

Exogenous Application of Salicylic Acid: Inducing Thermotolerance In Cotton (*Gossypium Hirsutum* L.) Seedlings

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Abstract. Improvement in thermotolerance of major cash crops by exogenous application of salicylic acid is an important way to promote economic utilization. Salicylic acid is an important plant growth regulator, commercially utilized to enhance tolerance in plants against harsh environments. The present study was designed to investigate the effects of foliar application of different salicylic acid concentrations (0. 0.5, 1.0, 1.5 mM) on thermotolerance of three cotton (*Gossypium hirsutum* L.) varieties - "Bt 703", 'Bt 131", and "Bt 3701"- at control (28± 2°C) and heat shocked (HS) (42±2 °C) cotton seedlings. Heat stress damages were quantified by H2O2 production, malondialdehyde content (MDA), osmolyte (Proline) accumulation, and total soluble proteins (TSP). Results revealed that foliar application of salicylic acid minimized heat shock damages; however, different concentrations showed varied responses. Heat stressed seedlings with salicylic application showed increased plant dry matter, free proline accumulation, and total soluble protein content as compared to minus salicylic acid stressed seedlings. The above mentioned responses were better in "Bt 703" among the cultivars, making its position thermotolerant. The best responses for most of the above parameters were with the application of 1.0 mM SA. These findings suggest that exogenous application of SA could mitigate the deleterious effects of HS on *Gossypium hirsutum* seedlings and offer an efficient, economic, and simple means to enhance SH tolerance of the cotton.

Keywords. salicylic acid, cotton, thermotolerance, membrane damage, proline, melondialdehyde, oxidative stress, global warming

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1. Introduction

Cotton is considered the world's leading textile fiber plant. It forms a vital part of the global agriculture economy. It is the main source of income for millions of human engaged in production, processing, ginning, textile, and trade-related activities, and contributes significantly to the GDP of many countries, including India, China, Pakistan, Uzbekistan, Australia, and Greece (FAO, 2005). Cotton is an important crop in several parts of the world, which is highly sensitive to environmental stresses (Saranga et al., 2001).

In the last century, carbon dioxide concentration (CO₂) has increased from about 350 mmol mol⁻¹ in 1980 to about 378 mmol mol⁻¹ at present due

to anthropogenic activities and population increase. It is predicted that continuous gas emissions of CO₂ will double by the end of this century. These environmental changes from CO₂ and other greenhouse gas accumulation are predicted to increase surface mean temperature ranging from 1.4–5.8 °C yearly, causing more frequent short episodes of high heat. Since the last decade, these altered climatic conditions, in terms of temperature and rainfall patterns, affect cultivation and yield of crop plants. The detrimental effects of high diurnal temperature on various physiological processes of crop yields are complicated. Physiological and metabolic

process are adversely affected due to high temperature (Wahid et al., 2007), such as well marked affects on membrane stability and its functions (Farooq et al., 2008) by peroxidation of membrane lipids resulting in ion leakage (Wen-Yue et al., 2001) under heat stress.

The optimum temperature range for cotton cultivation is (20 - 30°C) (Reddy et al., 1991) with an optimum for photosynthesis being 28°C (Wise et al., 2004). Improved thermotolerance of cotton germplasm is obviously needed to stabilize yields under stressful conditions. Currently, there is no feasible and economical technology to facilitate cotton crop production under heat stress conditions. However, development of thermo-tolerance in crop plants is considered a promising approach, which may satisfy the growing needs of population. Exogenous application of various organic solutes, such as salicylic acid, may significantly contribute to achieving thermo-tolerance with increased crop production under heat stress. SA controls the number of physiological processes that occur in plants by the activation of naturally present signaling molecules (Mutlu et al., 2009), enhances resistance against biotic and abiotic conditions (Karlidage et al., 2009), and establishes resistance against pathogenic attack (Kachroo et al., 2005). It is reported that SA successfully induced thermotolerance in mustard (Hayat et al., 2009), potato (Dat et al., 1998), tomato (Senaratna et al., 2000) and Arabidopsis (Pendle et al., 2005; Larkindale and Knight, 2002).

