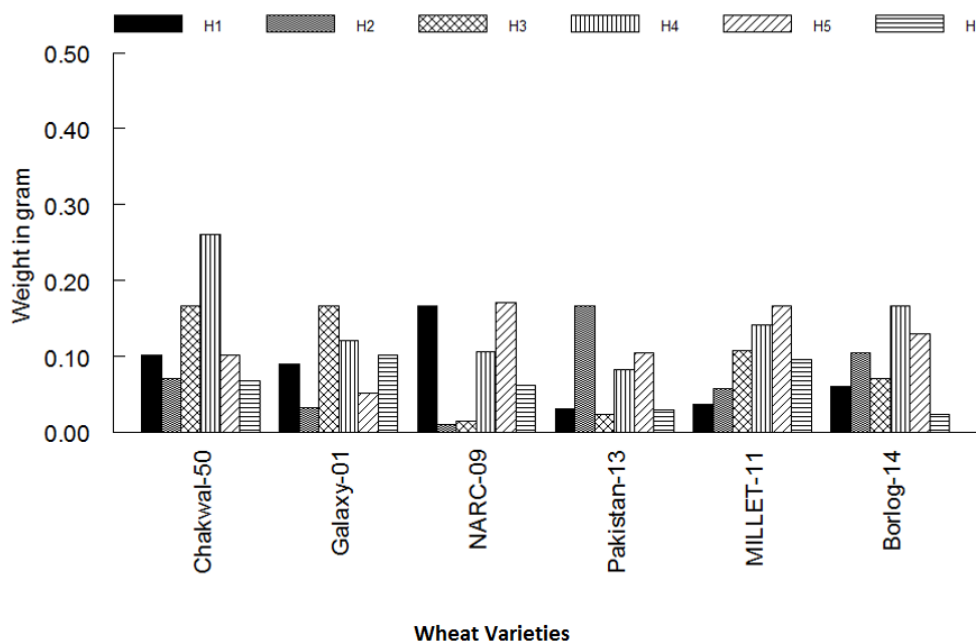


Figure 6. Callus weight of various wheat varieties in response to multiple 2,4-D concentrations

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

Variety	H1	H2	H3	H4	H5	H6	Mean
2, 4-D (mg/L)	0.5	1.5	2.5	3.5	4.5	5.5	(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	

by applying different growth regulators. The role of IAA alone for plant regeneration was not effective but combined effect of IAA and BAP together was vital in case of whole plant regeneration. These findings are consistent with Malik et al. (2004), they elaborated the combined effect of IAA and BAP on whole plant regeneration is necessary as compared to apply these hormones separately on wheat genotypes and found regeneration of plant step-up 84 and 52% in wheat cultivars Inqilab-91 and Pavon-76, respectively.

Pattern of callus development and tissue regeneration was varied in all studied genotypes by altering the IAA and Kinetin concentration. However, ability and efficiency of Millet-11 for callus development and plant regeneration

was highest in comparison to other genotypes, maybe because of high expression of callus formation and plant regeneration genes at genetics level. Highest amount of callus production was obtained at 2.5 mg/L 2,4-D concentration (Figure 5) while highest callus weight was generated at 3.5 mg/L 2,4-D concentration in wheat (Figure 6). High weight of callus is necessary to establish a good protocol for gene transformation and fast development of plantlets through tissue culture. By the way, the output of our current experiments for callus formation and plant regeneration are consistent with other researchers who also applied different combinations of hormones to establish a tissue culture protocol in wheat. For example, Malik et al. (2021) obtained the most

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	

noteworthy regeneration in Pakistani wheat varieties AS-2002 and Wafaq-2001 which yielded maximum embryogenic calli at concentration of 3.0 mg/L 2,4-D and 3.5 mg/L 2,4-D containing induction medium, respectively, and Mahmood et al. (2012) described perfect regeneration of plant tissues (41.19%) in wheat cultivars namely GA-2002 on culture media enhanced with 1.0 mg/L concentration of Kinetin. Shah (2023) tested 10 different Pakistani wheat genotypes with changeable concentrations of 2,4-D, IAA and Kinetin and observed maximum regeneration by variety Atta Habib at 0.1 to 0.4 mg/L (IAA- Kinetin) while maximum response of callus induction was 21 to 94%.

