



Mitigating Salt Stress in Cotton through Biochar Amendments: Effects on growth, Oxidative Stress, and Microbial Activity

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Abstract

Purpose This study aimed to evaluate the effectiveness of tobacco-derived biochar in mitigating salt stress in cotton (*Gossypium hirsutum* L.) by assessing its impact on physiological growth parameters, oxidative stress indicators, and soil microbial activity under greenhouse conditions.

Methods A greenhouse experiment was conducted using saline and non-saline soils, with biochar applied at three doses (8, 16, and 24 g kg⁻¹ soil) in a randomized complete block design with three replicates. Measurements included plant growth traits (root and shoot length, biomass, SPAD values, stomatal conductance), oxidative stress markers (H₂O₂, MDA, proline), antioxidant enzyme activities (APX, POD, CAT), and soil enzyme activities (catalase, urease, dehydrogenase).

Results Under saline conditions, biochar application at 24 g kg⁻¹ significantly improved cotton root and shoot biomass by 30% and 25%, respectively. SPAD values and stomatal conductance increased, while H₂O₂ and MDA levels decreased. Antioxidant enzyme activities and microbial enzyme levels were enhanced by up to 40%, demonstrating the positive role of biochar in alleviating salt-induced oxidative stress and improving soil health.

Conclusion Biochar, particularly at higher doses, enhances cotton resilience to salinity stress by improving physiological performance and microbial activity. These results highlight the potential of biochar as a sustainable soil amendment for saline agriculture.

Keywords Biochar · Cotton · Saline soil · Soil microbial activity

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1 Introduction

The cultivation of cotton (*Gossypium hirsutum* L.) in arid and semi-arid regions presents considerable challenges due to environmental stressors, among which soil salinity is particularly detrimental. Salinity impairs osmotic balance, restricts water and nutrient uptake, and disrupts overall plant metabolism, reducing growth and yield (Majeed and Muhammad 2019). Though economically crucial as a fiber, oil, and feed crop, cotton, is especially vulnerable to salinity during critical growth stages such as germination and early development (Rehman and Farooq 2019).

Globally, salinity affects approximately 20% of cultivated land, posing a serious threat to sustainable agriculture (Majeed and Muhammad 2019). In semi-arid climates with limited water availability and high evapotranspiration, salt accumulation in the root zone intensifies the stress on crops. This reduces photosynthetic capacity, chlorosis, and increases disease susceptibility (Yahya et al. 2020). Therefore, improving crop resilience to salinity is vital to

sustaining cotton production in salt-affected soils (YasminKhan et al., 2023).

One of the physiological consequences of salt stress is oxidative stress, caused by the overproduction of reactive oxygen species (ROS) that damage cellular membranes, proteins, and DNA (Hasanuzzaman et al. 2021). Although plants activate antioxidant systems including enzymes such as superoxide dismutase, catalase, and peroxidase these are often insufficient under severe salinity, leaving the plant vulnerable to oxidative injury (Gupta and Huang 2014). Strategies that can reinforce antioxidant defenses are essential for crop survival under saline conditions.

Among these strategies, biochar application has gained increasing attention. Biochar, a carbon-rich product of pyrolyzed organic material under limited oxygen, offers several benefits for soil quality and plant stress tolerance. Its porous structure improves soil aeration, water retention, and aggregation, while its chemical composition enhances nutrient retention and cation exchange capacity (Lehmann and Joseph 2024; Iqbal et al. 2024). These properties make biochar particularly suitable for ameliorating the adverse effects of salinity.

Biochar also supports microbial dynamics in the rhizosphere by providing a favorable habitat for beneficial microorganisms. These microbes contribute to organic matter decomposition, nutrient cycling, and symbiotic interactions with plant roots. Enzymes such as catalase, urease, and dehydrogenase, essential for soil biochemical functioning, have increased with biochar application (Pokharel et al. 2020; Zhu et al. 2022; Iqbal et al. 2023).

Furthermore, biochar has been linked to improved physiological responses in plants under stress. It can reduce ROS accumulation and enhance antioxidant enzyme activities, including ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT), thereby mitigating oxidative damage (Fujita and Hasanuzzaman 2022; Khan et al. 2023). It also improves photosynthetic performance by increasing stomatal conductance and chlorophyll content in plants grown in saline environments (Zhang et al. 2020; Khan et al. 2022).

Despite the expanding body of knowledge, the specific mechanisms by which biochar alleviates salinity stress in cotton remain insufficiently understood. The effects of biochar vary depending on its feedstock, pyrolysis conditions, application rates, and soil type. For example, high-temperature biochar tends to have greater surface area and alkalinity, potentially affecting soil pH and ion dynamics differently across soil systems (Khan et al. 2022; Yin et al. 2024). Understanding these interactions is critical to optimizing biochar use in saline agriculture.

Recent studies have reported that biochar improves root-zone conditions by enhancing soil aggregation, water-holding capacity, and nutrient availability, especially under

salt stress (Khan et al. 2023; Iqbal et al. 2023). Moreover, biochar modulates oxidative stress responses in plants and supports microbial activity that enhances plant-soil interactions (Wu et al. 2023; Naveed et al. 2020). These functions contribute to building a more resilient agroecosystem in saline environments (Sarioğlu et al. 2017; Li et al. 2022; Cevheri et al. 2022).

While the benefits of biochar have been increasingly documented, knowledge gaps remain regarding its dose-dependent effects on different stress indicators and its interaction with saline soil properties. To contribute to this expanding body of knowledge, therefore, this study seeks to answer the following research question: Can biochar application at varying doses alleviate salt-induced stress in cotton (*Gossypium hirsutum* L.) by improving plant physiological traits, oxidative stress responses, and soil microbial activity? To address this, we conducted a greenhouse experiment using three biochar doses (8, 16, and 24 g kg⁻¹ soil) in saline and non-saline soils and evaluated various plant and soil parameters.

This study aimed to evaluate whether biochar application at different rates can alleviate salt-induced stress in cotton (*Gossypium hirsutum* L.) by improving plant growth, physiological performance, and soil biological activity under saline and non-saline conditions. Specifically, the study investigated the effects of increasing biochar doses on root and shoot growth, biomass production, chlorophyll content, and stomatal conductance, as well as on oxidative stress indicators, including hydrogen peroxide, malondialdehyde, and proline accumulation. In addition, the responses of antioxidant enzyme activities (ascorbate peroxidase, peroxidase, and catalase) and soil microbial enzyme activities (catalase, urease, and dehydrogenase), together with changes in soil pH and electrical conductivity, were assessed. It was hypothesized that biochar application under saline conditions would enhance cotton growth and physiological performance, reduce oxidative damage through strengthened antioxidant defense systems, and stimulate soil microbial activity, thereby improving overall plant-soil interactions and contributing to sustainable cotton production in salt-affected soils.

2 Materials and Methods

2.1 Site Selection and Preparation of Materials

This study was conducted in the greenhouse of the Field Crops Department at the Faculty of Agriculture, Harran University, located in Şanlıurfa, Türkiye. Two distinct soil types were used: saline soil, collected from the Bozyazı village in the Harran district, and non-saline soil, obtained

Table 1 Characteristics of soils used in the experiment

Property	Saline Soil	Non-Saline Soil
EC (dS m ⁻¹)	3.40	0.88
pH	7.20	7.82
Lime (%)	26.62	10.96
Organic matter (%)	1.04	1.00

from the trial fields of Osmanbey Campus at a depth of 0–30 cm. Prior to the experiment, both soils were analyzed for their physical and chemical properties, and the results are presented in Table 1.

To quantify salinity stress, the soils collected soils' electrical conductivity (EC) was measured prior to planting. The saline soil had an EC of 3.4 dS m⁻¹, which is classified as moderately saline according to FAO guidelines, while the non-saline soil exhibited an EC of 0.8 dS m⁻¹. These values served as indicators of the salinity stress levels in the experiment. No additional salts were added, and the observed stress reflects the region's natural field conditions.

Biochar was applied at three rates (8, 16, and 24 g kg⁻¹ soil) to assess its potential to mitigate salinity-induced stress. The application was expected to alleviate the adverse effects of salinity through improved soil structure, pH moderation, nutrient retention, and stimulation of microbial activity.

