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RESEARCH ARTICLE

Identification of the responsiveness of some enzymes of the antioxidant system of the biotechnological cotton variety to salt stress

Nodira R Rakhmatova, Azadakhan S Imamkhodjaeva, Venera S Kamburova, Ilkhom B Salakhutdinov, Farkhod S Radjapov, Shakhnoza H Mamatkulova, Orastabonu L Isomiddinova, Dilshod E Usmanov, Jurabek K Norbekov, Khurshida A Ubaydullaeva, Adkham N Abdullaev, Shukhrat O Kushakov, Shakhnoza B Kadirova, Naim N Khusenov, Maftuna M Kholmuradova, Feruza I Babadjanova, Bakhtiyor K Rakhmanov*& Zabardast T Buriev

Center of Genomics and Bioinformatics, Academy of Sciences of Uzbekistan, University Street 2, Tashkent 111 215, Uzbekistan

*Correspondence email - bakhtiyor.rakhmanov@gmail.com

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Abstract

In this article, we studied the responsiveness of the antioxidant system of the biotechnological cotton variety to salt stress. We compared the reaction of two salt-resistant cotton varieties to elevated concentrations of sodium chloride (NaCl) and sodium sulfate (Na_2SO_4). To obtain more complete information about the resistance of the biotechnological variety to salt stress, we analyzed the activity of certain enzymes in the antioxidant system as well as the level of malondialdehyde (MDA). Plants respond to salt stress through the highly regulated protective enzymes such as MDA and superoxide dismutase (SOD), which enhance their ability to neutralize reactive oxygen species (ROS). Such abiotic stressors disrupt the ionic and osmotic balance of the cells. In this study, the activity of the antioxidant system of cotton (MDA and SOD) was determined in the leaves of seedlings grown in the laboratory under saline conditions. The stress resistance of plants and the accumulation of antioxidant enzymes in cotton have been thoroughly investigated in various experiments. The plant material used in this study was the Porloq-4 cotton variety, developed through individual selection of lines obtained by crossing the RNAi Coker-312 line with the commercial Namangan-77 variety. The object of this study was the modified Porloq-4 cotton genotype, while the parental Coker-312 genotype served as the control. Findings indicate that Porloq-4 and Coker-312 cotton varieties respond differently to stress induced by NaCl and Na_2SO_4 and their combination, with Porloq-4 exhibited a more active and responsive antioxidant enzyme system.

Keywords: abiotic stress; malondialdehyde; metabolomics; saline conditions; superoxide dismutase

Introduction

Salt resistance is defined as a plant's ability to grow, develop and reproduce under saline conditions with minimal damage. Every organism functions as a self-regulating system. To minimize oxidative damage caused by ROS production during salt stress, plants employ a complex system of interrelated mechanisms, including antioxidant enzymes, osmolytes and non-enzymatic antioxidants (1). To mitigate the adverse effects of salt stress on plants, two complementary approaches are commonly used: the development of salt-resistant varieties and the improvement of agrotechnological techniques (2, 3).

To obtain more comprehensive information about the biotechnological variety's tolerance to salt stress, we analyzed the activity of specific enzymes in the antioxidant system and the level of MDA. Plants respond to salt stress through highly regulated protective enzymes such as MDA and SOD, which enhance their capacity to neutralize ROS. Prolonged exposure to high salinity leads to the accumulation of toxic ions, such as sodium (Na^+), chloride (Cl^-) and sulfate (SO_4^{2-}), which induce ion toxicity and impair nutrient absorption, thereby exacerbating damage to plant cells and tissues (4). The detrimental effects of salt stress on plants are manifested at many levels: morphology (stunting, chlorosis, impaired seed germination), physiology (inhibition of photosynthesis and nutrient imbalance) and biochemistry (oxidative stress, electrolyte leakage and membrane disorganization) (5, 6). The adverse effects of salinity are particularly severe during the reproductive stage (7). Over the past 20 years, many studies have investigated the harmful effects of salinity on plants. Increasing plant tolerance to salt stress is a primary focus in the genetic improvement of agricultural crops. The study of salt tolerance is a crucial component of plant biology research, as it helps to understand the complex mechanisms of plant salt tolerance and explore strategies to mitigate the harmful effects of salt stress (8).

Objective of the study was to identify changes in certain metabolic parameters of Porloq-4 cotton seedlings under comparative conditions in a model experiment assessing the influence of chloride and sulfate salt solutions. Specifically, we aimed to determine responsiveness of the biotechnological Porloq-4 cotton variety, at the metabolite level, to abiotic stress in the form of salinization.

