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Biochar and Zinc Oxide Nanoparticles Partnership Mitigate Salinity Stress in Alfalfa by Boosting Antioxidant Defense, Regulating Hormonal Balance, and Stress-Responsive Genes

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ABSTRACT

Soil salinity is a major challenge to crop productivity and global food security, necessitating effective measures to counteract its toxic effects. Biochar (BC) and nanoparticles mitigate salinity stress (SS), yet the interactive effects of BC and zinc oxide nanoparticles (ZnO-NPs) remain underexplored. Therefore, we investigated the different mechanisms mediated by BC and ZnO-NPs in alfalfa under SS (8 dS m^{-1}) using five treatments: control, SS, SS + BC (2%), SS + ZnO-NPs (100 mg kg^{-1}), and SS + BC + ZnO-NPs. Results showed that SS alone increased oxidative damage, chlorophyll synthesis, hormone synthesis, and gas exchange characteristics, while enhancing Na and Cl availability, ultimately leading to reduced biomass. Co-application of BC + ZnO-NPs mitigated salinity-induced oxidative damage by decreasing hydrogen peroxide, malondialdehyde, abscisic acid, Na, and Cl, alongside increasing antioxidant activity. The combined treatment also restored osmotic balance through elevated proline, soluble protein, indole acetic acid, and gibberellic acid. Furthermore, BC + ZnO-NPs upregulated the expression of genes linked to antioxidant defense and Na extrusion from the cytoplasm into the external environment. Consequently, this improved antioxidant activities and reduced Na accumulation, thereby decreasing oxidative stress and boosting biomass production. Thus, the combined application of BC and ZnO-NPs represents a promising technique to enhance crop production in salt-affected soils.

Keywords: biochar, gene expression, hormonal balance, salinity, nutrient homeostasis.

INTRODUCTION

Salinity stress (SS) is a significant environmental constraint that severely hampers crop productivity, affecting approximately 1.3 million hectares globally exacerbated by intensive farming, unsustainable irrigation, and climate change (Lee et al., 2022; Kamal et al., 2024). SS diminishes growth via ionic toxicity, osmotic imbalance, oxidative bursts, and nutritional disorders (Abid et al., 2020; Shabbir et al., 2021). Ionic toxicity restricts nitrogen assimilation and protein biosynthesis, causing biomass losses (He et al., 2026). Moderate to high SS reduces water potential, creating osmotic gradients that impair stomatal conductance, chloroplast integrity, photosynthesis, and carbon fixation (Waqas et al., 2021). Excessive Na^+ and Cl^- accumulation damages membrane integrity, perturbs ionic balance, and accelerates oxidative damage (Li et al., 2022). This oxidative burst is fueled by reactive oxygen species (ROS), which cause cellular oxidation, degrade thylakoid membranes, decrease PS-II efficiency, and disrupt electron transport (Wei et al., 2016). SS also disturbs hormonal networks by upregulating abscisic acid (ABA) while downregulating indoleacetic acid (IAA) and gibberellic acid (GA), promoting leaf abscission and inhibiting meristematic activity (Hou et al., 2023; Gullap et al., 2024). Though plants deploy stomatal regulation, osmoprotectants, and antioxidant enzymes, these intrinsic responses are often insufficient, necessitating exogenous interventions to restore soil and plant functioning (Yuan et al., 2025).

Biochar (BC) is a recognized soil amendment to improve quality and induce salinity tolerance. Produced via pyrolysis, BC features an expansive surface area with functional groups that bolster soil fertility and yields (Wu et al., 2023; Liu et al., 2024). It

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alleviates SS by reducing Na^+ bioavailability, enhancing water retention, and stimulating microbial consortia (Yu et al., 2019). BC also improves stomatal conductance, photosynthesis, chlorophyll content, and antioxidant activities while reducing oxidative damage (Ali et al., 2017). However, the efficiency of BC varies considerably with pyrolysis temperature, feedstock type, and inherent soil properties, often resulting in inconsistent outcomes (Kapoor et al., 2022). Consequently, recent research has shifted toward integrating BC with complementary amendments to achieve more reliable and amplified stress mitigation (Wu et al., 2023).

Nanotechnology offers a revolutionary route for enhancing stress resilience. Nanoparticles (NPs), with high surface area, reactivity, and unique molecular interactions, can precisely modulate plant physiological and biochemical pathways (Ahmed et al., 2024a; Halshoy et al., 2025). NP applications elevate SS tolerance by promoting osmolyte accumulation, reinforcing antioxidant defenses, and finely tuning ion uptake dynamics (Lalarukh et al., 2022). Furthermore, NPs can directly scavenge ROS or indirectly stimulate endogenous enzymatic machinery, providing dual-layer protection while maintaining a favorable K^+/Na^+ ratio essential for cellular function. For example, TiO_2-NPs activate secondary metabolite production and defensive systems to curtail oxidative injury (Gohari et al., 2020), while also enhancing photosynthesis and upregulating genes for energy and water homeostasis (Wei et al., 2024).