The biochar used in this study was derived from the carbonization of tobacco stalks. It was characterized by high carbon content and alkaline pH (Table 2). The biochar was thoroughly mixed into both saline and non-saline soils at application rates of 8, 16, and 24 g kg⁻¹ soil to ensure homogeneous distribution. 5 kg of prepared soil mixture was filled into 7 kg capacity plastic pots for each treatment.

Five seeds of the cotton cultivar 'Fiona' were sown in each pot. Immediately after sowing, the pots were irrigated to ensure proper seed germination. Seven days after emergence, thinning was carried out to retain one healthy seedling per pot, providing uniform growth conditions across treatments.

The experiment began on July 1, 2023 and lasted for 60 days. A randomized complete block design (RCBD) was employed, with three replications per treatment, including controls without biochar application. In total, the setup comprised 24 pots.

Table 2 Chemical properties of tobacco Biochar

Property	Tobacco Biochar
Carbon (%)	46.21
Hydrogen (%)	2.88
Nitrogen (%)	1.68
Sulphur (%)	0.00
pH	10.00
EC (μS cm ⁻¹)	1515
CaCO ₃ (%)	27.57

2.2 Soil and Biochar Properties

The characteristics of the saline and non-saline soils used in the study are detailed in Table 1. The saline soil exhibited higher electrical conductivity (EC) and lime content compared to the non-saline soil, highlighting its challenging growing conditions.

The biochar used in this study was derived from tobacco stalks and exhibited high carbon content, a strongly alkaline pH, and a significant calcium carbonate concentration (Table 2). These properties suggest its potential to improve soil fertility and mitigate salinity stress.

2.3 Experimental Design and Treatments

The experiment was conducted under greenhouse conditions using a randomized complete block design (RCBD) to minimize experimental variability and ensure statistical reliability. The experimental layout consisted of eight treatment combinations: non-saline control (Control), biochar at 8 g kg⁻¹ soil (Bio8), 16 g kg⁻¹ soil (Bio16), and 24 g kg⁻¹ soil (Bio24); saline stress (SS); and saline stress combined with biochar at 8 g kg⁻¹ (SS+Bio8), 16 g kg⁻¹ (SS+Bio16), and 24 g kg⁻¹ (SS+Bio24).

Each treatment was replicated three times, resulting in a total of 24 experimental units. Pots were randomly assigned within each block to reduce spatial variation inside the greenhouse. Control pots containing untreated soil served as baseline comparisons. Throughout the experimental period, uniform environmental conditions were maintained, including controlled temperature and humidity, and plants were irrigated regularly to avoid water stress. This experimental design allowed the evaluation of the independent and interactive effects of salinity and biochar application and ensured reproducibility of the study.

2.4 Plant Growth and Physiological Measurements

Plant physiological responses were evaluated by measuring chlorophyll content, stomatal conductance, and biomass accumulation. Leaf chlorophyll content was determined using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Japan). Three readings were taken from fully expanded upper leaves of each plant and averaged.

Stomatal conductance was measured using a SC-1 Leaf Porometer (Decagon Devices, USA) between 10:00 and 12:00 h under ambient greenhouse conditions. These physiological measurements were conducted at 45 and 60 days after sowing to assess temporal responses to salinity and biochar application.

At harvest (60 days after sowing), plants were separated into shoots and roots. Dry biomass was determined after

oven-drying samples at 65 °C for 72 h until constant weight was achieved (Ben-Gal et al. 2009).

2.5 Oxidative Stress Indicators and Antioxidant Enzyme Activities

At the end of the experiment, fresh leaf samples were collected to determine oxidative stress markers and antioxidant enzyme activities. Hydrogen peroxide (H_2O_2) content was measured in 0.5 g leaf samples following the method described by Loreto and Velikova (2001). Malondialdehyde (MDA) content was determined using 0.1 g leaf samples according to Weisany et al. (2012), and proline concentration was analyzed from 0.5 g leaf tissue following the protocol of Bates et al. (1973).

For antioxidant enzyme assays, 0.5 g of fresh leaf tissue was homogenized in 50 mM sodium phosphate buffer containing 1% soluble polyvinylpyrrolidone. The homogenates were centrifuged at $20,000 \times g$ for 15 min at 4 °C, and the resulting supernatants were used for enzyme activity analyses. Ascorbate peroxidase (APX) activity was measured spectrophotometrically at 290 nm following Nakano and Asada (1981). Peroxidase (POD) activity was determined by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation (Chance and Maehly 1955). Catalase (CAT) activity was measured by recording the decrease in absorbance at 240 nm over 60 s, according to Kraus and Fletcher (1994).

2.6 Soil Chemical Properties and Microbial Enzyme Activities

Soil pH and electrical conductivity (EC) were determined using a 1:2.5 soil-to-water suspension. Air-dried soil samples (10 g) were mixed with 25 mL of deionized water, stirred for 30 min, and measured using a calibrated digital

pH meter. Electrical conductivity was measured in the same suspension after equilibration using a conductivity meter, following standard procedures for saline soils (Sparks et al. 2020).

Soil microbial enzyme activities were analyzed at the end of the experiment. Catalase activity was determined using a gas volumetric method with hydrogen peroxide (Beck 1971). Urease activity was assessed based on the color intensity of the filtrate following Hoffman and Teicher (1961), and dehydrogenase activity was measured spectrophotometrically according to Tabatabai (1982).

2.7 Statistics Analysis

Statistical analyses were conducted using SPSS software (Version XX, IBM Corp., USA). The normality of data distributions was tested using the Shapiro–Wilk test and the homogeneity of variances was assessed using Levene’s test. After confirming that the assumptions for parametric testing were met, a one-way analysis of variance (ANOVA) was performed to evaluate the effects of treatments. When statistically significant differences were observed, Duncan’s Multiple Range Test (DMRT) was used for pairwise comparisons among treatment means at a significance level of $p < 0.001$. All results are presented as means $\pm$ standard deviation.

3 Results

3.1 Effects of Biochar and Salinity on Root and Shoot Length

The study results regarding the root length under different doses of biochar (bio8, bio16, bio24) and salt stress are presented in Fig. 1A. Compared to the control group, biochar

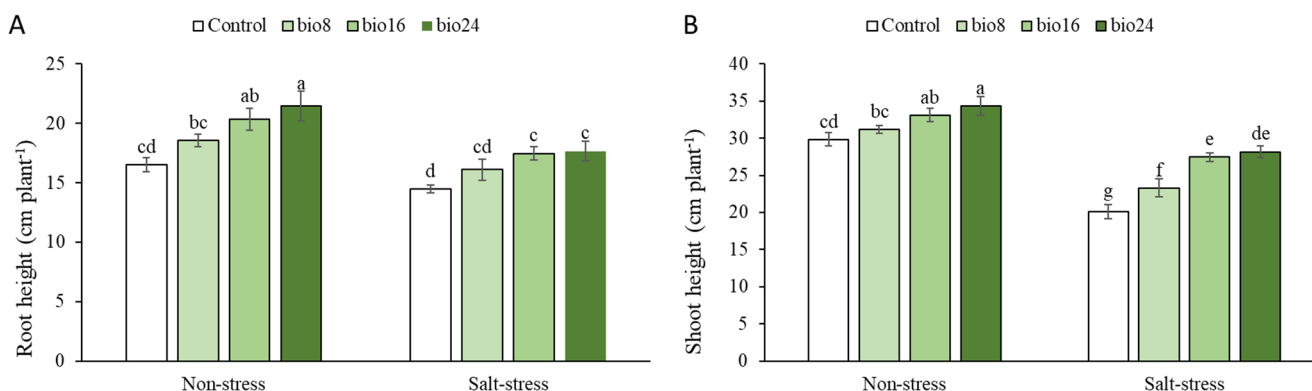


Fig. 1 Effects of biochar application on root (A) and shoot (B) length in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate statistically significant

differences among treatments according to Duncan’s multiple range test ($p < 0.001$) Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg⁻¹ biochar applied; bio16: 16 g kg⁻¹ biochar applied; bio24: 24 g kg⁻¹ biochar applied

applications significantly increased root length. Specifically, the bio24 application showed the highest increase of 30.11% in root length, while bio16 and bio8 applications resulted in increases of 23.49% and 12.34%, respectively.

