

EFFECTS OF BIOCHAR ON THE DIVERSITY AND COMMUNITY STRUCTURE OF SOIL FUNGI IN INTERCROPPING SYSTEM

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Abstract. In order to investigate the long-term effects of biochar application on the diversity and community structure of soil fungi, different amount of biochar were applied to the *Machilus pauhoi-Ilex asprella* intercropping system under field conditions. The results showed that biochar significantly increased the pH and the content of organic matter, total nitrogen, total phosphorus, available phosphorus and available potassium in 0-15 cm soils while it had no significant effect on 15-30 cm soil physicochemical properties. The results of species annotation indicated that each biochar treatment increased the relative abundance of *Mortierellomycotina*, *Chytridiomycota*, *Glomeromycota* and *Ascomycota* in 0-15 cm soils. The T2 and T3 treatments increased the abundance of *Ascomycota* in 15-30 cm soils and reduced the abundance of *Basidiomycota*, which were opposite to the results of T1 treatment. At the genus level, in the soil with the depth of 0-15 cm, the application of biochar reduced the relative abundance of *Clitocybula* while significantly increased the relative abundance of *Parascutellinia*, *Glomus*, *Mortierella*, *Funneliformis* and *Plectosphaerella*. In 15-30 cm soils, all biochar treatments increased *Clitocybula* and *Mortierella* relative abundance, but reduced *Glomus* and *Gyroporus* relative abundance. In summary, biochar application has a significant effect on soil physicochemical properties after 3 years of application.

Keywords: biochar, physicochemical properties, fungal community structure, high-throughput sequencing, RDA analysis

Introduction

Soil microbes are the basis for material recycling and utilization in ecosystems, and are also a guarantee for ecosystems to maintain stability and normal functioning (Chapin et al., 1997). Soil, as a site for soil microbial activity, has an important impact on microbial composition and activity (Kuramae et al., 2011; Jeanbille et al., 2016). As an important member of soil microbes, fungi play an important role in the decomposition of soil, especially in the degradation of plant residues in the soil (Barbi, et al., 2016). Some soil fungi play a huge role in improving plant stress resistance and maintaining normal plant growth (Mukerji et al., 1996; Filion et al., 1999). Fungi drive the material cycle and energy flow in soil ecosystem. The diversity of soil microbial community structure can reflect the change process of soil environmental quality in advance (Sharma et al., 2010). All soil physical and chemical properties, temperature, moisture, etc., have an effect on soil microbial communities, leading to changes in microbial composition and structure (Alvarado et al., 2010).

Biochar is a stable soil conditioner that biomaterials undergo pyrolysis in a high temperature and oxygen-free environment to form organic carbon-rich (Johannes et al.,

2015). Biochar porosity and adsorption increase soil nutrients and water content (Schulz et al., 2013), providing a direct habitat for soil microbes (Gul et al., 2015). Biochar plays an important role in regulating soil microbial community structure and diversity due to its special physical and chemical properties (Xu et al., 2016; Kolton et al., 2011). Yao et al. (2017) found that the addition of biochar to black soil significantly increased the relative abundance of *Guehomyces* and reduced the relative abundance of *Fusarium*. Zheng et al. (2016) found that biochar significantly increased the relative abundance of Zygomycota and Mortierella fungi in paddy soils, and reduced the relative abundance of *Penicillium* and *Cyphellophora* fungi degree. In addition, biochar can affect the colonization of mycorrhizal fungi in crop roots. Matsubara et al. (2002) found that adding coconut shell biochar to soil increased the abundance of asparagus root mycorrhizal fungi. Studies have shown that activated carbon components of biochar can be degraded at the beginning of application, providing a source of carbon for microorganisms and promoting microbial growth (Sagrilo et al., 2015; Smith et al., 2010). Kuzyakov et al. (2009) found that biochar-derived carbon in soil microbes decreased by 42.31% after biochar application for 20 months. Over time, the physical and chemical properties of biochar would change, and its long-term interaction with soil particles would create a new soil-biochar system (Jones et al., 2012). However, the current impact on soil microbes after long-term application of biochar is not clear. Therefore, research on the long-term effects of biochar on soil fungi is of great significance.

In this study, the southern red soil was used as the research object, and the effects of biochar on soil physical and chemical properties and fungal community structure after 3 years of biochar application were measured. The changes of soil physical and chemical properties and soil fungal community succession were analyzed. The study aimed to clarify the effects of biochar on fungal communities and explore the relationships between soil properties and soil fungal communities, hoping to provide a theoretical basis for biochar to improve soil biological characteristics.

Material and method

Experimental material

The soil used in this study was the surface soil (0-20 cm soil layer) in the trees garden of South China Agricultural University in Guangzhou, Guangdong province, and the Guangzhou red soil at lower layer, which were mixed according to the soil thickness of the actual woodland. The basic physical and chemical properties of the soil are shown in *Table 1*.

Biochar was supplied by Shaanxi Yixin Bioenergy Technology Development Co., Ltd., and was milled to pass a 3 mm sieve by thermal cracking of discarded fruit tree trunks and branches in a cracking furnace and oxygen-limited environment (450 °C).

Table 1. Basic physical and chemical properties of soil

Soil samples	pH	Organic matter (g/kg)	Total N (g/kg)	Total P (g/kg)	Total K (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
Woodland surface soil	5.65	15.25	0.305	0.248	2.763	19.32	9.95	36.67
Layer red soils	6.05	3.16	0.203	0.165	1.702	11.59	3.20	20.08

The tested plants were 20 cm *Machilus pauhoi* seedlings and 35 cm *Ilex asprella* seedlings, and both plants were selected with good growth and consistency.