In this study, we examined the effects of NaCl and Na_2SO_4 solutions at low, medium and high concentrations, both separately and in combination, on the total chlorophyll index, MDA as well as the activity of Porloq-4 at the early stage of vegetation, in comparison with the Coker-312 variety.

Materials and Methods

Plant materials

Cotton variety

The material for this study was the Porloq-4 cotton variety, developed by individually selecting lines obtained from a cross between the RNAi line Coker-312 and the commercial Namangan-77 variety.

Growing conditions

To study the salt resistance of Porloq-4, plants were treated with Na_2SO_4 solutions at concentrations of 2.0 %, 2.5 % and 5.0 %. The work was carried out under laboratory conditions. Seed germination in soil was conducted according to standard procedures (9, 10). The soil was pre-washed and dried. Small pots with drainage were filled with a soil-sand mixture in a 70 : 30 ratio. The experiment was performed using four concentrations of the stress factor (salt) with three replications. Ten cotton seeds were sown in each replication. Small pots, containing 250 g of soil were watered daily with 50 mL of water. During sowing, the laboratory temperature was maintained at 25-28 °C. In all treatments, watering with NaCl solution (100-200 mM) lasted 21 days, until seedlings reached the two-true-leaf stage, while Na_2SO_4 solutions (concentration 1.5 %, 2.0 % and 2.5 %) were applied for 30 days during the vegetative stage. Additionally, an experiment was conducted to study the effect of Na_2SO_4 salt solutions at three concentrations on cotton seedlings at the four-leaf stage.

In this experiment, the stress factors were chloride and sulfate salts dissolved together in a single solution (a complex solution), as is most often found in nature. This choice was based on their known toxicity to most plants. Three treatment options were selected for the combined use of these salts.

1. Medium concentration of chloride salt and low concentration of sodium sulfate- representing a low total solution concentration (150 mM NaCl + 1.5 % Na_2SO_4)

2. High concentration of chloride salt and low concentration of sodium sulfate- representing a medium total solution concentration (200 mM NaCl + 1.5 % Na_2SO_4)

3. High concentration chloride salt and sodium sulfate- representing a high concentration of the total solution (200 mM NaCl + 2.0 % Na_2SO_4).

Malondialdehyde (MDA) determination

The amount of MDA in plant samples was determined according to a modified protocol (11). Briefly, 100 mg of plant material was homogenized in 2 mL of 20 % trichloroacetic acid (TCA), with 0.5 mL additives. The homogenate was centrifuged at 10000 g for 15 min at 4 °C. Then, 1.5 mL of 0.5 % thiobarbituric acid (TBA) dissolved in 20 % TCA was added to 0.5 mL of the supernatant. The experimental samples were incubated in a water bath at 95 ° C for 30 min. To account for the effect of the extracts' inherent coloration, a control was prepared without TBA, in which 1.5 mL of 20 % TCA was added to 0.5 mL of the supernatant.

After incubation, the samples were cooled in an ice bath and absorbance measurements of both control and experimental samples were carried out at 532 nm and 600 nm using a Synergy NT microplate reader (BioTek Instruments, USA).

The concentration of MDA was calculated using the formula:

$C = (D_{532} - D_{600}) / (155*0.1) \times 1000$

Where, C is the concentration of MDA, µmol/g of crude weight;

155 –Thiobarbituric acid extinction coefficient, $mM^{-1}cm^{-1}$.

0.1 – weight of plant material (g).

Superoxide dismutase (SOD) determination

The activity of the enzyme SOD was determined based on the inhibition of superoxide radical formation during the autoxidation of adrenaline in an alkaline medium *in vitro* at a wavelength of 347 nm with some modifications (12). Briefly, 0.1 mL of distilled water and 0.1 mL of 0.1 % (5.46 mM) epinephrine hydrochloride solution were added to 2 mL of 0.2 M bicarbonate buffer (pH = 10.65), thoroughly and rapidly mixed and placed in a Cary UV-60 spectrophotometer. Optical density was recorded every 30 sec for 5 min at 347 nm using a 10 mm quartz cuvette. After that, 0.1 mL of the enzyme source (vegetable homogenate) and 0.1 mL of 0.1 % epinephrine hydrochloride were added to 2 mL of buffer (pH = 10.65), mixed and the optical density was measured.

