Effects of Corn Straw Biochar on Microbial Functional Diversity and Bacterial Community in Cinnamon Soil: Postprint

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Date: 2017-11-07T00:00:00+00:00

Abstract

Biochar application to soil is considered an effective measure for carbon sequestration and emission reduction, which can increase soil organic carbon and mineral nutrient contents, and improve soil water-holding capacity and nutrient retention capacity. To investigate its effects on soil microbial activity and diversity after application, this study, under pot experiment conditions, used a combination of Biolog and high-throughput sequencing methods to examine the effects of CK (no biochar) and applications of 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ corn straw biochar on soil microbial carbon source utilization capacity (AWCD), functional diversity indices, and the abundance and diversity of soil bacteria. The results showed that with increasing biochar application rate, the AWCD value, which characterizes soil microbial activity, showed a decreasing trend, expressed as: 5 $g \cdot kg^{-1}$ treatment CK > 10 $g \cdot kg^{-1}$ treatment > 30 $g \cdot kg^{-1}$ treatment > 60 $g \cdot kg^{-1}$ treatment, with no significant difference between CK and 5 $g \cdot kg^{-1}$ treatment (P>0.05), while the AWCD values of 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments were significantly lower than CK treatment throughout the incubation period (P<0.05); soil microbial community metabolic functional diversity index (H) and carbon source utilization richness index (S) both showed decreasing trends with increasing biochar application rate, but the evenness index (E) showed the opposite trend, with H of 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments increasing by 0.16%, -0.88%, -3.14%, and -11.09% compared to CK treatment, respectively, S increasing by -2.82%, -11.27%, -18.31%, and -47.89%, respectively, and E increasing by 1.14%, 3.00%, 3.73%, and 13.76%, respectively. Principal component analysis indicated that compared with CK treatment, 5 $g \cdot kg^{-1}$ treatment had no significant effect on soil microbial community carbon source utilization patterns (P>0.05), while 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments had significant effects on soil microbial community carbon source utilization patterns

($P<0.05$). With increasing biochar application rate, soil bacterial OTU number and richness index (Chao1) showed increasing trends, with no significant difference between 5 $g \cdot kg^{-1}$ treatment and CK treatment, while OTU numbers of 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments increased by 1.09%, 5.26%, and 24.42% compared to CK treatment, respectively, and Chao1 increased by 5.73%, 10.21%, and 37.68%, respectively. After biochar application to soil, the abundance of soil bacterial phylum Proteobacteria showed no significant difference between CK and 5 $g \cdot kg^{-1}$ treatments ($P>0.05$), while 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments increased by 32.3%, 21.1%, and 16.7% compared to CK treatment, respectively; the abundance of Bacteroidetes in 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments decreased by 22.1%, 55.3%, 66.8%, and 50.5% compared to CK treatment, respectively. Biochar application to soil reduced the activity of soil culturable microorganisms, decreased or altered the types of soil microbial carbon source utilization, changed the composition of the original soil microbial community, and also affected the abundance of various soil bacterial groups in soil, reducing their distribution uniformity. To avoid affecting microbial community structure and function, the single application rate of biochar in calcareous cinnamon soil should not exceed 5 $g \cdot kg^{-1}$ (dry soil).

Full Text

Chinese Journal of Eco-Agriculture, Jun. 2016, 24(6): 736−743
DOI: 10.13930/j.cnki.cjea.151212

Effect of Corn-Stalk Biochar on Soil Microbial Functional Diversity and Bacterial Community in Cinnamon Soils