Many reports exist on the heat tolerance potential of crop plants, but studies on improvement in heat tolerance threshold value of cotton by the exogenous application of salicylic acid are lacking and need to be established on firm grounds. In this regard, better understanding of the possible mechanism of heat tolerance (physiological, morphological, and yield processes) would not only be helpful in mitigating the adversaries of heat stress but also useful in developing reliable field-screening tools.

In view of the increasing adversaries due to global warming, it is necessary to discover indicators and mechanisms of inducing thermo-tolerance with exogenous application of salicylic acid for sustainable agriculture in warmer areas

of the country. In this regard, the following research was conducted to monitor the outcome of salicylic acid application on cotton under heat stress.

2. Materials and Methods

2.1 Crop Husbandry and Treatments

The experiment was carried out at a greenhouse at the Department of Agriculture & Agribusiness-Management, University of Karachi, Karachi, Pakistan and the Agriculture Biotechnology section at the Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi, Pakistan. Seeds of three cotton varieties (*Gossypium hirsutum* L.) "Bt 703", "Bt 131", and "Bt 3701" were collected from a soil and water testing laboratory Sahiwal, Punjab. The surface of the seeds was sterilized with sodium hypochlorite solution (5%) for five minutes and rinsed with sterile distilled water. The seeds were transferred into polyethylene bags (6*4 cm) filled with 300g soil. Plants were grown in a naturally illuminated greenhouse. After 16 hours of heat stress, the plants were harvested along with their roots and removed soil particles. Fresh leaves were stored at -80 °C for the study of physiological and biochemical parameters.

2.2 Relative Membrane Permeability (RMP)

A half gram of fresh leaf was added in 25 ml distilled water and vortexed. Electrical conductivity, EC₀, was recorded by EC Meter (Adwa AD3000). The solutions were kept at 4 °C for 24 hours, and EC₁ was determined. The tubes were autoclaved and the electrical conductivity (EC₂) was measured again. Relative membrane permeability in terms of percentage was determined by using the formula $(EC_1 - EC_0 / EC_2 - EC_0) * 100$ according to Yang et al., 1996.

2.3 Hydrogen Peroxide (H₂O₂) Concentration

The concentration of hydrogen peroxide was determined according to Jessup et al., (1994). For this, 0.1 gram of leaves was homogenized in 1 ml of 0.1% TCA. The homogenate were centrifuged at 12000 rpm for 15 minutes. One ml of supernatant was taken in another tube, and a 1 ml potassium buffer (pH 7.0) and 2 ml of 1M potassium iodide was added. The absorbance was recorded

at 390 nm to a standard calibration curve, standard in the range from 10 to 200 nmol/4 ml cuvette.

2.4 Lipid Peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) contents (Heath and Packer, 1968); 0.1 gram leaf samples were homogenized in 1 ml of 5% trichloro acetic acid (TCA) and centrifuged at 12,000 rpm for 15 minutes. One ml supernatant was mixed with 1ml of 0.5% TBA in 20% TCA. Solutions were incubated at 95°C for 30 minutes. The mixture was cooled and centrifuged at 7500rpm for five minutes. The absorbance was recorded at 532 nm and 600nm (5% TCA was used as a blank).

2.5 Free Proline Content

A 0.1 g leaf sample was homogenized in 1ml of 3% salphosalicylic acid. The homogenate was centrifuged at 12000rpm for 15 minutes, and the supernatants (2 ml) were mixed with 2 ml acid-ninhydrin solution (1.25 g of ninhydrin, 30 ml of glacial acetic acid, 20 ml of 6M orthophosphoric) and incubated at 100 °C for one hour. After incubation, it was cooled and 3 ml toluene was added. Proline was determined spectrophotometrically at 520 nm using the ninhydrin method.

2.6 Protein Quantification.

Leaf samples were homogenized in a mortar and pestle, and the grinded tissue was mixed with an appropriate amount of PBS buffer. The extract was centrifuged at 12000 rpm for 15 minutes.

Supernatant was used to quantify total soluble protein. The amount of total proteins was quantified using the Bradford assay method (Bradford, 1976).