The homogenate for determining SOD activity was prepared as follows: 100 mg of plant leaves were ground in a porcelain mortar with 1 mL of 10 mM Tris-HCl buffer (pH 7.8). The homogenate was centrifuged at 7000 g for 15 min at 4 °C and the resulting supernatant was used as the enzyme source.

SOD activity is calculated using the following formula:

$A = [(D_1 - D_0) \times 54.6 \times V] / t$

Where, A is the activity of SOD, mM of decomposed adrenaline per min, D_1 is the optical density of the experimental sample, D_0 is the optical density of the control sample, 54.6 mM is the concentration of adrenaline in the cuvette, V is the source of the enzyme in the sample, t is the inhibition time of autoxidation of adrenaline (5 min).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using OriginPro 2022 software. Results are presented as an average ± standard error (SE) of three biological and three technical replicates. Significant differences between mean values were determined using the Tukey's test. Differences at $p < 0.05$ were considered statistically significant.

Results

Modulating antioxidants activity may enhance the salt resistance in plants and serve as a marker for the effective selection of salt-tolerant varieties. The content of MDA reflects the degree of lipid peroxidation. In our results, salt stress influenced lipid peroxidation, as evidenced by both increases and decreases in MDA content under stress conditions.

In this experiment, we also observed an increase in MDA levels when plants were exposed to chloride and sulfate salts, with some variation depending on concentration. Specifically, relatively low concentrations (100 mM NaCl and 1.5 % Na_2SO_4) and higher concentrations (200 mM NaCl and 2.5 % Na_2SO_4) in the irrigation solutions led to an increase in MDA content relative to the control irrigation (plain water) by 15 % and 11 % and by 31 % and 17 %, respectively (Fig. 1A, B).

Different dynamics of MDA content and antioxidant enzyme activity were observed under separate and combined effects of chloride and sulfate salts at varying concentrations across the three experimental treatments. As mentioned earlier, the MDA concentration is considered an indicator of oxidative damage to cellular components resulting from the excessive production of ROS associated with high sodium salts. The average MDA values recorded for six experimental treatments in the leaves of Porloq-4 seedlings were as follows: under chloride salt exposure, MDA increased by 15 %, decreases by 22.36 % (relative to the control) and increased by 11.58 %. Under sulfate salt exposure, MDA content in leaf tissues exceeded that of the control by 32 %, 5.1 % and 117.9 %, respectively, corresponding to the increasing concentrations of the stress solution.

For the Coker-312 variety, the changes in MDA content were as follows: an increase over the control variant by 21.1 %, a decrease by 15.53 % and in the third treatment, an increase by 23.72 % under higher NaCl concentrations. When Na_2SO_4 was applied, MDA increased by 17.1 %, 4.65 % and 22.34 % in the three treatments, respectively. Overall, the results indicate a relatively lower MDA response to moderate salt concentrations compared with exposure to either low or higher concentrations of sodium salts (Fig. 1A, B).

The response of seedlings at the early vegetation stage to watering with combined NaCl and Na_2SO_4 solutions is shown in Fig. 2. MDA values increased with rising salt concentrations. In the first treatment of the NaCl and Na_2SO_4combination, MDA level exceeded the control by 30.6 % in Porloq-4 seedlings and 34.4 % in the Coker-312 variety. In the second treatment, these values increased to 54 % and 54.83 %, respectively. In the third treatment, MDA content in the leaves of Porloq-4 and Coker-312 seedlings increased by 57 % and 64.5 %, respectively.

It is noteworthy that, in the control variant, MDA levels in Porloq-4 exceeded those in Coker-312 by a factor of 1.1. Across the treatments, these ratios were 0.97 for the first treatment and 0.99 for the seconds and 0.95 for the third. This pattern also suggests that the medium salt concentration treatment produces a more pronounced MDA response.

The effect of medium concentration solutions (150 mM NaCl and 1.5 % Na_2SO_4) led to a decrease in MDA content relative to both the first treatment and, in the case of chloride salt, even the control. This decrease was reflected in the activity of SOD. SOD regulates the overproduction of ROS and higher SOD activity indicates a stronger antioxidant response. Consistently, our results showed a greater SOD activity under salt stress conditions compared to the control. A significant increase in SOD activity was observed in the leaves of Porloq-4 seedlings under salt stress. Since the SOD enzyme catalyzes the conversion of superoxide into molecular oxygen and H_2O_2, it is considered the most effective intracellular enzymatic antioxidant.