Numerous researchers have normalized optimal levels of 2,4-D for genetically dissimilar varieties of wheat. Iqbal et al. (2016) recovered utmost callus on MS media together with 4 and 6 mg/L 2,4-D. Similarly, Naz et al. (2021) recorded the highest proliferation and callus induction at concentration of 2 mg/L 2,4-D, in wheat, while highest plants regeneration was observed at concentration of IAA 6 mg/L and 6 mg/L Kinetin. Afzal et al. (2010) mentioned the highest callus formation at 3 mg/L of 2,4-D. Miroshnichenko et al. (2017) observed 10 shoots per explant in wheat by applying 3 mg/L Dicamba concentrations, 50 mg/L concentration of Daminozide, and with TZD concentration of 0.25 mg/L. Callus formation activity of wheat varieties to 2,4-D explained that genetic makeup of each variety have different demands of 2,4-D for maximal callus formation. Different cultivars of wheat have different behavior toward various amounts of plant growth hormones. Callogenesis and regeneration of varieties and variation among them were found to be genotype-dependent (Kagami et al., 2016; Ahmad et al., 2021).

Conclusion

Optimize concentration of the plant growth controlling hormones is crucial for maximal plantlets regrowth for most researches involving tissue development techniques. Among six researched wheat cultivars, high potential for tissue regeneration and callus development

was observed by the variety Millet-11. The results also suggest that regeneration potency of a genotype can speed up to a particular level by combining 1.5 mg/L concentration of IAA and 2 mg/L Kinetin concentration. Similarly, 2,4-D is a necessary plant growth hormones and appropriate amount is crucial for different plant species for callus formation and in wheat, 3.5 mg/L 2,4-D concentration was described best for high weight callus induction, so these results might be useful in future for gene transformation for the improvement of agronomic traits. Maximum potential for tissue culture of Millet-11 suggests the opportunity to use it for wheat improvement programs. These findings provide precise knowledge for plant breeders to develop new potential varieties of wheat having strong biotic and abiotic stress resistance in order to get maximum yield to feed growing population.

Author Contributions

Conceptualization, BC, MT, and F; designed the experiments, BC, and MT; performed the experiments BC, MT, SZ, F, HL, JL, HMA, and CMG; analyzed the data, BC, MT, MA, and AJR; wrote the manuscript, AJR, MT, BC, and MA. All authors reviewed the manuscript.

ACKNOWLEDGEMENTS

The authors are very grateful to the kind administration of Yibin University, Yibin 644000, China for providing us such a prestigious and well-equipped platform for research and development.