2.7 Statistical Analysis.

All the data were statically analyzed by two-way ANOVA through SPSS version 17, taking $P < 0.05$, according to the least significant difference (LSD) test.

3. Results and Discussion

Salicylic acid is mostly considered a tolerance enhancing stimulus in plants under unfavorable environmental conditions. Inter-accessional as well as intra-accessional variations, in response to salicylic inducing tolerance for abiotic stresses, are reported. Recently, the scientific community has eagerly explored a mode of salicylic acid aided tolerance mechanisms, especially in cash crops, to fill the yield loss due to global warming. Therefore, in this study, SA was applied exogenously to explore whether; SA could improve thermotolerance in cotton at specific concentrations.

3.1 Seedling Growth Analysis

At the seedling stage, the statistical analysis of data revealed that root length declined significantly ($p \leq 0.01$) due to heat shock although there were differences among the varieties (Table 1).

Table 1

Analysis of variance showing for root length shoot length, fresh to dry shoot weight ratio, relative electrical conductivity (RMP), H_2O_2 , MDA, Proline and total proteins to control and heat shock of cotton plants sprayed with salicylic acid

Variable sources	Root Length	Shoot Length	Dry Weight Ratio	RMP	MDA	H_2O_2	Proline	Total Proteins
Varieties (V)	***	***	***	***	***	***	*	***
Temperature (T)	***	***	ns	***	ns	***	***	***
Concentrations (C)	***	***	ns	***	***	***	***	***
V × T	***	***	**	***	***	***	***	***
C × T	***	***	***	***	***	***	***	***
V × C	***	***	***	***	***	***	***	***
V × C × T	***	***	***	***	***	***	***	***

ns= non-significant *, **, ***= significant at $p \leq 0.05$, 0.01 and 0.001, statistically respectively

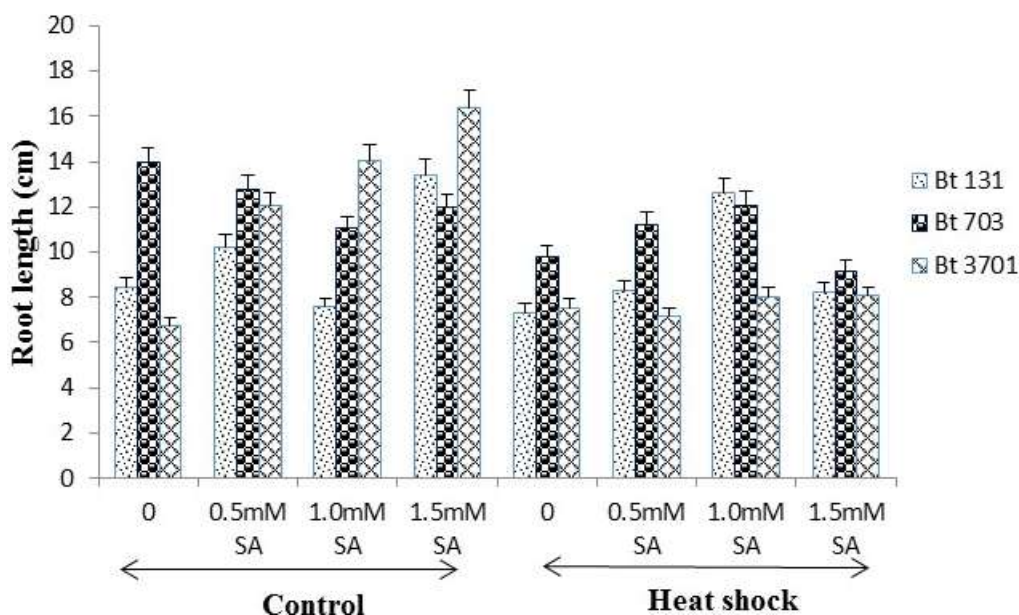


Figure 1: Root length of control and heat shocked cotton plants sprayed with salicylic acid

