Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2025.059315

ARTICLE

Impact of Seed Halopriming on Germination, Morphological Traits, and *Cry1Ac* Gene Expression in *Bt* Cotton (*Gossypium hirsutum*)

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Received: 04 October 2024 Accepted: 12 December 2024 Published: 24 January 2025

ABSTRACT

Seed priming is an effective seed pretreatment technology that enhances germination and overall crop performance by optimizing seed hydration and metabolic processes before planting. Seed quality is a critical determinant of cotton (*Gossypium hirsutum*) crop performance, influencing germination, plant vigor, and yield. This study evaluates the effects of seed priming with potassium salts (1% and 2% KCl and K_2SO_4) on germination, morphological traits, and *Cry1Ac* gene expression in three *Bt* cotton cultivars (IUB-2013, NIAB-878B, FH-142) as *Cry1Ac* enhance the pest resistance in *Bt* cotton and reduce the plant's dependence on chemical insecticides. Seeds were primed for six hours, air-dried, and sown in the field. Germination rates, plant height, number of bolls per plant, boll weight, seed cotton yield, and ginning outturn (GOT) were assessed at crop maturity. *Cry1Ac* gene expression was quantified to explore the influence of priming treatments on transgene activity. Results demonstrated that 1% K_2SO_4 priming significantly enhanced germination and yield-related traits, with *Cry1Ac* expression peaking in the IUB-2013 cultivar under 1% K_2SO_4 treatment. These findings suggest that potassium-based halopriming improves cotton seedling establishment and *Bt* gene expression. This study addresses the critical gaps in understanding the effects of seed halopriming on morphological traits, germination, and expression of the *Cry1Ac* gene in *Bt* cotton while providing a novel eco-friendly and cost-effective halopriming approach, offering the potential to improve cotton production.

KEYWORDS

Cotton; seed priming; halopriming; *Cry1Ac*; *Bt* cotton; germination; yield traits

1 Introduction

Cotton (*Gossypium hirsutum*) is the most widely cultivated natural fiber crop and ranks third among global oilseed crops. With an estimated annual production of 25 million tons, cotton is critical to agricultural economies and plays a pivotal role in the textile industry [1–3]. Projections suggest that global cotton production will increase by 1.5% annually, reaching approximately 30 million tons by 2029 [4]. Cotton significantly contributes to the economic development of many nations, especially in countries like Pakistan, where it accounts for 50% of industrial employment and over 60% of agricultural exports [5]. It is primarily cultivated in tropical and subtropical regions across the globe. The leading cotton-producing countries include China, India, the United States, Brazil, Pakistan, Australia, Turkey, and Uzbekistan [6]. Meeting the rising industrial demand and improving the economic conditions of cotton farmers require expanding cotton cultivation into new areas and enhancing crop productivity [7]. Growth in cotton production is expected to stem from higher global yields and the expansion of cultivation areas. However, achieving sustainable yield growth remains challenging due to climate change, poor seed quality, genetic limitations, and pest pressures, including whitefly infestations, cotton leaf curl virus (CLCuV), and pink bollworm attacks [5,8]. Addressing these challenges necessitates the development of resilient genetic varieties and the adoption of improved agronomic practices to secure the future of cotton production.

Biotic and abiotic stressors substantially threaten cotton productivity [9], leading to the development of transgenic cotton varieties like *Bt* cotton, which express *Cry1Ac* genes from *Bacillus thuringiensis* [10]. The *Cry1Ac* gene encodes an endotoxin toxic to lepidopteran pests such as cotton bollworms [11]. In *Bt* cotton, constitutive plant promoters regulate *Cry1Ac* gene expression, ensuring endotoxin production throughout the plant life cycle. However, variations in gene expression can occur due to environmental factors, genetic backgrounds, and plant developmental stages [12]. Agronomic practices, including nutrient management and seed priming, have been reported to influence *Cry1Ac* gene expression and pest resistance [13]. These findings underscore the need for targeted strategies to maximize the benefits of *Bt* cotton in diverse agricultural systems.

