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The effects of the combined applications of biochar and nitrogen fertilizer on the nitrogen-fixing microorganisms and mineral nutrients in saline-alkali soil

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Abstract

To address the aggravated soil salinization and low productivity degrading farmland in Tianjin, the synergistic mechanisms of biochar and nitrogen fertilizer application for ameliorating such soils require elucidation. Based on this, the present study investigated the effects of combined biochar and nitrogen fertilizer application on nitrogen-fixing microorganisms and mineral nutrients in saline-alkali soil by establishing four treatments: Low concentrations of nitrogen fertilizer (H1), biochar plus low concentrations of nitrogen fertilizer (NH1), high concentrations of nitrogen fertilizer (H2), and biochar plus high concentrations of nitrogen fertilizer (NH2). The results showed that the combined applications of biochar and different concentrations of nitrogen fertilizer had significant effects on the nitrogen-fixing microorganisms in maize rhizosphere. Biochar application increased the abundance and Operational Taxonomic Unit （OTU） number of nitrogen-fixing microorganisms in maize rhizosphere, which was specifically shown as NH2 > H2 > NH1 > H1. The Shannon and Simpson indices indicate that the combined application of biochar and nitrogen fertilizer enhances the diversity of soil nitrogen-fixing microorganisms. Biochar application increased the content levels of available potassium and available phosphorus in maize rhizosphere soil. Increasing the application of nitrogen fertilizer significantly increased the urease, alkaline phosphatase and sucrase activity in the soil. The combined applications of biochar and nitrogen fertilizer increased the yields of the maize and the thousand-seed weight. The maize yields and thousand-seed weight of the NH2 treatment were 29% and 16% higher than that of the H1 treatment, respectively. In conclusion, this study determined that for salinized cultivated soil, combined applications of biochar and nitrogen fertilizer can improve the diversity of soil nitrogen- fixing microorganisms, improve soil micro-environment, and increase maize yield, which are conducive to the improvement and fertilization of saline soil.

Keywords: Biochar, Maize, Nitrogen-fixing microorganisms, Saline-alkali soil, Available nutrients

Introduction

Soil salinization is a global environmental issue that severely reduces nitrogen fertilizer efficacy through multiple pathways due to high salt concentrations (Munns and Gilliham, 2015). Osmotic stress and sodium ion toxicity induced by salinity directly inhibit crop root uptake of nutrients such as ammonium nitrogen (Munns and Tester, 2008). The accompanying high pH environment (>8.5) significantly promotes the volatilization loss of urea and ammonium nitrogen as ammonia (Sommer *et al.*, 2004). Furthermore, saline-alkali stress inhibits the activity of key functional microbial groups, such as nitrifying bacteria and nitrogen-fixing microorganisms, leading to inefficient nitrogen transformation (Zhang *et al.*, 2024). Consequently, conventional nitrogen management in saline-alkali soils is inefficient, urgently requiring integrated strategies that simultaneously alleviate salt stress and enhance nitrogen use efficiency.

Biochar, as a multifunctional soil amendment, offers potential to address these challenges due to its unique properties. Its highly porous structure can adsorb NH^+, reduce leaching, and provide physical refuge for microorganisms (Bolan *et al.*, 2024). The abundant surface functional groups can increase soil cation exchange capacity, enhancing nutrient retention. Additionally, biochar has the potential to regulate soil pH, neutralizing alkalinity and directly suppressing ammonia volatilization (Mandal *et al.*, 2019). By improving soil aggregate structure and aeration, and serving as a slow-release carbon source (Zhu *et al.*, 2025), biochar can create a

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more favorable microenvironment for microbial activity, thereby stimulating nutrient cycling processes, including biological nitrogen fixation.

However, existing research has primarily focused on the macroscopic effects of biochar on crop yield or general soil properties. The systematic mechanisms by which biochar regulates the key biological driver—rhizosphere nitrogen-fixing microorganisms—and synergistically enhances mineral nutrient availability under saline-alkali stress remain unclear. Therefore, this study proposes the central hypothesis that the combined application of biochar and nitrogen fertilizer can improve the rhizosphere microenvironment, thereby promoting the diversity of nitrogen-fixing microbial communities, enhancing soil enzyme activity, and ultimately increasing maize productivity and nutrient use efficiency. Through field experiments, this study aims to elucidate the effects of biochar combined with different nitrogen fertilizer rates on the structure of nitrogen-fixing microbial communities, key soil enzyme activities, and mineral nutrients in coastal saline-alkali soil. The findings are expected to reveal the synergistic mechanisms and provide a theoretical basis for enhancing the fertility of salinized cultivated land.