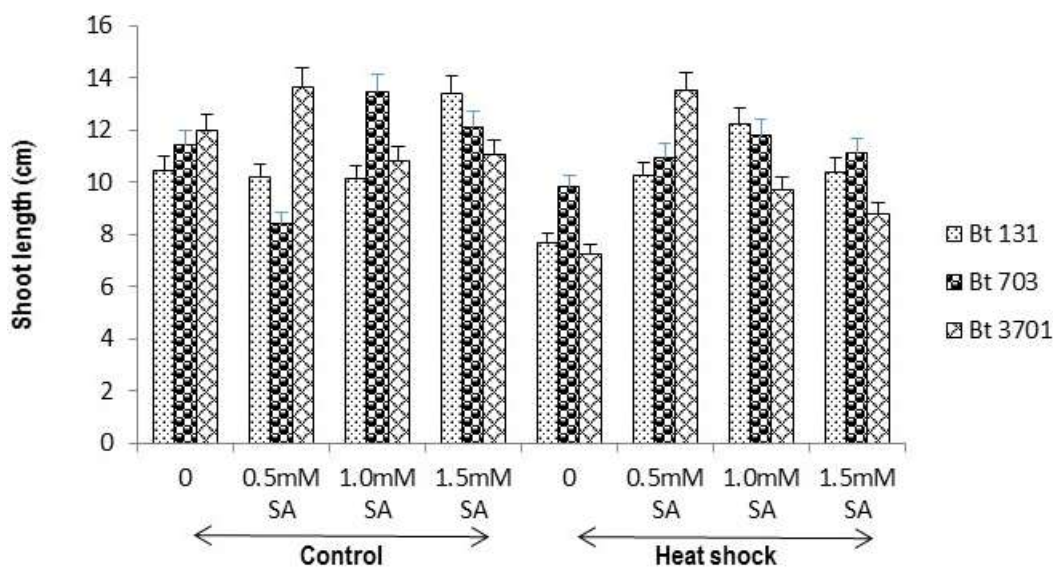


Figure 2: Shoot length of control and heat shock of cotton plants sprayed with salicylic acid

But the exogenous application of salicylic acid at heat stress conditions exhibited relatively increased root length among all varieties. Bt-131 and Bt-703 showed improved behavior at 1.0 mM concentration of salicylic acid coping heat stress (Figure 1) while shoot length increased significantly under heat shock due to application of salicylic acid. Inter-accessional variation of shoot length was not so much different, although there were significant ($p \leq 0.01$) differences for

interaction of varieties, concentration, and temperature (Table 1). Cultivar Bt-3701 showed maximum shoot length at 0.5 mM SA whereas cv. Bt-703 responded at 1.0 mM SA (Figure 2). This means Bt-3701 is more responsive to salicylic acid. Salicylic acid applications overcome damaging effects on growth under saline conditions in barley (El-Tayeb, 2005) and enhanced production of wheat under drought conditions (Singh and Usha 2003). As in our study, salicylic

foliar application improved cotton growth under heat stress.

The shoot fresh-to-dry weight ratio of cotton varieties was least affected by heat stress, having temperature and concentrations with no significant ($p \geq 0.05$) difference, while there were significant ($p \leq 0.01$) differences among interaction of varieties, concentration, and temperature (Table 1), which clearly shows that temperature alone has minimal effects on the fresh-to-dry weight ratio. However, salicylic acid application can have a major difference. Moreover, shoot fresh-to-dry weight ratios were high because of foliar application of SA in “Bt-131” and “Bt-703” under control condition. Pronounced results were observed for foliar application of higher concentration of SA. Bt- 703 exhibited maximum shoot

fresh-to-dry weight ratio at 1.5 mM concentration as compared to the other treatments (Figure 3). Maximum dry weight showed greater photosynthetic area, which means photosynthesis process was high in leaves (Karim et al., 2000; Huve et al., 2006; Suárez and Medina, 2008). This study revealed that heat stress restricts the photosynthetic capacity of cotton, which ultimately leads to altered dry matter production at the seedling stage, while foliar application of SA can trigger the cell division at meristem regions (Villanueva 1992; Vanacker et al., 2001) and exhibited maximum shoot length and root length. Many reports indicate that foliar application of 5-10M SA showed maximum growth of *Brassica juncea* (Fariduddin et al., 2003) and better biomass accumulation in wheat (Hayat et al., 2005) while foliar spray of SA (5-10M) improved root length and biomass (Sandoval-Yepiz 2004).

