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ARTICLE

Responses of Osmotic Adjustment and Antioxidant Enzyme Activities in Alfalfa (*Medicago sativa* L.) Seedlings to Biochar Amendment under Salt Stress

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ABSTRACT: Salt stress has been demonstrated to trigger the overaccumulation of reactive oxygen species (ROS) within plant tissues. This process, in turn, has been shown to induce oxidative damage and further impair the physiological and metabolic processes. As a promising organic soil conditioner, biochar has proven effective in mitigating the adverse effects of salt stress in crops. Nevertheless, the synergistic and interactive effects of salt stress and biochar application on the osmotic adjustment system and antioxidant defense system of alfalfa (*Medicago sativa* L.) have not been fully elucidated. This study employed a completely randomized design, comprising four NaCl-induced salt stress levels (0, 3, 6, and 9 dS m^{-1}) and three biochar application rates (0, 30, and 60 g kg^{-1}). To evaluate the role and underlying mechanism of biochar in alleviating oxidative damage caused by salt stress in alfalfa, we determined the growth indicators (shoot biomass [SB], root biomass [RB], and total biomass [TB]), physiological indicators (total chlorophyll content and relative water content), and biochemical indicators (osmotic adjustment substances, antioxidant enzyme activities, and oxidative stress-related substances). Results indicated that salt stress significantly suppressed alfalfa biomass accumulation by 16%–84%. With increasing salt stress intensity, the contents of proline (Pro), glycine betaine (GB), soluble sugars (SS), and soluble proteins (SP), along with the activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and polyphenol oxidase (PPO), significantly increased in alfalfa leaves; yet, these defensive responses failed to prevent the decrease in leaf relative water content and the significant accumulation of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) contents. These results indicated that the endogenous defense system alone is insufficient to counteract salt stress-induced oxidative damage. In contrast, compared with the non-biochar control, biochar application significantly increased alfalfa chlorophyll content (by ~40%) and biomass accumulation (by ~12%). Notably, under the 30 g kg^{-1} biochar treatment, the antioxidant enzyme activities and osmotic adjustment substance contents of alfalfa seedlings significantly decreased, accompanied by a marked reduction in $O_2^{\bullet-}$ (by ~13%), H_2O_2 (by ~21%), and MDA (by ~30%) contents. These results indicated that biochar could maintain the dynamic equilibrium of osmotic regulation and antioxidant enzyme systems in alfalfa leaves, and promote the synthesis of photosynthetic pigments and photosynthates, thereby enhancing alfalfa biomass accumulation by 54%–68%. However, under severe salt stress (9 dS m^{-1}), excessive biochar application rate (60 g kg^{-1}) failed to exert a positive effect on alfalfa growth, with only approximately a 1% increase in total biomass of alfalfa seedlings. Collectively, our findings demonstrated that appropriate biochar application can effectively alleviate salinity-induced oxidative damage in alfalfa and thus promote its growth in saline soils. To alleviate salt stress-induced phytotoxicity and optimize the efficacy of soil amendments, it is imperative to rationally regulate the application amount of biochar in saline soils.

KEYWORDS: Salt stress; biochar amendment; *Medicago sativa* L.; osmotic adjustment substances; antioxidant enzyme activities

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1 Introduction

Globally, an estimated 1.4 billion hectares of terrestrial land, accounting for more than 10% of the global total land area, is threatened by soil salinization [1]. In China, there are approximately 7.6 million hectares of salt-affected soils (over 5% of the total arable land area), which is projected to expand continuously in the coming years, driven by unsustainable water and land management practices, along with the intensifying impacts of global climate change [2]. Salt stress is defined as an adverse condition caused by the excessive accumulation of soluble salts in soil, which typically induces a series of adverse effects, including the disruption of ion balance, induction of osmotic stress, and excessive production of reactive oxygen species (ROS) [3,4]. These stress effects severely disturb the normal physiological and biochemical metabolic processes in plants (e.g., impairing chloroplast structure and thereby reducing chlorophyll content), thus inhibiting plant growth and development, and ultimately leading to a significant decline in crop yield and quality [5,6]. Numerous investigations have shown that abiotic stresses, especially salt stress, disrupt the dynamic equilibrium between ROS generation and scavenging in plant cells, and excessive ROS accumulation is the core reason for the decline in global crop yields [3,7–9]. Excessive ROS can impair multiple cellular physiological functions by damaging nucleic acid structures, oxidatively modifying proteins, and inducing lipid peroxidation, with the most prominent consequence being the impairment of cell membrane structural integrity [10]. This not only hinders substance transport across cell membranes but also disrupts cellular osmotic homeostasis and ultimately seriously affects the normal physiological functions in plants [11].

The major ROS that accumulate at high concentrations in plants include hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and superoxide anion ($O_2^{\bullet -}$) [7]. Given the frequent occurrence of water and salt stresses, which can affect the majority of habitats, plants have evolved a series of sophisticated adaptive strategies to cope with these abiotic stresses [12,13]. Plants possess efficient ROS scavenging systems to counteract oxidative damage induced by excessive ROS accumulation [14]. Studies have confirmed that under salt stress, plants can scavenge stress-induced ROS through the enzymatic antioxidant system, whose core components are superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO) [15,16]. Specifically, SOD converts $O_2^{\bullet -}$ into H_2O_2 to alleviate cellular damage, while POD, CAT, and APX further catalyze the decomposition of H_2O_2 into water and oxygen, achieving the cascade scavenging of ROS [7]. Meanwhile, plants can maintain cellular ion homeostasis by limiting excessive Na^+ accumulation and sustaining a stable Na^+/K^+ ratio, thereby mitigating ion toxicity-induced damage to plant tissues, which is primarily because K^+ functions as an important cofactor/activator for key antioxidant enzymes (e.g., SOD, POD, CAT, and APX) [17]. Therefore, maintaining cellular ion homeostasis can prevent a marked reduction in antioxidant enzyme activity, which would otherwise lead to the accumulation of ROS and exacerbate oxidative damage. As reported by Urbanavičiūtė et al., this process is coordinately regulated by the Salt Overly Sensitive (SOS) pathway genes (e.g., SOS3, SOS2, and SOS1), the vacuolar Na^+/H^+ antiporter gene (e.g., *NHX1*), high-affinity K^+ transporters (HKT), and the calcineurin B-like protein (CBL)–CBL-interacting protein kinase (CIPK) signaling complex, which collectively limit intracellular Na^+ buildup while sustaining K^+ acquisition [18]. Apart from the enzymatic antioxidant system, plant cells can also maintain osmotic homeostasis by accumulating osmotic adjustment substances to combat osmotic damage induced by salt stress [19,20]. The core mechanism involves increasing the concentration of compatible organic solutes in tissues to minimize intracellular water loss, enhance water absorption capacity in saline environments, and thereby avoid metabolic function disruptions from osmotic dehydration [21]. Relevant research has demonstrated that under salt stress, plants can significantly elevate tissue levels of osmotic adjustment substances, such as endogenous glycine betaine (GB), free proline (Pro), soluble sugars (SS), and soluble proteins (SP) [12,19]. This optimization of osmotic balance ultimately alleviates the symptoms of saline-alkaline stress [11]. Furthermore, these substances can protect the bio-membrane integrity, stabilize protein conformations, scavenge ROS, and maintain cellular redox homeostasis, playing a pivotal role in enhancing plant salt stress tolerance [4,22].

Alfalfa (*Medicago sativa* L.) represents a key high-value species in the economic forage industry. This long-lived leguminous forage is characterized by a deep root system, superior biomass yield, excellent palatability, and excellent digestibility. Its rich nutritional composition, particularly in terms of protein, vitamins, and mineral elements, further enhances its significance in modern agriculture [23]. Meanwhile, alfalfa can adapt to mild saline-alkaline environment, which can effectively lower the groundwater level, remove soil salt, and improve the physicochemical properties of saline soils, thus creating a suitable soil environment for the subsequent cultivation of other economic crops [16]. Previous research by Al-Farsi et al. [19] has shown that under mild saline-alkaline stress, alfalfa can significantly increase fresh forage yield by upregulating antioxidant enzyme activities and enhancing osmotic adjustment substance accumulation. However, this species is relatively sensitive to moderate salt stress, with its growth potential and productivity are significantly inhibited. Research has shown that under either moderate (6 dS m^{-1}) or severe (10 dS m^{-1}) salt stress, alfalfa accumulates higher amounts of free proline in leaves, increases soluble phenolic substances, enhances CAT activity, and raises potassium ion levels in leaves, yet forage fresh weight exhibits no significant difference compared with the control group [24,25]. Therefore, enhancing alfalfa's soil adaptability and tolerance to salt stress in salt-alkaline environments is a critical issue that urgently requires addressing in current production [23]. Currently, saline-alkaline soil improvement techniques such as substrate drainage, sand mixing and mulching, and field management have limited applications due to their shortcomings, such as high cost, low remediation efficiency, and potential risk of secondary pollution [26]. Therefore, it is imperative to develop efficient, low-cost, and eco-friendly management strategies to remediate saline-alkaline soils, alleviate the adverse impacts of ROS-induced damage by salt stress on alfalfa, and thereby improve its productivity in saline environments.