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Abstract
Biochar application is considered an effective soil carbon sequestration strategy that improves soil water and nutrient holding capacity. As key indicators of soil fertility, microorganisms play crucial roles in soil ecosystems. Understanding biochar' s influence on microbial communities and functional diversity in calcareous cinnamon soils is essential for rational biochar utilization. A pot experiment with five biochar application rates [0 $g \cdot kg^{-1}$ (control), 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$] was conducted to investigate soil microbial activity and diversity using Biolog analysis and high-throughput sequencing (HiSeq). Results showed that average well color development (AWCD), reflecting microbial activity, decreased with increasing biochar rate, following the order: 5 $g \cdot kg^{-1}$ control > 10 $g \cdot kg^{-1}$ > 30 $g \cdot kg^{-1}$ > 60 $g \cdot kg^{-1}$. No significant difference in AWCD existed between control and 5 $g \cdot kg^{-1}$ treatments. Soil microbial community diversity index (H) and richness

index (S) also decreased, while evenness index (E) increased with biochar rate. Compared with the control, biochar applications at 5, 10, 30, and 60 g · kg^{-1} changed H by +0.16%, −0.88%, −3.14%, and −11.09%; changed S by −2.82%, −11.27%, −18.31%, and −47.89%; and changed E by +1.14%, +3.00%, +3.73%, and +13.76%, respectively. Principal component analysis revealed that carbon substrate utilization was significantly affected by biochar rates $ $10g \cdot kg^{\{-1\}}.High-throughputsequencingshowedthatsoilbacterialOTUnumberssignificantlyincreasedby1.09\{-1\}$ treatments, respectively, with Chao1 richness index increasing by 5.73%, 10.21%, and 37.68% compared with the control. Biochar enhanced bacterial abundance but decreased distribution evenness. Proteobacteria abundance significantly increased by 32.3%, 21.1%, and 16.7% under 10, 30, and 60 g · kg^{-1} biochar treatments, respectively, while Bacteroidetes abundance decreased by 22.1%, 55.3%, 66.8%, and 50.5% across all biochar treatments compared with the control. These results indicate that to maintain microbial community structure and activity in calcareous cinnamon soil, the rational biochar application rate should not exceed 5 g · kg^{-1} (dry soil).

Keywords: Biochar; Calcareous cinnamon soil; Microbial activity; Microbial diversity; Biolog; High-throughput sequencing; Bacterial community

Introduction

China is rich in crop straw resources, accounting for approximately 25% of the global total [1]. From 2001 to 2010, China' s average annual production of grain crop straw was about 5.1$×10^{8}$ t, with annual burning accounting for approximately 21.6% of the total straw yield, emitting 1.15$×10^{7}$ t of CO, 1.57$×10^{8}$ t of CO_2, and 4.77$×10^{7}$ t of total carbon. This not only wastes resources but also severely pollutes the environment [2]. Biochar (or biomass charcoal) has attracted considerable research attention in recent years, referring to the product formed from pyrolysis of biological organic materials under low-oxygen or oxygen-limited conditions [3−4]. Producing biochar from crop straw waste through high-temperature pyrolysis and applying it to soil can achieve carbon sequestration [5] and reduce CO_2 emissions [6].

Biochar contains essential macro- and micronutrients for crop growth, features well-developed pore structures and a large specific surface area, and possesses strong adsorption capacity [7], making it widely applicable in agricultural production. Numerous studies have investigated biochar' s effects on soil physicochemical properties, demonstrating that biochar can increase soil organic carbon and mineral nutrient contents [8−12], improve soil water-holding and nutrient-retention capacities [13−15], and thereby enhance fertilizer nutrient use efficiency [16−17]. However, in soil ecosystems, nearly all nutrient transformation processes depend on soil microorganisms, which directly or indirectly participate in soil organic matter decomposition, inorganic nutrient cycling, and soil structure improvement [18−19]. The porous structure of biochar and its adsorption

of water and nutrients provide a favorable habitat for microbial growth and reproduction [20]. Application of biochar alters the community structures of soil bacteria, fungi, and archaea; for instance, bacterial diversity in biochar-rich Amazonian dark earth increased by 25% compared to untreated soil, while archaeal and fungal diversity decreased [21−22].