On the other hand, salt stress caused a 12.31% reduction in the root length of cotton plants ($p < 0.001$), demonstrating salt stress's adverse effects on plant development. However, biochar doses of bio8, bio16, and bio24 applied against salt stress resulted in increases in root length compared to the salt stress alone. These increases were calculated as 11.29%, 20.74%, and 22.14% for bio8, bio16, and bio24, respectively ($p < 0.001$). These results indicate that biochar application, particularly at high doses (bio24), effectively enhances root development in cotton plants under salt stress.

The study also found that the applications led to differences in the shoot length of cotton plants (Fig. 1B). Compared to the control group, biochar applications increased in shoot length. Specifically, the bio24 application showed the highest increase in shoot length at 14.97%, while bio16 and bio8 applications resulted in increases of 10.95% and 4.58%, respectively. Salt stress caused a 32.63% reduction in the shoot length of cotton plants. However, biochar doses of bio8, bio16, and bio24 applied against salt stress resulted in increased shoot length compared to salt stress alone. These increases were 15.92%, 36.48%, and 40.14%, respectively. These results were statistically significant ($p < 0.001$).

3.2 Effects of Biochar and Salinity on Root and Shoot Dry Biomass

The dry weights of the plant roots and shoots are shown in Fig. 2. Compared to the control group, biochar applications

led to significant increases in root weight (Fig. 2A). The bio24 application showed the growth increase in root weight at 86.99%. In comparison, bio16 and bio8 applications resulted in increases of 61.88% and 22.98%, respectively.

Salt stress caused a 58.55% reduction in the root weight of cotton plants. However, biochar doses of bio8, bio16, and bio24 applied to ameliorate salt stress resulted in increases in root weight compared to salt stress alone, with increases of 48.87%, 67.32%, and 102.82%, respectively ($p < 0.001$).

The study showed that biochar applications significantly affected shoot dry weight (Fig. 2B). Compared to the control group, bio8, bio16, and bio24 applications increased shoot dry weight under non-stress conditions by 66.8%, 67.6%, and 77.9%, respectively, and these increases were found to be significant ($p < 0.001$).

On the other hand, salt stress reduced shoot dry weight by 65.7%. Although biochar applications increased in shoot dry weight under salt stress conditions, these increases were not statistically significant ($p < 0.001$).

3.3 Physiological Responses: Chlorophyll Content and Stomatal Conductance

SPAD values indicate chlorophyll concentration in plant leaves and are generally directly related to plant health. The SPAD values measured in the study are presented in Fig. 3A. Compared to the control group, biochar applications (bio8, bio16, and bio24) increased SPAD values by 0.3%, 8.8%, and 6.2%, respectively ($p < 0.001$). Salt stress caused a 9.4% decrease in SPAD values in cotton plants. This result indicates that salt stress leads to chlorophyll degradation and reduced in photosynthetic capacity in plants. Under salt stress, biochar applications (bio8, bio16, and

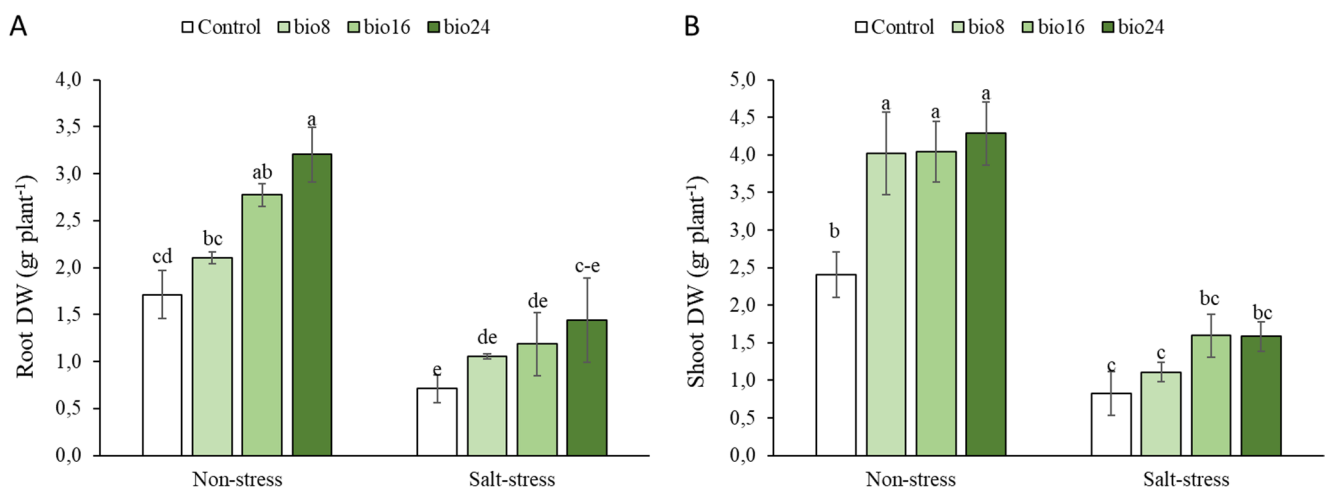


Fig. 2 Effects of biochar application on root (A) and shoot (B) dry weight in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate statistically

significant differences among treatments according to Duncan's multiple range test ($p < 0.001$) Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg⁻¹ biochar applied; bio16: 16 g kg⁻¹ biochar applied; bio24: 24 g kg⁻¹ biochar applied

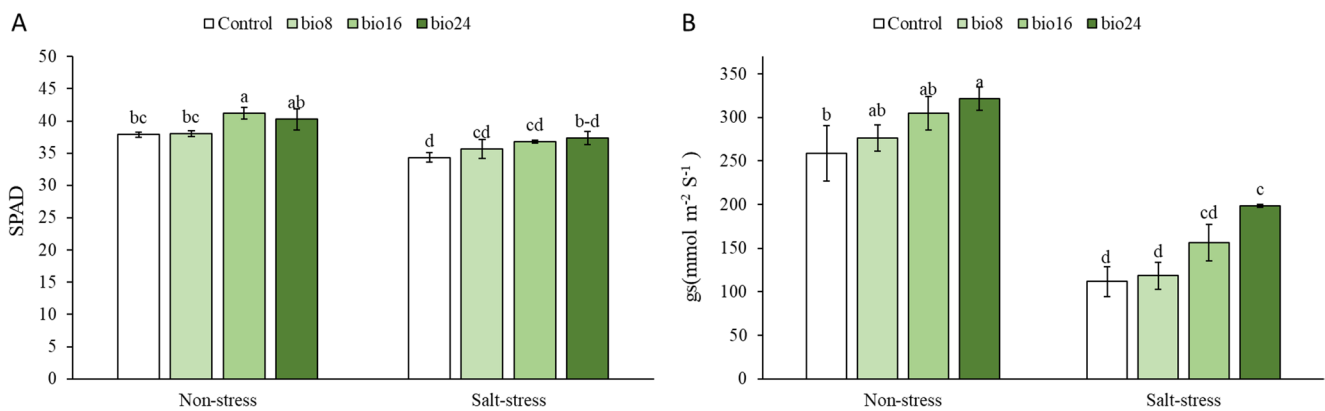


Fig. 3 Effects of biochar application on SPAD values (A) and stomatal conductance (gs) (B) in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate statistically significant differences among treatments according to

bio24) increased SPAD values by 5.9%, 2.8%, and 1.5%, respectively, and these results were statistically significant ($p < 0.001$).

At the end of the study, stomatal conductance (gs) was measured in the plants (Fig. 3B). Stomatal conductance is an important physiological parameter related to gas exchange and water loss in plants. Biochar applications (bio8, bio16, and bio24) increased stomatal conductance by 6.8%, 17.8%, and 24.3%, respectively ($p < 0.001$). Salt stress caused a 56.7% decrease in stomatal conductance. Under salt stress, biochar applications (bio8, bio16, and bio24) increased stomatal conductance by 5.8%, 39.9%, and 77.4%, respectively, and these results were statistically significant ($p < 0.001$). These results suggest that biochar promotes plant growth by improving plant water use efficiency and gas exchange.

