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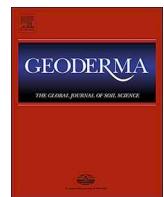
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Legacy of soil health improvement with carbon increase following one time amendment of biochar in a paddy soil – A rice farm trial



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ABSTRACT

“4 per 1000 Initiative” launched at COP21 has strongly recommended that increasing soil organic carbon (SOC) is a global imperative. However, the strategies to improve agricultural soil functioning and health by an increase in soil C have been poorly addressed. In a rice farm trial, topsoil samples were collected in 2015 respectively in a field amended with biochar (BA) (equivalent to 10 t ha⁻¹) in 2009 and an adjacent field with no biochar (NB). Soil aggregation, biochemical activities and microbial communities of the samples were analyzed with microscopic, micro-biochemical and molecular assays respectively. Compared to NB field, SOC storage increased by 45%, total and available nitrogen pool enhanced by approximately 30% while the bulk density decreased and soil pH unchanged in BA field. A 25% increase in mean weight diameter of water stable aggregates was observed in the BA compared to NB field. Correspondingly, microbial biomass nitrogen and enzyme activities in BA field was enhanced both by approximately 30% compared to NB field. Furthermore, with community structure altered, a moderate (32%) increase in total bacterial abundance and a significant decrease in microbial abundance of amino acid metabolism and fungal pathotrophs were observed in BA field compared to NB field. This could link to the higher (10%) grain yield with lower yield inter-annual variability in BA field compared to NB field, reported previously. All these changes clearly demonstrated a legacy of paddy soil health improvement over years following one time biochar amendment. Overall, addition of biochar to the clayey nutrient rich paddy soil could sustain an increase in SOC, soil aggregation and soil health functioning, with positive changes in microbial community over years. Thus, carbon increase as per required by the “4 per 1000 Initiative” could be a mechanistic driver to enhance soil fertility and improve soil-(plant) health for ensuring food production in world rice agriculture facing land degradation and climate change impacts, which could be assisted by biochar from crop residue in an approach of circular economy.

1. Introduction

Soil degradation and climate change had been the dilemma greatly challenging global food security for the rapidly growing population (Bajželj et al., 2014; Smith et al., 2015). Climate-smart soils had been urged to build up soil resistance against climate change impacts for stabilizing crop productivity (Paustian et al., 2016). In action, enhancing soil organic carbon (SOC) sequestration had been advocated to improve soil fertility and hence crop productivity while to mitigate climate change (Lal, 2004). Launched following the Paris Agreement in

2015, the “4 per 1000 Initiative” urged sequestering carbon in world soils by recycling waste organic residues into soil carbon amendments in agricultural soils with emphasis on food security and climate change neutral (Chabbi et al., 2017; Baveye et al., 2018; Rumpel et al., 2018). With potential multiple ecosystem co-benefits (Sohi, 2012), biochar soil amendment (BSA) had been recommended as a key measure to enhance SOC storage in terrestrial ecosystems (Lehmann et al., 2006) and agricultural lands (Schmidt et al., 2019). Besides, biochar could increase crop productivity (Jeffery et al., 2011; Liu et al., 2013) and improve soil microbial growth and activity (Lehmann et al., 2011; Zhou et al., 2018),

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and potentially improve soil-plant health through system acquainted resistance (Mehari et al., 2015; Kolton et al., 2017). But, if and how soil carbon increase following a biochar addition, could help long term improvement of soil fertility and health had been poorly addressed.

There had been increasing concerns with long term changes in soil fertility or properties of biochar following addition (Zhang et al., 2016; Hardy et al., 2017a,b; Yi et al., 2020). Soil carbon increase induced by biochar could persist over a long period of time (Kuzyakov et al., 2014; Wang et al., 2016) as the stable aromatic backbone of biochar carbon had a turnover time over hundreds of years (Lehmann and Joseph, 2015). As shown in a meta-analysis by Liu et al., (2013), a single biochar application could improve crop productivity by approximately 11% generally within two years/crop seasons, mostly in dry croplands and/or in pot experiments. However crop yield increase in short term was not parallel to the improvement of soil nutrient supply throughout the crop seasons following a single BSA (Liu et al., 2014a; Pandey et al., 2016; Arif et al., 2017). In a wood biochar treated temperate soil, crop yield tended smaller or unchanged though soil nutrients enhanced throughout a consecutive production of three crop seasons (Kloss et al., 2014). However, in a moderately salt-stressed maize soil, the positive changes in crop growth were found corresponding to those in abiotic and biotic attributes of the soil-plant system following a single BSA (Lashari et al., 2013). The results of these experiment pointed to a non-linear response of the changes in soil system functions (Sietz et al., 2017) albeit there were positive changes concurrently among abiotic soil quality attributes such as SOC, nitrogen, pH, bulk density, and available nutrients following a BSA. As SOC had been well recognized as the core for soil health (Maharjan et al., 2020), an issue could be raised if soil carbon increase could bring about a legacy in soil for better performance of synergic functioning. Although this was preliminarily addressed in a brown field following decade-long large application of an organic amendment (Lord and Sakrabani, 2019), it remained unclear if soil carbon increase could improve soil biotic properties and functioning of soil-plant system while improved abiotic properties in biochar amended soils.

