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Contrasting acidic and alkaline biochar reprogram alfalfa metabolism and rhizosphere microbiomes in saline-alkali soils

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Abstract

Soil salinization severely limits crop productivity and ecosystem sustainability, particularly in arid and semi-arid regions. Here, we investigated the potential of targeted biochar amendments to rehabilitate saline-alkali soils and promote alfalfa (*Medicago sativa*) performance through integrative analyses of soil, plant, and rhizosphere microbiome. Two contrasting biochars—acid-modified biochar (AC-biochar, pH 2.3) and alkaline biochar (AL-biochar, pH 8.8)—were applied at 1%, 2%, and 5% (w/w) in controlled pot experiments. Our findings identified low-dose AC-biochar (1%) and high-dose AL-biochar (5%) as the most effective treatments for ameliorating soil geochemical constraints, including enhancing nutrient levels, reducing electrical conductivity, and modulating pH. These amendments significantly promoted alfalfa biomass and forage quality. A closer examination showed that AL-biochar primarily stimulated plant growth by enhancing amino acid metabolism, reactive oxygen species detoxification, and nitrogen assimilation, coupled with increased bacterial diversity and enrichment of beneficial taxa linked to nutrient cycling (e.g., *Rhizobium* and Firmicutes). In contrast, AC-biochar enhanced root development via activation of secondary metabolite biosynthesis (e.g., flavonoids and alkaloids) and recruitment of Actinobacteria known for pathogen suppression and organic matter decomposition. Integrative metabolic and microbiome analyses reveal that biochar-induced plant benefits transcend soil amelioration, exerting targeted regulation on plant physiology and rhizosphere ecology. These findings uncovered a previously underappreciated functional dichotomy in biochar effects and demonstrated the potential of precision biochar application as a scalable, sustainable approach for rehabilitating saline-alkali soils and improving crop resilience under environmental stress.

Highlights

- Tailored biochar amendments improved soil traits and alfalfa yield under saline-alkali stress.
- Metabolome–microbiome analyses revealed biochar-type-specific stress resilience pathways.
- Alkaline biochar enhanced alfalfa growth by activating amino acid and ROS detox pathways.
- Acid-modified biochar promoted secondary metabolites and root defense under stress.
- Alkaline biochar enriched nutrient cyclers but acid-modified biochar suppressed pathogens.

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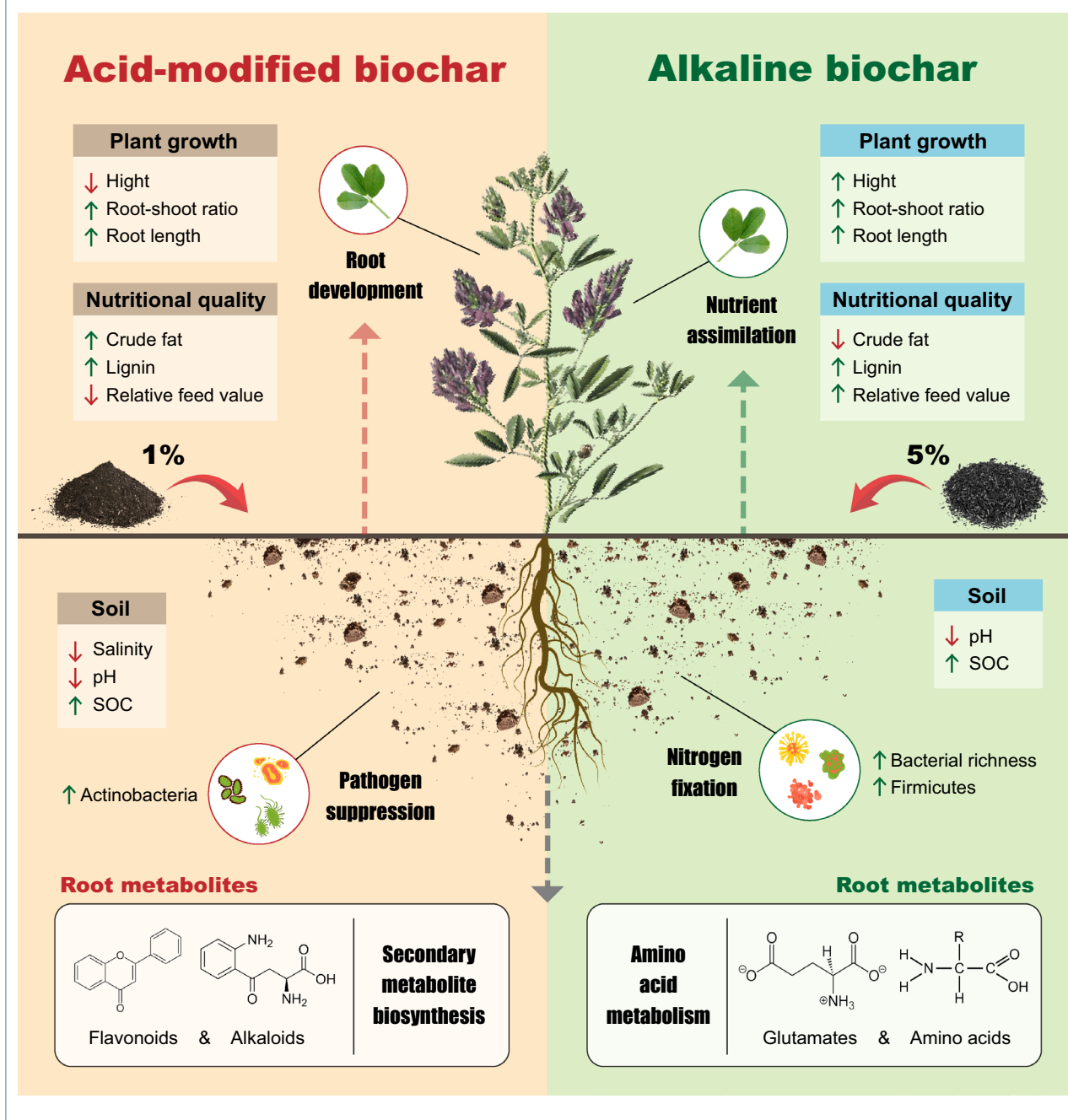
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Graphical Abstract



1 Introduction

Soil salinization has emerged as a critical global threat to agricultural sustainability, food security, and ecological stability, particularly in arid and semi-arid regions (Hassani et al. 2021; Singh 2022; Shokri et al.

2024). Driven by inappropriate irrigation, poor drainage, and excessive use of agrochemicals, salinization deteriorates soil structure and disrupts nutrient cycling, leading to severe land degradation (Kopittke et al. 2019; Singh 2022). High concentrations of

sodium ions (Na^+) displace essential cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), undermining soil aggregation and hydraulic conductivity, while promoting clay dispersion and surface crusting (Zhao et al. 2020; Sahab et al. 2021). These physicochemical constraints restrict root development, intensify water and nutrient stress, and ultimately suppress crop productivity (Mukhopadhyay et al. 2021; Wang et al. 2024).

In saline-alkali soils, excessive pH and salt concentrations impair plant physiological functions by inducing osmotic and ionic stress, oxidative damage, and photosynthetic inhibition (Munns et al. 2020; Muhammad et al. 2024). These stressors disrupt the metabolic homeostasis of plants, resulting in reduced biomass, diminished nutritional quality, and decreased resilience to environmental fluctuations (Munns et al. 2020; Wang et al. 2024). Alfalfa (*Medicago sativa* L.), a symbiotic nitrogen-fixing forage species, serves as a sensitive model for evaluating soil–plant–microbe interactions under saline-alkali stress and for testing soil remediation strategies (Long et al. 2019).

A broad range of interventions—including gypsum amendments, organic inputs, phytoremediation, and leaching—have been implemented to mitigate salinization (Hassani et al. 2021; Wang et al. 2024; Huang et al. 2025; Song et al. 2025). Among these, biochar has gained prominence as a sustainable amendment capable of enhancing soil structure, moderating pH, improving cation exchange capacity, and supporting microbial activity (Bolan et al. 2024; Song et al. 2025; Zhang et al. 2025; Zhao et al. 2025). Biochar mediates plant salt tolerance by reducing Na^+ accumulation, improving K^+/Na^+ homeostasis, and fostering beneficial microbial communities (Liu et al. 2020; Haider et al. 2024; Li et al. 2024). Given these multifunctional attributes, we hypothesized that biochar modulates rhizosphere conditions in ways that promote nutrient acquisition and microbially mediated stress alleviation.

