Optimization of *Agrobacterium tumefaciens*-mediated transformation in wheat using mature embryos

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Abstract

Wheat is the world's most widely grown staple food, meeting the nutritional needs of more than 35% of the human population. The development of transgenic wheat is slow worldwide due to the lack of a suitable transformation system. The availability of a reproducible regenerative system through tissue culture is a major bottleneck in the wheat improvement program. The present study deals with the efficient and reproducible *Agrobacterium*-mediated transformation system in wheat is established. The mature embryos of three commercially bred wheat genotypes from Pakistan i.e., Faisalabad 2008, Galaxy 2012 and NARC-443, were used to establish an efficient and stable transformation system. In callus induction, each variety responded differently to different concentrations of 2,4-D. Faisalabad 2008 and Galaxy2012 showed the highest callus induction of 83% and 71%, respectively, at three mg/l of 2, 4-D, while NARC-443 showed 65 % callus induction at 3.5 mg/l of 2, 4-D with different BAP concentrations were used to develop shoots and roots. Faisalabad 2008 and Galaxy 2012 showed significantly higher regeneration rates of 61% and 54% with 3.0 mg/l of BAP, while NARC-443 had the highest regeneration at 41% with 2.0 mg/l. The average fresh calli weights for Galaxy 2012, Faisalabad 2008, and NARC-443 were 0.58 g, 0.46 g, and 0.43 g, respectively. Fresh calli weights correlated significantly and positively with dry weights. The expression of GUS in the transgenic leaves was confirmed by histochemical analysis of β-glucuronidase. Tissue culture protocol optimization in wheat varieties, Faisalabad 2008, Galaxy 2012, and NARC-443 showed transformation efficiencies of 1.3%, 0.8%, and 0.3%, respectively. The current results will help in the selection of wheat cultivars that best respond to tissue culture and crop improvement through transgenic development.

Keywords: *Triticum aestivum*, Callus induction, Regeneration, *Agrobacterium tumefaciens*

1. Introduction

Wheat (*Triticum aestivum*) is Pakistan's most important staple food and is essential to the country's food security (1). Wheat is grown primarily for food, but seed, straw and bran can be used as industrial products and animal feed. Wheat straw can also be used as fuel, animal bedding and soil organic matter (2). Wheat production contributes 9.2% of the agricultural value and 1.8% to GDP. Wheat is sown on 9.178 million hectares of land in 2020-2021, with a growth rate of 8.1% and high production level of 27.293 million tonnes (3). With the increasing population of Pakistan, wheat demand is also increasing rapidly. The average yield per hectare is very low compared to world production, which is an alarming situation for an agricultural country. Several factors affect wheat yield in Pakistan despite management and efforts. Among these, many biotic and abiotic factors that lead to a reduction in crop yield, like unfavorable weather conditions, traditional style of cultivation, lack of high-yielding varieties, drought, improper use of fertilizers, soil fertility levels, and occurrence of diseases and insect pests (4).

Pakistan is an agricultural country, and wheat is the most important cereal crop, accounting for 36% of the total cropland. Traditional breeding methods are used in Pakistan to increase wheat crop production and improve quality (5). However, its average yield per hectare is meager and uncertain due to limited gene pool availability, conventional breeding methods, the long time required in traditional breeding, species barrier, and other biological limitations (6). Research has recently concentrated on in vitro culture and regeneration as cereal breeding tools. Furthermore, it is well-established that cereal genetic engineering relies on tissue culture and regeneration (5). Genetic engineering techniques are gaining popularity because the desired gene can be introduced into the wheat genome without a species barrier in a short time to improve desired characteristics (7). Wheat transformation depends entirely on tissue culture and regeneration of transformed explants (8).

Consequently, reliable tissue culture protocols for callus induction and regeneration are required to increase wheat yield through genetic transformation (9). Wheat tissue culture is influenced by genotype (10), culture medium (11), and growth regulators (12). Explants such as mature and immature embryos, leaves, shoot bases, root tips and whole seeds have all been used for this purpose (13). For callus and regeneration, immature embryos are preferred over mature embryos (14), but their availability is limited throughout the year.