According to the data obtained in this experiment, the activity of SOD in Porloq-4 cotton seedling increased by 18.9 %, 79 % and 34 %, respectively, with rising concentrations of the stressor. In the Coker-312 variety, the dynamics of this enzyme's activity followed a somewhat different pattern. When plants were watered with a solution of 100 mM NaCl, SOD activity increased slightly (3 %), while in the second and third treatments, the increase were 69 % and 11.5 %, respectively. It should be noted that these increases did not exceed those observed in Porloq-4 (Fig. 2).

As a glycophyte, cotton is more resistant to abiotic

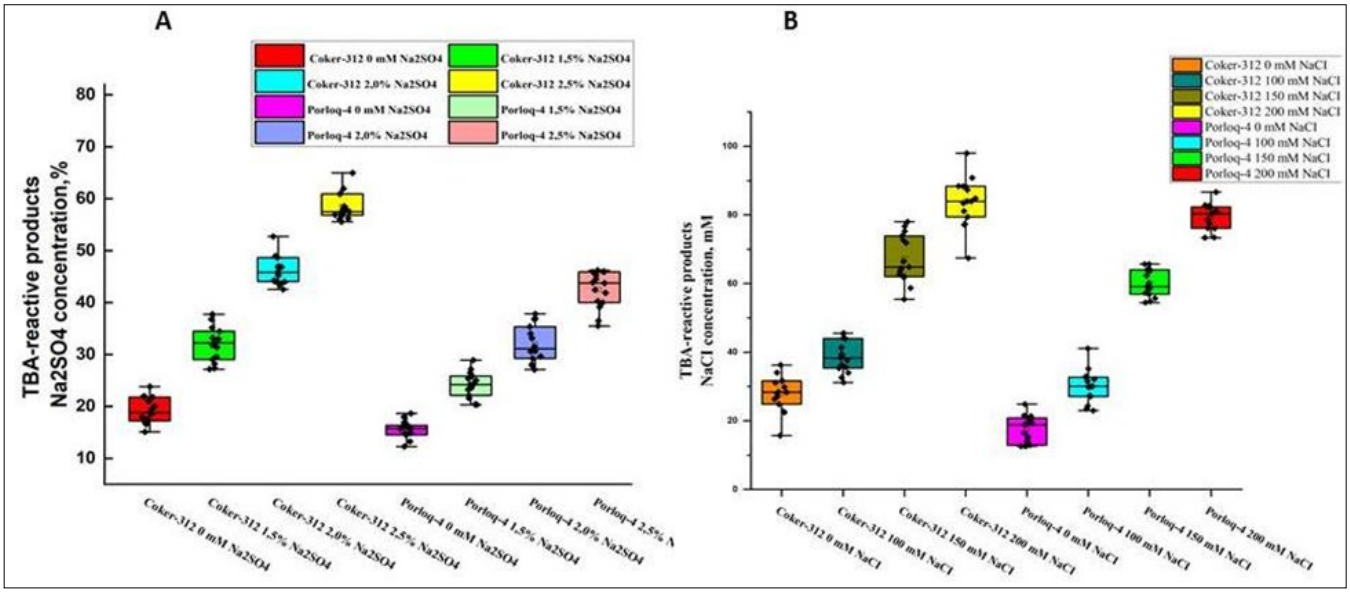

Fig. 1. MDA in the leaves of cotton seedlings when exposed to different concentrations of salt Na_2SO_4 (A) and NaCl (B). On the X-axis - the salt concentration (in % for Na_2SO_4 and in mM for NaCl); the Y-axis is the accumulation of TBA-active products, in μmol MDA/mg wet weight. Data are means ± standard errors (n = 15). $p \leq 0{,}0001$.