However, most biochars are inherently alkaline, which can aggravate high-pH saline-alkali soils (Bolan et al. 2024; Che et al. 2024). Furthermore, the heterogeneity of biochar's physicochemical properties—governed by feedstock type, pyrolysis conditions, and post-treatment processes—limits the predictability and consistency of its performance across soil types and cropping systems (Manya 2012; Zhang et al. 2025). Crucially, mechanistic insights into biochar-mediated improvements in plant performance remain insufficiently resolved, particularly regarding how biochar-induced shifts in rhizosphere microbial communities interact with plant metabolic adjustments under salt stress (Zhao et al. 2020; Anzano et al. 2022; Demiwai et al. 2024).

Advances in green chemistry have recently enabled the development of functionally biochars designed specifically for soil rehabilitation (Hao et al. 2025; Wang et al. 2025). Acid-modified biochar, produced through environmentally benign chemical activation, offers enhanced surface functionalization, greater sorption capacity, and reduced pH, making it especially suitable for ameliorating alkaline soils (Liu et al. 2020; Zhang et al. 2025). These function-specific biochars have the potential to not only ameliorate saline-alkali soil conditions but also drive microbial community restructuring and plant metabolic reprogramming, thereby enhancing legume resilience under salt stress (Xiang et al. 2022; Sree et al. 2025). Yet, systematic comparison of acid-modified versus conventional alkaline biochars—particularly their capacity to drive microbial reassembly and metabolic reprogramming in crops exposed to saline-alkali stress—remains limited.

Here, we conducted a comprehensive pot experiment to evaluate the effects of two feedstock types of biochar—alkaline and acid-modified—on ameliorating saline-alkali soil and promoting alfalfa growth. We aimed to (1) assess how different biochar types and application rates affect soil physicochemical properties, including salinity, pH, and nutrient availability, along with alfalfa growth traits, physiological responses, and nutritional quality; (2) investigate the relationship between plant performance and soil improvement parameters under biochar amendment; and (3) integrate rhizosphere microbiome profiling with root metabolomics to elucidate the biotic and metabolic mechanisms underpinning biochar-induced salt tolerance. We hypothesized that acid-modified biochar would outperform conventional alkaline biochar in enhancing alfalfa resilience by simultaneously improving soil chemistry, enriching symbiotic microbial taxa, and activating metabolic pathways associated with stress tolerance.

2 Materials and methods

2.1 Experimental design and soil preparation

A controlled pot experiment was conducted to assess the impact of biochar amendments on alfalfa (*Medicago sativa* L., cultivar Zhonglan 1) growth and rhizosphere microbial communities in saline-alkali soils. The experimental soil was collected from the upper 0–20 cm layer of a saline-alkali grassland located at the Linze Grassland Agricultural Experiment Station of Lanzhou University (100° 06' E, 39° 11' N). The site is characterized by a strongly alkaline soil profile, with an average pH of 8.6 and a salinity of 1.21%. Soil samples were obtained from ten randomly selected plots (5 m × 5 m) within a 500 m × 100 m area, following the removal of plant debris and stones. Collected samples were air-dried, homogenized, and sieved through a 2 mm mesh before use.

Two distinct biochar types were employed: (i) alkaline biochar (AL-biochar, pH 8.8) derived from corn straw, and (ii) acid-modified biochar (AC-biochar, pH 2.3) produced through acid functionalization. Both were pyrolyzed at 400 °C and characterized for elemental composition and physicochemical properties (Table S1). Biochar was incorporated into 1.0 kg of prepared soil at application rates of 0% (the control, CK), 1%, 2%, and 5% (w/w), representing low to high amendment levels based on common agronomic practices (Joseph et al. 2021; Zhang et al. 2025).

Seven treatments were established: CK (without biochar), AC1, AC2, AC5 (acid-modified biochar at 1%, 2%, 5%), and AL1, AL2, AL5 (alkaline biochar at 1%, 2%, 5%). Each treatment was replicated 12 times. Prior to sowing, biochar-treated soils were equilibrated for two weeks in a greenhouse. Pots (13 cm diameter × 15 cm height) were maintained under controlled conditions (24 °C, 50% relative humidity, and a 16-h photoperiod) and irrigated every two days to sustain uniform moisture. Twenty seeds were sown per pot at a depth of approximately 1 cm without pre-germination treatment. Seedling emergence was recorded after 7 days to calculate the germination rate. To ensure consistent plant density, seedlings were thinned to five uniformly growing individuals per pot, which were then cultivated for 90 days under identical greenhouse conditions. Rhizosphere soil was collected following a previously established method (Tang et al. 2022), while root and leaf tissues were flash-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

2.2 Analysis of soil physicochemical properties

Following harvest, soil from each pot was thoroughly homogenized and sieved (2 mm). Fresh subsamples were used to quantify gravimetric water content, nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+). Air-dried samples were analyzed for pH, salinity, soil organic carbon (SOC), total nitrogen (TN), and available phosphorus (AP).

Soil pH was measured in a 1:2.5 soil-to-water suspension using a calibrated pH meter (PHSJ-3F, INESA). Salinity was determined gravimetrically by evaporating a known volume of soil extract. NH_4^+ was quantified using the indophenol blue method, whereas NO_3^- and NO_2^- were assessed using the VCl_3 -Griess assay (Zhang et al. 2025). Soil organic carbon was determined by dichromate oxidation ($\text{K}_2\text{Cr}_2\text{O}_7$), total nitrogen by the Kjeldahl method, and available phosphorus by the sodium bicarbonate extraction–Mo–Sb colorimetric method.

2.3 Plant morphology, physiology, and nutritional evaluation

Germination was assessed by recording the number of surviving seedlings per pot. At the experiment's conclusion, plant height, stem diameter, and leaf area were measured. Roots were gently washed and scanned using a root analysis system (WinRhizo Pro LA2400, Regent Instruments) to evaluate total root length, surface area, volume, average diameter, and root tip count. Biomass was recorded after drying shoots and roots at 105 °C for 30 min, followed by oven drying at 65 °C to a constant weight. Root-to-shoot ratios were calculated on a dry-weight basis.

To assess physiological status, markers of oxidative stress and metabolic adaptation were analyzed. Frozen tissues (100 mg) were homogenized in 1 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone. Malondialdehyde (MDA) was quantified via the thiobarbituric acid assay; proline by ninhydrin colorimetry; soluble sugars by the anthrone method; and soluble proteins by the Coomassie brilliant blue G-250 assay. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) were determined using commercial assay kits (Comin Bio, Suzhou, China) and measured with a microplate reader (Infinite 200 PRO, TECAN, Switzerland).

Leaf samples were also analyzed for mineral content and forage quality. Sodium (Na^+) and potassium (K^+) were quantified by flame photometry (Model 410, Sherwood Scientific Ltd, UK). Crude protein, fat, lignin, acid detergent fiber (ADF), and neutral detergent fiber (NDF) were assessed using Fourier-transform infrared spectroscopy (FTIR). Relative feed value (RFV) was calculated as previously established by Kamran et al. (2022):

$$\text{RFV} = \text{DMI} \times \text{DDM} \div 1.29 \quad (1)$$

$$\text{DMI} = 120 \div \text{NDF} \quad (2)$$

$$\text{DDM} = 88.9 - 0.779 \times \text{ADF} \quad (3)$$

where DMI is dry matter intake, and DDM is digestible dry matter.

2.4 Metabolite profiling

To enable mechanistic interpretation, treatment groups for omics analysis were selected based on their distinct physiological and soil responses. Because the highest biochar addition rate (5%) produced the most pronounced and contrasting improvements in soil salinity, nutrient status, and plant growth, root tissues and rhizosphere samples from the control (CK), AC5, and AL5 were

subjected to metabolomic and genomic analyses, respectively. This selection allowed us to compare untreated plants with the two maximally responsive biochar treatments and thereby identify biochar-type-specific metabolic and microbial regulatory pathways. Root samples (100 mg) were ground in liquid nitrogen and extracted in 80% methanol following the protocol of Want et al. (2013) and Hou et al. (2021). Extracts were centrifuged, and supernatants were diluted for analysis using a Vanquish UHPLC system coupled to a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany).

Metabolites were separated on a Hypersil GOLD column (100 × 2.1 mm, 1.9 µm) using a 17-min linear gradient. The instrument was operated at a spray voltage of 3.5 kV and a capillary temperature of 320 °C. Peak alignment and quantification were performed using Compound Discoverer 3.1, and metabolites were annotated with the mzCloud, mzVault, and MassList databases. Functional pathway enrichment was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Human Metabolome Database (HMDB), and LIPID Maps. Significant differential metabolites were identified via partial least squares discriminant analysis (PLS-DA) and DESeq2 in R, using variable importance in projection (VIP) > 1, $p < 0.05$, and fold change > 4. Pathway analysis was conducted on MetaboAnalyst (<https://www.metaboolanalyst.ca>).

2.5 Rhizosphere microbial community analyses

To evaluate the effects of biochar on microbial dynamics, 16S rRNA gene sequencing was performed on rhizosphere soil from the CK, AC5, and AL5 groups. DNA was extracted from 0.5 g of soil using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using primers 338F and 806R. Amplicons were purified, quantified, and sequenced on the Illumina NovaSeq 6000 platform (BMKGENE, Qingdao, China).