The callus is a mass of undifferentiated cells that proliferates after explants are grown in nutrient media containing growth hormones (15). 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin widely used in cereal tissue cultures as a growth regulator (16). Callus formation in wheat has been achieved using 2,4-D alone or in combination with cytokinins (17). According to research, genotypes respond differently to callus induction at varied concentrations of 2,4-D (18). The current study is focused on optimizing an efficient *Agrobacterium*-mediated transformation system in wheat. These results will assist local wheat breeders to increase transgenic efficiency with enhanced expression.

2. Material and methods

2.1 Source of explant

Mature seeds of three wheat (*Triticum aestivum*) cultivars Galaxy 2012, Faisalabad 2008 and NARC-443 were used in this study. These cultivars were selected after consultation with Agriculture Extension Department Gujrat as these cultivars were grown locally. Faisalabad 2008, Galaxy 2012 and NARC-443 mature seeds were obtained from the National Agriculture Research Center (NARC) Islamabad.

2.2 Seed sterilization

Healthy seeds from selected wheat genotypes were used as explant sources and surface sterilized to remove contamination. Wash the seeds 2-3 times with running tap water. The seeds were sterilized with 60 % Clorox and one drop of Tween-20 for 20 minutes with vigorous shaking. The seeds were then rinsed 4-5 times with autoclaved water to remove excess Clorox and dried on autoclaved filter paper.

2.3 Callus induction and regeneration

Sterilized seeds were inoculated into test tubes containing MS media (19) supplemented with various concentrations of 2, 4-D (1.5 mg/L, two mg/L, 2.5 mg/L, three mg/L, 3.5 mg/L, and four mg/L). The pH of the medium was adjusted to 5.8 before autoclaving. To solidify the media, 8 g/L agar was used. The cultures were grown in a growth room at a temperature of 27 °C, photoperiod of 16 hours of light and eight hours of dark and 5400 lux light intensity. Callus proliferation rate and callus quality were recorded after 2-3 weeks. Their fresh and dry weights were measured. The callus was separated from the seeds and transferred to a fresh maintenance medium for further

proliferation and growth. To achieve maximum regeneration, calli were placed in regeneration media, which consisted of MS with varying BAP concentrations (1 mg/L, two mg/L, three mg/L, four mg/L, and five mg/L). The percentage of calli regenerated was recorded for each genotype. Regenerated plants were hydroponically grown for root elongation and healthy root development. When the roots had grown sufficiently, they were transferred to soil-filled pots.

2.4 Inoculation, Cocultivation and Plant Regeneration

Calli were placed in the center of the plate for inoculation and *Agrobacterium tumefaciens* containing pCAMBIA 1301 culture was poured over and left for 15-20 minutes. The excess culture was removed and transferred to MS medium containing three mg/L 2,4-D and 200 uM acetosyringone. The calli were incubated in the dark for three days at 21±1°C. After three days of cocultivation, calli were transferred to a callus induction medium with the best 2,4-D concentration as previously optimized for four weeks. After this, they were shifted to a regeneration medium containing optimized BAP concentration. After regeneration, plants were grown on soil.

2.5 Histochemical GUS assay

Histochemical staining of GUS analysis was used to confirm the GUS expression in wheat seedlings according to the procedure used by (20). 4-5 seedlings were selected from each cultivar and cut into small pieces. These green pieces of seedlings were then submerged in GUS staining solution. GUS staining solution was prepared by mixing 15 ml of 0.2 M sodium phosphate buffer (pH 7.2), 750 µl of 20 mM potassium ferrocyanide, 750 µl of 20 mM potassium ferricyanide, 600 µl of 0.1 M EDTA pH 7, 30µl of 0.1 % Triton X-100 and 30µl of 20 mM 5-bromo-4-chloro-3-indolyl-glucuronic acid (X-Gluc substrate) in 30 ml of distilled water. The seedlings were incubated for 10-12 hours at 37 ºC. Stained tissues were immersed in 90 % ethanol for 24 hours for easy visualization. GUS expression was observed in transformed wheat cultivars by blue color.