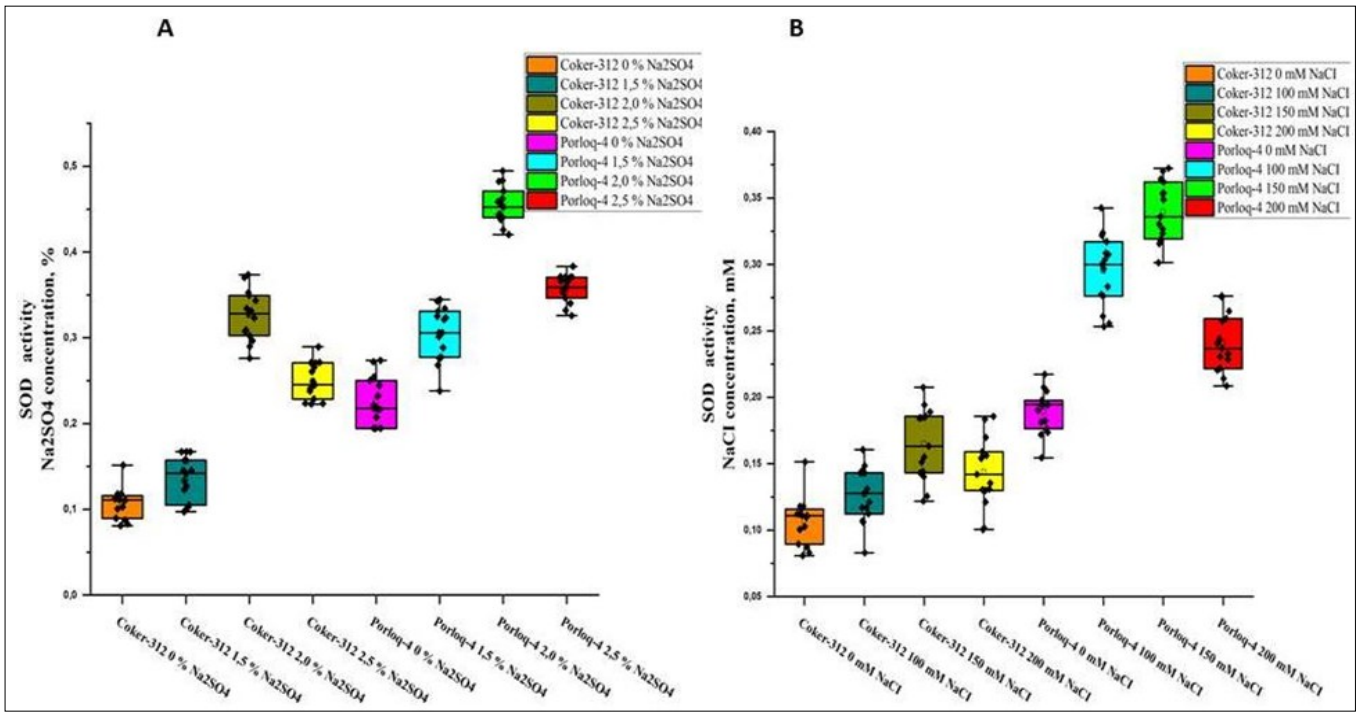

Fig. 2. The dynamics of SOD in the leaves of cotton seedlings under the influence of different concentrations of salt NaCl and Na_2SO_4. The Y-axis indicates SOD activity (U/ mg of protein). Data are means ± standard errors (n = 15); $p \leq 0{,}0001$.

stresses than many other major agricultural crops, regardless of the type of salt. However, it was important to examine the activity of SOD under the abiotic stress induced by sulfate salts. In the experiment, when the seedlings reached the four-leaf stage, they were watered Na_2SO_4 solutions at three different concentrations.

This dynamic was also observed in our experiment. Compared with the control, in the first treatment, where the Na_2SO_4 concentration in the irrigation solution was 1.5 %, SOD activity increased by 2.31 fold, reaching 231.4 %, i.e., 131.4 % higher than the control. In the second treatment, with a Na_2SO_4 concentration of 2.0 %, SOD activity rose to 260 %, which was 12 % higher than in the first treatment. When a 2.5 % solution was applied, SOD activity decreased slightly compared with the second treatment, though it still remained higher than in the control treatment (plain water).

Discussion

To assess the biotechnological genotype of Porloq-4 cotton under salinization during the early stages of vegetation, experiments were conducted in which seedlings the four-leaf stage were exposed to NaCl solutions at concentrations of 100 mM, 150 mM and 200 mM. Plants were watered for 21 days, after which the concentrations of MDA and the activity of SOD were determined in the collected leaves.

Abiotic stress induces changes in key physiological components and functions of green plants. As noted in the scientific literature, increased activity of antioxidant enzyme can be considered one of the possible mechanisms of resistance to abiotic stresses (13).

It is well established in the literature that, due to its function, SOD is considered the main antioxidant enzyme, as it regulates O_2 and H_2O_2 concentrations (14). It was also reported that in cotton tissues, increasing NaCl concentrations lead to a rise in SOD activity. This trend was also observed in our experiments. In a comparative context, we present the changes in the activity of this key antioxidant enzyme.