Experiment design

This experiment was carried out in outdoor concrete pools in the Resource and Environment College of South China Agricultural University, Guangzhou (N04°09'44.20", E113°21'57.75") from 2014 to 2017, with a marine climate of the south subtropical monsoon, a annual average temperature of 21.7 °C and a annual precipitation of 1689.3 mm to 1876.5 mm. The concrete pool has a volume of 1 m × 1 m × 2 m and an area of 2 m² (It was about 1 m wide, 2 m long and 1 m deep). There are 12 concrete pools, and each concrete pool serves as a experimental plot. The experiment adopted a randomized block design, and a total of 4 gradients of biochar treatments were set: 0 kg (CK), 1.2 kg (T1), 2.4 kg (T2), 4.8 kg (T3) (the forest charcoal application standard is 12 t/hm², which is equivalent to 1.2 kg in this experiment). First, each concrete pool was filled with 80 cm of Guangzhou red soil at the bottom, and then added with the woodland surface soil in the upper part. In 2014, biochar was applied to the surface soil followed by tumbling with tools. Each process was repeated 3 times and all processes were randomly arranged. The soil was pressed to the predetermined height and the conditions of soil were close to that of the actual forest soil. Urea, superphosphate and potassium sulfate was used as N fertilizer, P fertilizer and K fertilizer, respectively, with fertilization ratio at 261:150:181. After conversion according to the number of seedlings, base fertilizer which was composed of 41.76 g urea, 24 g superphosphate and 28.96 g potassium sulphate was added in one concrete pool. Topdressing was carried out with the same fertilizer composition one year later. Each concrete pool was equipped with automatic sprinkler irrigation system. It was irrigated every 3 days at 18:00 in dry season (October-March next year). According to the monthly irrigation allocation, the amount of irrigation was about the average daily rainfall of that monthly rainfall (600 mm). In case of rainy days, the irrigation time was adjusted artificially.

After biochar application, the concrete pools were provided with intercropping system of *Machilus pauhoi* and *Ilex asprella* at a ratio of 9:6. According to the plum planting pattern, the seedlings of *Machilus pauhoi* and *Ilex asprella* were transplanted, as shown in *Figure 1*.

Soil sampling and physical and chemical properties determination

Collection and determination of soil samples: After the seedlings were planted, in March 2017, a 5-point sampling method based on S-shape curve (Smalla et al., 2001) was used to collect soil with the depth of 0-15 cm and 15-30 cm. The five samples from the same depth range were mixed into one. The fresh soil sample was removed from the crop residue and stone by 2 mm sieve, and then divided into two parts. One part was placed in a sterile centrifuge tube and quickly placed in liquid nitrogen for the extraction of soil total DNA and subsequent bioinformatics analysis. The other part was naturally dried in the room and used for the determination of other physical and chemical properties of the soil.

The pH of the soil (water: soil = 2.5:1) was measured with potentiometer. Soil organic matter content was measured by the potassium dichromate volumetric method. Soil total nitrogen mass fraction was determined by Kjeldahl apparatus. Soil total

phosphorus, total potassium, available phosphorus, available potassium, and alkaline nitrogen content were determined according to soil agrochemical analysis methods (Bao, 2000).

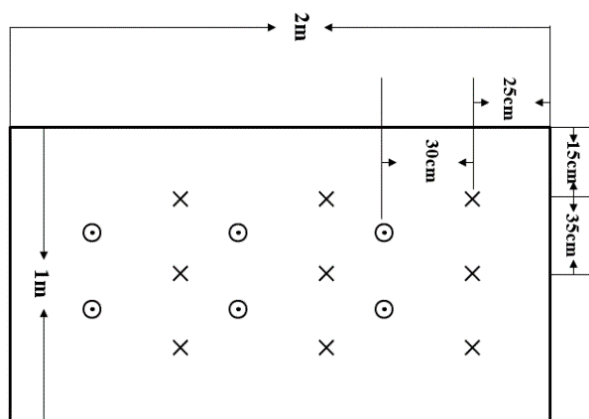


Figure 1. Experimental planting pattern. (X: *Machilus pauhoi*; O: *Ilex asprella*)

Determination of fungi community structure in the soil

Total DNA extraction and PCR amplification

The total DNA in the soil was extracted using a Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, U.S.A.) extraction kit. The integrity and concentration of the extracted DNA were examined using the Qubit 2.0 DNA kit. After passing the assay, the ITS2 region of the total DNA of the fungi was amplified using ITS3 and ITS4 primers (Huang et al., 2006).

Database construction and sequence processing

After purification of the PCR amplification product, all samples were mixed according to the DNA concentration in a ratio of 1:1, and a small fragment library was constructed for Paired-end sequencing using the Illumina (MiSeq PE300) sequencing platform of Guangdong Meige Gene Technology Co., Ltd. According to the similarity threshold of 97%, OTU were divided and clustered with the USEARCH (version 7.1) software for all qualified sequences. The sequence with highest abundance was selected as the representative sequence in each OTU. All representative sequences were species-annotated at the phylum and genus level using the RDP Classifier (classification threshold > 0.8) (Quast et al., 2013).

Data processing and analysis

The Shannon index, Chao1 index and sequencing coverage of samples were calculated using Mothur software (Pitta et al., 2014). Using SPSS software, soil physical and chemical indicators, α diversity of fungi communities and relative abundance of species at different taxonomic levels were analyzed by one-way ANOVA and Multiple comparisons were carried out by Duncan method ($P < 0.05$). The effect of environmental factors on the difference in fungi community structure between samples was analyzed by redundant ordering analysis (RDA). The visualization of RDA is plotted using the “vegan” package of the R software (version 3.3.1).

Results and analysis

Effect of biochar on soil physical and chemical properties

As can be seen from *Table 2*, the application of biochar significantly increased the soil pH in depth of 0-15 cm compared to the control group, while had no significant effect on the pH of soil in 15-30 cm. The contents of soil organic matter and available potassium in the depth of 0-15 cm and 15-30 cm increased with the increase of biochar addition. In general, biochar could significantly increase the content of total nitrogen, total phosphorus and available phosphorus in the soil depth of 0-15 cm, but no significant difference was observed under different biochar additions. In addition, biochar had no significant effect on the content of organic matter, total nitrogen, total phosphorus, total potassium, alkali nitrogen, available phosphorus and available potassium in the soil depth of 15-30 cm. The total nitrogen content of soil as well as total phosphorus, total potassium, alkali nitrogen and available phosphorus in 0-15 cm soil increased first and then decreased with the increase of biochar addition.