2.6 DNA Extraction from Leaves and PCR Analysis

Genomic DNA was extracted using FacroPrep^{TM} Plant Genomic DNA extraction Mini Kit (Cat # FAPGK-001, Favorgen Biotech Corp) from the leaves of wheat cultivars. The PCR reaction contains 1.5 µl (ng) template, one µl 25mM $MgCl_2$, 0.5 µl (2mM each) dNTPs mix, one µl (µM) of GUS forward primer (5-ACTATGCCGGAATCCATCG-3) and GUS reverse primer (5-ACGGTAGGAGTTGGCCCCAA-3), one µl 10X Taq buffer, 0.5 µl (U/µL) Taq polymerase and a final volume 25 µl by adding 13.5 µl nuclease-free water. The PCR program started with an

initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation (95 °C for 30 sec), annealing temperature was optimized (30 sec), extension (72°C for 45 sec) and a final extension of 5 minute at 72°C.

3. Results

3.1 Callus Induction

Three wheat cultivars Galaxy 2012, Faisalabad 2008 and NARC-443 were used to determine each cultivar's maximum callus induction at concentrations of 2.4-D. After 5-7 days, callus formation began in all wheat cultivars (Figure 1). Callus induction and shooting occurred concurrently, but by the third week, shoot growth had stopped while calli continued to grow in mass. Maximum callus induction was observed in Galaxy 2012 and Faisalabad 2008 at three mg/l of 2,4-D, while NARC-443 showed maximum callus induction at 3.5 mg/l of 2,4-D (Figure 2). Higher 2,4-D concentrations inhibited callus proliferation, while lower concentrations allowed for morphogenesis, affecting both callus quality and quantity (7). The callus was compact and nodular, characteristic of an embryogenic callus. Satyavathi et al., (13) have reported similar findings. The non-embryogenic callus was fleshy and whitish. These findings were also published by (14). According to the data, the cultivars' ability to produce callus under the same culture medium and conditions varied significantly. The callus induction response of cultivars to 2,4-D suggests that cultivars respond differently to 2,4-D concentration. The amount of 2,4-D required for maximum callus induction varies between varieties. After 30 days of callus induction, fresh and dry weights of whole calli were calculated for all varieties (Table 1). The average fresh weight for Galaxy 2012, Faisalabad 2008 and NARC-443 were 0.58g, 0.46g, 0.43 g, respectively. Fresh weights were found to be significant and highly correlated with dry weights in Galaxy 2012 ($r = 0.95$), Faisalabad 2008 ($r = 0.94$), NARC-443 ($r = 0.88$). Similarly, the average dry weights were 0.059g NARC-443, 0.062g Faisalabad 2008, and 0.067g of Galaxy 2012. Rahman et al. 2009 observed that an increase in 2,4-D concentration was negatively correlated with an increase in fresh weight and total cell number (21).

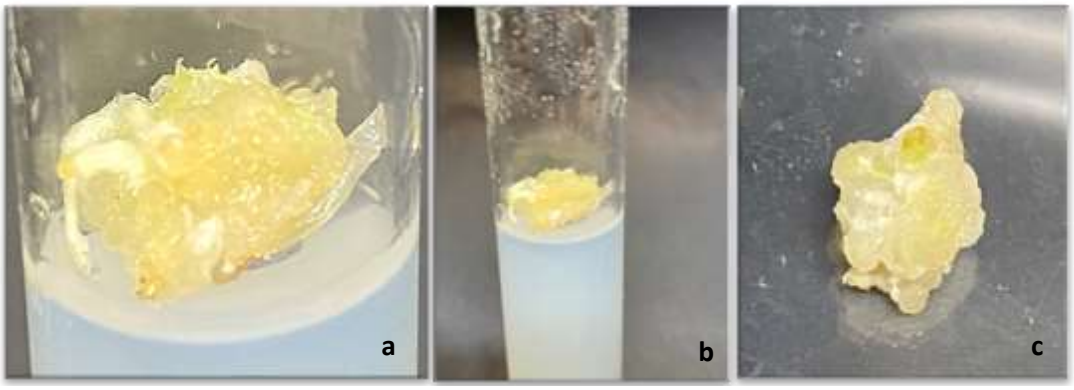

Figure 1: Callus induction of wheat cultivars a. Faisalabad 2008 (3mg/l), b. NARC-443 (3.5mg/l), c. Galaxy 2012 (3mg/l)