Alfalfa is a high-value leguminous forage with superior nutrition and adaptability to marginal conditions (Fan et al., 2025). As a nitrogen-fixer, it inherently enriches soil fertility and improves physical soil structure (Ghafoor et al., 2024). Although moderately salt-tolerant, alfalfa productivity drops substantially when electrical conductivity exceeds 6 dS m^{-1}, necessitating effective countermeasures to sustain forage production (Al-Farsi et al., 2020). While the individual effects of BC and NPs on salinity alleviation are well documented, their combined interactive effects on alfalfa physiology and stress defense remain critically underexplored. Therefore, we hypothesized that co-applying BC and zinc oxide nanoparticles (ZnO-NPs) would elicit a more pronounced mitigation of SS compared to singular treatments. The specific aims were: (1) to determine the restorative impacts of BC and ZnO-NPs on plant growth, physiological functioning (including gas exchange and chlorophyll synthesis), and ultimate biomass yield; (2) to elucidate their combined effects on phytohormonal equilibrium (ABA, IAA, GA) and ionic (Na^+/Cl^-) homeostasis; and (3) to evaluate their regulatory influence on the transcriptional expression of key antioxidant genes and Na^+-extruding genes responsible for cytoplastic Na^+ efflux. This investigation provides a scientific foundation for developing sustainable, integrated soil management strategies to boost forage productivity and resilience in salt-stressed agroecosystems.

MATERIAL AND METHODS

Location and experimental treatments

This study was conducted in a rain-protected shelter at Qingdao Agricultural University. Soil (0-20 cm topsoil) from the experimental field was used to fill pots. The soil had pH 5.58, EC 1.75 dS/m, total N 1.40 g/kg, available P 20.32 mg/kg, and available K 132.12 mg/kg. The study had five treatments: control, SS (8 dS/m), SS + BC (2%), SS + ZnO-NPs (100 mg/kg), and SS + BC + ZnO-NPs (100 mg/kg). Salinity was induced by dissolving NaCl in irrigation water and allowing two weeks of equilibration in pots. Biochar was prepared by pyrolyzing dried maize straw at 500°C, while ZnO-NPs were obtained commercially (Shanghai Xiaohuang Nanotech). Both BC and ZnO-NPs were thoroughly incorporated into the soil, which was then maintained at 70% field capacity for equilibration. Alfalfa seeds were surface-sterilized with 75% ethanol, rinsed, sown in seedling trays, and five uniformly sized seedlings at the cotyledon stage were subsequently transplanted into each pot. The experimental layout followed a completely randomized design with three replications per treatment.

Measurement of leaf gas exchange traits and physiological traits

Leaf gas exchange traits such as stomata conductance (gs), transpiration rate (E), carbon dioxide assimilation (A), and photosynthetic rate (Pn) were measured using the Li-6800 portable photosynthesis system. 2 g leaf slices were taken and immersed in 2 mL of acetone solution (95%) and subjected to shaking for 5 minutes to ensure the complete extraction of pigments and chlorophyll contents measured with methods of Arnon (1949). The relative water contents (RWC) were measured based on fresh, turgid and dry weight bases. The concentration H_2O_2 and MDA were measured with protocols of Velikova et al. (2000) and Heath and Packer (1968). The activities of SOD, and CAT were measured with techniques of Kono (1978) and Aebi (1984), while APX and POD were measured with methods of Nakano and Asada (1981).

Measure of osmolytes and hormones

The concentration of proline was measured with Bates et al. (1973) method, while FAA and SP were measured with protocols of Hamilton and Vanslyke (1943) and Bradford (1976). To measure different phyto-hormones, fresh leaves were taken and wrapped in aluminum foil, frozen in liquid nitrogen, and stored at -80°C. The concentration of IAA, GA, and ABA was determined by using Kit provided by Shanghai Jianglai Biotechnology, Limited, China by following the methods established by (Koshita et al., 1999).

Growth and biomass traits, and nutrient concentration in plant leaves

Five plants were selected to measure leaf area (LA), leaf length (LL), plant height (PH), and biomass yield (BY). After harvest, roots were separated from shoots to determine root length (RL) and root fresh weight (RFW). For leaf nutrient analysis, samples were washed to remove surface salts, blotted dry with tissue paper, dried at 80°C to constant weight, milled, and 0.1 g digested with HNO_3:$HClO_4$ (4:1) before dilution to 50 mL with water. Leaf Na and K were measured with flamephotometer; Cl using a chloride analyzer; N and P via spectrophotometry and flame photometry. Soil total nitrogen (TN) was assayed by the Kjeldahl method, available P and K by spectrophotometry and flame photometry, and soil organic carbon (SOC) by the potassium dichromate heating method with concentrated H_2SO_4.

Determination of gene expression

Briefly, 100 mg leaf samples were collected, and RNA extraction was done with the Trizol reagent technique. Following this extracted RNA was reverse transcribed into cDNA by using the eraser kit (Takara, Co. Ltd., Japan). Thereafter, SYBR premix Ex TaqII was used to analyze the cDNA with the aid of quantitative real-time polymerase chain reaction. Furthermore, Actin was used as a reference gene, and the expression level of targeted genes was measured by the methods of Livak and Schmittgen (2001).

Data analysis

The data collected on the traits were analyzed with one-way analysis of variance by using Statistix 8.1. Tukey's multiple comparison test was employed to compare difference among different treatments. The correlation among different traits were performed by using the ggplot2 packages in R software (version 4.2.2).

