DOI: https://doi.org/10.54393/fbt.v6i2.244

FUTURISTIC BIOTECHNOLOGY
https://fbtjournal.com/index.php/fbt
ISSN (E): 2959-0981, (P): 2959-0973
Vol 06 Issue 02, (April-June, 2026)

Original Article

OPEN ACCESS

Sodium Bicarbonate and Hydrogen Peroxide Irrigation Improves (Ex Vitro) Survival of Agrobacterium-Transformed Wheat. Plantlets: A Comparative Assessment of Post-Regeneration Interventions

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ARTICLE INFO

Keywords:
Triticum aestivum, Agrobacterium-Mediated Transformation, Ex Vitro Acclimatization, Sodium Bicarbonate, Hydrogen Peroxide

How to Cite:
Arshad, M. T. (2026). Sodium Bicarbonate and Hydrogen Peroxide Irrigation Improves (Ex Vitro) Survival of Agrobacterium-Transformed Wheat. Plantlets: A Comparative Assessment of Post-Regeneration Interventions: Na_2CO_3 Plus H_2O_2 Irrigation for Survival of Agrobacterium-Transformed Wheat. Futuristic Biotechnology, 6(2), 39-43. https://doi.org/10.54393/fbt.v6i2.244

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Received Date: 9th May, 2026
1st Revision Received: 17th May, 2026
2nd Revision Received: 18th June, 2026
Acceptance Date: 25th June, 2026
Published Date: 30th June, 2026

ABSTRACT

Low ex vitro survival rates of transgenic wheat plantlets following transfer from tissue culture to soil represent a major constraint limiting the efficiency of genetic transformation pipelines, with losses often exceeding 70-90%. **Objectives:** To compare the effectiveness of four simple, low-cost post-regeneration interventions PPM supplementation, photo-acclimatization, modified root-transfer technique, and irrigation with sodium bicarbonate Na_2CO_3 + H_2O_2 in improving the ex-vitro survival of Agrobacterium-transformed Borlaug-16 wheat plantlets, and to identify the most practical protocol modification for routine transformation workflows. **Methods:** Fifty putative transgenic wheat plantlets (*Triticum aestivum L.* cv. Borlaug-16), generated via Agrobacterium tumefaciens (strain AGL1)-mediated transformation of immature embryo-derived calli with a Bar gene selection cassette, were allocated to four treatment groups (n=10 each) and one untreated control (n=10). Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, and PCR analysis targeting the ubiquitin promoter–Bar gene junction confirmed transgene integration in all surviving plantlets. **Results:** The Na_2CO_3 + H_2O_2 irrigation treatment achieved the highest survival rate at 30%, representing a 20-percentage-point improvement over the untreated control (10%). In contrast, PPM supplementation showed no improvement (10%), while both photo-acclimatization and modified root-transfer resulted in 0% survival. PCR analysis confirmed the presence of the expected ~1 kb ubiquitin–Bar amplicon in all surviving plantlets. **Conclusions:** Supplementing irrigation water with Na_2CO_3 and H_2O_2 provides an inexpensive and effective strategy for ex-vitro survival of transgenic wheat plantlets, whereas PPM, photo-acclimatization, and tissue-wrapped root transfer offered no advantage under the conditions tested.

INTRODUCTION

Wheat (*Triticum aestivum L.*) serves as a staple food crop for over 2.5 billion people globally, providing approximately 20% of the world's dietary calories and protein [1]. In South Asia, particularly Pakistan, wheat contributes to nearly 62% of the national caloric intake, with the country ranking among the top ten wheat-producing nations [2]. However, wheat production in the region faces mounting challenges, including water scarcity, soil degradation, climate change-induced heat stress, and increasing urbanization of agricultural lands [3, 4]. These constraints necessitate the development of improved wheat varieties with enhanced stress tolerance, disease resistance, and nutritional quality [5]. Genetic transformation has emerged as a powerful tool for introducing desirable traits into wheat, complementing conventional breeding approaches [6]. Transgenic wheat lines with improved drought tolerance [7], enhanced salt tolerance [8], elevated provitamin A content [9], and fungal disease resistance [10] have been successfully developed. Additionally, CRISPR-Cas9-mediated genome editing has enabled precise trait modification in wheat, including the development of powdery mildew-resistant varieties [11] and lines with

