

*Original Research*

# Variations in Fungal Diversity in a Biochar-Treated Continuous Cotton-Cropped Soil Environment Through Metagenomics-Based Analyses

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## **Abstract**

Biochar amendment strategy has remained a good alternative for improving soil fertility and alleviating climate change challenges. There exists inadequate information on the role of biochar in influencing soil fungal community structure, especially up to a 40-year chrono-sequence cropping of plants such as cotton. This study was therefore purposed to determining the soil fungal constituents and their respective diversity in biochar-treated (0 t·ha<sup>-1</sup>, 20 t·ha<sup>-1</sup> and 40 t·ha<sup>-1</sup>) soils of different continuously cotton cropping years (5, 10, 20 and 40 years). The study applied high-throughput sequencing of the the 18S rRNA gene and integrated biostatistics-based techniques to screen the soil fungal community assemblage, key fungal species, soil fungal association structure and metabolic functions, the soil fungal constituents, their proportions and diversity in the biochar-treated cotton pots over the different continuous cropping years. The most abundant fungal phyla were Ascomycota and Basidiomycota. In addition, *Aspergillus*, a pathogenic fungus, was also detected in all our sequence data. Soil pH changes were also identified as a key factor in shaping soil fungal communities. Over the continuous cropping duration, the soil pH gradually declined. At 20 years of continuous cropping, the number of fungal OTU's started to increase and their highest overall quantity was achieved at 20 years of continuous cropping, when pH was 7.3±0.1. Overall, our study findings show that biochar application over a long continuous cropping system alters soil fungal diversity and is therefore an important factor in soil quality management.

**Keywords:** biochar, community structure, continuous cropping, diversity, soil fungi

## Introduction

Global warming phenomena is already causing serious effects that negatively impact agricultural production all over the globe [1]. Therefore, there is a need for urgent remedial action to strengthen the future of food security for the rapidly expanding human population. A good approach towards that is by finding sustainable mitigation ways to the hindered agricultural sector [2]. The trend in climatic change that is brought about by global warming has resulted in widespread famine conditions in many parts of the world [1]. The sequestration of major nutrients that are key to enhanced crop production are the current key techniques in combating the expanding impacts of global warming and climate change to agricultural productivity [2-3]. Currently, the most preferred nutrient-enhancing techniques are those with minimum interference with soil microbiota such as the ecologically significant fungi [4]. Some of these techniques include the use of inorganic fertilizers such as biochar, which has not only proved to be the best sustainable alternative way of sequestering carbon and other greenhouse gases from the environment but also has promoted soil nutrient quality [5-6]. This has rendered the application of biochar as a form of manure in crop production to be one of the best minimum tillage techniques with positive contributory effects to the soils' beneficial microbes in agricultural farms [7]. Studies by Han et al. [8] reported that continuous long-term biochar application has significantly impacted the diversity of soil bacteria within the cotton farms in China. However, there is still inadequate knowledge on the relationship between long-term biochar application and the soil fungal community within continuously cotton-cropped soil [5, 9].

Studies have also shown that the improvement of soil nutrient contents such as iron (Fe) and molybdenum (Mo) properties in agricultural farmlands highly depend on the inter and intra relationship between the different soil bacteria and fungi [4-5]. Most endophytic fungus have the ability to support ecosystem services through symbiotic relationships with the host and the abiotic environment, and such relationships can enhance the acquisition of important soil nutrients such as nitrogen. However, there is inadequate understanding regarding how the fungus influence soil properties and microbial communities [5, 9]. [5] reported that the mechanism of plant-soil feedback may be mediated by the plant-fungus interaction such as rice-endophyte interactions, especially in soils with nutrient limitations. Proper understanding of the impact of long-term biochar application to the soil beneficial fungal community is essential for promoting the production of essential agricultural products, especially in regions like China where the large human population has continued to add pressure to deteriorating agricultural produce [3, 7]. The few existing studies that have focused on the effects of biochar application on the soil microbial community assemblage have widely considered the influence of

long-term continuous biochar application (more than 20 years) on the bacterial community rather than on the fungal community [8]. Furthermore, the majority of these studies have not interpretively applied the highly reliable modern bioinformatic and biostatistical techniques in assessing either the availability of bacterial or fungal community and soil quality on long-term (up to 40 years) biochar application [2, 8, 10].

Our study hypothesized that there was no difference in the soil fungal community assemblage, key fungal species, soil fungal association structure and metabolic functions, soil fungal constituents, their proportions and diversity between the varied duration of cotton cropping years and different fertilizer application rates. Therefore, the current study investigated the impact of the application of biochar as a soil treatment additive in a varied duration of cotton cropping years (5, 10, 20 and 40 years) and application rates (0 t·ha<sup>-1</sup>, 20 t·ha<sup>-1</sup> and 40 t·ha<sup>-1</sup>) to the soil fungal community structure. The study applied high-throughput sequencing of the 18S rRNA gene and integrated biostatistical-based techniques to screen the soil fungal community assemblage, key fungal species, soil fungal association structure and metabolic functions, the soil fungal constituents, their proportions and diversity in the biochar-treated cotton pots over the different continuous cropping years. This study aimed to provide a highly reliable inference on the response of soil fungal community to the application of biochar in cotton soils with different continuous cropping years and application rates using the integrated bioinformatic analyses approach.

