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Full Length Article

Thiourea Priming Improves Salt Tolerance in Cotton (*Gossypium hirsutum*) by Modulating Gas Exchange, Antioxidant Defense System and Osmoprotection

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Abstract

Excessive salt is one of the many factors contaminating agricultural environments and reducing productivity. In the recent past, several strategies have been investigated to overcome yield losses due to salt stress. However, the effects of thiourea on the morphology, physiology, and yield of cotton plants, have not yet been clearly understood. Therefore, the experiment was done to estimate the effects of 10 and 20 m*M* thiourea application as pre-sowing seed treatment on the growth and antioxidative activities of two cotton varieties (FH-114 and FH-118) grown under normal (0 m*M*) and NaCl stress (150 m*M*). Salt stress significantly reduced the overall shoot length (47%), chlorophyll *a* and *b* (42%, 37%), CO_2 assimilation rate (17%), stomatal conductance (29%), sub-stomatal CO_2 concentration (30%) and seed weight (29%). On the other hand, salinity enhanced the superoxide dismutase (16%), peroxidase (34%), ascorbic acid (47%), glycine betaine (41%), H_2O_2 (24%) and malondialdehyde (33%). The 10 m*M* application of thiourea considerably enhanced plant growth and physiology under salt stress conditions but was more effective in improving the CO_2 assimilation rate (37%), catalase (13%), superoxide dismutase (28%), ascorbic acid (33%), glycine betaine (67%), Ca^{2+} (57%) and K^+ (64%) contents as compared to 20 m*M* thiourea. On the other side, the 20 m*M* application of thiourea was more effective in enhancing shoot length (45%), chlorophyll *a* and *b* (33%, 39%), stomatal conductance (42%), sub-stomatal CO_2 concentration (41%), total soluble proteins (41%), peroxidase (46%), seed weight (40%), number of bolls (43%) and weight bolls (31%). This research offers significant insights into strategies for mitigating the adverse effects of salt stress on cotton, a crop of critical economic importance. Importantly, the differential effects of 10 m*M* and 20 m*M* thiourea application offer practical guidance for optimizing seed priming techniques to enhance stress resilience. Thus, this study contributes valuable knowledge toward sustainable cotton production under saline environments, offering a potential, low-cost agronomic solution to address soil salinization challenges and ensure crop productivity.

Keywords: Cotton; Thiourea; Salinity; Antioxidants; Yield

Introduction

Agricultural productivity is greatly affected due to abiotic stresses (Ahmad *et al*. 2021; Zulfiqar *et al*. 2022, 2023). Salinity is an important environmental stressor that can negatively impact crop growth and development throughout all stages of life cycle, ranging from seed germination to the final harvest (Moukhtari *et al*. 2020). The problem of salinity is especially prominent in dry and semi-dry areas characterized by limited precipitation and high evapotranspiration rates. In such regions, salinity has been found to exert a substantial adverse influence on crop yields and overall agricultural productivity (Jha *et al*. 2019). Globally, salinity affects over 7% of total land area and 20% of arable land, with an annual rate of increase of 1-2% (Rizwan *et al*. 2015). In Pakistan, 23% of arable land is affected by salinity (Haq *et al*. 2010). It has been projected that by 2050, 50% of arable land will become unproductive due to increased toxic ion contents (Bianco and Defez 2009). Salinity stress is causing significant economic losses to the agricultural sector, with a reported loss of over US$ 27.3 billion per year in revenue, thereby exacerbating global food security concerns (Qadir *et al*. 2014). In order to address this challenge, it is crucial to prioritize the development of efficient and tolerant crop varieties that can thrive in infertile soils and saline conditions (Safdar *et al*. 2019). This would enable the exploitation of additional arable land and increased crop productivity, thereby contributing to the overall goal of enhancing food security.

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Cotton (*Gossypium hirsutum* L.) is a significant crop for both fiber and oilseed, accounting for 35% of the world's total fiber production (Abdelraheem *et al*. 2019). As reported by the International Cotton Advisory Committee (ICAC), cotton cultivation spans over 100 countries, supporting the livelihoods of approximately 250 million people, including around 25 million farmers who cultivate it (Voora *et al*. 2023). Likewise, cotton holds immense economic significance for Pakistan and makes a substantial contribution to the country's GDP. According to the Pakistan Cotton Ginners Association (PCGA), Pakistan is the fifth largest producer of cotton in the world, with a production of around 8.32 million bales in the year 2021-22 (GOP 2022).

One of the major environmental stresses affecting cotton production is salinity stress (Maryum *et al*. 2022). It affects various physiological and biochemical processes in cotton plants, which ultimately results in reduced growth and yield (Sharif *et al*. 2019). Salinity stress also affects the accumulation of important nutrients in cotton plants, such as nitrogen, phosphorus, and potassium (Maryum *et al*. 2022). When exposed to salt stress, the concentration of Na^+ and Cl^- tends to increase in cotton leaves, while the levels of K^+, Na^+, and Mg^{2+} decrease. This occurs because there is competition for uptake among these ions, leading to an increase in certain ions while decreasing others (Maryum *et al*. 2022).

Plant growth regulators and secondary metabolites are often applied to enhance salt stress tolerance by modulating metabolic processes (Lohani *et al*. 2020). One such compound is thiourea, which is a water-soluble compound that acts as a stress-alleviating agent by scavenging hydroxyl or superoxide radicals (Sahu 2017). Thiourea treatment has been shown to improve the redox potential, chlorophyll content, and antioxidant activity of leaves under heat stress (Srivastava *et al*. 2017) and to enhance the root and shoot ratio and tolerance index under salinity stress (Pandey *et al*. 2021). Seed priming serves as a promising strategy to enhance plant performance under various abiotic stress conditions. The regulated hydration and dehydration process during priming initiates key metabolic and physiological activities, thereby strengthening the seedling's ability to cope with temperature fluctuations and other environmental challenges (Waqas *et al*. 2019). Thiourea seed priming has been found to increase the concentration of signal molecules, including microRNA and hormones, which can improve crop growth and development under stressful conditions (Srivastava *et al*. 2017). Furthermore, thiourea can maintain the redox equilibrium within plant cells under stressful conditions, helping to maintain the redox potential of the plant (Patade *et al*. 2020).

Previously it has been shown that seed treatment with thiourea can enhance gas exchange attributes and increase photosynthetic pigment contents, leading to improved crop yield (Perveen *et al*. 2015). As a result, using thiourea for seed priming is a promising strategy for mitigating abiotic stress in plants and boosting crop productivity. Priming with thiourea may enhance salt stress tolerance in plants by regulating physiological and biochemical mechanisms associated with stress mitigation. Although the role of thiourea in stress tolerance has been established, specific studies on cotton especially regarding thiourea's role in alleviating oxidative stress under salt stress, are very limited. This study specifically aimed at to compare the differential effectiveness of 10 and 20 m*M* pre-sowing thiourea concentrations in mitigating salt-induced oxidative damage and promoting overall plant health and productivity in cotton.