Raw sequence data were processed using QIIME2 (Bolyen et al. 2019), including demultiplexing, denoising with DADA2, and removal of amplicon sequence variants (ASVs) with fewer than 10 reads. Taxonomic classification was performed using a Naive Bayes classifier trained on the RDP database (version 11.5) (Wang et al. 2007). Microbial alpha diversity (Shannon, Faith's phylogenetic diversity, species richness) and beta diversity (Bray–Curtis dissimilarity) were computed. Principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA, “*adonis*” function in the “vegan” package) were applied to assess treatment effects on community structure.

2.6 Statistical analyses

All statistical analyses were conducted in R version 4.2.2. Statistical comparisons between treatments were performed using a t-test (applied to account for paired comparisons) and the least significant difference (LSD) test (applied to account for multiple comparisons) using the “EasyStat” package. Two-way analysis of variance (ANOVA) was implemented to examine interactions between biochar feedstock type and application rate using the “agricolae” package. Pearson's correlation with false discovery rate (FDR) correction was computed using the “psych” package, while linear regression was performed with “ggpmisc”. Redundancy analysis (RDA) and Mantel tests were conducted using the “MASS” and “ggcor” packages to explore the influence of biochar application rate and soil physicochemical properties on the plant characteristics across AL- and AC-biochar treatments. Data visualization was carried out using the “ggplot2” package.

3 Results

3.1 Biochar amendments improve soil physicochemical properties

Application of both acid-modified (AC) and alkaline (AL) biochar significantly ameliorated key soil constraints in saline-alkali soils (Fig. 1). Soil salinity was significantly reduced following the application of AC-biochar. Both treatments led to marked reductions in soil pH relative to the unamended control (CK), with the extent of reduction positively correlated with increasing biochar application rates. The most pronounced effects were observed at the highest amendment level (5%), where salinity declined by 37.4% with AC-biochar and by 12.3% with AL-biochar ($p < 0.05$, LSD test). Across all rates, AC-biochar consistently outperformed AL-biochar in mitigating both salinity and pH ($p < 0.05$, t-test), likely due to its higher surface acidity and enhanced cation exchange capacity. In parallel, soil water content declined significantly following biochar addition, though response patterns varied by biochar type. AL-biochar led to a linear decrease in soil water content with increasing application rates, reaching a minimum of 15.8% at the 5% level. In contrast, AC-biochar induced a non-linear response, with the lowest water content (18.5%) recorded at the intermediate 2% application rate. Although biochar is generally associated with improved soil water retention, the reduction observed here likely reflects an indirect, plant-mediated effect. Both biochar types markedly enhanced alfalfa biomass accumulation and physiological performance, which collectively increased plant water demand and extraction from the soil. In

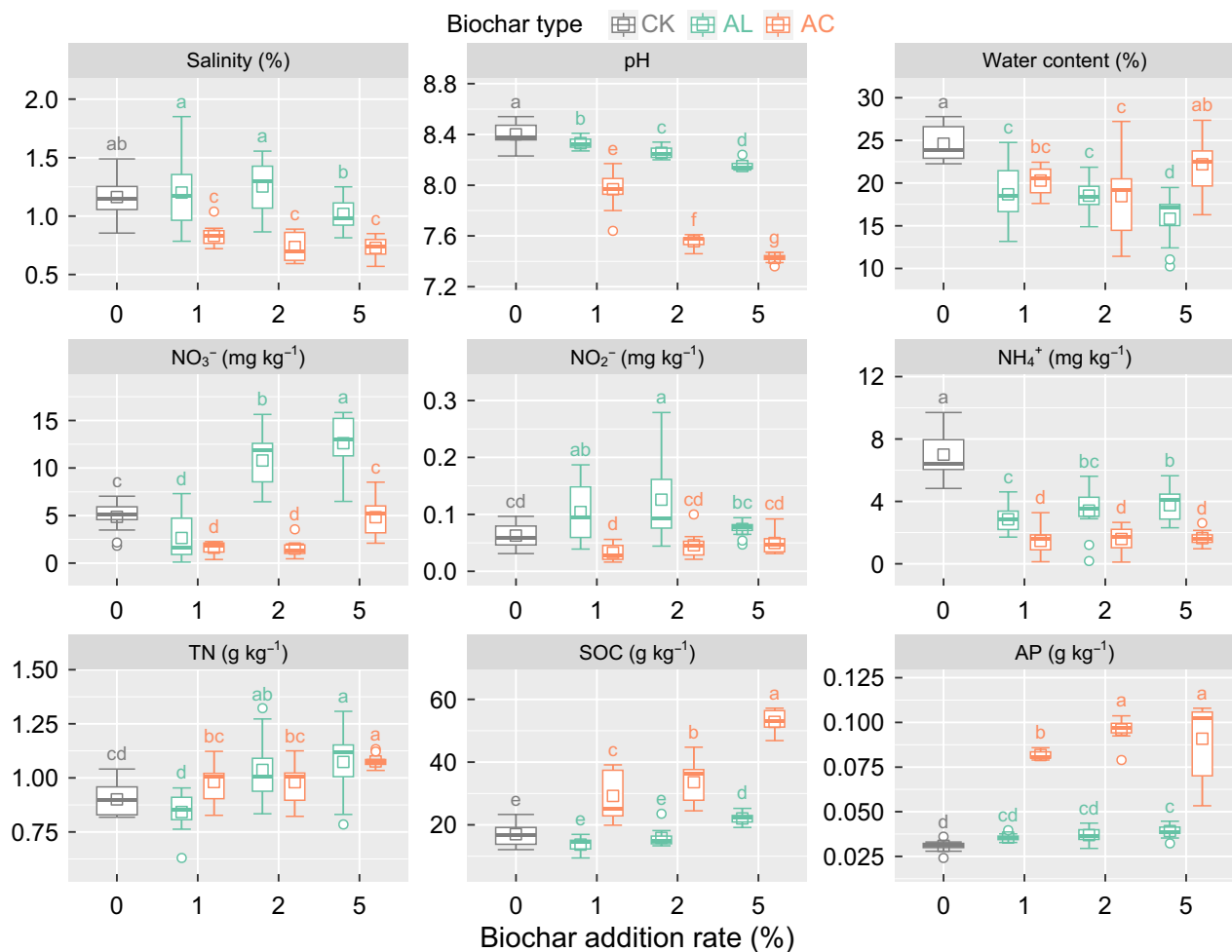


Fig. 1 Amending effect of biochar feedstock type and application rate on soil physicochemical properties in saline-alkali soils. NO_3^- : nitrate, NO_2^- : nitrite, NH_4^+ : ammonium, TN: total nitrogen, SOC: soil organic carbon, AP: available phosphorus. Different lowercase letters indicate statistically significant differences among treatments based on LSD tests ($p < 0.05$)

addition, biochar-amended soils—particularly with the larger porous size AL-biochar—exhibited improved infiltration and drainage characteristics (Murtaza et al. 2024).

Biochar amendments markedly improved soil nutrient status. The 5% AC-biochar treatment yielded the highest increase in soil organic carbon content, rising by 211.0% relative to the control (52.8 g kg⁻¹ vs. 17.0 g kg⁻¹), while the same rate of AL-biochar resulted in a modest 29.7% increase (22.0 g kg⁻¹ vs. 17.0 g kg⁻¹, Fig. 1). Total nitrogen levels increased comparably in both treatments, with both 5% AC- and AL-biochar elevating total nitrogen by approximately 19%. AC-biochar significantly enhanced the availability of phosphorus, with available P concentration rising by 194.1% under the 5% AC-biochar treatment—substantially greater than the marginal gains observed with AL-biochar.

3.2 Modulation of alfalfa traits by biochar amendments

3.2.1 Biochar application promotes alfalfa growth and development

Biochar amendments markedly improved alfalfa performance under saline-alkali stress. AC-biochar significantly enhanced seed germination rates compared to the unamended control ($p < 0.05$, LSD test, Fig. 2). The 5% AC-biochar treatment produced the highest germination rate (89.2%), much higher than the control (50.4%). Germination under AL-biochar increased in a dose-dependent manner. Specifically, the germination rate was reduced to a certain extent by 1% and 2% AL-biochar treatments, while it was increased by a 5% AL-biochar treatment.