## Materials and Methods

### Experimental Study Site and Cotton Variety

The study was designed in an experimental farm owned by Xinjiang Jin B Seed Centre in Bole City, Xinjiang, China (GPS coordinates: 44°46'56.0"N 82°23'57.0"E). The study site is located in the northwestern part of Xinjiang, which neighbors Kazakhstan. Xinjiang's regional climate is a local steppe climate. Bole is a continental arid semi-desert and desert climate with long sunshine hours and large temperature differences between day and night. The temperature in spring is warm and cold, summer is hot, the climate is hot, with dry and hot winds, autumn is cool, winter is long and cold, the annual average temperature is 5.6°C, average annual precipitation is 181 mm, and annual average evaporation is 1562.4 mm. The extreme maximum temperature is 44°C and the extreme minimum temperature is -36°C. The annual average sunshine hours are 2815.8, accumulated temperature of ≥10°C is 3137.9°C, and the frost-free period is 169 days. Bole has good light, heat and water elements, and is suitable for planting a variety of agricultural and melon crops.

Table 1. Treatment/Sample labels.

Years	0 t·ha <sup>-1</sup>			20 t·ha <sup>-1</sup>			40 t·ha <sup>-1</sup>		
	Rep 1	Rep2	Rep3	Rep 1	Rep2	Rep3	Rep 1	Rep2	Rep3
5	AT01	AT02	AT03	AT11	AT12	AT13	AT21	AT22	AT23
10	BT01	BT02	BT03	BT11	BT12	BT13	BT21	BT22	BT23
20	CT01	CT02	CT03	CT11	CT12	CT13	CT21	CT22	CT23
40	DT01	DT02	DT03	DT11	DT12	DT13	DT21	DT22	DT23

### Soil and Biochar Fertilizer

Soil samples were obtained from the top layer (0-15 cm depth) of cotton farm plots that had been continuously cropped at time intervals of 5, 10, 20 and 40 years under constant culturing and soil management. Based on Food Agriculture Organization (FAO) classification guidelines, the soil samples were categorized as acrisols with 66% clay content. Mineral fertilizers (NPK) used in these field tests were full element compound fertilizer. The total nutrient content is 45%, of which the total nitrogen content is 15%, available phosphorus content is 15%, and available potassium content is 15%. Biochar is a pyrolysis of farmland waste at high temperature (550°C). It does not have microbial activity itself, but it can provide a micro-environment for soil microbes and some carbon sources for microbial utilization. Biochar was made out of corncob feedstock using a traditional kiln reactor (Fengben Biological Technology Co., Ltd, Shandong, China) at a rising heating gradient of 10°C min<sup>-1</sup> up to 550°C. Firstly, the corncob feedstock was air-dried before being pyrolyzed under controlled conditions. Total carbon and nitrogen were established through combustion analysis (vario Macro CNS; Elementar, Germany). Ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) evaluations were performed through extraction with 0.5 M K<sub>2</sub>SO<sub>4</sub> and colorimetric analysis of NH<sub>4</sub><sup>+</sup>, and thereafter the extracts of NO<sub>3</sub><sup>-</sup> were established using an automated flow injection by means of a Skalar auto-analyzer (Skalar San Plus). Carbonate equivalence

assessment was performed as depicted by Rayment and Lyon. Electrical conductivity (EC) and pH were determined in a 1:5 (w/v; g cm<sup>-3</sup>) soil:water environment and in 0.01 M CaCl<sub>2</sub> mixtures. The basic chemical properties of the soil and biochar are described in Table 2.

### Experimental Design and Cotton Variety

Different continuously cotton-cropped research fields (5, 10, 20 and 40 years), each with measurements of 100 m<sup>2</sup>, were selected and marked for the study. Initial field preparation entailed removal of pebbles, stones and plant debris. Thereafter, appropriate calculated portions of the biochar were mixed in the field plots, with respective biochar application rates of 0 t·ha<sup>-1</sup>, 10 t·ha<sup>-1</sup> and 20 t·ha<sup>-1</sup>. In total, each of the four continuous cropped plots had three biochar application rates, resulting in 12 treatments. Each treatment was done in triplicate, leading to a total of 36 experimental plots (4 × 3 × 3 = 36) as shown in Table 1. A similar quantity of initial compound fertilizer (N:P:K = 15:15:15) was applied to all the plots. The cotton variety used in this study is Xinluzao 80, which has the characteristics of early maturity, high quality and suitability for mechanical harvesting. The growth period is 117 days, plant height is 75cm, pre-frost flower rate is 95.6%, clothing fraction is 43%, the upper half of the fiber is 30.0 mm, specific strength is 31.4 cN/tex, micronaire value is 4.7, and uniformity index is 85.3%. The average yield of lint cotton per

Table 2. Basic chemical properties of biochar and soil.