3.4 Oxidative Stress Indicators

In the study, H_2O_2 , MDA, and proline analyses were performed to determine the oxidative stress caused by salt in the plants (Fig. 4). The study observed that biochar applications resulted in significant reductions in H_2O_2 levels compared to the control (Fig. 4A). For instance, the bio24 application reduced H_2O_2 levels by 28.25%, although this reduction was not statistically significant ($p < 0.001$). Salt stress increased H_2O_2 levels by 195.68%. Under salt stress, biochar doses (bio8, bio16, and bio24) reduced H_2O_2 levels by 8.25%, 12.41%, and 16.94%, respectively, and these results were statistically significant ($p < 0.001$).

High MDA levels can cause damage to cell membranes. This study showed that biochar applications decreased MDA levels (Fig. 4B). The bio24 application reduced MDA levels by 5.07%, although this reduction was not significant ($p < 0.001$). Salt stress caused a 169.85% increase in MDA

Duncan's multiple range test ($p < 0.001$) Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg⁻¹ biochar applied; bio16: 16 g kg⁻¹ biochar applied; bio24: 24 g kg⁻¹ biochar applied

levels. Under salt stress, biochar applications (bio8, bio16, and bio24) reduced MDA levels by 6.43%, 12.86%, and 20.49%, respectively.

High proline levels improve plants' adaptation to stress conditions. This study observed that biochar applications increased proline levels (Fig. 4C). The bio24 application particularly increased proline levels by 17.79%. Salt stress caused a 131.31% increase in proline levels. Under salt stress, biochar applications (bio8, bio16, and bio24) further increased proline levels by 35.03%, 60.21%, and 72.72%, respectively.

3.5 Antioxidant Enzyme Activities

This study establishes biochar as a versatile and effective tool for mitigating salt stress in cotton cultivation. The significant improvements in plant growth, photosynthetic performance, and oxidative stress management, coupled with enhanced soil microbial activity, highlight biochar's potential as a sustainable solution for saline agriculture. The highest biochar dose (24 g kg⁻¹ soil) consistently demonstrated superior performance across all parameters, indicating a dose-dependent effect. These findings advance our understanding of biochar's mechanisms in stress mitigation and provide practical insights for its application in real-world agricultural systems. Future research should focus on field-scale applications and long-term impacts to fully harness biochar's potential in addressing the global challenge of soil salinization.

The study showed that biochar applications in the control group caused changes in APX levels (Fig. 5A). The bio16 application showed the highest increase of 25%, although this increase was not significant ($p < 0.001$). Salt stress, however, increased APX levels by 173.2% compared to the control group. Biochar applications under salt stress further

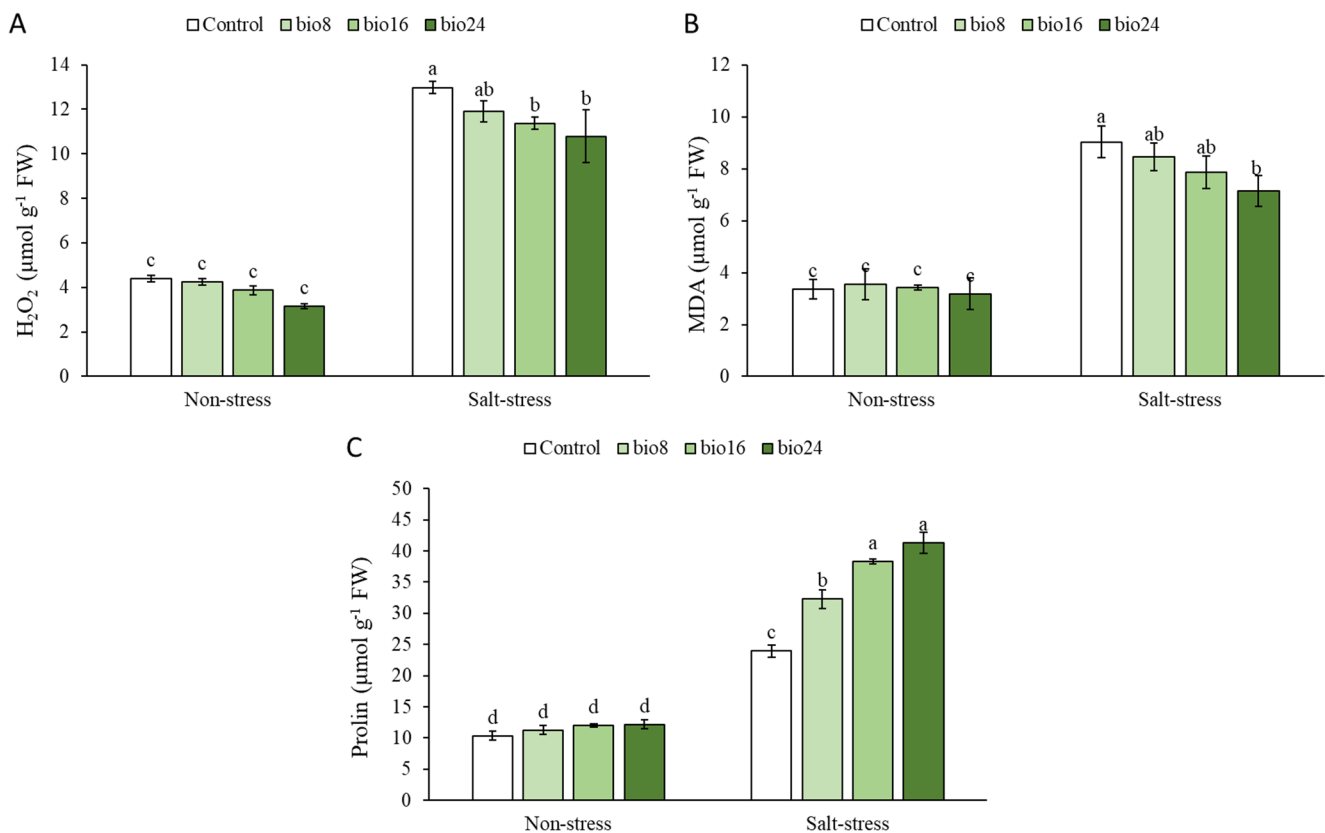


Fig. 4 Effects of biochar application on oxidative stress indicators, including hydrogen peroxide (H_2O_2) (A), malondialdehyde (MDA) (B), and proline (C), in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate

statistically significant differences among treatments according to Duncan's multiple range test ($p < 0.001$) Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg^{-1} biochar applied; bio16: 16 g kg^{-1} biochar applied; bio24: 24 g kg^{-1} biochar applied

increased these levels. Bio8, bio16, and bio24 applications under salt stress increased APX levels by 12.9%, 21.1%, and 38.8%, respectively. These results were significant ($p < 0.001$).

POD results are presented in Fig. 5B. Biochar applications in the control group increased POD levels, with the bio24 application showing the highest increase of 11%. Salt stress increased POD levels by 136.4% compared to the control group. Biochar applications under salt stress further increased these levels. Bio8, bio16, and bio24 applications under salt stress increased POD levels by 16%, 16.4%, and 37.5%, respectively. These results were statistically significant ($p < 0.001$).

Biochar applications in the control group increased CAT levels, although this increase was not significant ($p < 0.001$) (Fig. 5C). The bio24 application showed the highest increase of 24.6%. Salt stress increased CAT levels by 94.9% compared to the control group. Biochar applications under salt stress further increased these levels. Bio8, bio16, and bio24 applications under salt stress increased CAT levels by 9.6%, 49.5%, and 61.5%, respectively ($p < 0.001$).

3.6 Soil Microbial Enzyme Activities and Chemical Properties

Catalase is an important enzyme for soil microbial activity, reducing oxidative stress by breaking down hydrogen peroxide into water and oxygen. Soil microorganisms increase catalase production under stress conditions to protect against oxidative damage. The study showed that biochar applications significantly increased catalase activity compared to the control group (Fig. 6A). The bio24 application showed the highest increase of 41.9%, which was statistically significant ($p < 0.001$). Salt stress reduced catalase activity by 61.1% compared to the control group. Biochar applications under salt stress increased catalase activity. Bio8, bio16, and bio24 applications under salt stress increased catalase activity by 13.8%, 23.5%, and 31.4%, respectively ($p < 0.001$).