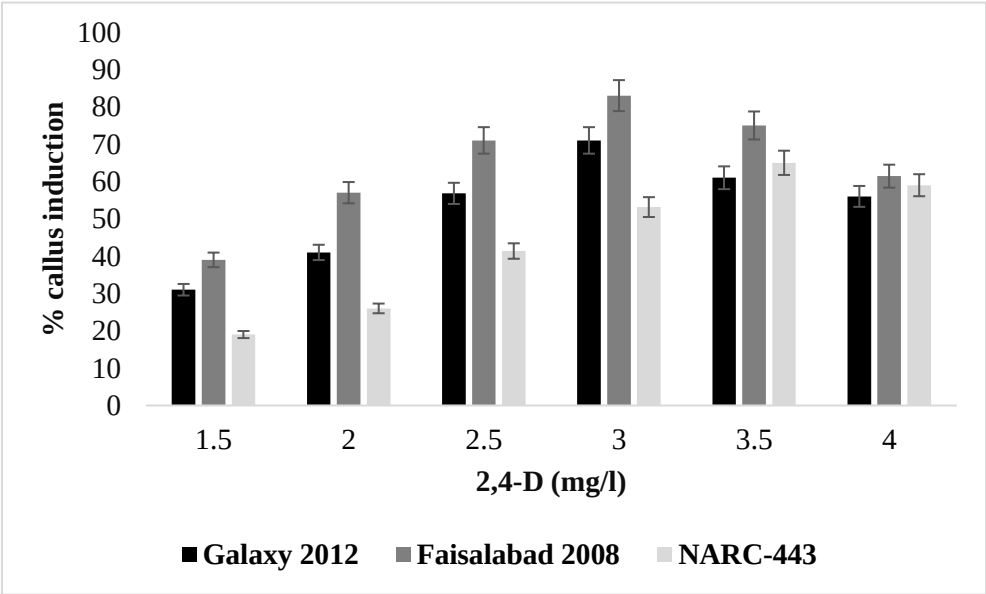

Figure 2: Callus induction of wheat cultivars at various concentrations of 2,4-D. Mean ± SE of mean represented by error bar.

The callus induction frequency variation in response to different levels of 2, 4-D could be explained by differences in callusing genes. This experiment's results suggested that the cultivars' variable response to tissue culture could be attributed to genotype and media interaction (10). These findings support the findings of (22). Farooq et al., (23) proposed that wheat genotype influences callus induction. Many studies have revealed that various levels of 2,4-D are required for maximum callus induction (24). Rashid et al., (14) also reported maximum callus induction in wheat cultivars at four mg/l and six mg/l 2, 4-D in MS medium. These outcomes could be attributed to variations in explant varieties, sources, media types, and tissue culture conditions. It was also

observed that when 2,4-D was low, the tendency of shooting increased while callus induction decreased, in contrast, when 2,4-D was raised, the tendency of shoot regeneration decreased and callus induction increased. Similar findings were reported by Alizadeh et al. 2004 (25).

Table 1: Fresh and Dry Weight of wheat cultivars callus

Sr. no.	Fresh weights (g)			Dry weights (g)		
	NARC-443	Faisalabad 2008	Galaxy 2012	NARC-443	Faisalabad 2008	Galaxy 2012
1	0.44	0.48	0.53	0.059	0.061	0.059
2	0.45	0.49	0.51	0.058	0.063	0.067
3	0.43	0.47	0.56	0.060	0.059	0.062
4	0.47	0.43	0.64	0.059	0.062	0.076
5	0.41	0.45	0.52	0.061	0.061	0.071
6	0.42	0.47	0.51	0.063	0.063	0.069
7	0.44	0.45	0.71	0.059	0.064	0.068
8	0.43	0.46	0.55	0.058	0.065	0.064
9	0.41	0.47	0.68	0.060	0.061	0.063
10	0.45	0.49	0.66	0.057	0.062	0.071
Average	**0.43**	**0.46**	**0.58**	**0.059**	**0.062**	**0.067**