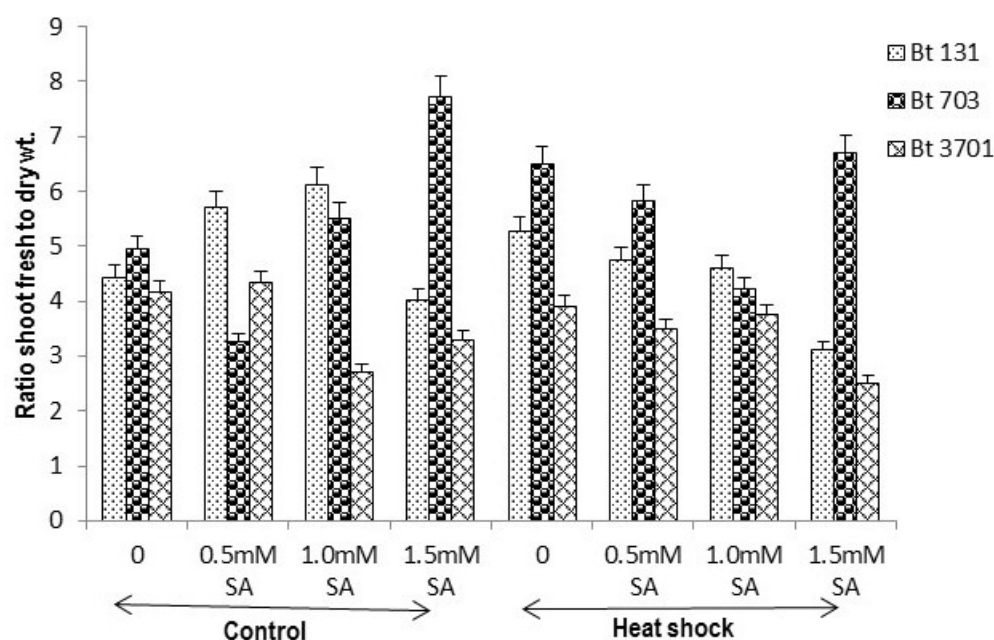


Figure 3: Shoot fresh to dry weight ratio of control and heat shock of cotton plants sprayed with salicylic acid

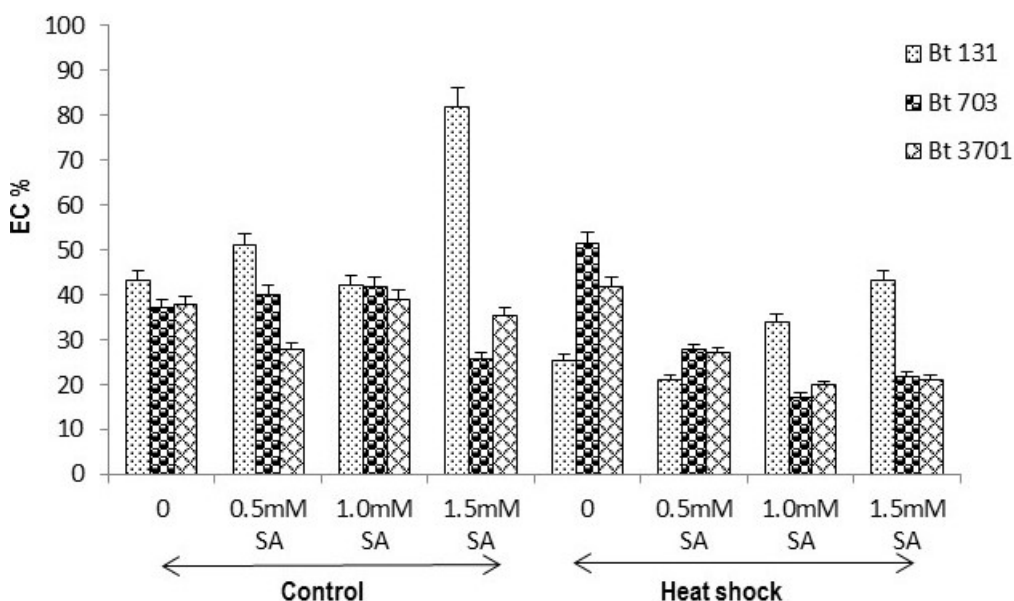


Figure 4: Relative membrane permeability (RMP) of control and heat shocked cotton plants sprayed with different concentration of salicylic acid

