



# Biochar-based organic fertilizer application rates for *Tetrastigma hemsleyanum* planted under Moso bamboo

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**Abstract** The under-forest economy has received increased attention in China. However, little is known about the effects of co-composted biochar on soil and plant biomass in under-forest planting systems. In this study, plant biomass, soil nutrient levels, and bacterial communities were evaluated after application of biochar-based organic fertilizer (BOF, derived from co-composted biochar-compost) at varying rates to soils supporting *Tetrastigma hemsleyanum* Diels & Gilg planted under a Moso bamboo (*Phyllostachys edulis*) forest. BOF treatment increased the biomass of *T. hemsleyanum*. Compared with the control, BOF application significantly increased soil pH and organic carbon (SOC). The high-throughput sequencing results showed significant differences in the

Bacteroidetes, Verrucomicrobia, Chlorofexi, and OD1 phyla among all groups. At the genus level, the control group was characterized by a preponderance of *Conexibacter*. *Rhodanobacter* was enriched in soils with a 3% BOF application and *Steroidobacter* and *Spirochaeta* were the most prominent phyla in the 5% BOF group. There was no biomarker selected in the 1% BOF group at the genus level. In conclusion, BOF application increased the biomass of *T. hemsleyanum* when intercropped under a Moso bamboo forest; this effect may be due to changes in the soil physicochemical properties and microbial communities after BOF application.

**Keywords** Co-composted biochar · Under-forest economy · Plant biomass · Soil microbiota

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## Introduction

*Tetrastigma hemsleyanum* Diels et Gilg, *Vitaceae*, is a herbaceous perennial climber species native to China. *T. hemsleyanum* is found in tropical to subtropical areas in Asia, mainly in the few provinces of south China, viz. Zhejiang, Jiangxi, Guangxi, and Hunan (Chinese Flora Commission 2007). The root tubers and whole herbs are used as raw materials in Chinese medicine for the treatment of high fever, infantile febrile convulsion, pneumonia, asthma, hepatitis, rheumatism, menstrual disorders, sore throat, and scrofula. Due to over-exploitation, environmental deterioration, and difficulties in cultivation (Dai et al. 2009), *T. hemsleyanum* has become an endangered species.

The under-forest economy is a sector of agroforestry, a green, low-carbon, three-dimensional, cyclic, and sustainable economic system (Miao et al. 2015). In recent years,

the under-forest economy has received increased attention in China and is being vigorously developed (Chen et al. 2015). Research has indicated that establishing under-forest medicinal plant farming can protect rare medicinal plant resources, speed up construction and certification of standardized planting bases, and promote sustainable development of the medicinal plant industry (Du et al. 2014). Moso bamboo (*Phyllostachys edulis*) is an important plantation resource in southern China, covering an area of 4.43 million ha and accounting for 70% of the total area of Chinese bamboo forest (Song et al. 2011). It has been reported that *T. hemsleyanum* could be intercropped under bamboo forests (Wu et al. 2015).

Biochar is a carbon-rich product from the pyrolysis of bio-wastes and its use as soil amendment provides multiple benefits, including improved soil fertility, crop productivity (Liu et al. 2013; Pandey et al. 2016; Gao et al. 2017), soil structure, water retention (Omondi et al. 2016), suppression of plant diseases (Rogovska et al. 2017), and stabilization of potentially toxic metals (Oustriere et al. 2017) and organic pollutants (Oustriere et al. 2017). Negative effects of biochar on crop yields have also been reported (Van Zwieten et al. 2010; Andrew et al. 2013; Borchard et al. 2014a). These effects include phytotoxicity and nutrient imbalance (Ding et al. 2010; Taghizadeh-Toosi et al. 2011; Borchard et al. 2014b). These limitations may be overcome by combining the application of biochar with compost (Steiner et al. 2010; Fischer and Glaser 2012). However, little is known regarding the effects of co-composted biochar on soil and plant biomass in under-forest planting systems.

In this study, biochar-based organic fertilizer (BOF), derived from the combination of biochar and compost, was applied to soils supporting *T. hemsleyanum*. Diels & Gilg planted under a Moso bamboo forest. The objectives of this study were to investigate the effects of different application rates of BOF on plant biomass, soil nutrient levels, and bacterial communities. To our knowledge, this is the first study to investigate these metrics after different BOF applications in under-forest intercropping systems.