Materials and Methods

Sample plot selection and sampling method

The experimental site was located in a seed multiplication farm in the Jinghai District of Tianjin. Jinghai District is situated in the southwestern section of Tianjin, with the specific coordinates of 116°42′-117°12′30″E and 38°35′-39°4′45″N. The soil type of the experimental site is fluvo-aquic soil. During the process of land formation, low-lying saline-alkali areas were formed due to natural reasons, such as marine transgression-regression and other human causes. In this study, maize was used as the experimental plant species. Maize was selected as the test crop in this study due to its status as a widely cultivated and climatically adapted staple crop in the Tianjin region. Low concentrations of nitrogen fertilizer (H1), biochar plus low concentrations of nitrogen fertilizer (NH1), high concentrations of nitrogen fertilizer (H2), and biochar plus high concentrations of nitrogen fertilizer (NH2) were applied in this according to the experimental settings. A randomized block design was adopted and each treatment was repeated three times. Each treatment block measured 6 m in length × 5 m in width, with a row spacing of 60 cm, and a density of 7.5×10^4 plants/ha (row spacing of 60 cm × plant spacing of 22.2 cm). Phosphorus fertilizer (P_2O_5) and potassium fertilizer (K_2O) were applied once each in the form of base fertilizers. The phosphorus application rate was 75.0 kg ha^{-1} and potassium application rate was 120 kg ha^{-1}. In this study, 60% of the nitrogen fertilizer was used as seed fertilizer, and 40% of nitrogen fertilizer was utilized as top-dressing at the flare opening stage. The field managements were consistent with the local field planting management processes. All field management operations for a given task were completed on the same day to ensure consistency. Among those, the nitrogen application rates of the H1 and NH1 were 90 kg ha^{-1}; nitrogen application rates of the H2 and NH2 were 135 kg ha^{-1}; and the biochar application rates of the NH1 and NH2 were 3×10^4 kg ha^{-1}. Then, after three months of maize growth, samples of the rhizosphere soil of maize were collected, one portion of which was placed into cold storage in order to determine the diversity of the nitrogen-fixing microorganisms in the samples. The other portion of the samples was taken back to the laboratory for air drying for the purpose of determining the physical and chemical indexes of the soil.

Physical and chemical properties of the experimental soil

The basic physical and chemical properties of the experimental soil were as follows: pH: 8.86; ESP:16%; soil salt content: 0.18%; ammonium nitrogen: 25.4 mg kg^{-1}; and available phosphorus: 10.6 mg kg^{-1}. The biochar used in this study was provided by the Shaanxi Yixin Bioenergy Technology Development Co., Ltd. This biochar was derived from corn straw through pyrolysis at a temperature of 450°C, Biochar produced at this temperature typically exhibits higher porosity and a greater abundance of surface functional groups, with a pH value of 9.01, specific gravity of 0.85 g cm^{-3}, ash content of 12.76%, and carbon content of 79.66%.

Methods for the determination of the soil physical and chemical indices

The soil pH values were determined using a Shanghai Rex multi-parameter water quality analyzer DZS-708 according to a soil/water ratio of 1:5. The total nitrogen content was determined using a Kjeldahl Azotometer. A molybdenum-antimony anti-spectrophotometric method was adopted to determine the total phosphorus content (Ho-Plágaro *et al.,* 2020). The available phosphorus content was determined using amolybdenum blue colorimetry method (Sharpley *et al.*, 2004). In addition, the alkali-hydrolyzable nitrogen content was determined using an alkali hydrolyzed diffusion method (Bao, 2000). In the present study, the activities of soil urease, alkaline phosphatase, sucrase, and protease (corresponding to the four enzymes shown in the figure) were determined using enzyme activity assay kits (Solarbio, Beijing) based on the visible spectrophotometry method, as described by Lian *et al.* (2025). All operations were performed strictly in accordance with the manufacturer's instructions. Specifically, one unit of soil urease activity was defined as the amount of enzyme that produced 1 μg NH_3-N per gram of dry soil per day under the assay conditions, and the enzyme activities for all four enzymes are expressed as units per gram of dry soil (U g^{-1}), consistent with the unit presented in the accompanying figure.

Determination of the soil microbial diversity, DNA extraction, PCR amplification, and Cloning

The collected soil samples were sent to Shanghai Omicsspace Bioteh Co. Ltd. for sequencing analysis using the Illumina MiSeq platform (PE300). A MIO-BIO Power Soil DNA Isolation Kit was used to extract the soil DNA according to the operational steps. The extraction quality and concentration of DNA were detected by 1% agarose gel. In addition, by using the diluted genome DNA as a template, Bi-directional sequencing was conducted according to the Illumina Miseq high-throughput sequencing requirements, in order to design a target area and fusion primers with "5'Miseq joint-barcode-sequencing primer-specific primer-3'". The library was constructed using a two-step PCR amplification method. The upstream primer of nifH was PolF5'-TGCGAYCCSAARGCBGACTC-3'. The downstream primer of nifH was PolR 5'-ATSGCCATCATYTCRCCGGA-3'. The PCR amplification procedure was as follows: pre-degeneration at 94℃ for 5 minutes; degeneration at 94℃ for 30 seconds; annealing at 58℃ for 30 seconds; extension at 72℃ for 60 seconds; 32 cycles, maintaining at 72℃ for 7 minutes after the end of each cycle; and then ending at 4℃ with data collected. The mixture of three PCR reaction products of the same sample was detected by 2% agarose gel electrophoresis. An AxyPrep DNA gel extraction kit (AXYGEN) was used for the gel extraction; Tris HCl was used for the elution; 2% agarose was utilized for the electrophoresis detection; QuantiFluor™-ST blue fluorescence quantitative system (Promega) was adopted for the quantitative detection process; and finally, corresponding proportions of mixing were conducted according to the sequencing requirements of each sample. The mixed samples were sequenced by Miseq, and the sequencing data were then uploaded to the NCBI SRA database (SRA accession: PRJNA542599).