3.2 Physiological Analysis

Relative Membrane Permeability (RMP) in terms of electrical conductivity (EC) measure and malondialdehyde (MDA) content was assessed in both control and heat treated, salicylic sprayed and non-sprayed cotton cultivars. The outcomes manifested that heat stress increased membrane permeability under high temperatures in all used cotton cultivars. However, improvement in cell membrane integrity was also observed significantly in SA treated cotton seedlings as compared to non-treated plants (Table. 1). Cotton varieties under the control condition produced low MDA compared to heat shocked plants. The highest MDA accumulation was evident in BT-131, exhibiting severe membrane damage by lipid peroxidation. High temperatures trigger more evapo-transpiration, leading to dehydration stress (Machado and Paulsen, 2001; Mazorra et al., 2002) and associated metabolic changes (Zhu, 2003; Farooq et al., 2009). As a result of these changes, loss of integrity of cellular membranes occurred, causing ion-leakage due to high temperature (Yang et al., 1996). Under heat shock condition, although H_2O_2 was maximum but gradually decreased in all three varieties by the exogenous application of SA. At

1.0 mM SA, H_2O_2 was remarkably increased for Bt- 131, showing maximum oxidative damages (Figure 5). Enhanced MDA and H_2O_2 levels is an indicator of oxidative damage (Sairam et al., 2000; Larkindale and Knight, 2002; Ozden et al., 2009).

In the present study, it is observed that exogenous application of 1.5 mM of salicylic acid had a drastic effect on Bt-703 and Bt-3701 by exhibiting reduced ion leakage (Figure 4) and declined MDA contents and H_2O_2 levels (Figures 5 and 6), leading to the development of thermotolerance. It is reported that SA helps to control membrane leakage, lipid peroxidation, and mineral uptake under stressful conditions (Gunes et al., 2007). Salicylates can promote H_2O_2 levels in plants (Rao et al., 1997, Dat et al., 1998). Therefore, it is evident from results that SA application caused low membrane damage and oxidative stress in cotton under high temperatures by triggering antioxidant metabolism to tolerate oxidative damage (Senaratna et al., 2000; Wang and Li 2006). Antioxidant metabolism capacity for the protection of cotton plants under high temperatures was also reported by Mahan and Mauget (2005).