3.2 Regeneration

The calli were transferred to the regeneration medium after one week of maintenance, which consisted of MS medium with varying concentrations of BAP. BAP was widely considered an effective cytokinin for the regeneration of cereal crops. All cultivars' regeneration responded significantly to different concentrations of BAP (Figure 3). Faisalabad 2008 and Galaxy 2012 showed the highest regeneration at 3mg/l of BAP, while NARC-443 showed maximum regeneration at 2mg/l of BAP (Figure 4). Because wheat regeneration was genotype-dependent, each genotype responded differently to different levels of BAP. Further, increase in BAP concentration decline the regeneration of wheat cultivars. Regeneration frequency was genotype-dependent and can be controlled by the genetic system, a growth regulator optimized for plant regeneration in one cultivar cannot develop plants in another cultivar of the same species, as shown in Figure. Many researchers have reported excellent regeneration when using various combinations of growth regulators. MS medium supplemented with 0.5 mg/L (Figure 4.32). BAP, 0.5 mg/L Kn and 25.0 mg/L tyrosine resulted in maximum regeneration (26).

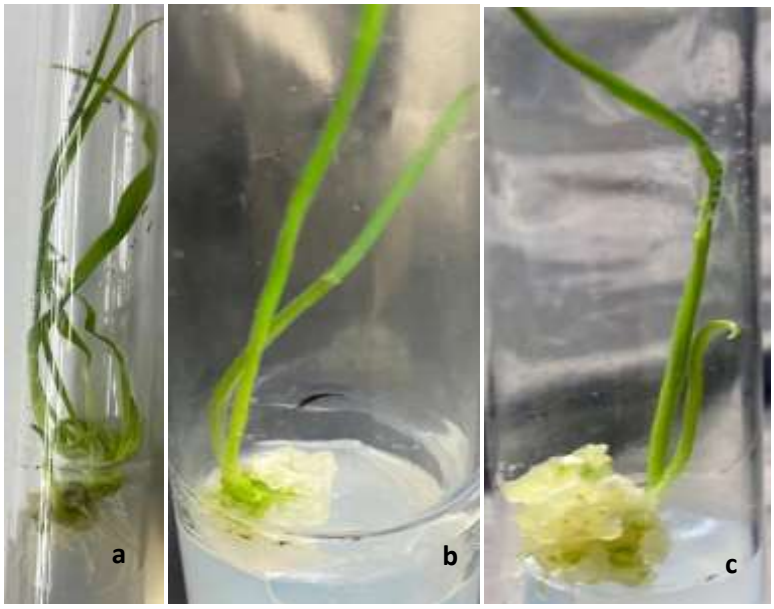

Figure 3: **Regeneration on different concentration of BAP** a. Faisalabad 2008 (3mg/l), b. NARC-443 (2 mg/l), c. Galaxy 2012 (3mg/l)