Soil health and ecosystem functioning could be largely mediated by soil biota (Copley, 2000). Biochar aging, through enhanced SOC stabilization (Mukherjee et al., 2014) and biochar-soil-root interaction (Lehmann et al., 2015), was found promoting soil reactivity with exotic toxic organic compounds (Spokas, 2013) and metals (Bian et al., 2014), and manipulating potent greenhouse gases production (Martin et al., 2012), in the amended soils. As such, changes in soil microbial community and activity over long run in biochar-aged fields could be a critical issue with BSA in agriculture (Lehmann et al., 2011). BSA induced changes in bacterial abundance and community composition took some months to years (Jenkins et al., 2017). In a salt-stressed soil, particularly, bacterial gene abundance and diversity as well as enzyme activities were all significantly increased and microbial community structure shifted, being even greater after two years than one year following wheat biochar addition (Lu et al., 2015). Other studies had shown a range of changes in abundance of different micro-organisms with the addition of biochar as a function of time (Khodadad et al., 2011; Yao et al., 2017). Biochar could induce short term positive changes in microbial growth and metabolic activity, but these changes were weakened in subsequent years (Zhou et al., 2017). Such positive biotic change could translate into soil health improvement. Liang et al. (2014) observed a BSA shifted fungal community in a tropical forest soil, which was linked mechanistically to drought resistance. Using global gene expression data for plants grown in soil amended with biochar, Viger et al. (2015) revealed a more important role of biochar in promoting plant defense than enhancing photosynthesis. Microbial community changes in genera level opened a window to look into the biochar induced system resistance related to soil health and thus plant defense, initially addressed by Elad et al., (2010). This was highlighted by the potential suppression on soil-borne pathogens (mostly fungal parasites) observed under BSA treatment (Bonanomi et al., 2015). Their

further work (Bonanomi et al., 2018) stressed the importance of plant-associated microbiome for crop productivity, potentially modified or modulated by introducing organic amendments and/or beneficial microbes. Thus, if soil carbon increase could provide a legacy for soil health improvement, particularly via manipulation of microbial community and activity, then the goal of “4 per 1000 Initiative” on soil carbon and human food could be reached.

Rice had been one of the world's most important stable crops and produced on 20% of the total world croplands (Kuenzer and Knauer, 2013). A major challenge with rice agriculture had been to increase yield while minimizing environment risks to water and air pollutions (Tillman et al., 2011). To avoid field burning and methane emission from incorporation in paddy, rice straw had been increasingly utilized for biochar production in China (Pan et al., 2015a). Indeed, biochar could counteract soil acidification, compaction and organic carbon decline, widely addressed with global soil degradation (Smith et al., 2015), particularly in rice producing regions from tropical and subtropical Asia (UNEP, 2014). In paddies across South China, BSA treatment had shown consistently positive effects in increasing rice yield and N use efficiency (Huang et al., 2013), in reducing nitrous oxide emission (Liu et al., 2012) and Cd concentration in rice production (Bian et al., 2013) in short term following addition. Meanwhile, BSA could increase total and active bacterial community abundance with community structure altered (Chen et al., 2013, 2015; Zheng et al., 2016) but decrease fungal community abundance (Chen et al., 2016). As shown by Wang et al. (2018), one time BSA increased rice productivity and fertilizer use efficiency over several years following application.