## Materials and methods

### Experimental site

This study was carried out at Quanwang Township (28°56'N, 118°55'E) in Qujiang District, Quzhou City, Zhejiang Province in southeast China. This region is a subtropical zone of humid monsoon climate with four distinct seasons. Annual rainfall is 1667 mm, and average annual temperature is 17.3 °C. The lowest recorded temperature is -10.4 °C and the highest is 40.5 °C. The

average annual frost-free period is 258 days and average cumulative annual daily sunshine is 1713 h. The elevation of the site is 500–560 m a.s.l. The soil is medium thick yellow brown earth and is fertile.

### Experimental design

Our BOF was produced by Zhejiang Raymond Agricultural Polytron Technologies Inc. (Hangzhou, Zhejiang). The BOF included nitrogen, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O at rates of 6%, 2.3%, and 2.5%, respectively. Organic matter content was greater than 40% and pH ranged from 5.8 to 6.0.

The sample site had been a moso bamboo forest for many years, with an average canopy density of 80%, average diameter of 9.8 cm, and an average plant height of 14.2 m. *T. hemsleyanum* was intercropped in 2014, and in 2015, was cut to 10 cm long stems for cuttage cultivation. At that point, the *T. hemsleyanum* plantlets were obtained. In April 2016, we dug soil from the moso forest, placed it in non-woven bags of approximately 10 L capacity, and then mixed it with 0% (control), 1%, 3%, and 5% BOF. Each group had three replicates, each replicate had ten bags, and each bag had two *T. hemsleyanum* plantlets. During the experiment, irrigation, grass and pest control were performed.

### Plant biomass

We collected roots, stems, and leaves of the *T. hemsleyanum* plantlets at the close of the study for analyses. The roots, stems and leaves are henceforth called dry matter. The dry matter was dried in an oven at 105 °C for 30 min, and then the temperature was set to 70–80 °C to obtain a constant weight.

### Soil physicochemical analysis

Soil samples from non-woven bags were analyzed for soil physicochemical properties and microbial communities in October 2016. Soil pH was determined with a glass electrode using a soil-to-water ratio of 1:2.5 (w/v). Determination of soil organic carbon (SOC) was performed by means of the K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> oxidation–reduction titration method. Determination of the soil total nitrogen (TN) was performed by the diffusion method using the forestry industry standard of China (LY/T 1228 – 1999). Available P (AP) was extracted using 0.5 mol·L<sup>-1</sup> NaHCO<sub>3</sub> at pH 8.5 (Olsen 1954) and measured using a colorimeter (UV2550; Shimadzu, Japan). Total phosphorus (TP) was measured by spectrophotometry after wet digestion with HClO<sub>4</sub>-H<sub>2</sub>SO<sub>4</sub>.

## DNA extraction, PCR amplification and Illumina sequencing

Soil bacterial DNA from each sample was extracted using a soil DNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The V3 and V4 regions were amplified using forward primers containing the sequence CCTACGGGNGGCWGCAG and reverse primers containing the sequence GACTACHVGGGTATCTAATCC. PCR was conducted in 20  $\mu$ L reactions with 1  $\times$  reaction buffer (Takara, Dalian, China), 2 mM  $Mg^{2+}$ , 0.2 mM dNTPs, 0.1  $\mu$ M of each primer, 1 U HotStarTaq polymerase (Takara, Dalian, China) and 2  $\mu$ L of template DNA. The cycling program was as follows: 95  $^{\circ}$ C for 2 min; 35 cycles of 94  $^{\circ}$ C for 20 s, 55  $^{\circ}$ C for 40 s, and 72  $^{\circ}$ C for 1 min; and then 72  $^{\circ}$ C for 2 min. To add specific tag sequences to each sample, the PCR reaction was performed in a total volume of 20  $\mu$ L containing 1  $\times$  reaction buffer (NEB Q5TM), 0.3 mM dNTPs, 0.25  $\mu$ M F primer, 0.25  $\mu$ M index primers, 1 U Q5TM DNA polymerase (NEB) and 1  $\mu$ L of diluted template. The PCR conditions were as follows: an initial denaturation step at 98  $^{\circ}$ C for 30 s, 11 cycles of denaturation at 98  $^{\circ}$ C for 10 s, annealing at 65  $^{\circ}$ C for 30 s, extension at 72  $^{\circ}$ C for 30 s, and a final extension step of 72  $^{\circ}$ C for 5 min. PCR products were visualized and cleaned using gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen). The DNA concentration of each PCR product was quantified using a UV-Vis spectrophotometer (NanoDrop ND1000, USA) and then pooled in an equimolar manner. Next, generation of the sequencing library preparations and Illumina MiSeq sequencing were conducted at G-BIO Inc. (Hangzhou, China).

