Effect of cotton seed priming on Bt gene expression and other traits

Thesis submitted in the partial fulfilment of requirements for the degree of

MASTER OF PHILOSOPHY

In

Plant Genomics and Biotechnology

By

Binyameen Bin Shafqat

Department of Plant Genomics and Biotechnology

PARC Institute of Advanced Studies in Agriculture

National Agriculture Research Centre, Islamabad

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Certificate

The thesis submitted by **Binyameen Bin Shafqat** to Plant Genomics and Biotechnology (PGB), PARC Institute of Advanced Studies in Agriculture (PIASA), National Agriculture Research Centre (NARC), Quaid-I-Azam University, Islamabad, Pakistan, is accepted in its current form. This thesis fulfills all the requirement for facilitating him with Degree of Master of Philosophy in **Plant Genomics and Biotechnology.**

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Chairman: _____

DECLARATION

I would like to declare that the data presented in this thesis "**Effect of cotton seed priming on Bt gene expression and other traits**" is generated myself from original research work under the supervision of **Dr. Shaukat Ali** at Department of Plant Genomics and Biotechnology (PGB), PARC Institute of Advanced Studies in Agriculture (PIASA), National Agriculture Research Centre (NARC), Quaid-I-Azam University Islamabad, Pakistan. The results and material used in this thesis never presented anywhere else earlier.

_____

Binyameen Bin Shafqat

Dated: ……..

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Dated: ……..

Binyameen Bin Shafqat

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LIST OF ABBREVIATIONS

Bt	*Bacillus thuringiensis*
GoT	Ginning out turn
CLCuV	Cotton leaf curl virus
KCl	Potassium chloride
K2SO4	Potassium Sulphate
CaCl2	Calcium Chloride
ABA	Abscisic acid
PEG	Polyethylene Glycol
APX	Ascorbate peroxidase
NIGAB	National Institute for Genomics and Advanced Biotechnology
Cm	Centimeter
ELISA	Enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic Acid
PPB	Parts per billion
Ml	Micro liter
O.D	Optical Density
ANOVA	Analysis of Variance
RCBD	Randomized Complete Block Design
Mg	Milligram
LSD	Least Significant Difference
NARC	National Agricultural Research Center
SEM	Standard error of mean

ABSTRACT

Cotton is the most significant fiber producing crop in the world. Cotton belongs to family Malvaceae and genus Gossypium. Globally, Pakistan is the major producer and exporter of cotton. Cotton production is affected by many factors and one of them is the non-availability of good quality seed. Low quality seed has direct impact on poor germination that prompts low harvest. Seed priming is a process in which plant germination and yield related parameters can be increased. Keeping in view the importance of cotton seed priming, the present study was designed to check the effect of halopriming treatments on three cotton cultivars as compared to control. For this purpose, each cotton seeds were primed with 1% and 2 % KCL and K2SO4 for six hours and air-dried seeds were sown in the field. Later on, seed germination response was checked while at crop maturity stage, other yield related parameters of primed plants and control were taken. Meanwhile, effect of priming on Cry1Ac gene expression was also checked in these cultivars. The results of halopriming on cotton morphological data revealed that germination percentage, plant height, number of bolls per plant, number of seeds per boll, boll weight, seed cotton yield and GOT were high at 1% K2SO4 followed by 2% KCl. While on the other hand, Cry1Ac gene expression was also high at 1% K2SO4 followed by 2% KCL in IUB-2013 cultivar followed by NIAB-878B and FH-142 respectively. It has been depicted from this study that potassium salts i.e. 1% K2SO4 and 2% KCL have positively contributed in cottonseed germination, morphological parameters and Bt gene expression in the tested cultivars as compared to 2% K2SO4, 1% KCL treatments and control. To the best of our knowledge, halopriming effect on Bt gene expression is not reported yet. For the first time we have checked Bt gene expression in cotton cultivars against halopriming.

Introduction

1.1 General importance of cotton

The word "cotton" has been derived from Sanskrit word "kapas." Globally cotton is grown on 2.3 percent of the total fertile land and it can grow in in diverse environmental and socio-economic conditions (Sahay, 2019). Cotton is the most significant fiber crop worldwide and is a member of the family Malvaceae and the genus Gossypium, this genus is comprised of almost 50 species. Cotton can be grown under a wide range of weather and soil conditions (Shah *et al*., 2017). It is a member of genus Gossypium comprising four domesticated species such as *Gossypium hirsutum* and *Gossypium barbadense* which are allopolyploids (2n = 52), and *Gossypium herbaceum* and *Gossypium arboreum* (2n = 26) which are diploid. Out of these, upland cotton (*G. hirsutum*) is the most cultivated (>90% area of over the world cotton cultivation) cotton and reason is its high yield. Cotton is a perennial plant originated from tropical and subtropical regions, but is usually cultivated as annual crop to get animal meal, seed oil, and primarily lint (Constable & Bange, 2015).

1.2 Production of cotton in Pakistan

Cotton (Gossypium hirsutum L.) is mostly called "white gold" in Pakistan due to its fiber which is the backbone of textile industry of Pakistan (Ali & Khan 2007a). Cotton is annually cultivated on about 3 million ha (13% of total cultivated area in Pakistan). Pakistan is in among few countries which have distinction of being home of diploid species of cotton.

Even though there has not been increase in area significantly since 1947, the major gains in cotton production have been achieved after 1990. In the years of 1992 and 2004-05, Pakistan was at 2nd position in cotton production after USA. In Pakistan, nearly over two decades, almost 90% of the upland cotton was cultivated in Punjab province. Globally, Pakistan is the fourth major producer and third major exporter of cotton. In Pakistan, the cotton industry has flourished to 1000 ginneries, >250,000 looms (non-mill sector), 8000 looms (mill sector), 4000 garment units, more than 13 million spindles and almost 700 knitwear units (Ali *et al.*, 2019).

In Pakistan there are about 1.6 million cotton dependent farmers. Pakistan is at 5th position with 1350 thousand metric tons cotton production (Statista, 2019/2020). Among the cash crops in Pakistan, cotton has significant importance by contributing 55% to foreign exchange earnings and 10% to the national gross domestic product (GDP). In Pakistan, Punjab and Sindh are the two particular cotton producing provinces, accounting for 80% and 19% of the entire national production (Kouser *et al*., 2019).

The plantation of cotton started in February to June and its harvesting time is from August to December. Major growing areas of cotton in Pakistan are mainly in South Punjab and Sindh Province (USDA 2016).

1.3 History and role of Bt cotton:

Moreover to these economic benefits, Bt cotton may provide health and environmental advantages by decreasing the demand for chemical pesticide sprays (Kouser & Qaim, 2011). Chemical pesticides are directly linked with pollution of soil and water supplies (Damalas & Eleftherohorinos, 2011), harm to useful insects and biodiversity (Brethour & Weersink, 2001) and health related issues for consumers and farmers (Gesesew *et al*., 2016). Throughout the globe, genetically modified cotton plants have received wide acceptance and earned great trust due to the revolutionary characters of insect-pest and herbicide resistance (Rathore *et al*., 2009).