Materials and Methods

Experiment design and arrangement

The experiment was performed in a greenhouse at University of Agriculture, Faisalabad, Pakistan. The seeds of two cotton varieties FH-114 (FH-925 × Bollgard-1) and FH-118 (FH-925 × NuCot-N33B) were obtained from Ayub Agriculture Research Institute (AARI), having 100% purity. The seeds were cultivated in pots with dimensions of 26 cm × 22 cm × 24 cm. The pots were filled with 8 kg thoroughly washed river sand. In each pot, four seeds were planted, and the thinning was performed at the three-leaf level, leaving only two plants in each pot. Each pot received 1000 mL of complete Hoagland's nutrient solution (Delden *et al*. 2020) weekly, from 3rd week of germination till the crop harvesting, to meet the plants' nutritional requirements. Salt stress was induced once a week by supplementing the full-strength Hoagland nutrient solution (Hamani *et al*. 2020) with 150 m*M* NaCl, starting from the third week after germination and maintained till the crop harvesting. The experimental design followed a completely randomized arrangement with six treatments and four replications. The treatments included a control group (T0), 10 m*M* thiourea treatment (T1), 20 m*M* thiourea treatment (T2), 150 m*M* NaCl treatment (T3), 150 m*M* NaCl with 10 m*M* thiourea treatment (T4) and 150 m*M* NaCl with 20 m*M* thiourea treatment (T5). The pots were watered as needed to maintain adequate moisture levels in the sand to support proper root growth. During the experimentation period, the average temperature, relative humidity, sunshine, wind speed and average rain fall were recorded 31.8°C, 53.9%, 8.7 h, 4.95 km/h and 9.3 mm, respectively based on daily observations collected from the nearest meteorological station.

Morphological parameters

After 6 weeks of sowing, 2 plants with maximum growth from each pot were uprooted carefully without damaging the long roots. Different growth attributes such as length, fresh weight, and dry weights of shoots as well as roots were measured and analyzed. The shoot and root parts of the cotton plants were carefully separated, and their lengths were measured using a measuring tape for accurate measurements. Fresh weight was noted using an electric balance (CBB-600). The plants were subjected to oven drying for 7 days at 60°C.

Physiological and gas exchange parameters

For estimation of the chlorophyll contents (Chl *a*, Chl *b* and carotenoids), Arnon (1949) technique was applied with slight modification. Fresh leaf samples (0.1 g) were finely chopped into small pieces and immersed in 5 mL of 80% acetone solution. The samples were kept at room temperature overnight to allow color extraction. The following day, the absorbance of the extract was measured at 663, 645 and 480 nm using a spectrophotometer (IRMECO, U2020). To estimate the gas exchange parameters such as CO_2 assimilation rate (*A*), transpiration rate (*E*), sub-stomatal CO_2 concentration (C_i), and stomatal conductance (g_s), a portable infrared gas analyzer (LCA-4, ADCADC BioScientific Ltd., UK) was used. From top of the plant, third fully expanded leaf was used for this purpose. Data regarding all parameters were noted 11 am to 1 pm by adjusting leaf chamber settings, *i.e*., 6.9–8.5 mbar vapor pressure, 395.5 mmol m^{-2} s^{-1} air flow, 99.8 kPa pressure of atmosphere, 1722 μmol m^{-2} s^{-1} maximum leaf surface PAR, 337 μmol mol^{-1} ambient CO_2 concentration, 27.2-30.3°C leaf temperature and 24-29°C temperature of ambient environment. The water use efficiency [*A*(CO_2 assimilation rate)/*E*(transpiration rate)] of the plants was also calculated.

Oxidants and antioxidants

To analyze the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content (Cakmak and Harst 1991), fresh plant material weighing 0.25 g was ground in 3 mL of 05% trichloroacetic acid (TCA) solution. The resulting mixture was transferred to Eppendorf tubes and then subjected to centrifugation at 12,000 rpm for 15 min. Following centrifugation, the supernatant was carefully collected to measure the levels of MDA and H_2O_2. One mL of the supernatant was added into 1 mL TBA solution (0.5% thiobarbituric acid) and 5 mL TCA solution (20%); water bathed at 95°C for 15 min. To stop the reaction, the sample solutions were ice bathed for 15 min, and the absorbance was taken at 600 and 532 nm. The hydrogen peroxide (H_2O_2) content was determined according to the method of Velikova *et al*. (2000). A 0.25 g of fresh cotton leaves were homogenized in 5 mL of 0.1% (w/v) TCA solution and centrifuged at 12,000 rpm for 15 min. The reaction mixture containing 0.5 mL of supernatant, 0.5 mL of phosphate buffer and 1 mL of 1 m potassium iodide was vortexed and the absorbance was measured at 390 nm using an IRMECO U2020 spectrophotometer.

For the estimation of antioxidative activities, fresh leaf sample weighing 0.25 g was crushed in a 5 mL phosphate buffer using a mortar and pestle. The crushing process took place in a chilled environment. Subsequently, the mixtures were centrifuged for 15 min at a speed of 12,000 rpm. The supernatant was collected for antioxidants activities determination. Catalase (CAT) and peroxidase (POD) activities were measured by following the method of Chance and Maehly (1955). Superoxide dismutase (SOD) activity was evaluated by digesting the leaves in phosphate buffer and measuring the inhibition of photoreduction of p-nitroblue tetrazolium (NBT) Spitz and Oberley (2001). CAT activity was estimated using a reaction mixture containing 1.9 mL phosphate buffer, 1.0 mL H_2O_2 and 0.1 mL plant extract in each cuvette. The absorbance was recorded at 240 nm at 30-second intervals for 2 min. For POD activity, each sample comprised phosphate buffer of standard volume, 20 m*M* guaiacol, 40 m*M* H_2O_2 and 50 μL plant extract. Absorbance readings were taken at 470 nm every 30 sec for 2 min. SOD activity was assessed using a 2.5 mL reaction mixture containing distilled water, phosphate buffer, L-methionine, Triton-X, nitroblue tetrazolium (NBT), riboflavin and 50 μL plant extract. The mixture was exposed to fluorescent light for 15–20 min. Absorbance was measured at 560 nm at 30 sec intervals for 2 min.