Bioinformatic analysis

This study's bioinformatic analyses were completed by Shanghai Omicsspace Biotech Co. Ltd. After the original data became available from the computer, first a data quality control process was carried out. The optimized sequences were obtained after sequence splicing, filtering, and the removal of mosaics. After that, OTU clustering and annotation were carried out, among which the OTU classifications were based on a 97% similarity level of the nucleotide sequences.

Statistical method

In this study, SPSS 17 statistical software was used to analyze experimental data. Multiple comparisons were performed using Duncan's multiple range test. The designed significance level was set as 5%.

Results and Analysis

A Venn diagram can be used to clearly and intuitively show the similarities and differences in the number of species within different groups. In the present study, the addition of biochar was found to have significant effects on the nitrogen-fixing microorganisms in maize rhizosphere under different nitrogen application levels (Figure 1). A total of 156 OTUs were shared among the four treatments, with the number of OTU in the four treatments shown as follows: NH2 (394) > H2 (375) > NH1 (345) > H1 (221). The number of OTU in the nitrogen-fixing microorganisms in the soil rhizosphere had increased with the increases in the nitrogen application rates. It was observed that on average, the numbers of OTU in the NH2 and H2 treatments were 100 higher than those observed in the NH1 and H1 treatments. The addition of biochar was confirmed to have increased the number of nitrogen-fixing microorganisms in the soil. This was particularly evident in the cases of low nitrogen applications. The effects of the biochar additions on the nitrogen-fixing microorganisms in the soil were particularly significant, among which the NH1 was 56.1% higher than the H1.

In this study, the sample sequencing depth index coverage reached more than 99.94%. The higher the coverage of the sample library was, the higher the probability of the sample sequences being detected would be, thereby more accurately representing the real situations of the microorganisms in the samples. As can be seen in Table 1, the Ace and Chao1 values indicated that the biochar had significant effects on the abundance of nitrogen-fixing microorganisms in the maize rhizosphere at different nitrogen application levels ($p < 0.05$), which was presented as NH2 > H2 > NH1 > H1. The Shannon and Simpson analysis results of the samples indicated that higher nitrogen applications and biochar synergism could significantly increase the diversity of the nitrogen-fixing microorganisms ($p < 0.05$).

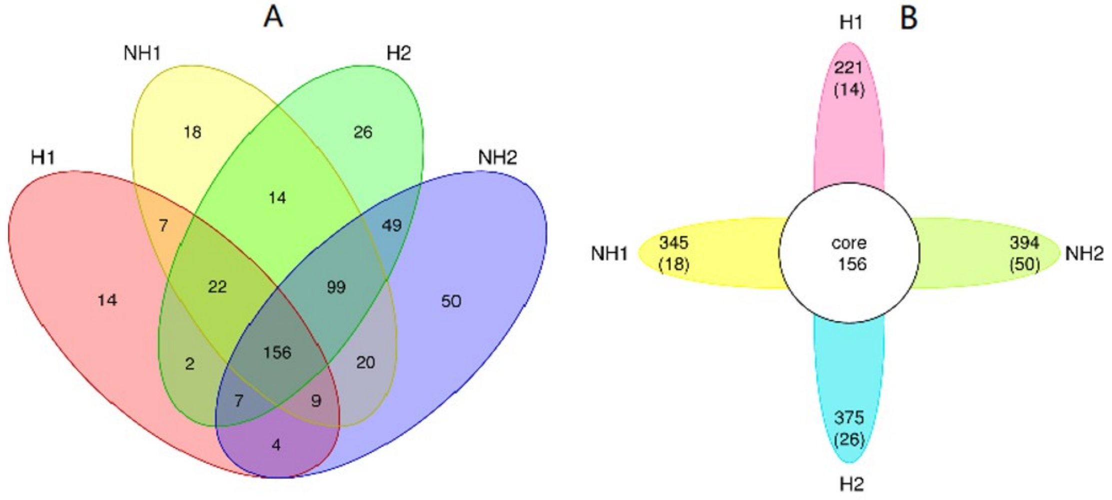

Figure 1: Visual analysis of soil microbial community distribution under different treatments

Table 1: Alpha diversity of nitrogen-fixing microorganisms in different treatments