Adverse biotic and abiotic conditions affected crop growth and production (Gomez *et al*., 2006). These problems suggested transgenic engineering of cotton plants, to furnish them with the ability to resist against adverse environmental conditions and stimulate higher yield. (Kranthi *et al*., 2009). Bt cotton is developed by taking cry genes from a soil bacterium *Bacillus thuringiensis*. (Kouser & Qaim, 2011).

In 1901, Japanese scientist Ishiwata for the first time isolated a gram positive, soil born bacterium named as *Bacillus thuringiensis* (Bt) from a silkworm larva. *Bacillus thuringiensis* is mostly used as biological pesticide agent. (Asree, Falih, Mahdi, AL-Maameri, & Khirallah).

Bacillus thuringiensis at their sporulation stage make parasporal crystals. Crystals are consisted of insecticidal proteins having one or more delta endotoxins. These Cry

proteins are proved as destructive to some of the insect order including Lepidoptera, Hymenoptera, Coleoptera and Diptera, but they are not harmful to most of organisms such as insects and wildlife. Whenever an insect larva is ingested by Bt toxin, it results in solubilization of crystal proteins in the insect midgut and digestive protease cause its activation. (Reinoso *et al*., 2018). In the midgut of coleopteran and lepidopteran larvae, *cry* protein attaches to the epithelial cells by making pores and eventually insect die (Whalon & Wingerd, 2003).

In 1996, America has introduced Bt cotton (Bollgard^{TM}) that contain Cry1Ac gene taken from *Bacillus thuringiensis* (Perlak *et al*., 1990). With the minimal or zero detrimental effects to human beings and other living organisms, transgenic cotton containing Cry endotoxin protein has brought a revolution in the field of pest control. It provides various direct and indirect advantages to farmers counting eco-friendly behavior and reduce pesticide usage, maximum yield and low production cost. Additionally, it has also assisted to get high production with virtuous sustainability (Godfray *et al.*, 2010).

Insecticidal efficiency of Bt cotton is higher in leaves as compared to other parts of plant and moreover new emerging leaves have high potential of resistance than old leaves (Shen *et al*., 2010). Although Bt cotton does not provide resistance to usual sucking pests like thrips, whiteflies, jassids and mealybugs or diseases including cotton leaf curl virus but on the other hand, it has the potential to control bollworm damage and reduce pesticide cost (Ma *et al*., 2017).

1.4 Cotton Production Constraints

Though breeding advancement has brought increase in cotton production, but sometimes yield decreases due to many reasons. One of the major constraints in cotton production is the non-availability of good quality seed. Low quality seed outcomes in poor germination that prompts low harvest. Due to seed germination issue in Pakistan and non-availability of good quality seeds federal seed certification and registration department has reduced seed germination standard to 50% which is 95% in other cotton producing countries. Low germination rate is reported in the cotton (Anonymous 2016). Furthermore, non-availability of good quality seed, laborious picking, unpredicted cotton estimate, dry season and heat stress are the significant hurdles (Ali *et al*., 2019). As

cotton plant is generally attacked by different sucking and chewing pests. Biotic components are predominantly playing role in low efficiency of cotton in Pakistan. These insects and bugs causes huge loss to cotton production up to 30% (Dhaliwal *et al*.,2010). But the development of genetically modified Bt cotton which have endotoxin of Bacillus thuringiensis is a wonderful success (Benedict *et al*., 1996). Cotton leaf curl virus (CLCuV) is one of the huge biotic limitations that influence the production of crops (Deepika & Asokhan, 2019).

Abiotic stresses effect cotton badly and reduces its growth. Drought is most prominent abiotic stress for cotton (Loka & Oosterhuis, 2012). Drought have negative effects on flowering and boll development of cotton which in turn reduces the yield (Han, 2001). Salinity stress has also adversely affected cotton production. Cotton is little bit salt tolerant (Chinnusamy *et al*., 2005) but when salinity increases it limit the seedling emergence of cotton. Moreover salinity also effected the Bt protein expression which reduced its insecticidal activity (Luo *et al*., 2017). Heat stress affect boll opening and flowering in cotton (Mao, 2010).

1.5 Importance of seed priming

Agricultural production systems are threatened by rapid human population growth, high food prices and fast dietary transitions. In future, energy requirements can be fulfilled by changing climate and increase the crop production (Fedoroff *et al*., 2010). Major hindrance in crop productivity is mainly considered as poor seedling emergence and inadequate plant establishment. Seed priming is a process in which increase the percentage of germinated seeds and decreases the time of emergence; as the chitting phase (seed absorb maximum water and complete all processes before germination during priming) of primed seed of plants is completed. During this process seeds are allowed to treat with different solutions by increasing the osmotic potential. For this purpose, different type of solutions are used according to the requirements. Seed priming enhances the protrusion of the radical, germination rate and plant performance (Shabbir *et al*., 2014). One of its application is to protect the disease attack by seed priming. Through seed priming germination percentage and seed vigor can be increased (Nawaz *et al*., 2013).

Priming of various crop seeds with KCl and CaCl2 improved seedling emergence and upgraded stand establishment, resulting in better crop performance due to a decrease in the time between seed sowing and seedling emergence and synchronization of emergence (Parera & Cantliffe, 1994). Seed priming is causes decline in the lag time to imbibition, enzyme activation, accumulation of germination-booster metabolites better osmotic adjustment (Lee & Kim, 2000) (Ullah *et al*., 2019). Moreover, seed priming brings tolerance to abiotic and biotic stresses (Harris *et al*., 2007) due to the buildup of latent defense proteins and improved stress response (Borges *et al*., 2014). Seed priming have been reported to uptake high level of nutrients, induce seed dormancy, improved water use efficiency and reflects maturity of crops (Hill *et al*., 2008).

Seed priming influences physiological and biochemical alterations in the seeds. Osmo-priming gave the maximum germination rate in delinted seed when contrasted with fuzzy seeds (Sadeghi *et al*., 2011). For getting high rate of germination and seed vigor, seeds are treated with water. Osmo-priming in oil seed crop is related with increased antioxidant enzyme activities (Bailly *et al*., 2000). One more treatment for seed priming is treated with polyethylene glycol (PEG). It has high molecular weight so can't pass the cell wall and thus used for water potential adjustments in different crops germination (Seyed & Seyed, 2008).

Halopriming in combination of HCl can be enhanced the chilling tolerance, improved final germination index and pre seedling growth (Farooq *et al*., 2008). Priming of the cotton seed for germination of seed, development of seed and seedling vigor is altered by genotypes. KNO3 is applied on cotton seed (usually 2-4%) that had elevated the rate of germination in the cotton genotypes. When Priming of cotton seeds with sodium chloride at 2% occur it can increase optimum rate of germination (Çokkizgin & Bölek, 2015).