Funding

This work was supported by Yibin University Research Initiation Project (NO. 2020QH06).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Aadel H, Ahansal K, Udupa SM, Gaboun F, Abdelwahd R, Douira A, Iraqi D (2016). Effect of genotypes and culture media on embryogenic callus induction and plantlet regeneration from mature and immature bread wheat embryos. *Romanian Agricultural Research* (33):61-69.
- Abbas M, Hussain SS, Abbas SK, Abbas S, Tahir M, Su Y, Li J, Ahmed N, Khan MS, Gou C (2023). Optimization of regeneration protocol and prospecting spectinomycin resistance in barley (*Hordeum vulgare* L.) cv Haider-93. *African Journal of Biotechnology* 22(9):172-182.
- Abbas M, Peszlen I, Shi R, Kim H, Katahira R, Kafle K, Xiang Z, Huang X, Min D, Mohamadamin M, Yang C (2020). Involvement of CesA4, CesA7-A/B and CesA8-A/B in secondary wall formation in *Populus trichocarpa* wood. *Tree Physiology* 40(1):73-89.
- Afzal A, Rashid H, Khan MH, Chaudhry Z, Malik SA (2010). High frequency regeneration system optimization for wheat cultivar Inqilab-91. *Pakistan Journal of Botany* 42(3):1857-1862.
- Ahmad A, ul Qamar MT, Shoukat A, Aslam MM, Tariq M, Hakimian M, Joyia FA (2021). The effects of genotypes and media composition on callogenesis, regeneration and cell suspension culture of chamomile (*Matricaria chamomilla* L.). *Peer J* 9:e11464.
- Bhatta M, Regassa T, Rose DJ, Baenziger PS, Eskridge KM, Santra DK, Poudel R (2017). Genotype, environment, seeding rate, and top-dressed nitrogen effects on end-use quality of modern Nebraska winter wheat. *Journal of The Science of Food and Agriculture* 97(15):5311-5318.
- Bull T, Michelmore R (2022). Molecular determinants of in vitro plant regeneration: prospects for enhanced manipulation of lettuce (*Lactuca sativa* L.). *Frontiers in Plant Science* 13:888425.
- Chaimae S, Khadija A, Rabha A, Ghizlane D, Fatima G, Mahabala US, Allal D, Driss I (2021). Development of an efficient regeneration system for mature bombarded calli of Moroccan durum wheat varieties. *Australian Journal of Crop Science* 15(3):431-437.
- El-Sappah AH, Rather SA, Wani SH, Elrys AS, Bilal M, Huang Q, Dar ZA, Elashokhy M, Soaud N, Koul M, Mir RR (2022). Heat stress-mediated constraints in maize (*Zea mays*) production: challenges and solutions. *Frontiers in Plant Science* 13:879366 doi: 10.3389/fpls.2022.879366.
- El-Sappah AH, Yan K, Huang Q, Islam MM, Li Q, Wang Y, Khan MS, Zhao X, Mir RR, Li J, El-Tarabily KA (2021). Comprehensive mechanism of gene silencing and its role in plant growth and development. *Frontiers in Plant Science* 12:705249.
- Eshagi SM, Dorani E, Aghazadeh R (2021). Effects of plant growth regulators and organic compounds on callus induction and plant regeneration in wheat (*Triticum aestivum* L.) using mature embryos. *Journal of Plant Molecular Breeding* 9(1):62-69.
- Hill K, Schaller GE (2013). Enhancing plant regeneration in tissue culture: a molecular approach through manipulation of cytokinin sensitivity. *Plant Signaling & Behavior* 8(10):212-224.
- Iqbal M, Raja NI, Asif S, Ilyas N, Hussain M, Yasmeen F, Ejaz M, Sultan MA, Aslam S, Javed H (2016). In vitro study of callogenesis and regeneration potential of elite wheat (*Triticum aestivum* L.) cultivars. *American Journal of Plant Sciences* 7(17):2515.
- Kagami H, Taguchi K, Arakawa T, Kuroda Y, Tamagake H, Kubo T (2016). Efficient callus formation and plant regeneration are heritable characters in sugar beet (*Beta vulgaris* L.). *Hereditas* 153(1):1-6.
- Kumar R, Mamrutha HM, Kaur A, Venkatesh K, Grewal A, Kumar R, Tiwari V (2017). Development of an efficient and reproducible regeneration system in wheat (*Triticum aestivum* L.). *Physiology and Molecular Biology of Plants* 23:945-954.
- Long Y, Yang Y, Pan, G, Shen Y (2022). New insights into tissue culture plant-regeneration mechanisms. *Frontiers in Plant Science* 13:926752.
- Mahmood I, Razzaq A (2017). Responses of explant type of wheat (*Triticum aestivum* L.) genotypes to different tissue culture media. *Journal of the National Science Foundation of Sri Lanka* 45(3). DOI: 10.4038/jnsfsr.v45i3.8191