DOI: https://doi.org/10.54393/fbt.v6i2.244

reduced immunogenic gluten content [12]. Despite these advances, the commercial deployment of transgenic wheat remains limited, partly due to challenges in the transformation and regeneration pipeline [13]. Plant tissue culture remains the cornerstone of wheat transformation, enabling the propagation of transgenic lines and recovery of transformed cells [14, 15]. However, the process is constrained by several factors, including microbial contamination of culture media, suboptimal regeneration frequencies, and most critically, the poor survival of plantlets during the transition from in vitro conditions to ex vitro environments [16]. This transition, known as acclimatization, subjects tissue-cultured plantlets to higher irradiance, lower relative humidity, and unsterile substrates—conditions to which they are poorly adapted [17]. The resulting physiological shock often leads to wilting, desiccation, and mortality rates that can exceed 70-90% [18]. This bottleneck substantially reduces the throughput of transformation programs, as the majority of confirmed transgenic events at the tissue culture stage fail to produce viable plants for agronomic evaluation [19]. Various strategies have been explored to improve ex vitro survival of micropropagated plants. These include the use of antimicrobial agents such as Plant Preservative Mixture (PPM) to control contamination [20], gradual light acclimatization to enhance photoautotrophic competence [21], and modified transplantation techniques to reduce root disturbance [22]. More recently, irrigation with hydrogen peroxide (H_2O_2) has been shown to improve root oxygenation and stimulate defense responses against pathogens [23], while sodium bicarbonate (Na_2CO_3 has demonstrated antifungal properties in horticultural systems [24].

However, these interventions have not been systematically compared in a single transformed wheat system, nor have they been evaluated in combination with antifungal and oxygenating irrigation strategies. Therefore, this study aimed to compare the effectiveness of four post-regeneration interventions- PPM supplementation, photo-acclimatization, modified root-transfer, and Na_2CO_3+ H_2O_2 irrigation- on the ex-vitro survival of Agrobacterium-transformed Borlaug-16 wheat plantlets. To identify a simple, cost-effective protocol modification that can be readily integrated into existing wheat transformation pipelines to improve overall regeneration efficiency. To molecularly confirm the transgenic status of surviving plantlets through PCR analysis to ensure the observed survival differences were not due to non-transgenic escapes.

METHODS

This experimental study was conducted at the Department of Biotechnology, Forman Christian College, University, Lahore, Pakistan. The study duration was from December 2022 to May 2023. Immature embryos (15-20 days post-anthesis) were excised from surface-sterilized developing seeds of wheat cultivar Borlaug-16 and cultured on Murashige and Skoog (MS) callus-induction medium (2,4-dichlorophenoxyacetic acid 2 mg/L) in darkness at 24°C for 2-3 weeks following the protocol of Abid *et al.* [18]. Three-week-old embryogenic calli were transformed with Agrobacterium tumefaciens strain AGL1 carrying the binary vector pSB219, containing the selectable marker Bar gene (conferring BASTA resistance) under the control of the ubiquitin promoter, using the standard protocol of the host laboratory [18]. Following co-cultivation (2 days on MS medium with acetosyringone 100 µM), calli were transferred to MS medium with Rocephin (250 mg/L) for Agrobacterium elimination, then to MS regeneration medium supplemented with kinetin (0.5 mg/L) for shoot induction (3 weeks). Regenerated plantlets were selected on MS medium containing BASTA (3 mg/L) for 10 days to eliminate non-transgenic escapes. From approximately 5,000 embryos processed, 50 putatively transgenic plantlets with healthy shoots and roots were selected and randomly allocated to five groups (n=10 each): (1) untreated control; (2) PPM—0.2% (v/v) plant preservative mixture in regeneration medium before transfer; (3) photo-acclimatization—stepwise photoperiod increase (2, 4, 6, 8 hours over 4 days); (4) modified root-transfer—roots wrapped in autoclaved tissue paper saturated with liquid regeneration medium; (5) Na_2CO_3+ H_2O_2—irrigation with 5% (v/v) H_2O_2 at each watering and 5% (w/v) Na_2CO_3 at every fifth watering. All plantlets were transferred to artificial soil (peat moss: cocopeat:humus, 3:1:1) and maintained under polyethylene covers at 24°C with a 16-hour photoperiod, with covers gradually vented over 3 weeks. Survival was assessed 4 weeks after transfer.

Genomic DNA was extracted from leaf tissue of all surviving plantlets using the CTAB method [19]. PCR was performed with primers UbiF2 (5'-...-3') and BastR2 (5'-...-3') targeting the ubiquitin promoter–Bar gene junction under standard conditions (94°C denaturation, 58°C annealing, 72°C extension, 40 cycles) to amplify a ~1 kb fragment. Products were resolved on a 1% agarose gel alongside positive (plasmid DNA) and negative (non-transformed wheat DNA) controls and a 1 kb DNA ladder.