Urease is an enzyme that breaks down urea into ammonia and carbon dioxide and plays a crucial role in the soil nitrogen cycle. Soil urease activity is critical for soil microbial health and fertility. Urease activity results are presented in Fig. 6B. Biochar applications in the control group increased

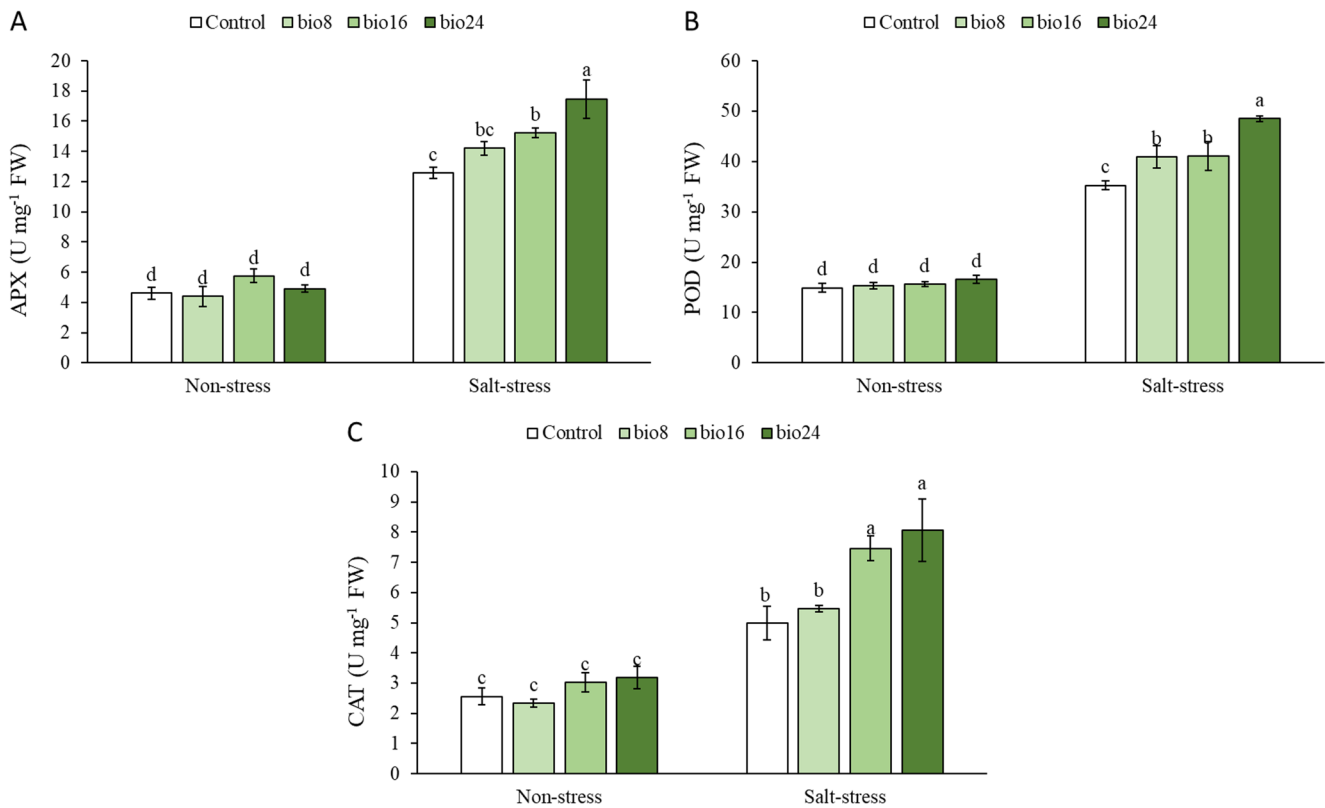


Fig. 5 Effects of biochar application on antioxidant enzyme activities, including ascorbate peroxidase (APX) (A), peroxidase (POD) (B), and catalase (CAT) (C), in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate

urease activity, with the bio24 application showing the highest increase of 71.2%. Salt stress reduced urease levels by 45.6% compared to the control group. Biochar applications to mitigate the effects of salt stress increased urease levels. Bio8, bio16, and bio24 applications increased urease levels by 22.3%, 54.5%, and 56.3%, respectively. These results were significant ($p < 0.001$).

Dehydrogenase (DHG) is used as an indicator of soil microbial respiration and is a crucial measure of soil microbial activity. DHG activity reflects the metabolic activity of microorganisms in the soil. DHG results are presented in Fig. 6C. Biochar applications in the control group increased DHG levels, with the bio24 application showing the highest increase of 23.3%. Salt stress reduced DHG activity by 12.5% compared to the control group. Biochar applications under salt stress increased DHG levels. Bio8, bio16, and bio24 applications under salt stress increased DHG levels by 5.3%, 4.4%, and 21.7%, respectively. These results were statistically significant ($p < 0.001$).

Soil pH affects the uptake of plant nutrients and microbial activity. In the study, biochar applications to the control group resulted in slight increases in pH levels (Fig. 7A).

statistically significant differences among treatments according to Duncan's multiple range test ($p < 0.001$). Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg⁻¹ biochar applied; bio16: 16 g kg⁻¹ biochar applied; bio24: 24 g kg⁻¹ biochar applied

The bio24 application to the control soil showed the highest increase of 1.6%, which was found to be statistically significant ($p < 0.001$). Similarly, biochar applications to saline soils also led to pH increases ranging from 0.5% to 1.4%. These increases can be attributed to the high pH of the biochar material used.

Biochar generally tends to neutralize soil pH. This is because of its high surface area and cation exchange capacity (CEC). The alkaline nature of biochar can neutralize soil acidity and increase pH (Lehmann and Joseph 2024). However, some types of biochar and application doses, especially those with low pH, can reduce soil pH (Joseph et al. 2021).

Soil EC (electrical conductivity) indicates the salt concentration in the soil, and high EC values can hinder water uptake in plant roots. In the study, biochar applications resulted in different outcomes for EC levels compared to the control group (Fig. 7B). In non-saline soils, biochar applications increased soil EC values by 12% to 17%. In saline soils, the bio16 application led to a 4% increase in EC, while the bio24 application reduced the EC value by 4.9%. These results were found to be statistically significant ($p < 0.001$).