- Mahmood I, Razzaq A, Khan ZD, Hafiz IA, Kaleem S (2012). Evaluation of tissue culture responses of promising wheat (*Triticum aestivum* L.) cultivars and development of efficient regeneration system. *Pakistan Journal of Botany* 44(1):277-284.
- Malik SI, Rashid H, Yasmin T, Minhas NM (2004). Plant regeneration by somatic embryogenesis from callus of mature seed explants of bread wheat (*Triticum aestivum* L.). *Pakistan Journal of Botany* 36(3):629-634.
- Malik WA, Mahmood I, Razzaq A, Afzal M, Shah GA, Iqbal A, Zain M, Ditta A, Asad SA, Ahmad I, Mangi N (2021). Exploring potential of copper and silver nano particles to establish efficient callogenesis and regeneration system for wheat (*Triticum aestivum* L.). *GM Crops & Food* 12(1):564-585.
- Mehmood K, Arshad M, Ali GM, Razzaq A (2013). Tissue culture responses of some wheat (*Triticum aestivum* L.) cultivars grown in Pakistan. *Pakistan Journal of Botany* 45:545-549.
- Miroshnichenko D, Chaban I, Chernobrovkina M, Dolgov S (2017). Protocol for efficient regulation of in vitro morphogenesis in einkorn (*Triticum monococcum* L.), a recalcitrant diploid wheat species. *PLoS One* 12(3):e0173533.
- Naz M, Nizamani F, Nizamani GS, Nizamani MR, Ahmed S, Ahmed N, Saif-Ur-Rehman SAS (2021). Callus induction and plant regeneration from immature embryos of spring wheat varieties (*Triticum aestivum* L.) under different concentrations of growth regulators through tissue culture technique. *Pure and Applied Biology* 5(3):378-385.
- Peng X, Pang H, Abbas M, Yan X, Dai X, Li Y, Li Q (2019). Characterization of *Cellulose synthase-like D* (*CSLD*) family revealed the involvement of *PtrCSLD5* in root hair formation in *Populus trichocarpa*. *Scientific Reports* 9(1):1452. doi: 10.1038/s41598-018-36529-3.
- Phogat S, Poudel A, Bhurta R, Kalwan G, Madhavan J, Padaria JC, Singh PK, Vinutha T, Mandal PK (2023). An improved in-vitro regeneration protocol using scutellum of mature and immature embryos of wheat. *Indian Journal of Genetics and Plant Breeding* 83(2):195-204.
- Rahman M, Shamsuddin A, Asad U (2008). In vitro regeneration from mature embryos in spring wheat. *International Journal of Sustainable Crop Production* 3(2):76-80.
- Rashid H, Khan MH, Chaudhry Z, Bano R, Raja NI (2012). An improved Agrobacterium mediated transformation system in wheat. *Pakistan Journal of Botany* 44(1):297-300.
- Roesler EA, Manfroi E, Morás A, Didoné DA, Grando MF, Lau EY (2018). Adaptation of in vitro regeneration protocol for Brazilian wheat genotypes. *Ciência Rural* 48(11):e20170806.
- Shah M (2023). Pakistani wheat cultivars depict genotype dependent callus and regeneration response. *Journal of Agriculture and Food* 3(2):46-61.
- Singh CK, Singh D, Taunk J, Chaudhary P, Tomar RSS, Chandra S, Singh D, Pal M, Konjengbam NS, Singh MP, Singh Sengar R (2021). Comparative inter-and intraspecies transcriptomics revealed key differential pathways associated with aluminium stress tolerance in lentil. *Frontiers in Plant Science* 12:693630.
- Sugimoto K, Meyerowitz EM (2013). Regeneration in Arabidopsis tissue culture. *Plant Organogenesis: Methods and Protocols* pp. 265-275.
- Wei X, Fan X, Lu X, Han L, Zhang H, Liu S, Qu J, Guan S, Ma Y (2022). Optimization of callus induction conditions from Immature embryos of maize under stress. *Phyton* (0031-9457) 91(9).
- Xu L, Huang H (2014). Genetic and epigenetic controls of plant regeneration. *Current Topics in Developmental Biology* 108:1-33.
- Yu Y, Wang J, Zhu M, Wei Z (2008). Optimization of mature embryo-based high frequency callus induction and plant regeneration from elite wheat cultivars grown in China. *Plant Breeding* 127(3):249-255. doi: 10.1111/j.1439-0523.2007.01461.x.
- Yu Y, Yu H, Peng J, Yao WJ, Wang YP, Zhang FL, Su YH (2023). Enhancing wheat regeneration and genetic transformation through overexpression of TaLAX1. *Plant Communications* 5:100738.