Table 2. *Effects of biochar addition on soil physical and chemical properties*

Soil samples	Treatment	pH	Organic matter (g/kg)	Total nitrogen (g/kg)	Total phosphorus (g/kg)	Total potassium (g/kg)	Alkali nitrogen (mg/kg)	Available phosphorus (mg/kg)	Available potassium (mg/kg)
0-15 cm	CK	6.07±0.08a	9.56±2.00a	0.45±0.05a	0.42±0.07a	4.92±1.11a	31.66±5.76a	20.25±7.72a	56.47±11.65a
	T1	6.17±0.01ab	15.9±4.13bc	0.64±0.17b	0.54±0.06b	6.73±1.7ab	43.1±10.24a	28.8±6.53b	80.81±2.67ab
	T2	6.31±0.05ab	17.12±4.15bc	0.65±0.15b	0.49±0.02ab	6.72±0.3ab	43.48±4.12a	25.82±1.58ab	85.59±27.14ab
	T3	6.52±0.07bc	17.99±4.63bc	0.59±0.11ab	0.5±0.08ab	5.12±1.07a	36.81±6.87a	31.33±13.73b	89.67±24.29ab
15-30 cm	CK	6.57±0.08abc	3.77±0.36a	0.20±0.07a	0.26±0.02a	2.47±0.06a	16.02±0.57a	3.32±0.38a	35.53±9.05a
	T1	6.44±0.07ab	4.02±0.5a	0.21±0.01a	0.27±0.03a	2.46±0.2a	16.02±1.51a	3.57±1.38b	34.63±11.11a
	T2	6.47±0.02ab	4.47±1.32ab	0.22±0.03a	0.28±0.01a	2.76±0.2a	19.07±2.58a	3.55±0.76ab	48.62±12.17ab
	T3	6.57±0.03abc	5.63±3.13ab	0.19±0.09a	0.30±0.09ab	2.65±0.59a	16.97±5.5a	8.18±8.85a	69.59±19.1bc

Different letters indicate significant differences ($p < 0.05$) among different treatments.

The OTU of soil samples

A total of 1,193,794 sequences were obtained from 24 soil samples. After optimization, the sequence sequencing amount of each sample was between 40,000 and 55,000. The OTU was clustered at 97% similarity level and the dilution curve of each sample was prepared (*Fig. 2*), and 9,265 OTUs were obtained. The curve gradually becomes flat and the sequencing become saturated, indicating that the library construction was reasonable, and the number of sample sequences was sufficient to reflect the species diversity.

The Venn map can reflect the number of common and unique OTUs between groups or between samples, visually showing the similarity and overlapping between groups or samples. The OTU was analyzed with 97% similarity, and the number of OTUs could also represent the number of strains.

Figure 3 presents the mixed clustering result of the OUT representative sequences of 4 groups of samples in the depth of 0-15 cm and 15-30 cm. It can be seen from *Figure 3* (left) that 744 OTUs can be identified for the soils in the depth of 0-15 cm, of which 440, 448, 545, and 511 OTUs fall in the CK, T1, T2, and T3 communities, respectively. The number of fungi OTUs shared by different treatments was 279. Each treated soil had its unique fungi OTUs, the number of which in CK, T1, T2 and T3 group was 39, 28, 83 and 70, respectively. The number of unique fungi OTUs under T2 and T3

treatments was larger than that of the control group, indicating that the application of biochar could lead to the change of fungi specificity in the soil to a certain extent. Although biochar treatment can increase the unique fungi species in the soil, the increased species varies with the application amount of biochar.

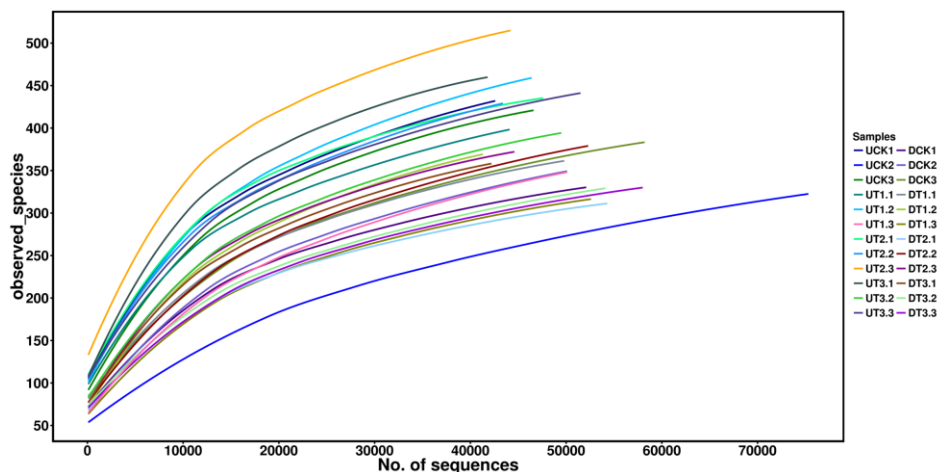


Figure 2. Rarefaction curve of fungal community in soils. CK, T1, T2 and T3 represent different amounts of biochar, U and D represent soil depths of 0-15 cm and 15-30 cm, respectively

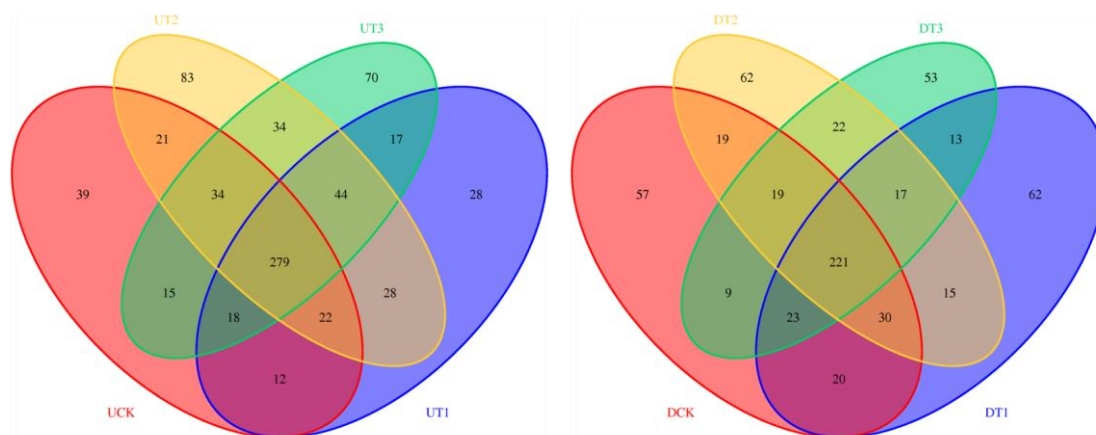


Figure 3. Venn diagram of fungi in 0-15 cm (left) and 15-30 cm (right) of soil layers with biochar addition

It can be seen from *Figure 3* (right) that 642 OTUs can be identified in the soils with the depth of 15-30 cm, of which 398, 401, 405, and 377 OTUs fall in the CK, T1, T2, and T3 communities, respectively. The number of fungi OTUs shared by different treatments was 221. Each treated soil had its unique fungi OTUs, the number of which in CK, T1, T2 and T3 group was 57, 62, 62 and 53, respectively. The number of unique fungi OTUs under T1 and T2 treatments was larger than that of the control group, indicating that the application of biochar can lead to the change of fungi specificity in

the soil with the depth of 15-30 cm to a certain extent as well. Although biochar treatment can increase the unique fungi species in the soil, the increased species varies with the application amount of biochar.