RESULTS AND DISCUSSION

Growth and biomass, photosynthetic pigments and relative water contents

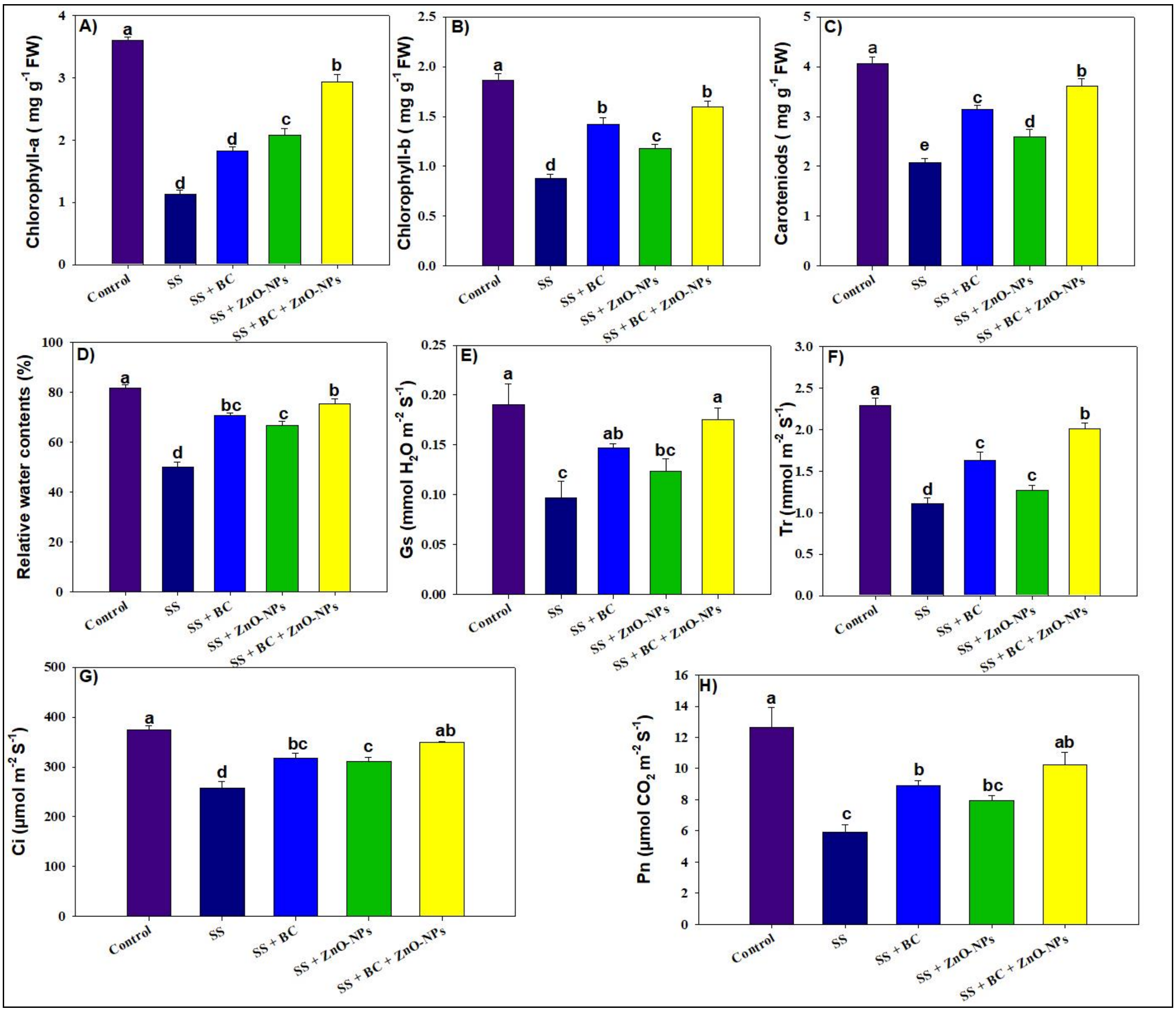
Biochar and ZnO-NPs significantly mitigated the adverse impacts of SS and caused a significant increase in growth traits (Table 1). Under SS integrated BC + ZnO-NPs enhanced the RL, RFW, PH, LL, LA and BY of alfalfa by 30.57%, 63.49%, 45.24%, 51.91%, 49.82% and 78.26% respectively (Table 1). Salinity stress markedly reduced chlorophyll (Chl-a/b), carotenoids, relative water content (RWC), and gas-exchange traits (gs, Tr, Ci, Pn) vs. control (Figure 1).

Biochar and ZnO-NPs individually mitigated these adverse effects, but their combined application proved most beneficial. Under SS, application of BC + ZnO-NPs raised Chl-a, Chl-b, carotenoids, and RWC by 159.29%, 80.68%, 42.65%, and 50.66%, respectively, while enhanced gs, Tr, Ci, and Pn by 80%, 81%, 35%, and 74% (Figure 1).