## 16S rRNA data processing

PANDAseq (Masella et al. 2012) was used for quality filtering and the assembly of the two ends of each read into contigs, with parameters including a 400 bp minimum and 500 bp maximum. Chimeric reads were filtered using the "identity\_chimeric\_seqs.py" module of USEARCH (Edgar 2010) in QIIME v.1.9 (Caporaso et al. 2010). The chimera-filtered sequences from each sample were combined into one file using the QIIME script add\_qiime\_labels.py. Operational taxonomic units (OTUs) were picked based on 97% identity using the open-reference OTU-picking workflow pipelines (Caporaso et al. 2010) and the UCLUST algorithm (Edgar 2010) against the Greengenes reference database (DeSantis et al. 2006) August 2013 release. OTUs with an abundance below 0.005% of the total number of sequences were discarded (Bokulich et al. 2013). Alpha diversity measurements were calculated

using the alpha\_rarefaction.py script in QIIME. Weighted and unweighted unifracs distances (Lozupone and Knight 2005) were calculated from the rarefied OTU table using the beta\_diversity\_through\_plots.py script in QIIME.

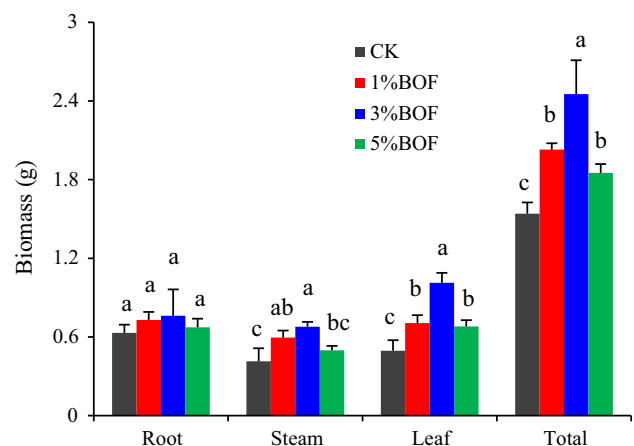
## Statistical analysis

The linear discriminant analysis (LDA) effect size (LEfSe) method was used (Segata et al. 2011) to compare the microbial composition of the BOF treatments and the control groups. The relative abundance of bacterial groups in samples from the BOF and control groups were compared using one-way ANOVA (Statistical Package Social Science, SPSS, version 19.0). The compare\_alpha\_diversity.py script was used to compare the alpha diversity (within sample diversity) metrics in the QIIME pipeline, which implements a nonparametric two-sample *t* test with 999 Monte Carlo permutations. Beta diversity (between sample diversity) comparisons were completed using ANOSIM (compare\_categories.py; QIIME). A *p*-value < 0.05 was considered statistically significant.

## Results

### Plant biomass allocation

Total biomass showed significant increase in plants grown under BOF treatments in the following order: 3% > 1% > 5% BOF (Fig. 1). For stem biomass (Fig. 1), significant increase was recorded in the 1% BOF and 3% BOF treatments, but no difference was recorded between the 5% BOF treatment and the control. All BOF treatments significantly increased leaf biomass (3% > 1% > 5% BOF, Fig. 1). *T. hemsleyanum* exhibited no significant



**Fig. 1** Variation in plant biomass allocation in *Tetrastigma hemsleyanum* among the four treatments

increase in plant root biomass in the presence of BOF (Fig. 1).