Alpha diversity analysis

The Observed species index and the Chao1 index are used to measure the richness of the community in the sample, i.e., the number of single species (the number of OTUs), without involving the abundance of each species in the community. The larger the value is, the richer the species in the sample. Shannon index, Simpson index and PD whole tree reflect the diversity of the community, which is affected by the species richness and species uniformity in the sample community. The larger the value of Shannon index, PD whole tree and Simpson index indicates the higher diversity of the community in the samples.

It can be seen from *Table 3* that in the 0-15 cm soils, the fungi community richness under biochar treatment is higher than that of the control group, which is consistent with the dilution curve. The sequence of chao1 index and the Observed species (OTUs index) were respectively $T2 > T1 > T3 > CK$ and $T2 > T3 > T1 > CK$; the chao1 index of 15-30 cm soils under each biochar treatment was slightly lower than that of biochar treatment, but the difference was not significant ($P > 0.05$). The difference in Observed species (OTUs index) was also not significant as well. In general, the difference in the abundance of fungi community in the soil with the depth of 15-30 cm was not significant under different treatment conditions.

For different biochar treatment groups, the order of PD whole tree value and Shannon index of soils with the depth of 0-15 cm were $T2 > T3 > T1 > CK$. T2 had a higher Simpson index than T3, and both T2 and T3 treatment had a higher Simpson index than T1 and CK. The diversity of soil fungi community under charcoal treatment was higher than that of the control group, and it reaches the peak value under T2 treatment. In the 15-30 cm soils, the PD whole tree value, Shannon index and Simpson index under T1 treatment were slightly higher than those of the control group. Overall, there was no significant difference in fungi community diversity among the 15-30 cm soils.

The fungi dominance index of the 0-15 cm soil after T1 treatment was higher than that of the control group while the index after T2 and T3 treatment were smaller than that of control group. For 15-30 cm soils, the conclusions were just the opposite.

Comparing fungal Alpha diversity index of the 0-15 cm soils with that of 15-30 cm soils, it can be seen that in the 0-15 cm soils, except for the dominance index, other indexes were higher than those of 15-30 cm soils.

Table 3. Alpha diversity index of soil fungal with biochar application treatment

Soil depth	Treatment	PD whole tree	Chao1 index	Dominance index	Observed species (OTUs index)	Shannon index	Simpson index
0-15cm	CK	18.00±4.36ab	516.74±85.92ab	0.24±0.11ab	363.33±96.13ab	3.54±1.21a	0.76±0.11ab
	T1	18.67±3.06ab	536.85±44.15ab	0.27±0.32ab	387.33±59.58ab	3.91±1.72a	0.74±0.32ab
	T2	22.00±1.73b	566.89±67.11b	0.08±0.03b	451.33±49.08b	5.07±0.48a	0.92±0.03b
	T3	20.00±2.65ab	516.88±34.05ab	0.18±0.12ab	416.00±42.51ab	4.33±0.94a	0.82±0.12ab
15-30cm	CK	16.33±0.58a	460.14±40.35a	0.28±0.04a	327.33±18.5a	3.41±0.23a	0.72±0.04a
	T1	17.00±1.73a	446.53±42.89a	0.26±0.11ab	336.67±37.54a	3.48±0.77a	0.74±0.11a
	T2	16.67±2.52a	441.40±57.63a	0.38±0.04a	333.67±41.2a	3.00±0.21a	0.62±0.04a
	T3	15.67±2.08a	434.77±23.22a	0.29±0.08ab	319.67±32.39a	3.40±0.52a	0.71±0.08a

Soil fungi community structure under different treatments

Relative abundance of fungal in the soil at phylum level under different treatments

Species annotation results showed that 13 of the fungi were detected in the 24 soil samples, including: *Mortierellomycotina*, *Chytridiomycota*, and *Glomeromycota*. *Ascomycota*, *Basidiomycota* and some fungi classified as undetermined. The sequence of relative abundance from high to low was 50.389% of *Ascomycota*, which was the dominant species, 32.01% of *Basidiomycota*, 10.17% of *Glomeromycota*, and 1.69% of *Mortierellomycotina*, and 0.62% of *Chytridiomycota*. Their relative abundances together accounted for more than 90% of the annotated species.

It can be seen from *Figure 4* that biochar has different effects on the abundance of different fungi at phylum level in soils with different depth. In 15-30 cm depth soil layer, *Basidiomycota* had the highest relative abundance, and *Ascomycota* ranked the second place. Biochar could increase the relative abundance of *Mortierellomycotina*, *Chytridiomycota*, *Glomeromycota* and *Ascomycota*, all of which peaked the maximum under T2 treatment. However, this phenomenon was not found in 15-30 cm depth soil. In 15-30 cm depth soil layer, the relative abundance of *Ascomycota* was the highest, followed by *Basidiomycota*. Compared with CK treatment, the abundance of *Ascomycota* increased while the abundance of the *Basidiomycota* decreased after T2 and T3 treatment, which was the opposite to T1 treatment. The decrease degree of the *Glomeromycota* increased with the amount of biochar application. And there was little difference in relative abundance of the *Chytridiomycota* under different treatment.