Chemical Properties	Biochar	Contiuous cropped soil			
		5 Years	10 Years	20 Years	40 Years
Total N (g·kg <sup>-1</sup> )	2.7	1.51±0.02 <sup>a</sup>	1.23±0.02 <sup>b</sup>	1.01±0.02 <sup>c</sup>	0.93±0.05 <sup>c</sup>
NH <sub>4</sub> <sup>+</sup> (mg·kg <sup>-1</sup> )	<0.1	16.2±0.1 <sup>a</sup>	14.1±0.1 <sup>b</sup>	12.2±0.1 <sup>c</sup>	10.8±0.1 <sup>c</sup>
NO <sub>3</sub> <sup>-</sup> (mg·kg <sup>-1</sup> )	<0.2	2.4±0.1 <sup>a</sup>	2.4±0.0 <sup>a</sup>	1.9±0.1 <sup>b</sup>	1.80±0.1 <sup>b</sup>
Total C (g·kg <sup>-1</sup> )	680.0	11.5±0.3 <sup>a</sup>	10.2±0.2 <sup>b</sup>	10.1±0.1 <sup>b</sup>	10.0±0.2 <sup>b</sup>
pH (CaCl <sub>2</sub> )	8.3	8.3±0.3 <sup>a</sup>	8.1±0.4 <sup>ab</sup>	7.6±0.1 <sup>b</sup>	7.3±0.1 <sup>b</sup>
EC (μS·cm <sup>-1</sup> )	526.3	43.3±1.8 <sup>a</sup>	43.1±1.6 <sup>a</sup>	40.8±2.0 <sup>a</sup>	37.8±2.7 <sup>b</sup>

The data are expressed as mean±SD (n = 3). Superscript letters that differ within rows indicate significant differences between treatments (p<0.05).

hectare is 1830-22. Cotton seedling sowing was done on 15 April 2017 at a planting gap of 10 x 50 cm. Constant weeding was manually conducted whenever necessary during the experiment. Watering of the plots was also done whenever necessary.

### Soil Sampling

Soil sampling in all the treatments was performed at the boll-opening stage on 25<sup>th</sup> July 2017. Briefly, the soils samples were randomly collected in replicates of 10 in a range of 5cm from the cotton trunk at soil depths of 0-15 cm. Thereafter, all the replicates were mixed to produce homogenous sample. They were transported to the lab in cold-chain and all the physico-chemical parameters analyzed within a week from the collection time. One part of all study samples was cryopreserved (-80°C) for later use in soil microbiological and chemical analysis.

### DNA Extraction and PCR Amplification of 16S rRNA

Genomic DNA was extracted from the soil samples using an E.Z.N.A. soil DNA kit (Omega Bio-Tec, Inc., USA) as per the manufacturer's guidelines. The obtained DNA was assessed by loading 10 µL of the samples and running them on 1% agarose gel. The barcoded fusion primers (forward primers: 341F: CCTACACGACGCTCTTCCGATCTNCCTACGGGNGGCWGCAG, reverse primers: 805R GACTGGAGTTCTTGGCACCCGAGAATTCCAGACTACHVGGGTATC-TAATCC) were used in the amplification of the V3-V4 hypervariable regions of 16S rRNA via PCR from the microbial genomic DNA. The PCR reaction mixture (50 µl) comprised of 5 µl of 10 × PCR reaction buffer (TakaRa, Japan), 10 ng of DNA template, 0.5 µl of each primer, 0.5 µl of dNTPs and 0.5 µl of Platinum Taq DNA polymerase (TakaRa, Japan). Thermocycler amplification conditions were set at: initial 94°C for 3 min, 94°C for 30 s, annealing at 45°C for 20 s and 65°C for 30 s, which was repeated for 5 cycles, followed by 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, which was repeated for 20 cycles, before a final elongation at 72°C for 5 min. Specificity assessment of the PCR amplification product was assessed on 1.5% agarose gel and thereafter purified using a QIAquick Gel Extraction Kit.

### Illumina Sequencing and Data Processing

Paired-end sequencing was conducted using an Illumina MiSeq (Illumina, San Diego, CA, USA) system. Sequencing amplicons were barcoded into two (V3 and V4 amplicon). Quality check was performed on the sequence data and poor-quality reads were cut off using Prinseq software (PRINSEQ-lite 0.19.5). The V3 and V4 paired-end reads were assembled with respect to a 10-base pair reads overlap with no mismatch via

Flash software (FLASH v1.2.7), and afterwards the non-assembled reads were discarded. Uclust software (Uclust v1.1.579) was used to cluster sequences and assign them to operational taxonomic units (OTUs) at a 3% dissimilarity level. Assigning of taxonomic ranks to each sequence was done using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier v.2.2 trained on the green genes database (October 2012 version).