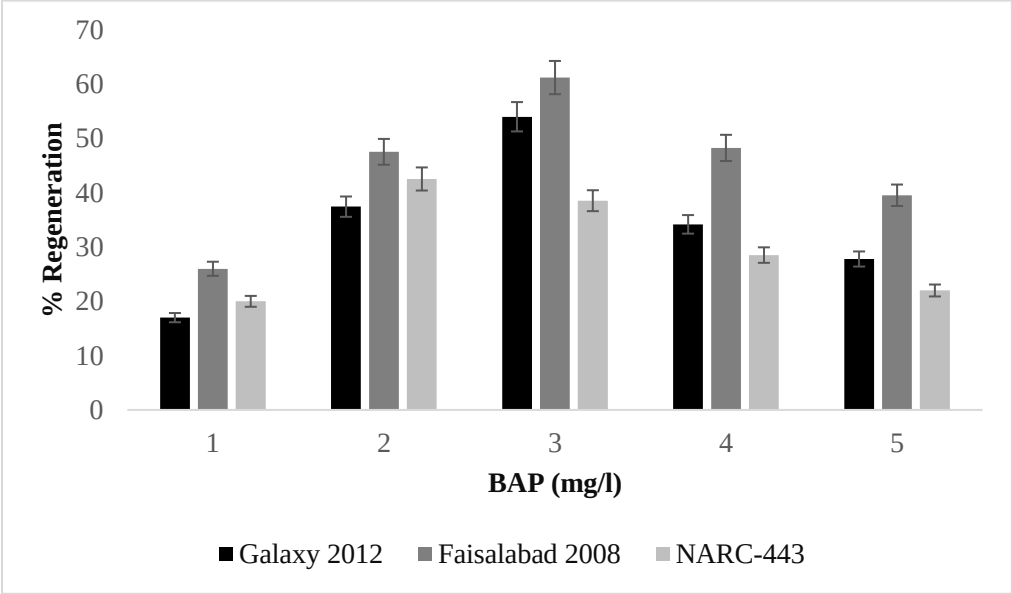

Figure 4: Effect of BAP on regeneration of different wheat cultivars. Data is represented as Mean ± SE of mean represented by error bar.

3.3 Inoculation, Cocultivation and Plant Regeneration

The optimized concentration of 2,4D gave maximum callus induction for Galaxy 2012, Faisalabad 2008 and NARC-443 were used for callus induction. The callus was co-cultivated with inoculum containing *A. tumefaciens* with transformed pCAMBIA 1301 containing the GUS gene. After cocultivation, the callus was shifted to regeneration media containing BAP which showed maximum results. The multiple shootings were induced and then shifted in soil for growth.

3.4 GUS assay

Histochemical staining of GUS analysis was used to confirm the GUS expression in wheat seedlings. These green pieces of seedlings were then submerged in GUS staining solution. GUS expression was observed in blue color in transformed wheat cultivars as shown in figure 5. The GUS-positive seedlings were shifted in pots for further growth and genomic DNA was extracted for PCR confirmation of transgenic.

Figure 5: Transient GUS expression of wheat cultivars. a. Faisalabad 2008, b. Galaxy 2012, c. NARC-443

3.5 DNA Extraction of Transformed Wheat Plants

The transformed wheat cultivars were analyzed for integration of the GUS using PCR on randomly selected five plants from wheat cultivars that were GUS positive. GUS primers were used in the PCR to confirm transgenic wheat verities. The 485 bp band was visualized, confirming the integration of the GUS gene, as shown in figure 6a. It confirmed the presence and integration of the GUS gene in wheat cultivars. Very low transformation efficiency was observed for all cultivars. Faisalabad 2008 showed maximum transformation efficiency (1.3%), while the transformation efficiency of Galaxy 2012 and NARC were 0.8% and 0.3 %, respectively, as shown in figure 6b.