RESULTS

Overall survival of the 50 putative transgenic Borlaug-16 plantlets transferred to artificial soil varied significantly among the treatment groups. These results showed that the photo-acclimatization and modified root-transfer groups did not have any plantlets after the treatment, while

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the Na_2CO_3+ H_2O_2 group had a 30% survival rate, a 20% improvement over the control group (Table 1).

Table 1: Survival Rate of Putative Transgenic Wheat Plantlets Four Weeks After Transfer to Artificial Soil, by Treatment Group

Treatment Group	n (plantlets)	Survivors (n)	Survival Rate (%)	Change vs. Control
Control (No Treatment)	10	1	10	—
PPM (0.2% v/v)	10	1	10	No change
Photo-Acclimatization	10	0	0	-10 pp
Modified Root-Transfer	10	0	0	-10 pp
Na_2CO_3+ H_2O_2 (5% v/v each)	10	3	30	+20 pp

Interpretation: There was no increase in survival in groups receiving antimicrobial supplementation, with 0% survival in the abrupt light or root-handling stress groups, and a survival advantage in the groups receiving the combination of antifungal/oxygenating irrigation compared to the standard irrigation group. Plantlets in the Na_2CO_3 + H_2O_2 group exhibited visibly improved root establishment and shoot growth compared with the control and PPM groups. Scale bars = 2 cm (Figure 1).

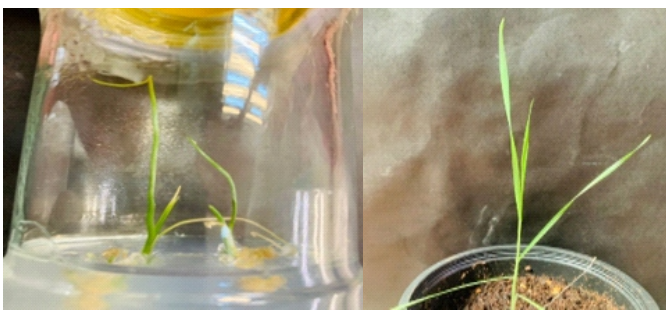

Figure 1: Ex Vitro Survival of Transgenic Wheat Plantlets Following Different Post-Regeneration Interventions

(A) Representative plantlets during BASTA (3 mg/L) selection on regeneration medium before transfer to artificial soil. Only plantlets carrying the Bar gene remained healthy. (B) Representative surviving plantlets four weeks after transfer to artificial soil: (i) untreated control (10% survival), (ii) PPM treatment (10% survival), and (iii) Na_2CO_3+ H_2O_2 irrigation (30% survival). Photo-acclimatization and modified root-transfer groups exhibited 0% survival and are not shown. The presence of the expected ~1 kb amplicon in all lanes confirms the transgenic status of every surviving plantlet, ruling out the possibility of BASTA escapees (Figure 2).

Figure 2: PCR Confirmation of Transgene Integration in Surviving Plantlets

Genomic DNA was extracted from leaf tissue of all surviving plantlets and amplified using primers targeting the ubiquitin promoter–Bar gene junction (~1 kb fragment). Lanes: M, 1 kb DNA ladder; P, positive control (pSB219 plasmid); N, negative control (non-transformed wheat); lane 1, control survivor; lane 2, PPM survivor; lanes 3–5, Na_2CO_3+ H_2O_2 survivors.

DISCUSSION

The low plantlet survival rates from in vitro to ex vitro conditions are a major hurdle in genetic transformation pipelines, but the losses due to transfer of the tissue culture to the soil have not been a significant problem [7, 16]. In the present study, four simple, low-cost interventions of low cost were systematically compared to improve ex vitro survival, and irrigation with Na_2CO_3+ H_2O_2 proved to be most effective, producing a 20-percentage-point higher survival rate (30%). The higher mortality rate of PPM supplementation (10% survival) compared to the control does not indicate that microbial contamination was a significant cause of mortality in this system. Physiological stress of the ex-vitro transition (such as different light intensity, less humidity, or root disturbance) has been reported to cause a loss of microbial contamination in Murashige and Skoog media at 0.25 mL/L or less [20]; however, the present data suggest that these stresses may be the primary source of loss. This has been confirmed by recent research on cereal transformation, which has revealed that most of the plantlet mortality is due to acclimatization stress and not to contamination [7, 16]. Likewise, the lack of an increase in survival rate after photoacclimatization (0%) does not agree with other species in which progressive exposure to light increased photosynthetic capacity. The current results indicate that adding stepwise light exposure to the stresses imposed by the BASTA selection procedure and root disturbance may surpass the wheat plantlet's adaptive capacity and that this strategy was beneficial in other micropropagation systems [21]. It has been recently reported that there is a need to optimize light intensity, CO_2 concentration, and nutrient availability for successful photoautotrophic micropropagation, which were not optimized in the present protocol [21]. The complete failure of the modified root