Biochar is a stable carbonaceous material produced by heating organic materials in an oxygen-limited environment [27]. It exhibits outstanding application prospects for improving the physical and chemical characteristics as well as nutrient availability of salt-affected soils [28]. Its unique porous structure, conferred by a high specific surface area, endows it with outstanding physicochemical stability and a strong ability to adsorb and retain essential soil elements [29,30]. Relevant studies have shown that biochar application could regulate plant growth and alleviate stress damage through direct and indirect dual pathways under saline-alkaline stress: (1) Directly, biochar is rich in mineral elements such as nitrogen (N) and phosphorus (P), which directly enhance plant nutrient uptake efficiency and provide material support for plant growth and development [31]. (2) Indirectly, its regulatory effects mainly lie in two aspects: First, its porous structure can effectively improve soil physicochemical properties; increased application doses of biochar markedly raise the fraction of soil macroaggregates and soil porosity, thereby enhancing soil aeration and optimizing soil water-holding capacity (WHC) [32,33]. Second, biochar modulates the structure, metabolic activity, and diversity of soil bacteria and fungi, thereby indirectly affecting the availability and supply efficiency of soil nutrients (e.g., soil available N and available P), and creating a favorable microenvironment for plant growth [30,34]. Overall, biochar application synergistically optimizes nutrient supply, soil physicochemical properties, and the microecological environment, thereby significantly promoting plant growth in salt-stressed soils and effectively alleviating saline-alkaline stress-induced growth inhibition in plants [29,35]. This provides important technical support for vegetation restoration in saline soils. However, the existing research on the effects of biochar on the osmotic adjustment system and antioxidant enzyme regulation system of alfalfa leaves under gradient salt stress remains relatively limited.

Therefore, the purpose of this study was to investigate the responses of osmotic adjustment and antioxidant enzyme systems in alfalfa seedlings to biochar amendment under salt stress. Specifically, the determined parameters included growth traits (total biomass [TB], shoot biomass [SB], and root biomass [RB]), leaf physiological characteristics (total chlorophyll [Chl] content and relative water content [RWC]), osmotic adjustment substances (Pro, GB, SS, and SP), antioxidant enzyme activities (CAT, POD, SOD, APX, and PPO), along with oxidative stress indicators (H_2O_2, $O_2^{\bullet -}$, and malondialdehyde [MDA]). We hypothesized two predictions based on earlier reports and our prior pilot test findings: (1) salt stress can inhibit the physiological traits and growth of alfalfa, but alfalfa would mitigate oxidative damage caused by excessive ROS accumulation through the active regulation of its osmotic adjustment and antioxidant enzyme systems; (2) appropriate biochar application rates could significantly enhance the contents of osmotic adjustment substances and activities of antioxidant enzymes in alfalfa, thereby improving its growth performance. This study is expected to provide empirical data support and a theoretical basis for developing a green regulatory strategy to enhance alfalfa salt tolerance under saline conditions.

2 Materials and Methods

2.1 Biochar and Soil Preparation

The biochar employed in this study was derived from wheat straw via a 500°C pyrolysis process. The experimental soil was collected from the uppermost layer of soil (0–25 cm depth) of a loam soil in Jiaozhou City, Shandong Province, China (120°04′ E, 36°26′ N, 37 masl). The fundamental physicochemical characteristics of the examined soil and biochar are presented in Table 1.

Table 1: Soil and biochar characteristics in this study. Data are presented as the arithmetic mean of five independent replicates.

	TC	**TN**	**TP**	**TK**	**OC**	**AN**	**AP**	**AK**	**pH**	**EC**
Biochar	582.39	1425.17	1357.33	19.45	524.15	134.71	697.26	13.78	8.91	2.96
Soil	23.23	673.29	818.43	8.54	20.91	41.63	17.82	2.63	7.85	1.24

Note: TC, total carbon (g kg^{-1}); TN, total nitrogen (mg kg^{-1}); TP, total phosphorus (mg kg^{-1}); TK, total potassium (g kg^{-1}); OC, organic carbon (g kg^{-1}); AN, available nitrogen (mg kg^{-1}); AP, available phosphorus (mg kg^{-1}); AK, available potassium (g kg^{-1}); EC, electrical conductivity (dS m^{-1}).

2.2 Study Site and Experimental Design

The experiment was conducted within an artificial climate greenhouse at Qingdao Agricultural University (120°39′ E, 36°31′ N, 50 masl), adopting a two-factor fully randomized block design including salt stress (S) and biochar amendment (B). Alfalfa seedlings (*Medicago sativa* L. cv. Zhongmu No.3) were subjected to four levels of salt stress (0 dS m^{-1}, 3 dS m^{-1}, 6 dS m^{-1}, and 9 dS m^{-1}, induced by NaCl) and three biochar application rates (0 g kg^{-1}, 30 g kg^{-1}, and 60 g kg^{-1}). The seeds of *M. sativa* were subjected to a process of surface sterilization, employing 75% ethanol as the primary agent, followed by thorough rinsing with deionized (DI) water. The seeds were then subjected to a germination process on moist filter paper at 20°C for a period of 48 h. Before the transplanting of alfalfa seedlings, the weighed biochar was fully mixed with the test soil, and the mixture was filled into plastic pots (24 cm × 23 cm), each containing with 5 kg of soil-biochar mixture in each pot. Five alfalfa seedlings with consistent growth vigor and uniform size were selected and transplanted into each pot, with no seedling injury during the transplanting process. Salinity treatments were initiated two weeks after seedling transplantation, with NaCl-supplemented irrigation water used to ensure uniform application of salt stress. To mitigate acute salt injury to alfalfa seedlings, a gradual increment method was adopted for salt stress imposition. Specifically, the NaCl concentration in the irrigation water was increased by one-third of the final target concentration every 3 days until the predetermined salt stress level was reached. During the seedling establishment period, daily irrigation was applied to maintain the soil water content at 70% of field capacity, ensuring water did not become a limiting factor for growth. The greenhouse environment was controlled throughout the experiment, adhering to a day/night temperature regime of 25/20°C and a relative humidity of 70%.

After five weeks of continuous treatment, alfalfa fresh leaf samples were collected, and leaf RWC measurement and biomass determination were conducted concurrently [36]. Fresh leaf samples were rapidly frozen with liquid nitrogen (LN) to halt metabolic activity and prevent enzymatic degradation, the breakdown of proteins caused by enzymes, which maximized the retention of their inherent physiological and biochemical properties. The samples were subsequently divided into two portions for the purpose of determining the physiological and biochemical parameters: one portion was freeze-dried to constant weight using a freeze dryer, while the other portion was promptly transferred to an ultra-low temperature freezer set at a temperature of −80°C for the purpose of temporary storage. The leaf samples were subjected to a process of freeze-drying, after which they were ground into a uniform fine powder using a ball mill (MM 400, Retsch, Haan, Germany). The powder was then sieved through a 100-mesh sieve for the determination of glycine betaine (GB), proline (Pro), soluble sugar (SS), and malondialdehyde (MDA) contents. The samples temporarily stored in the ultra-low temperature freezer were used for assaying total chlorophyll (Chl) content, soluble protein (SP) content, antioxidant enzyme activities (including catalase [CAT], peroxidase [POD], superoxide dismutase [SOD], ascorbate peroxidase [APX], and polyphenol oxidase [PPO]), and ROS-related indicators (hydrogen peroxide [H_2O_2] and superoxide anion radical [$O_2^{\bullet-}$]).

2.3 Determination of Biomass

In the present study, the shoots and roots of each plant in each treatment group were carefully harvested. Subsequently, the roots were rinsed through mesh sieves (0.2 mm) until free of soil particles. All harvested shoot and root tissues were heat-fixed at 105°C for 30 min to inactivate endogenous enzyme activity in plant tissues, thereby preserving the compositional quality and physicochemical stability of plant samples. Following this, the tissues were dried to a constant weight in a forced-air oven at 65°C, and the weight was subsequently determined using an electronic analytical balance. Shoot biomass (SB) and root biomass (RB) were weighed separately, with total biomass (TB) calculated as the sum of the two components [37].