Patterns of biomass accumulation mirrored the aforementioned responses. AL-biochar significantly boosted both shoot and root biomass, with 5% amendments yielding increases of 130.4% and 335.6%, respectively. AC-biochar also increased shoot biomass

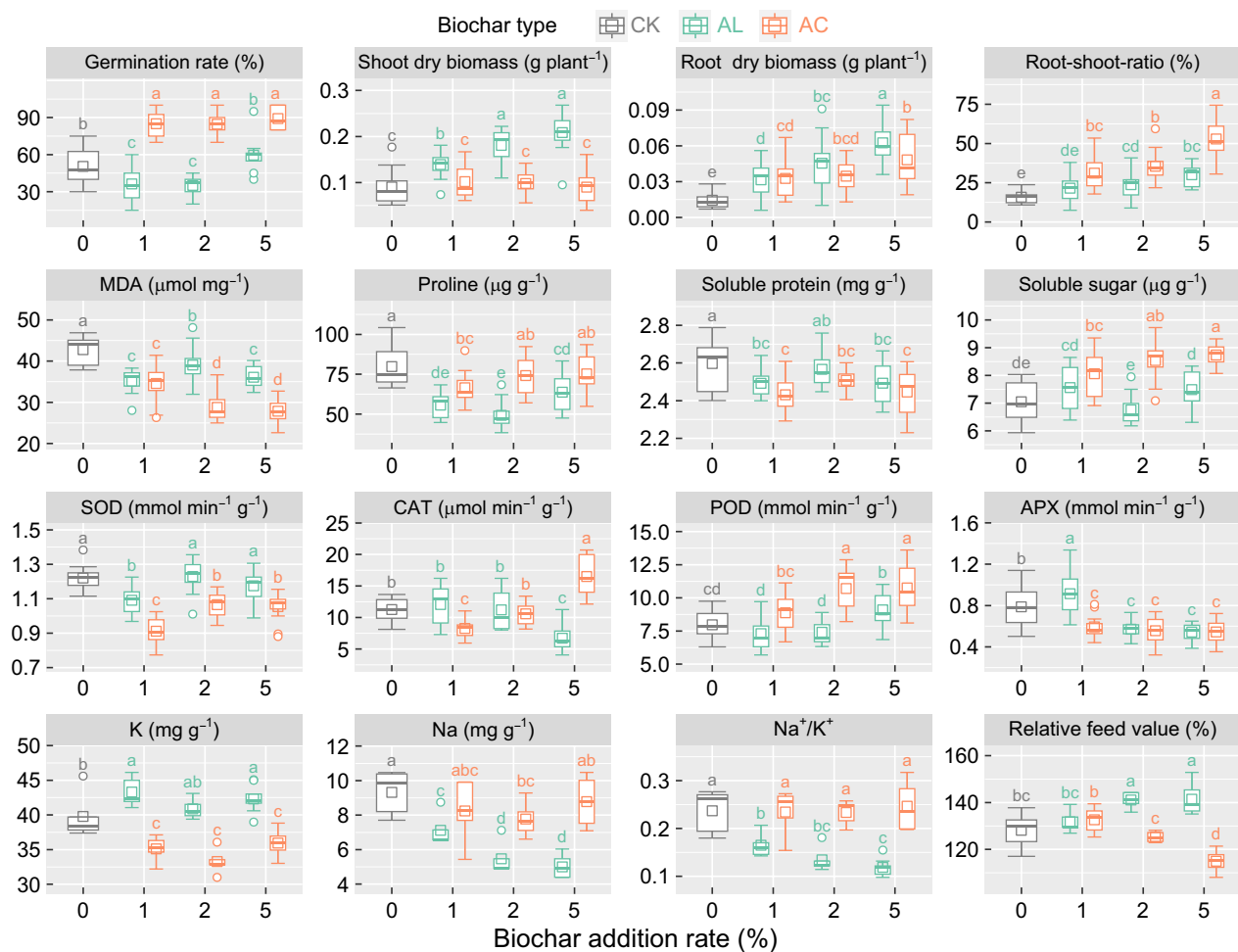


Fig. 2 Enhancement of alfalfa seed germination, seedling growth, physiological properties, and nutrient quality under biochar amendment. Assessed biochemical markers include malondialdehyde (MDA), proline, soluble protein and sugar content, and antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). Different lowercase letters indicate statistically significant differences among treatments based on LSD tests ($p < 0.05$)

in a dose-responsive fashion ($p < 0.05$, LSD test), but its effect on root biomass followed an unimodal pattern, peaking at the 1% rate. Despite its generally lower promotive effect on total biomass, AC-biochar significantly elevated the root-to-shoot ratio ($p < 0.05$, LSD test), suggesting a strategic shift in carbon allocation favoring root investment under certain amendment levels.

Plant morphological traits—such as height, branching, stem diameter, leaf area, root length, and root surface area—were most strongly enhanced by AL-biochar, particularly at the 5% application rate (Fig. S1). In contrast, AC-biochar showed positive effects at the 1% level, but exhibited inhibitory trends at higher rates, even falling below control levels. Collectively, these findings indicate a broader and more consistent stimulatory effect of AL-biochar on alfalfa growth and

development, while the performance of AC-biochar is more variable and dose-sensitive.

3.2.2 Biochar regulates plant physiological responses to saline stress

Biochar amendments significantly mitigated oxidative stress markers in alfalfa (Fig. 2). Malondialdehyde content—a proxy for lipid peroxidation—decreased significantly across all treatments, with AC-biochar showing a clear dose-dependent reduction ($p < 0.05$, LSD test). Proline content exhibited a biphasic response, reaching minima at 1% AC- and 2% AL-biochar before rising again with higher rates, potentially reflecting a transient alleviation of osmotic stress under moderate amendment levels. Soluble protein content declined across treatments, with the exception of a modest increase under 2% AL-biochar. Soluble sugar content, in contrast, progressively

increased with rising AC-biochar concentrations, suggesting enhanced carbon metabolism or osmotic adjustment. No significant changes in soluble sugar levels were observed in response to AL-biochar ($p > 0.05$, LSD test).

Among antioxidant enzymes, ascorbate peroxidase activity declined under both biochar treatments ($p < 0.05$, LSD test, Fig. 2), whereas peroxidase activity rose steadily, particularly under AC-biochar, reaching a 35.0% elevation at the 5% level. Superoxide dismutase and catalase activities initially declined under low AC-biochar rates but subsequently rebounded and surpassed control levels at higher doses, notably under the 5% treatment. These results demonstrate biochar's capacity to modulate antioxidant defense systems, contributing to improved physiological resilience in saline environments.

3.2.3 Biochar influences alfalfa ion homeostasis and nutritional quality

Biochar amendments also altered nutrient uptake and forage quality parameters (Figs. 2 and S2). AL-biochar significantly reduced sodium accumulation while enhancing potassium uptake in alfalfa tissues ($p < 0.05$, LSD test), thereby improving K^+/Na^+ homeostasis. Conversely, AC-biochar tended to suppress potassium absorption while having a negligible effect on sodium content, suggesting divergent ion-regulatory capacities between the two biochar types. Crude fat content increased significantly under AC-biochar, peaking at a 17.7% rise at the 5% application rate ($p < 0.05$, LSD test). However, this benefit was offset by a concomitant decline in crude protein content, which reached its lowest level under the same treatment. AL-biochar elicited minimal effects on both crude fat and protein concentrations, indicating a more conservative influence on basic forage composition.

Both biochars increased lignin content in a dose-dependent manner, reaching 14.1% and 12.3% above control levels under 5% AC- and AL-biochar, respectively ($p < 0.05$, LSD test, Fig. S2). Similarly, acid detergent fiber and neutral detergent fiber levels rose under AC-biochar, whereas AL-biochar generally reduced neutral detergent fiber. Consequently, relative feed value—which inversely correlated with acid detergent fiber and neutral detergent fiber—declined under AC-biochar but improved with AL-biochar application, highlighting contrasting effects on forage digestibility and nutritional value.

3.3 Linking soil improvements to plant performance

Redundancy analysis was conducted to assess the interactive influence of biochar-induced soil changes on alfalfa performance. The first two RDA axes collectively explained over 80% of the total variation in plant traits as influenced by biochar rate and soil parameters

(AL-biochar: $F = 5.16$, $p < 0.001$; AC-biochar: $F = 3.65$, $p < 0.001$, Fig. 3a). Biochar application rate emerged as the most influential factor, accounting for 14.3% and 13.55% of the explained variance under AL- and AC-biochar treatments, respectively (Fig. 3b). Soil pH and nutrient availability, particularly soil organic carbon and available phosphorus, were also key contributors to plant response patterns.

Nitrogen dynamics differed between biochar types: AL-biochar predominantly modulated nitrate concentrations, while AC-biochar affected ammonium levels (Fig. 3b, c). Moreover, total nitrogen and soil water content had greater explanatory power for plant performance under AL-biochar, whereas soil salinity exerted a more pronounced influence under AC-biochar. These findings reveal the distinct modes of action by which AC- and AL-biochars modulate soil environments and, in turn, plant physiological and developmental responses.