Bt cotton offers several advantages, such as reduced pesticide use, enhanced pest resistance, and improved yield potential [14]. It has demonstrated high efficacy against bollworms, with insecticidal efficiency higher in leaf tissues, particularly in younger leaves [15]. However, poor access to high-quality seeds remains a significant challenge, negatively affecting germination, crop establishment, and yield potential [16]. Enhancing seed quality and availability is therefore essential to optimize *Bt* cotton performance and ensure sustainable production.

Seed priming, a low-cost pre-sowing technique, has shown potential in improving seed germination and seedling vigor under stress conditions. By enabling controlled hydration, seed priming activates pre-germinative metabolic processes without initiating germination, enhancing metabolic activity and nutrient uptake [17]. This technique is effective in improving tolerance to biotic and abiotic stresses [18], promoting uniform germination [19], and increasing water-use efficiency [20]. Halopriming, which involves treating seeds with salt solutions, has been found to enhance chilling tolerance, improve germination indices, and boost seedling vigor [21]. Potassium-based priming, such as with KNO_3, has demonstrated significant improvements in germination rates, growth, and hormonal responses in cotton [22,23].

Furthermore, seed priming has been linked to gene expression changes influencing germination and stress responses. For example, priming can modulate the balance of plant hormones like gibberellins (GA) and abscisic acid (ABA) [24] and enhance the expression of stress-related genes [25]. Studies in other crops have shown that priming alters the expression of over 1300 genes, affecting key metabolic events such as DNA replication [26] and protein synthesis [27]. However, the specific impact of priming on transgene expression, particularly *Cry1Ac*, in *Bt* cotton remains underexplored. The present study aims to evaluate the effects of potassium-based halopriming on germination, morphological traits, and *Cry1Ac* gene expression in three *Bt* cotton cultivars (*IUB-2013, NIAB-878B, FH-142*). By investigating how stress mitigation techniques like halopriming enhance seedling establishment and *Cry1Ac* gene expression, this research seeks to develop sustainable strategies for improving *Bt* cotton performance in stress-prone environments. These findings will contribute to optimizing *Bt* cotton cultivation across diverse agroclimatic conditions, promoting resilient and sustainable cotton production.

2 Methodology

2.1 Plant Samples

In this study, seeds from three cotton (*Gossypium hirsutum*) varieties, FH-142, NIAB-878B, and IUB-2013 were obtained from the Crop Sciences Institute (CSI), National Agriculture Research Council (NARC), Islamabad, Pakistan, during the 2022–2023 growing season.

2.2 Cotton Seed Priming Treatments

Seeds from each cotton variety were subjected to halopriming treatments. The seeds were immersed for six hours in priming solutions of 1% and 2% concentrations of potassium chloride (KCl) and potassium sulfate (K_2SO_4). The temperature of the priming solution was maintained at room temperature (approximately 25 ± 1°C) to avoid thermal stress and the pH of the priming solution was maintained near neutral (approximately 6.5–7) to avoid Ph-induced stress. After the priming treatment, seeds were air-dried on filter paper to remove excess moisture. Once completely dried, the seeds were prepared for sowing under field conditions.

2.3 Sowing of Primed Seeds

Field plots were prepared and the primed seeds were manually sown in field tunnels using the dibbing method for precise seed placement. The experiment followed a randomized complete block design (RCBD), with three replicates per treatment. Uniform agronomic practices, including fertilizer application and plant protection measures, were applied to all treatments. Seeds treated with the priming solutions and control seeds treated with normal water were sown for comparative analysis.

2.4 Data Collection of Morphological and Yield-Related Traits

Germination data (%) for all treatments were recorded on the 15–20 days after sowing. At maturity, morphological parameters were assessed for both primed and control plants of the cotton cultivars. From control and each seed primed plant, randomly selected the five full-length cotton plants to measure the plant height (cm), internode distance (cm), number of bolls per plant, boll weight (g), seeds per boll, ginning outturn (%), and yield (kg/acre).