Other studies have reported decreased bacterial diversity in forest soils after biochar application [22], suggesting that biochar exerts certain control over microbial community distribution [23]. Currently, most reports on biochar's effects on soil microbial community diversity have focused on acidic soils [24], while its effects on microbial community diversity in calcareous cinnamon soils remain scarce. This study investigated changes in soil microbial community characteristics following biochar application in calcareous cinnamon soil using a combination of Biolog and high-throughput sequencing methods, aiming to clarify the effects of straw biochar on microbial communities in calcareous soils and provide a theoretical basis for rational biochar utilization in calcareous cinnamon soils.

Materials and Methods

1.1 Experimental Materials

The soil used in this experiment was collected from the 0–20 cm plow layer at the Dongyang Experimental Base of the Shanxi Academy of Agricultural Sciences. The soil type was calcareous cinnamon soil with a sandy clay loam texture (19.8% clay <0.002 mm, 22.3% silt 0.02–0.002 mm, and 57.9% sand 2–0.02 mm). The test soil was air-dried and passed through a 2 mm sieve before use. The chemical properties of the test soil were: organic carbon 3.71 $g \cdot kg^{-1}$, total nitrogen 0.48 $g \cdot kg^{-1}$, available phosphorus 3.08 $mg \cdot kg^{-1}$, available potassium 83.82 $mg \cdot kg^{-1}$, CEC 13.14 $cmol \cdot kg^{-1}$, pH 8.3, and EC 0.16 $mS \cdot cm^{-1}$. The test biochar was produced by Shanxi Gongxiao Commercial Co. through carbonization of corn straw at 300–500 °C under micro-oxygen conditions. The biochar was passed through a 2 mm sieve before use. The basic chemical properties of the biochar were: total carbon 369.87 $g \cdot kg^{-1}$, total nitrogen 6.56 $g \cdot kg^{-1}$, CEC 59.2 $cmol \cdot kg^{-1}$, pH 10.22, and EC 10.97 $mS \cdot cm^{-1}$. The test crop was 'Jinza 34' sorghum.

1.2 Experimental Design

The experiment was conducted using a pot culture method with five biochar application levels: 0 (CK), 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ (equivalent to 0, 13.5, 27, 81, and 162 $t \cdot hm^{-2}$, respectively), with three replicates per treatment. Nitrogen, P_2O_5, and K_2O were applied at rates of 0.2 $g \cdot kg^{-1}$, 0.15 $g \cdot kg^{-1}$, and 0.15 $g \cdot kg^{-1}$, respectively. These fertilizers were thoroughly mixed with the soil before potting, and then the biochar was thoroughly mixed in. The mixture was placed in plastic pots measuring 18 cm in height and 21 cm in width. Each pot contained 4 kg of soil, and equal amounts of water were added to all pots for a 3-week soil incubation period. The experiment was

conducted in a solar greenhouse, with sowing on April 15, 2014, and harvest on July 2, 2014. At the end of the experiment, soil samples were collected by gently removing the top 2 cm of soil and collecting fresh soil from below 2 cm. Samples were immediately placed in ice boxes, transported to the laboratory, and stored at 4 °C until analysis.

1.3 Biolog Analysis

Ten grams of fresh soil sample were placed in a triangular flask containing 90 mL of sterilized physiological saline (0.85% NaCl), shaken at 200 r $\cdot$ min^{-1} for 30 min, and left to stand for 10 min. The solution was then diluted to 10^{-3} with physiological saline (0.85% NaCl). One hundred fifty microliters of the diluted soil suspension were inoculated into each well of a Biolog-Eco plate (BIOLOG, Hayward, USA). The inoculated Eco plates were incubated at 25 °C. Absorbance values at wavelengths of 590 nm and 750 nm were measured every 24 h using a Biolog Reader (BIOLOG, Hayward, USA). The method of Glassen et al. [25] was used to analyze the average absorbance per well on the Eco-MicroPlate. Specifically, the absorbance values of each well at 590 nm and 750 nm were subtracted from their respective control well absorbance values, and then the 750 nm value was subtracted from the 590 nm value for each corresponding well to obtain the actual absorbance of the color reaction. Using this data, the method of Garland and Mills [26] was applied to calculate the average well color development (AWCD) value:

$$\mathrm{AWCD} = \frac{\sum(A_i - A_0)}{n} \quad (1)$$

where A_i is the actual absorbance value of each well, A_0 is the absorbance value of the control well (negative values of $A_i - A_0$ were set to 0), and n is the number of carbon sources (31 for Eco-MicroPlate). AWCD values were averaged across three replicates.