3.4 Biochar-induced shifts in root metabolomic profiles

Given the pronounced plant-promoting effects observed under the 5% amendment rate for both AC- and AL-biochar treatments, we performed a metabolomic profiling of alfalfa roots. A total of 2,067 metabolites were identified across treatments. Partial least squares discriminant analysis revealed distinct metabolomic signatures between the treatments (Anosim: $R^2 = 0.471$, $p < 0.001$; Adonis: $R^2 = 0.341$, $p < 0.001$; Fig. 4a, Table S3). Differential abundance analysis identified 188 significantly altered metabolites across treatments, spanning 10 superclasses, 36 classes, and 49 subclasses based on HMDB classification (Fig. S3, Table S4). Compared with the control (CK), AL-biochar treatment upregulated 13 and downregulated 52 metabolites, whereas AC-biochar upregulated 25 and downregulated 82 compounds (Figs. 4b and S4). Notably, the upregulated metabolites were entirely distinct between the two biochar types, although they shared 22 downregulated compounds.

AL-biochar specifically upregulated metabolites involved in stress tolerance and nitrogen metabolism, including oridonin, agmatine, catechol, DL-arginine, 4-methylhippuric acid, nicotinamide *N*-oxide, geranylacetone, ornithine, and vitamin B12, as annotated in the KEGG database. In contrast, AC-biochar uniquely upregulated several compounds, including chlordinazepoxide, glycerol 3-phosphate, spinosin, polypodine B, artemether, medicarpin, isoliquiritigenin, ginglycolipid B, irisflorelin, angelol B, cryptotanshinone, acetylcysteine, and schisanhenol (Figs. 4b, S5–S6).

Pathway enrichment analysis revealed that AL-biochar predominantly modulated pathways related to arginine and proline metabolism (ko00330), arginine

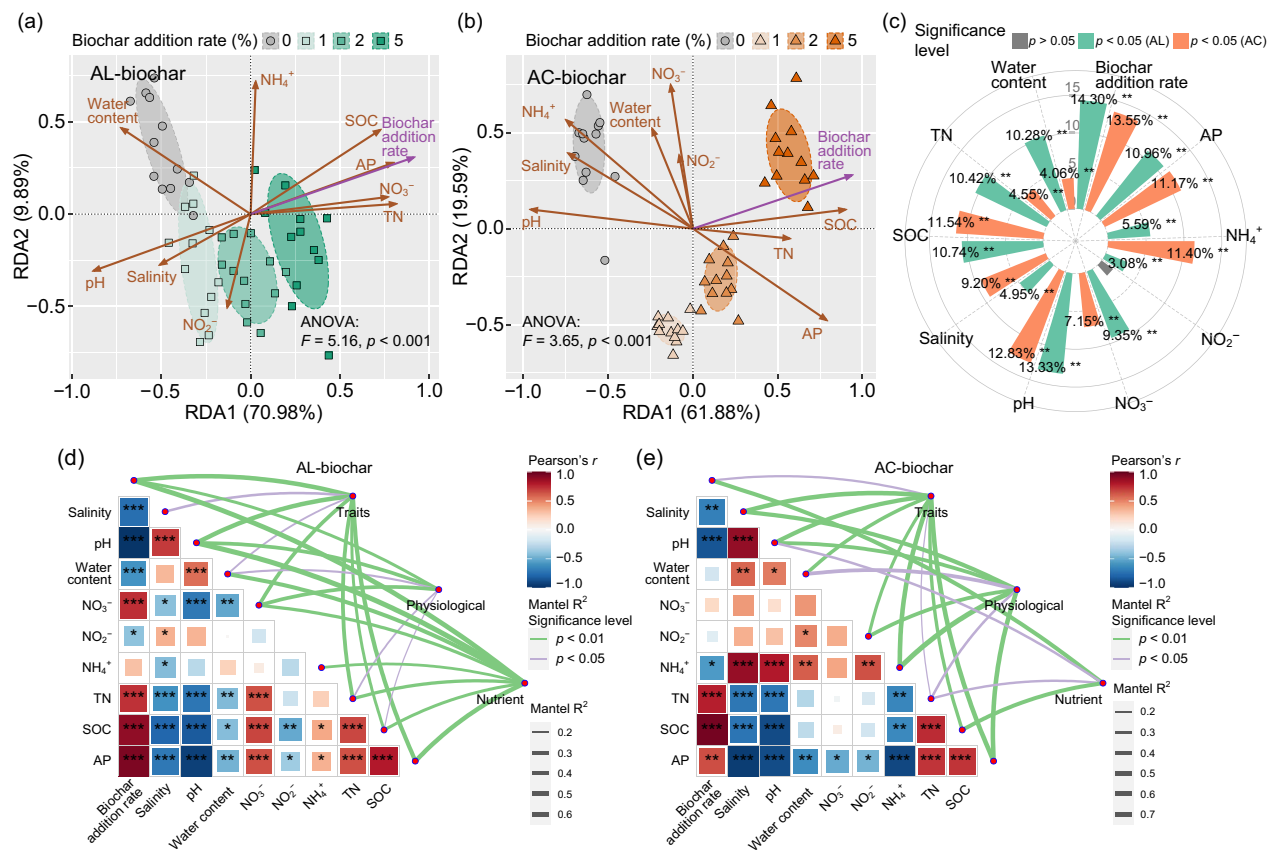


Fig. 3 Relationships between biochar-amended soil properties and alfalfa biomass and morphological traits. **a, b** Redundancy analysis (RDA) biplot illustrating the multivariate correlations between soil nutrient variables and plant growth parameters. **c** The variance in plant traits explained by individual soil factors. **d, e** Mantel test assessing the overall correlation between soil properties and plant biomass, indicating significant linkages driving plant performance under biochar treatments. The color gradient represents Pearson's correlation coefficient (r), with significance levels denoted as *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ (FDR-adjusted). TN: total nitrogen, SOC: soil organic carbon, AP: available phosphorus, NO_3^- : nitrate, NO_2^- : nitrite, NH_4^+ : ammonium

biosynthesis (ko00220), alanine, aspartate and glutamate metabolism (ko00250), and glutathione metabolism (ko00480) (Fig. 4c). In contrast, AC-biochar was more strongly associated with specialized secondary metabolism, particularly betalain biosynthesis (ko00965) and isoquinoline alkaloid biosynthesis (ko00950).

Correlation network analysis further revealed key metabolites related to alfalfa growth. DL-arginine (Pearson's $r_{\text{root}} = 0.788$, Pearson's $r_{\text{shoot}} = 0.706$), ornithine (Pearson's $r_{\text{root}} = 0.784$, Pearson's $r_{\text{shoot}} = 0.705$),

and sibiricose A5 (Pearson's $r_{\text{root}} = 0.743$, Pearson's $r_{\text{shoot}} = 0.639$) showed strong, positive correlations with both root and shoot biomass (Figs. 4d, S7). Additionally, nicotinamide *N*-oxide (Pearson's $r = 0.710$) and 5,6-dimethoxy-2-(2-methoxyphenyl)-4*H*-chromen-4-one (Pearson's $r = 0.658$) were positively correlated with root biomass. AC-biochar-specific upregulation of metabolites, such as isoliquiritigenin, medicarpin, palmaturbine, polypodine B, and acetylcysteine, may contribute to enhanced root development by regulating antioxidant and hormone-like pathways.

(See figure on next page.)

Fig. 4 Metabolic profile alterations in alfalfa roots following biochar amendments. **a** Partial least squares discriminant analysis (PLS-DA) score plot differentiating metabolic fingerprints among treatment groups. **b** Summary statistics of significantly differential metabolites identified. **c, d** Enrichment analysis highlighting key metabolic pathways affected by biochar application. **e** Networks showing associations between metabolite concentrations and plant biomass, providing insights into biochemical mechanisms underlying growth promotion