Treatments	Coverage	Sobs	Chao	Ace	Shannon	Simpson
H1	0.999674	221±5.86d	236.94±17.64d	230.39±9.23d	3.643±0.019d	0.086±0.0022a
H2	0.999927	375±9.45b	369.54±9.63b	377.11±2.92b	4.771±0.013b	0.017±0.0003c
NH1	0.999768	345±8.33c	355.79±12.3c	351.26±6.39c	4.481±0.014c	0.027±0.0005b
NH2	0.999482	394±10.21a	410.92±15.54a	408.08±10.91a	4.909±0.012a	0.013±0.0002d

Note: In the table, different lowercase letters indicate significant differences among treatments at $p < 0.05$. The same convention applies to the figures/tables below

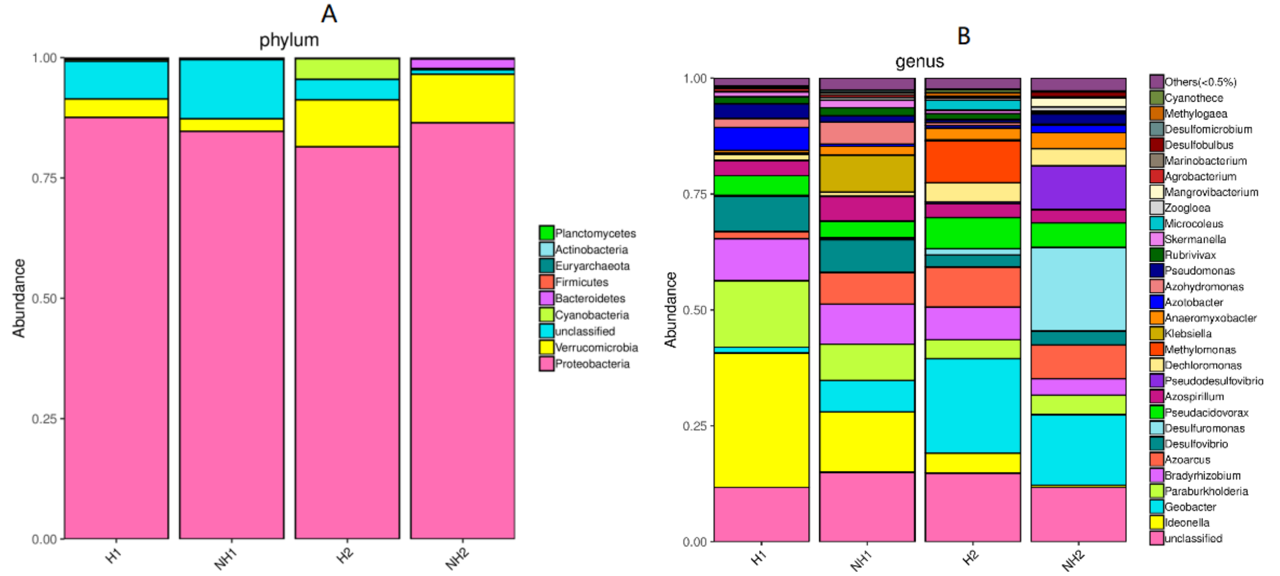

Figure 2 : Effects of biochar-nitrogen fertilizer co-application on the community composition of soil nitrogen-fixing microorganisms. (A) Composition at the phylum level. (B) Composition of dominant genera (relative abundance > 1%)

The OTUs representative sequences of twelve samples from four treatments were compared and identified in this experimental investigation, and the information of species of soil nitrogen-fixing microorganisms in thirty genera and nine phyla were obtained（Figure 2）. At the phylum level, the nitrogen-fixing microorganisms with relative abundance less than 0.5%, and no annotated results at that level, were classified as "Others". Among the four types of treatments, it was found that the nitrogen-fixing microorganisms mainly belonged to eight groups. The two phyla with higher relative abundance were Proteobacteria (with an abundance range of between 81.5% and 87.6%) and Verrucomicrobia (with an abundance range of 2.6% to 10.1%). Meanwhile, it was found that Bacteroidetes was unique to the NH2 treatment, with an abundance maintained at 2%. At the genus level, the nitrogen-fixing microorganisms with relative abundance less than 0.5%, and no annotated results at that level, were classified as " Others ". At the genus level, it was found that Ideonella, Geobacter, Paraburkholderia, Bradyrhizobium, Azoarcus, and Desulfovibrio were the most abundant genera. In total, 29 genera of NH2 and H2; 27 genera of NH1; and 26 genera of H1 were identified. In addition, Geobacter was determined to be the dominant genus of NH2 and H2, and Ideonella was the dominant genus of NH1 and H1. The results obtained in this study revealed that the addition of biochar and the application of different amounts of nitrogen fertilizer had affected the abundance of nitrogen-fixing microorganisms at the phylum and genus levels.

