



Novel environmental factors affecting microbial responses and physicochemical properties by sequentially applied biochar in black soil

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Abstract

Biochar was increasingly used in agriculture soil amendment and has received widespread attention due to its potential to improve soil micro-ecological environment and crop growth. The raw material of the biochar used in this study is peanut shell, which is mixed with other organics and minerals to become a mineral-enhanced biochar under heating conditions (220 °C). When the third season crop is finished, we evaluated black soil physicochemical properties, microbial communities, and crop growth in long-term agricultural trials. Four treatments were set up: no amendment (control CK), nitrogen fertilizer only (70 kg ha⁻¹ N), enhanced biochar only (5 t ha⁻¹ B), and nitrogen fertilizer (70 kg ha⁻¹) + enhanced biochar (5 t ha⁻¹) (NB). The enhanced biochar promotes crop growth and increased the richness of the bacterial community, while reducing the richness of the fungal community. Nitrogen fertilizer + enhanced biochar increased soil microbial biomass carbon, nitrate nitrogen, and ammonium nitrogen by 43.75, 7.25, and 19.28%. In addition, we found changes in bacterial community were closely related to soil organic carbon, while changes in fungal community structure were closely related to soil carbon to nitrogen ratio. And the soil organic carbon and soil carbon to nitrogen ratio of biochar treatment were increased by 5.64 and 6.25% compared with fertilizer treatment, respectively. We concluded that enhanced biochar improved the soil more effectively and made the soil more conducive to crop growth. Regulating the microbial community by improving the physicochemical properties of soil was an important way to improve the stability and condition of the soil system with biochar. An enhanced biochar was of great significance for circular development of agriculture and soil improvement in Northeast China.

Keywords Biochar · Soil micro-ecological environment · Physicochemical properties · Microbial communities · Tobacco

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Introduction

Black soil is the result of the evolution of surface vegetation after long-term corrosion to form humus. Black soil formed in cold climates has high organic matter content, fertile soil, and loose soil, which is most suitable for farming. Only the Mississippi River Basin in the United States, the Great Plains of Ukraine, and Northeast China have cold black soil. Northeast China is one of only three belts of black soil in the world (Wang et al. 2002). Most of the black soil has been cultivated for decades or even hundreds of years. China's famous commodity grain base is grown on the black soils of the Songnen Plain. With an increase in the black soil opening period and unreasonable management methods, soil fertility levels have decreased rapidly and the black soil layer is becoming thinner, to the extent that the Loess parent material is exposed in a few areas. The black soil has also shown a

decrease in the carbon/nitrogen ratio and a decline in microbial populations. These soil degradation trends have seriously affected the productive capacity of black soil (Han et al. 2010). In the 100 years to date, the soil organic carbon content of the 0–20-cm black soil layer in China has decreased by 66.64% and total nitrogen has decreased by 64.99% (Atkinson et al. 2010); in the 20–40-cm layer, organic carbon has decreased by 44.92% and total nitrogen has decreased by 33.33% (Atkinson et al. 2010). In order to curb the degradation of black soil and improve soil fertility, sustainable development of agriculture in the black soil area is key.

Biochar is a kind of highly aromatic carbonization of biomass under complete or partial anoxic conditions (Atkinson et al. 2010). In the past 10 years, biochar in agricultural production was receiving an increasing attention due to the need for more sustainable and better soil management materials and methods. The physicochemical properties of biochar itself can increase soil fertility while reducing the harmful effects of fertilizers, such as reduced organic carbon and acidification in soil (Sohi et al. 2010). The chemical properties of biochar are very stable (Fang et al. 2015), depending on biochar pyrolysis temperature, soil type, and type of raw material (Kloss et al. 2012; Van et al. 2010; Grunwald et al. 2017). This trend stems from lower soil respiration and interdecadal stability of rhizosphere sediments (Weng et al. 2015; Weng et al. 2017). In fact, a meta-analysis has shown that biochar increased the soil microbial biomass, resulting in reduced metabolic quotient (qCO_2) and reduced respiration (Zhou et al. 2017).

The latest approach to biochar applications is to blend traditional fertilizers with biochar to improve the efficiency of nutrient use, reducing agricultural investment. The acquisition of agricultural waste is simple and the cost is low. The role that biochar can play here is (1) to improve soil physicochemical properties (Van et al. 2015; Macdonald et al. 2014) (e.g., by addressing limiting factors such as soil pH), thereby increasing crop yield and increasing the amount of fertilizer applied (Zheng et al. 2017); (2) to contribute to the maintenance of fertilizer efficiency in the soil, such as cation exchange capacity; and (3) to through the physicochemical properties on the fertilizer itself improve fertilizer utilization efficiency (Singh et al. 2014; Yao et al. 2015). This biochar was enhanced by chemical mixing, which provides a way to improve the effect of biochar (Mandal et al. 2016; Joseph et al. 2013). More nutritious enhanced biochar was made by increasing nutrients (such as cow dung, sesame cake) and minerals (such as medical stones) to biochar (pyrolyzed) and then baking the mixture to produce complex organic mineral phases on the submicroscale (Ye et al. 2017; Joseph et al. 2013). The enhanced biochar can increase the soil redox activity and thereby increase the activity of beneficial microorganisms in the soil (Ye et al. 2017; Sun et al. 2017). In fact, the small use of enhanced biochar (1.35 t ha^{-1}) has been studied to have the same effect as the chemical fertilizers on crop growth and

development while altering the community structure of soil microorganisms (Nielsen et al. 2014).

Black soil degradation is not only reflected in the degradation of the characteristics of vegetation communities, but also in the degradation of the structure and function of soil microbial communities (Kaur et al. 2009). Fertilization practices could directly affect the soil microorganisms (Traoré et al. 2016) and had direct impacts on agricultural soil through nutrient cycling and conversion and interactions with crop. There are complex processes, such as nitrification and denitrifying bacteria, which control the balance of nitrate and ammonium in the soil through their own metabolism as well as some rhizosphere bacteria that promote root growth; they provide nutrients to the root system and promote the absorption of nutrients by the root system as well as crop growth promoting factors provided by rhizobacteria (Geisseler et al., 2014); these factors together affect crop growth performance. Most crop-microbe interactions occur near the root system; microbial products and exudates are transferred on the root system, which results in a unique microbial community compared with soil parts lacking the root system (Van et al. 2010; Baudoin et al. 2003;). This experiment, as an ongoing agricultural field trial, studied the performance of mineral-enhanced biochar in tobacco production; we examined the microbial communities and physicochemical properties within black soil. More specifically, we explored the use of combined biochar and nitrogen fertilizers, as well as the effects of enhanced biochar or nitrogen fertilizers alone, on soil productivity and plant growth. We use 3 years of continuous use of enhanced biochar or nitrogen fertilizer to cultivate soil and tobacco, and at the end of the third season, we measure the pH, soil bulk density, microbial biomass C (C_{mic}) and N (N_{mic}), nitrate nitrogen and ammonium nitrogen, and other physical and chemical indicators and tobacco growth status. At the same time, we measured the soil microbial diversity using 16S and 18S technologies and analyzed the correlation between microbial diversity and soil physicochemical properties. And several soil physicochemical indicators that have a greater impact on soil microbial diversity were identified. This study uses conventional experimental methods to explore ways to improve the quality of black soil in Northeast China and at the same time provides a theoretical reference for agricultural production. But the long-term effects of enhanced biochar on agricultural soils need to be further studied.