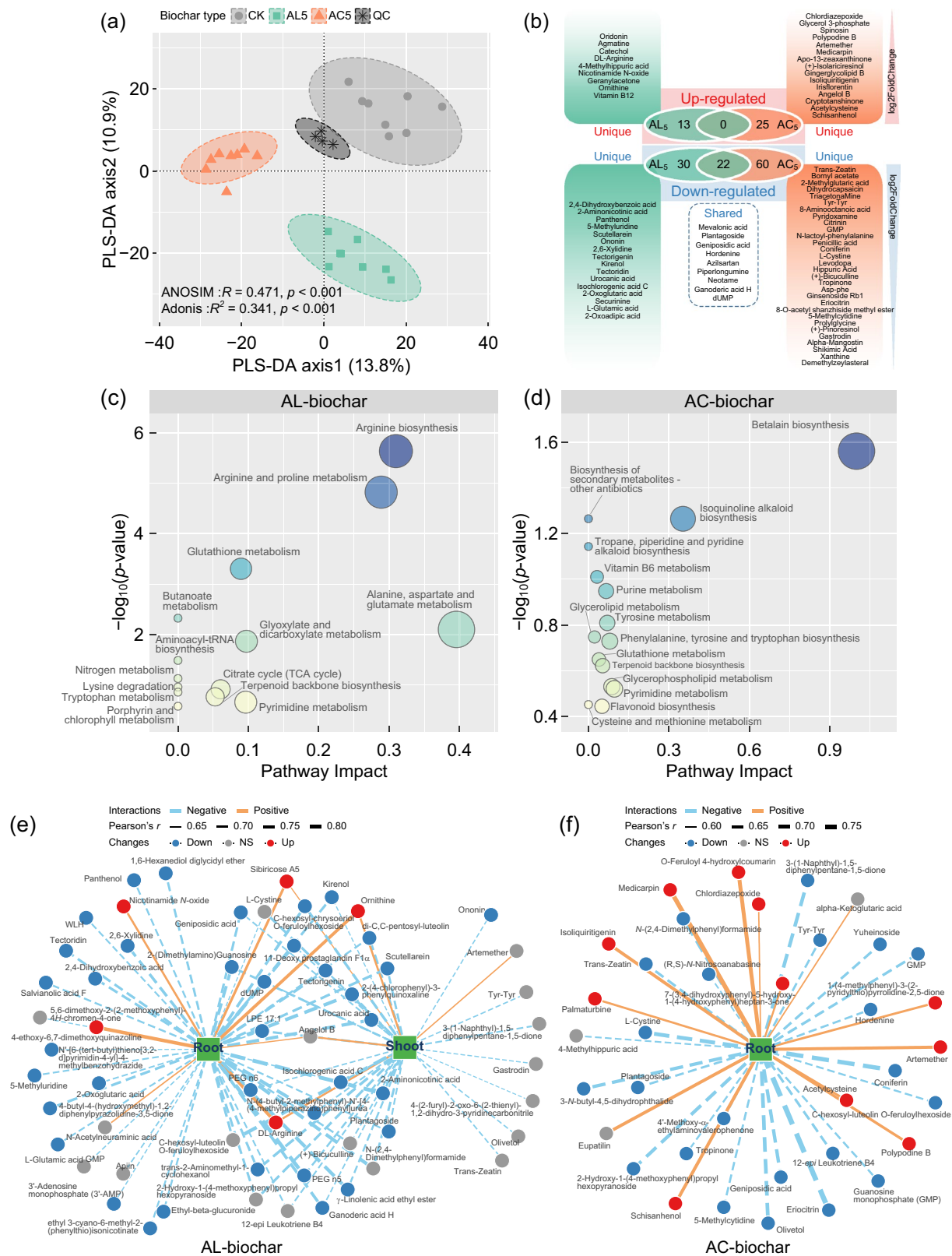


Fig. 4 (See legend on previous page.)

3.5 Alterations in rhizosphere bacterial community structure following biochar application

Rarefaction curves plateaued across all samples, indicating that sequencing depth was sufficient to capture the full extent of rhizosphere bacterial diversity (Fig. S8). After quality filtering, a total of 5,494 bacterial ASVs were retained. Among them, only 562 ASVs were shared among the control, AC-biochar, and AL-biochar treatments, representing 68.4%, 67.6%, and 59.7% of their respective communities (Fig. 5a). Each treatment also harbored a considerable number of unique ASVs: 1,049 in the control, 1,304 in AL-biochar, and 1,388 in AC-biochar, accounting for 10.9–17.8% of total richness. Alpha diversity indices (Shannon index and richness) indicated that AL-biochar significantly enhanced bacterial diversity compared to both the control and AC-biochar (Fig. 5b; $p < 0.05$, LSD test). Principal coordinate analysis based on Bray–Curtis distances further confirmed the clear separation in bacterial community composition among treatments (Anosim: $R = 0.994$, $p < 0.001$; Adonis: $R^2 = 0.631$, $p < 0.001$; Fig. 5c, Table S5).

At the phylum level, AL-biochar significantly enriched Acidobacteria and Firmicutes, while AC-biochar increased the relative abundance of Actinobacteria and *Candidatus* Saccharibacteria (Figs. 5d and S9). Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes showed similar responses to both treatments. At the genus level, 2,658 of the 5,494 ASVs (accounting for 61.9–81.7% of total abundance) could be taxonomically annotated. AL-biochar significantly enhanced the abundance of genera such as *Neobacillus*, *Pseudoxanthomonas*, and *Rhizobium* (Figs. 5e and S10). Several other genera, including *Agrobacterium*, *Devosia*, *Ensifer*, *Pseudomonas*, *Sphingomonas*, *Arenimonas*, *Marinobacter*, *Pontibacter*, *Rheinheimera*, and *Salinimicrobium*, responded positively to both AC- and AL-biochar.

DESeq2 analysis identified 82 genera with significant changes across treatments ($VIP > 1$, $|\log_2FC| > 2$, $p < 0.05$, Figs. S11–S12, Table S6). AL-biochar-specific enrichment was mainly associated with members of Firmicutes, including *Anaerovorax*, *Neobacillus*, *Sedimentibacter*, *Clostridium sensu stricto*, *Romboutsia*, *Geosporobacter*, *Fonticella*, *Cellulosilyticum*, and *Desulfosporosinus* (Figs. 5f, S13–S14). In contrast,

AC-biochar-specific upregulated genera were dominated by members belonging to Actinobacteria, including *Catelliglobospora*, *Glycomyces*, *Yinghuangia*, *Rhodococcus*, *Nocardiopsis*, *Saccharothrix*, *Geodermatophilus*, *Aeromicrobium*, *Blastococcus*, *Catellatospora*, *Streptomyces*, *Nocardioides*, *Pseudonocardia*, *Solirubrobacter*, and *Gaiella*. Six genera (i.e., *Sandaracinus*, *Massilia*, *Terrimonas*, *Akkermansia*, *Pedobacter*, and *Aciditerrimonas*) were upregulated by both biochar types, suggesting a shared core microbial response to biochar addition.

Correlation network analysis revealed that biochar amendments reshaped the rhizosphere microbiome in taxonomically distinct ways and that these shifts were tightly linked to changes in soil properties (Figs. 5f, S15). Importantly, metabolome–microbiome integration showed that biochar-induced microbial restructuring was directly associated with root metabolic reprogramming. Under AL-biochar, several growth-promoting metabolites—including sibiricose A5, ornithine, nicotinamide *N*-oxide, DL-arginine, and 4-ethoxy-6,7-dimethoxyquinazoline—exhibited significant positive correlations with diverse bacterial genera such as *Acidovorax*, *Agrobacterium*, *Anaerovorax*, *Cellulosilyticum*, *Chitinophaga*, *Desulfosporosinus*, *Dyadobacter*, *Fonticella*, *Neobacillus*, *Pedobacter*, *Rhodococcus*, *Sandaracinus*, and *Terrimonas* (Fig. S16). Many of these taxa are associated with nitrogen cycling, osmolyte biosynthesis, or organic matter turnover, suggesting that AL-biochar promotes a microbially mediated metabolic environment favorable for stress alleviation and nutrient acquisition.

In contrast, AC-biochar stimulated a distinct set of metabolite–microbe associations. AC-biochar-upregulated compounds—such as schisanhenol, polypodine B, palmaturbine, O-feruloyl-4-hydroxycoumarin, medicarpin, isoliquiritigenin, and acetylcysteine—were positively correlated with genera including *Aeromicrobium*, *Agrobacterium*, *Akkermansia*, *Catellatospora*, *Chitinophaga*, *Dyadobacter*, *Ensifer*, *Massilia*, *Methylopila*, and *Rhodococcus* (Fig. S17). Notably, several of these metabolites (e.g., medicarpin, isoliquiritigenin) have documented antimicrobial or defense-related activities, and the associated microbial taxa include genera known for xenobiotic degradation and detoxification. These integrated patterns indicate that AC-biochar primarily

(See figure on next page.)