Osmoprotectants

Using the Bradford (1976) technique, the total soluble protein concentration was measured. A 0.1 mL of supernatant (collected by the centrifugation of 0.5 g leaf in phosphate buffer) was mixed into 5 mL of Bradford reagent and vortexed for 30 sec. The absorbance was documented at 595 nm using a spectrophotometer. Glycinebetaine content was quantified according to the method of Grieve and Grattan (1983) with some modifications. Dried leaf powder (0.5 g) was extracted with 20 mL of deionized water at 25°C for 24 h and filtered. 1 mL of the filtrate was mixed with 1 mL of 1 N H_2SO_4, cooled in an ice bath for 1 h, and then 0.5 mL of cold KI_3 reagent was added. After incubation at 0-4°C for 90 min, the mixture was centrifuged and 1 mL of the upper phase was mixed with 4 mL of 1, 2-dichloroethane. The absorbance of the resulting pink layer was measured at 365 nm to estimate the GB concentration. For determination of leaf ascorbic acid content in fresh leaf samples, Mukherjee and Choudhuri (1983) method was employed. To do this, 0.1 g of leaf samples was grinded in 5 mL of 60% TCA solution and centrifuged for 15 min at 12000 rpm. The supernatant was collected and mixed with 1 mL of 2% dinitrophenyl hydrazine solution and a drop of 10% thiourea solution. The sample mixture was placed in the water bath at 90°C for 15 min. After adding 5 mL of 80% sulphuric acid to each sample, an observance was recorded at 530 nm.

Mineral ions

Following the method proposed by Wolf (1982), for each 0.1 g of dried shoot sample 3 mL of sulphuric acid was added. The samples were left in the darkness overnight. The next day, these sample solutions were switched on the hot plate and H_2O_2 was mixed till the sample appeared transparent and the concentration of different ions *i.e*., K^+, Ca^{2+} and Na^+ was estimated using a flame photometer (Sherwood 410).

Yield

Different yield-related parameters were estimated by the method of Rehman *et al*. (2020). The number of bolls/plant was taken by calculating the average number of opened bolls in each replicate. Seed cotton yield was estimated by weighing the opened bolls per plant. The seed weight was obtained by weighing the 100-seed weight of each plant.

Statistical analysis

The experiment was laid out in Completely Randomized Design (CRD) with four replicates per treatment. The data recorded for various analysis were subjected to three-way ANOVA using Statistix software 8.1. Cluster Heatmap and Principal Component Analysis (PCA) were performed by statistical software "R (v. 4.0.1)". Mean treatment values were compared by using Tukey HSD at a 5% significance level. Microsoft Excel (v. 365) was used for creating graphs.

Results

Growth parameters

The roots and shoots length and their total biomass of the cotton varieties reduced more under salinity stress as compared to control conditions (Fig. 1). Salt stress (150 m*M*) reduced the shoot length, fresh, and dry weight by 47, 38, and 50% in FH-114 and by 36, 32, and 53% in FH-118, respectively. Similarly, a decrease in root length was 26.6 and 26.5%, fresh weight was 58 and 34%, and dry weight was 46 and 29% in FH-114 and FH-118. However, the application of thiourea caused a visible improvement in length, fresh weight and dry weights of roots, as well as shoots of both varieties. Application of 20 m*M* thiourea with salt stress showed a more positive effect on the length, fresh and dry weights of FH-114 shoots and roots i.e., 50 and 26, 28 and 35, and 52 and 35% as well as FH-118 shoots and roots *i.e*., 34 and 29, 30 and 35 and 53 and 25%, respectively, as compared to 10 m*M* thiourea treatment (Fig. 1).

Photosynthetic pigments and gas exchange attributes

Photosynthetic pigments and gas exchange attributes were strongly affected by the NaCl treatment in comparison to no treatment plants. The salt toxicity reduced chlorophyll *a* by 42 and 37%, chlorophyll *b* by 37 and 27%, and carotenoids by 52 and 41% in FH-114 and FH-118, respectively. The salinity stress led to a reduction in the total chlorophyll content by 40 and 34% in FH-114 and FH-118, respectively as compared to the control (Fig. 2). Reduction recorded in the *A*, *E*, C_i, and g_s was 17, 20, 29 and 30% in FH-114 and 14, 15, 29 and 15% in FH-118, correspondingly. The *A*/*E* was increased in both FH-114 and FH-118 up to 6 and 2%, respectively, under salinity. The application of thiourea in salt-stressed plants significantly amended photosynthetic

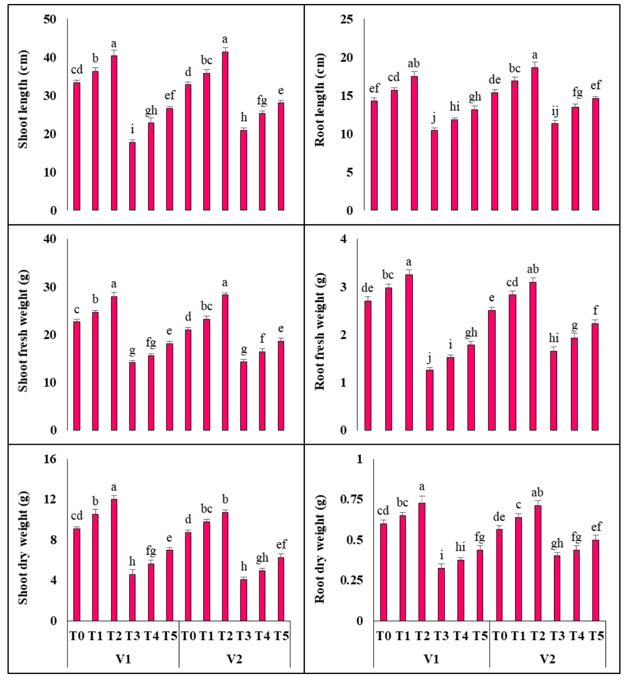

Fig. 1: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on growth attributes of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

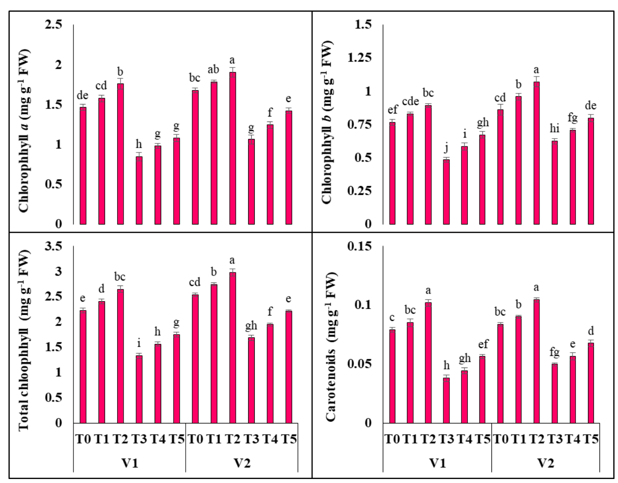

Fig. 2: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on the chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