### Classification and Taxonomic Status Identification

Orthogonal taxonomic units (OTU) status and identification was achieved by using QIIME software that compared the OTU representative sequence to the template sequence of the corresponding database to get the taxonomic information corresponding to each OTU. Each specific database is used as the template sequence of OTU classification status identification for different categories of sequences. The phylogenetic and taxonomic trees were constructed on MEGAN software (<http://ab.inf.uni-tuebingen.de/software/megan6/>), which involved mapping the abundance information of OTU and Taxonomy composition data contained in each sample to the microbial taxonomy hierarchy tree provided by the NCBI website (<https://www.NCBI.nlm.nih.gov/Taxonomy>) to present the concrete composition of all samples at each classification level in a standard classification hierarchy. A comparative analysis was done through GraPhlAn and Krona software.

### Community Diversity and Taxonomic Composition

Community diversity was calculated through Chao 1, Ace, Shannon and Simpson indices using QIIME software. The specific composition of each sample at genus level was calculated based on the results on OTU classification and taxonomic status identification.

### Screening of Key Species

This was carried out through principal component analysis (PCA) and multi-dimensional scaling analysis (MDA). PCA was used to analyze the community composition structure at the genus level, and the natural distribution between the samples was described with two-dimensional and three-dimensional images by R software. The unweighted pair group method with arithmetic means (UPGMA) was used in MDA using QIIME software. Comparative analysis of bacteria and screening of key species was done through PLS-DA (partial least squares discriminant analysis).

### Cluster Distribution and Prediction of Microbial Metabolic Function

The number of unique and shared functional group was calculated by R software through Venn graph

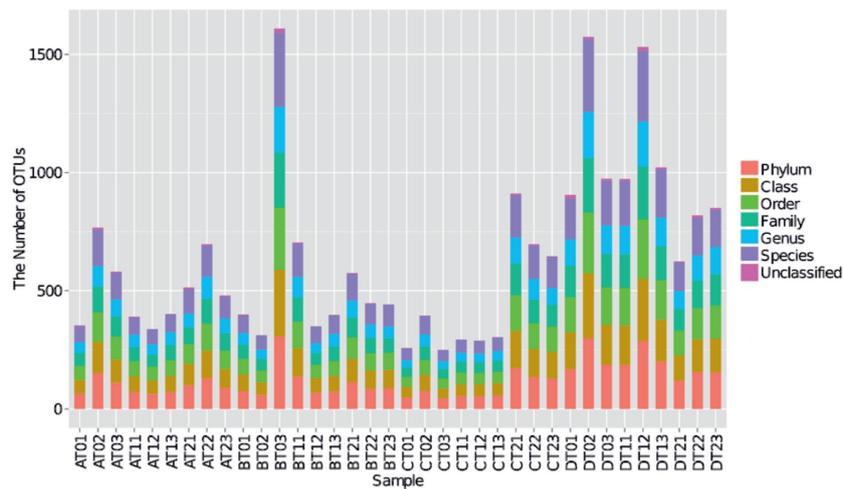


Fig. 1. OTU abundance in the generated sequences.

analysis. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST) was used in predicting the significant microbial metabolic functions using the KEGG database (<http://www.genome.jp/kegg/pathway.html>).

Statistical Data Analysis

Data was analyzed using SPSS, QIIME and R software. Mean differences were calculated at a p value of 0.05. Significant differences among the means were

determined using the LSD test. T-tests and metastats (<http://metastats.cbcb.umd.edu/>) in Mothur were used to compare the differences, and all p-values were adjusted with the false discovery rate (FDR) using the BH method with the mt.rawp2adjp function in R.

Results and Discussion

Soil physicochemical properties including total N ( $\text{g}\cdot\text{kg}^{-1}$ ),  $\text{NH}_4^+$  ( $\text{mg}\cdot\text{kg}^{-1}$ ),  $\text{NO}_3^-$  ( $\text{mg}\cdot\text{kg}^{-1}$ ), total C