Table 1. Impacts of biochar and zinc oxide on the growth traits of alfalfa subjected to salinity stress

Treatments	RL (cm)	RFW (g)	PH (cm)	LL (mm)	LA (mm)	BY (g/pot)
Control	24.65a±1.73	1.14a±0.09	29.94a±1.72	15.29a±0.97	159a±4.11	20.97a±2.57
SS	17.01c±0.85	0.63d±0.02	17.57d±0.49	8.90d±0.28	93d±5.35	9.66d±0.42
SS + BC	20.21bc±0.82	0.87bc±0.05	21.20c±0.82	11.90bc±0.26	132bc±5.32	14.65bc±0.60
SS + ZnO-NPs	18.87bc±1.25	0.76cd±0.04	19.03cd±0.60	10.56cd±0.29	118c±4.32	12.37cd±0.74
SS + BC + ZnO-NPs	22.21ab±0.82	1.03ab±0.06	25.52b±0.86	13.52ab±0.67	139b±4.92	17.22ab±0.81

RL: root length, RFW: root fresh weight, PH: plant height, LL: leaf length, LA: leaf area, BY: biomass yield. The data represent the mean with ±SD (n=3) and different letters with mean values represent the different differences (p≤0.05).



The data represent the mean with ±SD (n=3) and different letters with bars represent the different differences (*p≤0.05*). Gs: stomata conductance, Tr: transpiration rate, Ci: intercellular CO_2, Pn: photosynthetic rate.

Figure 1. Impacts of biochar and zinc oxide on photosynthetic pigments (a-c), leaf water contents (d) and leaf gas exchange properties (E-H) of alfalfa subjected to salinity stress.

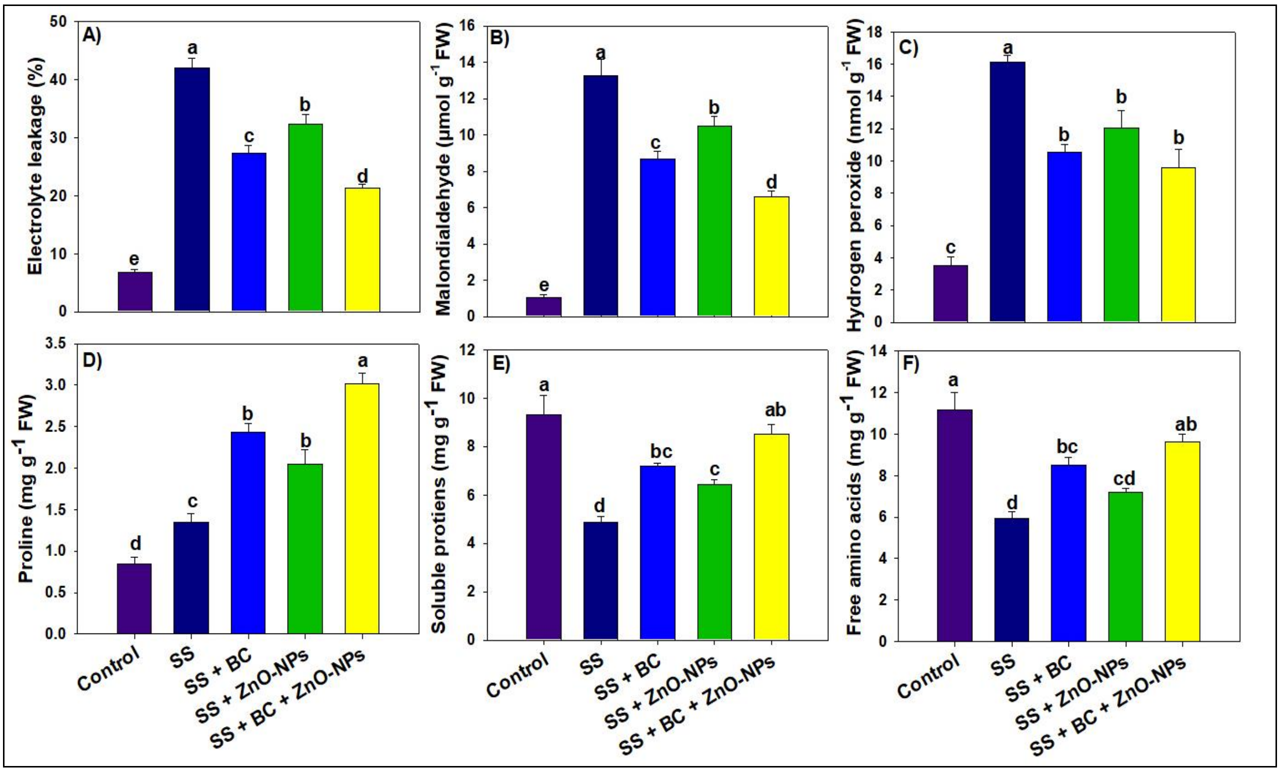
Oxidative markers and osmolytes

Salinity stress (SS) elevated EL, H_2O_2, and MDA, but this rise was less pronounced under BC + ZnO-NPs than individual treatments. Under SS, combined BC+ ZnO-NPs reduced EL, H_2O_2, and MDA by

96.66%, 68.16%, and 101.36%, respectively. SS raised proline but lowered soluble SP and FAA. Both BC and ZnO-NPs enhanced proline, SP, and FAA, with their combination yielding the greatest increases of 123.68%, 75.51%, and 61.57%, respectively under SS (Figure 2, Table 1).