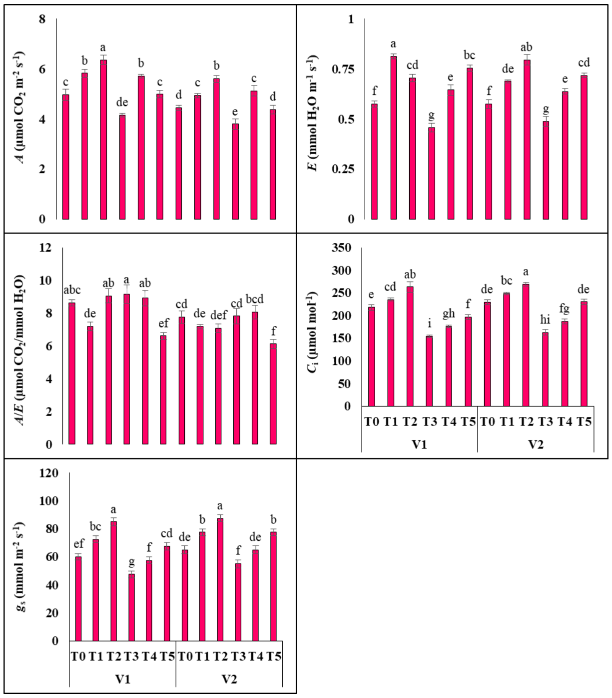

Fig. 3: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on the gas exchange parameters of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

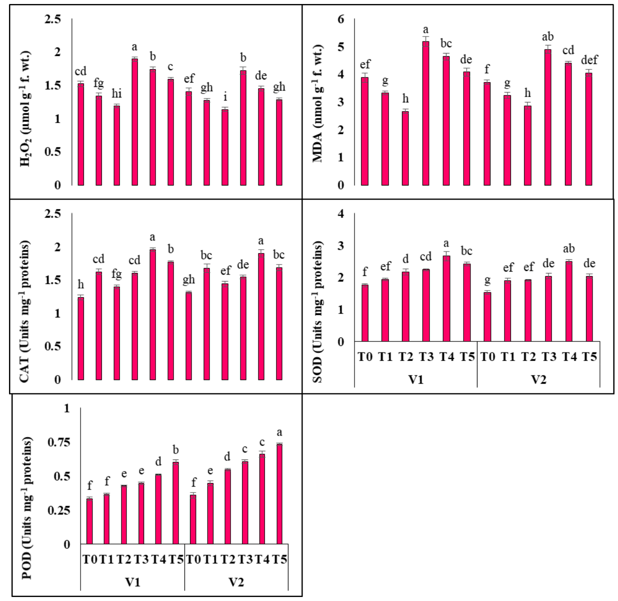

Fig. 4: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on the H_2O_2, MDA, CAT, SOD and POD of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

and gas exchange characteristics of both cotton varieties. Highest chlorophyll *a* (33%), chlorophyll *b* (39%), carotenoids (48%), transpiration rate (65%), sub-stomatal CO_2 (41%) and stomatal conductance (42%) were recorded in 20 m*M* thiourea treatment followed by 10 m*M* thiourea treatment and control conditions (Fig. 3).

Oxidants and antioxidants

The oxidants and the activities of antioxidants were significantly affected by salt stress. Results highlighted that NaCl considerably increased the H_2O_2 and MDA by 24 and 33% in FH-114 and 22 and 32% in FH-118. The thiourea (20 m*M*) treatment was more effective in decreasing the H_2O_2 and MDA content by 16, 25 and 25 and 21% in FH-114 and FH-118, respectively (Fig. 4).

The salt stress increased the antioxidant activities of CAT, SOD, and POD as compared to control plants. The highest activities of CAT, SOD and POD under salt stress, were recorded in FH-118 *i.e*., 1.8, 34 and 68%, respectively. Thiourea treatment showed positive effect on the CAT, SOD, and POD activities in FH-114 cotton plants under salt stress. The improvement was recorded as 1, 8 and 34% under 20 mM thiourea under salt stress. However, in FH-118, the trend in the antioxidant activities of CAT and SOD varied. CAT was reduced by 1% whereas SOD activities were reduced by 0.15% under 20 m*M* thiourea application (Fig. 4). Treating cotton plants with NaCl (150 m*M*) resulted in an increase in the activities of AsA and GB, in both varieties. A highest increase under salt stress in FH-114 was observed for GB i.e., 41% and in FH-118 was for AsA *i.e*., 47% as compared to the control. The application of thiourea further enhanced the GB and AsA contents in both varieties under salinity stress. With 20 m*M* thiourea treatment, FH-114 showed an increase of 35 and 33%, whereas FH-118 experienced an increase of 67 and 19% in AsA and GB, respectively. Treatment of cotton plants with 150 m*M* NaCl showed an increase in the amount of total soluble proteins (TSP). Highest value of 25% was recorded in FH-118 as compared to FH-114 under saline conditions. However, under 20 m*M* thiourea treatment, FH-114 showed an increase of 41% whereas FH-118 experienced an increase of 25% in TSP, respectively (Fig. 5).

Mineral ions

Salt stress increased Na^+ content whereas decreased the K^+ and Ca^{2+} content of the shoots in both varieties. The FH-118 Na^+ content (35%) was higher than in FH-114. The maximum reduction in K^+ and Ca^{2+} content was observed in FH-118 as 40 and 38%, respectively. The application of thiourea reduced the Na^+ and increased the K^+ and Ca^{2+}

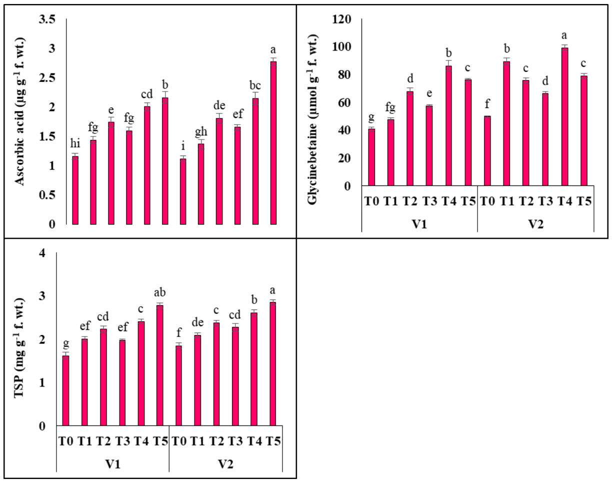

Fig. 5: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on AsA, GB and TSP of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