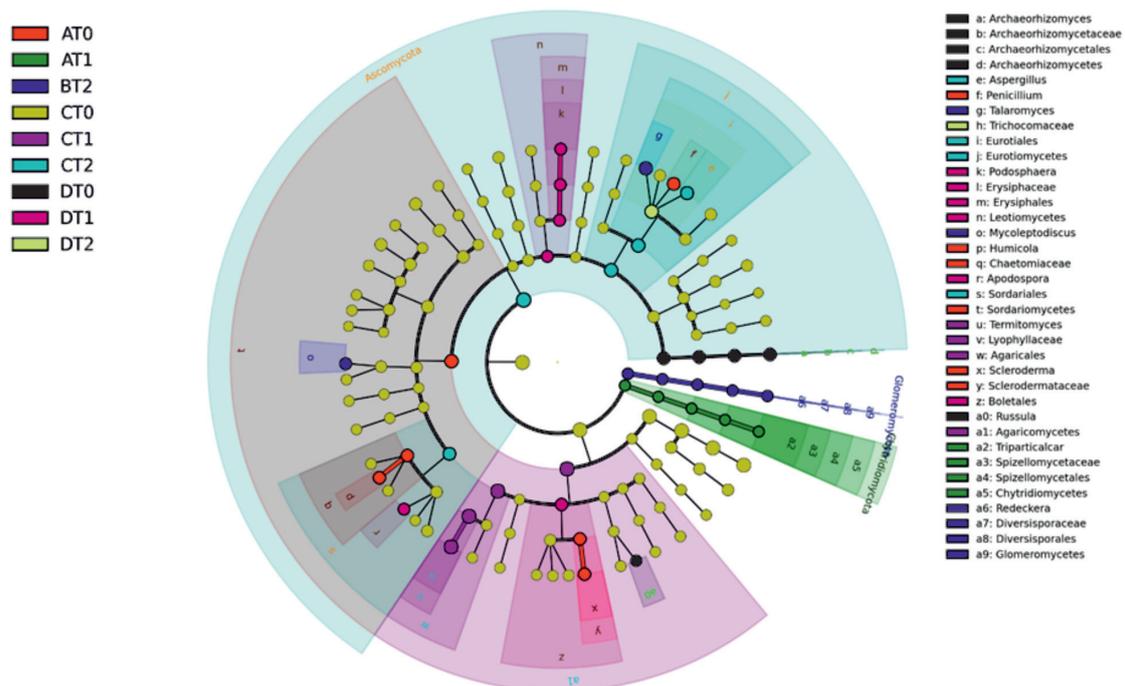


Fig. 2. Cladogram indicating the phylogenetic distribution of fungal lineages under the fertilization regimes, with each circle's diameter proportional to the given taxon's relative abundance; differences are represented in the color of the highest abundant taxa (AT0, AT1, BT2, CT0, CT1, CT2, DT0, DT1, and DT2 are all indicated with varying colors).

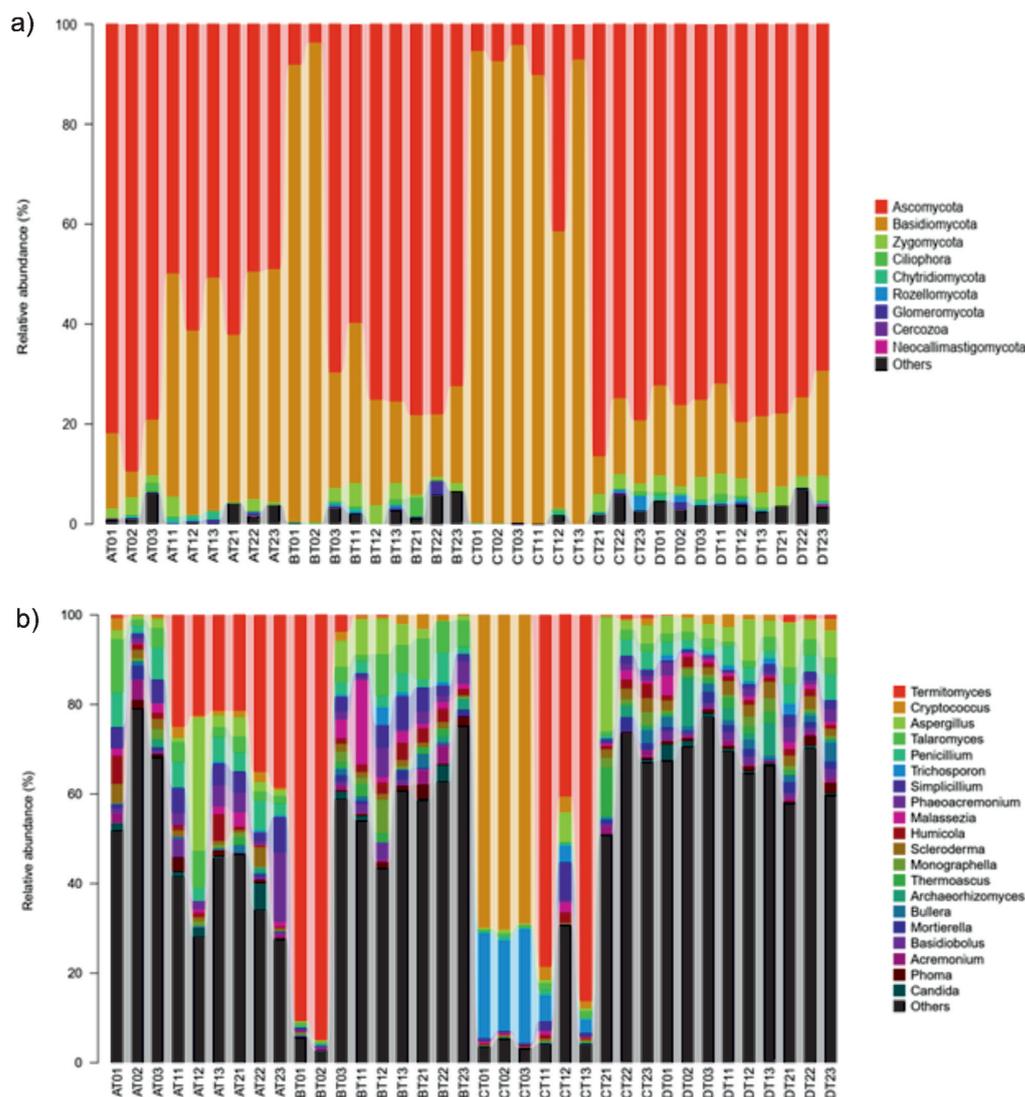


Fig. 3. Relative abundances of fungal phyla a) and genera b) under different fertilizer treatment regimens.