Hormones and antioxidants

Different treatments, including SS, BC, and ZnO-NPs, significantly affected IAA, GA, and ABA production in alfalfa plants (Figure 3). Salinity declined IAA and GA contents of alfalfa leaves, while it increased ABA contents (Figure 3). Additionally, BC, ZnO-NPs, and their combination significantly increased the IAA, and GA contents while decreased the ABA content in alfalfa plants facing the SS (Figure 3). Under SS, BC + ZnO-NPs treatment caused an increase of 92.42%, and 82.06% in IAA, GA contents while BC + ZnO-NPs decreased the ABA contents by 46.22% (Figure 3). The results depicted that SS caused a little increase in antioxidants activity showing that alfalfa plants activated their antioxidant defense to counter SS. However, BC + ZnO-NPs increased the antioxidants, which helped in mitigating the adversities of salinity (Figure 4). Under SS, combined use of BC + ZnO-NPs caused an appreciable increase of 52.09%, 22.31%, 88.42% and 26.43% respectively, in APX, CAT, POD, and SOD activities (Figure 4).

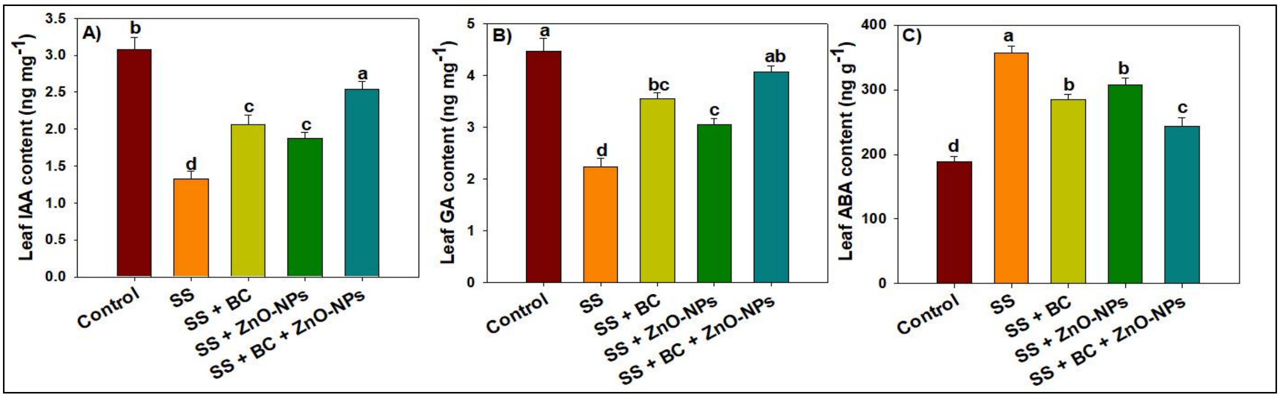


The data represent the mean with ±SD (n=3) and different letters with bars represent the different differences (*p≤0.05*).

Figure 2. Impacts of biochar and zinc oxide on oxidative markers (a-c), and osmolytes (D-F) of alfalfa subjected to salinity stress.

Nutrient accumulation in plant parts

Under SS, alfalfa leaves exhibited reduced concentrations of K, N, P, Ca, and Mg compared to the control (Table 2). Furthermore, relative to the control, SS drastically reduced N, P, Ca, and Mg by 92.42%, 92.42%, 82.06%, and 46.22%, respectively. Both BC and ZnO-NPs reversed these declines, and their co-application further augmented N, P, Ca, and Mg by 38.81%, 30.03%, 42.21%, and 47.14%, respectively, under SS (Table 2). Salinity stress increased Na and Cl accumulation while BC + ZnO-NPs a more pronounced reduction of 55.57% for Na and 68.59% for Cl in leaf tissues.