2.5 Expression Analysis of Bt Gene

Quantification of Cry1Ac protein expression in *Bt* cotton plants was performed using a sandwich ELISA kit. A 0.05 g leaf sample was homogenized in 0.5 mL of sample extraction buffer and mixed for 5 min. The extract was centrifuged at 4000 rpm for 3 min, and 100 μL of the supernatant was added to ELISA wells. Samples were incubated in the dark for 45 min, after which the wells were washed 4–5 times with washing buffer. After washing, 100 μL of enzyme solution was added to each well and incubated in the dark for 30 min. The washing step was repeated, and 100 μL of chromogenic substrate was added. Samples were incubated for 15 min at room temperature in the dark. To quantify Bt protein expression, 100 μL of stop buffer was added to each well to terminate the enzymatic reaction. Absorbance (O.D.) was then measured at 450 nm using a microplate reader, following the protocol provided by Envirologix Inc., USA.

2.6 Data Analysis

Data were recorded in triplicate for each treatment and control group, and mean values were subjected to analysis of variance (ANOVA) using Statistix 8.1 software. The experiment followed a randomized complete block design (RCBD). ANOVA was performed on all morphological parameters and *Cry1Ac* gene expression data to assess the effects of individual treatments and cotton varieties. A two-way factorial ANOVA was conducted to analyze treatment-by-variety interactions.

3 Results

3.1 Statistical Analysis of Morphological Traits

Significant differences among treatments were observed when compared to the control group ($p < 0.01$). The impact of halopriming on the morphological traits was robust, as demonstrated by the two-factor ANOVA (Table 1).

Table 1: Analysis of variance table for overall morphological parameters

Source	DF	Germination	PH	ID	NBPP	BW	NSPB	Yield	GOT
Replication	2								
Treatment	4	35.96**	47.43**	27.48**	39.98**	19.96**	18.71**	54.66**	14.26**
Variety	2	35.39**	3.94*	0.15^{NS}	32.61**	2.14^{NS}	4.74*	25.31**	6.61**
Treatment* variety	8	3.45**	12.95**	2.71*	1.61^{NS}	2.07*	5.14**	2.20*	2.88*
Error	28								
Total	44								

Notes: DF = Degrees of freedom; PH = Plant height; ID = Internode distance; NBPP = Number of bolls per plant; BW = Boll weight; NSPB = Number of seeds per boll; GOT = Ginning outturn. *Significant at 5%; **Significant at 1%; NS = Non-significant.

3.2 Effect of Halopriming on Germination

Germination rates were significantly influenced by halopriming treatments. The cultivar FH-142 exhibited the highest germination percentage at 82.6% under the 1% K_2SO_4 treatment, followed by NIAB-878 at 80%. Conversely, the cultivar IUB-2013 had the lowest germination percent (60%) with the same treatment (Fig. 1). The results indicated significant differences in germination percentages both between treatments and among cultivars ($p < 0.01$).

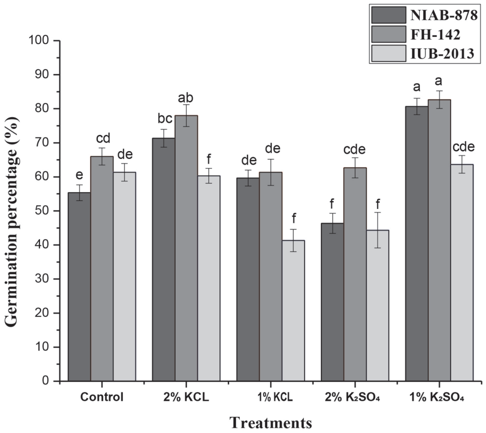

Figure 1: Effect of seed priming on cotton germination and selected morphological parameters in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). The mean values for each parameter are shown with error bars representing the standard error of the mean (SEM). Bars marked with the same letter indicate no significant difference between treatments according to Tukey's test at the 5% significance level ($p < 0.05$)

3.3 Effect of Halo-Priming on Morphological Data and Number of Bolls per Plant

The mean plant height for all treatments and varieties was assessed in comparison to the control. Results indicated that the variety NIAB-878 exhibited the highest plant height across all treatments. Specifically, 2% KCl and 1% K_2SO_4 treatments resulted in the greatest plant heights of 167 and 166 cm, respectively, in the NIAB-878 cultivar. In contrast, the other two varieties, FH-142 and IUB-2013, displayed shorter plant heights compared to NIAB-878. Overall, all cultivars performed best under the 1% K_2SO_4 treatment, followed closely by 2% KCl, compared to the other treatments (Fig. 2A). The number of bolls is directly correlated with yield. In this study, after calculating the mean number of bolls per plant for all treatments and the control, results indicated that the 1% K_2SO_4 treatment produced the highest number of bolls per plant across all varieties. Specifically, NIAB-878 had the greatest number of bolls, reaching 78 at 1% K_2SO_4. However, under all other priming conditions, FH-142 exhibited the highest number of bolls. The effect of cultivar on boll number was significant at the 1% level ($p < 0.01$). A significant interaction was observed between treatment and variety in terms of boll number per plant (Fig. 2B).