Fig. 5 Impact of biochar amendments on rhizosphere bacterial community structure and diversity in alfalfa. **a** Venn diagram displaying unique and shared amplicon sequence variants (ASVs) across treatments with corresponding relative abundances. **b** Diversity indices including Shannon diversity, species richness, and Faith's phylogenetic diversity (PD), revealing microbial diversity shifts. **c** Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity illustrating community compositional changes. Taxonomic composition at the **(d)** phylum and **(e)** genus levels demonstrating shifts in dominant bacterial taxa. **f** Differentially abundant genera identified by DESeq2 analysis, revealing taxa responsive to biochar amendments

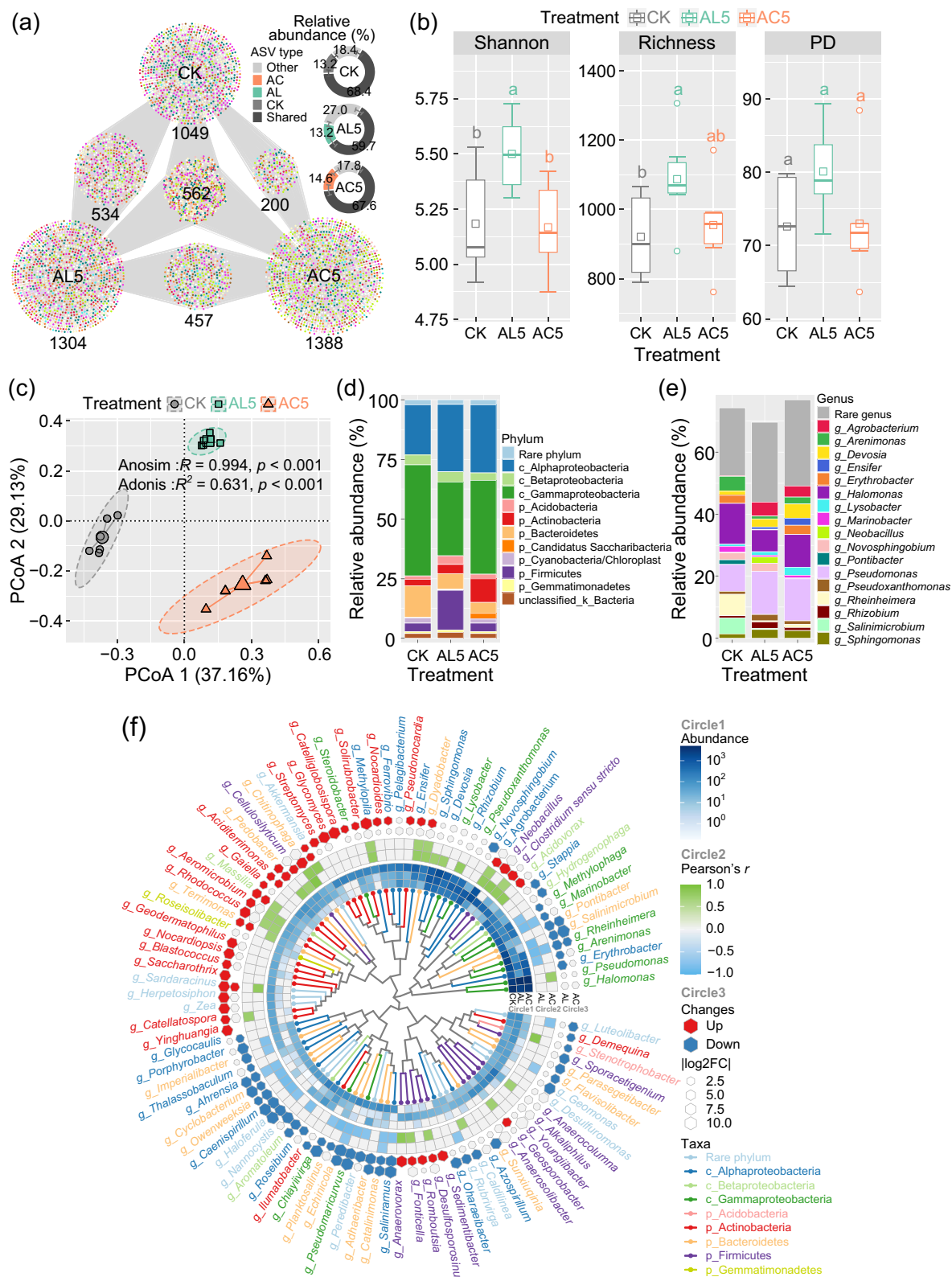


Fig. 5 (See legend on previous page.)

stimulates root defense metabolism in conjunction with microbial taxa linked to pathogen suppression and secondary metabolite turnover.

4 Discussion

4.1 Biochar-specific modulation of ionic and redox–osmotic processes enhances alfalfa stress resilience

Our findings show that both acid-modified and alkaline biochars enhanced alfalfa performance under saline-alkali stress by jointly improving ionic homeostasis and strengthening metabolic stress-defense pathways. These effects were biochar-type-specific. Biochar application reduced Na^+ accumulation and improved K^+/Na^+ selectivity, largely due to its high porosity and ion-exchange capacity (Abbas et al. 2018; Saifullah et al. 2018), which facilitate Na^+ adsorption and displacement by beneficial cations such as K^+ , Ca^{2+} , and Mg^{2+} (Ghassemi-Golezani and Abdoli 2023). These interactions contributed to a more favorable ionic environment and reduced ion-induced metabolic disruption.

Unlike generalized descriptions of oxidative stress responses, our results reveal distinct antioxidant and osmotic adjustments induced by each biochar type. Both AC- and AL-biochar reduced malondialdehyde accumulation and elevated antioxidant enzyme activities (Fig. 2)—including catalase and peroxidase—indicating strengthened reactive oxygen species detoxification (Fujii et al. 2011; Waszczak et al. 2018; Alvarez et al. 2022). However, AC-biochar triggered a stronger osmotic adjustment response, reflected in higher proline and soluble protein contents. This elevated osmolyte accumulation likely stemmed from enhanced phosphorus availability under AC-biochar, as its lower pH promoted the release of phosphorus from insoluble mineral complexes that can support osmolyte and antioxidant biosynthesis (Alkharabsheh et al. 2021; Alvarez et al. 2022; Anzano et al. 2022).

Importantly, the distinct physiological responses observed here reflect not only differences in biochar chemistry but also differences in the buffering interactions between biochar and the saline-alkali soil matrix. The moderately alkaline AL-biochar (pH 8.8) integrated smoothly with the already alkaline soil, gradually improving cation exchange capacity and moderating Na^+ toxicity without sharply perturbing pH. In contrast, the extreme acidity of AC-biochar (pH 2.3) induced a rapid but localized neutralization reaction, quickly lowering microsite pH and enhancing phosphorus mobilization, but also raising the risk of over-acidification when applied at higher rates (Cui et al. 2021; Guo et al. 2022).

Together, these observations highlight a coordinated yet divergent set of stress-mitigation processes: AL-biochar primarily strengthens ionic regulation and cellular

redox balance, whereas AC-biochar intensifies osmotic adjustment and antioxidant metabolism in response to rapid microenvironmental pH shifts. Notably, the intensified physiological reprogramming induced by AC-biochar also coincided with increased lignin and acid detergent fiber contents at higher doses, suggesting that aggressive stress-defense activation may be linked to diminished forage quality. This quality–resilience trade-off underscores the need for careful dose optimization when deploying modified biochars in forage systems, ensuring that gains in stress tolerance do not compromise nutritional value.

4.2 Metabolic reprogramming as a central mechanism of biochar-induced stress alleviation

This study provides mechanistic insights into how acid- and alkaline-modified biochars induce distinct yet complementary metabolic reprogramming in alfalfa roots under saline-alkali stress. The differentiated metabolomic responses observed here indicate that biochar amendments can be strategically tailored to enhance plant resilience by selectively modulating key stress-response pathways. These metabolomic adjustments likely arise from the concurrent improvement of soil chemical attributes, such as reductions in salinity and pH, and increases in available nitrogen and phosphorus—following biochar incorporation (He et al. 2020, 2022).

Application of AL-biochar produced the most pronounced shifts in root metabolic profiles, especially in pathways related to amino acid biosynthesis and osmotic regulation. Metabolites including DL-arginine, ornithine, and nicotinamide *N*-oxide were significantly upregulated (Fig. 4), mapping primarily to the arginine and proline metabolism (ko00330) and arginine biosynthesis (ko00220) pathways. These pathways play central roles in osmotic regulation, redox homeostasis, and cellular proliferation under stress. DL-arginine serves as a precursor of polyamines, which stabilize membranes, support cell division, and mitigate osmotic stress (Khare et al. 2018; Chen et al. 2019). Its strong positive association with biomass further suggests that AL-biochar enhances alfalfa growth by stimulating polyamine-mediated root elongation and shoot development (Blázquez 2024). Likewise, ornithine, an intermediate in proline biosynthesis and the urea cycle, contributes to osmotic adjustment and cellular protection under saline conditions (Winter et al. 2015). AL-biochar also enriched the glutathione metabolism pathway (ko00480), which is central to reactive oxygen species detoxification (Sakhno et al. 2019). Such coordinated increases in osmolyte and antioxidant production are consistent with previous reports showing

that biochar enhances amino acid metabolism and stress tolerance in crops such as maize (Nafees et al. 2024).

The observed metabolic patterns align closely with biochar-induced changes in rhizosphere microbial communities. AL-biochar significantly enriched functional bacterial genera involved in nitrogen fixation and mobilization—such as *Methylopila* and *Rhizobium* (Fig. 5f)—whose activity enhances root nitrogen availability (Saifullah et al. 2018; Alkharabsheh et al. 2021). This microbial nitrogen provision directly supports the synthesis of nitrogen-rich metabolites, including arginine, proline, polyamines, and glutathione, which collectively strengthen alfalfa's osmotic adjustment and antioxidant capacity. Likewise, enrichment of phosphate-solubilizing taxa may enhance phosphorus acquisition, thereby supporting amino acid metabolism, ATP production, and cellular energy balance essential for stress adaptation.