2.4 Determination of Total Chl Content and RWC in Alfalfa Leaves

The total Chl content was determined and calculated according to the methodology established by Zhong et al. [9]. Briefly, 0.2 g of alfalfa leaf samples were punched into small pieces and transferred to test tubes, to which 2 mL of 95% acetone was added. In order to ensure full extraction and prevent chlorophyll degradation, the test tubes were subjected to an incubation process at 37°C in conditions of darkness for a period of 4 h. After incubation, the mixture was subjected to a centrifugation procedure, and the supernatant was collected to determine absorbances at 663 nm and 645 nm, respectively. The total Chl content was determined through the utilization of the following formula:

$$C_{\text{total}}\ (\text{mg/L}) = 8.02 \times A_{663} + 20.29 \times A_{645}$$

$$\text{Total Chl content} = \frac{C_{\text{total}} \times V}{FW \times 1000}$$

where C_{total} denotes the concentration of total chlorophyll in the 95% acetone extraction solution; V represents the volume of 95% acetone employed for chlorophyll extraction; and FW stands for the fresh weight of alfalfa leaf sample used for the assay. FW is the same as described below.

The RWC of alfalfa leaves was determined using the method of Barrs and Weatherley [38]. A fresh leaf sample measuring 0.1 g was selected for the purpose of the experiment, and its FW was recorded at the outset. The samples were then soaked in DI water until fully saturated, after which the saturated weight (SW) was measured. Subsequently, the leaf samples were oven-dried at 65°C to a constant dry weight (DW). The leaf RWC of alfalfa was calculated using the following equation: RWC (%) = [(FW − DW)/(SW − DW)] × 100%.

2.5 Determination of Pro, GB, SS, and SP Contents in Alfalfa Leaves

The Pro concentration was determined according to the method previously described by Bates et al. [39]. The absorptivity of the upper organic phase was measured (520 nm), and the Pro content was calculated on the basis of the standard curve, which was generated from a proline standard solution.

$$\text{Pro content} = \frac{(A_{520} - b) \times V_{\text{total}}}{a \times V_{\text{used}} \times DW}$$

where A_{520} represents the absorbance of the proline-ninhydrin complex in the upper organic phase at 520 nm; V_{total} denotes the total volume of the extract obtained from alfalfa leaf samples; V_{used} denotes the volume of extract added to the reaction system for absorbance measurement; a denotes the slope of the standard curve (linear regression equation: $y = ax + b$); b denotes the intercept of the standard curve; DW denotes the freeze-dried weight of the alfalfa leaf sample used for the assay. a, b, V_{total}, V_{used}, and DW are the same as described below.

The GB content was determined following the method established by Grieve and Grattan [40]. The absorbance of the reaction solution was measured at 365 nm using a microplate reader (Infinite M Plex, Tecan Trading Co., Ltd., Salzburg, Austria). The calculation of GB content was performed utilizing the following formula:

$$\text{GB content} = \frac{(A_{365} - b) \times V_{\text{total}}}{a \times V_{\text{used}} \times DW}$$

where A_{365} represents the absorbance of the GB-titration reagent complex measured at 365 nm.

The SS content was determined according to the anthrone-sulfuric acid colorimetric method of Yoshida et al. [41]. In summary, 0.5 g of mature alfalfa leaves was homogenized in 5 mL of 80% anthrone solution. Subsequently, 10 mL of anthrone reagent (2 g anthrone dissolved in 1 L of 95% H_2SO_4 w/v) was slowly added to the homogenate. The mixture was heated in a water bath at 80°C for 30 min to ensure complete extraction, and then centrifuged at 8000× g for 10 min at 4°C to collect the supernatant. The degree of absorption of the extract was determined at a wavelength of 620 nm using a microplate reader. A calibration curve was generated with glucose as the standard, and the total SS content in alfalfa leaves was consequently quantified. The calculation of SS content was determined by the following formula:

$$\text{SS content} = \frac{(A_{620} - b) \times V_{\text{total}} \times DF}{a \times V_{\text{used}} \times DW}$$

where A_{620} represents the absorbance of the sugar-anthrone complex at 620 nm.

The SP content in alfalfa leaf tissue was assayed following the method reported by Bradford [42]. Following centrifugation (12,000× g, 4°C, 15 min), the supernatant was collected and subjected to absorbance detection at 595 nm via a microplate reader. SP content was quantified via a calibration curve established with bovine serum albumin (BSA) as the standard.

2.6 Determination of CAT, POD, SOD, APX and PPO Activities in Alfalfa Leaves

Briefly, 0.5 g of fresh alfalfa leaf tissue was ground in an ice-cold mortar with 6 mL of 50 mmol L^{-1} sodium phosphate buffer (pH 7.0), which was pre-supplemented with 0.2 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP) to scavenge phenolic compounds and maintain enzymatic activity. After filtration through four layers of sterile cheesecloth, the homogenate was centrifuged (15,000× g, 4°C, 20 min). The resulting supernatant was harvested as the crude enzyme extract for later activity measurements. Using the method of Maehly and Chance [43], the activities of CAT (the absorbance at 240 nm) and POD (the absorbance at 470 nm) were measured.

CAT activity was calculated using the formula:

$$\text{CAT activity} = \frac{\Delta A_{240} \times V_{\text{total}}}{\varepsilon \times L \times V_{\text{used}} \times \Delta \text{t} \times W_{\text{SP}}}$$

where ΔA_{240} represents the linear absorbance decrease at 240 nm reflecting the decomposition rate of H_2O_2; ε represents the molar extinction coefficient of H_2O_2 at 240 nm (classical value: 43.6 $\text{mM}^{-1}\cdot\text{cm}^{-1}$); L the light path length of the cuvette employed for absorbance measurement; V_{total} represents the total volume of crude enzyme extract from alfalfa leaves; V_{used} represents the volume of crude enzyme extract added to the reaction system; Δt represents the reaction duration during the linear phase of absorbance change; W_{SP} represents the total soluble protein content in the crude enzyme extract. L, V_{total}, V_{used}, Δt, and W_{SP} are the same as described below.

POD activity was calculated using the formula:

$$\text{POD activity} = \frac{\Delta A_{470} \times V_{\text{total}}}{\varepsilon \times L \times V_{\text{used}} \times \Delta \text{t} \times W_{\text{SP}}}$$

where ΔA_{470} represents the linear absorbance increase at 470 nm reflecting the oxidation rate of guaiacol; ε represents the molar extinction coefficient of oxidized guaiacol at 470 nm (classical value: 26.6 $\text{mM}^{-1}\cdot\text{cm}^{-1}$).

SOD activity was determined following the method described by Geng et al. [44]. This method quantifies SOD activity by measuring its capacity to suppress the photochemical reduction of nitro-blue tetrazolium (NBT). SOD activity was computed using the equation below:

$$IR = \frac{\left(A_0 - A_{\text{sample}}\right)}{A_0} \times 100\%$$

$$\text{SOD activity} = \frac{\left(\frac{IR}{50-IR}\right) \times V_{\text{total}} \times DF}{V_{\text{used}} \times W_{\text{SP}}}$$

where A_0 represents the absorbance of the control group (without enzyme extract, NBT fully reduced) at 560 nm; A_{sample} represents the absorbance of the sample group (with enzyme extract) at 560 nm after illumination; Inhibition rate (*IR*) represents the percentage of NBT photochemical reduction inhibited by SOD. *DF* represents the correction factor if the enzyme extract is diluted to adjust the inhibition rate to the linear range.

APX activity was assayed according to the method described by Nakano and Asada [45], with activity calculated according to the linear region of the absorbance decrease and the molar extinction coefficient of ascorbate. APX activity was calculated using the formula:

$$\text{APX activity} = \frac{\Delta A_{290} \times V_{\text{total}}}{\varepsilon \times L \times V_{\text{used}} \times \Delta \text{t} \times W_{\text{SP}}}$$

where ΔA_{290} denotes the linear reduction in absorbance detected at 290 nm, representing the oxidation rate of the substrate ascorbic acid (AsA); ε represents the molar extinction coefficient of ascorbic acid at 290 nm (classical value: 2.8 $\text{mM}^{-1}\cdot\text{cm}^{-1}$).

PPO activity was assayed according to the protocol established by Gauillard et al. [46]. The absorbance at 495 nm was recorded at 30-s intervals for 3 min using a spectrophotometer. PPO activity was estimated from the linear change in absorbance (ΔOD min^{-1} mg^{-1} protein) and normalized to the corresponding protein content.