( $\text{g}\cdot\text{kg}^{-1}$ ), pH ( $\text{CaCl}_2$ ) and EC ( $\mu\text{S}\cdot\text{cm}^{-1}$ ) were all tested and results are represented in Table 2. A total of 1,662,207 reads with an average length of 300bp were obtained after Miseq Illumina sequencing. Using QIIME, a total of 4339 OTU's were generated, with 97% identity as the cutoff. BT03, DT02 and DT12 were the three most abundant OTU's (Fig. 1 and Supplementary Fig. 1). Furthermore, a considerably higher number of OTU's was in DT01, DT01, DT02, DT03, DT11, DT12, DT13, DT21, DT22 and DT23 – all which fall on 40 years of continuous cotton cropping. Using a GraphAn toolkit, hierarchical tree on taxonomic composition and abundance distribution of the soil mycobiome was generated (Fig. 2). Ascomycota was the most dominant fungus phyla, followed by Basidiomycota. Furthermore, under Ascomycota, Sordariomycetes, Dothideomycetes and Eurotiomycetes were the top three most dominant groups while Agaricomycetes and Tremellomycetes were the most abundant groups under Basidiomycota (Fig. 2).

Through QIIME, Chao 1, ACE and Shannon indices was used to assess the alpha diversity of the microbial communities present in the soil samples used in this present study (Table 3). In brief, Chao 1 and Ace indices quantify the microbial community richness, whereas Shannon and Simpson indices give more sensitivity to the microbial richness together with rare OTU's and microbial evenness in addition to dominance. Fungal richness and diversity continuously increased with respect to more addition of biochar (Table 3). The characterized sequences for each fertilizer treatment were associated with the dominant fungal phyla: Ascomycota, Basidiomycota and Zygomycota were the leading group in almost all the fertilizer treatments. Specifically, Ascomycota was the most predominant group in DT01 to DT23 treatments, while other remaining sequences were associated with other less dominant fungal phyla (Fig. 3a). The main genera across all the treatment groups were Termitomyces, Cryptococcus, Aspergillus and Talaromyces, among

Table 3. Diversity index values by Chao 1, ACE, Simpson and Shannon methods for every fertilizer treatment.

Years/ Indices	0 t·ha <sup>-1</sup>			20 t·ha <sup>-1</sup>			40 t·ha <sup>-1</sup>		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
5	AT01	AT02	AT03	AT11	AT12	AT13	AT21	AT22	AT23
Chao 1	72.00	163.43	119.00	75.43	65.50	76.11	110.33	138.17	97.00
ACE	72.08	163.54	119.90	76.43	66.21	76.78	111.13	138.58	97.00
Simpson	0.96	0.76	0.98	0.91	0.86	0.92	0.93	0.87	0.83
Shannon	5.23	4.19	6.03	4.47	3.88	4.54	5.09	4.99	4.07
10	BT01	BT02	BT03	BT11	BT12	BT13	BT21	BT22	BT23
Chao 1	80.31	64.00	334.47	146.67	71.60	81.50	120.50	94.33	94.86
ACE	81.91	65.68	333.69	147.97	72.11	81.78	120.63	93.71	96.18
Simpson	0.18	0.10	0.98	0.97	0.96	0.97	0.97	0.98	0.97
Shannon	0.95	0.56	7.06	6.17	5.04	5.64	5.84	5.79	5.66
20	CT01	CT02	CT03	CT11	CT12	CT13	CT21	CT22	CT23
Chao 1	52.60	79.11	47.00	56.25	57.11	59.25	185.60	150.50	135.00
ACE	52.97	79.49	47.20	57.00	60.26	59.50	185.52	151.02	135.35
Simpson	0.46	0.47	0.46	0.38	0.80	0.26	0.92	0.98	0.98
Shannon	1.47	1.71	1.40	1.63	3.41	1.21	5.26	6.26	6.26
40	DT01	DT02	DT03	DT11	DT12	DT13	DT21	DT22	DT23
Chao 1	190.20	320.15	197.30	199.67	318.36	221.55	126.00	169.38	165.00
ACE	190.09	319.48	198.07	200.41	318.09	218.13	126.28	170.03	165.27
Simpson	0.98	0.99	0.98	0.99	0.98	0.98	0.98	0.99	0.99
Shannon	6.63	7.26	6.73	6.94	6.95	6.53	6.16	6.68	6.74