1.7 Effect of primed seed on gene expression

Seed priming is an agronomic trait in which it enhances germination and seed vigor by involving multiple genetic and environmental factors (Rajjou *et al*., 2012). Seed priming increased growth and hormonal change in plant. Priming effects on biosynthesis and signaling which have been reported to enhance the gibberellins (GA)/abscisic acid

(ABA) ratio (El-Araby *et al*., 2006) and this could be a direct result of a priming impact on gene expression pattern (Schwember & Bradford, 2010). According to (Buitink *et al*., 2006) more than 1300 genes regulation were alteres after the addition of seed priming with PEG in Medicago truncatula. Expression of many stress related genes is very much rapid in primed seeds as compared to unprimed seeds (Falourd *et al*., 2014). Certain metabolic events, i.e., replication of DNA (Lanteri *et al*., 1994); DNA, RNA and proteins synthesis (Bailly *et al*., 2000); and piling of beta-tubulin are stimulated by seed priming (Paparella *et al*., 2015). Seed priming induced better solubility of storage proteins in seeds which in turn increased anti-oxidative enzymatic activities (Randhir & Shetty, 2005). Protein concentration in primed seeds is increased due to two reasons. First, protein synthesis increased and second one is the lysis of storage protein by protease action. Seed priming can activate proteases (de Lespinay, 2009).

Still so far, molecular mechanism underlying halopriming is not fully understood but most probably it has been recognized that many transcription factors are being accumulated and play their respective role (Conrath *et al*., 2006). Furthermore, priming effect on seed is considered to perform role in chromatin remodeling that could facilitate fast response to priming (Bruce *et al*., 2007).

It has been reported that priming treatment in sugarcane plant has significantly increased Ascorbate peroxidase (APX) gene after priming stress (Patade *et al*., 2012). The application of halopriming on rice seed has enhanced the expression of Cu/Zn-SOD of rice seedlings as compared control (Sen, Challabathula, & Puthur).

1.8 Aims and Objectives

The main objectives of this study are given below;

- Evaluation of specific treatments of cotton seed priming
- Comparison of seed germination, morphological data and yield parameters between primed and non-primed cultivars
- Estimation of Bt gene expression pattern under primed condition

Methodology

2.1 Plant Sample

In this study, three varieties of cotton FH-142, NIAB-878B and IUB-2013 seeds were obtained from National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC Islamabad during the year 2020-21.

2.2 Cotton seed priming treatments

Seeds of three varieties mentioned above were taken separately for the specific treatment of halopriming. Seeds were dipped for six hours in the primed solution. In this experiment, 1% and 2% of KCL and K2SO4 priming solution applied on three cotton cultivars respectively. After the completion of dipping cottonseeds in their respective salt treatments, seeds were air dried by putting on filter paper. Completely dried seeds of cotton cultivars were then taken for sowing purpose under field conduction.

Fig. 2.1 Application of halopriming on cotton seeds

2.3 Sowing of primed seeds

Cotton field tunnels were prepared where primed seeds of each treatment were sown by dibbing it manually. Uniform agronomic practices were applied for all the treatments and replications. All the treated seeds were sown in triplicate and in RCBD format. Recommended dose of fertilizers and plant protection measures were applied. Meanwhile control seeds were also sown for comparison with primed seeds.

2.4 Germination Percentage (%) Recording

After 15-20 days after sowing, germination data (%) for all the treatments were recorded.

2.5 Morphological data recording

At plant maturity stage, the following morphological parameters were recorded for the primed and control plants of cotton cultivars.

Fig. 2.2. Representative pictures of cotton primed plants

2.5.1 Plant height

Random full-length plants (five plants per treatment/replication) of primed and control treatments were selected for plant height and their height was measured in (cm).

2.5.2 Internode distance

At maturity, internode distance (cm) was measured for all the selected plants of primed and control.

2.5.3 Number of bolls

Total number of bolls of five plants selected plants per treatment and control were manually counted separately for comparison.

2.5.4 Boll weight

At boll maturity stage, boll weight (g) was measured through electric balance.

2.5.5 Number of seeds/bolls

Total number of seeds in individual boll of five plants of each treatment were counted separately.

2.5.6 Ginning out turn percentage (GoT %)

Ginning outturn (%) was measured for all the treated and control lint having seeds. Individual fiber and seeds were separated manually and fiber was measured through electric balance. Then GoT in percentage was calculated one by one for all the treatments and control.

Fig. 2.3 Cotton plants at maturity stage

2.5.7 Yield

Finally, yield was calculated as (kg/acre) by calculating for all treated and control cultivars of cotton.

2.6 Expression analysis of Bt gene

For quantification of Bt protein, we have used sandwich ELISA kit which is simple, rapid and a sensitive method. Sandwich ELISA kit was used to measure Cry1Ac protein of genetically modified cotton samples. Materials and reagents that were used as Standards of Cry1Ab/Ac, concentrated enzyme solution, sample extraction buffer, enzyme diluent, chromogenic agent, washing solution and stop solution. Equipment and instruments involved as microplate reader, incubator, centrifuge machine, centrifuge tubes, shaker and micropipette.

First of all, we have prepared washing solution by using 19 part of distilled water and 1 part of concentrated washing liquid in a ratio of 19:1. Secondly, we prepared standard solutions of 16 ppb,8 ppb, 4 ppb and 2 ppb respectively. For 16 ppb standard solution preparation, dissolve standard of 3 ml sample extract. Then add 500 ml of 16ppb and 500 mL sample extract the resulting solution is 8ppb. To make 4ppb took 500 mL of 8ppb and add 500 ml sample extract. Similarly, 2ppb solution was made by adding 500 mL 4ppb and 500 ml sample extract. Then prepared enzyme working solution by using 1 part of concentrated enzyme liquid and 10 part of enzyme diluent in ratio of 1:10 according to manufacturer instructions.

2.6.1 Procedure of sandwich ELISA

- Take 0.05 g leaf sample and put in centrifuge tube and grind them
- Then 0.5 ml of sample extraction was added and mix it for 5 minutes
- Centrifuge the tube at 4000 rpm for 3 minutes and took 100 mL of supernatant
- Add sample extract to micro wells, rotate pleasantly and put it in dark for 45 minutes for reaction
- After 45 minutes wash the wells by washing solution 4-5 times with the interval of 10 seconds and then dry the wells by pat on absorbent paper till bubbles were removed
- Added 100 ml enzyme solution to wells, mix it and put it in dark for 30 minutes

- Repeat the process of washing
- After washing added 100 mL chromogenic agent and kept in for 15 minutes in dark at room temperature
- Finally add 100 ml of stop solution, mix well and set the microplate at 450 nm and check the O.D value of every well.

2.7 Data Analysis

Data of all treatments and control plants were recorded in three replication and their mean values were taken for analysis of variance (ANOVA) through software statistix 8.1. Our experimental data were designed in RCBD format. ANOVA was applied for all morphological parameters and Bt gene expression with respect to individual treatment and variety, while in combination i.e. treatment*variety interaction was also determined through two factorial ANOVA.

Results

In this study the effect of cottonseed primed with KCL and K2SO4 were checked on three commercial cultivars of cotton. Primed and non-primed seeds were then sown under field condition of NARC during the month of May, 2020 by following RCBD design.