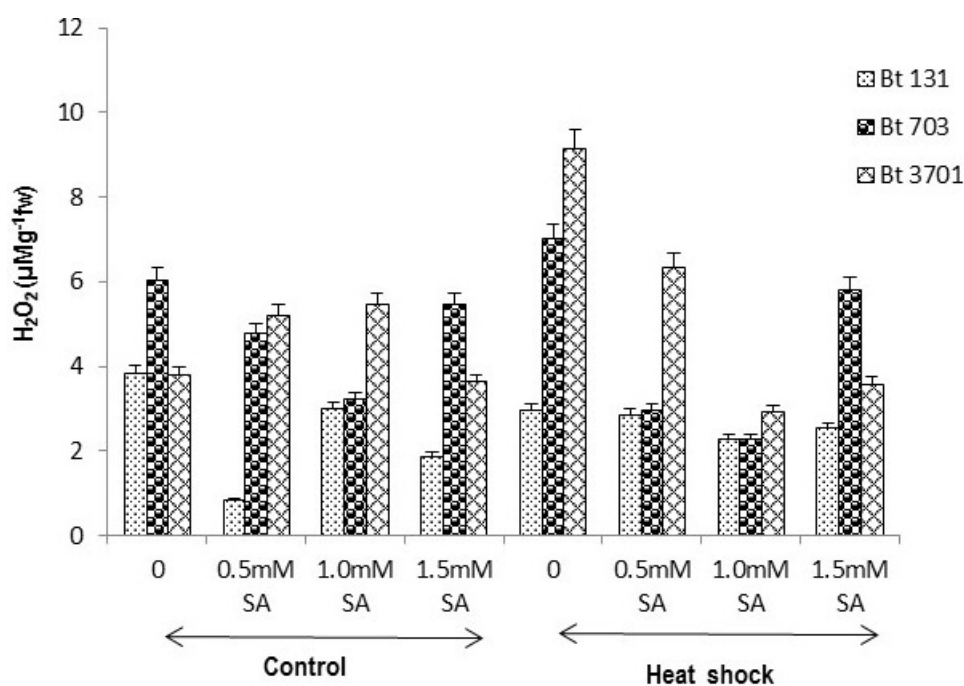


Figure 5: H_2O_2 of control and heat shock of cotton plants sprayed with salicylic acid

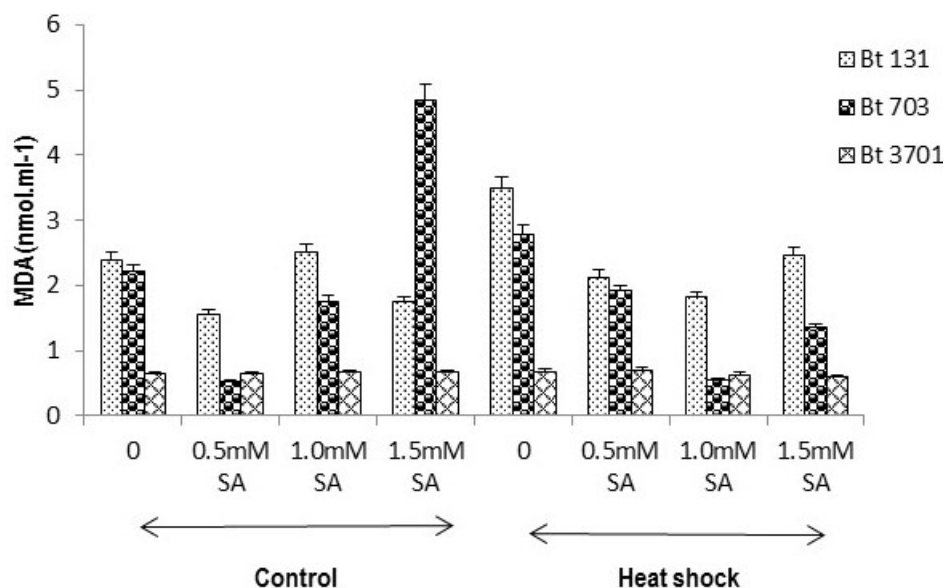


Figure 6: MDA of control and heat shock of cotton plants sprayed with salicylic acid

3.3 Biochemical Analysis

Data showed significant ($p \leq 0.01$) difference in the varieties $\times$ concentration $\times$ temperature and significant interaction of the factors (Table. 1) for proline and protein contents. However, all three cotton varieties exhibit varietal differences under control conditions by exogenous application of SA. Significantly low proline content was observed under heat shock without any SA

spray. Moreover, Bt 3701 and Bt 703 gave maximum proline content at 1.0mM concentration of salicylic acid under heat shock, and it maintained higher content comparatively (Figure 7). Proline accumulated in cells as an osmoprotectant under abiotic stress (Hare and Cress, 1997; Ozden et al., 2009) helps maintain osmotic potential (Wahid, 2007) and stabilize membrane structures to mitigate dehydration losses.