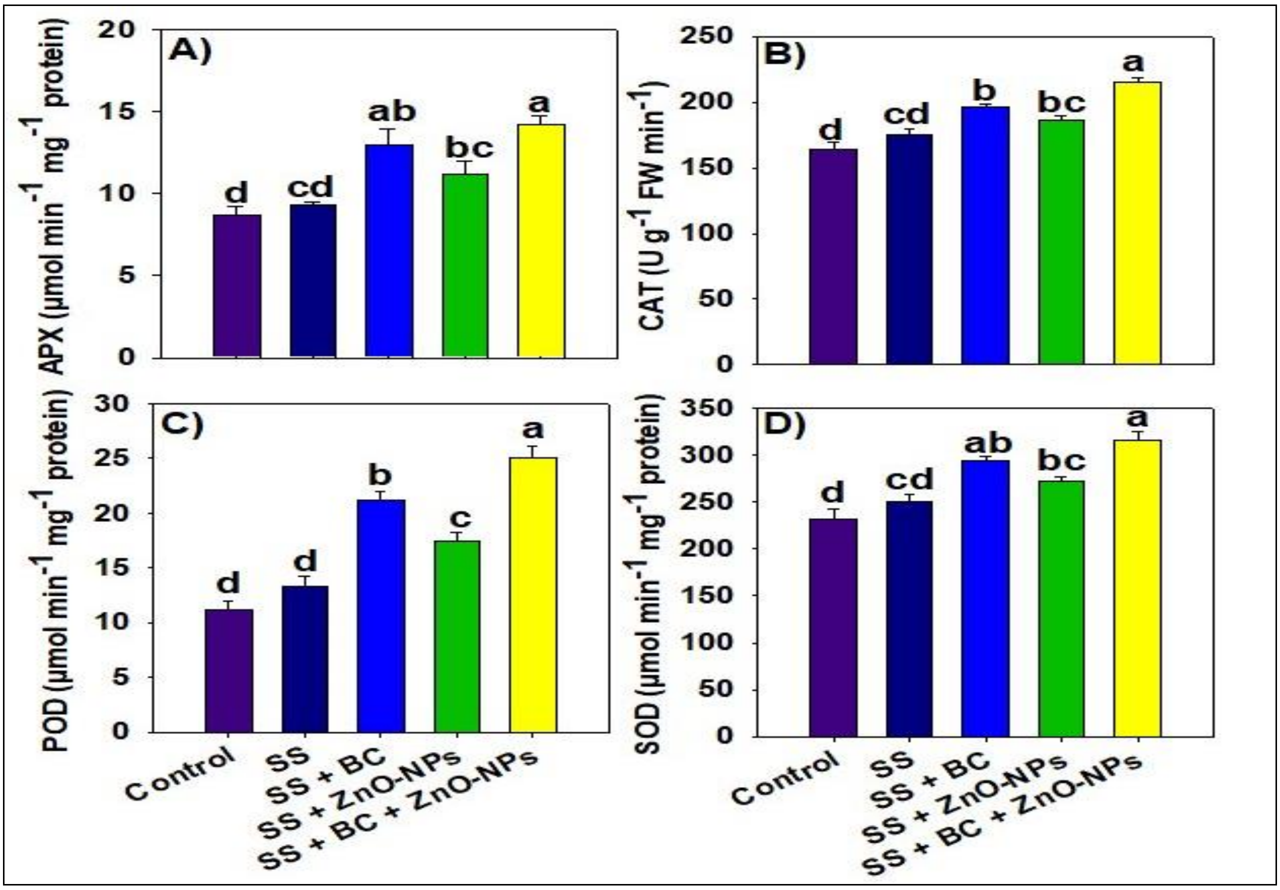


The data represent the mean with ±SD (n=3) and different letters with bars represent the different differences ($p \leq 0.05$). IAA: indole acetic acid, GA: gibberellic acid, ABA: abscisic acid.

Figure 3. Impacts of biochar and zinc oxide on phyto-hormones synthesis (a-c) of alfalfa subjected to salinity stress.

Expression pattern of antioxidant and ion transport genes

Antioxidant gene expression was increased in response to SS, indicating alfalfa plants activated their defense system in response to SS (Figure 5). Biochar and ZnO-NPs enhanced *MtAPX*, *MtCAT*, *MtPOD*, and *MtSOD* gene expression (Figure 5). We witnessed that BC + ZnO-NPs caused an increase of 39.77%, 76.67%, 89.92% and 63.94%, respectively in *MtAPX*, *MtCAT*, *MtPOD* and *MtSOD* gene expression under SS (Figure 5). Moreover, *SOS1* and *HKT1* expression also increased in response to soil salinity (Figure 5). The maximum increase in expression of both *SOS1* and *HKT1* (63.95% and 35.82%) was observed with BC + ZnO-NPs under SS (Figure 5).