In contrast, AC-biochar predominantly stimulated the production of secondary metabolites, including isoquinoline alkaloids and flavonoids (e.g., isoliquiritigenin and medicarpin). These compounds function in both biotic and abiotic stress responses (Winkel-Shirley 2002; Shen et al. 2022). Enhanced isoquinoline alkaloid biosynthesis (ko00950) suggests that AC-biochar strengthens defense-related mechanisms, improving root vigor and resilience to environmental stressors (Nabavi et al. 2020). Flavonoids such as isoliquiritigenin and medicarpin contribute to reactive oxygen species scavenging while also serving as signaling molecules that coordinate stress response pathways (Demiwal et al. 2024). Furthermore, increased accumulation of acetylcysteine, a glutathione precursor, indicates that sulfur-containing antioxidants also play an integral role in maintaining redox homeostasis under AC-biochar treatment (Islam et al. 2024).

Correlation network analyses further validate the physiological importance of these metabolic shifts. DL-arginine, ornithine, and sibiricose A5 showed strong positive associations with both root and shoot biomass, underscoring their roles in stress adaptation and growth promotion (Blázquez 2024). Nicotinamide *N*-oxide, linked to NAD⁺ biosynthesis and cell redox regulation, also correlated positively with root biomass, suggesting sustained effects on energy metabolism and cellular maintenance during salinity stress (Tannous et al. 2021). Collectively, these results indicate that AL- and AC-biochars do not merely alleviate stress through soil chemical regulation but through targeted metabolic pathway activation that reflects their contrasting chemical properties. AL-biochar primarily enhances nitrogen-dependent amino acid biosynthesis, osmotic balance, and antioxidant metabolism, whereas AC-biochar intensifies defense-related secondary metabolism and sulfur-mediated redox regulation. This mechanistic divergence also explains the

greater dosage sensitivity of AC-biochar, which produced beneficial metabolic responses at moderate levels but exhibited inhibitory effects at higher addition rates, likely due to salt release, pH perturbation, or microstructural alterations in the soil matrix (Cui et al. 2021; Guo et al. 2022).

4.3 Functional divergence in rhizosphere microbiomes and their links to alfalfa metabolic responses

Our findings demonstrate that acid- and alkaline-modified biochars restructure rhizosphere microbiomes through distinct recruitment pathways, ultimately contributing to enhanced alfalfa performance under saline-alkali stress. This shows that biochar functions not only as a soil amendment but also as a selective microbial habitat modifier, offering new opportunities for precision microbiome engineering in saline-alkali agroecosystems. Both AL- and AC-biochars significantly altered alfalfa growth and rhizosphere bacterial community structure (Fig. 5), corroborating the rhizosphere's critical role as a mediating interface for plant–microbe interactions (de Vries et al. 2020; Shang et al. 2023). AL-biochar notably increased bacterial α -diversity, likely reflecting its favorable physicochemical properties—such as pH buffering capacity, higher surface area, and nutrient supply (Singh et al. 2022; Bolan et al. 2024). Principal coordinate analysis confirmed that AL- and AC-biochars shaped markedly distinct microbial community structures (Fig. 5c), consistent with earlier studies showing that biochar composition determines microbial recruitment patterns (Singh et al. 2022; Bolan et al. 2024).

Taxonomic shifts further revealed clear functional divergence between biochar types. AL-biochar increased Acidobacteria and Firmicutes, phyla associated with organic matter turnover, nutrient cycling, and improvement of soil physical structure (Kerner et al. 2023; Fan et al. 2024). The proliferation of these groups is likely strengthened by enhanced soil aeration and more stable microaggregate formation (Tsai et al. 2012; Kim et al. 2021), as well as increased nitrogen and phosphorus availability following AL-biochar application (He et al. 2020, 2022). In contrast, AC-biochar enriched Actinobacteria and *Candidatus* Saccharibacteria—taxa known for xenobiotic degradation and antimicrobial compound synthesis (van Bergeijk et al. 2020; Jose et al. 2021). These results suggest a potential enhancement of rhizosphere defensive capacity rather than nutrient-oriented functions.

Genus-level patterns reinforced these functional distinctions. AL-biochar promoted *Rhizobium*, *Neobacillus*, and *Pseudoxanthomonas*, consistent with improved nitrogen fixation, nutrient mobilization, and rhizosphere nutrient turnover (Li et al. 2022). Conversely, AC-biochar

enriched *Streptomyces*, renowned for antibiotic production and suppression of soil-borne pathogens (van Bergeijk et al. 2020; Jose et al. 2021). While these microbial profiles imply pathogen-suppressive potential, the current study did not quantify pathogen abundance or perform suppression assays. Thus, AC-biochar's effects should be interpreted as enhancing microbial defense potential rather than demonstrating direct disease suppression.

Integration of metabolomic and microbiome datasets provided strong mechanistic support for these divergent pathways. AC-biochar upregulated metabolites with antimicrobial or detoxification functions—such as medicarpin, isoliquiritigenin, and acetylcysteine. These compounds were strongly correlated with Actinobacteria, including *Streptomyces*, *Aeromicrobium*, and *Catellatospora*, linking AC-biochar to a rhizosphere metabolic environment oriented toward defense and xenobiotic processing (Zheng et al. 2018; Gong et al. 2023). In contrast, AL-biochar exhibited strong associations between nitrogen-transforming bacteria and metabolites involved in amino acid biosynthesis, osmolyte accumulation (e.g., arginine, ornithine), and glutathione-mediated antioxidant pathways.

Taken together, these results demonstrate that AC- and AL-biochars activate fundamentally different rhizosphere functional trajectories that correspond to their plant metabolic impacts. AL-biochar fosters nutrient cycling, ionic homeostasis, and amino acid–redox pathways that promote sustained growth, whereas AC-biochar reinforces defense-oriented microbial networks and xenobiotic detoxification, enhancing resilience but with narrower dosage tolerance. These mechanistic insights underscore the practical implications of biochar selection: AL-biochar is better suited for large-scale saline-alkali soil remediation, while AC-biochar may be advantageous for rapid pH correction or in systems requiring elevated stress-defense capacity. Future field-based studies should evaluate long-term microbial legacy effects, ecological safety, and cumulative impacts of repeated biochar additions to refine dosage recommendations and ensure sustainable deployment in forage production systems.

5 Conclusion

This study provides valuable insights into how precision biochar amendments can effectively rehabilitate saline-alkali soils and enhance alfalfa growth through distinct biochemical and microbial pathways. Specifically, we identified low-dose AC-biochar (1%) and high-dose AL-biochar (5%) as optimal treatments, each offering unique functional benefits. Both biochars improved key soil physicochemical properties, such as reducing pH and electrical conductivity, while enhancing nutrient

availability, thereby alleviating salt-induced constraints on plant growth. However, their functional impacts diverged markedly. AL-biochar promoted shoot biomass accumulation and enhanced stress resilience by stimulating amino acid biosynthesis, reactive oxygen species detoxification, and enriching nutrient-cycling microbial taxa. Conversely, AC-biochar favored root development and defense-related metabolic pathways, including flavonoid and alkaloid production, while fostering microbial communities associated with pathogen suppression and organic matter degradation. These findings demonstrate the importance of selecting biochar types tailored to specific agronomic goals. AL-biochar is particularly effective for enhancing productivity and microbial-driven nutrient dynamics, whereas AC-biochar provides advantages in stress mitigation and soil health restoration. Overall, this study highlights the potential of precision biochar amendments as a scalable, sustainable strategy for reclaiming saline-alkali soils and improving legume crop performance under challenging environmental constraints.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s42773-026-00595-y>.

Supplementary Material 1.

Author contributions

Jie Liu: Conceptualization, Formal analysis, Visualization, Writing—Original Draft, Funding acquisition. Ziyue Shi: Investigation, Formal analysis, Writing—Original Draft, Data curation, Visualization. Lan Zhang: Investigation, Methodology, Data curation. Runqiu Feng: Investigation. Guorui Zhang: Investigation. Hao Zou: Investigation. Gangsheng Wang: Conceptualization, Writing—Review and Editing. Yunfeng Yang: Conceptualization, Supervision, Writing—Review and Editing, Validation.

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Data availability

The sequencing data generated in this study have been deposited in the Genome Sequence Archive at the National Genomics Data Center, part of the China National Center for Bioinformation (CNCB-NGDC), under BioProject accession number PRJCA026895 (accession: CRA027032).

Declarations

Competing interests

The authors declare no competing interests.

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