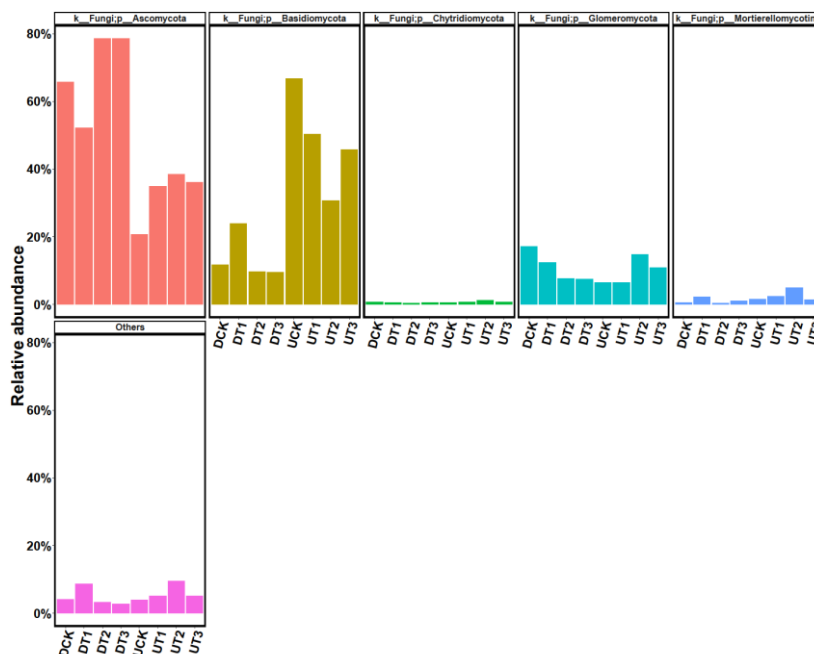


Figure 4. Relative abundance of fungal communities in soil at phylum level

Relative abundance of soil fungi at genus level under different treatments

As shown in *Figure 5*, the species annotation results at genus level indicated that the relative abundance of 12 genera was more than 1%. The relative abundance of *Parascutellinia* was the highest, accounting for 42.68% in all the species, followed by

clitocybula, accounting for 32.71%. The relative abundance of these two species was significantly higher than that of other species. In 0-15 cm soil, the application of biochar reduced the relative abundance of *Clitocybula*, which was the smallest under T2 treatment. However, biochar application significantly improved the relative abundance of *Parascutellinia*, *Glomus*, *Mortierella*, *Funneliformis*, *Plectosphaerella*. The relative abundance of *Parascutellinia*, *Glomus*, and *Mortierella* under T2 treatment were the highest, while that of *Funneliformis* and *Plectosphaerella* increased with the amount of biochar application.

In 15-30 cm soil, the variation of relative abundance of *Parascutellinia* was consistent with that of soil with the depth of 0-15 cm. The relative abundance of *Clitocybula* and *Mortierella* increased significantly under biochar treatment and peaks the maximum value under T1 treatment. The relative abundance of *Phaeococcomyces* and *Plectosphaerella* were the largest under T2 treatment. However, the relative abundance of *Glomus* and *Gyroporus* decreased under biochar treatment. The relative abundance of *Mycotribulus* and *Ostropa* were not significantly changed under different treatments.

For soils in different depth, the abundance of *Parascutellinia*, *Gyroporus*, *Glomus* and *Phaeococcomyces* in the soils with the depth of 15-30 cm were higher than that in the soils with the depth of 0-15 cm. On the contrary, the 0-15 cm soils contained more *Clitocybula*, *Funneliformis*, *Mortierella*, *Mycotribulus*, *Ostropa*, and *Plectosphaerella*.

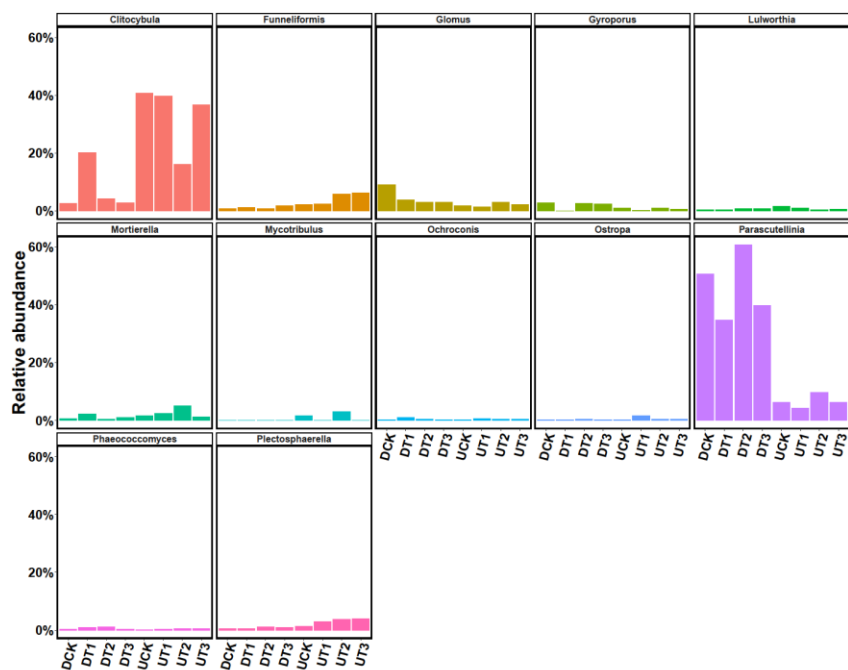


Figure 5. Relative abundance of fungal communities in soil at genus level

Cluster analysis on species relative abundance

Cluster analysis on species relative abundance at phylum level

Figure 6 is a species heat map of soil fungi at phylum level. The relative abundances of the species at each level are ranked, and the bacteria in the top 13 are selected (all the species are selected if the number of phylum is less than 13). The heat map is used to

cluster the abundance similarity between different treatments. The similarity and color gradient are used to reflect the difference and similarity of fungal community composition at each level. The darker the red is, the greater the abundance, while darker the blue is, the lighter the abundance. A color patch represents the abundance of a phylum in a sample. In the figure, the horizontal direction is the sample information, and the vertical direction is the species annotation information. The sample clustering tree lies in the upper part and the species clustering tree lies in the left part of the figure.