The combined application of biochar and nitrogen fertilizer had little effect on alkali-hydrolyzable nitrogen content in saline soils, with no significant differences observed among treatments (Figure 3). However, this

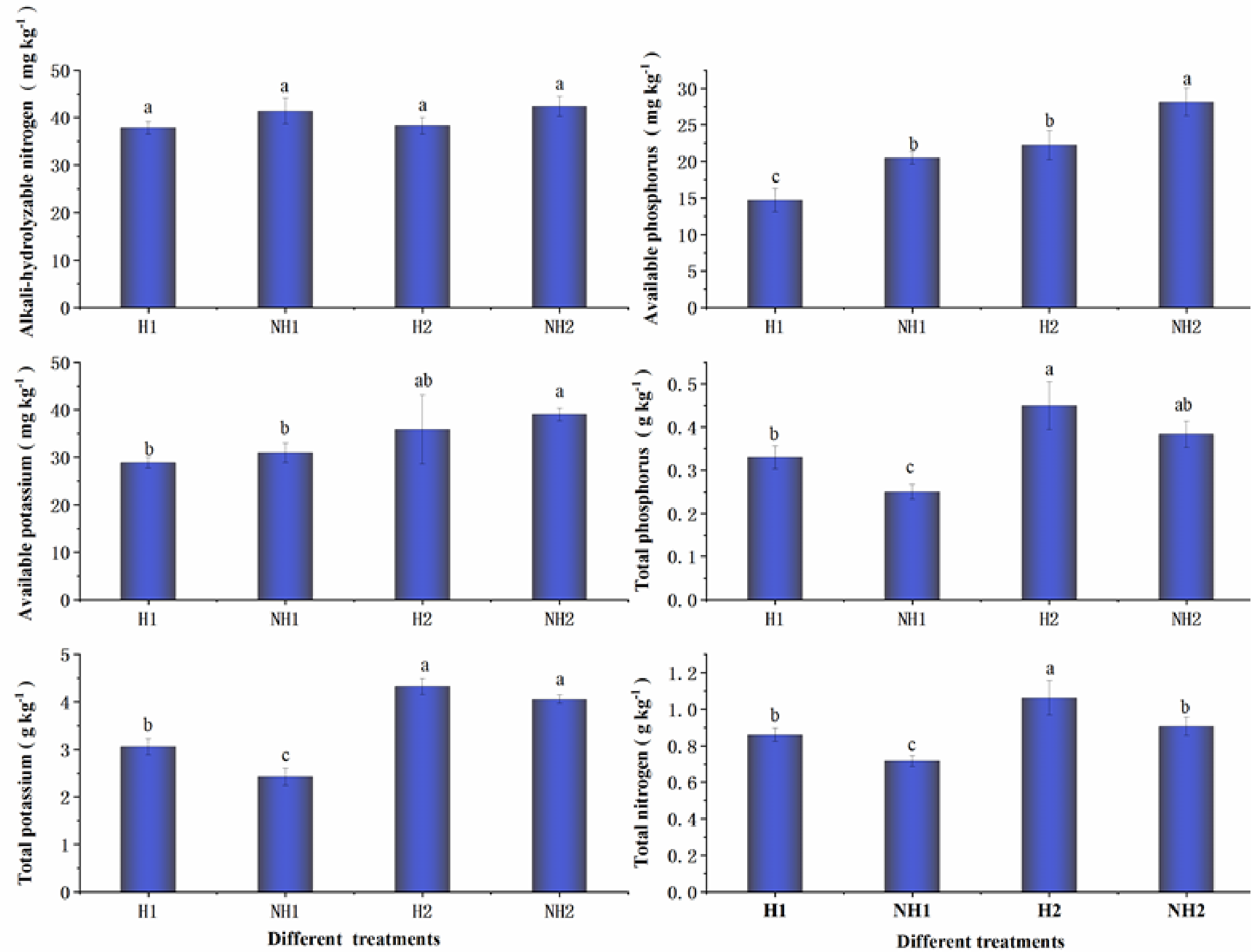

Figure 3: The impact of biochar-nitrogen fertilizer co-application on mineral nutrients in saline-alkaline soils

combined application significantly increased the contents of available potassium and available phosphorus in the soil. Under the same nitrogen application rate, the addition of biochar enhanced the levels of available potassium and phosphorus in saline soils. In high-nitrogen treatments, the contents of available potassium and phosphorus were higher than those in low-nitrogen treatments, specifically following the order: NH2 > H2 > NH1 > H1.

In saline soils, increasing the application of nitrogen fertilizer significantly enhances the total nitrogen, total phosphorus, and total potassium content in the soil (Figure 3). Specifically, the average values of total nitrogen, total phosphorus, and total potassium in NH2 and H2 are 24.9%, 43.7%, and 13.2% higher than those in NH1 and H1, respectively. The addition of biochar results in lower total nitrogen, total phosphorus, and total potassium contents compared to treatments without biochar, with the specific order being H1 > NH1 and H2 > NH2. Notably, there are significant differences in total nitrogen, total phosphorus, and total potassium content between the H1 and NH1 treatments, while no significant differences are observed in total phosphorus and total potassium content between the H2 and NH2 treatments.