In Porloq-4 plants, SOD activity increased by 51 %, 124 % and 71 %, whereas the in Coker-312 variety, activity increased by 81.1 %, 198.6 % and 95.9 % under the same treatments. The ratio of SOD activity between Porloq-4 and Coker-312 was 0.62, 0.63 and 0.74, respectively, under chloride salt exposure.

Watering the seedlings with sulfate salt solutions led to a change in the SOD activity that were somewhat lower than those observed with chloride salt. In Porloq-4 plants, SOD activity in the leaves increased by 14 % at a relatively low Na_2SO_4 concentration (1.5 %), by 9 % at the second concentration and by 6 % at the third concentration (Fig. 2). In contrast, in the Coker-312 variety, the increases were 1.1 %, 10.37 % and 39.3 %, respectively.

The effect of solutions of combined salts of NaCl and Na_2SO_4 in a single irrigation treatment on seedlings caused changes in SOD activity in the following order. In the first treatment, where relatively low concentrations of NaCl and Na_2SO_4 were applied, SOD activity increased by 44.9 % in Porloq-4 and by 27.2 % in Coker-312. In the second treatment, activity increased by 99.1 % in Porloq-4 and 69.3 % in Coker-312. In the third treatment, with relatively high concentrations of both salts applied at the four-leaf stage, SOD activity increased compared with the control by 83.5 % and 65.8 % for the two varieties, respectively. Thus the second treatment elicited the strongest antioxidant enzyme response suggesting that this concentration combination was more toxic than the others (Fig. 2).

Experiments to assess the tolerance potential of different cotton genotypes have also been conducted by foreign researchers. For example, one study evaluated five cotton varieties under NaCl concentrations of 0, 50, 100 and 200 mM, to better understand varietal responses to salinization stress (15). Parameters such as chlorophyll content were used to determine the tolerance of a particular genotype and select the most

resistant ones. This selection criterion is directly related to increases yield, higher photosynthetic yield and greater dry matter production (16).

Since excessive concentrations of various salts-both chloride and sulfate-gradually accumulate in soils for several reasons, it is necessary to conduct research to identify the tolerance to these stressors in different combinations. However, most studies that quantify salt tolerance in plants have used NaCl as the predominant salt (17). Studies of biochemical and physiological response parameters have confirmed the existence of genetic variations in salinity tolerance in cotton (18). At the highest stressor concentration, there was a noticeable decrease in activity compared with the response of the medium concentration of saline solution. Previous studies have noted that the high activity of antioxidant enzymes is associated with both salt resistance and salt sensitivity (19).

Conclusion

In this study, we experimentally assessed the potential of the Porloq-4 knockout gene variety and the conventional Coker-312 variety under simulated salinization conditions created by two types of sodium salt (NaCl and Na_2SO_4) for some physiological and biochemical parameters. Under artificially induced salinization stress in laboratory conditions, an increase in the MDA content and the activity of antioxidant enzymes were noted. As the concentration of stress salt increases, a general rise in all indicators relative to the control variant was observed. However, in some cases, a decrease was also noted, particularly in the third treatment, where the salt concentration was relatively high. Nevertheless, the levels remained higher than those in the corresponding control treatments. Based on these results, it can be concluded that both Porloq-4 and Coker-312 genotypes possess adaptive abilities, with the Porloq-4 biotechnological variety showing a more active response than Coker-312. Thus, our findings indicate that Porloq-4 and Coker-312 cotton varieties respond differently to stress induced by NaCl and Na_2SO_4 and their combination, with Porloq-4 exhibiting a more active and responsive antioxidant enzyme system. Future studies should investigate the molecular basis of the antioxidant response in Porloq-4 through gene expression analysis and metabolic profiling under field conditions, particularly when salinity stress is combined with other abiotic stresses such as drought. Such work may contribute to a broader understanding of cotton adaptation mechanisms under complex stress environments.

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Authors' contributions

NRR, ASI, VSK and IBS carried out the experiments and wrote and revised the manuscript and performed the statistical analysis. FSR, SHM, OLI, DEU, JKN, KAU, ANA, SOK, SBK, NNK, MMK, FIB and BKR participated in the experiments, collected the data and prepared the draft of the manuscript. ZTB edited and approved the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest declare.

Ethical issues: None

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