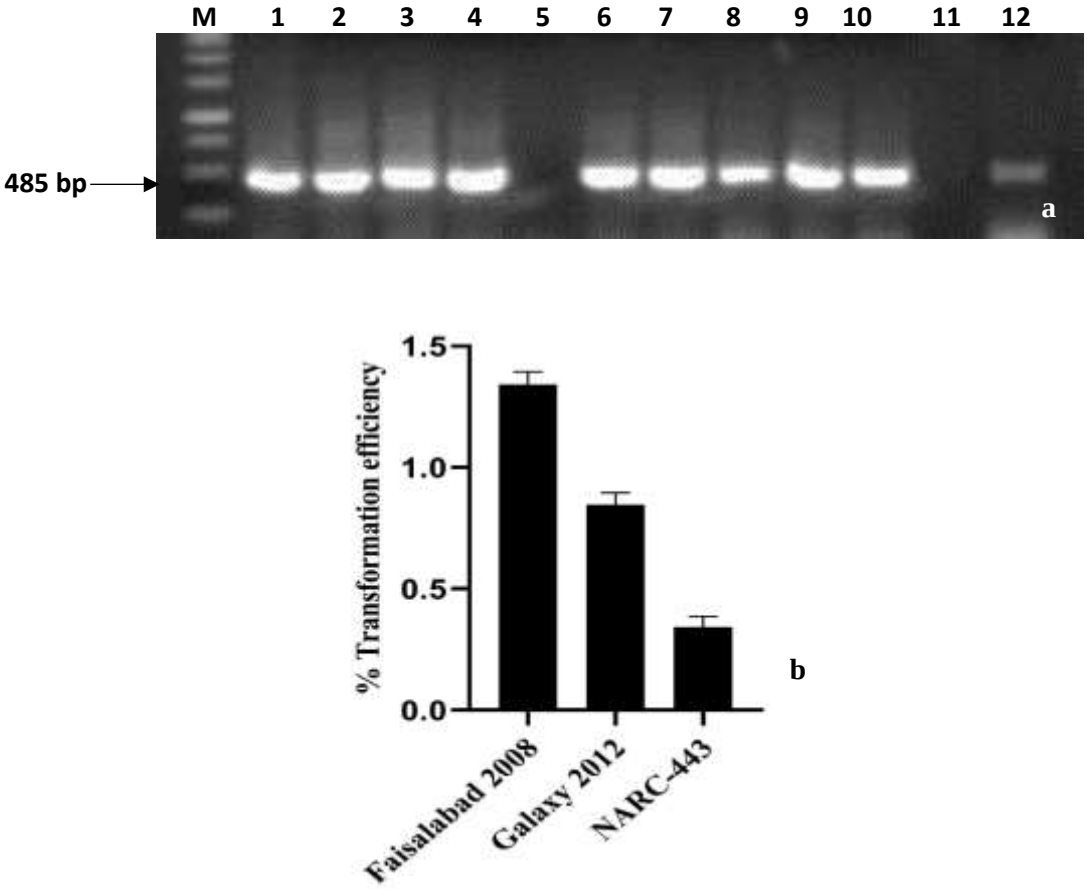

Figure 6: Confirmation of transformation and *Agrobacterium* mediated transformation efficiency in different wheat cultivars. a. Selection of transgenic wheat cultivars with PCR using GUS primers M1: 1 kb ladder, 1-3 Faisalabad 2008, 4,6-7 Galaxy 2012, 8-10 NARC-443, 11 Negative control, 12 Positive control, b. % Transformation efficiency of wheat cultivars through tissue culture. Error bar shows ± SE of mean within group.

4. Conclusions

This study shows that by using seeds as an explant source, high-frequency callus induction and regeneration can be achieved for selected wheat cultivars. In wheat cultivars, callus induction and regeneration potential are genotype and media-dependent. A combination of auxins and cytokinins in an MS-based medium can significantly increase the transformation efficiency, thus overcoming

callus induction and regeneration problems in wheat. Growth regulators, light, and temperature conditions must be optimized for maximum callus induction and regeneration in wheat cultivars. The current findings will aid in selecting the most tissue-culture-responsive wheat cultivars for further genetic transformation against various biotic and abiotic stressors and improve wheat crop agronomic traits.