### Soil properties

BOF treatments increased soil pH by 0.68–1.34 compared with the control group (Table 1). There were significant differences in soil pH between the control (CK) and BOF-treated soils. SOC content increased by 22.03%, 14.39%, and 43.73% ( $p < 0.05$ ) in BOF applications of 1%, 3%, and 5%, respectively. SOC content differed by BOF group and ranked in the following order: 5% > 1% > 3% BOF. TN content in the 1% and 5% BOF treatments was 18.46% and 32.82% higher ( $p < 0.05$ ), respectively, than in the control. C/N was similar in the control and BOF groups. Total phosphorus and available phosphorus were significantly higher in the 5% BOF group and significantly lower in the 1% BOF group than in the control. There was no difference in total nitrogen, total phosphorus and available phosphorus between the 3% BOF and control groups.

### Sequencing data and taxonomic assignments

A total of 262,296 sequences passed the quality check and were considered for further analysis. These sequences were classified to the genus level at a 97% sequence identity threshold.

A total of 10 phyla with an abundance > 0.5% were identified in the soil samples. Figure 2 shows the relative abundance of the community compositions (bacterial phylum and family) in the control and BOF-treated groups. The most abundant phylum in all soil samples was Proteobacteria, which accounted for approximately 41.83% of all sequences, followed by Acidobacteria (16.39%), Actinobacteria (10.69%), Chloroflexi (7.59%), Planctomycetes (4.06%), Bacteroidetes (4.20%), Verrucomicrobia (4.30%), OD1 (3.81%), Gemmatimonadetes (1.48%), TM7 (0.94%), and Firmicutes (0.69%) (Fig. 2a). Among the 23 known families detected in the present study with an abundance > 0.5%, the most commonly identified families were Xanthomonadaceae (19.87%), Acidobacteriaceae (11.48%), Sinobacteraceae (3.21%), Thermogemmatimonadaceae

(2.47%), Rhodospirillaceae (2.43%), Acetobacteraceae (2.22%), Chitinophagaceae (2.07%), Solibacteraceae (1.51%), auto67\_4 W (1.12%), Comamonadaceae (1.09%), Hyphomicrobiaceae (1.03%), Pirellulaceae (0.91%), Alcaligenaceae (0.86%), Caulobacteraceae (0.73%), Streptomycetaceae (0.71%), Gemmataceae (0.69%), Opitutaceae (0.69%), Koribacteraceae (0.64%), Isosphaeraceae (0.63%), Mycobacteriaceae (0.61%), Sphingobacteriaceae (0.61%), Cytophagaceae (0.60%), and Conexibacteraceae (0.57%) (Fig. 2b).

### Bacterial alpha-diversity and beta-diversity

Alpha and beta diversity analysis was performed, and plots were created specifying a sampling depth of 17,189. Rarefaction curve analysis suggested that the sequencing depth in this study was adequate (Fig. 3). There were no significant differences in Shannon, Chao1, Faith's phylogenetic diversity, or observed species indexes (Table 2).

A comparison of the similar communities found in each sample is shown in Fig. 2. Communities differed by treatment when analyzed using the weighted Unifrac matrix (ANOSIM,  $p = 0.001$ ) and unweighted Unifrac matrix (ANOSIM,  $p = 0.001$ ) (Fig. 4).