Under the same nitrogen fertilizer application, the addition of biochar significantly increased the contents of urease, alkaline phosphatase, and sucrase in saline soils (Figure 4). The protease activity in the NH2 treatment was significantly higher than that in the H2 treatment, while no significant difference in protease activity was observed between the H1 and NH1 treatments. The nitrogen fertilizer application rate had a considerable impact on alkaline phosphatase and protease activity, with higher nitrogen

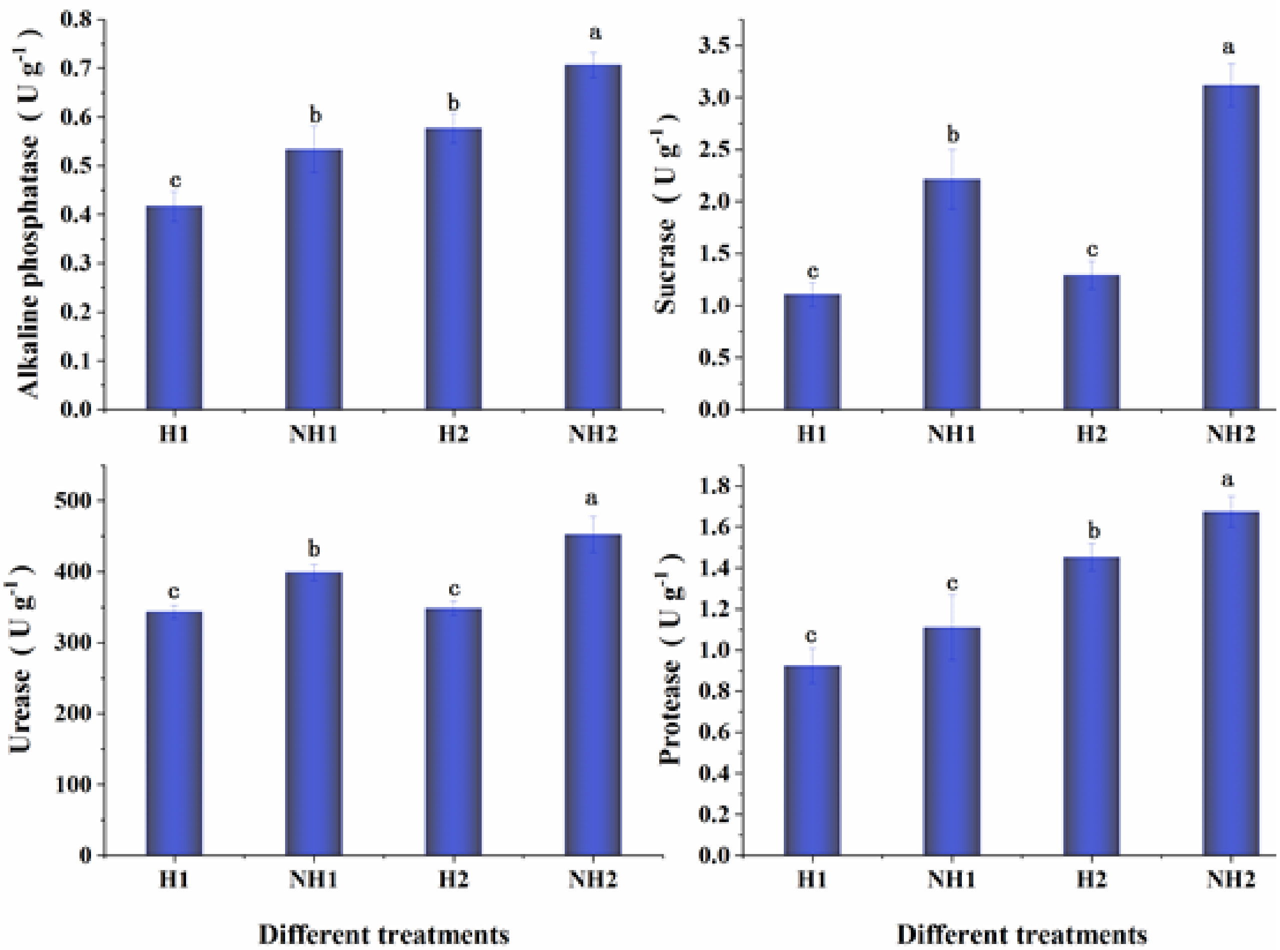

Figure 4: The impact of biochar-nitrogen fertilizer co-application on enzyme activity in saline-alkaline soils

treatments showing greater activity than lower nitrogen treatments. The combined application of biochar and nitrogen fertilizer significantly influenced soil enzyme activities. Specifically, the activities of sucrase and urease followed the order: NH2 > NH1 > H2 > H1, while the activities of alkaline phosphatase and protease followed the order: NH2 > H2 > NH1 > H1.