Materials and methods

Experimental materials

The basic physicochemical properties of the black soil are as follows: pH 6.52, total nitrogen 0.74 g kg^{-1} , available phosphorus 35.27 mg kg^{-1} , soil organic matter 9.54 g kg^{-1} ,

alkaline nitrogen 88.64 mg kg^{-1} , and available potassium $315.74 \text{ mg kg}^{-1}$. The experimental field consisted of 12 plots ($10.75 \times 7.35 \text{ m}$), which were arranged in the random complete block design (repetitions = 3). The enhanced biochar used in this research was hot cracked as the method used by Nielsen et al. (2014). Biochar of peanut shell was mixed with medical stone (Medical stone is a natural silicate mineral that is nontoxic. And it is also a compound mineral or medicinal rock that is nontoxic, harmless, and biologically active. The main chemical component of medical stone is inorganic aluminosilicate.), sesame cake, phosphate rock, and cow dung and baked at $220 \text{ }^\circ\text{C}$ for 3 h. The complete chemical characterization of the enhanced biochar used in this research is given in Table 1, based on the following method.

The agricultural land was added with enhanced biochar (5 t ha^{-1}) or traditional nitrogen fertilizer (70 kg ha^{-1}) and cross-designed, with uncorrected controls every year. This experimental design allowed us to study the interaction with current fertilization regimes. At the start of the tobacco cycle,

5 t ha^{-1} of enhanced biochar and 70 kg ha^{-1} of traditional nitrogen fertilizer (as urea) were used to lots of plots, resulting in four fertilization systems: (a) no amendment (CK), (b) nitrogen fertilizer only (N), (c) enhanced biochar only (B), and (d) nitrogen fertilizer and enhanced biochar (NB). The experimental farmland was recycled to apply the material into the 0–15-cm soil layer. The above operations are repeated annually before the start of the crop cycle. Samples were collected at the end of the third year of crop growth cycle. Previous research have shown the effect of enhanced biochar on agriculture soil improvement over time; therefore, this experiment began to collect data at the end of the third crop growth cycle.

Prior to tobacco harvest (September 14, 2017), 5 soil samples were selected from every experimental farmland for microbial diversity analysis and five soil samples were mixed to get composite samples for physicochemical analysis; five replicates were randomly taken from the mixed soil samples to determine microbial diversity. For soil samples, homogenize and collect about 0.5 kg of soil in a 0–15-cm soil layer. For root samples, the tobacco plants were carefully picked up from the soil layer, and the loose soil was taken out from tobacco roots (Barillot et al. 2013). The rhizosphere soil sample was put in sealed bags and placed in the liquid nitrogen before analyzing of microbial community. The sample of tobacco plants was heated at $110 \text{ }^\circ\text{C}$ for 20 min and heated at $65 \text{ }^\circ\text{C}$ until completely dried, and then weighed. Three middle leaves were selected from each tobacco plant sample (the bottom one was the first leaf; the eighth, the ninth, and the tenth leaves were used as samples), and their maximum length and width were measured with a tape measure. The leaf area calculation formula is $d = (L + 2W)/3$, where d , L , and W represent the leaf area, the maximum length of the blade, and the maximum width of the blade, and the average of the three leaf area is calculated. The root tip was measured by a root structure measuring instrument (produced by Shanghai Zequan Technology Co., Ltd., model: CI-600); after the tobacco root system was completely taken out, it was washed with water and scanned on the instrument, and the images were analyzed. Then the number of tobacco root tips was calculated by these images.

Table 1 The complete physicochemical properties of the enhanced biochar

Variable	Limit of detection	Enhanced biochar
Specific surface area (m^2/g)	0.7	16.72
pH	0.05	8.6
EC (Ds/m)	0.02	2.3
Total Nitrogen (mg kg^{-1})	0.2	7.9
Total Carbon (mg kg^{-1})	4	583.4
Ammonium-N (mg kg^{-1})	0.2	587
Nitrate-N (mg kg^{-1})	0.2	0.38
Aluminum (g kg^{-1})	0.004	1.7
Arsenic (mg kg^{-1})	4	<4
Boron (mg kg^{-1})	5	13
Calcium (g kg^{-1})	0.002	56
Cadmium (mg kg^{-1})	0.3	<0.15
Cobalt (mg kg^{-1})	0.5	4.3
Chromium (mg kg^{-1})	0.2	7.9
Copper (mg kg^{-1})	0.3	26
Iron (g kg^{-1})	0.0004	5
Potassium (g kg^{-1})	0.005	4.1
Magnesium (g kg^{-1})	0.0007	12.2
Manganese (mg kg^{-1})	0.2	1893
Molybdenum (mg kg^{-1})	0.4	1.8
Sodium (g kg^{-1})	0.006	1.6
Nickel (mg kg^{-1})	0.5	5.8
Phosphorus (g kg^{-1})	0.0002	2.3
Lead (mg kg^{-1})	2	3.7
Sulfur (g kg^{-1})	0.0007	0.39
Selenium (mg kg^{-1})	3	<3
Zinc (mg kg^{-1})	0.6	117

Physicochemical analysis of biochar and soil

The nitrate, ammonium, and conductivity (EC), Bray and pH, Colwell phosphorus, total nitrogen and carbon of soil samples, and broken biochar were measured by the way of Van Zwielen (Grunwald et al. 2017). In short, using Elementar vario MAX CN (the instrument from Shanghai Haichao Optoelectronics Technology Co., Ltd. in Shanghai, China, and made in Germany), an analyzer with a heating chamber temperature set at $800 \text{ }^\circ\text{C}$, total carbon and nitrogen are determined by Dumas combustion. The oxygen and C flow rates were 125 mL min^{-1} . The pH was determined in 0.01 M CaCl_2 (1:5) according to the Method 4B2 (Rayment et al. 1992).