As can be seen from *Figure 6*, the species in each sample are: Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycotina, and Chytridiomycota, Zoopagomycotina, Mucoromycotina, Arthropoda, Blastocladiomycota, Kickxellomycotina, Entomophthoromycota, Cryptomycota and Annelida. The similarity between UT1 and UCK was the highest, and UCK, UT1, and UT3 were also clustered at the top. These three groups had higher abundances of Basidiomycota and Zoopagomycotina, but were far away from UT2, which indicated that, compared with the control group, the effects of T1 and T3 treatment on the fungi community structure of 0-15 cm soil were not obvious while the effect of T2 treatment on soil fungal community structure was significant. UT2 was clustered as a specific group, and, compared to UCK, the red colors corresponding to Mortierellomycotina, Chytridiomycota, Arthropoda, Cryptomycota, Glomeromycota, Mucoromycotina, and Kickxellomycotina in 0-15 cm depth soils under T2 treatment were much darker than other treated samples. However, the color representing Basidiomycota was shallower, indicating that the abundance of fungi community in 0-15 cm soils under T2 treatment was the highest, and the abundance of the above seven fungi could be significantly increased while the abundance of Basidiomycota was reduced.

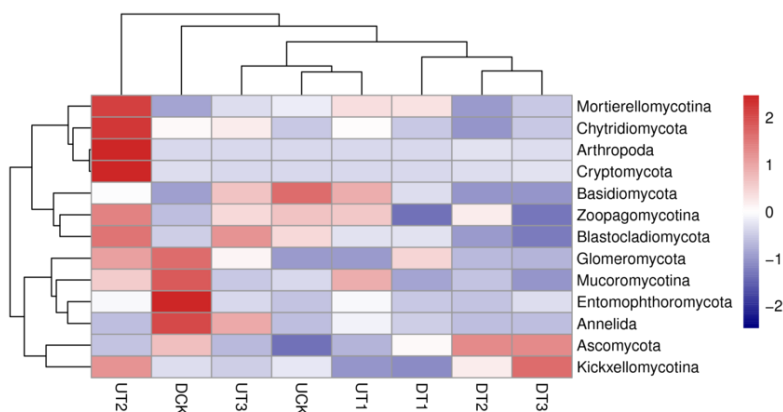


Figure 6. Thermal map of relative abundance of different fungal phylum in soil. (The sample clustering tree is the clustering tree above and the species clustering tree is the left cluster tree. Similarly hereinafter)

DT1, DT2 and DT3 are clustered at the top, but they are far away from DCK, indicating that biochar has a great influence on the fungal community structure of 15-30 cm soils. However, different biochar application rates resulted in similar fungi community in 15-30 cm soils. Compared with DCK, the color of *Ascomycota* and *Kickxellomycotina* in the DT2 and DT3 groups were relatively dark while that of *Glomeromycota*, *Mucoromycotina*, *Entomophthoromycota* and *Annelida* was relatively shallow, indicating that the T2 and T3 biochar treatment could increase the abundance

of *Ascomycota* and *Kickxellomycotina* while decrease the abundance of *Glomeromycota*, *Mucoromycotina*, *Entomophthoromycota* and *Annelida*.

It can be seen that the effects of different biochar treatments on the abundance of the same species are different. The same biochar treatment had different effects on the abundance of different types of species. Generally, the biochar treatment could increase the abundance of most fungi communities in 0-15 cm soils to some extent. And the effect of T2 treatment effect was the most obvious. Biochar application could reduce the abundance of most fungi communities in the 15-30 cm soils to some extent. In general, the abundance fungi in 0-15 cm soils were higher than that in 15-30 cm soils, which was consistent with the analysis of the relative abundance distribution in the previous section.

Cluster analysis on species relative abundance at genus level

Figure 7 is a species heat map of soil fungi at genus level. The relative abundances of the species at each level are ranked, and the species in the top 30 are selected (all the species are selected if the number of phylum is less than 30).

As can be seen from Figure 7, for different treatments, the clustering result at genus level is consistent with that at phylum level. Comparing the aggregation degree of fungi in 0-15 cm soils under different treatments, it could be found that the abundance of *Ostropa*, *Ochroconis*, *Capnobotryella*, *Muyocopron* and *Hydropisphaera* under T1 and T3 treatment were higher than those under CK treatment. The abundance of most fungi in T3 group were significantly higher than those in CK group, and were also higher than those in other groups. Compared to CK treatment, T1, T2 and T3 treatment had seen more abundant *Funneliformis*, *Plectosphaerella*, *Chaetomella*, *Peziza*, and *Hyaloraphidium*. Nevertheless, CK treatment resulted in higher abundance of *Lulworthia* and *Thamnocephalis*.

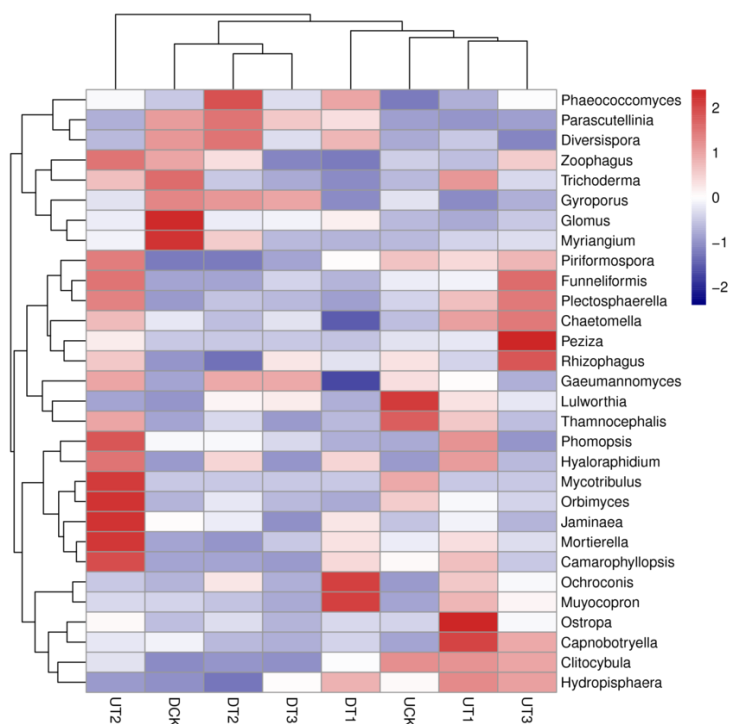


Figure 7. Thermal map of relative abundance of different fungal genus in soil

In 15-30 cm soil layer, the abundance of *Glomus*, *Myriangium*, *Capnobotryella*, *Zoophagus*, and *Trichoderma* were relatively low under the treatment of biochar. However, biochar could increase the abundance of certain fungi species. For example, compared with CK, the abundance of *Mortierella*, *Ochroconis*, *Muyocopron*, *Camarophylloopsis*, *Hyaloraphidium*, and *Jaminaea* were relative high under T1 treatment, which indicated that the application of low-dose biochar to the soil could increase the abundance of some fungi in the 15-30 cm soil layer.