3.4 Number of Seeds per Boll and Boll Weight

The number of seeds per boll was highest in NIAB-878 under the 1% K_2SO_4 treatment, with a mean of 38 seeds per boll, while the lowest number (25 seeds) was observed at 2% K_2SO_4 compared to the control and KCl treatments. Similarly, variety FH-142 showed the highest number of seeds per boll at 1% K_2SO_4 and the lowest at 1% KCl. The cultivar IUB-2013 consistently produced fewer seeds per boll. Statistical analysis revealed significant differences between varieties at the 5% probability level ($p < 0.05$). Additionally, there was a highly significant treatment × variety interaction effect ($p < 0.01$) on the number of seeds per boll, as illustrated in Fig. 3A. The boll weight for variety NIAB-878 was highest with 2% KCl and 1% K_2SO_4 treatments, recording weights of 5.2 and 4.8 g, respectively, compared to the control. For variety NIAB-878, the 1% K_2SO_4 treatment resulted in the highest boll weight of 5.2 g relative to the FH-142 and IUB-2013 with 5 g boll weight. The lowest boll weight was at 2% K_2SO_4 in variety NIAB-878 with 3.7 g. Varieties IUB-2013 and NIAB-878 exhibited similar boll weights across all primed treatments. Results indicated that the individual effects of varieties on boll weight were not significant; however, the interaction between variety and treatment showed highly significant differences at the 5% probability level ($p < 0.05$), as depicted in Fig. 3B.

3.5 Internode Distance

In this study, cotton cultivars NIAB-878, FH-142, and IUB-2013 exhibited the lowest internode distances of 3, 3, and 2.7 cm, respectively, under the 1% K_2SO_4 treatment. Both the 1% K_2SO_4 and 2% KCl treatments resulted in significantly lower mean internode distances compared to the other treatments. Conversely, the 2% K_2SO_4 treatments produced the highest internode distances of 4.6 cm for variety IUB-2013 in comparison to all other varieties and treatments. The lowest internode distance of 2.7 g was seen for 1% K_2SO_4 treatment in IUB-2013 variety. While most internode distance measurements were non-significant, the interaction between variety and treatment revealed significant differences at the 5% LSD level, as depicted in Fig. 4.

3.6 Seed Cotton Yield (SCY) and Ginning Outturn (OUT)

Halopriming exhibited promising effects on seed cotton yield. The results indicated that the 1% K_2SO_4 treatment yielded the highest results in the NIAB-878 cultivar, with a yield of 920 kg/acre, surpassing all other treatments and cultivars. This was followed by FH-142, which achieved yields of 880 kg/acre under 1% K_2SO_4 and 810 kg/acre under 2% KCl. In contrast, IUB-2013 recorded the lowest yield under the 1% KCl treatment. Statistical analysis revealed significant differences in the interaction between priming and variety, with yield differences being significant at the 1% level ($p < 0.01$). Additionally, seed cotton yield exhibited significant differences at the 5% LSD level for the variety × treatment interaction, as shown in Table 2.