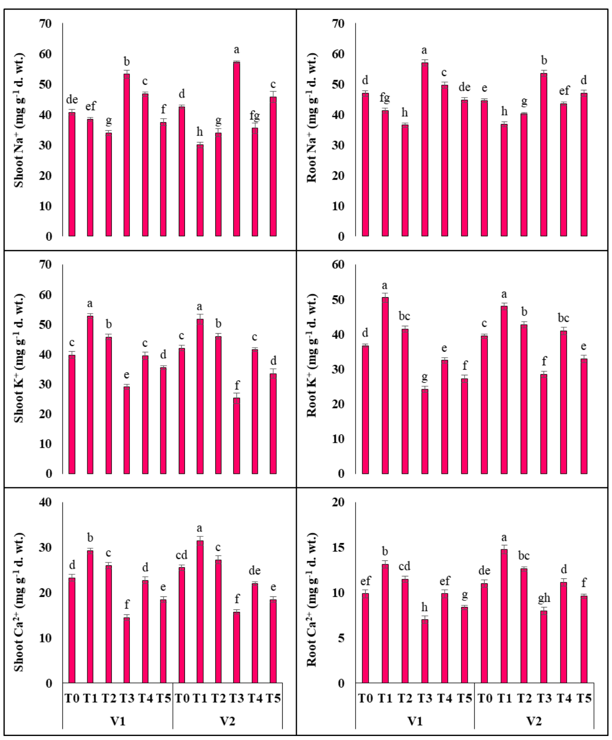

Fig. 6: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on mineral ions of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

contents in both FH-114 and FH-118 shoots. In this regard, the highest improvement was observed under 10 m*M* thiourea in the K^+ content of FH-118 shoots (64%). Likewise, 20 m*M* thiourea application enhanced the K^+ and Ca^{2+} content by 23 and 28% in FH-114 and 33 and 17% in FH-118, respectively. Mineral ions (Na^+, K^+ and Ca^{2+}) also showed significant variations in the roots of the plants under salt stress as compared to the control. Na^+ concentration increased by 21 and 20%, whereas K^+ (34 and 28%) and Ca^{2+} (29 and 27%) concentrations decreased in FH-114 and FH-118, respectively. Thiourea application helped in redeeming the harmful effects of salt stress by reducing the Na^+ and enhancing the K^+ and Ca^{2+} content of the roots of cotton plants. The highest increase in K^+ and Ca^{2+} was observed in FH-118 roots (44% and 39%) under 10 m*M* thiourea treatment (Fig. 6).

Yield attributes

The salinity stress showed a significantly negative effect on the yield-related attributes of both cotton varieties *i.e*., seed weight, number of bolls, and weight of bolls (Fig. 7). Results exhibited that excessive amounts of salts decreased the seed weight, number of bolls and weight of bolls by 29, 30 and 28% in FH-114 and by 28, 28 and 32% in FH-118, respectively. However, the application of thiourea was helpful in enhancing the cotton yield and other yield-related attributes as compared to control and salt stress plants. In this critique, 20 m*M* thiourea was even more effective than the 10 m*M* thiourea treatment. The highest seed weight (40%), the number of bolls (43%) and the bolls weight (31%) were recorded under 20 m*M* thiourea (Fig. 7).

Multivariate analysis

Principal component analysis (PCA) and Pearson correlation matrix: The effect of salt stress treatments was illustrated by principal components analysis in two different cotton varieties (Fig. 8). The Eigenvector closely associated with less distance demonstrate the significant association with positive eigenvalues. The photosynthetic traits responded to more influence on growth and photosynthetic pigments. These traits showed a cumulative variation of PC1 82.6% and PC2 8.8% (91.4%). *A/E* showed a higher positive eigenvalue (± 2.5) and was associated with the PC2 positive side. The biomass production (SFW, SDW, RFW, RDW) were closely clustered with *A* in both of the cotton varieties. These traits were more corresponded with the absolute saline control (0 m*M*). However, the photosynthetic pigments were closely related to g_s and *E* with negative eigenvalues (Fig. 8a). The antioxidants, compatible osmolytes and ions showed a variation of 83.7% for PC1 and PC2. Under a higher salt regime (150 m*M*) with thiourea treatments the antioxidants (POD, SOD, CAT) and organic osmolytes significantly enhanced to mitigate salt influence and strongly associated with each other. However, the level

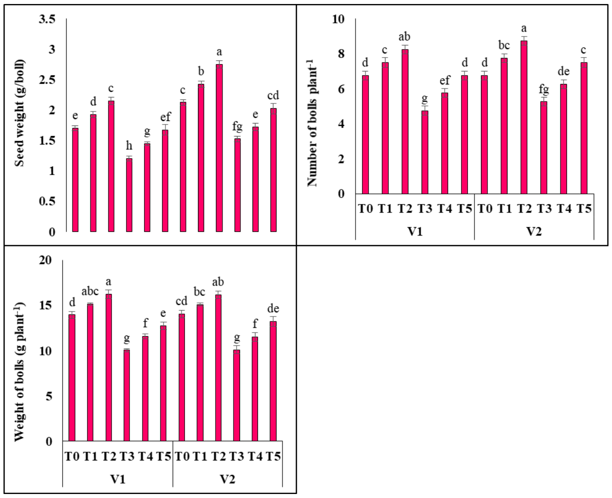

Fig. 7: Impact of salt stress and thiourea application (T0: control; T1: 10 m*M* thiourea; T2: 20 m*M* thiourea; T3: 150 m*M* NaCl; T4: 150 m*M* NaCl + 10 m*M* thiourea and T5: 150 m*M* NaCl + 20 m*M* thiourea) on the seed weight, number of bolls and weight of bolls of the two different cotton varieties *viz*., V1 (FH-114) and V2 (FH-118). The error bars above means represents standard error (± S.E.). For a parameter, means that share same letters do not differ significantly at $P < 0.05$

of ROS (H_2O_2) and lipid peroxidation in terms of MDA and root and shoot Na^+ were decreased under thiourea treatments (Fig. 8b).

The Pearson correlation matrix shows a significant ($P \leq 0.05$) correlation between growth, photosynthesis and antioxidant enzymes under various salt stress and thiourea treatments (Fig. 9). The growth traits (SFW, SDW, RFW, RDW, RL) and pigments (Chl *a*, *b* and Caro.) showed a strong positive correlation with g_s, *Ci, A, E,* R.K, R.Ca, WB, SW and NB. However, these traits exhibited a strong negative correlation with ROS. The ROS also strongly negatively correlated with antioxidants and compatible osmolytes.

Clustered heatmap: The clustered heatmap was constructed to summarize the response of both cotton varieties under salt stress with counterpart treatments of thiourea (Fig. 10). The traits CAT, SOD, GB, POD, AsA, *A/E,* R.NA, S.NA and ROS were strongly linked with high salt stress (150 m*M*) and thiourea (T1, T2, T3). This indicated a positive influence of thiourea under saline conditions in both cotton varieties. However, all of these traits corresponded negatively toward biomass production and photosynthetic pigments. These traits corresponded positively in both genotypes under S1 (0 m*M* NaCl) and thiourea treatments. The traits CAT, SOD, GB, POD, AsA, *A/E,* R.NA, S.NA and ROS were positively associated with high salt stress (0 m*M*) and thiourea [T1 (0 m*M* TU), T2 (10 m*M* TU) and T3 (20 m*M* TU)].