### Microbial differential abundance

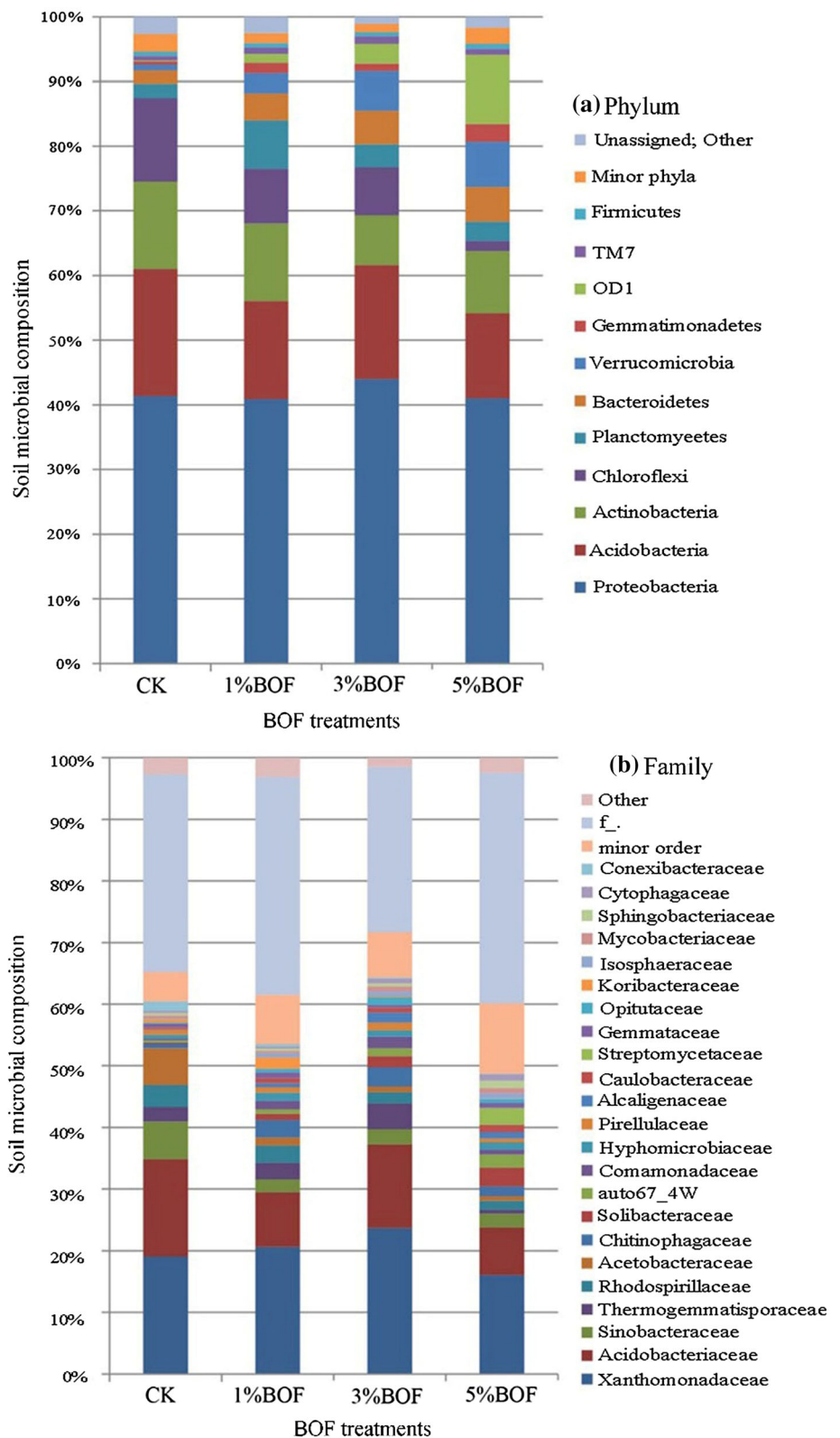
Microbial abundance differed by BOF treatment for phyla Bacteroidetes, Verrucomicrobia, Chloroflexi, and OD1 (Fig. 5). Compared with the control, the 3% BOF treatment yielded significantly higher abundance of sequences affiliated with Verrucomicrobia and Bacteroidetes. The 3% BOF treatment had a decreased abundance of Verrucomicrobia, Bacteroidetes, and OD1, relative to the 5% BOF treatment. No difference was observed between the 3% BOF and 1% BOF groups.

To further detect major differences between the BOF-treated and control groups, the LEfSe method was used to analyse the metagenomic data of bacterial taxa. There were 47 differentially abundant taxonomic clades with an LDA score higher than 3.5: 15, 3, 7, and 22 clades representing the control, 1% BOF, 3% BOF, and 5% BOF groups,

**Table 1** Selected soil properties from the control and BOF treatments

Treatment	pH	SOC (g kg <sup>-1</sup> )	TN (g kg <sup>-1</sup> )	C/N	TP(g kg <sup>-1</sup> )	AP(mg kg <sup>-1</sup> )
CK	4.22 ± 0.13c	20.15 ± 0.72d	1.95 ± 0.09c	10.34 ± 0.11a	1.20 ± 0.05b	185.94 ± 5.56b
1% BOF	4.90 ± 0.24b	24.59 ± 0.76b	2.31 ± 0.14ab	10.65 ± 0.32a	1.02 ± 0.09c	137.38 ± 2.05c
3% BOF	4.91 ± 0.55ab	23.05 ± 0.46c	2.04 ± 0.29b	11.41 ± 0.41a	1.22 ± 0.07b	180.95 ± 2.56b
5% BOF	5.56 ± 0.33a	28.96 ± 0.26a	2.59 ± 0.11a	11.19 ± 0.38a	1.57 ± 0.04a	225.21 ± 3.07a