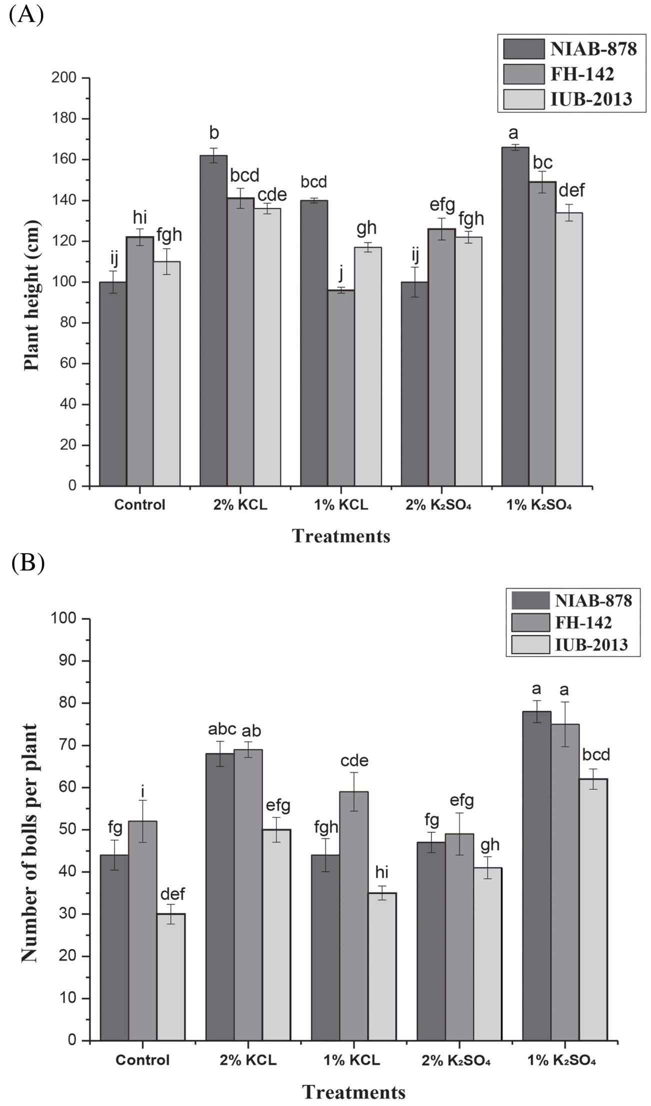

Figure 2: Effect of seed priming on plant height (A) and number of bolls per plant (B) in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). Panel A shows the mean plant height for each cultivar under different priming treatments, with NIAB-878 exhibiting the maximum height, particularly under 2% KCl and 1% K_2SO_4 treatments. Panel B displays the number of bolls per plant, with 1% K_2SO_4 resulting in the highest boll count across all cultivars. NIAB-878 recorded the greatest number of bolls at 1% K_2SO_4, while FH-142 outperformed under other priming conditions. Error bars represent the standard error of the mean (SEM), and bars with the same letter indicate no significant difference ($p < 0.05$)

The results of the mean ginning outturn (GOT) percentage indicated that the cultivar NIAB-878 achieved the highest GOT values, recording 40.4% and 39.6% with 2% KCl and 1% K_2SO_4 treatments, respectively, compared to the other varieties, treatments, and control. Conversely, the treatments with 1% KCl and 2% K_2SO_4 resulted in lower GOT percentages. The cultivar FH-142 showed no significant variation in GOT across all treatments. The minimum GOT was observed in

IUB-2013 under the 2% K_2SO_4 treatment. Statistical analysis revealed significant differences in GOT among the varieties at the 1% level ($p < 0.01$). Additionally, significant differences in GOT were found at the 5% level for the interaction between variety and treatment, as illustrated in Table 2.