Discussion

Nitrogen-fixing microorganisms are widely present in agricultural soils and are influenced by a complex interplay of environmental factors. The form of nitrogen fertilizer (e.g., Urea), fertilization practices (e.g., split application), and the addition of exogenous substances can all exert significant effects on the community structure of soil diazotrophs (Li *et al.*, 2019; Wei *et al.*, 2025). This study revealed that the combined application of biochar and nitrogen fertilizer significantly increased the OTU number, Chao1 index, and Shannon index of diazotrophic microorganisms, with the most pronounced effect observed in the NH2 treatment. This phenomenon may be attributed to unique microbial response strategies within saline-alkali soil ecosystems. Conventional theory posits that readily available nitrogen (e.g., ammonium, nitrate) inhibits nitrogenase activity. However, our findings in this saline-alkali stressed environment showed that a high nitrogen treatment combined with biochar amendment enhanced diversity of nitrogen-fixing microorganisms. Under saline-alkali stress, microorganisms face primary challenges from osmotic stress and ion toxicity (Li *et al.*, 2025), where nitrogen may first serve as a limiting resource for survival and stress resistance (Martínez-Espinosa, 2020). The input of exogenous nitrogen might be prioritized to alleviate fundamental metabolic stress. Concurrently, biochar addition provides physical refuge via its porous structure and serves as a slow-release carbon source, modulating the soil C/N ratio. Together, these factors support the construction of a more complex microbial community (Xu *et al.*, 2021). Consequently, the increased diversity of the nifH gene in high nitrogen treatments (H2 and NH2) likely reflects a strategy where microorganisms in this nitrogen-limited saline-alkali environment utilize acquired nitrogen resources to mitigate environmental stress, thereby supporting community development. This aligns with the conclusions of Li *et al.* (2023) regarding biochar-mediated improvement of coastal saline-alkali soils, where biochar effectively altered the community structure of nifH harboring bacteria and stimulated their growth. Further analysis of community composition revealed Proteobacteria and Verrucomicrobia as the dominant phyla. Notably, the relative abundance of Bacteroidetes significantly increased in the NH2 treatment, suggesting that biochar may selectively enrich microbial groups with specific functions, such as organic matter degradation (Deshoux *et al.*, 2023; Hu *et al.*, 2024).

The positive response of the community of nitrogen-fixing microorganisms forms a crucial biological foundation for the synergistic effects observed between biochar and nitrogen fertilizer. This synergy primarily manifests in two aspects: improvement of physical structure and regulation of chemical properties (Zhang *et al.*, 2024). Due to its porous structure and large specific surface area, biochar provides suitable habitats for soil microorganisms while adsorbing nitrogen nutrients, reducing leaching losses and thereby prolonging fertilizer efficacy. In this study, the combined application significantly regulated the soil nutrient pool, particularly enhancing the content of available potassium and available phosphorus. This demonstrates the dual mechanism of biochar as an efficient “nutrient buffer/reservoir”: for available potassium, retention relies mainly on the high cation exchange capacity of biochar; for available phosphorus, biochar likely influences phosphorus fixation and release processes- thereby increasing its availability—through mechanisms such as pH modulation and provision of adsorption sites (Hagemann *et al.*, 2017). In saline-alkali soils, nitrogen fertilizer supplies essential nitrogen for crop growth, while biochar amendment, via its extensive surface area and functional groups, optimizes the adsorption-desorption balance of nutrients, leading to a more gradual and sustained release. This synergistic interaction peaked in the NH2 treatment, resulting in the highest nutrient availability and maize yield. Furthermore, the stimulation of soil enzyme activity by biochar intensified nutrient transformation processes. Soil enzyme systems are highly sensitive to environmental changes, and their activity is closely linked to soil physicochemical properties and biological activity (Iqbal *et al.*, 2025). This study observed significant increases in urease, alkaline phosphatase, and sucrase activities, with a particularly notable surge in sucrase activity following biochar addition. This indicates that enzymes associated with the carbon cycle are more responsive to biochar, potentially due to the direct or indirect provision of readily utilizable carbon sources. The enhanced enzyme activity directly promotes the decomposition of soil organic matter and nutrient transformation (Wang *et al.*, 2022), supplying more abundant available nutrients for maize and microorganisms, which ultimately led to higher maize yields (Figure 5).

In summary, biochar not only directly ameliorates soil physicochemical properties but also shapes a more diverse and functionally specific microbial community by modulating the microenvironment and C/N ratio. This community, in turn, further drives nutrient cycling by