Discussion

Plants encounter numerous challenges throughout their

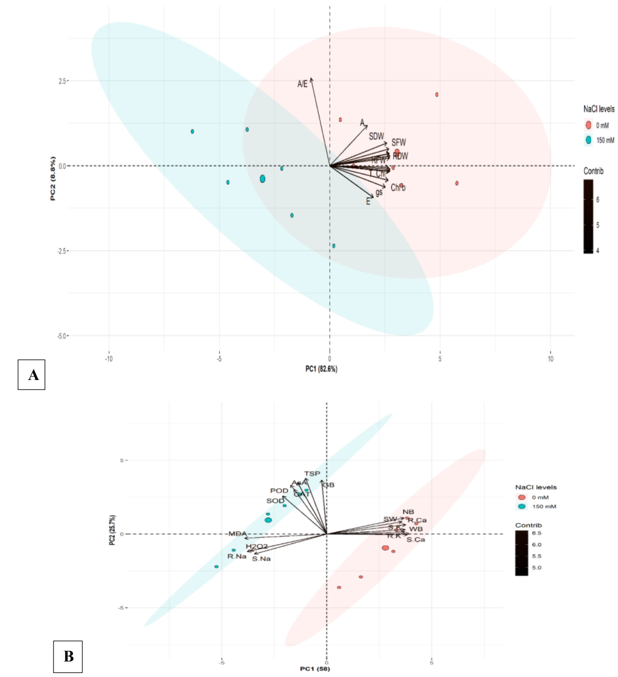

Fig. 8: (A) PCA biplot for growth, chlorophyll contents, gas exchange and photosynthetic pigments. **(B)** Root and shoot ions, osmoprotectants, ROS and antioxidant enzymes of two cotton genotypes under salt stress and thiourea treatments. SFW = shoot fresh weight, SDW = shoot dry weight, SL = shoot length, RFW = root fresh weight, RDW = root dry weight, RL = root length, A = carbon dioxide assimilation rate, E = transpiration rate, Ci = sub-stomatal CO_2 concentration, gs = stomatal conductance, A/E = water use efficiency, CAT = catalase, POD = peroxidase, SOD = Superoxide dismutase, H_2O_2 = hydrogen peroxide, MDA = malondialdehyde, AsA = ascorbic acid, GB = glycinebetaine, TSP = total soluble protein, R.Ca = root calcium, R.K = root potassium, R.Na = root sodium, S.Ca = shoot calcium, S.K = shoot potassium, S.Na = shoot sodium, WB = weight of bolls, SW = seed weight, NB = number of bolls

development as a result of climate change, which negatively affects their growth. Among these challenges, salinity stress is recognized as a major factor limiting plant development (Imran *et al*. 2022). Globally, salt stress has been shown to substantially reduce both the growth and yield of cotton crops (Chaudhary *et al.* 2024; Zhang *et al.* 2024). The results obtained from this study provide validation to our hypothesis that the application of thiourea has a mitigating effect on the adverse consequences of salinity on the growth and yield of cotton. In the current study, salt stress reduced the shoots/root length, fresh and dry weights of both roots and shoots, of both cotton varieties (Fig. 1). It is well-established fact that a majority of cotton roots are concentrated within the upper 35-45 cm of soil, and that excessive salt concentration within this root zone ultimately impairs plant growth (Yang *et al.* 2017). Overall reduction in growth is also attributed to the toxic effects of salinity on photosynthesis, nutrient balance and antioxidant activities (Talaat and Shawky 2014; Sumaiya *et al.* 2020). The studies

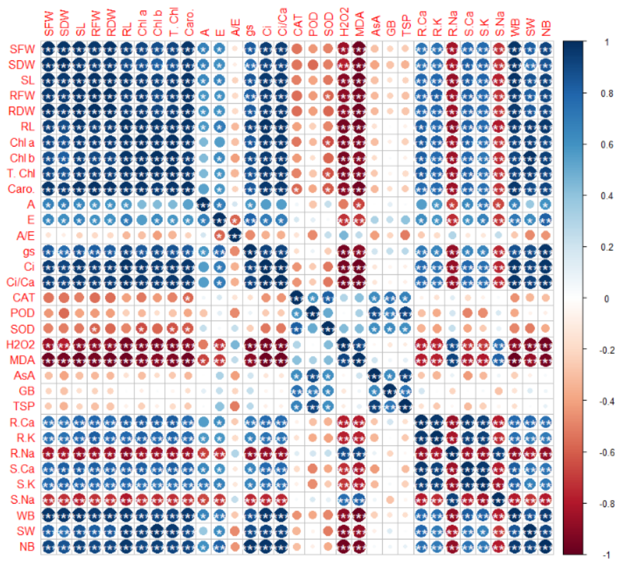

Fig. 9: Pearson correlation of two cotton genotypes under salt stress and thiourea treatments. SFW = shoot fresh weight, SDW = shoot dry weight, SL = shoot length, RFW = root fresh weight, RDW = root dry weight, RL = root length, A = carbon dioxide assimilation rate, E = transpiration rate, Ci = sub-stomatal CO_2 concentration, gs = stomatal conductance, A/E = water use efficiency, CAT = catalase, POD = peroxidase, SOD = Superoxide dismutase, H_2O_2 = hydrogen peroxide, MDA = malondialdehyde, AsA = ascorbic acid, GB = glycinebetaine, TSP = total soluble protein, R.Ca = root calcium, R.K = root potassium, R.Na = root sodium, S.Ca = shoot calcium, S.K = shoot potassium, S.Na = shoot sodium, WB = weight of bolls, SW = seed weight, NB = number of bolls