Under the various treatments, there was significant difference in the fungi at genus level in 0-15 cm soil layer. The effect of biochar was prominent. The difference of fungi in 15-30 cm soil layer was relatively small.

Correlation between fungi community structure and soil environmental factors

Figure 8 shows the correlation between fungi at the genus level and soil environmental variables. The first and second axes of the RDA ordering accounted for 53.14% and 19.37% of the fungal genus variables, respectively. 0-15 cm soil and 15-30 cm soil samples were clearly separated in the direction of RDA1 axis. In the 0-15 cm soil samples, DCK and DT3 were separated from DT1 and DT2, indicating there was huge difference between T1 and T2 treatment and CK treatment. In the 15-30 cm soil samples, UCK and UT1 were clearly separated from UT2 and UT3, indicating that T2 and T3 treatments were significantly different from CK. In general, compared with soils with different depths, the points of 15-30 cm soils under different treatments were relatively close and concentrated while the points of 15-30 cm soils were far away from each other and the distribution was relatively scattered, indicating that response of fungi abundance to biochar in 0-15 cm soil was more sensitive.

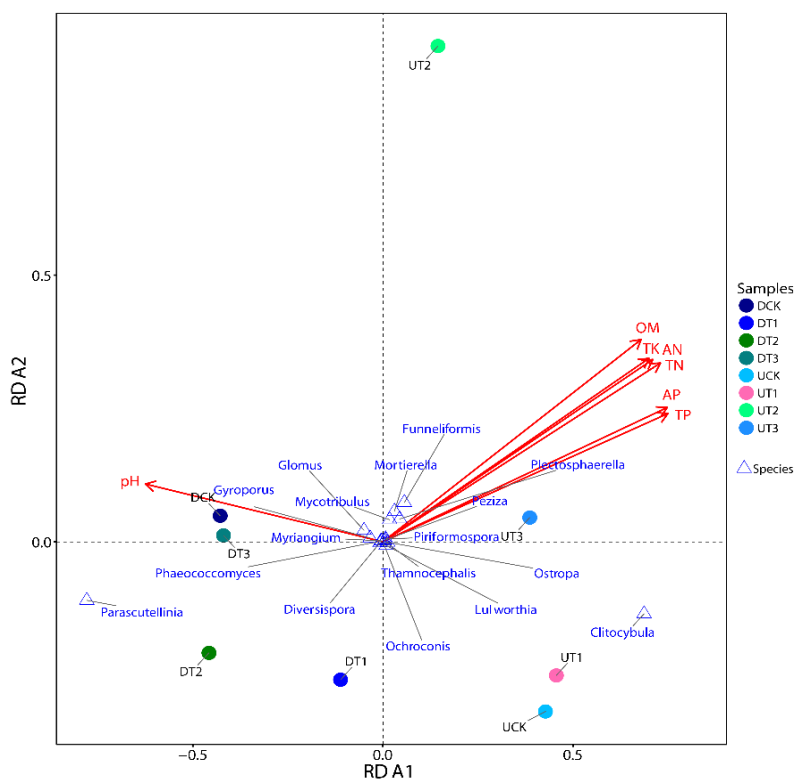


Figure 8. RDA triplot showing the relationship between the relative abundance of major soil fungal genus in different soil samples and the environmental variables

There was a significant positive correlation between the content of organic matter, total nitrogen, total phosphorus, total potassium, alkali nitrogen, available phosphorus and available potassium in the soil, but they were negatively correlated with soil pH. The abundance of *Mortierella*, *Funneliformis*, *Plectosphaerella*, *Peziza*, *Piriformospora*, *Ostropa*, *Lulworthia*, *Clitocybula* and *Thamnocephalis* were positively related to content of organic matter, total nitrogen, total phosphorus, total potassium, alkali nitrogen, available phosphorus, and available potassium, while were negatively correlated with soil pH. Among them, the abundance of *Plectosphaerella* had the largest correlation with the content of total phosphorus and available phosphorus, which corresponded to the farthest point from the origin, indicating that it was the most sensitive to the environment. *Piriformospora* was closest to the origin, indicating that it was the least sensitive to the environment. The abundance of *Glomus*, *Mycotribulus*, *Gyroporus*, *Myriangium*, *Phaeococcomyces*, *Parascutellini* were positively related to soil pH, where the *Gyroporus* was the most relevant and *Parascutellinia* was the most sensitive to the environment.

Discussion

After the biochar was applied, the soil microorganisms were directly or indirectly affected by changing the physical and chemical properties of the soil. Most studies had shown that biochar promotes microbial communities (Kolb et al., 2009; Ameloot et al., 2013). In this study, it was clear that after 3 years of application, the physical and chemical properties of the soil had undergone major changes. Soil pH, the content total nitrogen, total phosphorus and available phosphorus were increased. The abundance and diversity of fungi in the soil changed significantly with the application of different amounts of biochar.

From the number of unique fungi OTU and the relative abundance distribution of fungi communities as shown in the Venn diagram (Figs. 4 and 5), the application of biochar had changed the original fungal community structure, inhibited the growth of some original fungi species and increased the population of specific microorganisms that are more adaptable to the environment. Biochar had a void structure and a large specific surface area, as a result, the oxygen content was significantly increased as the application of biochar. The soil fungi had both anaerobic and aerobic groups, and their respective abundance was reduced or increased as the change of soil bulk density. The results of Marluthi et al. (2010) also showed that the application of biochar in soil changed the type of carbon source used by soil microbes. There were also differences in the number of fungi species when different amounts of biochar was applied, but the difference was more prominent in 0-15 cm soils than in 15-30 cm soils. The dilution curve also showed that biochar could increase the amount of soil OTUs, which was more significant in 0-15 cm soils, indicating that the effect of biochar on soil fungal community was mainly concentrated in the directly contacted soil layer (0-15 cm) while the impact on the lower soil layer was relatively slight. This might be related to the porosity and large specific surface area of biochar, which could provide a good attachment site and growth environment for soil fungi to promote the growth and reproduction of fungi and avoid predators as well (Thies et al., 2009).