Acid-extractable elements and metals were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Varian 720-EC ICP-OES according to USEPA 6010. The soot value of enhanced biochar was appointed as the carbonate equivalent by the Method 19A1 (Rayment et al. 1992). The bulk density of soil was determined by the ring knife method, using the formula:

$$\text{soil porosity (\%)} = (1 - \text{volume density/specific gravity}) \times 100,$$

and the calculation formula of porosity was calculated as:

$$\text{soil porosity (\%)} = (1 - \text{bulk density/specific gravity}) \times 100$$

Soil microbial biomass C (C_{mic}) and N (C_{mic}) were obtained using the flow analyzer method and chloroform fumigation direct extraction method and calculated (Chen et al. 2013) as:

$$\text{soil microbial biomass C (} C_{\text{mic}} \text{)} = (\text{fumigated soil organic carbon} - \text{unfumigated soil organic carbon})/0.45;$$

$$\text{soil microbial biomass N (} C_{\text{mic}} \text{)} = (\text{fumigated soil microbial biomass nitrogen} - \text{unfumigated soil microbial biomass nitrogen})/0.25 \text{ (Zhou et al. 2017).}$$

The soil moisture content and temperature were measured using a tripartite meter (conductivity, temperature, water content) (model WET-3).

Microbial community analysis with 16S rRNA gene sequencing

The gene of the microbial community of the soil surrounding the root was subjected to DNA extraction, sequencing, and PCR. The soil around the roots represents the rhizosphere soil microbial community (i.e., in the soil that adheres tightly to the roots) (Schloss et al. 2009). The obtained gene sequences were subjected to mass screening and clustering into operational classification units (OTUs) with the following changes. After clustering OTUs with 97% similarity, abundance filters were used to remove OTUs with a total abundance < 60. In addition, the OTU counts within the subsample ($n = 5$) were averaged over the graph, yielding the samples and rounding to integers. This not only reduces the complexity of the model but also simplifies data analysis (Bolker et al. 2009).

The SPSS 20.0 software was used to analyze the physicochemical properties of the soil samples, and correlation analysis and one-way analysis of variance (ANOVA) were performed ($P < 0.05$). The principal component analysis (PCA) was used to analyze changes in microbial community composition. The distribution of community composition among multiple samples was observed using the ratio of community composition (bar) method. The comparisons of microbial community composition and the evaluation of differences among microbial communities were carried out using sample stratification analysis, and redundant analysis (RDA) was used to assess the impact of environmental factors on the differences in bacterial community between samples. Visualization of the PCA, RDA, bar, and hierarchy analysis

was performed using the “vegan” package of R software (version 3.3.1).

Results and analysis

Variation in soil physicochemical properties among treatments

The pH of soil treated with enhanced biochar was higher than that of soil without it; soil pH treated with enhanced biochar only (B) was the highest (Fig. 1A). and the pH of soil treated with nitrogen fertilizer only (N) was lower than that of soil with no amendment (CK) (Fig. 1A). Enhanced biochar reduced soil bulk density, while N had the opposite effect (Fig. 1B and C). Soil bulk density and porosity were significantly negatively correlated (related coefficient -97.64) (Table 2), which is consistent with the soil bulk density and porosity differences observed for the four different fertilizer regimes (Fig. 1B and C). Enhanced biochar also affected soil temperature and water content, with an obvious positive effect on soil water content (Fig. 1D and E).

Enhanced biochar also affected soil nitrate nitrogen, ammonium nitrogen, and microbial biomass carbon and nitrogen. Among the treatments, nitrogen fertilizer and enhanced biochar (NB) had the most obvious effects on the above indicators (Fig. 1F, G, J, and K). Interestingly, although the soil pH showed a significant negative correlation with ammonium nitrogen (correlation coefficient -69.54) (Table 2), the soil pH reached a maximum in the treatment with B, while the maximum value for ammonium nitrogen was observed in soil treated with NB (Fig. 1G). There was a significant positive correlation between soil nitrate nitrogen and soil microbial biomass C (C_{mic}) and soil microbial biomass N (N_{mic}), and the correlation coefficient with N_{mic} reached 91.32 (Table 2), which was also related to the four different fertilizers. Enhanced biochar effectively increased the total carbon content of the soil (Fig. 1I). Although soil total carbon showed a significant positive correlation with total nitrogen (correlation coefficient of 89.64), the above two indicators showed inconsistent patterns in the four different fertilizer regimes. Soil C/N was lower in treated soils, and the application with nitrogen fertilizer alone was lower, but not significantly different from the control (Fig. 1).

Variation in soil microbial communities among treatments

The relative abundances of fungi and bacteria differed among the four different fertilizer regimes (Fig. 2A). For example, the Proteobacteria were more abundant in N (nitrogen fertilizer only) treatment soils, while in CK (no amendment) soil, the abundance of Proteobacteria was relatively low. In NB