In this study, we hypothesized that one time BSA could build up a legacy of soil health improvement through enhanced soil aggregation and in turn microbial activity with SOC increase over years following an addition in rice paddy. And, changes in microbial community and activity could be linked to the positive abiotic changes with enhanced SOC sequestration and with nutrient input in management adaptation to biochar altered system, over years following a BSA in the rice soil. In this study, soil water stable aggregate distribution, carbon/nitrogen transforming activities and microbial community and functional groups of topsoil were compared between paddy fields non-biochar treated and biochar treated over years in a single rice farm. The study aimed to provide insights into how soil carbon increase, as demanded by the “4 per 1000 Initiative”, could link to a long term legacy of soil health improvement with biochar in rice paddy.

2. Materials and methods

2.1. Site and soil

In this study, a field trial comparing biochar amendment to no biochar amendment was conducted in a rice farm located at Yifeng village ($31^{\circ}24'10''N$, $119^{\circ}41'28''E$), Yixing Municipality, Jiangsu Province, China. Situated at southwestern Tai Lake plain, the local area used to be important for rice agriculture in China, conventionally under a farming system of summer rice rotated with winter wheat or rape seed for hundreds years. The area was prevailed by a subtropical monsoon climate, with a mean annual temperature of $17.1^{\circ}C$ and precipitation of 1242 mm over 2009–2015. Derived from mostly lacustrine deposits, the soil in the area is clay in texture with mixed clay mineralogy of illite-kaolinite-chlorite. The rice paddy soil had been developed under long history of rice cultivation and classified as a hydroagric Stagnic Anthrosol (Gong, 1999). As in a traditional high-yielding rice production region, the rice paddies in this area generally contained an available phosphorus pool over 30 mg kg^{-1} and potassium over 80 mg kg^{-1} (Liang, 2004; Xiao, 2010). The basic topsoil properties of the rice farm before experiment are shown in Table 1.

Table 1

Basic properties of topsoil of the rice farm fields before trial and of the wheat biochar before amendment.

Sample	pH (H ₂ O)	BD (g cm ⁻³)	CEC (cmol kg ⁻¹)	Total OC (g kg ⁻¹)	Total N (g kg ⁻¹)	Total P (g kg ⁻¹)	Clay (%)
Topsoil	6.50	1.01	18.10	23.5	1.78	0.40	39.0
Biochar	10.4	0.65	40.78	467	5.92	1.61	n/a

BD, bulk density; CEC, cation exchange capacity. n/a, not applicable.

2.2. Biochar used

As described by [Zhang et al., \(2010\)](#), biochar used in this study was provided by the Sanli New Energy Co. Ltd., Henan, China. Chopped wheat straw was pyrolyzed in a continuous vertical kiln made of refractory bricks with the temperature in a range of 350–550 °C within the kiln. The wheat straw biomass was converted to biochar in a recovery of 35% as the solid residue released from the kiln. After cooling, the biochar material was milled into granular particles of about a 0.3 mm in diameter. For the field study, the biochar mass was again passed through a 2 mm sieve, and mixed thoroughly to obtain a powder consistency that would mix more uniformly with the soil. Following the protocol described by [Lu \(2000\)](#), the biochar's properties were analyzed with similar methods as for soil, detailed below. The production process of the biochar is described in [Fig. S1](#) and the biochar properties provided also in [Table 1](#). In addition, the biochar had a surface area of 8.92 m² g⁻¹, electric conductivity of 139.75 μS cm⁻¹, total P of 1.61 g kg⁻¹ and total K of 26 g kg⁻¹, citric acid extractable Si of 718 mg kg⁻¹, and a dissolvable organic carbon of 0.5 g kg⁻¹ ([Zhang et al., 2010, 2012; Liu et al., 2014a](#)) in addition to a total ash content of 20.8%. The information of organic functional groups and other metal elements in the wheat biochar was detailed by [Bian et al. \(2014\)](#).

2.3. The farm trial

A rice farm managed by a senior rice farmer (Supplement Information 1) was selected for this study. One field (866.6 m²) in this farm was treated with biochar amendment after wheat harvest in 2009 ([Zhang et al., 2010, 2012](#)). As designed, biochar was amended at 0, 10, 20 and 40 t ha⁻¹ of biochar respectively with or without N fertilization. For this, biochar material was spread over soil surface and mixed into plow layer (ca 0–15 cm) with a wooden ranker to keep consistency in a plot. Triple replicated plots (each in area of 5 m × 4 m) were then randomly distributed across the field as per a complete random block design ([Figs. S2](#)). In 2012, however, the biochar treated field was machine ploughed to a depth of 15 cm after wheat harvest and thoroughly homogenized with repeated cycles of ploughing followed by raking to minimize field variability for routine rice cultivation (shown in Supplement [Figs. S2](#) and S3). This gave an overall biochar amendment rate equivalent to a dose of 10 t ha⁻¹ over the whole field. Another adjacent field in similar size in the same farm but never treated of biochar, was used for comparison for the biochar effect. As owned and managed by the same farmer, all the agronomic practices for rice production were consistent between no biochar (NB) and biochar amended (BA) fields over the time span of the trial. However, fertilizers and pesticides were applied often at a lower rate in BA field than in NB ([Table S1](#)). In this area, crop straw used to be burnt in field but since 2011 incorporation into soil had been operated while ploughing before the next crop season, following the provincial regulation for crop residue utilization.