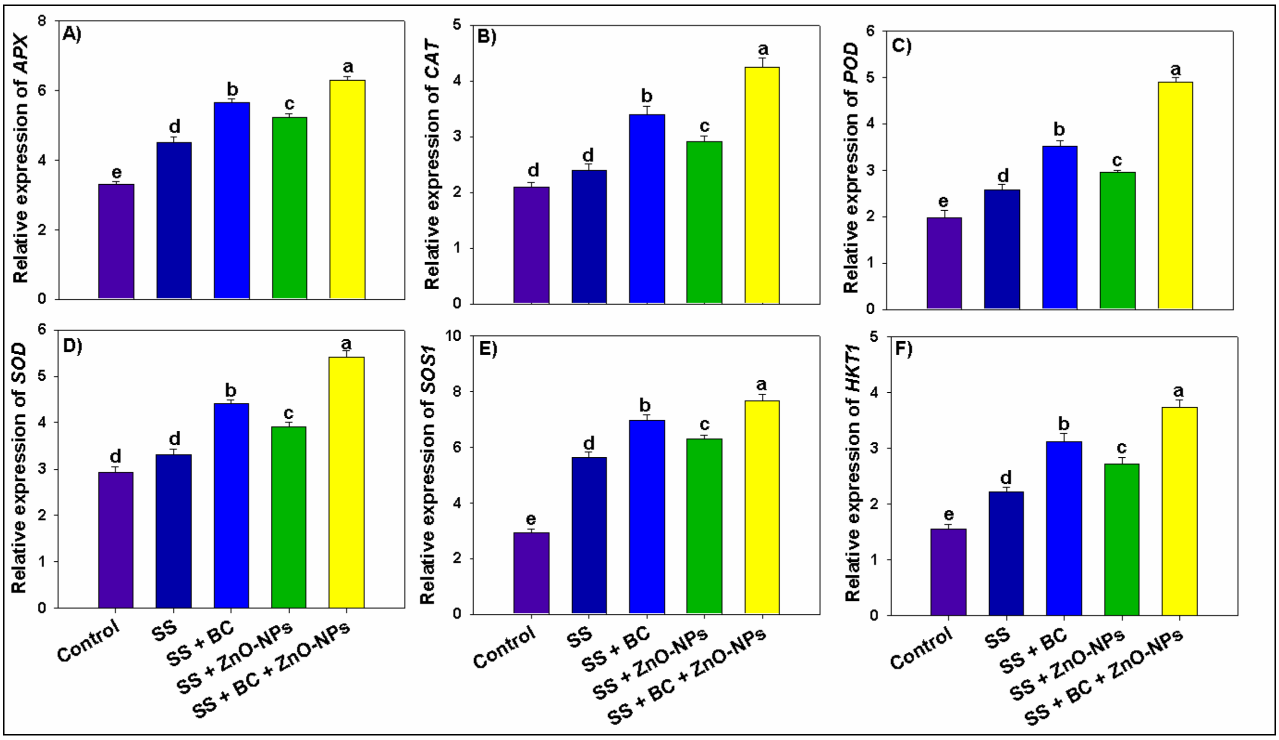


The data represent the mean with ±SD (n=3) and different letters with bars represent the different differences (*$p \leq 0.05$*). APX: ascorbate peroxidase, CAT: catalase, POD: peroxidase, SOD: superoxide dismutase.

Figure 4. Impacts of biochar and zinc oxide on antioxidants activities (a-d) of alfalfa subjected to salinity stress.

Soil nutrient and carbon availability

The availability of soil TN, AP, and AK was significantly curbed in SS, indicating an intense competition between salt and nutrient ions (Table 3). Biochar and ZnO-NPs restored the effects of salinity and increased soil TN, AP, and AK availability (Table 3). Notably, BC + ZnO-NPs caused a marked increase of 24.73%, 57.97% and 27.72% in soil TN, AP, and AK availability under SS (Figure 6). The results also reported that SS decreased soil SOC availability, while BC considerably increased the availability of SOC in saline conditions (Table 3). The results showed that BC enhanced the SOC by 11%, while BC + ZnO-NPs enhanced the SOC availability by 11% in saline soil (Table 3).



The data represent the mean with ±SD (n=3) and different letters with bars represent the different differences ($p \leq 0.05$).

Figure 5. Impacts of biochar and zinc oxide on antioxidants genes expression (a-d) and ion transport gene expression of alfalfa subjected to salinity stress.

Salinity stress significantly reduced alfalfa growth and biomass production, primarily through heightened Na availability that provoked oxidative stress, membrane destabilization, impaired hormone synthesis, diminished photosynthetic efficiency, and reduced soil nutrient availability. SS also decreased root length and plant height, attributable to inhibited cell division and elongation (Zarat et al., 2020; Barzana et al., 2021). Salinity also decreased the leaf area and leaf number. This SS-mediated decline in leaf area and length limits photosynthetic carbon gain, further suppressing biomass accumulation. Collectively, SS retards growth via ionic and osmotic imbalances. In contrast, BC and ZnO-NPs, particularly in combination, markedly improved both below- and above-ground traits. BC+ZnO-NPs boosted nutrient absorption, light interception, and photosynthetic efficiency (Figure 1), thus enhancing growth and yield. BC alone elevates soil water-holding capacity, porosity, and SOC content (Hou et al., 2023; Lin et al., 2024), while NPs reinforce plant function by heightening antioxidant activity and curbing Na/Cl accretion (Table 2). Consequently, BC + ZnO-NPs yielded superior alfalfa growth and biomass. This combined treatment further augmented soil nutrient availability and plant nutrient uptake, attributable to the abundant nutrients inherent in BC (Figure 1) and Zn supplementation from ZnO-NPs, which collectively favor growth (Kapoor et al., 2022). Notably, improved growth correlated with diminished Na accumulation and subsequent oxidative damage (Faizan et al., 2021). Salinity stress significantly decreased carbon assimilation, transpiration, and photosynthetic rates, linked to stomatal closure, reduced chlorophyll (Figure 1), and inhibited enzyme activity (Giannakoula et al., 2025). However, BC+ZnO-NPs enhanced photosynthesis and gas exchange by boosting antioxidant defense, nutrient availability (Figures 5, 7), and chloroplast membrane stability (Chatzistathis et al., 2025). This improvement arose from enhanced soil properties, essential nutrient provision, improved antioxidant defense, and reduced Na availability (Table 2). Additionally, BC+ZnO-NPs elevated IAA and GA while lowering ABA (Wang et al., 2023), improving root growth (Table 1) and stomatal conductance (Figure 1), thus maintaining optimal gas exchange (Ahmed et al., 2024b).