2.7 Determination of H_2O_2, and $O_2^{\bullet-}$ and MDA Contents in Alfalfa Leaves

The H_2O_2 concentration in alfalfa leaves was determined according to the protocol of Velikova et al. [47]. Absorbance of the reaction solution was recorded at 390 nm, and the H_2O_2 concentration was quantified based on a standard curve prepared with known standards. The calculation was performed using the equation shown below:

$$H_2O_2 \text{ content} = \frac{(A_{390} - b) \times V_{\text{total}} \times DF}{a \times V_{\text{used}} \times FW}$$

where A_{390} represents the absorbance of the H_2O_2-titanium complex at 390 nm.

The generation rate of $O_2^{\bullet-}$ was measured with reference to Yan et al. [48]. Since the extraction procedure for $O_2^{\bullet-}$ was identical to that of H_2O_2, the crude extract prepared above was directly used for subsequent reactions. The absorbance of the reaction solution was detected spectrophotometrically at 530 nm. The $O_2^{\bullet-}$ concentration was calculated against a calibration curve established with sodium nitrite as the standard, combined with the reaction time. The corresponding value was derived using the equation below:

$$O_2^{\bullet-}\text{content} = \frac{(A_{530} - b) \times V_{\text{total}} \times DF}{a \times V_{\text{used}} \times \Delta t \times FW}$$

where A_{530} represents the absorbance of the nitrite-color complex (formed by $O_2^{\bullet-}$ oxidizing hydroxylamine) at 530 nm.

MDA content in alfalfa leaves was measured using a modified thiobarbituric acid (TBA) method reported by Heath and Packer [49]. In brief, 0.5 g of fresh leaf tissue was homogenized with 5 mL of 5% TBA solution. After centrifugation at 1790× g for 10 min, a 2 mL supernatant fraction was mixed with TBA solution. The mixture was incubated in a 98°C water bath for 10 min, then cooled to ambient temperature to stop the reaction. After a second centrifugation to remove precipitates, the absorbance of the supernatant was measured at 532 nm. Background correction was performed at 600 nm to eliminate nonspecific interference, and the MDA concentration was quantified accordingly. MDA content was computed according to the equation below:

$$\text{MDA content} = \frac{(A_{532} - A_{600} - b) \times V_{\text{total}} \times DF}{a \times V_{\text{used}} \times DW}$$

where A_{532} represents the absorbance of the MDA-TBA complex at 532 nm; A_{600} represents the background absorbance at 600 nm to eliminate interference from sugars and pigments.

2.8 Statistical Analysis

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, USA), while graphical visualization was conducted with SigmaPlot 12.5 (Systat Software, San Jose, USA). Normality and homogeneity of variances were tested using the Shapiro–Wilk and Levene's tests, respectively. Differences among treatments for all determined indices were assessed by one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$). Furthermore, two-factor ANOVA was applied to evaluate the individual impacts of salinity (S) and biochar application (B), together with their interactive influence (S × B). Correlation analysis of all quantified indicators was carried out with the GGally and ggplot2 libraries in R (v4.2.2; R Core Team, Vienna, Austria). All experimental data are expressed as mean ± standard deviation (SD) from four replicates (n = 4).

3 Results

3.1 Impacts of Biochar Application on Biomass, Total Chl Content, and RWC of Salt-Stressed Alfalfa

Our results revealed that both salinity exposure and biochar application exerted substantial effects on the growth status of alfalfa seedlings, including shoot biomass (SB), root biomass (RB), total biomass (TB) of alfalfa seedlings, and total Chl content ($p < 0.05$, Table 2), but biochar amendment had no significant effect on leaf relative water content () ($p > 0.05$, Table 2). Compared with non-saline control, SB, RB, TB, total Chl content, and RWC decreased significantly as salt stress intensified (Figs. 1 and 2). In the present study, biochar application alleviated this salt-induced decline, increasing the values of these parameters, except for RB and RWC under the 9 dS m^{-1} NaCl stress condition. Based on the average values of all measured indices, the 3% biochar amendment exhibited significantly higher values than the other biochar treatments ($p < 0.05$). Under the same salt stress levels, the 3% biochar amendment treatment enhanced SB by 65%–73%, RB by 41%–61%, TB by 54%–68%, total Chl content by 8%–23%, and RWC by 1%–7% compared with no biochar addition treatment (non-biochar). However, under the 9 dS m^{-1} NaCl stress condition, the 6% biochar treatment decreased RB and RWC by 1% and 2% relative to the non-biochar treatment, respectively (Figs. 1B and 2B). Among all treatments, the 3% biochar treatment under non-saline conditions exhibited the highest values for SB, RB, TB, total Chl content, and RWC. In contrast, the lowest values for these parameters were observed in the non-biochar treatment under the 9 dS m^{-1} NaCl stress condition.

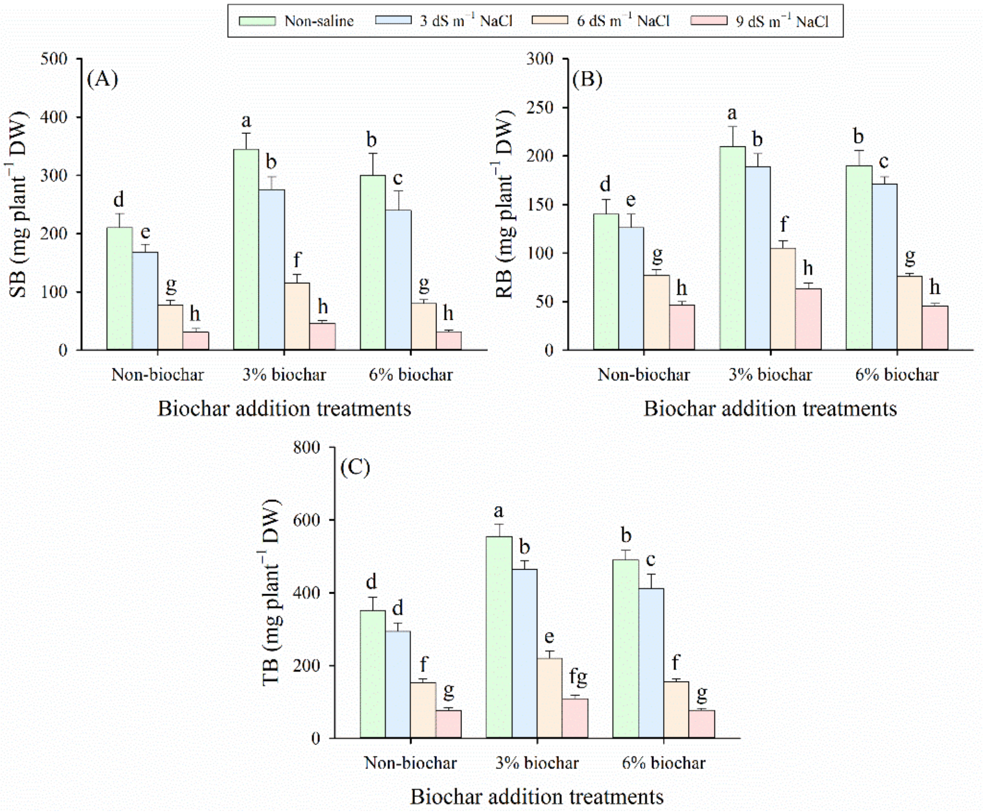


Figure 1: (**A**) Shoot biomass (SB), (**B**) root biomass (RB), and (**C**) total biomass (TB) in alfalfa plants exposed to diverse salinity levels and biochar application regimes. Distinct lowercase letters represent statistically significant variations across all treatment combinations ($p < 0.05$). Salt stress treatments included NaCl concentrations of 0 dS m^{-1} (Non-saline), 3 dS m^{-1} (3 dS m^{-1} NaCl), 6 dS m^{-1} (6 dS m^{-1} NaCl), and 9 dS m^{-1} (9 dS m^{-1} NaCl). Biochar amendment treatments included application regimes of 0 g kg^{-1} (Non-biochar), 30 g kg^{-1} (3% biochar), and 60 g kg^{-1} (6% biochar). This treatment notation applies to all subsequent figures.