Shannon-Weiner diversity index, Shannon-Weiner evenness index, and carbon source utilization richness index were used to characterize soil microbial community functional metabolic diversity [27]. Absorbance values from 96 h of Eco-MicroPlate incubation were used to calculate these indices:

Shannon-Weiner diversity index (H'):

$$H' = -\sum P_i \ln P_i \quad (2)$$

where $P_i = (A_i - A_0)/\sum(A_i - A_0)$, with A_i and A_0 as defined above. P_i represents the difference between the absorbance of a well with carbon source and its control well, divided by the total difference across the entire microplate.

Shannon-Weiner evenness index (E):

$$E = \frac{H'}{\ln S} \quad (3)$$

where H' is the Shannon-Weiner diversity index and S is the number of wells showing color change.

Carbon source utilization richness index (S):

$$S = \text{Total number of utilized carbon sources} \quad (4)$$

The richness index represents the number of wells showing color change; absorbance values less than 0.25 were considered as no color change.

1.4 Soil Bacterial High-Throughput Sequencing

Total genomic DNA of soil microorganisms was extracted using the OMEGA (D5625-01) kit according to the manufacturer' s instructions. The extracts were stored at −20 °C until use. Universal primers (341F-805R) were used to amplify the V3-V4 region of the microbial 16S rDNA gene. Primer sequences were: 341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC. The PCR reaction system consisted of: 5 L of 10×PCR Buffer, 0.5 L of dNTP (10 mmol · L^{-1} each), 10 ng of genomic DNA, 0.5 L of Bar-PCR primer F (50 mol·L^{-1}), 0.5 L of primer R (50 mol·L^{-1}), 0.5 L of Platinum Taq (5 U· L^{-1}), and sterile water to a total reaction volume of 50 L. PCR conditions were: 94 °C for 3 min, followed by 5 cycles of (94 °C for 20 s, 55 °C for 30 s), then 20 cycles of (94 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s), and a final extension at 72 °C for 5 min. PCR products were subjected to agarose gel electrophoresis, and DNA was recovered by gel extraction. The recovered DNA was precisely quantified using the Qubit 2.0 DNA assay kit, mixed in equal amounts, and sequenced.

1.5 Statistical Analysis

High-throughput data analysis involved the following steps: 1) samples were separated and barcodes removed based on barcode sequences; 2) short sequences (<200 bp) and low-quality sequences (average quality value <20) were removed; 3) 16S rDNA gene sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using Uclust software; 4) representative sequences from each OTU were selected and classified using RDP classifier software (classification threshold >80%) to obtain bacterial taxonomic information. Soil bacterial α-diversity indices were calculated using mothur software. The Chao1 richness index was calculated as:

$$S_{\text{chao1}} = S_{\text{obs}} + \frac{N_1^2}{2(N_2+1)} - \frac{N_{1N}2}{2(N_2+1)^2} \quad (5)$$

where S_{obs} is the total number of OTUs detected, N_1 is the number of OTUs with only one sequence, and N_2 is the number of OTUs with only two sequences.

The Shannon diversity index was calculated as:

$$H' = -\sum \frac{n_i}{N} \ln\left(\frac{n_i}{N}\right)$$

where n_i is the number of sequences in each taxonomic unit and N is the total number of sequences.

SPSS 18.0 was used for variance analysis (Duncan' s test) and principal component analysis, and Microsoft Excel 2007 was used for graphing. The significance level was set at α=0.05.