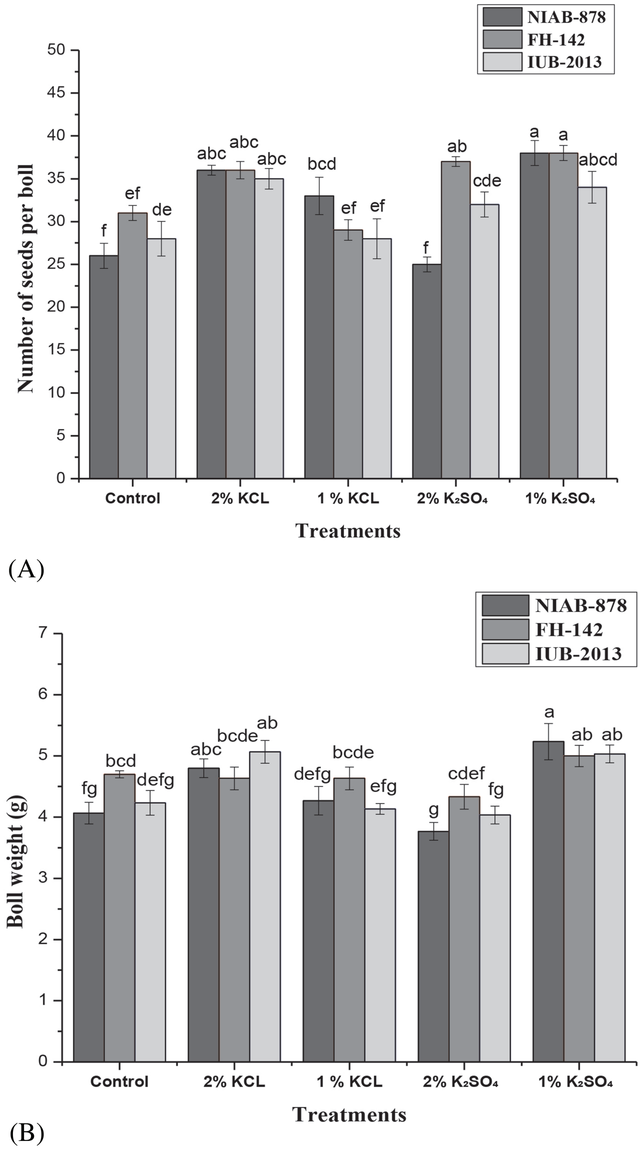

Figure 3: Effect of seed priming on the number of seeds per boll (A) and boll weight (B) in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). Panel A illustrates the mean number of seeds per boll for each cultivar under different priming treatments, with NIAB-878 demonstrating the highest seed count at 1% K_2SO_4. Panel B shows boll weight, where NIAB-878 exhibited maximum weight with 2% KCl and 1% K_2SO_4 treatments. Error bars represent the standard error of the mean (SEM). Bars labeled with the same letter indicate no significant difference between treatments ($p < 0.05$)

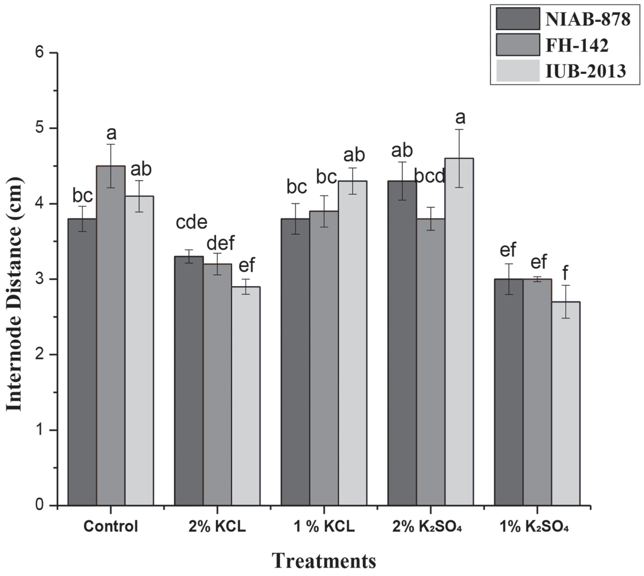

Figure 4: Effect of seed priming on internode distance in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). The graph displays the mean internode distance for each cultivar under different priming treatments, with the 1% K_2SO_4 treatment resulting in the shortest distances. Error bars indicate the standard error of the mean (SEM). Bars labeled with the same letter indicate no significant difference between treatments ($p < 0.05$)

Table 2: Effect of seed priming on seed cotton yield (SCY) and Ginning outturn (GOT) in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). The table illustrates the means of SCY and GOT for each cultivar under various priming treatments, with the maximum yield achieved by NIAB-878 with the 1% K_2SO_4 treatment

Treatment	NIAB-878-SCY (kg/acre)	NIAB-878-GOT (%)	FH-142-SCY (kg/acre)	FH-142-GOT (%)	IUB-2013-SCY (kg/acre)	IUB-2013-GOT (%)
Control	597.3333^{gh}	37.6^{cd}	688.3333^{ef}	688.3333^{cd}	418^{i}	418^{f}
2% KCL	860.3333^{bc}	40.43333^{a}	810.6667^{cd}	810.6667^{cd}	760.3333^{de}	760.3333^{bcd}
1% KCL	602.6667^{gh}	37.03333^{de}	772^{de}	772^{def}	470.6667^{hi}	470.6667^{cd}
2% K_2SO_4	571.6667^{ghi}	36.86667^{def}	694.6667^{fg}	694.6667^{de}	535.6667^{ghi}	535.6667^{ef}
1% K_2SO_4	920.3333^{a}	39.6^{ab}	880.6667^{ab}	880.6667^{bc}	800.6667^{cd}	800.6667^{bc}