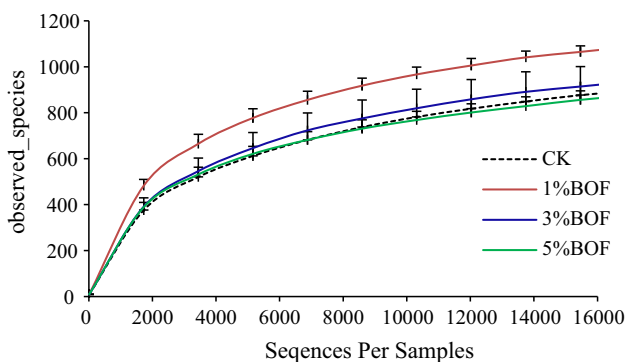
**Fig. 2** The soil microbial composition in the control and BOF groups at the phylum (a) and family (b) levels



respectively (Fig. 5). At the genus level, *Steroidobacter* and *Spirochaeta* were the most prominent phyla in the 5% BOF group, and *Rhodanobacter* was enriched in soils with

a 3% BOF application. The control was characterized by a preponderance of *Conexibacter*. There was no biomarker associated with the 1% BOF group at the genus level.





**Fig. 3** Rarefied plot for the relationship between the number of sequences per sample (x axis) and the number of observed species (y axis)

## Discussion

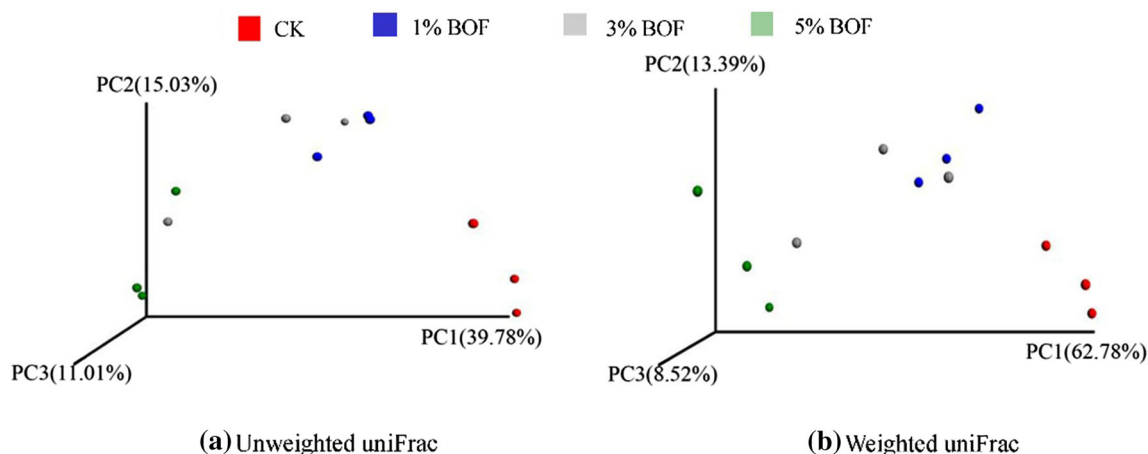
Our results show that soil, plants, and bacterial communities were influenced by the application of BOF. BOF significantly increased *T. hemisleyanum* total biomass. This is consistent with previous studies demonstrating that the same application of BOF significantly increased the growth of *Pistacia chinensis* Bunge (Wu et al. 2015). Kammann et al. (2015) showed that plant growth exhibited up to a fivefold increase with composted biochar compared to untreated biochar, and up to a threefold increase compared to the control. Luo et al. (2017) reported that biochar-compost addition at a lower rate (e.g., 1.5%) promoted the

growth of *Sesbania*, but inhibited its growth at rates of 5 and 10%.