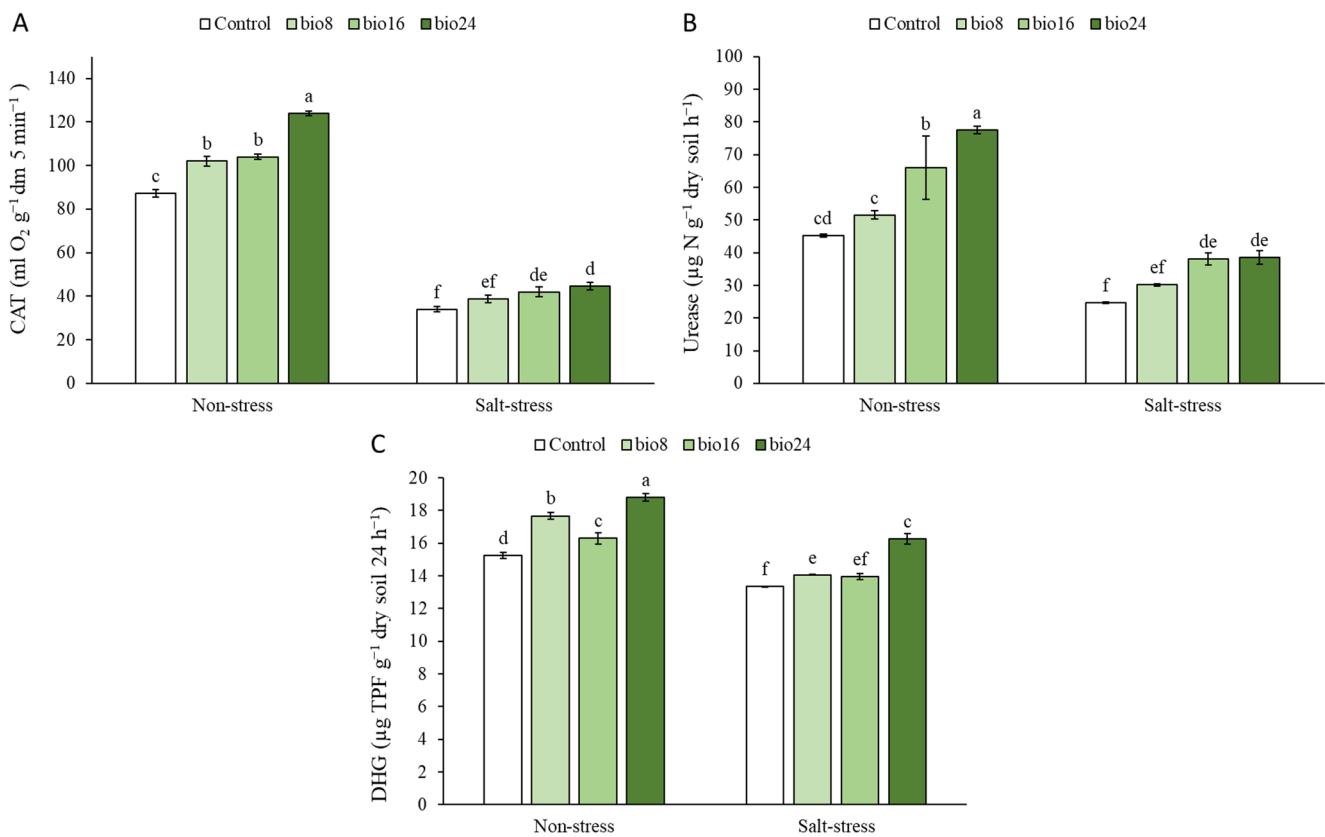


Fig. 6 Effects of biochar application on soil microbial enzyme activities, including catalase (CAT) (A), urease (B), and dehydrogenase (DHG) (C), in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate statistically

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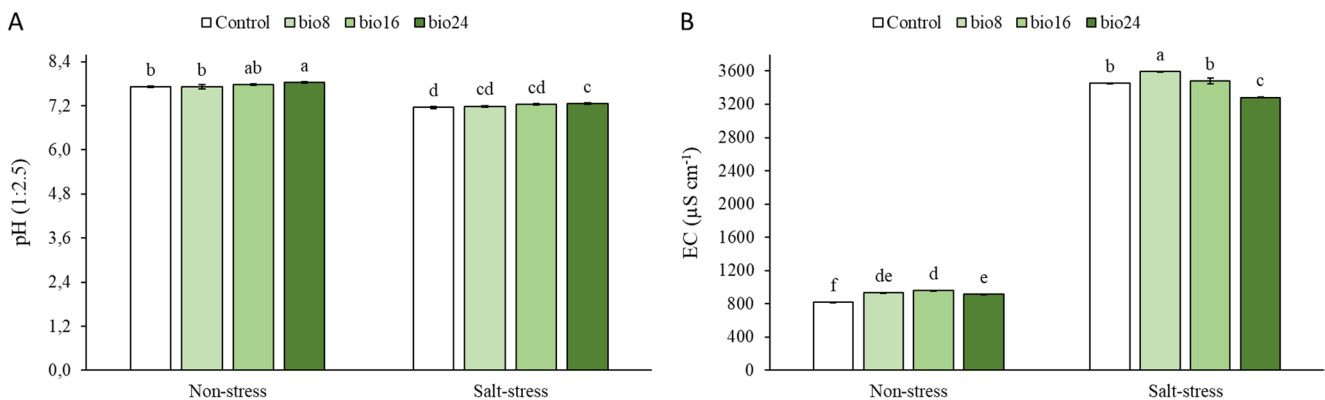


Fig. 7 Effects of biochar application on soil pH (A) and electrical conductivity (EC) (B) in cotton (*Gossypium hirsutum* L.) grown under non-saline and saline conditions. Values are presented as means $\pm$ standard error ($n=3$). Different lowercase letters above the bars indicate statistically significant differences among treatments according to

Duncan's multiple range test ($p < 0.001$) Non-saline: salt-free soil; Saline: saline soil; Control: no biochar applied; bio8: 8 g kg^{-1} biochar applied; bio16: 16 g kg^{-1} biochar applied; bio24: 24 g kg^{-1} biochar applied

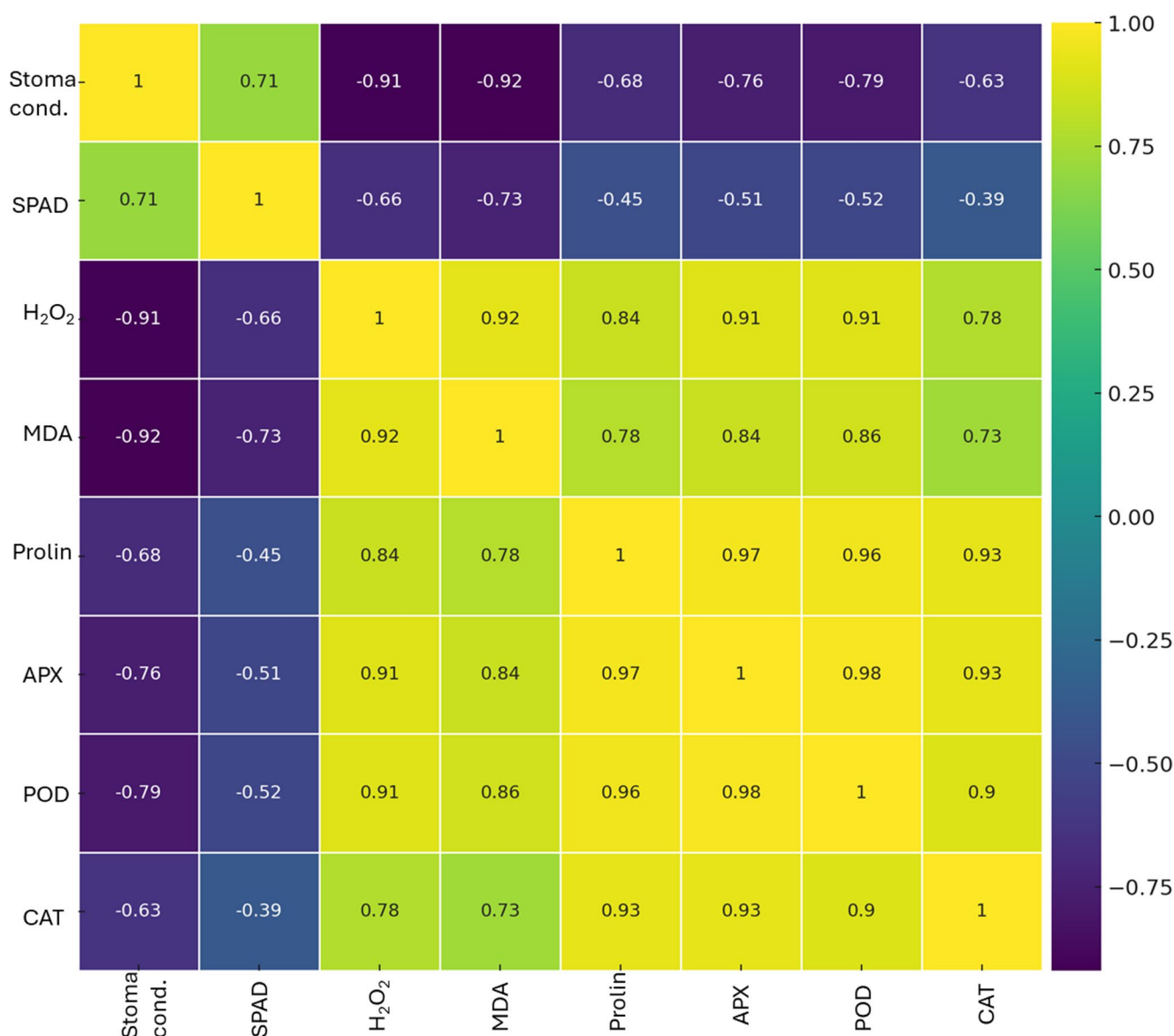


Fig. 8 Correlation Matrix of Cotton Plant Parameters with Biochar Treatment (stoma cond.: stomatal conductance, SPAD: chlorophyll content, H₂O₂: Hydrogen peroxide, MDA: Malondialdehyde, APX: Ascorbate peroxidase, POD: Peroxides, CAT: catalase)

3.7 Correlation Analysis

Correlation analysis was performed on the results obtained. This correlation matrix examines the effects of different biochar doses applied to cotton plants on various physiological and microbial parameters (Fig. 8). The matrix shows the relationships between these parameters and the strength of these relationships. Positive correlations are shown in yellow and green tones, while negative correlations are in purple and blue.