3.7 Effect of Halopriming on Bt Gene Expression

Analysis of variance (ANOVA) was conducted to assess the effects of various treatments and cultivars on Bt protein expression, including the interaction between treatment and variety, using Statistix 8.1 software. Significant differences were observed among all treatments, with a high level of significance at the 1% probability level ($p < 0.01$) compared to the control. The results indicate a strong correlation between halopriming treatment and Bt protein levels as determined through two-factorial ANOVA. Additionally, the effect of cultivar on Bt toxin levels was also highly significant. However, no significant differences were found in Bt toxin levels concerning the treatment × variety interaction (Fig. 5).

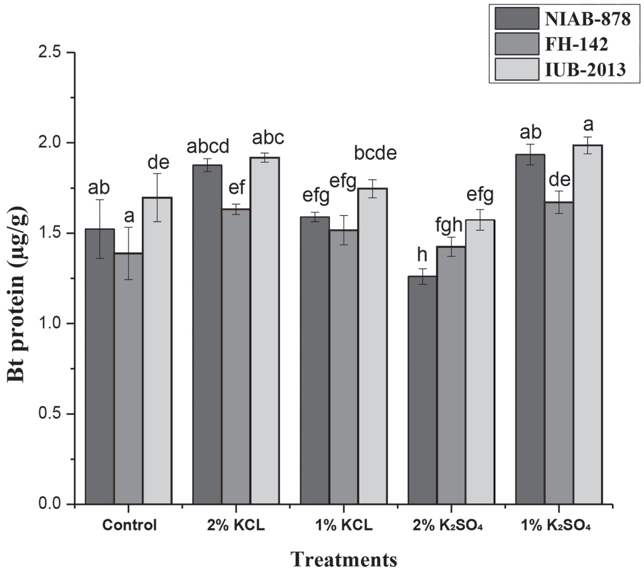

Figure 5: Effect of seed priming on *Bt* gene expression in three cotton cultivars (NIAB-878, FH-142, and IUB-2013). The graph illustrates the mean levels of Bt protein (Cry1Ac) for each cultivar under various priming treatments, highlighting the maximum expression observed with the 1% K_2SO_4 and 2% KCl treatments. Error bars represent the standard error of the mean (SEM). Bars labeled with the same letter indicate no significant difference between treatments ($p < 0.05$)

The cultivar IUB-2013 exhibited the highest level of Bt toxin at 1.9 µg/g with the 1% K_2SO_4 treatment. IUB-2013 and NIAB-878 showed comparable levels of Bt toxin under the 2% KCl treatment. Results of the *Bt* gene (*Cry1Ac*) expression indicate that the 1% K_2SO_4 and 2% KCl treatments resulted in the highest concentrations of Bt protein compared to the control. Conversely, the 1% KCl and 2% K_2SO_4 treatments exhibited lower concentrations of Cry1Ac protein compared to the control. Overall, the data on *Bt* gene expression suggest that the 1% K_2SO_4 and 2% KCl treatments yield the highest Bt protein contents, as illustrated in Fig. 5.

4 Discussion

The global cotton industry faces numerous challenges, with poor seed germination and low seedling vigor being significant constraints on production. Seed priming has emerged as an effective strategy to enhance seedling growth and ultimately increase cotton yield. Previous studies have demonstrated that halopriming, which involves the treatment of seeds with various salts, can significantly improve the rate of seedling emergence and overall seed vigor under stress conditions [16,28]. Potassium (K), an essential macronutrient, plays a pivotal role in osmotic regulation and is integral to the development of cotton plants. The response of crops to priming is highly dependent on the concentration, composition of the priming medium, and exposure duration [29].