References

1. Ahmad F. Food security in Pakistan. Pak J Agri Sci. 2009;46(2):83-9.
2. Awika JM. Major cereal grains production and use around the world. Advances in cereal science: implications to food processing and health promotion. 2011;1089:1-13.
3. Batool A, Aleem S, Nawaz A, Khan MI, Arshad W, Aslam M, et al. Evaluating the Impact of Variable Seed Rates on Growth, Productivity and Yield attributes of different Wheat (Triticum aestivum L.) Genotypes of Barani Areas. Pakistan Journal of Agricultural Research. 2022;35(230).
4. Ahmad I, Akhtar MJ, Zahir ZA, Jamil A. Effect of cadmium on seed germination and seedling growth of four wheat (Triticum aestivum L.) cultivars. Pak J Bot. 2012;44(5):1569-74.
5. Mendoza MG, Kaeppler HF. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (Triticum aestivum L.). In Vitro Cellular & Developmental Biology-Plant. 2002;38(1):39-45.
6. Rashid H, Khan MH, Chaudhry Z, Bano R, Raja NI. An improved Agrobacterium mediated transformation system in wheat. Pakistan Journal of Botany. 2012;44(1):297-300.
7. Malik SI, Rashid H, Yasmin T, Minhas NM. Effect of 2, 4-dichlorophenoxyacetic acid on callus induction from mature wheat (Triticum aestivum L.) seeds. Int J Agric Biol. 2003;6:156-9.
8. Yu Y, Wang J, Zhu ML, Wei ZM. Optimization of mature embryo-based high frequency callus induction and plant regeneration from elite wheat cultivars grown in China. Plant breeding. 2008;127(3):249-55.
9. Noor S, Ali GM, Rashid U, Arshad M, Ali S, Zafar Y. Optimization of callus induction and regeneration system for Pakistani wheat cultivars Kohsar and Khyber-87. African Journal of Biotechnology. 2009;8(20).
10. Yasmin S, Khan IA, Khatri A, Seema N, Nizamani GS, Arain MA. In vitro plant regeneration in bread wheat (Triticum aestivum L.). Pak J Bot. 2009;41(6):2869-76.
11. Vendruscolo ECG, Schuster I, Negra ES, Scapim CA. Callus induction and plant regeneration by Brazilian new elite wheat genotypes. Crop Breeding and Applied Biotechnology. 2008;8(3).
12. Iqbal M, Asif S, Ilyas N, Raja NI, Hussain M, Shabir S, et al. Effect of plant derived smoke on germination and post germination expression of wheat (Triticum aestivum L.). American Journal of Plant Sciences. 2016;7(6):806-13.
13. Satyavathi V, Jauhar P, Elias E, Rao M. Effects of growth regulators on in vitro plant regeneration in durum wheat. Crop science. 2004;44(5):1839-46.
14. Rashid U, Ali S, Ali GM, Ayub N, Masood MS. Establishment of an efficient callus induction and plant regeneration system in Pakistani wheat (Triticum aestivum) cultivars. Electronic Journal of Biotechnology. 2009;12(3):4-5.

15. Özgen M, Türet M, Avci M. Cytoplasmic effects on the tissue culture response of callus from winter wheat mature embryos. Plant cell, tissue and organ culture. 2001;64(1):81-4.
16. He D, Tanner G, Scott K. Somatic embryogenesis and morphogenesis in callus derived from the epiblast of immature embryos of wheat (Triticum aestivum). Plant Science. 1986;45(2):119-24.
17. Mathias R, Fukui K, Law C. Cytoplasmic effects on the tissue culture response of wheat (Triticum aestivum) callus. Theoretical and applied genetics. 1986;72(1):70-5.
18. Poddar S. WHEAT BREEDING USING TISSUE CULTURE TECHNOLOGY.
19. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum. 1962;15(3):473-97.
20. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO journal. 1987;6(13):3901-7.
21. Islam F, Xie Y, Farooq MA, Wang J, Yang C, Gill RA, et al. Salinity reduces 2, 4-D efficacy in Echinochloa crusgalli by affecting redox balance, nutrient acquisition, and hormonal regulation. Protoplasma. 2018;255(3):785-802.
22. Shah M, Khalid Q, Khan U, Shah S, Shah S, Hassan A, et al. Variation in genotypic responses and biochemical analysis of callus induction in cultivated wheat. Genet Mol Res. 2009;8(3):783-93.
23. Farooq M. Comparative Tissue Culture Response of Wheat Cultivars and Evaluation of Regenerated Plants Muhammad Farooq," Hamid Rashid," Ihsanullah, Zubeda Chaudhry and Khan Bahadar Marwat. Pakistan Journal of Biological Sciences. 2004;7(3):406-8.
24. Alizadeh H, Naghavi M, Omidi M, Saatian B, editors. Effect of plant growth regulators on direct shoot regeneration of wheat (Triticum aestivum). New direction for a diverse planet: Proceeding of the 4th International Crop Congress Brisbane, Australia; 2004.
25. Raja NI, Bano A, Rashid H, Khan MH, Chaudhry Z. Effect of age of embryogenic callus on plant regeneration in local cultivars of wheat (Triticum aestivum L.). Pak J Bot. 2009;41(6):2801-6.
26. Sarker R, Biswas A. In vitro plantlet regeneration and Agrobacterium-mediated genetic transformation of wheat (Triticum aestivum L.). Plant Tissue Cult. 2002;12(2):155-65.

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