Statistical analysis of variety, treatment and variety * treatment interactions of Germination percentage (%) and morphological data of cotton

Source	DF	Germination (%)	PH (cm)	ID (cm)	NBPP	BW (g)	NSPB	Yield (kg/acre)	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**

Table 3.1 Analysis of variance table for overall morphological parameters

DF represents degree 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 *Significance at 5%, **Significance at 1%, NS= Non-Significance

Treatment *Variety	**8**	**3.45****	**12.95****	**2.71***	**1.61^{NS}**	**2.07***	**5.14****	**2.20***	**2.88***
Error	**28**								
Total	**44**								

Overall morphological data through ANOVA with respect to individual treatment and variety, while also in combination i.e. treatment*variety interaction was also determined through Statistix 8.1 software. Among all the treatments, there were highly significant differences recorded at 1% probability ($p<0.01$) as compared to control. Halopriming treatment effect on morphological traits is strongly correlated through 2 factorial ANOVA analysis. It shows that treatments responded well on morphological data in terms of statistical analysis carried out in this study as shown in table 3.1.

3.1 Effect of halo-priming on Germination percentage (%)

The fully germinated plantlets data after 15-20 days were taken for all treatments and control cultivars of cotton. ANOVA results indicated that priming has significant effects on germination (%) of treatments. Varieties effect on germination percentage demonstrated that there were significant differences at 1% ($p<0.01$). Moreover, treatment*variety interaction of germination percentage also showed highly statistical difference (1%).

Cultivar FH-142 has the highest germination percentage i.e. 82.6% followed by NIAB-878 i.e. 80% at 1% K2SO4. Similarly, cultivar FH-142 has high germination (78%) at 2% KCL and IUB-2013 cultivar has the lowest germination i.e. 60%.

Cultivar FH-142 has shown the highest germination percentage at all priming treatments. However, cultivar IUB-2013 has lower germination percentage among the tested treatments. Moreover, 1% KCL and 2% K2SO4 have shown less germination percentages as compared to other treatments as shown in figure 3.1.

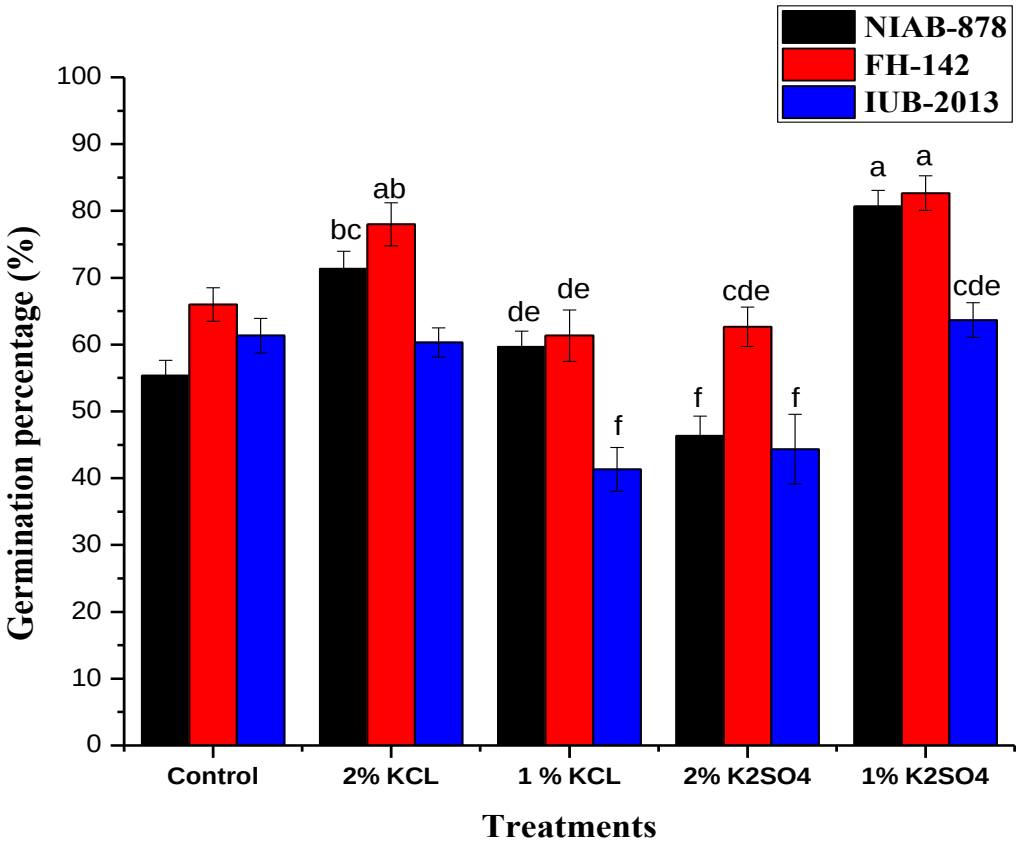

Fig. 3.1 Effect of priming on cotton germination. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2 Effect of halo-priming on Morphological data

3.2.1 Plant height

Mean plant height data of all treatments and varieties were determined as compared to control. Results of plant height showed that variety NIAB-878 has maximum plant height at all treatments. Among the treatments, 2% KCL and 1% K2SO4 have maximum plant heights of 167cm and 166 cm respectively in NIAB-878 cultivar. Other two varieties FH-142 and IUB-2013 have less plant height as compared to NIAB-878. All cultivars performed well on 1% K2SO4 followed by 2% KCl as compared to other two treatments.

Data of plant height of different salts treatment have illustrated that significant differences were seen at ($p<5\%$) among the treatments and cultivars except 1% and 2 % KCL in NIAB-878B and FH-142 cultivars. On the other hand, results clearly showed highly statistical difference (1%) among the treatment*variety interaction of plant height (Fig 3.2).

Fig.3.2. Effect of priming on plant height. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.2 Number of bolls per plant

Number of bolls are directly linked with yield. In this study, after getting the mean of number of bolls per plant of all treatments and control, results showed that treatment i.e. 1% K2SO4 on all varieties have maximum number of bolls per plant as compared to other treatments and controls. NIAB-878 have a greater number of bolls which is 78 at 1% K2SO4 but at all other priming conditions FH-142 have highest number of bolls.

In conclusion like all other parameters, treatment 1% K2SO4 and 2% KCL have performed very well as compared to treatments 1% KCL, 2% K2SO4 and control as well.

Cultivars effect on number of bolls per plant that there were significant differences at 1% ($p<0.01$). Meanwhile, number of bolls per plant did not show any significant response with respect to treatment×variety interaction as shown in figure 3.3.