Results

2.1 Carbon Source Utilization by Soil Microorganisms

The average well color development (AWCD) reflects the ability of culturable microorganisms to utilize different substrates (carbon sources) and indicates soil microbial physiological metabolic activity to some extent. Dynamic monitoring of AWCD values for 31 carbon sources (every 24 h) showed that AWCD values for all treatments increased with incubation time. During the first 24 h, AWCD values were very low for all treatments, but increased rapidly after 24 h, indicating gradual carbon source utilization and enhanced microbial metabolic activity. No significant differences in AWCD values were observed among CK, 5 $g \cdot kg^{-1}$, and 10 $g \cdot kg^{-1}$ treatments before 72 h of incubation (P>0.05). After 72 h, the 10 $g \cdot kg^{-1}$ treatment was significantly lower than CK and 5 $g \cdot kg^{-1}$ treatments (P<0.05). The AWCD values of 30 $g \cdot kg^{-1}$ and 60 $g \cdot kg^{-1}$ treatments were significantly lower than other treatments throughout the entire incubation period (P<0.05), indicating that high biochar application rates impaired microbial carbon source utilization capacity and reduced metabolic activity. At the end of incubation, AWCD values followed the order: 5 $g \cdot kg^{-1}$ CK > 10 $g \cdot kg^{-1}$ > 30 $g \cdot kg^{-1}$ > 60 $g \cdot kg^{-1}$. Compared with CK, the 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments showed changes of +1.57%, −9.67%, −27.84%, and −53.73%, respectively [Figure 1: see original paper].

2.2 Diversity of Culturable Soil Microorganisms

As shown in Table 1 , the diversity index and carbon source utilization richness index of soil microbial metabolic function decreased with increasing biochar application rate, while the evenness index showed an increasing trend. The 5 $g \cdot kg^{-1}$ biochar treatment had the highest diversity index. No significant difference was observed between CK and 5 $g \cdot kg^{-1}$ treatments (P>0.05), but the remaining three treatments differed significantly from each other and were all significantly lower than both 5 $g \cdot kg^{-1}$ and CK treatments (P<0.05). Compared with CK, the 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments showed changes in diversity index of +0.16%, −0.88%, −3.14%, and −11.09%, respectively. The evenness index increased with biochar application rate, with the 60 $g \cdot kg^{-1}$ treatment being significantly higher than CK, 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, and 30 $g \cdot kg^{-1}$ treatments. Compared with CK, the 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$,

30 g · kg^{-1}, and 60 g · kg^{-1} treatments showed increases in evenness index of 1.14%, 3.00%, 3.73%, and 13.76%, respectively. The carbon source utilization richness index decreased with increasing biochar rate, though no significant differences were observed among CK, 5 g · kg^{-1}, 10 g · kg^{-1}, and 30 g · kg^{-1} treatments ($P>0.05$). The 60 g · kg^{-1} treatment was significantly lower than all other treatments ($P<0.05$).

2.3 Principal Component Analysis of Microbial Carbon Utilization

Principal component analysis (PCA) is a dimensionality reduction method that transforms multiple variables into several comprehensive variables. Garland [28] suggested that differences in spatial positions among treatments are associated with carbon sources clustered on coordinate axes. PCA of carbon source utilization was performed based on 96 h AWCD values. Four principal components were extracted from 31 variables, with a cumulative contribution rate of 97.5%. The first principal component (PC1) contributed 54.06%, the second (PC2) contributed 24.40%, and the third and fourth components contributed 11.74% and 7.55%, respectively. Analysis of the first two principal components plotted treatment scores with PC1 and PC2 as the x- and y-axes [Figure 2: see original paper]. On the PC1 axis, treatments were divided into two groups: CK, 5 g · kg^{-1}, and 10 g · kg^{-1} treatments were distributed on the positive side, with CK and 5 g · kg^{-1} clustering together, indicating similar carbon source utilization patterns and metabolic functions. In contrast, 30 g · kg^{-1} and 60 g · kg^{-1} treatments were distributed on the negative side of PC1. On the PC2 axis, treatments were again divided into two groups: CK, 5 g · kg^{-1}, and 30 g · kg^{-1} treatments were on the positive side, while 10 g · kg^{-1} and 60 g · kg^{-1} treatments were on the negative side.