Table 2. Impacts of biochar and zinc oxide on the leaf nutrient concentration of alfalfa subjected to salinity stress

Treatments	Na	Cl	N	P	K	Ca	Mg
	mg kg^{-1} (DW)						
Control	3.62d±0.43	3.82d±0.32	40.72a±1.27	13.33a±0.90	31.24a±0.77	9.22a±0.80	8.67a±0.42
SS	10.19a±0.81	14.60a±0.67	26.41d±0.71	8.59c±0.47	17.84c±1.30	5.78d±0.41	4.90d±0.12
SS + BC	8.26b±0.16	9.67c±0.33	32.92c±1.38	10.81b±0.43	23.97b±1.35	7.16bc±0.08	6.07c±0.13
SS + ZnO-NPs	8.91ab±0.34	12.11b±0.69	30.81c±0.44	10.11bc±0.41	21.20bc±0.82	6.81c±0.26	5.36cd±0.33
SS + BC + ZnO-NPs	6.55c±0.48	8.66c±0.24	36.66b±1.08	11.17b±0.82	27.82a±1.07	8.22ab±0.20	7.21b±0.46

Na: sodium, Cl: chloride, N: nitrogen, P: phosphorus, K: potassium, Ca: calcium, Mg: magnesium. The data represent the mean with ±SD (n=3) and different letters with mean values represent the different differences ($p \le 0.05$).

Proline works as an osmotic regulator and cellular protectant, and its synthesis was increased in response to SS. BC + ZnO-NPs enhanced the proline synthesis, which could be linked with up-regulation of carboxylate synthase enzymes, which play a crucial role in proline synthesis (Rai and Penna, 2013). Soluble proteins and FAA are important osmo-protective compounds that play a crucial role against stress (Xu et al., 2021). In the current study, salinity decreased the SP and FAA by aggravating the cellular injuries, increasing ROS production, and decreasing N availability (Table 2). Phyto-hormones also play a critical role in plants' adaptation to SS. Salinity stress decreased IAA and GA and increased ABA. BC + ZnO-NPs restored hormonal balance by enhancing IAA and GA and reducing ABA via improved water availability and down-regulated ABA signaling pathways (Fahad et al., 2015). Salinity stress also enhanced the ROS production while BC and ZnO-NPs decreased ROS production by increasing antioxidants activity. Both zinc and BC control the Na and Cl absorption; therefore, they can mitigate the potential damages such as lipid peroxidation and ROS production (Ahmed et al., 2023). The antioxidants activities increase with BC and ZnO-NPs application. The gene expression results also showed that BC + ZnO-NPs enhanced the expression of antioxidant genes, which prevented ROS damage via increasing the defense mechanisms.

Table 3. Impacts of biochar and zinc oxide on the soil nutrients and carbon availability

Treatments	TN (g kg^{-1})	AP (mg kg^{-1})	AK (g kg^{-1})	SOC (mg kg^{-1})
Control	1.53±0.074	19.87±1.25	3.72±3.72	11.33±0.80
SS	1.12±0.057	10.85±0.96	4.19±4.19	9.59±0.31
SS + BC	1.29±0.029	15.47±0.90	1.92±1.92	12.13±0.73
SS + ZnO-NPs	1.19±0.054	11.87±0.47	2.05±2.05	9.94±0.28
SS + BC + ZnO-NPs	1.39±0.052	17.14±0.37	1.88±1.88	13.53±0.97

The data represent the mean with ±SD (n=3) and different letters with mean values represent the different differences ($p \le 0.05$). TN: total nitrogen, AP: available phosphorus, AK: available potassium, SOC: soil organic carbon.

Under SS, Na^+ and Cl^- contents increased, whereas N, P, K, Ca, and Mg decreased, indicating that Na competitively inhibits uptake of essential ions, provoking ionic imbalance (Kaymak Bayram et al., 2025). Biochar and ZnO-NPs effectively reduced Na^+ uptake while enhancing K^+ and other nutrient acquisitions, consistent with prior reports (Mukhtar et al., 2023; Sultan et al., 2024). This Na^+ reduction stems from adsorption onto BC's large surface area (Sudratt and Faiyue, 2023) and Zn^{2+}-mediated limitation of Na^+ entry (Mazhar et al., 2023), collectively stabilizing membranes and improving growth. Plants counter abiotic stress via complex gene-expression reprogramming (Miryeganeh, 2021). Our results showed that BC + ZnO-NPs markedly augmented expression of antioxidant-related genes, (Vighi et al., 2017). Additionally, this combined treatment upregulated *SOS1* and *HKT1* genes, which encode Na^+-extruding transporters, promoting efflux of Na^+ from the cytoplasm to the external environment. This dual action enhancing antioxidant capacity and facilitating Na^+ extrusion effectively alleviates salt toxicity, restores ion homeostasis, and supports better plant performance under saline conditions.

CONCLUSIONS

Salinity diminished the alfalfa growth and biomass by increasing oxidative damages, sodium availability, abscisic acid synthesis and decreasing chlorophyll pigments, osmolytes production, hormones synthesis and soil nutrient and carbon availability. However, biochar and ZnO-NPs maintained the cell membrane structure, chlorophyll synthesis, gas exchange properties, antioxidant activities and osmotic balance and decreased the sodium availability and oxidative damages leading to increase in growth and biomass yield. The integrated biochar and ZnO-NPs also modulated the expression of antioxidants and ion transporter genes thus, decreasing the oxidative damages and augmenting the biomass production. This indicates the potential of biochar and ZnO-NPs as important practices to enhance alfalfa productivity in salt affected soil. However, further studies are needed to assess the efficacy of this practices in varying climate conditions and saline conditions. Moreover, studies should also focus on nano-particles accretion and its impacts on plant functions and growth.

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