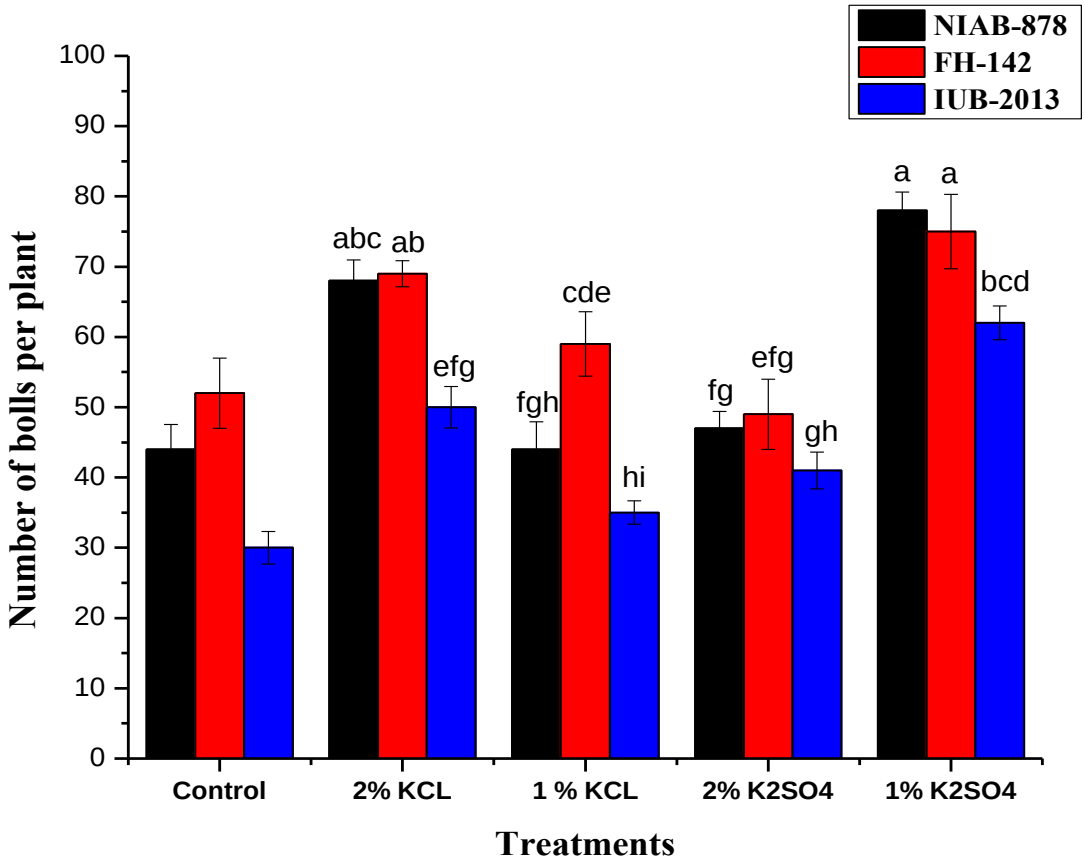

Fig. 3.3. Priming effect on number of bolls per plant. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.3 Number of seeds per boll

In this case, variety NIAB-878B with 1% K2SO4 have shown maximum mean number of seeds per boll i.e.38 and at 2% K2SO4 has shown lower number of seeds per boll (25) as compared to control and KCL treatments. Variety FH-142 has also shown maximum number of seeds per bolls at 1% K2SO4 and lowest numbers at 1% KCL.

Cultivar IUB-2013 with treatments have less numbers of seeds as compared to other cultivars but performed well against control. Varieties effect on number of seeds per boll showed statistical differences at 5% probability ($p<0.05$). On the other hand, results showed highly statistical difference (1%) among the treatment*variety interaction of number of seeds per boll as shown in fig.3.4.

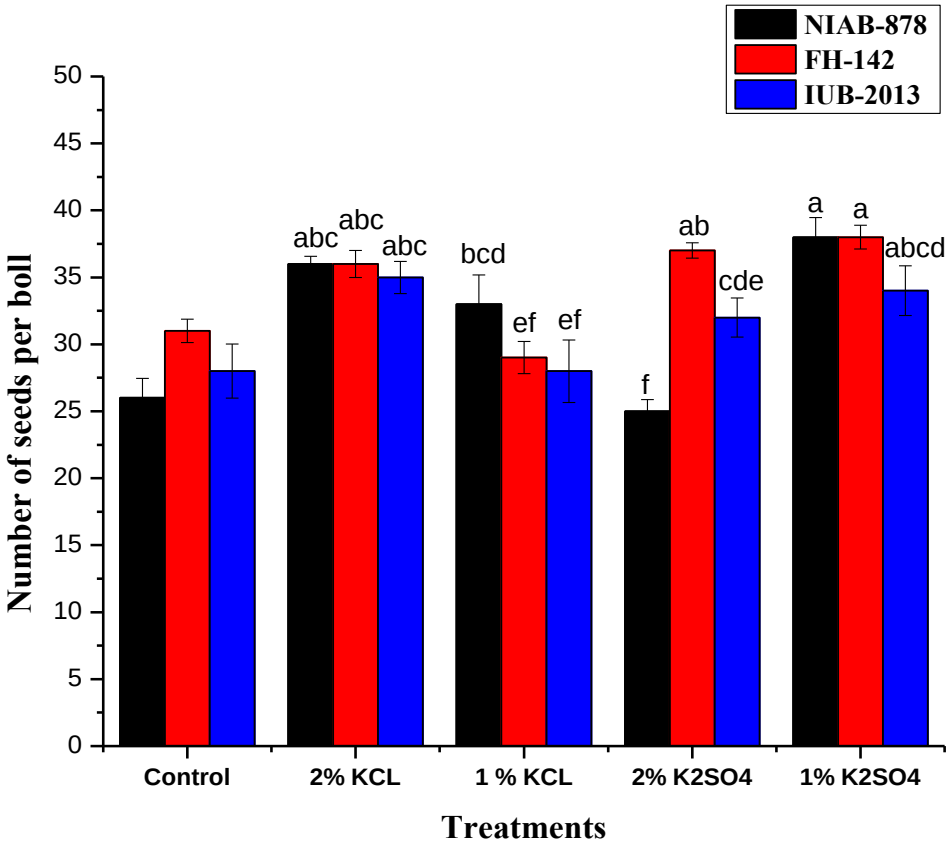

Fig.3.4. Priming effect on number of seeds per boll per plant. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.4 Boll weight

Boll weight of variety NIAB-878 has high value treated with 2% KCL and 1% K2SO4 5.2g and 4.8g respectively as compared to control. In case of variety FH-142 only 1% K2SO4 gave high weight of bolls i.e. 5g with respect to all other treatments and control. Varieties IUB-2013 and NIAB-878B have got nearly the same boll weight in all primed treatments.

ANOVA results indicated that variety individual effect on boll weight did not show any significant responses But in variety*treatment interaction highly significance differences at 5% probability ($p<0.05$) as shown in fig 3.5.