2.4 Bacterial Diversity Based on High-Throughput Sequencing

As shown in Table 2 , OTU numbers generally increased with biochar application rate. No significant differences were observed among CK, 5 g · kg^{-1}, and 10 g · kg^{-1} treatments ($P>0.05$), while 30 g · kg^{-1} and 60 g · kg^{-1} treatments were significantly greater than the above treatments ($P<0.05$). Compared with CK, OTU numbers in 5 g · kg^{-1}, 10 g · kg^{-1}, 30 g · kg^{-1}, and 60 g · kg^{-1} treatments changed by −2.57%, +1.09%, +5.26%, and +24.42%, respectively, indicating that biochar application rates greater than 5 g · kg^{-1} increased soil bacterial OTU numbers. The Chao1 index estimates total species richness in a community and showed an increasing trend with biochar application rate, following the order: 60 g · kg^{-1} > 30 g · kg^{-1} > 10 g · kg^{-1} > CK > 5 g · kg^{-1}. No significant difference was observed between CK and 5 g · kg^{-1} treatments ($P>0.05$), but all other treatments differed significantly from each other and were significantly greater than CK and 5 g · kg^{-1} treatments ($P<0.05$). Compared with CK, the treatments showed changes in Chao1 index of −0.16%, +5.73%, +10.21%, and +37.68%, respectively. The Shannon index describes the uncertainty of individual occurrence in a community, with higher values indicating greater

community diversity. The 60 $g \cdot kg^{-1}$ biochar treatment showed the highest Shannon index, which was significantly different from 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, and 30 $g \cdot kg^{-1}$ treatments ($P<0.05$) but not significantly different from CK ($P>0.05$). No significant differences were observed among 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, and 30 $g \cdot kg^{-1}$ treatments ($P>0.05$).

2.5 Bacterial Community Structure at Phylum Level

Figure 3 [Figure 3: see original paper] shows that the dominant bacterial phyla in calcareous cinnamon soil included Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Gemmatimonadetes, Planctomycetes, Verrucomicrobia, Firmicutes, Chloroflexi, as well as unclassified and rare bacterial groups. Proteobacteria was the most dominant group, accounting for 39.68% of total reads. No significant difference in the abundance of this group was observed between 5 $g \cdot kg^{-1}$ and CK treatments. However, when biochar application exceeded 5 $g \cdot kg^{-1}$, the abundance of Proteobacteria increased significantly, with 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments showing increases of 32.3%, 21.1%, and 16.7% compared with CK, respectively. Bacteroidetes was the second most abundant phylum, with a relative abundance of 13.16% in CK treatment. The abundance of this group decreased significantly with increasing biochar application rate, showing reductions of 22.1%, 55.3%, 66.8%, and 50.5% in 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments compared with CK, respectively. The abundances of other bacterial groups also varied among treatments [Figure 4: see original paper], demonstrating that biochar application affects the abundance of various bacterial phyla in soil. Cluster analysis in the heatmap showed that CK and 5 $g \cdot kg^{-1}$ treatments clustered together first, while 30 $g \cdot kg^{-1}$ and 60 $g \cdot kg^{-1}$ treatments clustered together before joining with 10 $g \cdot kg^{-1}$ treatment, indicating that CK and 5 $g \cdot kg^{-1}$ treatments had similar bacterial communities, whereas 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments shared similar bacterial community structures.

Discussion and Conclusion

Methods for evaluating soil microbial communities primarily include community-level physiological profiling (Biolog method) and biomarker methods (PLFA) based on biochemical techniques, as well as DNA length polymorphism (T-RFLP), DNA composition polymorphism (DGGE), and high-throughput sequencing technologies based on modern molecular biology. The Biolog microplate method reflects differences in soil microbial community metabolic capacity through microbial utilization of different carbon sources. Average well color development (AWCD) and soil microbial metabolic functional diversity indices can reflect soil microbial activity and diversity to some extent [29]. Our results showed that AWCD values, soil microbial metabolic functional diversity indices, and carbon source utilization richness indices all decreased with increasing biochar application rate, generally following the order: 5 $g \cdot kg^{-1}$ CK > 10 $g \cdot kg^{-1}$ > 30 $g \cdot kg^{-1}$ > 60 $g \cdot kg^{-1}$. No significant difference was observed