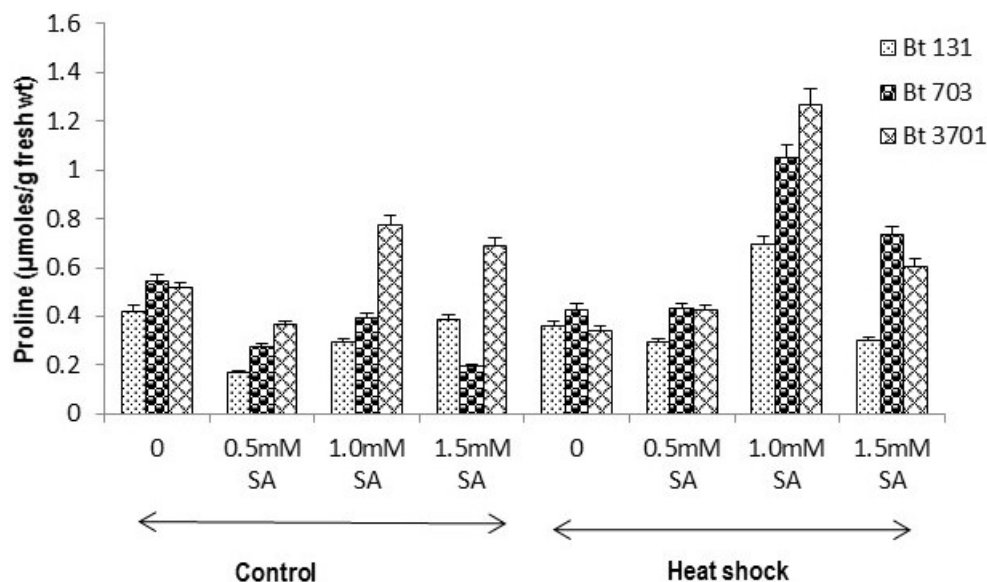


Figure 7: Free proline content of control and heat shock of cotton plants sprayed with salicylic acid

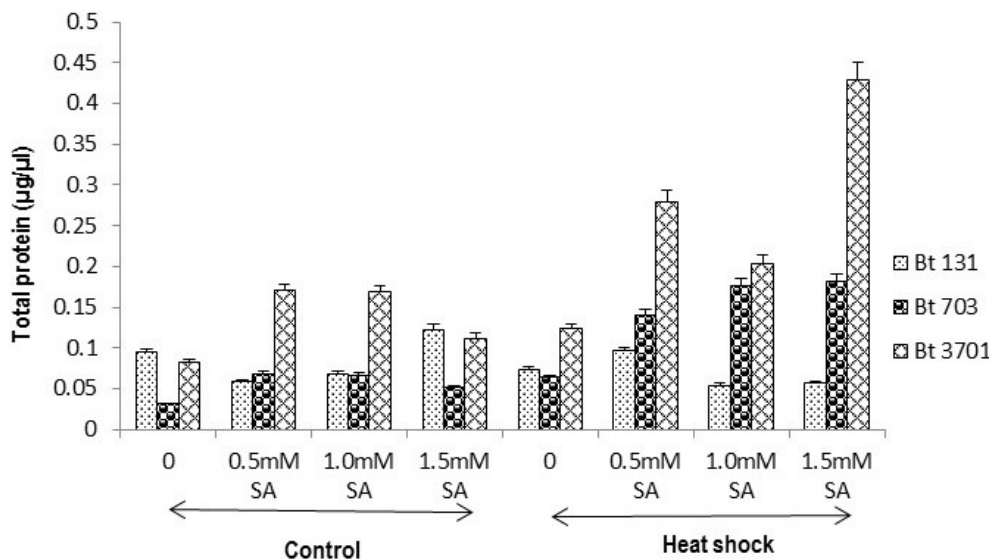


Figure 8: Total protein of control and heat shock of cotton plants sprayed with salicylic acid

As presented in Figure 8, total protein content was increased under heat stress as compared to un-treated control by the exogenous application of salicylic acid. SA treatment under heat stressed and non-stressed conditions further augmented the soluble protein of cotton plants. Significantly high total protein contents were found at 1.5mM concentration of SA under heat stressed condition for Bt-703 and Bt-3701.

4. Conclusion

It is concluded that exogenous application of 1.0 mM SA could be a very promising approach for the development of thermotolerance of cotton to mitigate the damaging effects of heat stress by inducing a wide range of stress tolerance mechanism. It directly induces protection from the cell membrane damage (reduced electrical leakage, H_2O_2 and MDA contents in leaves) and dehydration losses by triggering the antioxidant defense system and free proline and protein accumulation.

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