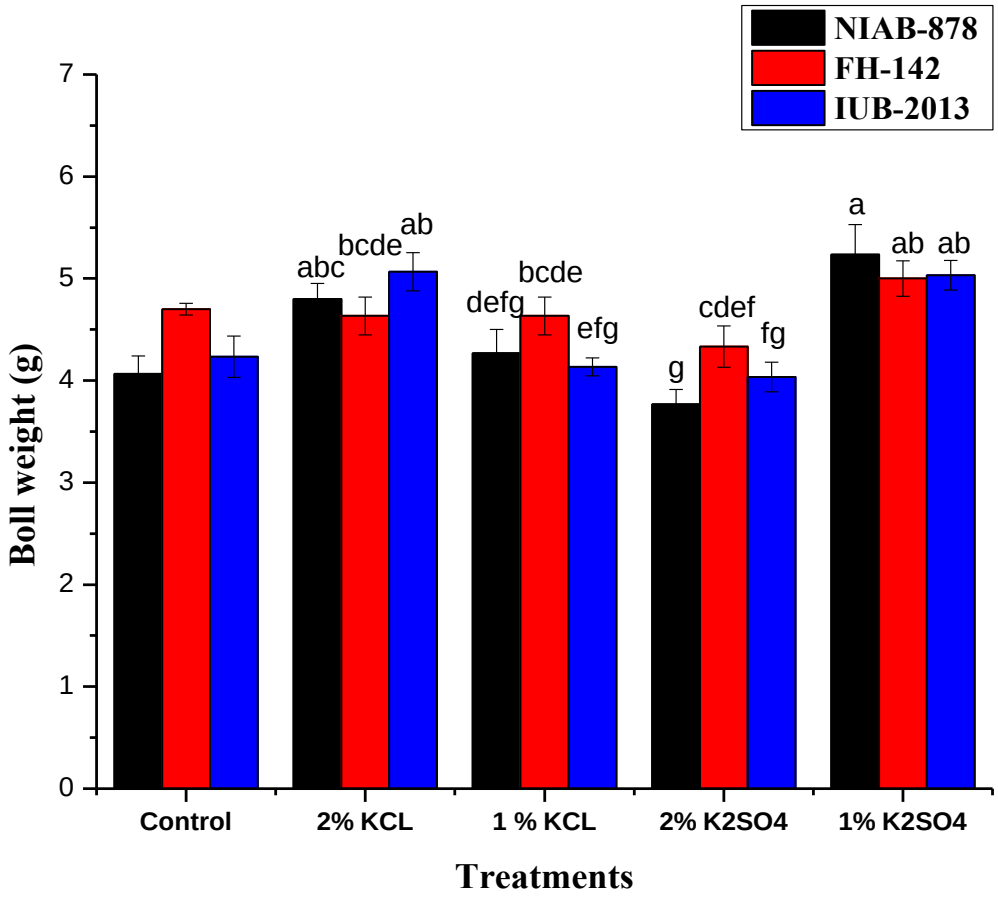

Fig. 3.5. Priming effect on boll weight. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.5 Internode distance

In the present study, cotton cultivars NIAB-878, FH-142 and IUB-2013 all have less internode distance at 1% K2SO4 i.e. 3cm, 3cm and 2.7cm respectively. Treatments with 1% K2SO4 and 2% KCL of varieties have the lowest mean internode distance as compared to other two treatments. Treatments 1% KCL and 2% K2SO4 of all varieties have highest internode distance as compared to control. Most of the internode distance data of treatments were non-significant. While internode distance has showed significantly at 5% LSD in variety*treatment interaction as shown in fig.3.6.

Fig. 3.6. Priming effect on internode distance. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.6 Seed cotton yield

Halopriming have shown promising effects on seed cotton yield. The results indicated that treatment 1% K2SO4 and N-878 have maximum yield of 920kg/acre as compared to other treatments and cultivars followed by FH-142 with 880kg/acre at 1% K2SO4 and 810kg/acre at 2% KCL. IUB-2013 has got the minimum yield on 1% KCL.

Statistical differences were recorded in priming and varieties interaction. Cultivars effect on yield demonstrated that there were significant differences at 1% ($p<0.01$). While and yield showed significantly at 5% LSD in variety*treatment interaction as shown in figure 3.7.

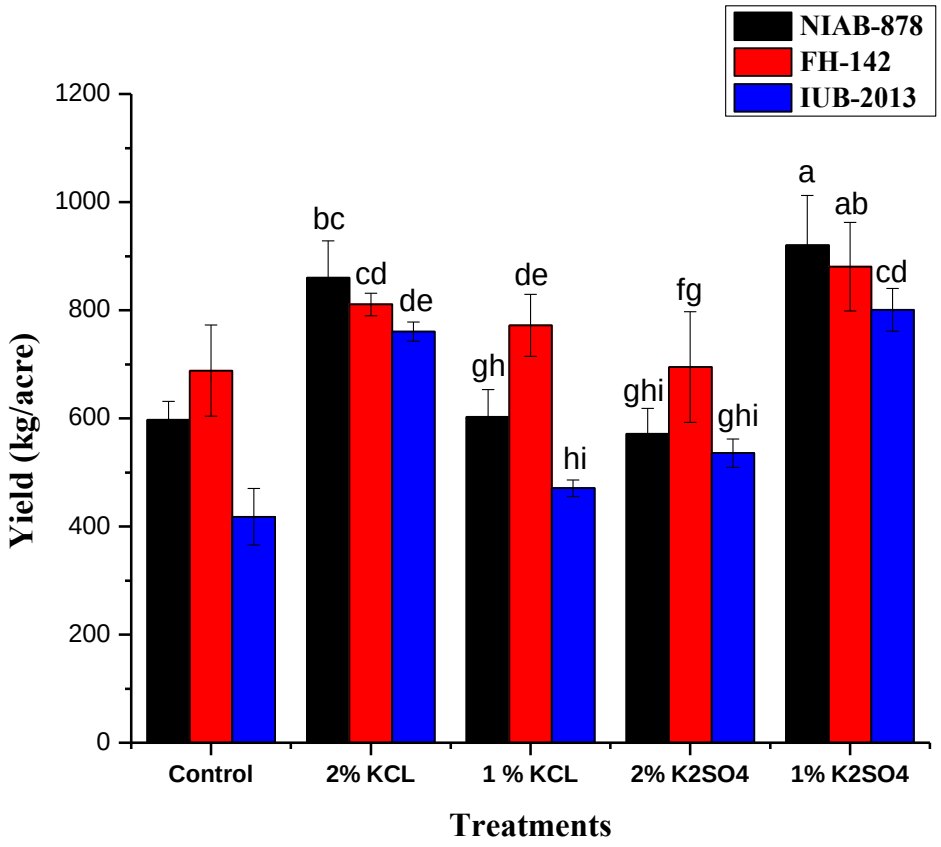

Fig. 3.7. Priming effect on seed cotton yield. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.2.7 Ginning outturn (GOT)

Results of mean GOT percentage indicated that cultivar NIAB-878 has maximum GOT value when treated with 2% KCL and 1% K2SO4 i.e. 40.4% and 39.6% respectively as compared to other varieties, treatments and control. While with treatments 1% KCL and 2% K2SO4 have lower GOT.

Cultivar FH-142 at all treatments did not affect GOT values. Minimum GOT was secured in IUB-2013 at 2% K2SO4. Varieties effect on GOT demonstrated that there were significant differences at 1% ($p<0.01$). Statistically, GOT has showed significant differences at 5% in variety*treatment interaction as shown in fig. 3.8.

Fig. 3.8. Priming effect on GOT. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

3.3 Effect of halopriming on Bt gene expression

Analysis of variance (ANOVA) of Bt protein with respect to individual treatment and variety, while in combination i.e. treatment*variety interaction was also determined through Statistix 8.1 software as shown in table 2.