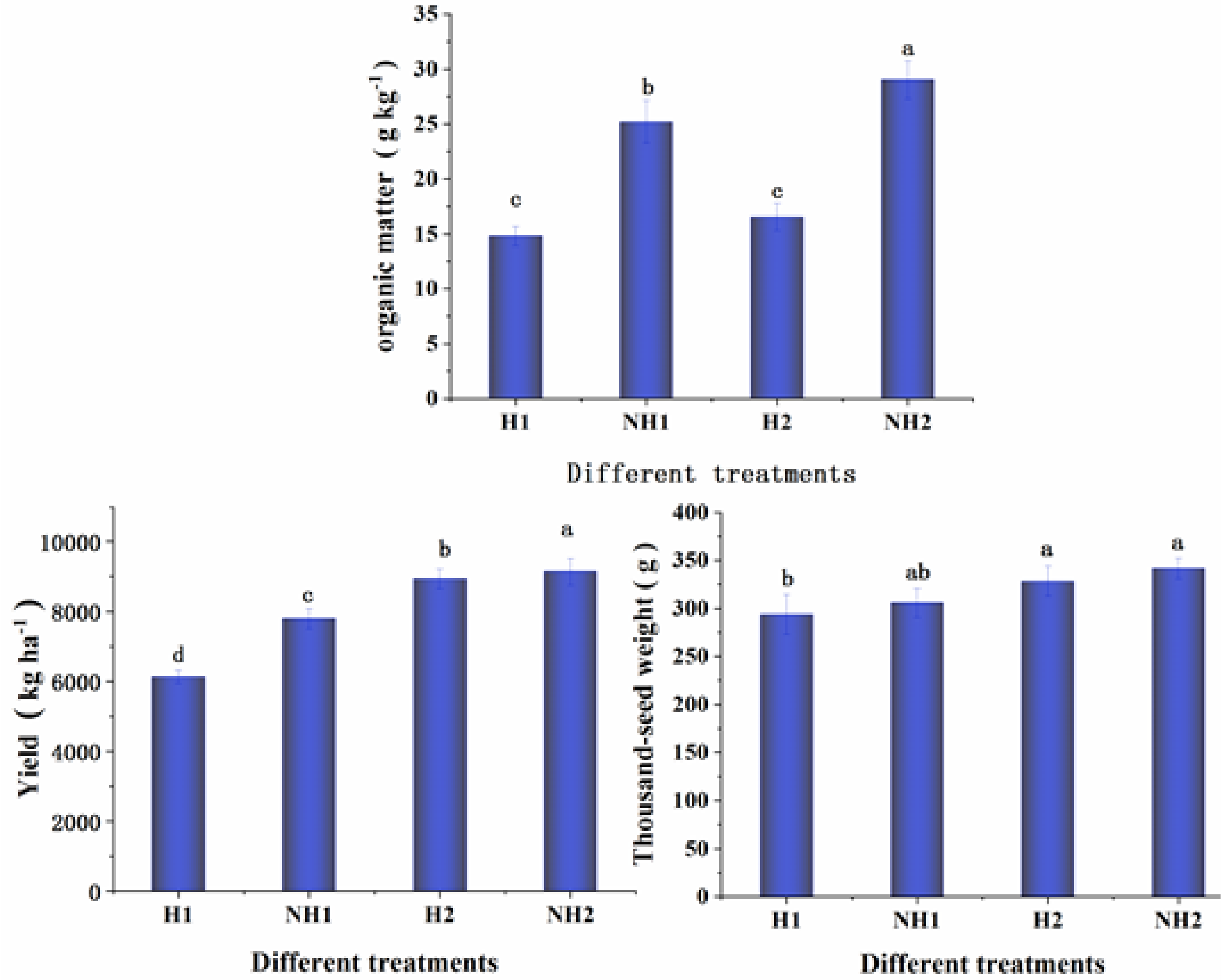

Figure 5: The impact of biochar-nitrogen fertilizer co-application on organic matter and maize yield in saline-alkaline soils

enhancing nitrogen fixation potential and soil enzyme activity. This tripartite interactive model of "biochar - nitrogen fertilizer - microorganisms" offers a novel green and efficient strategy for saline-alkali agriculture. From a practical perspective, our results suggest that a combined application strategy of nitrogen fertilizer and biochar in saline-alkali lands, particularly at appropriate or higher nitrogen levels, can comprehensively improve soil fertility, microbial activity, and maize yield. However, to achieve sustainable agricultural development, future research should further explore the feasibility of combining biochar with reduced nitrogen input, aiming to maintain yield while enhancing resource use efficiency. It is important to note that the effects of biochar application are contingent upon its properties (feedstock, pyrolysis temperature) and soil type, and its long-term environmental impacts (e.g., potential effects on greenhouse gas emissions) require continuous monitoring. Future studies could focus on the functional matching mechanisms between biochar characteristics and the microbiome, and validate long-term ecological effects at the field scale to optimize the technical framework for saline-alkali soil remediation.

Conclusions

This study demonstrates that the co-application of biochar and nitrogen fertilizer exerts multiple ameliorative effects on saline-alkali soil. It significantly enhances the diversity of nitrogen-fixing microorganisms, effectively improves soil nutrient status, markedly increases soil enzyme activities, and ultimately boosts maize yield. Thus, the combined application of biochar and chemical fertilizer can serve as an effective strategy for the improvement and fertilization of saline-alkali land, leading to enhanced land productivity in such areas.

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Note: Shaopeng Li and Jin Du contributed equally to this work and should be considered co-first authors.

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