between CK and 5 $g \cdot kg^{-1}$ treatments, while AWCD values and diversity indices of 10 $g \cdot kg^{-1}$, 30 $g \cdot kg^{-1}$, and 60 $g \cdot kg^{-1}$ treatments were significantly lower than CK throughout the incubation period. The evenness index increased with biochar application rate, likely because large biochar additions increased soil C/N ratio. The speed and final extent of enzymatic reactions in soil microbial communities are related to the number and types of microorganisms capable of utilizing single carbon sources (substrates) [28]. Without nitrogen fertilizer input, biochar application may have reduced soil microbial numbers on one hand, and inhibited the growth of certain microbial types on the other, decreasing species richness. This indicates that biochar application altered the original soil microbial community composition, inhibited the growth of indigenous soil microbial species, and increased populations of specific microorganisms adapted to the new environment. Dempster et al. [30] reported that high biochar application rates significantly reduced microbial biomass carbon compared with the control. Studies by Wu Yingga [31] and Marluthi et al. [32] also demonstrated that biochar application changed the types of carbon sources utilized by soil microorganisms, consistent with our findings. However, other studies showed that in albic soil, fluvo-aquic soil, gray desert soil, and brown soil, AWCD and diversity indices were higher in treatments without biochar during the early experimental stage, while treatments with biochar applied at 40 $t \cdot hm^{-2}$ (equivalent to 16 $g \cdot kg^{-1}$ in our experiment) showed the highest AWCD values and diversity indices in the later stage [33]. These discrepancies may be related to differences in biochar feedstock and production processes, leading to different physicochemical properties (particularly total carbon content and pH), as well as soil texture and acidity/alkalinity. Additionally, different crop species and their root exudates may influence soil microbial diversity [34].

Given that the Biolog microplate method only detects culturable microorganisms and cannot fully reflect soil microbial metabolic diversity, this study combined high-throughput sequencing to investigate biochar effects on soil bacterial diversity at the genetic level. Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Gemmatimonadetes, Planctomycetes, Verrucomicrobia, Firmicutes, and Chloroflexi were the dominant bacterial phyla in calcareous cinnamon soil. Biochar application significantly increased the abundance of Proteobacteria but decreased that of Bacteroidetes, demonstrating that biochar affected the distribution of various bacterial phyla in soil. The 5 $g \cdot kg^{-1}$ and CK treatments had similar bacterial communities, indicating that a 5 $g \cdot kg^{-1}$ biochar application rate does not affect soil bacterial diversity in calcareous cinnamon soil. In contrast to Biolog results, soil bacterial OTU numbers and richness indices increased with biochar application rate, while diversity indices were higher in CK and 60 $g \cdot kg^{-1}$ treatments and significantly lower in other treatments. This discrepancy occurs because the Shannon index is determined by two factors: species richness (the number of populations) and evenness (the uniformity of individual distribution among populations). Since richness increased with biochar application rate, the lower diversity in 5 $g \cdot kg^{-1}$, 10 $g \cdot kg^{-1}$, and 30 $g \cdot kg^{-1}$ treatments may be attributed to uneven distribution of bacterial communities,

as illustrated in the community abundance distribution heatmap [Figure 4: see original paper]. Differences between the two diversity analysis methods may arise from their different emphases and the fact that high-throughput sequencing only analyzed soil bacteria without considering other microbial types, which warrants further investigation. Both Biolog and high-throughput sequencing methods demonstrated that biochar application altered the original soil microbial community composition, affected the abundance of various bacterial groups, and reduced the evenness of their distribution. To avoid impacting microbial community structure and function, the application rate of straw biochar in calcareous cinnamon soil should not exceed 5 $g \cdot kg^{-1}$ (dry soil) per application.

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Note: Figure translations are in progress. See original paper for figures.