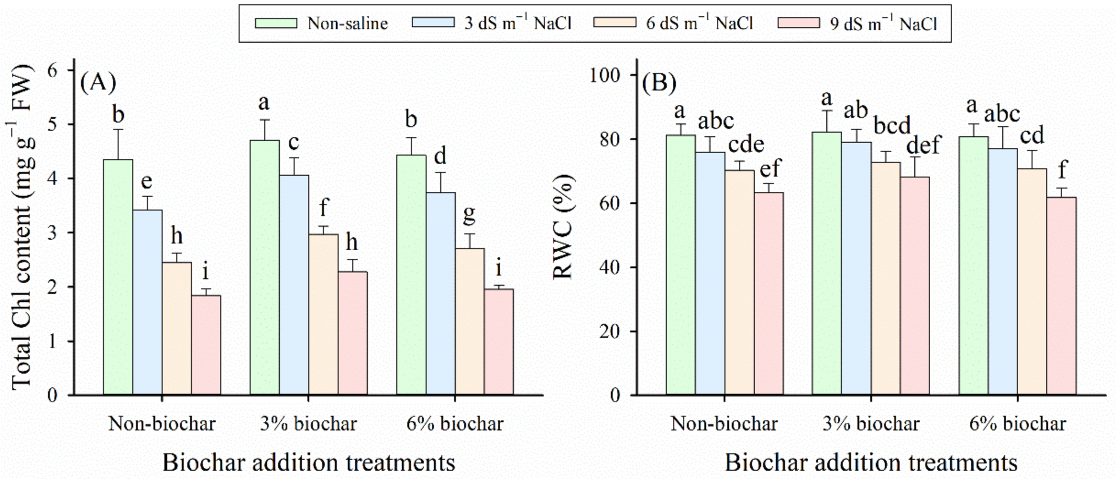


Figure 2: (**A**) Total chlorophyll (Chl) content and (**B**) relative water content (RWC) in alfalfa foliage subjected to different salinity levels and biochar application doses. Distinct lowercase letters represent statistically significant variations across all treatment combinations ($p < 0.05$).

3.2 Effects of Biochar Application on the Concentrations of Pro, GB, SS, and SP in Alfalfa Leaves under Salt Stress

Salinity levels, biochar application, and their interaction significantly affected the concentrations of proline (Pro), glycine betaine (GB), soluble sugar (SS), and soluble protein (SP) in alfalfa leaves ($p < 0.05$, Table 2), whereas no significant interactive effect was observed between salt stress and biochar amendment on SP content ($p > 0.05$, Table 2). Specifically, with the intensification of salt stress, the concentrations of Pro, GB, SS, and SP in alfalfa leaves gradually increased (Fig. 3). Notably, biochar application alleviated the salt-induced accumulation of these osmotically active solutes and reduced their levels. Under the same salt stress level, the 3% biochar treatment resulted in significantly lower concentrations of these osmotically active solutes compared with the non-biochar and 6% biochar treatments ($p < 0.05$). At all salinity levels, compared with non-biochar controls, 3% biochar treatment reduced the concentrations of Pro, GB, SS, and SP by 12%–20%, 12%–36%, 18%–24%, and 7%–12%, respectively. Among all treatments, the highest concentrations of Pro, GB, SS, and SP were detected in the 6% biochar treatment under 9 dS m^{-1} NaCl stress condition, while the lowest concentrations were recorded in the 3% biochar treatment under non-saline conditions. This further confirmed the dose-dependent regulatory role of biochar in mediating alfalfa's osmotic adjustment response under salt stress.

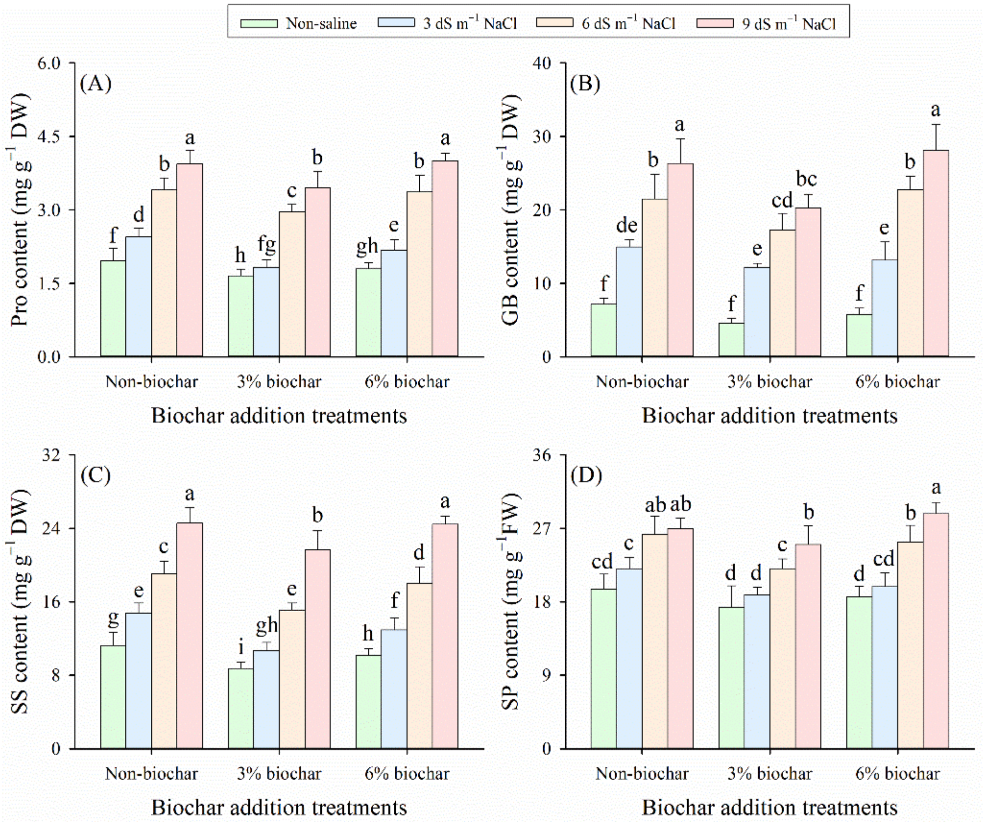


Figure 3: (**A**) Proline (Pro), (**B**) glycine betaine (GB), (**C**) soluble sugar (SS), and (**D**) soluble protein (SP) contents in alfalfa foliage under different saline conditions and biochar application rates. Distinct lowercase letters represent statistically significant variations across all treatment combinations ($p < 0.05$).

3.3 Effects of Biochar Addition on the Activities CAT, POD, SOD, APX and PPO in Alfalfa Leaves under Salt Stress

Two-way ANOVA verified that salinity level and biochar addition exerted highly significant effects on the antioxidant enzyme profiles including CAT, POD, SOD, APX and PPO in plant leaves ($p < 0.01$, Table 2). Specifically, salt stress and biochar amendment had significant interactive effects on the activities of CAT, SOD, and APX ($p < 0.01$), while no significant interactive effect was observed on the activities of POD and PPO ($p > 0.05$; Table 2). Under all biochar application regimes, salinity significantly increased the activities of all tested antioxidant enzymes (Fig. 4). Among the biochar treatments, the 3% biochar amendment resulted in the lowest CAT, POD, SOD, APX, and PPO activities under the same salt stress level. Notably, the minimum values of all these enzyme activities were observed in the 3% biochar treatment under non-saline conditions. Specifically, under 9 dS m^{-1} NaCl stress condition, the 3% biochar treatment reduced CAT, POD, SOD, APX, and PPO activities by 19%, 12%, 24%, 18%, and 2%, respectively, compared with the non-biochar control. However, the 6% biochar treatment increased the activities of CAT, POD, APX, and PPO by 12%, 4%, 7%, and 16% under the same severe salt stress condition, which indicates that excessive biochar application weakened the mitigating effect on salt-induced disorders of antioxidant enzyme activities, thereby exerting a negative regulatory role.

Table 2: Results of two-way analysis of variance (ANOVA) showing *F*-values and *p*-values for the effects of salt stress (S), biochar amendment (B), and their interaction (S × B) on the growth, physiological, and biochemical indicators of alfalfa seedlings.