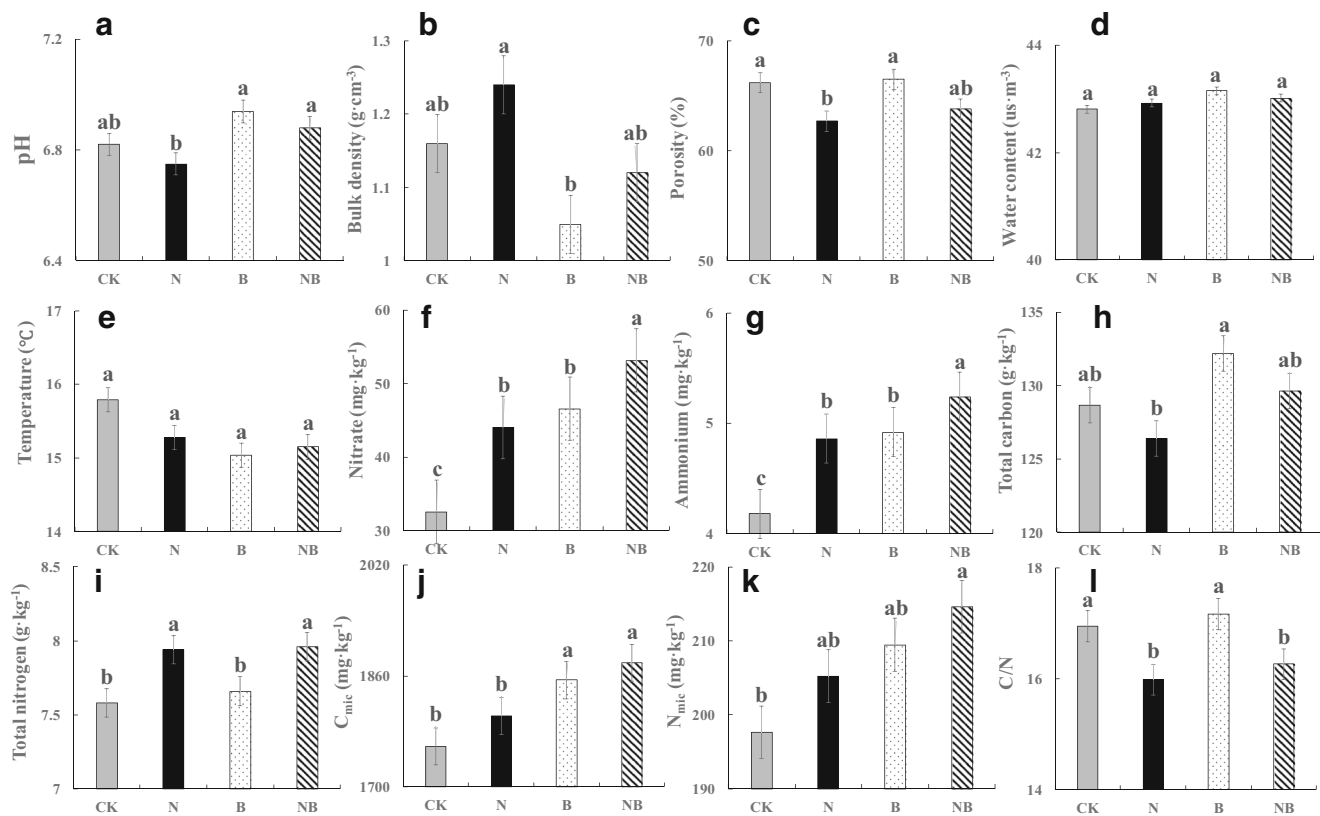


Fig. 1 Effect of enhanced biochar and nitrogen fertilizer on the physicochemical properties of black soil. The bars are standard deviation ($n = 12$); the different letters on the error lines indicate significant differences between the different treatments ($\alpha = 0.05$); C_{mic} ,

N_{mic} , C_{soil} , and N_{soil} indicate soil microbial biomass carbon, soil microbial biomass nitrogen, soil carbon content, and soil nitrogen content, respectively

(nitrogen fertilizer and enhanced biochar) soil, the abundance of Proteobacteria was higher than that of B (enhanced biochar only) soil, but lower than that of N soil. The Acidobacteria were significantly more abundant in the CK than in the treated

soil (N, B, NB). The Actinobacteria were least abundant in the soil with nitrogen fertilizer only and were at lower abundances in treated soils than in the control; the Chloroflexi and Actinobacteria showed similar trends. Fungi were analyzed

Table 2 Results of Pearson's correlation analysis between soil physical and chemical properties

Parameter	pH	Bulk density	Porosity	Water content	Temperature	C_{soil}	N_{soil}	C_{mic}	N_{mic}	NO_3^-	NH_4^+
Bulk density	-15.36										
Porosity	10.87	-97.64**									
Water content	21.33	-34.67*	44.37**								
temperature	-29.84*	78.64**	69.54**	-34.95*							
C_{soil}	7.85	-11.34	6.89	5.92	22.17						
N_{soil}	15.36	-5.78	9.31	7.89	19.26*	89.64**					
C_{mic}	36.79**	-45.91**	39.54**	51.94**	28.64*	95.31**	94.37**				
N_{mic}	41.25**	-37.59**	35.21**	48.27**	15.57	96.34**	97.28**	87.24**			
NO_3^-	-11.36	-21.05	6.54	27.58*	9.27	59.58**	92.45**	60.48**	91.32**		
NH_4^+	-69.54**	-33.78*	-40.21**	15.64	15.66	45.29**	87.31**	71.29**	95.19**	-56.47**	
C/N	53.79**	-54.92**	70.48**	47.26**	50.92**	78.64**	-54.32**	88.54**	-44.87**	-48.94**	-50.67**

*Significantly correlated at the 0.05 level, **significantly correlated at the 0.01 level. C_{mic} , N_{mic} , C_{soil} , and N_{soil} indicate soil microbial biomass carbon, soil microbial biomass nitrogen, soil carbon content, and soil nitrogen content, respectively

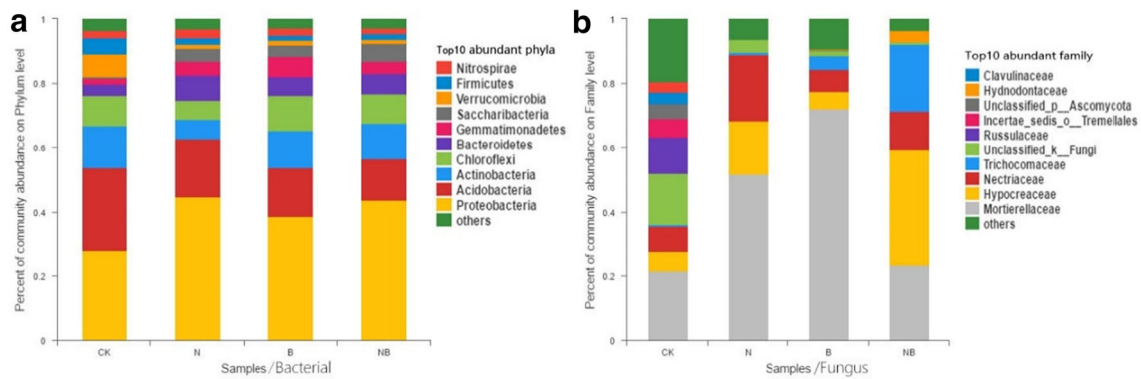


Fig. 2 (A) Taxonomic profiles of soil microorganisms. Top abundant phyla were defined as those that were > 2% of total sequences across all samples combined; the remainder were combined and lumped in a category designated as “Other.” (B) Taxonomic profiles of fungi sample

groups at the family level. Top abundant phyla were defined as those that were > 3% of total sequences across all samples combined; the remainder were combined and lumped in a category designated as “Other”

at the level of family (Fig. 2B). the Mortierellaceae were the dominant order in soils treated with NB, constituting more than 50% of the sample in soils treated with enhanced biochar only. The abundance of Mortierellaceae was lower in CK and NB soils. The proportion of Hypocreaceae in NB samples was relatively large, while the proportion in CK and B soils was much lower. The Nectriaceae was relatively most abundant in N soils and was significantly more abundant than the Hypocreaceae or Trichocomaceae. In the N and B soils, the abundances of Nectriaceae were relatively high, and the Trichocomaceae were also present in treatments containing biochar (B and NB), while they were found at low levels in CK and N soils (Fig. 2B).