2.4. Soil sampling and processing

Soil sampling of topsoil (0–15 cm) was done at rice harvest in late October 2015. In the field respectively of BA and NB, 3 composite bulk samples were collected (each about 1 kg, of 5 undisturbed soil cores

randomly taken with an Eijkelkamp core sampler in a Z-shaped protocol). After initial homogenization, a composite sample was stored and sealed in a stainless steel can, and kept in an ice box before shipping to laboratory within 24 h after sampling. After removal of plant litters and detritus, a composite sample was hand crashed, homogenized and divided into three portions, with one air-dried for analysis of basic physicochemical properties, one left undisturbed and stored at 4 °C for aggregate fractionation and one for bioassays. The subsample for bioassays was further gently squeezed to pass a 2 mm sieve, homogenized and further divided into 3 sub-portions, stored at 4 °C, –20 °C and –80 °C respectively for biochemistry analyses, DNA extraction and RNA extraction.

2.5. Soil aggregate size fractionation and SEM observation

Particle size fractionation of soil aggregates was performed using a low energy water dispersion and wet sieving, developed by [Cambardella and Elliott \(1994\)](#) and modified by [Six et al. \(2000\)](#). In a device with a series of sieves with sequential sizes, a 100 g equivalent portion of an undisturbed sample was weighed onto a 2000 μm sieve and submerged under water. With the sieves moving up and down 50 times with the distance of 3 cm for 2 min, soil particles passed through the sieves sequentially in size of 2000 μm, 250 μm and 53 μm. The soil mass over a sieve was separately collected as a fraction in a size range between the upper and lower size of the sieves. Thus, macroaggregates in size of 2000–250 μm and microaggregates in size of 250–53 μm were obtained, respectively. Then the soil mass passing through a sieve of 53 μm were sequentially centrifuged to obtain coarse silt fraction in size of 53–20 μm, fine silt fraction in size of 20–2 μm and clay particle fraction in size < 2 μm, respectively. All the obtained soil aggregate fraction samples were freeze-dried using a vacuum freeze drier (FD-1E-80, Beijing Biocool Lab Instruments Co Ltd, China) prior to analysis.

Mean weight diameter (MWD, μm) of soil aggregates was calculated with the mass percentage of individual size fractions, using the following equation ([van Bavel, 1950](#)):

$$MWD = \sum_{i=1}^5 P_i * S_i \quad (1)$$

where P_i is the mass percentage of a size fraction i to the sum of all the fractions; S_i is the average diameter (μm) of the fraction i .

Using a Hitachi S-3000 N SEM, scanning electron microscope (SEM) analysis of macroaggregate (2000–250 μm) fraction was performed for the three topsoil samples respectively collected from NB and BA fields. For this, a portion of the freeze-dried macroaggregate fraction sample was randomly selected and mounted on a stub, and coated with gold prior to observation. Images were taken by automatic image capturing software, marked with magnifications and liner scale.

2.6. Analysis of abiotic and biotic soil properties

Soil bulk density was measured with a 100 cm³ volume stainless steel cylinder, in a similar way to soil core sampling in the field. Soil pH was measured using a soil-to-water ratio of 1:2.5 (m/v) with a Mettler-Toledo pH meter. Contents of SOC and TN were determined using a CNS Element Analyzer (Elementary, Germany) while soil available nitrogen (AN) using incubation with 1 M NaOH for 24 h at 40 °C ([Lu, 2000](#)). Microbial biomass carbon (MBC) and nitrogen (MBN) were analyzed using a modified fumigation-extraction method ([Vance et al., 1987](#)). For this, samples unfumigated and fumigated with ethanol-free chloroform were kept in dark for 24 h at 25 °C, followed by extraction with 0.5 mM K₂SO₄ for 30 min on a shaker. Carbon content of the extracts was analyzed by a multi N/C analyzer (Jena, Germany), but nitrogen by a micro-Kjeldahl method. The difference between fumigated and unfumigated sample was converted to MBC using a Kec of 0.38, while MBN with 0.45. In addition, C isotopic abundance ($\delta^{13}\text{C}$ %)

PDB) of soil organic carbon of bulk sample and the separated soil aggregate size fractions was determined using isotope mass-spectroscopy of Finnegan MAT 253, at the Nanjing Institute of Soil Science, Chinese Academy of Sciences.