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transfer technique (0% survival) was unexpected since similar techniques were found to be beneficial in other ornamental species. The tissue wrap probably kept too much moisture around the roots, which would have allowed the fungus to grow and would have caused a lack of oxygenation at the roots, but not acclimatization. Recently, it has been shown that root health is an important factor in ex vitro survival; hypoxic conditions cause rapid deterioration of roots and plant death [16]. A discussion of the superior performance of the irrigation treatment using Na_2CO_3+ H_2O_2 is warranted (30% survival). Hydrogen peroxide has been demonstrated to influence a variety of physiological functions in plants, including cell division, germination, resistance to fungal entry, and as a source of molecular oxygen in the root tissue [22]. Furthermore, H_2O_2 is a signaling molecule in plant defense responses, inducing systemic acquired resistance, an anti-pathogen response [22]. Sodium bicarbonate has been reported to be fungicidal against spore germination and mycelial growth of fungal pathogens in horticultural crops [20]. These two agents most probably acted synergistically, with H_2O_2 enhancing root oxygenation and triggering defense reactions, and Na_2CO_3 inhibiting root-zone fungal pathogens. This appears to be an interpretation in line with recent studies on cereal systems in which both oxidative and alkaline treatments were found to enhance the establishment of seedlings [22]. All surviving plantlets must be molecularly confirmed as transgenic. All 5 survivors contained evidence of the ~1 kb ubiquitin–Bar amplicon, thus demonstrating that the survival differences seen were not the result of non-transgenic escapes from BASTA selection and therefore validating the transformation efficiency [7]. Although the 30% survival rate with irrigation of Na_2CO_3+ H_2O_2 is high, it is not sufficient to achieve scale transformation pipelines with a survival rate greater than 70%. The number of plants per treatment (10) is rather small for statistical power, and the single wheat genotype (Borlaug 16) may not be representative of other wheat varieties that vary in regeneration ability [16]. In addition, the concentrations of Na_2CO_3 (5% w/v) and H_2O_2 (5% v/v) used in this study were determined from preliminary trials and may not be optimal. Concentrations may be phytotoxic at higher concentrations and may be sub-optimal for fungal suppression at lower concentrations. Studies conducted recently have shown that the best concentration of H_2O_2 to use for root oxygenation in cereal crops is 0.5–3% (v/v) [22], depending on the species and growth stage. Likewise, soil-borne pathogens have been controlled at 1-3% (w/v) Na_2CO_3 levels without affecting plant growth [20].

The current study has some limitations to be noted. There are two limitations with the results: firstly, the small size of the sample and its single genotype restrict the generalizability of the results and, secondly, the results are not directly applicable to other populations. Second, this study lacked detailed physiological measurements (such as photosynthetic efficiency, stomatal conductance, root viability assays) to explain the survival differences seen. Third, molecular characterization only involved PCR confirmation of the transgene and did not involve either assessing transgene expression level or copy number. Future studies may involve quantitative PCR (qPCR) to determine the copy number of the transgenes and phenotypic characterization of regenerated plants to confirm that the plants have the desired traits.

CONCLUSION

In conclusion, the present findings demonstrate that Na_2CO_3+ H_2O_2 irrigation is a simple, low-cost strategy for improving *ex vitro* survival of transgenic wheat plantlets, whereas PPM, photo-acclimatization, and modified root transfer confer no benefit under the tested conditions. These results have practical implications for wheat transformation pipelines and provide a foundation for further optimization studies aimed at achieving higher survival rates.

Author's Contribution

Conceptualization: MTA
Methodology: MTA
Formal analysis: MTA
Writing and Drafting: MTA
Review and Editing: MTA

The author approved the final manuscript and take responsibility for the integrity of the work

Conflicts of Interest

The author declare no conflict of interest.

Source of Funding

The author received no financial support for the research, authorship and/or publication of this article.

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