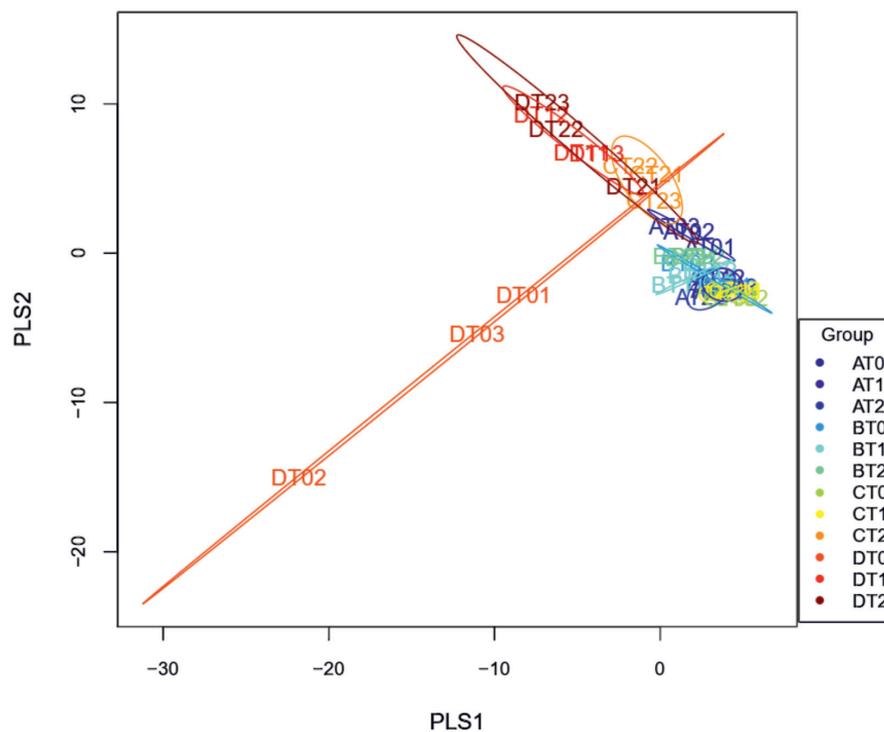


Fig. 4. PLS-DA discriminant analysis chart.

others (Fig. 3b). The partial least squares discriminant analysis (PLS-DA) indicated that AT0, AT1, AT2, BT0, BT1, BT2, CT0, Ct1 and CT2 scattered closely on the right, whereas the DT01, DT02 and DT03 groups scattered diagonally across the x and y axis (Fig. 4). There was an overlap observed between the DT and CT groups, while also a second overlap and cluster was seen in the At and BT groups, indicating a similarity degree of fungal diversity between the groups.

Generally, soil harbors diverse microbiome. This microbiome structure and diversity varies from one environ to another. This variation state of soil community structure can be influenced by the type of soil [11-12], soil pH [13-16], environmental climate [7, 17], soil electrical conductivity (EC) [13, 18], nutrient availability [19] and plant diversity [20]. Equally, soil microbiome has been linked to playing a significant part in defining some soil functions such as nitrogen (N) mineralization, carbon (C) turnover rate and pest control [21-23]. Biochar, a pyrolysis product of organic material, has got extensive interest as a way to enhance soil nutrient availability and subsequently improve crop productivity. Based on various previously conducted studies, soil biochar treatments have been shown to improve soil microbial communities by enriching the physical and chemical characteristics of the soil [24-26], providing conducive habitats for microorganisms against predators [27], contributing labile C substrates for degradation [28-29], boosting macronutrient availability such as N and P [25, 30], or even adsorbing compounds that would hinder microbial growth [31].

All along the chrono-sequence age, variations in the soil fungi types was apparent at taxonomic levels. Our findings demonstrate that the application of biochar on continuously cropped cotton soils (5,10,20 and 40 years) significantly altered soil myco-biota communities. From Chaol and ACE indices, our results indicate that continuous and quantity increase of biochar in cropped cotton soil treatment significantly increased the fungal richness (Table 1). Previously conducted studies have reported on the positive effect of organic matter application in cropped soils through improvement of soil pH, organic C and the major soil nutrients of N, P and K [32]. Sequence analyses of our data revealed that the dominant recovered fungal sequences were the phyla Ascomycota and Basidiomycota (Figure 2a). The relative predominance of Ascomycota (57.9%) was higher in fields with continuous high biochar quantity treatment compared to fields with lower treatment quantities, while for Basidiomycota (36.5%) it was less. In agreement with our results, the majority of saprotrophic microfungi are Ascomycota and their growth tempo is associated with the availability of nitrogen [14, 33]. Basidiomycetes are generally identified as lignin decomposers, hence they are vital for soil carbon cycling [34]. Furthermore, a study conducted by Schadt et al. also identified a large percentage of Ascomycota as the most common fungal sequences in tundra soils [35]. In addition, *Aspergillus*, a pathogenic

fungus, was also detected in all our treatments (Fig. 3b). *Aspergillus* is a known plant pathogen responsible for various plant rot cases [36]. Since fungal species vary with respect to their pH tolerance, soil pH changes could shape fungal community composition. Over the continuous cropping duration, the soil pH gradually declined (Table 2). At 20 years of continuous cropping, the number of fungal OTU's started to increase and their highest overall quantity was achieved at 20 years of continuous cropping when the pH was  $7.3 \pm 0.1$ . Our findings are in line with previously conducted studies that have described the association of higher fungal diversity increases to a declining soil pH gradient [37].