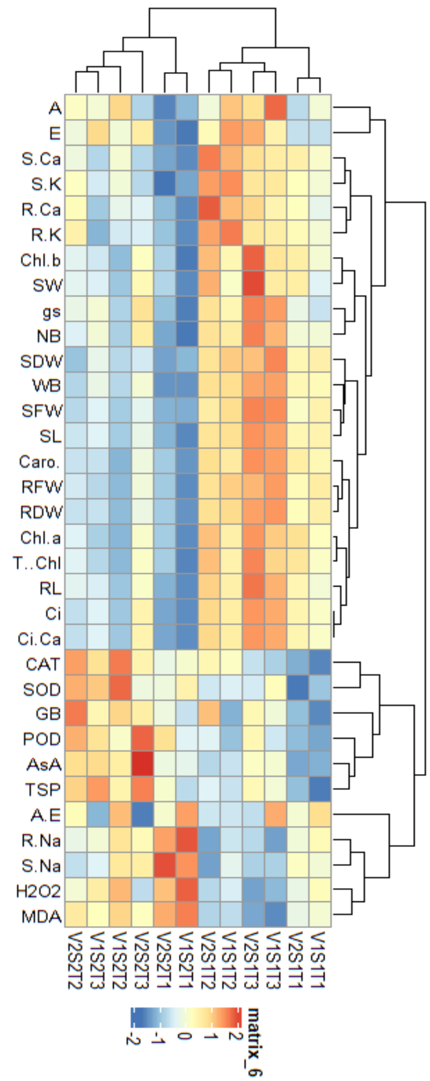

Fig. 10: Clustered heatmap among growth, chlorophyll contents and gas exchange, osmolytes, ROS and antioxidant enzymes of two cotton genotypes under salt stress and thiourea treatments. V1- Variety 1, V2- Variety 2, S1- 0 m*M*, S2- 150 m*M*, T1- 0 m*M*, T2- 10 m*M*, T3- 20 m*M*. SFW = shoot fresh weight, SDW = shoot dry weight, SL = shoot length, RFW = root fresh weight, RDW = root dry weight, RL = root length, A = carbon dioxide assimilation rate, E = transpiration rate, Ci = sub-stomatal CO_2 concentration, gs = stomatal conductance, A/E = water use efficiency, CAT = catalase, POD = peroxidase, SOD = Superoxide dismutase, H_2O_2 = hydrogen peroxide, MDA = malondialdehyde, AsA = ascorbic acid, GB = glycinebetaine, TSP = total soluble protein, R.Ca = root calcium, R.K = root potassium, R.Na = root sodium, S.Ca = shoot calcium, S.K = shoot potassium, S.Na = shoot sodium, WB = weight of bolls, SW = seed weight, NB = number of bolls

have revealed that elevated salinity in the plant growing regions, significantly reduces growth and productivity of several important crops like wheat (Saddiq *et al.* 2021), rice (Jan *et al.* 2019) and maize (Zahra *et al.* 2020). Among the different treatments evaluated, the application of 20 m*M* thiourea emerged as the most effective in alleviating the adverse impacts of salinity, leading to marked improvements in the root and shoot development of cotton plants (Fig. 1). Thiourea, a compound rich in nitrogen and sulfur, serves dual functions as a fertilizer and a plant growth regulator. By alleviating salt-induced stress, thiourea promotes improved seed germination and supports enhanced overall plant growth (Wahid *et al.* 2017; Patade *et al.* 2020). TU application improves the absorption and assimilation of essential nutrients such as nitrogen and phosphorus, which are critical for plant growth and yield. This nutrient optimization helps improve plant vigor under stress (Waqas *et al.* 2019).

The present study demonstrated that NaCl-induced stress adversely affected the photosynthetic parameters and gas exchange characteristics in both cotton varieties, FH-114 and FH-118 (Fig. 2–3). A decline in chlorophyll content is likely attributed to an increased activity of chlorophyllase enzyme under saline conditions, which promote degradation of chlorophyll pigments (Hamani *et al.* 2020), consequently inducing cellular oxidative damage (Jiang *et al.* 2017; Sumaiya *et al.* 2020). Salt stress causes osmotic stress leading to a disturbance in nutrient uptake which negatively affects the stomatal opening, CO_2 concentration and photosynthesis rate (Ren *et al.* 2021). In the present study, salinity stress led to a decline in chlorophyll content, whereas the foliar application of thiourea improved chlorophyll levels by strengthening the source-to-sink relationship and enhancing osmoregulation, as already reported in tomato varieties (Majeed *et al.* 2024). These results are consistent with increase in growth attributes. Thus, thiourea shows potential for use in exogenous treatments to alleviate abiotic stresses such as salinity. This thiourea-based improvement in photosynthetic pigments and gas exchange attributes has already been cited in wheat (Sher *et al.* 2021), maize (Shoukat *et al.* 2022) and camelina (Ahmad *et al.* 2021). Thiourea enhances metabolic activities and delays chloroplast aging, thereby promoting increased chlorophyll production (Pandey *et al.* 2013; Zahra *et al.* 2018). In addition, TU acts as a reactive oxygen species (ROS) scavenger, alleviating oxidative stress that normally degrades chlorophyll molecules under saline conditions (Li *et al.* 2024).

Plants exposed to toxic amounts of salts accumulate Na^+ and Cl^- reducing the uptake of other essential minerals by the roots. This disturbs the plant-water relationship and leads to the overproduction of reactive oxygen species (Kamran *et al.* 2019; Jalil and Ansari 2020). In this study, NaCl treatment significantly elevated the levels of H_2O_2, and MDA in cotton seedlings (Fig. 4). An elevated H_2O_2 and MDA contents suggested that excessive production of reactive oxygen species under salt stress caused oxidative damage to membrane lipids (Jiang *et al.* 2020). The production of oxidants (MDA and H_2O_2) during salinity stress is directly related to the generation of enzymatic and non-enzymatic antioxidants (Hameed *et al.* 2021; Nawaz *et al.* 2021). Antioxidants together hunt out the oxidants and protect plants from oxidative damage (Hasanuzzaman *et al.* 2020). SOD work as the first line defense and transforms the O_2^- to H_2O_2 which is further detoxified by ascorbic acid (Liebthal *et al.* 2018). The antioxidative activities are enhanced under salt stress as an endogenous defense mechanism (Bhantana *et al.* 2021; Javed *et al.* 2021). Application of thiourea results in lower levels of H_2O_2 and MDA, reflecting a reduction in lipid peroxidation and protection against membrane damage due to enhanced antioxidant activity (Uzma *et al.* 2025). By modulating the redox state, TU helps maintain cellular homeostasis, which is vital for plant survival under saline conditions. Moreover, AsA, GB and soluble proteins also play a major role to shield plants against salts stress (Athar *et al.* 2022). Similar reports indicated an increase of ascorbate activity in wheat (Rady *et al.* 2019), total soluble proteins, ascorbic acid and glycine betaine contents in maize (Perveen and Hussain 2021), enzymatic and non-enzymatic antioxidants in *Brassica juncea* (Khator and Shekhawat 2020), and enzymatic antioxidants in cotton (Farooq *et al.* 2022). The present findings also showed an increase in metabolites such as glycine betaine, total soluble proteins and ascorbic acid in cotton, highlighting responses that differ from previously reported traits in this crop (Fig. 5). Although salinity stress elevates enzymatic antioxidant levels, non-enzymatic antioxidants such as ascorbic acid (AsA) further enhanced the activities of superoxide dismutase, CAT and POD. This increase was accompanied by a reduction in malondialdehyde content under salt stress. These findings suggest an improved antioxidant defense with thiourea contributing to salt tolerance in plants (Hamani *et al.* 2020).