2.7. Biochemical assay for extracellular enzyme activities

Extracellular enzyme activities (EEAs) of a sample were measured in triplicates with fluorimeter using MUB (methylumbelliferon)-linked substrates and L-DOPA (L-3, 4-dihydroxyphenylalanine) following DeForest (2009) and German et al. (2011). Enzymes measured here were α -glucosidase (EC 3.2.1.20), β -glucosidase (EC 3.2.1.21), β -xylosidase (EC 3.2.1.37), β -cellobiosidase (EC 3.2.1.91), N-Acetyl-glucosaminidase (EC 3.2.1.30), phosphatase (EC 3.1.3.1), sulfatase (EC 3.1.6.1), phenol oxidase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7). In detail, 200 μ l of a homogenized aggregate slurry and 50 μ l of 200- μ M MUB-linked substrate were dispensed into a 96-well and 300 μ l microplate of black polystyrene. The substrate plates in six replicates were incubated in dark at 25 °C for 3 h and measured with SpectroMax M5 (Molecular Devices, Inc., USA) using 365 nm excitation and 450 nm emission filters. Negative control and MUB standards in a range of 0–100 μ M were prepared for each sample to compute the EEA values.

Meanwhile, the substrate of L-DOPA was used for measuring phenol oxidase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) activities, following Saiya-Cork et al. (2002) and DeForest. (2009). In brief, 1.0 ml of a soil slurry was pipetted into a 2 ml PE plastic centrifuge tube. To the tube was added 250 μ l L-DOPA (25 mM) for phenol oxidase analysis and 250 μ l L-DOPA plus 50 μ l 0.3% H₂O₂ solution for peroxidase analysis, with prepared negative control and sample control. The tubes were incubated waveringly in dark at 30 °C for 6 h and subsequently centrifuged. Finally, a 250 μ l supernatant of each sample was pipetted into a 96-well clear microplate and the EEA was measured with a microplate spectrophotometer using the absorbance at 460 nm.

Finally, the enzyme activities individually measured were normalized and an overall EEA assessed using the following equation:

$$x_i' = x_i / \sum_{i=1}^6 x_i \quad (2)$$

where x_i' is a single individual EEA measured of a sample i (triplicates for a treatment of BA or NB, totally 6 samples), x_i is the normalized (dimensionless) value of the individual EEA of the sample. Subsequently, the arithmetic average of nine measured enzyme activities was obtained as the normalized overall enzyme activity (NEA) value for each sample (Jin et al., 2012).

2.8. Gene extraction, real-time quantitative PCR assay and high throughput sequencing

For gene extraction, a portion of moist soil (10 g) stored at -20 °C of each sample was used for DNA extraction using the PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories Inc., CA) following the manufacturer's instructions. Whereas, a portion of moist soil (2 g) with three replicates stored at -80 °C of each sample was protected by LifeGuard™ Soil Preservation Solution (Mo Bio Laboratories Inc., CA) before RNA extraction. Total RNA was isolated using the RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories Inc., CA)

according to the manufacturer's protocol. RNA was transcribed into complementary DNA (cDNA) using PrimeScript™ RT reagent Kit (Takara Shuzo, Shiga, Japan).

Copy numbers of bacterial 16S rRNA (primer set 338F/518R) and fungal 18S rRNA genes (primer set NS1F/FungR) were quantified by My-IQ1 thermocycler (Bio-Rad, USA), as described by Muyzer et al. (1993) and May et al. (2001), respectively. The plasmid DNA possessing the target genes of known copy numbers was used to generate the standard curve ranging from 10² to 10⁷ copies of 10-fold serial dilution. The 20 μ l PCR mixture contained 10 μ l of SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan), 0.5 μ M each primer and 1 μ l extracted DNA. To obtain the final quantities of bacterial 16S rDNA and fungal 18S rRNA genes, calibration with dedication was done against total DNA and RNA concentrations extracted and soil moisture contained.