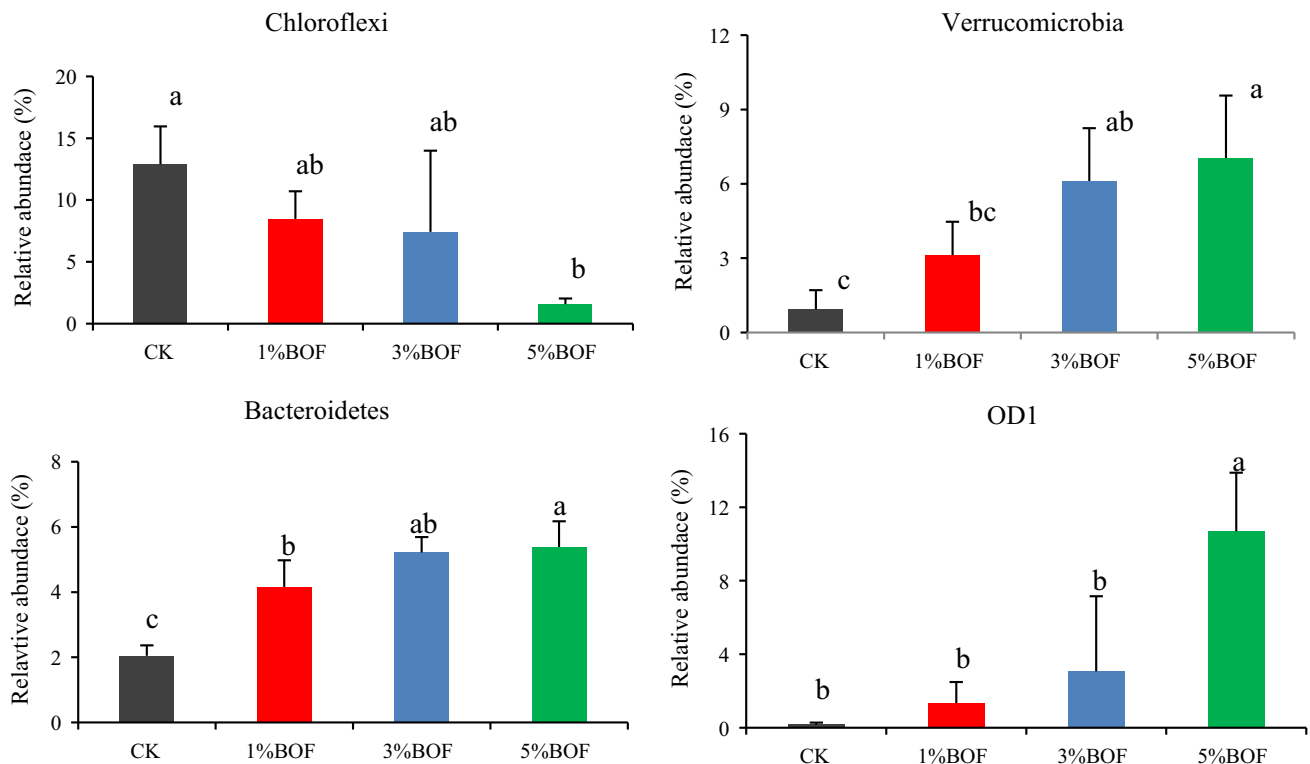
Both biochar and compost have been shown to improve soil properties (Zhang et al. 2012; Abujabhah et al. 2016; Agegnehu et al. 2017), although the effects are dependent on factors such as soil type and the amount/characteristics of the amendment. In our study, BOF application increased soil pH. Schulz et al. (2013) showed that composted biochar had no significant effect on pH in sandy substrate. This result may be explained by the fact that the composted biochar showed pH values similar to the pH values of the soil substrate. Therefore, the increased soil pH after BOF application may be due to the higher pH of the BOF than that of the soil substrate. Bass et al. (2016) reported that no difference was observed between the pH of the control and the compost-biochar treatments, probably due to low biochar application rates. We found that BOF application significantly increased the SOC content. Previous studies reported that total SOC significantly increased due to the application of various types of biochar (Kimetu and Lehmann 2010; Xie et al. 2013; Angst et al. 2014). Agegnehu et al. (2016) reported that SOC and TN increased by 14–29% and 59–117%, respectively, compared to the initial nutrient content of the soil. Glisczynski et al. (2016) showed that biochar-compost substrate applications containing 15% and 30% of biochar significantly increased SOC stocks.