There is a positive correlation (0.71) between stomatal conductance (gs) and SPAD. This indicates that as stomatal conductance increases, chlorophyll content also increases. There is a robust positive correlation (0.92) between H₂O₂

and MDA. Both parameters are indicators of oxidative stress and are directly related to each other. There is a strong positive correlation (0.97) between proline and APX. As proline responds to plant stress, APX also increases as part of the antioxidant defense mechanism. There is a very strong positive correlation (0.98) between APX and POD. This indicates that antioxidant enzyme activities are closely related to each other.

There is a negative correlation (−0.66) between SPAD and H₂O₂. High chlorophyll content (SPAD) is generally associated with low H₂O₂ levels, indicating lower oxidative stress in the plant. A negative correlation (−0.73) exists between SPAD and MDA. This shows that as chlorophyll content increases, lipid peroxidation decreases. There is a

strong negative correlation (-0.91) between stomatal conductance and H_2O_2 . This indicates that as stomatal conductance increases, cellular H_2O_2 levels decrease.

A negative correlation (-0.39) between catalase (CAT) and SPAD exists. This suggests that plants with higher chlorophyll content may have slightly lower CAT activity. Antioxidant enzyme activities (APX, POD, CAT) increased with biochar applications, and the strong positive correlations between these enzymes indicate that the defense mechanisms of plants under stress work in coordination.

This correlation matrix clarifies how different biochar doses influence physiological and microbial parameters in cotton plants. Such analyses are essential for understanding the mechanisms by which biochar alleviates plant stress.

4 Discussion

The results of this study underscore biochar's role as an effective soil amendment in mitigating salt stress in cotton. The observed improvements in root and shoot biomass are attributed to biochar's ability to enhance soil water retention and nutrient availability, thereby alleviating osmotic stress. Furthermore, the reduction in oxidative stress markers (e.g., H_2O_2 and MDA) and the increase in antioxidant enzyme activities (e.g., APX, POD, CAT) suggest that biochar strengthens the plant's intrinsic defense mechanisms. These findings align with previous studies highlighting biochar's ability to enhance antioxidant responses under stress conditions. Additionally, the stimulation of microbial activity, reflected in increased soil enzyme activities, reinforces the hypothesis that biochar fosters a supportive rhizosphere environment, crucial for nutrient cycling. This integrated plant and soil health improvement demonstrates biochar's dual role in stress mitigation and productivity enhancement.

The study's findings regarding the dry weights of plant roots and shoots (Fig. 2) underscore the positive effects of biochar applications on cotton growth under both normal and saline conditions. Biochar applications significantly increased root weight, with the bio24 dose showing an 86.99% increase, consistent with its known benefits on soil properties and nutrient availability (Agegehu et al. 2016).

It should be noted, however, that while the highest dose of biochar (24 g kg^{-1} soil) resulted in significant improvements in root and shoot biomass, the long-term implications of high biochar applications should be evaluated with caution. Although biochar is largely stable and does not readily decompose, excessive application may alter soil carbon dynamics or nutrient balance long-term, particularly in non-saline or carbon-rich soils. Therefore, the optimal dose should be selected based on soil type, crop species, and environmental conditions to maximize benefits without

compromising soil health. Future studies should explore the threshold levels beyond which biochar may negatively affect sensitive crops or soil ecosystems.

Salt stress resulted in a 58.55% reduction in root weight, highlighting its adverse impact on plant biomass (Obadi et al. 2024). However, biochar applications mitigated these effects, with root weight increases of 48.87%, 67.32%, and 102.82% for bio8, bio16, and bio24, respectively, under salt stress. In conclusion, biochar applications significantly enhance plant biomass and mitigate the negative effects of salt stress on cotton plants, highlighting its potential as a valuable soil amendment in saline-prone agricultural areas.

At the end of the study, stomatal conductance (gs) and SPAD (Fig. 3) were measured in the plants (Fig. 3). These findings suggest that biochar applications improve photosynthetic efficiency by increasing plant chlorophyll content. Similar findings are reported in the literature. For instance, Cong et al. (2023) reported that biochar applications increased chlorophyll content and photosynthesis rates in maize plants. Similarly, Pokovai et al. (2020) found that biochar applications increased chlorophyll content in pepper plants. Vaccari et al. (2011) also found that biochar positively affected chlorophyll content in tomato plants.

Additionally, Akhtar et al. (2015a) observed that biochar increased chlorophyll content in faba bean plants. Parida and Das (2005) reported that salt stress reduces chlorophyll content in plants. Similarly, Ashraf and Harris (2013) reported that salt stress decreases chlorophyll content and reduces photosynthesis rates in plants. These findings suggest that biochar effectively mitigates the adverse effects of salt stress and maintains plant health. Chan and Xu (2009) reported that biochar increased chlorophyll content in plants exposed to salt stress. Quilliam et al. (2013) also reported that biochar applications increased plant resilience to salt stress. Stomatal conductance is an important physiological parameter related to plant gas exchange and water loss. Similar studies support our results. Zhang et al. (2020) reported that biochar applications increased stomatal conductance in soybean plants, improving water use efficiency. Similarly, Haider et al. (2015) reported that biochar applications increased stomatal conductance in wheat plants. Gul et al. (2015) also found that biochar positively affected stomatal conductance in barley plants. Another study by Major et al. (2010) observed that biochar increased stomatal conductance in maize plants.

Salt stress caused a decrease in stomatal conductance. This result indicates that salt stress leads to stomatal closure and reduced plant gas exchange to prevent water loss. Munns and Tester (2008) reported that salt stress causes stomatal closure and reduced plant gas exchange. Ashraf (2004) also reported that salt stress decreases stomatal conductance in plants. These findings suggest that biochar effectively

mitigates the adverse effects of salt stress and improves plant water management. Jeffery et al. (2011) reported that biochar applications increased plant resilience to salt stress. Another study by Schulz et al. (2013) observed that biochar increased stomatal conductance in plants exposed to salt stress.

When comparing the H_2O_2 results (Fig. 4A) with previous studies, similar findings have been reported in the literature. Liang et al. (2006) stated that biochar applications increase soil water-holding capacity and microbial activity, thereby enhancing plant resistance to stress. Haider et al. (2022) observed that biochar reduced H_2O_2 levels in maize plants and promoted growth. Additionally, biochar is known to stabilize plant cell membranes, thus reducing oxidative stress (Lehmann and Joseph 2024). Elad et al. (2010) also reported that biochar enhances resistance against plant pathogens and decreases oxidative stress indicators. Rizhsky et al. (2004) noted that biochar applications effectively reduce oxidative damage.

When the literature is examined, the values obtained from MDA results (Fig. 4B), Similarly, Lehmann and Joseph (2024) noted that biochar reduces MDA formation by stabilizing plant cell membranes. Chan and Xu (2009) also reported that biochar applications decrease MDA levels by reducing plant stress. Shi et al. (2021) demonstrated that biochar helps plants cope with oxidative stress by reducing lipid peroxidation. Zhang et al. (2014) stated that biochar effectively reduces plant stress and prevents lipid peroxidation. Zhang et al. (2024) also reported that biochar reduces MDA levels by protecting plant cell membranes.

Proline values (Fig. 4C) increased because of stress. This increase provides support to the plant. Farooq et al. (2009) noted that biochar applications increase proline levels by enhancing plants' osmoregulation capacity. El Nahhas et al. (2021) reported that biochar applications promote proline accumulation in response to plant stress. Additionally, Ashraf (2004) and Hafez et al. (2020) emphasized that biochar improves plants' adaptation to stress conditions by increasing proline accumulation. Szabados and Savouré (2010) also highlighted the significant role of proline in plant stress tolerance. Hossain et al. (2014) demonstrated that proline enhances survival under stress conditions by protecting plant cells. The results are consistent with similar studies in the literature and show that biochar applications effectively reduce oxidative stress and improve osmoregulation in cotton plants. Biochar applications improved plant health and stress tolerance by reducing H_2O_2 and MDA levels while increasing proline levels.