According to the Alpha diversity analysis, biochar treatment could increase the abundance and diversity of soil fungal communities, which reached the maximum value under T2 treatment. When the biochar application rate was high (T3 treatment), the soil

fungal community abundance and diversity did not increase with the increase of the amount of biochar applied. The reason might lie in the fact that the pH of the applewood after carbonization was alkaline, thus adding biochar to the soil would increase the soil pH. It was reported that the higher soil pH could reduce the abundance of soil fungi (Rousk et al., 2010). Biochar with low addition could increase the dominance of fungi in 0-15 cm soil layer, but the effect was not increased as the application rate was increased to T2 and T3. High-volume biochar treatment was not conducive to improving the abundance of soil fungal communities, and the appropriate amount of application was beneficial to the construction of soil micro-ecological environment. Compared with the 15-30 cm soil layer, the fungi abundance and diversity in 15-30 cm soil layer were low, and the difference between the treatments was not obvious. This was also consistent with the OTU quantitative analysis results.

In the soils tested in this experiment, *Mortierellomycotina*, *Chytridiomycota*, *Glomeromycota*, *Ascomycota*, and *Basidiomycota* were dominant species at phylum level. Among them, *Ascomycota* and *Basidiomycota* accounted for more than 80%, which was consistent with previous studies (Roesch et al., 2007). In 0-15 cm soil, the relative abundance of *Mortierellomycotina*, *Chytridiomycota*, *Glomeromycota* and *Ascomycota* under the application of different amounts of biochar were higher than that of control group and were the highest under T2 treatment. Studies have shown that nitrogen application can increase the relative abundance of *Ascomycota* (Paungfoo-Lonhienne et al., 2015), probably because biochar's adsorption to nitrogen fertilizer indirectly increased soil nitrogen content (Clough et al., 2013), thereby increasing the relative abundance of *Ascomycota* in the soil. Different from 0-15 cm soils, the application of biochar could reduce the abundance of certain species, such as *Basidiomycota*, *Glomeromycota*, while increase the abundance of *Ascomycota* in 15-30 cm soils to some extent. This phenomenon was particularly significant under T2 and T3 treatment. It indicated that the dominant fungi in red soil were sensitive to biochar addition. Similarly, at the genus level, biochar also increased the relative abundance of dominant genus such as *Parascutellinia* and *clitocybula*.

Soil chemistry plays an important role in affecting soil microbial community structure (Teague et al., 2011). Previous studies have shown that soil fungi are closely related to many soil physical and chemical properties, such as soil pH (Fierer et al., 2006) and available nitrogen (Frey et al., 2004). Due to the different biological characteristics of the fungi, the soil factors affecting different fungi are also different (Avander et al., 2006). RDA is used to analyze the relationship among environmental factors (pH, soil nutrients), dominant species and different treatments. Due to the composition and structural specificity of biochar, different microbial communities often respond differently to the addition of biochar. In this experiment, the abundance of *Funneliformis*, *Ostropa*, *Piriformospora* and *Plectosphaerella* in soil were positively correlated with the content of organic matter, total nitrogen, total phosphorus, total potassium, alkali nitrogen, and available phosphorus in the soil while were negatively related to soil pH. The abundance of *Glomus*, *Mycotribulus*, and *Gyroporus* were positively correlated with soil pH, where *Gyroporus* had the maximum correlation. In general, neutral and alkaline conditions were considered to be beneficial to bacterial growth but were not conducive to fungi growth (Rousk et al., 2009). Xu et al. (2018) found that soil phosphorus closely affects fungal growth, and insufficient or excessive available phosphorus would inhibit fungi growth and development. In this study, soil total phosphorus and available phosphorus were significantly positively correlated with

Plectosphaerella and *Peziza*, while negatively correlated with *Parascutellinia* and *Phaeococcomyces*, which indicated that the fungi was selective for phosphorus levels, providing a valuable insight into the relationship between fungi diversity and phosphate fertilizer, as well as studies on fungal functional genes (such as genes controlling the phosphorus cycle). In addition, in this study, the interpretation rates of the first and second axes for sample changes were mostly lower than 65.0%, indicating that in addition to the soil physical and chemical indicators analyzed in this study, there might be other soil physical and chemical properties that jointly drove the changes in microbial structure.

The promotion of soil microbial abundance by short-term application of biochar is mainly caused by absorption of soluble carbon in biochar through soil microbe activities (Quilliam et al., 2013). However, although this study has not detected the soluble carbon content of biochar after application, many scholars have shown that the soluble carbon will be depleted by soil microbes within one year (Cheng et al., 2008; Kuzyakov et al., 2009; Wang et al., 2016). In addition, in this study, we found that changes in fungi community structure in soils were closely related to the physical and chemical factors including content of organic matter, total nitrogen, total phosphorus, total potassium, alkali nitrogen, available phosphorus, available potassium, and pH. And these factors were also significantly correlated with the amount of biochar (*Table 3*). In summary, the increase in soil abundance and the change in community structure in this study were indirectly driven by changes in soil physical and chemical properties caused by biochar application.

Conclusions

(1) After 3 years of biochar application, soil pH and nutrient content increased to varying degrees. Especially, soil pH, organic matter and available potassium increased proportionally to the biochar addition. Besides, the nutrient content in the upper layer of soil was significantly higher than that of the lower layer, which illustrated that biochar has a significant long-term effect on soil nutrient retention.

(2) Biochar could increase the amount of soil OTU. Different biochar treatments can lead to specific changes of fungi to a certain extent and increased the abundance and diversity of fungi communities, which reached the maximum value under T2 treatment.

(3) Biochar significantly changed (increased or decreased) the relative abundance of some soil fungi, which was reflected at both phylum and genus level.

(4) RDA analysis showed that the addition of biochar could significantly change the physical and chemical properties of soil pH and nutrient content after 3 a. The changes of these environmental factors further affected the soil fungal community structure. And at different depths, different biochar application addition and soil physical and chemical factors affected the abundance of different fungi species.

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