Table 3.2 Analysis of variance table for Bt protein

Source	DF	*Bt* protein
Replication	**2**	
Treatment	**4**	**15.94****
Variety	**2**	**15.85****
Treatment*Variety	**8**	**1.20 ^{NS}**
Error	**28**	
Total	**44**	

Grand Mean = 1.6492 CV = 8.46 *Significance at 5%, **Significance at 1%, NS= Non-Significance

Among all treatments, there were highly significant differences recorded at 1% probability ($p<0.01$ %) as compared to control. Halopriming treatment effect on Bt protein is strongly correlated through 2 factorial ANOVA analysis. Similarly, varieties effect on Bt toxin level was also highly significant. Bt toxin did not show any significance differences with respect to treatment*variety (table 2).

IUB-2013 has maximum level of Bt toxin i.e. 1.9µg/g with treatment 1% K2SO4. IUB-2013 and NIAB-878 have almost the same level of Bt toxin with treatment 2% KCL.

Results of Bt gene (Cry1Ac) expression suggests that with treatment 1% K2SO4 and 2% KCL of all varieties have showed maximum concentration of Bt protein as compared to control. But treatments 1% KCL and 2% K2SO4 have low concentration of Cry1Ac protein as compared to control. Overall data of Bt gene expression showed that 1% K2SO4 and 2% KCL have maximum Bt protein contents as shown in fig. 3.9.

Fig. 3.9. Priming effect on Bt gene expression. Mean data having error bar represents standard error of mean (SEM). Mean data of bars followed by same letter do not differ significantly at ($p<0.05$).

Discussion

In Pakistan, cotton plant is facing many challenges and poor germination is a major constraint to cotton production. To improve the cotton germination and vigor, the technique of cotton seed priming is used that ultimately increased the yield. Previous study indicated that halopriming improved rapid and uniform cotton seedling emergence (Batool *et al*., 2015).

Halopriming in which seeds were treated with different salts have major effects on germination and metabolism of different crops (Afzal *et al*., 2008). Potassium (K) is third macronutrient after Nitrogen (N) and Phosphorus (K) that have special osmotic role in plants. Response of a crop to priming is dependent on composition or percentage of priming medium and duration. Priming with potassium salts have showed maximum germination of cotton (Nazir *et al*., 2014).

In the current study, we have determined the effect of halopriming on cotton seeds. Results indicated that different dosage of salts i.e. 1% and 2% of KCL and K2SO4 each have different effects on germination of cotton. It has been revealed from our results that treatment 2% KCL and 1% K2SO4 of three cotton varieties have maximum germination percentage as compared to control and other treatments. Similar to our findings the effect of different dosage of K2SO4 on crop like rice has endorsed that 1% K2SO4 treatment performed well as compared to 2% and 4% of K2SO4 (Ramesh & Singh, 2006). Positive results were also reported by applying seed priming at early stages of germination and hence increase the rate of replication in primed seeds (Hassanpouraghdam *et al*., 2009).

It has also been find out that K2SO4 had significant role in plant height of mungbean and other crops (Abbas *et al*., 2011). Our results also demonstrated that potassium salts i.e. K2SO4 and KCL have significantly increased cotton plant height. Treatments with 1% K2SO4 and 2% KCL have performed more as compared other treatments.

In the present study, potassium salts effect the internode distance. Treatment 1% K2SO4 has low internode distance as compared to other treatments. Leaf area and internode distance also effected by halopriming (Hamidi *et al*., 2013).

The results of the current study have confirmed the positive effects of potassium salts on yield related parameters in cotton. Treatment with 1% K2SO4 and 2% KCL have maximum number of bolls, maximum number of seeds per boll, boll weight and high GOT percentage which in turn produce maximum yield as compared to 1% KCL, 2% K2SO4 and control. Among the varieties, NIAB-878 has secured maximum yield as compared to IUB-2013 and FH-142 which is due to more may be some genetic reason. In conclusion, all measured traits were significantly affected by seed priming. Number of bolls of cotton is directly linked with yield. Potassium salts increased the plant yield (Abbas *et al*., 2011). Positive influence of K2SO4 might also be due to its accompanying anion ($SO4^{-2}$) in the Sulphur deficient areas of Pakistan. Cotton yield depends on different components such as number of bolls, number of seeds per boll, boll weight of cotton and GOT percentage. Likewise our results, K2SO4 and KCL have improved the yield and their related parameters in cotton (Zheng *et al*., 2014).

Research on Bt cotton indicated that it has reduced the use of pesticides, decrease crop damage and increase farmers income. Cotton yields are increased with the introduction of Bt gene as compared to non-Bt (Carpenter, 2010). It has been investigated that seed priming alter the gene expression of many crops which in turn improve plant health. Many genes are involved in carbohydrate metabolism and protein biosynthesis that are effected by seed priming (Cheng *et al*., 2017). RNA stimulation and consequently protein synthesis as well as hormone concentration are increased due to seed priming (Hamidi *et al*., 2013). Moreover, seed priming enhance DNA repair, gene expression and synthesis of new mRNA and protein synthesizing machinery are enhanced (Varier *et al*., 2010).

In present study it is clearly indicated that treatments of potassium salts have positive effects on Bt protein contents. The effect of halopriming on Bt gene expression is not reported before. For the first time we have investigated Cry1Ac gene expression in cotton under K2SO4 and KCl.

Among four Treatments, treatment with 1% K2SO4 and 2% KCL have high Bt protein concentration as compared to control and other treatments. In plants like Arabidopsis the quantity and solubility of proteins increased with priming conditions (Gallardo *et al*., 2001).

Future Perspectives

Application of halopriming i.e. 1% and 2% of K2SO4 and KCL each on cotton seed of three cultivars NIAB-878B, FH-142 and IUB-2013 and their subsequent effects on cotton morphological and Bt gene expression have portrayed positive effects on yield related parameters and Bt gene expression as well. Out of four treatments, special emphases have been oBtained on 1% K2SO4 and 2% KCL. For future point of view, other priming methods and their treatments should be applied on cotton seed to check their effects on cotton yield related attributes. Besides this, it is also recommended that further Bt cotton high yielding approved varieties need to be tested against priming treatments. In this study we have checked for the first time the effect of halopriming on Bt gene expression. Furthermore, molecular based research is needed for more authentication of Bt gene expression at transcriptomic, metabolomic and proteomic level as well.