## Conclusion

This work highlights results on changes of soil fungi during long continuous cropping patterns, specifically for cotton as the crop of use. Our findings demonstrate that the application of biochar on continuously cropped cotton soils (5,10,20 and 40 years) significantly altered soil fungal communities. The most abundant fungal phyla were Ascomycota and Basidiomycota. In addition, *Aspergillus*, a pathogenic fungus responsible for various plant diseases, was also detected in all our sequence data. Soil pH changes were also identified as a key factor in shaping soil fungal communities. Over the continuous cropping duration, the soil pH gradually declined. At 20 years of continuous cropping, the number of fungal OTU's started to increase and their highest overall quantity was achieved at 20 years of continuous cropping, when the pH was  $7.3 \pm 0.1$ . Overall, our study findings show that biochar application over a long continuous cropping system alters soil fungal diversity.

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## Conflict of Interest

The authors declare that there is no conflict of interest between them or their various institutions.

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### Supplementary

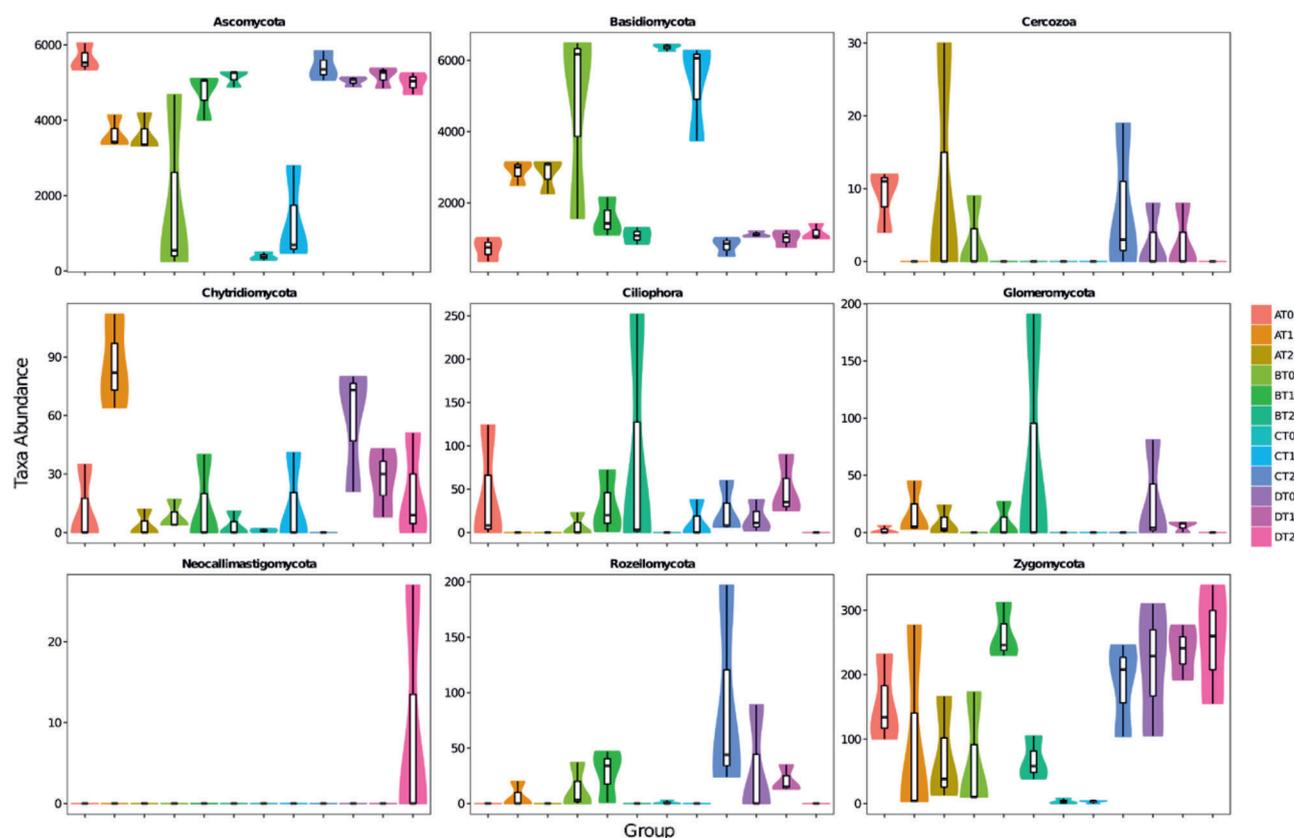


Fig. 1. The most dominant phyla across all the treatment groups.