In our study, we explored the effects of halopriming on various cotton cultivars and found that treatments with 1% KCl and 2% K_2SO_4 significantly improved germination rates, consistent with findings by Singh et al. [30] and Khalequzzaman et al. [28], where lower concentrations of potassium salts also enhanced germination in other crops such as rice. Moreover, seed priming accelerated germination and improved plant growth, as demonstrated in other studies [31,32]. Our results showed that the 1% K_2SO_4 treatment led to the shortest internode distance, suggesting that potassium salts not only enhance germination but also influence other growth parameters such as plant height, leaf area, and internode distance, which aligns with previous reports on cotton and other crops [19,33,34]. Further, potassium salts significantly impacted yield-related parameters in cotton. Treatments with 1% K_2SO_4 and 2% KCl led to a higher number of bolls, seeds per boll, boll weight, and ginning outturn (GOT) percentage, ultimately improving overall yields. Among the cotton cultivars tested, NIAB-878 showed the highest yield, confirming the positive role of potassium salts in enhancing cotton productivity [33]. The beneficial effects of potassium sulfate may also be attributed to the presence of sulfate ions (SO_4^{2-}), which have been shown to improve plant growth and yield [35].

In addition to growth and yield improvements, our study highlights the potential for seed priming to influence gene expression. Specifically, seed priming with potassium salts enhanced the expression of the *Cry1Ac* gene in *Bt* cotton, which is critical for pest resistance. The introduction of the *Bt* gene has been shown to lead to reduced pesticide usage and increased yields [3,36]. Our findings suggest that halopriming not only improves cotton physiology but may also enhance the expression of key traits, such as the Cry1Ac protein, contributing to better pest resistance and improved yield outcomes. This is consistent with research showing that seed priming can influence gene expression in various crops, improving plant health through enhanced RNA stimulation, protein synthesis, expression at the transcriptional and translational level by various internal and external factors and hormone regulation [37–39]. Moreover, seed priming has been linked to enhanced DNA repair and the stimulation of mRNA and protein synthesis machinery, further supporting its role in improving plant growth and stress resistance [40,41]. Finally, our study underscores the potential of halopriming as a strategy to enhance cotton germination, growth, and yield. The positive effects of potassium salts on cotton physiological traits, combined with the improved expression of pest resistance genes like *Cry1Ac*, offer promising avenues for enhancing cotton productivity while managing pest threats in an environmentally sustainable manner.

5 Conclusion

The application of halopriming, specifically using 1% K_2SO_4 and 2% KCl, on cotton seeds from three cultivars, NIAB-878B, FH-142, and IUB-2013 demonstrated significant positive effects on various morphological traits and *Bt* gene expression. Among the treatments, 1% K_2SO_4 and 2% KCl emerged as particularly effective in enhancing yield-related parameters and boosting Bt protein levels. To further advance cotton cultivation, future studies should explore additional priming methods and their respective treatments to assess their impact on yield-related attributes. Additionally, it is essential to evaluate other high-yielding *Bt* cotton varieties in conjunction with priming treatments. This study represents the first investigation into the effects of halopriming on *Bt* gene expression in cotton. Therefore, further molecular-based research is warranted to provide a more comprehensive understanding of *Bt* gene expression at the transcriptomic, metabolomic, and proteomic levels.

Acknowledgement: Thanks to Crop Sciences Institute (CSI), National Agriculture Research Council (NARC), Islamabad, Pakistan, for providing Seeds.

Funding Statement: This project was supported by National Natural Science Foundation of China (32160456; 32360474; 32360486), grants from the Provincial Basic Research Program (Natural Science) ([2020] 1Z018), Provincial Key Technology R&D Program ([2021] YiBan272).

Author Contributions: Study conception and design: Luhua Li and Muhammad Arif; data collection: Wenqi Shi, Binyameen Bin Shafqat and Muhammad Arif; analysis and interpretation of results: Wenqi Shi, Binyameen Bin Shafqat and Muhammad Arif; writing original draft preparation: Wenqi Shi, Binyameen Bin Shafqat and Muhammad Arif; review and editing: Ayesha Fazal Nawaz, Muhammad

Amir Zia, Xu Ling, Dingli Hong, Ruhong Xu and Luhua Li; funding: Luhua Li and Ruhong Xu. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: All the data that support the findings of this study are available in the manuscript.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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