Studies on APX in literature report similar findings (Fig. 5A). For example, Helaoui et al. (2023) noted that biochar increased APX activity in maize plants, providing protection against salt stress. Additionally, Akhtar et al. (2015c)

observed that biochar applications reduced H_2O_2 levels and increased APX activity in pepper plants. Hafez et al. (2021) reported that biochar applications increased APX activity in rice plants, enhancing resistance to salt stress. Kapoor et al. (2024) found that biochar increased APX activity in pepper plants, facilitating oxidative stress management. Jindo et al. (2012) also showed that biochar improved plant stress responses by increasing soil microbial activity.

Recent studies on POD activity highlight the effectiveness of biochar in combating oxidative stress (Fig. 5B). For instance, Liang et al. (2006) reported that biochar increased POD activity in barley plants, reducing the effects of salt stress. Mehmood et al. (2020) demonstrated how POD activity increases as a plant stress response and how biochar applications optimize this process. Hafeez et al. (2019) reported that biochar applications increased POD activity in rice plants, protecting against salt stress. Zulfiqar et al. (2022) found that biochar applications increased POD activity in wheat plants, helping them cope with oxidative stress. Akhtar et al. (2015b) showed that biochar increased POD activity in faba bean plants, improving their stress tolerance.

Current literature on CAT activity supports the effectiveness of biochar (Fig. 5C). Sharma et al. (2012) showed that biochar significantly increased CAT activity in wheat plants under salt stress. Smith et al. (2014) reported that biochar applications improved overall stress tolerance by increasing CAT activity in various plant species. Reddy et al. (2004) noted that biochar increased CAT activity in maize plants, reducing oxidative stress. Hafez et al. (2020) reported that biochar provided protection against salt stress by increasing CAT activity in barley plants. Gao et al. (2019) also demonstrated that biochar improves oxidative stress responses in plants.

When microbial activity results are examined (Fig. 6), Similar results have been observed in the literature. Zhang et al. (2016) reported that biochar increased catalase activity in wheat plants, enhancing resistance to salt stress. Li et al. (2022) observed that biochar improved soil catalase activity, supporting plant growth. Liang et al. (2006) noted that biochar increased soil enzyme activities, improving soil health. For instance, Chen et al. (2019) showed that biochar applications increased soil catalase activity, supporting microbial activity in the soil. Wang et al. (2015) reported that biochar increased soil enzyme activities, improving soil fertility. Laird et al. (2010) indicated that biochar applications improved plant health by increasing soil enzyme activities. Glaser et al. (2002) found that biochar improved agricultural productivity by increasing soil fertility and microbial activity. The same applies to urease results (Fig. 6B). Similar studies support these findings. Nelissen et al. (2014) reported that biochar applications increased soil urease activity, supporting plant growth. Song et al. (2020) and

Sakin et al. (2021) reported that biochar positively affected soil enzyme activities, improving soil nutrient cycling. Peng et al. (2019) noted that biochar improved plant nitrogen uses by increasing soil urease activity. Contrary results have also been reported. For example, Ameloot et al. (2013) observed that some biochar types did not increase or even decrease urease activity. These differences may depend on the properties of the biochar material used and application methods.

Studies on DHG activity (Fig. 6C) in the literature generally report that salt stress reduces microbial activity and enzyme activities. However, some studies have reported that salt stress can increase DHG activity under certain conditions by causing changes in microbial community structure. For example, Luo et al. (2011); Sakin et al. (2024b) reported that biochar applications increased soil DHG activity, supporting microbial activity in the soil. Paz-Ferreiro et al. (2014) and Cevheri et al. (2022) reported that biochar improved soil fertility by increasing enzyme activity. Gul et al. (2015) and Sakin et al. (2021) noted that biochar supported soil health by increasing soil microbial respiration and enzyme activities.

Biochar can be effective in reducing salt concentrations in saline soils. This is due to biochar's high surface area and porous structure, which can help remove salts from the soil solution (Leng et al. 2021). Additionally, the organic matter content in biochar can help move salts away from plant roots (Lee et al. 2022). However, in some cases, biochar applications can increase soil EC. This situation can occur if the biochar contains salts or increases the dissolved organic matter in the soil (Amini et al. 2016; Sakin et al. 2024a).

Studies in the literature report a wide range of effects of biochar on soil pH and EC (Fig. 7). For instance, Dai et al. (2014) noted that biochar applications increased soil pH and neutralized soil acidity. Hossain et al. (2020) observed that biochar improved the uptake of plant nutrients by increasing soil pH. Additionally, Sheng and Zhu (2018) reported that biochar supported soil microbial activity by increasing soil pH.

Studies on EC values generally report the salt-reducing effects of biochar in soils. For example, Farhangi-Abriz and Torabian (2018) noted that biochar supported plant growth by reducing salt concentrations in saline soils. Akhtar et al. (2015b) reported that biochar applications reduced soil EC values, improving water uptake in plants under salt stress. Das et al. (2020) observed that biochar increased plant health and productivity by reducing soil EC.

Although the experiment was conducted in a greenhouse under controlled conditions, it is important to acknowledge potential confounding factors that may have influenced the outcomes. Minor variations in environmental parameters

such as temperature gradients, light exposure, or humidity across different positions in the greenhouse may have impacted plant responses. Additionally, slight heterogeneity in soil composition, water distribution, or salt accumulation within the pots could also have contributed to variability in some measurements. While using a randomized complete block design helped mitigate these effects, future studies may benefit from more refined environmental control and sensor-based monitoring to minimize such sources of experimental noise.

Additionally, attention should be given to the chemical composition of biochar, particularly the presence of volatile organic compounds (VOCs) such as acetic acid. Although no phytotoxic effects were observed in the present study, certain biochar feedstocks or high application rates may pose a risk due to the accumulation of weak organic acids. Therefore, further investigations are warranted to determine safe application thresholds and assess the long-term effects of these compounds under diverse soil and environmental conditions.

Limitations: Besides environmental variability, several broader limitations of this study should be acknowledged. The experiment was conducted under greenhouse conditions with a relatively small sample size ($n=3$), which may limit the extrapolation of the findings to open-field conditions. Moreover, the study duration of 60 days may not fully capture the long-term effects of biochar on soil structure or plant physiology. The findings demonstrate strong treatment-related associations; however, the underlying biochemical and molecular mechanisms were not explored. Future research should consider longer-term field experiments, larger replicates, and molecular-level analyses to further validate and expand on the present results.

5 Conclusion

This study demonstrates that biochar application effectively mitigates the adverse effects of salinity on cotton growth by simultaneously improving plant performance and soil functionality. Biochar-treated plants exhibited enhanced root and shoot development, improved photosynthetic capacity, and greater physiological stability under saline conditions, indicating an overall improvement in plant resilience to salt stress.

The findings clearly support the tested hypotheses by showing that biochar reduces salt-induced oxidative damage while strengthening plant defense mechanisms and stimulating soil biological activity. Improvements in soil chemical properties and microbial enzyme activities further

indicate that biochar promotes a more favorable soil environment for plant growth under salinity stress. Among the tested doses, the highest application rate consistently produced the strongest responses, highlighting a dose-dependent effect of biochar on both plant and soil systems.

Beyond its effects on individual physiological and biochemical traits, the results emphasize the role of biochar in enhancing plant soil interactions under challenging environmental conditions. By improving soil quality while supporting plant stress tolerance, biochar represents a multifunctional amendment capable of addressing multiple constraints associated with saline agriculture.

Overall, these findings contribute to a broader understanding of sustainable strategies for managing salt-affected soils and improving crop productivity. The use of biochar may offer practical benefits for agricultural systems facing increasing soil salinization, particularly in arid and semi-arid regions. This study provides a scientific basis for integrating biochar into sustainable soil management practices and supports its potential application in environmentally resilient crop production systems.

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Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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