	Salt Stress (S)		**Biochar (B)**		**S × B**	
	F	***p***	***F***	***p***	***F***	***p***
SB	392.96	$<0.001^{***}$	53.64	$<0.001^{***}$	8.94	$<0.001^{***}$
RB	368.18	$<0.001^{***}$	65.01	$<0.001^{***}$	8.16	$<0.001^{***}$
TB	665.50	$<0.001^{***}$	99.81	$<0.001^{***}$	14.82	$<0.001^{***}$
Total Chl	635.27	$<0.001^{***}$	43.31	$<0.001^{***}$	0.84	$0.548^{\text{n.s.}}$
RWC	29.56	$<0.001^{***}$	2.01	$0.140^{\text{n.s.}}$	0.29	$0.940^{\text{n.s.}}$
Pro	816.41	$<0.001^{***}$	76.32	$<0.001^{***}$	3.60	0.007^{**}
GB	177.32	$<0.001^{***}$	17.16	$<0.001^{***}$	2.37	0.049^{*}
SS	965.21	$<0.001^{***}$	109.93	$<0.001^{***}$	2.40	0.047^{*}
SP	57.76	$<0.001^{***}$	12.14	$<0.001^{***}$	1.23	$0.315^{\text{n.s.}}$
CAT	731.20	$<0.001^{***}$	174.39	$<0.001^{***}$	11.98	$<0.001^{***}$
POD	210.36	$<0.001^{***}$	9.29	0.001^{**}	0.64	$0.695^{\text{n.s.}}$
SOD	1184.64	$<0.001^{***}$	227.59	$<0.001^{***}$	7.49	$<0.001^{***}$
APX	1577.24	$<0.001^{***}$	149.00	$<0.001^{***}$	6.50	$<0.001^{***}$
PPO	132.06	$<0.001^{***}$	15.54	$<0.001^{***}$	1.30	$0.281^{\text{n.s.}}$
H_2O_2	682.45	$<0.001^{***}$	102.60	$<0.001^{***}$	5.92	$<0.001^{***}$
$O_2^{\bullet-}$	43.25	$<0.001^{***}$	9.79	$<0.001^{***}$	0.66	$0.685^{\text{n.s.}}$
MDA	582.85	$<0.001^{***}$	204.47	$<0.001^{***}$	4.63	0.001^{**}

Notes: SB, shoot biomass; RB, root biomass; TB, total biomass; Chl, chlorophyll; RWC, relative water content; Pro, proline; GB, glycine betaine; SS, soluble sugar; SP, soluble protein; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; APX, ascorbate peroxidase; PPO, polyphenol oxidase; H_2O_2, hydrogen peroxide; $O_2^{\bullet-}$, superoxide anion radical; MDA, malondialdehyde. Levels of significance are indicated as: n.s. = non-significant ($p > 0.05$); * = significant at $p < 0.05$; ** = significant at $p < 0.01$; *** = significant at $p < 0.001$.

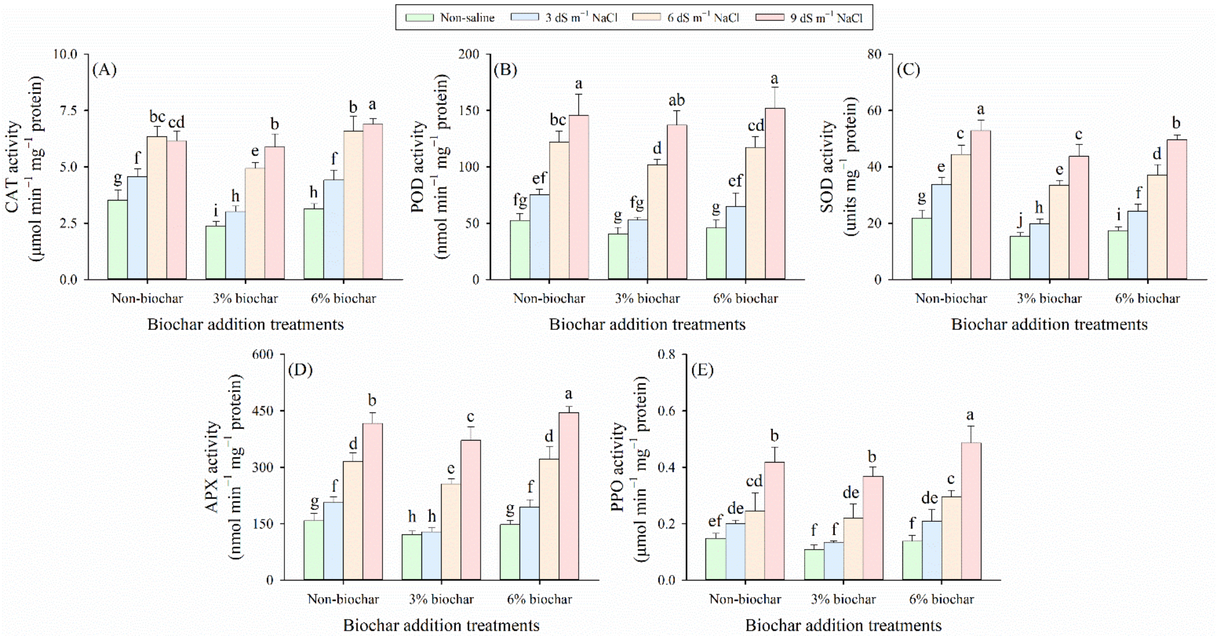


Figure 4: (**A**) Catalase (CAT) activity, (**B**) peroxidase (POD) activity, (**C**) superoxide dismutase (SOD) activity, (**D**) ascorbate peroxidase (APX) activity, and (**E**) polyphenol oxidase (PPO) activity in alfalfa foliage under different saline levels and biochar application rates. Distinct lowercase letters represent statistically significant variations across all treatment combinations ($p < 0.05$).

3.4 Effects of Biochar Addition on the Contents of H_2O_2, $O_2^{\bullet-}$, and MDA in Alfalfa Leaves under Salt Stress

H_2O_2, $O_2^{\bullet-}$, and MDA contents were significantly elevated with the intensification of salt stress, in contrast, biochar application lowered their concentrations at the identical salt stress level ($p < 0.05$, Fig. 5). Salt stress, biochar amendment, and their interaction exerted a significant effect on the concentrations of H_2O_2, $O_2^{\bullet-}$, and MDA in alfalfa leaves ($p < 0.05$, Table 2). However, no significant interactive effect between salt stress and biochar amendment was detected on $O_2^{\bullet-}$ content ($p > 0.05$, Table 2). Specifically, under non-saline conditions, the contents of H_2O_2, $O_2^{\bullet-}$, and MDA treated with 3% biochar were the lowest, which were 23%, 12%, and 32% lower than the non-biochar group, respectively ($p < 0.05$). On the contrary, under 9 dS m^{-1} NaCl stress conditions, the concentrations of H_2O_2 and $O_2^{\bullet-}$ were the highest in 6% biochar treatment, showing a significant increase of 85% and 63% in non-saline conditions compared with non-biochar controls. In contrast, the highest MDA concentration was found in the non-biochar treatment under 9 dS m^{-1} NaCl stress condition, a significant 76% increase compared to the non-biochar control under non-saline conditions. Overall, based on the average of all treatments, 3% biochar treatment exhibited the lowest oxidative stress solute concentrations among all biochar treatments, which further confirmed its best mitigating effect on salt-induced oxidative damage in alfalfa.