**Table 2** Analysis of soil microbial  $\alpha$ -diversity in the different treatments

Treatment	shannon	chao1	observed_species	PD_whole_tree
CK	7.26 $\pm$ 0.48	1083.77 $\pm$ 100.11	901 $\pm$ 149	49.16 $\pm$ 5.96
1% BOF	7.83 $\pm$ 0.39	1237.09 $\pm$ 30.57	1094 $\pm$ 27	57.04 $\pm$ 0.82
3% BOF	7.19 $\pm$ 0.32	1106.00 $\pm$ 155.89	936 $\pm$ 120	50.76 $\pm$ 5.39
5% BOF	7.42 $\pm$ 0.42	1074.01 $\pm$ 41.95	876 $\pm$ 54	48.37 $\pm$ 1.57



**Fig. 4** PcoA analysis based on Unweighted and Weighted UniFrac in the different treatments



**Fig. 5** Differences in phyla of the soil microbes in the four treatments

Soil bacterial diversity is considered to be critical for the integrity, function, and long-term sustainability of soil ecosystems (Kennedy and Smith 1995). Moreover, greater biodiversity in the soil can lead to a more stable ecosystem and enhance the combination of vital microbial functions and processes (Chaer et al. 2009). Loss of microbial diversity is the general consequence of long-term chemical fertilization. The return of crop residues can mitigate the negative effects of chemical fertilization on bacterial diversity (Ramirez et al. 2010; Sun et al. 2015). In our study, no difference was observed in the bacterial richness and diversity between the BOF and control treatments, possibly due to the short-term BOF application of only 6 months.

High-throughput sequencing results showed significant increases in abundances of microbes of the phyla Bacteroidetes, Verrucomicrobia, Chloroflexi, and OD1 for all BOF treatments. Bacteroidetes preferentially consume soil organic C, have high nutrient requirements, and are more abundant when biogenic resources increase (Fierer et al. 2007). Chloroflexi and Acidobacteria are slow-growing bacteria (Davis et al. 2011) and generally prefer an oligotrophic environment (Hallam et al. 2004; Fierer et al. 2012). Representatives of the phylum Acidobacteria are typically abundant in conditions of low nutrient status due to their higher substrate affinities (Fierer et al. 2007). In our study, the relative abundance of Bacteroidetes increased

significantly in BOF treatments compared to the control, and the abundance of Acidobacteria and Chloroflexi declined. These results indicate that the soil favoured copiotrophs over oligotrophs following BOF application. To the best of our knowledge, only one prior study used cultures isolated from soils to document the capacity of Verrucomicrobia to degrade polysaccharides (Chin et al. 1999). OD1 has mainly been detected in sulphur-rich, anoxic environments (Elshahed et al. 2005; Briée et al. 2007; Barberán and Casamayor 2011), and the partial genome revealed genes known only from anaerobic or facultative anaerobic microorganisms with genomic organization similar to that of methanotrophic Archaea (Elshahed et al. 2005) that are found in anoxic marine sediments (Hallam et al. 2004).

The LEfSe results showed that the genus *Conexibacter* was the most differentially abundant taxon in the control soil. *Conexibacter* are strictly aerobic, can reduce nitrate to nitrite, and might function in carbon cycling in soil ecosystems (Monciardini et al. 2003; Pukall et al. 2010; Seki et al. 2012). Our 3% BOF application significantly increased the abundance of genus *Rhodanobacter*. Members of the genus *Rhodanobacter* are Gram-negative, rod-shaped and aerobic, with most strains being catalase- and oxidase-positive (Madhaiyan et al. 2014). *Steroidobacter* and *Spirochaeta* were significantly influenced by the 5% BOF application. The genus *Spirochaeta* was found to be

positively correlated with the removal of CIP (Huang et al. 2017), secreted seven glycoside hydrolases for the degradation of plant biomass (Schiefner et al. 2016), and was a dominating bacterium for hydrolysis of lignocelluloses (Pandit et al. 2016). Some *Steroidobacter* spp. are strictly aerobic and have soil catalase activity (Sakai et al. 2014).

In conclusion, *T. hemsleyanum* biomass was increased by BOF application because (1) soil pH and SOC increased significantly after BOF application and (2) soil bacterial communities were increased by BOF application, and the responses differed among phyla and/or genera.

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