The PCA (principal component analysis) revealed significant differences in the bacterial composition of the soil samples from the four different fertilizer regimes, indicating that the bacterial microbial community showed significant gradient changes with the addition of biochar or nitrogen fertilizer. The bacterial community was sensitive to the addition of biochar (Fig. 3A). The fungal community composition of the soil samples from the three different fertilizer regimes were significantly different from that of the CK; however, differences among the treated soil fungal communities were not apparent. The difference with the CK indicates that the addition of potent biochar or nitrogen fertilizer or both to soil significantly affects the composition of soil fungal communities (Fig. 3B). The bacterial sample dispersion was concentrated within each treatment (Fig. 3C), while for the fungal communities, the dispersion was broader (Fig. 3D).

Enhanced biochar and nitrogen fertilizer altered environmental characteristics and microbial communities, possibly through changes in soil geochemical characteristics, primarily. Through redundant analysis (RDA), we found that the microbial community was mainly determined by the characteristics of the ecological environment (including total nitrogen, ammonium nitrogen, organic carbon, nitrate nitrogen, moisture, and pH). After removing redundant variables, the RDA

selected nine environmental features as shown in Fig. 4: total nitrogen (p D 0.002), organic carbon (p D 0.03), and electrical conductivity (p D 0.03) significantly affected the bacterial community of soil, while soil C/N (p D 0.03) significantly affected the fungal community structure (Fig. 4 A and B). The correlation heat map shows the relationships between bacterial phyla and environmental factors. The Ktedonobacteria appeared to have highly significant positive correlation with the soil electrical conductivity (EC), while the Deltaproteobacteria appeared to have very significant negative correlation with (EC). The KD4-96 appeared to have very significant negative correlation with organic carbon, GS37-AG-4, and the Anaerolineae appeared to have a very significant negative correlation with nitrate and ammonium (Fig. 4C). The Microbotrya oomycetes and unclassified Ascomycota appeared to have a very significant negative correlation with nitrate (Fig. 4D).

For comparison, a core OTU analysis was performed on the soil samples of the control (CK) (i.e., the OTU shared among all samples in a given group), including four groups of the control (CK), nitrogen fertilizer only (N), enhanced biochar only (B), and enhanced biochar and nitrogen fertilizer (NB) (Fig. 5A). There were 496, 107, 154, and 438 (CK, NB, B, N) core OTUs restricted to each of these four separate bacterial groups, with a total of 1549 core OTUs in all groups. All treated OTUs share the vast majority of B and NB OTUs (107/3067, 154/3573 OTU). Of these, 1778 OTUs were only shared with B and 100 were only shared with the enhanced biochar and nitrogen fertilizer. Also, there were 678, 90, and 257, and in these four separate fungal groups, 174 (CK, NB, B, N) core OTUs were limited, with a total of 83 core OTUs common to all groups. Unlike bacteria, only the vast majority of enhanced biochar and nitrogen fertilizer OTU (345/435 OTU) is shared with all treatment OTUs. In contrast, the OTUs of NB, B, and N soil fungi were much less than that in the other treatments: 57.56, 32.29, and 44.20% less than no amendment, enhanced biochar only, and nitrogen fertilizer

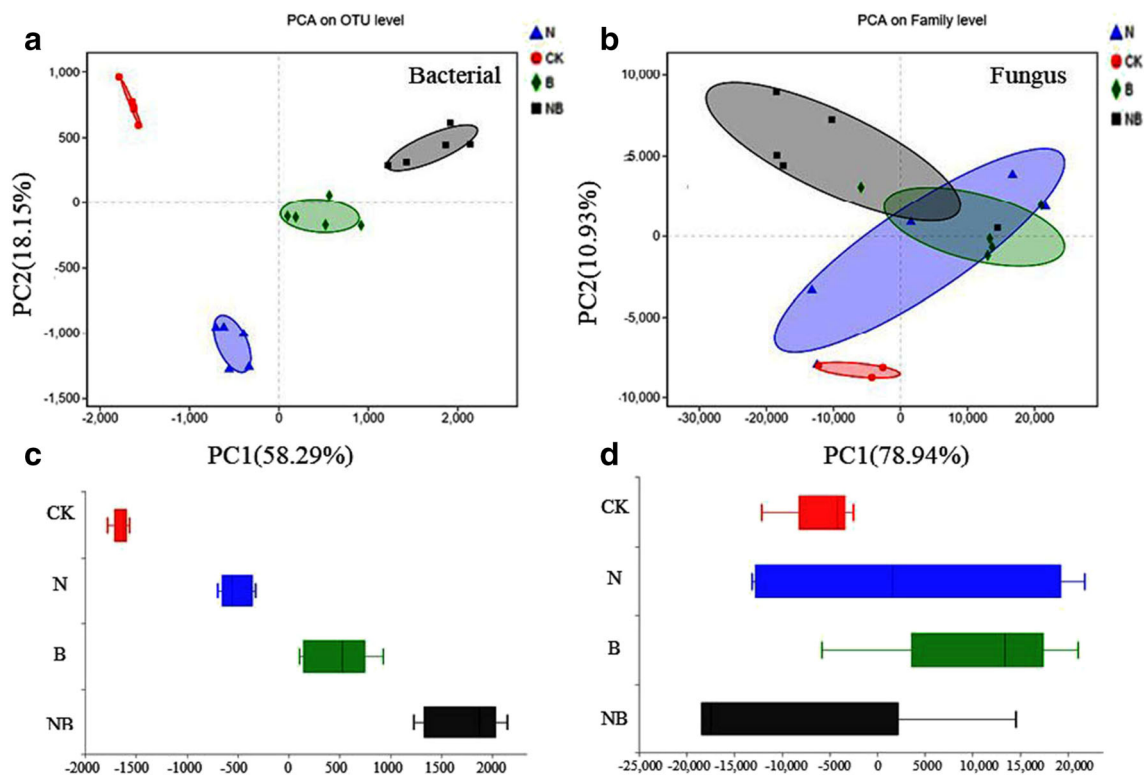


Fig. 3 Principal coordinates analysis of bacterial (A) and fungal (B) communities. The values of axes 1 and 2 are the percentages that can be explained by the corresponding axis. The box plots of bacteria (C) and fungi (D) represent the dispersion of the distribution of different sets of samples on the PC1 axis

only (Fig. 5B), respectively. As can be seen from the above situation, there was greater similarity between soil microbial communities in enhanced biochar-treated soils than with soil without enhanced biochar. However, treatment with nitrogen fertilizer only produced quite different results from that in the CK soils (Fig. 5A). These results were also seen in the fungi. However, the fungi in enhanced biochar treatment soils showed greater similarity. Relative to the no amendment control, fungal communities of the enhanced biochar treatment showed a stronger similarity with treatments using nitrogen fertilizer only (Fig. 5B).