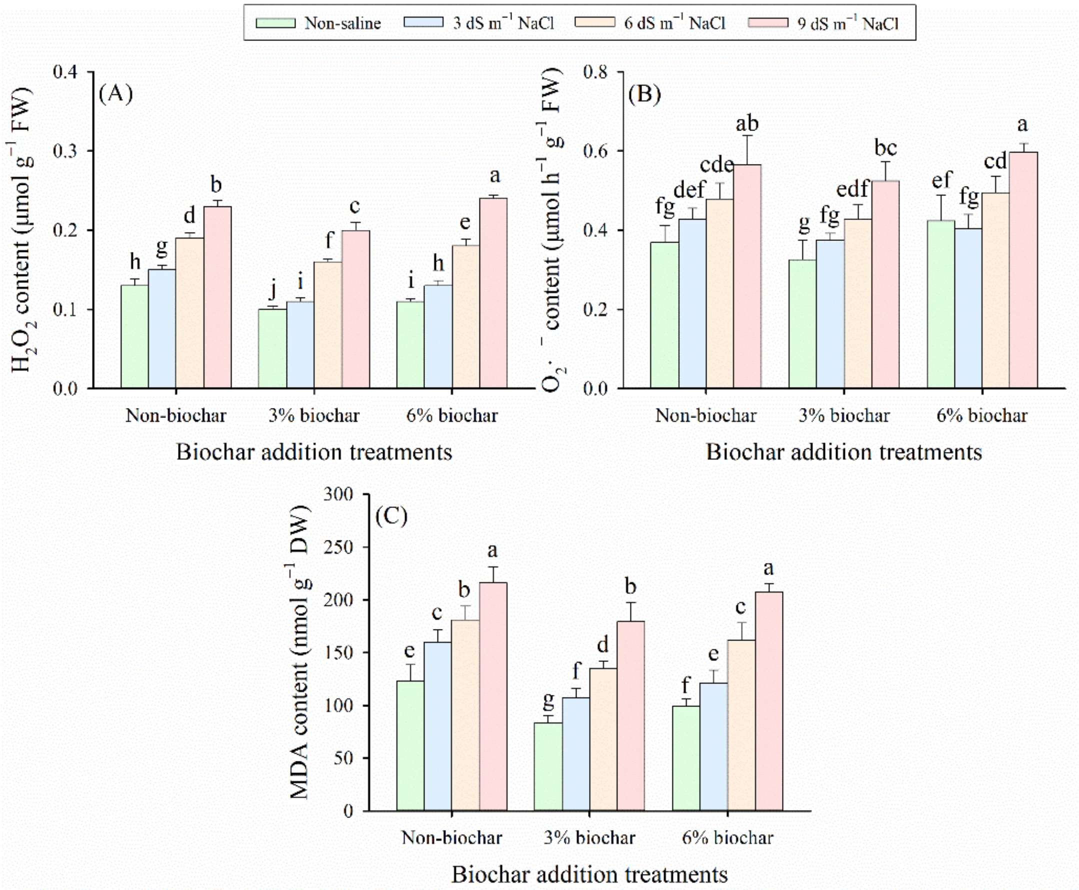
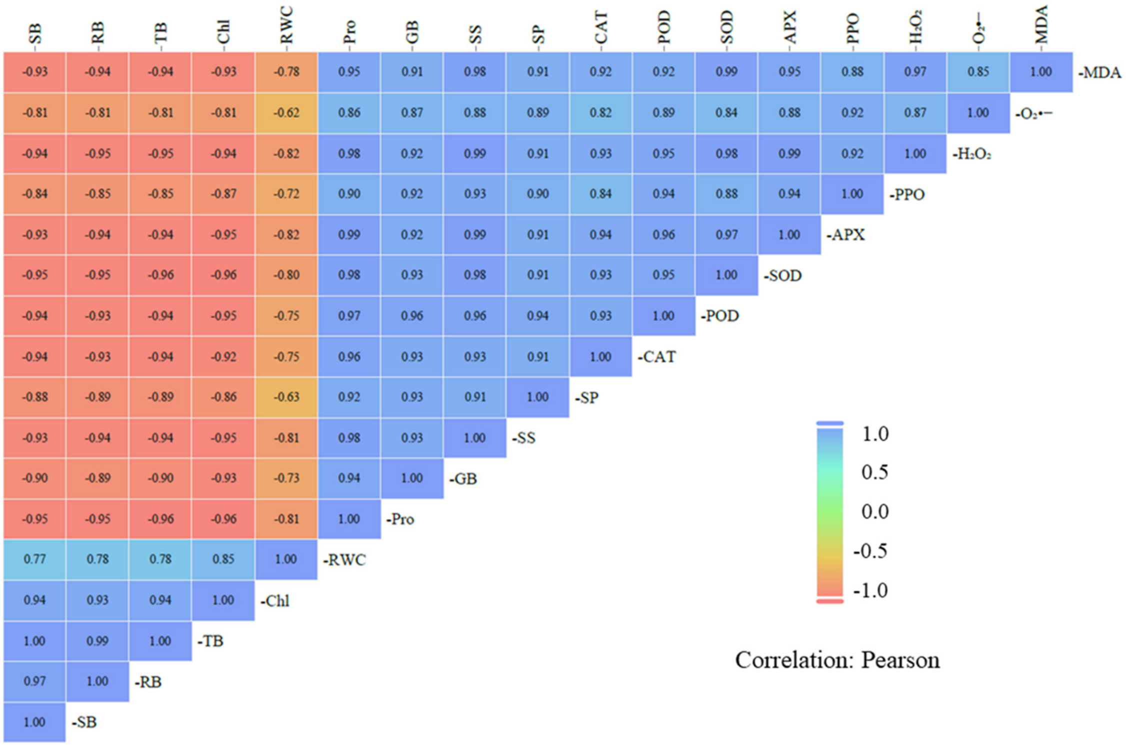


Figure 5: (**A**) Hydrogen peroxide (H_2O_2), (**B**) superoxide anion radical ($O_2^{\bullet-}$), and (**C**) malondialdehyde (MDA) contents in alfalfa leaves exposed to different salt stress levels and biochar application rates. Distinct lowercase letters represent statistically significant variations across all treatment combinations ($p < 0.05$).

3.5 The Correlation between Different Growth, Physiological, and Biochemical Indicators

We explored the effect of biochar on alfalfa growth, analyzed the correlations between its growth, physiological, and biochemical indicators (Fig. 6). The TB was significantly positively correlated with both total Chl content and RWC, but negatively correlated with osmotic adjustment substances (Pro, GB, SS, and SP), antioxidant enzyme activities (CAT, POD, SOD, APX, and PPO), and oxidative stress-related substances (H_2O_2, $O_2^{\bullet-}$, and MDA). Furthermore, total Chl content was positively correlated with RWC and negatively correlated with oxidative stress-related substances. Osmotic adjustment substances and antioxidant enzyme activities were positively correlated with oxidative stress-related substances. Based on these analyses, our results indicated that biochar amendment could enhance RWC and reduce ROS accumulation in alfalfa seedlings under salt stress by regulating the osmotic adjustment and antioxidant enzyme systems.



SB	RB	TB	Chl	RWC	Pro	GB	SS	SP	CAT	POD	SOD	APX	PPO	H_2O_2	$O_2^{\bullet-}$	MDA	
-0.93	-0.94	-0.94	-0.93	-0.78	0.95	0.91	0.98	0.91	0.92	0.92	0.99	0.95	0.88	0.97	0.85	1.00	MDA
-0.81	-0.81	-0.81	-0.81	-0.62	0.86	0.87	0.88	0.89	0.82	0.89	0.84	0.88	0.92	0.87	1.00		$O_2^{\bullet-}$
-0.94	-0.95	-0.95	-0.94	-0.82	0.98	0.92	0.99	0.91	0.93	0.95	0.98	0.99	0.92	1.00			H_2O_2
-0.84	-0.85	-0.85	-0.87	-0.72	0.90	0.92	0.93	0.90	0.84	0.94	0.88	0.94	1.00				PPO
-0.93	-0.94	-0.94	-0.95	-0.82	0.99	0.92	0.99	0.91	0.94	0.96	0.97	1.00					APX
-0.95	-0.95	-0.96	-0.96	-0.80	0.98	0.93	0.98	0.91	0.93	0.95	1.00						SOD
-0.94	-0.93	-0.94	-0.95	-0.75	0.97	0.96	0.96	0.94	0.93	1.00							POD
-0.94	-0.93	-0.94	-0.92	-0.75	0.96	0.93	0.93	0.91	1.00								CAT
-0.88	-0.89	-0.89	-0.86	-0.63	0.92	0.93	0.91	1.00									SP
-0.93	-0.94	-0.94	-0.95	-0.81	0.98	0.93	1.00										SS
-0.90	-0.89	-0.90	-0.93	-0.73	0.94	1.00											GB
-0.95	-0.95	-0.96	-0.96	-0.81	1.00												Pro
0.77	0.78	0.78	0.85	1.00													RWC
0.94	0.93	0.94	1.00														Chl
1.00	0.99	1.00															TB
0.97	1.00																RB
1.00																	SB

Figure 6: Correlation plots between all measured variables in the experiment. Colour intensity represents the absolute value of the Pearson correlation coefficient.

4 Discussion

Salinity stress is one of the primary abiotic stressors threatening global food security [50]. It impairs plant water uptake by establishing an osmotic gradient between the rhizosphere and plant cells, disrupts Na^+/K^+ homeostasis, perturbs plant physiological and metabolic processes, and ultimately inhibits plant growth, thus culminating in compromised crop yield and quality [17]. Our findings showed that total Chl content in alfalfa leaves decreased significantly with increasing salt stress intensity (Fig. 2A). Consistently, Jabborova et al. [35] also confirmed that total Chl and carotenoid contents in alfalfa were significantly reduced under salt stress conditions. This phenomenon has been widely documented across diverse plant species: Shaheen et al. [51] and He et al. [52] observed significant reductions in Chl content of wheat and maize, respectively, with escalating salt stress, which aligns with the present study's findings. Alfalfa adopted a defensive strategy against salt stress by upregulating the accumulation of osmotic adjustment substances, including leaf Pro, GB, SS, and SP in alfalfa leaves (Fig. 3). Such accumulation increases intracellular solute concentration and reduces cellular water potential, thereby maintaining osmotic balance and preventing irreversible damage induced by excessive cellular dehydration [22]. Despite this adaptive response, the RWC of alfalfa leaves still decreased significantly under salt stress in our research (Fig. 2B). The reduction in alfalfa Chl content is closely associated with two key factors: the decline in leaf water content triggered by osmotic stress and excessive production and accumulation of ROS under salt stress [4]. Relevant studies have corroborated that ROS overaccumulation is typically induced by water and ionic imbalances caused by salt stress [53]. Although our findings confirmed that alfalfa could activate its enzymatic antioxidant system as a defensive strategy by increasing the activities of CAT, POD, SOD, APX, and PPO to scavenge ROS (Fig. 4), the contents of H_2O_2 and $O_2^{\bullet-}$ exhibited a substantial increase (Fig. 5A,B), which further exacerbated chlorophyll degradation (Fig. 2A). Collectively, our findings confirm that the salinity-induced reduction in alfalfa chlorophyll content results from the synergistic effects of decreased leaf RWC (Fig. 2B) and excessive ROS accumulation (Fig. 5). However, the dominant contributor to chlorophyll reduction in salt-stressed alfalfa—whether osmotic imbalance or ROS-mediated damage—requires further clarification in follow-up research.