Under salt stress, excessive amounts of toxic ions (Na^+ and Cl^-) impair the K^+ and Ca^{2+} uptake, thus resulting in nutrient imbalances (Joshi *et al.* 2022). An increase in Na^+ triggers the activation of NHX-type Na^+/H^+ antiporters (NHXs) present on the plasma membrane, promoting the removal of excess Na^+ from the cytoplasm or its storage in vesicles. This process also facilitates the release of K^+ from the vesicles, thereby helping to maintain the Na^+/K^+ balance within the cytoplasm (Bassil and Blumwald 2014). Thiourea regulates the uptake of mineral ions and their storage to resist the changes that occur during stressful environment (Choudhary *et al.* 2017; Waqas *et al.* 2019). This improvement in the uptake of K^+ and Ca^{2+} after the thiourea treatment was observed in both cotton varieties (Fig. 6). These outcomes are in line with the other studies done on coriander (Meena *et al.* 2015), cluster beans (Meena *et al.* 2016) and *Vigna mungo* (Choudhary *et al.* 2017). Previous studies show that TU can enhance K^+ uptake in plants experiencing salinity stress, thereby helping to maintain Na^+/K^+ balance (Pandey *et al.* 2021; Li *et al.* 2024). In this experiment, we observed a substantial increase in Na^+ content in cotton leaves when exposed to salinity stress. Simultaneously, K^+ levels significantly decreased under the same conditions, indicating a disruption in the Na^+/K^+ balance. However, the application of TU resulted in a reduction of Na^+ accumulation and a lesser decline in K^+. These results imply that TU can effectively limit Na^+ absorption and boost K^+ uptake, helping to restore Na^+/K^+ balance in wheat under saline stress. Another reason for this improved ion balance could be an enhanced overall water status of the plant due to thiourea application (Kunrath *et al.* 2018). Thiourea application promotes the activation of potassium transport mechanisms and lowers the Na^+ buildup by regulating the activity of sodium transporters, thereby enhancing the uptake of K^+ ions and improving the plant's capacity to compartmentalize Na^+ ions respectively (Liu *et al.* 2019). Furthermore, TU application enhances the uptake of essential nutrients such as N, P and K (Korat *et al*., 2020). It additionally supports the optimization of the photosynthetic apparatus (Seleiman and Kheir 2018). The concurrent enhancement in the uptake of N, P, K^+ and Ca^{2+}, along with a reduction in Na^+ content, indicates that thiourea application facilitates salt exclusion mechanisms in plants under salinity stress.

Salt stress adversely affects the water status, minerals uptake, photosynthetic pigments, and the growth of plants, which ultimately reduces the overall crop yield (Helaly *et al*. 2017; Abdelaal *et al.* 2020). A significant reduction in the number of bolls, their weight, and seed weight was observed under salinity in this study (Fig. 7). These results are closely related to already reported findings of Ren *et al.* (2021), Zhang *et al.* (2021) and Zafar *et al.* (2022) and on cotton under saline conditions, but the novelty of current study lies in the specific genotypes evaluated, the salinity levels tested, and the physiological and biochemical parameters studied under controlled and stress conditions. Thiourea can provide tolerance against salinity in different ways (Waqas *et al.* 2019). At the physiological level, TU application has been shown to enhance photosynthetic activity and assimilate translocation, thereby improving source–sink dynamics and contributing to better crop productivity under salinity stress (Waqas *et al.* 2019). At molecular level, it regulated cellular energy metabolism, coordinated Ca and ABA signaling, and maintained water balance. Additionally, TU strengthened antioxidant defense mechanisms and optimized sulfur assimilation pathways. Thiourea-based increase in the yield of lentils (Premaradhya *et al.* 2018), maize (Waqas *et al.* 2017) and wheat (Seleiman and Kheir 2018) have already been studied.

At lower concentrations (*e.g.*, 10 m*M*), thiourea can promote growth parameters due to enhanced cell division and protection against mild oxidative stress (Srivastava *et al.* 2011). However, at higher concentrations (*e.g.*, 20 m*M*), it may be more effective in inducing stress-related responses such as proline accumulation and antioxidant enzyme activity due to stronger stimulation of plant defense systems (Rady *et al.* 2013; Ishfaq *et al.* 2024). This biphasic or hormetic effect of thiourea is well documented in plant stress physiology. Therefore, the observed changes in optimal concentrations under different parameters reflect the complexity of plant responses to exogenous chemical treatments.

The PCA demonstrated that growth parameters as well as photosynthetic parameters have similar pattern of variation, which showed an inclined regarding these parameters on PC1 (Fig. 8). It shows that as a result of any environmental stimulus such as salt stress, these parameters are affected simultaneously, a trend also emphasized by Hussein (2014). The other set of ionic and oxidative pattern were deviated towards PC2, indicating that this pattern of change was different in relation to growth and photosynthetic traits. The same trend in ionic and stress related parameters were reported by Abbas *et al.* (2025). This suggested that salt stress disrupted ion homeostasis and induces oxidative damage. Antioxidants were negatively correlated with stress (Sanaullah *et al.* 2016) as identified by correlation matrix. The link between photosynthesis and productivity was highlighted by positive correlation and similar trends were also explained by (Anjum *et al.* 2011). In addition to PCA and correlation, cluster heat map elaborated the overall variation pattern, which explains that high salt concentration enhanced the stress markers (MDA and H_2O_2) suppressed the growth and photosynthesis (Perveen *et al.* 2016). The present study advocates that application of thiourea mitigated these effects of salt stress and exhibited higher stress tolerance by enhancing the osmoprotectants (GB and TSP) and antioxidant activity (Faryal *et al.* 2022). Collectively, these analyses elucidated thiourea's role in ameliorating salt stress by modulating ionic balance, oxidative defense, and metabolic pathways, with varietal-specific responses to acquire the resilience. Collectively, these analyses elucidated thiourea's role in ameliorating salt stress by modulating ionic balance, oxidative defense, and metabolic pathways, with varietal-specific responses to acquire the resilience (Fig. 9-10).

Conclusion

Salt stress considerably reduced the plant growth and development, while exogenous thiourea application positively influenced the growth, physiology and yield-related attributes of salinity affected cotton plants and also improved the antioxidative properties in cotton plants. Of the two varieties, FH-118 performed better than FH-114, which may be attributed to its enhanced gas exchange characteristics, higher amount chlorophyll pigments and stronger antioxidant defense under salt stress. With 20 m*M* thiourea pre-sowing treatment, morphological traits (growth), photosynthetic traits and yield showed better results particularly in FH-118. On the contrary, 10 m*M* thiourea enhanced enzymatic antioxidants (SOD, CAT and POD) and AsA and GB in FH-118, compared to other variety of cotton studied in current experiment. This study revealed a novel, variety-specific response of cotton to different thiourea concentrations under salt stress, enabling targeted optimization for effective stress mitigation. Additionally, these findings offer a practical and cost-effective strategy to enhance salt tolerance and productivity in cotton, contributing to sustainable crop management in saline environments.

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Author Contributions

ZR and MS planned the experiment, analyzed the data and write original draft.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

All data generated or analyzed during this study are included in this article and will be provided on demand.

Ethics Approval

Not applicable for this paper.

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