Variation in crop growth among treatments

The leaf area was significantly higher in all treated soils (N, B, NB) than in the control (CK) soil and the leaf area was the highest in soils treated with NB (34.94% increase compared with CK), and simultaneously, the leaf area of N and NB is also significantly higher than that of B (Fig. 6A). Interestingly, for the different treatments, total biomass, root activity, and root tips showed similar patterns to the changing trend of root tips (Fig. 6 B, C, and D). The total biomass reached its peak with NB, an increase of 12.38% relative to the CK (Fig. 6B). Root activity of each treatment was not significantly different (Fig. 6C). However, the root tips of each treatment was significantly different, NB had the highest number of tobacco root tips, and the root tips of CK was the least (Fig. 6D).

Discussion

The enhanced biochar improved the physical properties of the experimental black soil and significantly affected the pH, bulk density, and porosity of the soil, which may be due to the increase in soil microbial activity after the application of enhanced biochar (Birk et al. 2009). Agglomeration will be enhanced, thereby improving soil structure (Glaser et al. 2002; Brodowski et al. 2006). Piccolo et al. (1996) found that the enhanced biochar reduces the soil bulk density, which means that the total porosity and macroporosity increase and may increase the soil water infiltration rate. Laird (Laird et al. 2010; Steinbeiss et al. 2009) thought that the application of enhanced biochar can improve soil water retention. Only treatment with nitrogen fertilizer had obvious side effects including a decrease in soil porosity and pH and an increase in soil bulk density, which is consistent with previous findings (Masulili et al. 2010). No obvious effect of enhanced biochar or nitrogen fertilizer alone on soil temperature was observed. Soil microbial biomass C, ammonium nitrogen, and nitrate nitrogen of biochar were higher than in soil treated with nitrogen fertilizer. Although the amount of nitrogen applied was not large, the enhanced biochar could increase the nitrogen mineralization efficiency in soil and increase the available nitrogen content. When using nitrogen fertilizer, the total nitrogen content in soil increased,

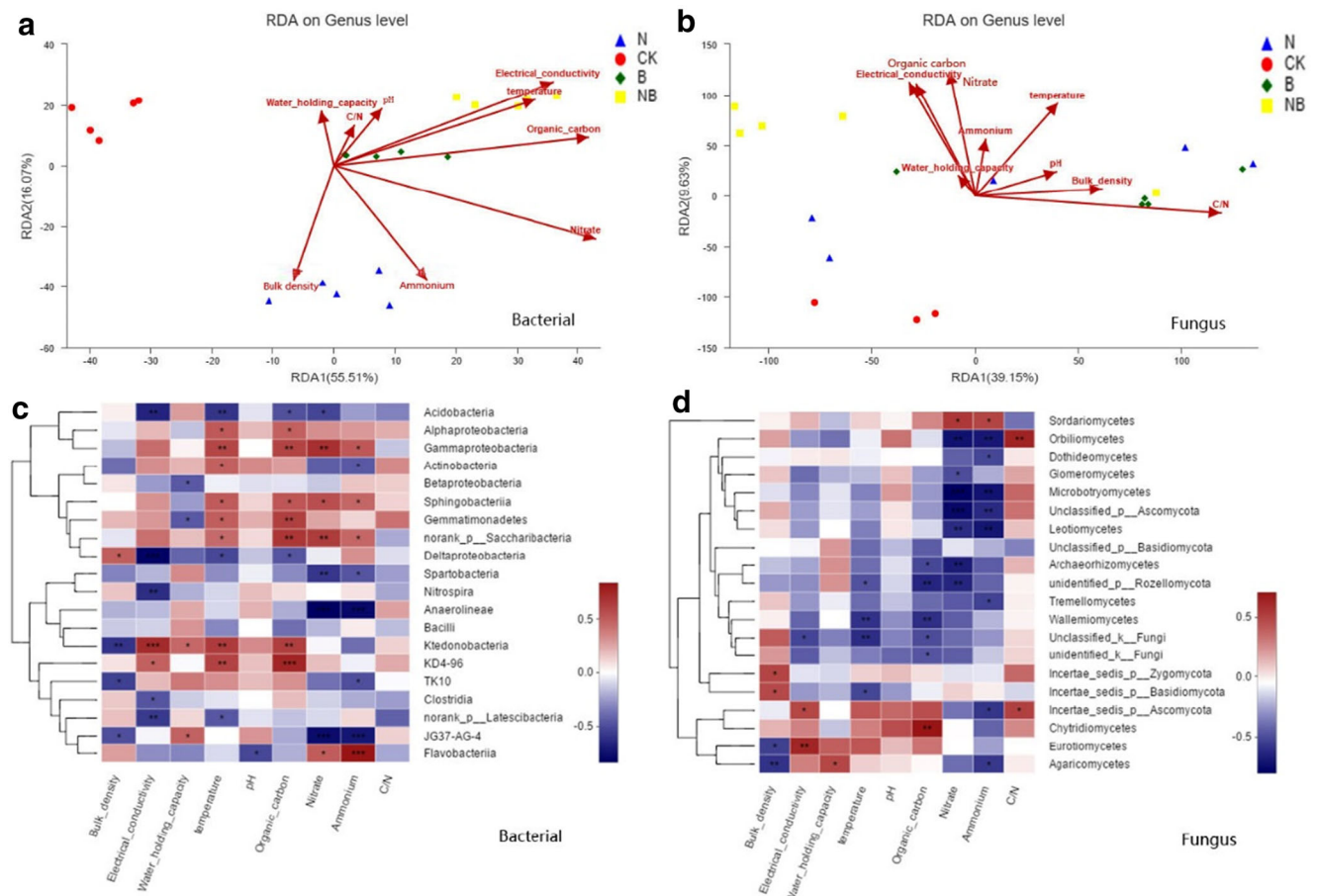


Fig. 4 Redundancy analysis (RDA) of MiSeq data (symbols) and environmental characteristics (arrows). Bacterial and fungal communities are shown in A and B, respectively. The values of axes 1 and 2 are the percentages explained by the corresponding axis. Correlation heat map of the top twenty phyla and soil properties. X and Y axes are the

environmental factors and phyla, respectively. The right side of the legend is the color range depicting different R values. The value of $P < 0.05$ is marked with “*”, $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, and $*** P \leq 0.001$. Bacterial and fungal communities are shown in C and D, respectively

while the total carbon remained the same. When the enhanced biochar was applied, the total carbon in the soil increased, and the total carbon was higher than when soil was treated with the mixture of nitrogen and enhanced biochar. This result was observed possibly because the mixed treatment improves the microbial respiration in the soil and, thus, reduces total carbon content in soil to some extent.