Notably, our study showed that biochar application significantly increased leaf Chl content (Fig. 2A) and significantly enhanced alfalfa's shoot, root, and total biomass (Fig. 1), a finding that is consistent with previous studies [29,35]. As a core component of plant photosynthesis, elevated Chl content can enhance photosynthetic capacity, providing material and energy supplies for plant growth and development, thereby promoting biomass accumulation [9,16]. Notably, an interesting observation was that biochar-treated alfalfa showed significantly higher leaf RWC but lower contents of osmotic adjustment substances (Pro, GB, SS, and SP) than the control group (non-biochar) (Figs. 2B, 3 and 6). Under salt stress, alfalfa accumulates osmotic adjustment substances to lower osmotic potential and avoid dehydration [19]. The reduced demand for osmotic adjustment following biochar application indicates biochar improves soil water conditions to alleviate salt-induced physiological drought and reduce plant dependence on these substances [28,54]. The underlying mechanism is primarily attributed to biochar's distinctive physicochemical properties, where its large specific surface area and well-developed porous structure enhance the water retention capacity of saline soil and optimize the rhizosphere microdomain, thereby promoting plant water uptake and alleviating physiological drought [9]. Meanwhile, biochar application decreased the activities of antioxidant enzymes (CAT, POD, SOD, APX, and PPO) under salt stress (Fig. 4). Biochar is speculated to reduce ROS generation and accumulation by improving rhizosphere water status and regulating ionic absorption balance, thus diminishing the need for enzymatic antioxidant system activation and alleviating ROS-induced metabolic damage [32,55]. In addition, biochar is rich in plant-available mineral nutrients, and its application can optimize soil nutrient supply to support alfalfa growth—consistent with previous studies [31,54]. Furthermore, the porous structure of biochar improves the rhizosphere microenvironment, promotes root morphological development and function, and stimulates plant-microbe interactions, these effects synergistically improve alfalfa's nutrient and water acquisition capacity under salt stress, ultimately promoting plant salt stress tolerance and growth performance [55].

A significant finding of this study was the marked capacity of biochar to mitigate the deleterious effects of salt stress on alfalfa (Figs. 1B, 2B, 3, 4A,B,D,E and 5). Moderate biochar application can remarkably optimize plant osmotic adjustment capacity, maximize the defensive function of the enzymatic antioxidant system, and effectively alleviate salt stress damage [54]. In contrast, high-rate biochar application inversely attenuates these positive effects [56]. This result aligns well with the well-documented finding that biochar exerts a concentration threshold-dependent regulatory effect on plants, thus further confirming that an optimal application rate is critical for maximizing biochar's remediation efficacy in saline soils [9]. Regarding the mechanism underlying the adverse effects of high-rate biochar application, this study proposes that biochar produced via pyrolysis at high temperature (e.g., >500°C) exhibits strong alkalinity [30]. Excessive application tends to cause a significant increase in rhizosphere microdomain pH, disrupting the original soil acid-base homeostasis. Consequently, the diminished bioavailability of essential mineral nutrients, including phosphorus, zinc, and iron, which inhibits their uptake and translocation by alfalfa roots, is coupled with alterations in soil wettability and pore architecture that induces heterogeneous rhizosphere water distribution, ultimately impairing root water absorption [57]. The combined deficiency of nutrient and water supply synergistically inhibit the synthesis and accumulation of photosynthetic pigments such as chlorophyll, impairs plant photosynthetic efficiency, and decreases photosynthate accumulation, ultimately leading to reduced biomass [5]. Additionally, the strong adsorption capacity of high-rate biochar may excessively immobilize soil available ions, further disrupting ionic homeostasis (e.g., Na^+/K^+ balance) in plants and exacerbating salt stress-induced cellular damage [56]. The conclusions of this investigation provide significant practical advice concerning the wide-ranging implementation of biochar in the context of forage production in saline-alkaline environments. In practice, to amend saline-alkaline soils and enhance alfalfa productivity with biochar, the application rate should be precisely adjusted according to soil salinization degree and biochar pyrolysis characteristics to determine the optimal threshold and avoid adverse effects from excessive application [58]. This approach not only ensures the practical application efficacy of biochar amendment technology but also reduces biochar resource waste and production costs, significantly enhancing its promotion value and economic viability in saline-alkaline forage cultivation, while improving the economic and practical efficiency of its field application.

The present study was conducted via pot experiments under controlled greenhouse conditions, and it may not fully align with those prevailing in actual field production systems. Nevertheless, through the precise control of experimental variables and sufficient experimental replication, this study ensured the reliability and reproducibility of the results. Consequently, this provided a substantial theoretical and experimental foundation for the extrapolation of these conclusions to actual field production. In fact, the conclusion that optimal biochar application rates can effectively alleviate salt stress and enhance crop yield has been widely validated in numerous field trials conducted on saline-alkali soils [32,56,59]. The present study further provides a clear mechanistic understanding into such regulatory effects at the physiological and biochemical levels of both the osmotic adjustment and antioxidant enzyme systems, thereby deepening the mechanistic understanding of biochar-mediated amelioration in saline-alkali soils. However, we should also acknowledge that environmental conditions (e.g., climate, soil texture, and pH) differ substantially across regions, which is a key limitation of the present study. Furthermore, the physicochemical properties of biochar are dependent on factors such as the type of feedstock and the conditions under which the pyrolysis process occurs [26,30]. In addition, different crop species and even distinct genotypes within the same crop may exhibit different responses to biochar application [16,19]. Therefore, long-term multi-year in situ field positioning trials should be the focus of future research. Through the integration of regional environmental characteristics, biochar physicochemical properties, and crop genotypic differences, researchers should establish and optimize a sustainable biochar application strategy—encompassing optimal application rates, application timings, and combined application methods. This regime holds great potential to markedly enhance the saline-alkaline stress resistance of forage crops, enhancing forage productivity in saline-alkali soils. It also provides a solid technical foundation for restoring saline-alkali lands and efficiently using forage cultivation in such areas.

5 Conclusion

The experimental findings of this study confirmed that salt stress significantly reduced the RWC and induced a marked accumulation of ROS in alfalfa leaves, along with a pronounced decline in leaf chlorophyll content in alfalfa, ultimately resulting in severe inhibition of plant growth. Our results revealed that alfalfa could partially alleviate these adverse effects by increasing antioxidant enzyme activities and activating osmotic adjustment mechanisms. However, increasing salt stress intensity further promoted the accumulation of ROS (i.e., H_2O_2 and $O_2^{\bullet-}$) and MDA, which intensified oxidative damage, reduced photosynthetic pigment content, and ultimately impeded the biomass accumulation of alfalfa seedlings. Biochar amendment has been demonstrated to effectively alleviate salt stress in alfalfa by maintaining the stability of the leaf antioxidant enzyme and osmotic adjustment systems. Furthermore, biochar application promoted the synthesis of photosynthetic pigments and photosynthates, thereby enhancing biomass accumulation, with this mitigative effect most pronounced at an application rate (30 g kg^{-1}). Nevertheless, under severe salt stress levels (9 dS m^{-1}), a higher biochar rate (60 g kg^{-1}) did not promote alfalfa growth, potentially because of osmotic stress induced by excessive application. Therefore, appropriate biochar application is essential for mitigating salt toxicity and promoting alfalfa growth in saline soils. Against the backdrop of the pressing demand for environmentally friendly and sustainable approaches to addressing soil salinization, biochar application is a promising agricultural practice to alleviate moderate oxidative stress and improve alfalfa yield, offering theoretical and practical guidance for the scientific and rational use of biochar in saline-alkali soil restoration and sustainable forage production.

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Availability of Data and Materials: The authors confirm that the data supporting the findings of this study are available within the article.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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