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APPENDICES

Appendix 1

LSD All-Pairwise Comparisons Test of Germination percentage (%) for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	71.33^{bc}	60.33^{de}	78.00^{ab}	69.88^{b}
1% KCL	59.66^{de}	41.33^{f}	61.33^{de}	54.11^{d}
2% K2SO4	46.33^{f}	44.33^{f}	62.66^{cde}	51.11^{d}
1% K2SO4	80.66^{a}	63.66^{cde}	82.66^{a}	75.66^{a}
Control	55.33^{e}	61.33^{de}	66.00^{cd}	60.88^{c}
Overall Mean	62.66^{b}	54.20^{c}	70.13^{a}	

LSD value for treatment = 5.01, LSD value for variety = 3.88, LSD value for Treatment*variety = 8.67. Means followed by same letters do not differ significant

Appendix 2

LSD All-Pairwise Comparisons Test of Plant height (cm) for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	152.00^{b}	136.33^{cde}	141.33^{bcd}	143.22^{a}
1% KCL	139.67^{bcd}	117.00^{gh}	96.33^{j}	117.67^{b}
2% K2SO4	100.67^{ij}	122.33^{fgh}	125.67^{efg}	116.22^{b}
1% K2SO4	166.67^{a}	134.33^{def}	149.00^{bc}	150.00^{a}
Control	100.33^{ij}	110.33^{hi}	122.33^{fgh}	111.00^{b}
Overall Mean	131.87 ^{a}	124.07 ^{b}	126.93^{ab}	

LSD value for treatment = 7.4347, LSD value for variety = 5.7589, LSD value for Treatment*variety = 12.877. Means followed by same letters do not differ significantly

Appendix 3

LSD All-Pairwise Comparisons Test of Number of bolls per plant for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	68.333^{abc}	50.333^{efg}	69.333 ^{ab}	62.667^{b}
1% KCL	44.333^{fgh}	35.667 ^{hi}	59.000^{cde}	46.333^{c}
2% K2SO4	47.333^{fg}	41.667^{gh}	9.667^{efg}	46.222 ^{c}
1% K2SO4	78.333^{a}	62.333^{bcd}	75.000^{a}	71.889^{a}
Control	46.000^{fg}	30.667^{i}	52.333^{def}	43.000 ^{c}
Overall Mean	56.867^{a}	44.133^{b}	61.067 ^{a}	

LSD value for treatment = 5.7745, LSD value for variety = 4.4729, LSD value for Treatment*variety = 10.002. Means followed by same letters do not differ significantly.

Appendix 4

LSD All-Pairwise Comparisons Test of Number of seeds per boll for Variety, treatment and variety*treatment				
Treatment	NIAB-878	IUB-2013	FH-142	Overall Mean
2% KCl	36.00^{abc}	35.667^{abc}	36.000^{abc}	35.889^{a}
1% KCL	33.667^{bcd}	28.667^{ef}	29.333^{ef}	30.556^{bc}
2%K2SO4	25.33^{f}	32.333^{cde}	37.000^{ab}	31.556^{b}
1%K2SO4	38.667^{a}	34.667^{abcd}	38.667^{a}	37.333^{a}
Control	26.667^{f}	28.667^{ef}	31.333^{de}	28.889^{c}
Overall Mean	32.067^{b}	32.000^{b}	34.467^{a}	

LSD value for treatment = 2.41 LSD value for variety = 1.87 LSD value for Treatment*variety = 4.18 Means followed by same letters do not differ significantly.

Appendix 5

LSD All-Pairwise Comparisons Test of boll weight (g) for Variety, treatment and variety*treatment				
Treatment	NIAB-878	IUB-2013	FH-142	Overall Mean
2% KCL	4.80^{abc}	5.06^{ab}	4.60^{bcde}	4.8222^{a}
1% KCL	4.26^{defg}	4.13^{efg}	4.63^{bcde}	4.3444^{b}
2% K2SO4	3.76^{g}	4.03^{fg}	4.33^{cdef}	4.0444^{c}
1% K2SO4	5.23^{a}	5.03^{ab}	5.00^{ab}	5.0889^{a}
Control	4.06^{fg}	4.23^{defg}	4.70^{bcd}	4.3333^{bc}
Overall Mean	4.4267^{a}	4.5000^{a}	4.6533^{a}	

LSD value for treatment = 0.2956 LSD value for variety = 0.2290 LSD value for Treatment*variety = 0.51 Means followed by same letters do not differ significantly.

Appendix 6

LSD All-Pairwise Comparisons Test of Internode Distance (cm) for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	3.36^{cde}	2.90^{ef}	3.23^{def}	3.16^{b}
1% KCL	3.83^{bc}	4.30^{ab}	3.90^{bc}	4.01^{a}
2% K2SO4	4.30^{ab}	4.63^{a}	3.80^{bcd}	4.24^{a}
1% K2SO4	3.03^{ef}	2.76^{f}	3.03^{ef}	2.94^{b}
Control	3.83^{bc}	4.10^{ab}	4.50^{a}	4.14^{a}
Overall Mean	3.67^{a}	3.74^{a}	3.69^{a}	

LSD value for treatment = 0.33 LSD value for variety = 0.25 LSD value for Treatment*variety = 0.57 Means followed by same letters do not differ significantly.

Appendix 7

LSD All-Pairwise Comparisons Test of yield (kg/acre) for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	860.3^{bc}	760.3^{ef}	810^{cd}	810.4^{b}
1% KCL	602.7^{gh}	470.7^{hi}	872.0^{de}	648.4^{c}
2% K2SO4	571.7^{ghi}	535.7^{ghi}	694.7^{fg}	600.7^{c}
1% K2SO4	920.3^{a}	880.7^{cd}	800.7^{ab}	866.9^{a}
Control	597.3^{gh}	418.0^{hi}	688.3^{ef}	567.2^{c}
Overall Mean	710.87^{b}	612.87^{c}	772.27^{a}	

LSD value for treatment = 100.94 LSD value for variety = 78.19 LSD value for Treatment*variety = 174.84 Means followed by same letters do not differ significantly.

Appendix 8

LSD All-Pairwise Comparisons Test of GOT percentage (%) for Variety, treatment and variety*treatment				
Treatment	**NIAB-878**	**IUB-2013**	**FH-142**	**Overall Mean**
2% KCL	40.433[a]	38.200[bcd]	37.667[cd]	38.767[a]
1% KCL	37.033[de]	37.767[cd]	36.967[def]	37.256[b]
2% K2SO4	36.867[def]	35.633[ef]	37.000[de]	36.500[b]
1% K2SO4	39.600[ab]	38.567[bc]	38.767[bc]	38.978[a]
Control	37.600[cd]	35.500[f]	37.700[cd]	36.933[a]
Overall Mean	38.307[a]	37.133[b]	37.620[b]	

LSD value for treatment = 0.8574 LSD value for variety = 0.6641 LSD value for Treatment*variety = 1.4850 Means followed by same letters do not differ significantly.

Appendix 9

LSD All-Pairwise Comparisons Test of Bt toxin (µg/g) for Variety, treatment and variety*treatment			
Treatment	NIAB-878	IUB-2013	FH-142
2% KCL	1.5229^{efg}	1.3880^{gh}	1.6967^{cde}
1% KCL	1.8768^{abcd}	1.6319^{ef}	1.9178^{abc}
2% K2SO4	1.5900^{efg}	1.5166^{efg}	1.7463^{bcde}
1% K2SO4	1.9349^{ab}	1.6710^{de}	1.9854a
Control	1.2603^{h}	1.4251^{fgh}	1.5738^{efg}

LSD value for Treatment*variety = 0.2335 Means followed by same letters do not differ significantly.

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