The enhanced biochar increases the pH of the soil. This may be because the enhanced biochar contains more salt-based ions, such as potassium, sodium, calcium, magnesium, etc., which can reduce the exchangeable hydrogen ions and exchangeable aluminum ions in the soil. In addition, most of the biochar is alkaline, so the enhanced biochar can promote an increase in pH of soil. The pH of the soil can significantly affect the content of ammonium ions in the soil. When soil pH is closer to neutral, it is beneficial to the growth of most microorganisms. Therefore, when the enhanced biochar makes the soil pH more neutral (Kolb et al. 2009), the microbial abundance in the soil increases, which correspondingly increases the microbial biomass carbon and nitrogen in the soil.

At the same time, the enhanced biochar has a large specific surface area and after being applied to the soil, it can adsorb a plurality of ions (Kimetu et al. 2010). However, it is a selective absorption of nutrients (Glaser et al. 2002), showing strong adsorption of ammonium and nitrate ions (Zech et al. 2007). When the soil bulk density is reduced, the soil permeability increases, which accelerates the metabolism and reproductive rate of soil microorganisms. When the soil has suitable temperature and humidity conditions, most microorganisms show increased activity, thereby increasing soil nitrogen mineralization rate and efficiency, which was why the enhanced biochar could increase the content of nitrate and ammonium in the soil.

In terms of plant growth and development, the advantages of enhanced biochar and nitrogen fertilizer are clear. The apex is the most active part of the root, and the root tips was the highest when the enhanced biochar and nitrogen were used simultaneously, which is related to the improvement in soil physical and chemical properties. The root system is an active absorbing organ and a synthetic organ. The growth and

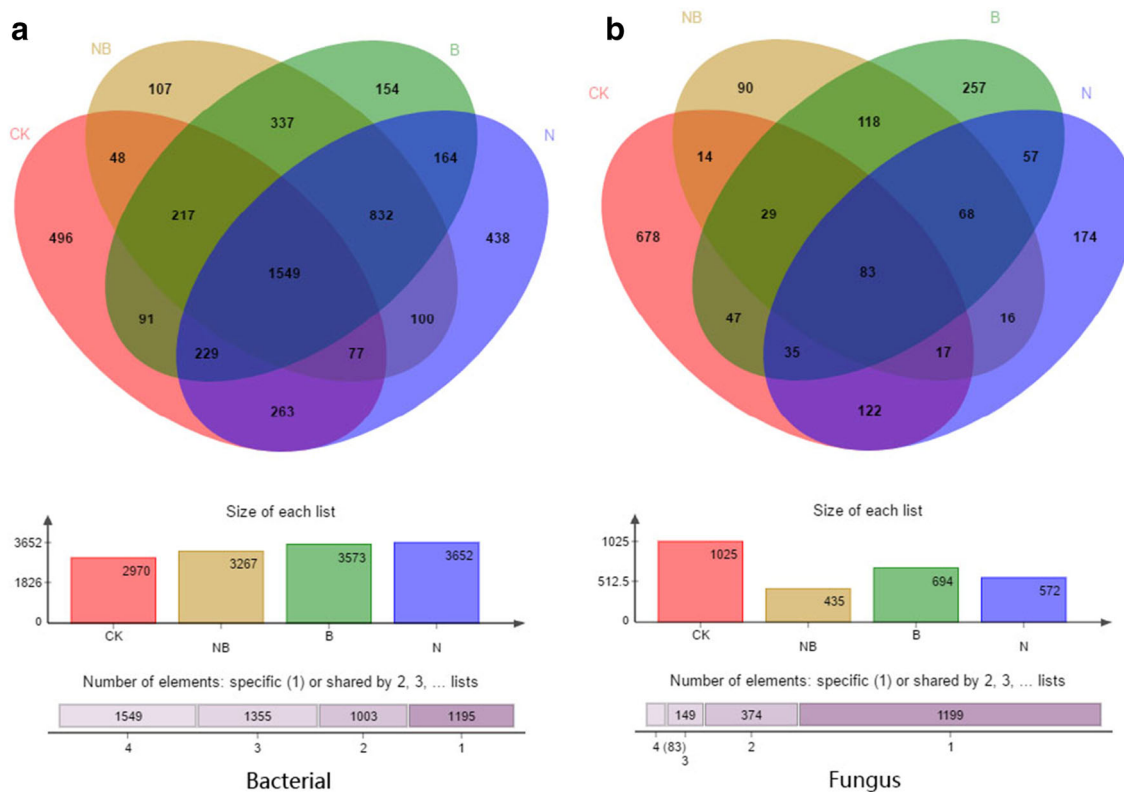


Fig. 5 Venn graph analysis of the core OTUs in soil treated with enhanced biochar and nitrogen fertilizer. Different colors represent different samples, overlapping parts represent species shared among

multiple samples, non-overlapping parts represent species unique to the sample, and numbers indicate the number of corresponding species

vitality level of the root directly affect the nutrient status and yield level of the shoot. The root activity characterizes the root system of the plant. In this study, changes in the root activity were related to changes in the number of root tips, an indicator that reflects the vitality of the root system. When the roots are sufficiently active, nutrient uptake is greater. Previous studies have shown (Liu et al. 2009) that the application of enhanced biochar can significantly promote crop growth and increase the accumulation of dry matter in the shoots and crop yield. Therefore, the combination of enhanced biochar and nitrogen fertilizers can increase plant biomass.

Changes in soil microbial diversity are an important concern in the study of biochar-improved soils. Within a certain dosage range, the enhanced biochar applied increased the diversity and distribution of the bacterial species in the rhizospheric soil, which is consistent with the results of Graber (Graber et al. 2010; Matthias et al. 2010). This may be due to the porosity of enhanced biochar, which provides more space for bacteria to grow and multiply in the soil, thereby increasing the number of bacteria. The addition of biochar can also regulate the physicochemical properties of soil and affect and regulate soil microbial growth, development, and metabolism and eventually improve soil fertility. We also found that with an increase in the application rate of enhanced

biochar, the abundance, species abundance, and uniformity of fungal OTUs in rhizosphere soil decreased, which is consistent with the findings of Van Zwieten (Grunwald et al. 2017). The enhanced biochar promotes the proliferation of bacteria, which increases the ratio of bacteria to fungi and induces a shift toward “bacterial” soil. Bacterial soil is generally considered to be a marker of good soil fertility.

The reduction of soil fungal species diversity (OTU) may be due to the different pH requirements of bacteria and fungi. Bacteria tend to prefer neutral soil, while fungi prefer acidic soils. Studies have shown that enhanced biochar can not only improve soil fertility and increase soil pH (Rondon et al. 2007) but also increase soil disease resistance by changing soil pH (Yamato et al. 2006), because fungi are considered to be the main pathogens causing plant-borne diseases (Li et al. 2008). By increasing soil pH and the number of bacteria, reducing the number of fungi, the enhanced biochar may prove to be a suitable soil additive to improve tobacco growth (Grossman et al. 2010).

The PCA indicated that there was a significant difference in bacterial species composition between soils with no treatment and those that received enhanced biochar, which was similar to the opinion of Grossman (Chen et al. 2018), and the soil with only nitrogen fertilizer was significantly different from the other

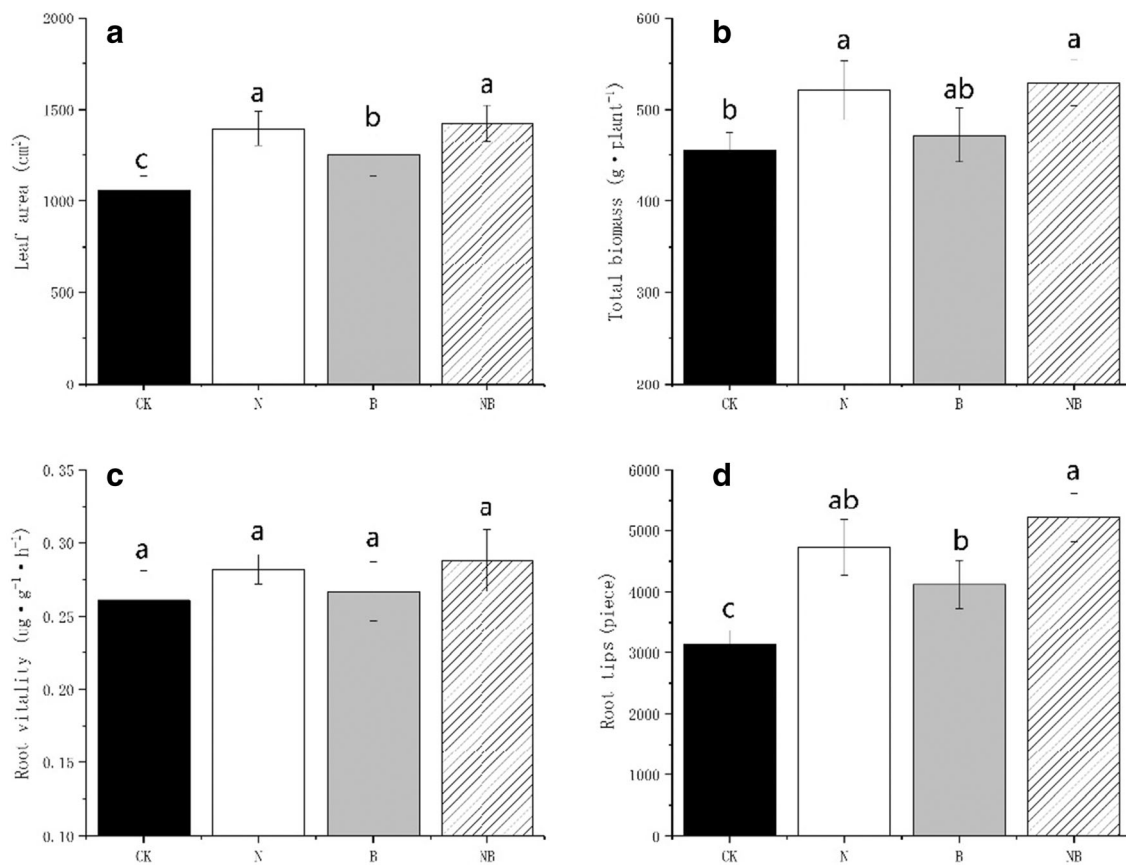


Fig. 6 Tobacco plant growth and root development. The bars are standard deviation; the different letters on the error lines indicate significant differences between the different treatments ($\alpha = 0.05$)

three treatments, indicating that enhanced biochar had a certain control effect on the microbial community distribution. And nitrogen fertilizer had some influence on bacterial composition.

The composition of bacterial species in the control treatment soil was different from that of enhanced biochar treatments. After treatment with enhanced biochar and nitrogen fertilizer, the abundance of Proteobacteria increased. This is inconsistent with previous research results (Graber et al. 2010). Treatment with a single application of nitrogen fertilizer reduced the abundance of Actinobacteria and Chloroflexi, while the other treatments resulted in little difference in the abundance of these phyla. The application of nitrogen fertilizer also increased the abundance of Bacteroidetes. For fungi, the nitrogen fertilizer increased the abundance of Mortierellaceae; treatment with enhanced biochar only resulted in a more obvious increase. The combination of enhanced biochar and nitrogen clearly increased the abundance of Hypocreaceae. Treatment with nitrogen fertilizer had a high effect on the abundance of Nectriaceae.

Conclusions

We discovered a series of effects on soil nutrients, microbial communities, and crop development combined with enhanced biochar and nitrogen fertilizers. In our field trials, soil nitrogen,

carbon, and pH were the main drivers of the observed pattern, both of which were affected by nitrogen fertilizers and enhanced biochar. Microorganism communities responded in different ways to the fertilization regimes, and these results have important implications for microbes in the soil and should be further studied in future research. At the same time, the degree and trend of different biochar degradation on the soil are different. We need to do more experiments on different biochar consumption. Therefore, whether enhanced biochar can be used as a long-term and effective soil amendment, it can only be solved by long-term positioning experiments and investigations.

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