Furthermore, 16S rRNA and ITS genes amplification and high throughput sequencing were performed using the primer sets 515F/806R (Bates et al., 2011) and ITS3F/ITS4R (White et al., 1990), to amplify bacterial 16S rRNA gene (V4) and fungal ITS region (ITS2), respectively. A sample-specific 12-bp barcode was added to the reverse primer. Each DNA sample was amplified in duplicates and pooled before purification and quantification, and then PCR products were prepared for Illumina MiSeq sequencing.

Basic bioinformatics analysis of sequencing data was detailed in Supplement Information 2. Following the protocol reported by Liu et al (2007), UniFrac-based principal-coordinate analysis (PCoA) was performed to explore the microbial community structure difference between the two fields. In addition, functions of microbial community were predicted using PICRUSt for bacteria (Langille et al., 2013) and FUNGuild for fungi (Nguyen et al., 2016) with OTU tables. And bacterial functions were shown with KEGG Orthology classification scheme, which is a database resource for understanding high-level functions and utilities of the biological system. All raw sequences had been deposited in the NCBI with SRA accession of SRP144526.

2.9. Data processing and statistical analysis

All data of a field was expressed as the mean plus/minus standard deviation (Mean \pm Sd) of 3 composite samples. Data processing was performed with Microsoft Excel Version 2013. All statistical analyses were conducted using the programming language R (version 3.1.0, R Foundation for Statistical Computing). Pearson method was used for correlation analyses. Differences between BA and NB fields were analyzed with One-way ANOVA and the individual means were compared using Turkey HSD test, with the significance defined at $p < 0.05$.

3. Results

3.1. Soil organic carbon, nutrient and aggregation

Edaphic properties of topsoil of NB and BA fields are given in Table 2. Content of SOC was 23.2 g kg⁻¹ in NB and 33.7 g kg⁻¹ in BA, with a very significant but great increase ($p < 0.001$) by 10.5 g kg⁻¹ in BA over NB field. Meanwhile, total N was 2.24 g kg⁻¹ in NB field and 3.02 g kg⁻¹ in BA field, being significantly ($p < 0.01$) increased by 0.8 g kg⁻¹ with biochar addition. There was a similar trend for other

Table 2

Selected basic properties of topsoil from no biochar (NB) and biochar amended (BA) fields sampled and measured in 2015.

Field	pH(H ₂ O)	BD (g cm ⁻³)	SOC (g kg ⁻¹)	Total N (g kg ⁻¹)	Available N (mg kg ⁻¹)	Aggr-MWD (μ m)	MBC (mg kg ⁻¹)	MBN (mg kg ⁻¹)
NB	6.04 \pm 0.08a	0.98 \pm 0.01a	23.16 \pm 0.66b	2.24 \pm 0.01b	167.8 \pm 2.48b	197.32 \pm 18.32b	804.1 \pm 14.11b	65.68 \pm 5.68b
BA	5.96 \pm 0.11a	0.90 \pm 0.04b	33.73 \pm 0.43a	3.02 \pm 0.37a	191.2 \pm 3.70a	247.38 \pm 20.54a	887.9 \pm 48.53a	85.64 \pm 6.87a

BD, bulk density; Aggr-MWD, mean weight diameter of soil aggregates as per the water-stable aggregate fractionation described in Section 2.5. Different letters in a single column indicate significant differences ($p < 0.05$) between the two fields.

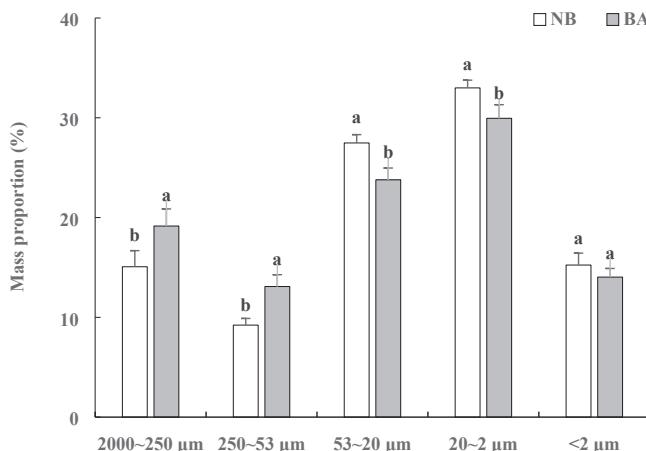


Fig. 1. Mass proportion (%) of water-stable aggregate size fractions of topsoil in no biochar (NB) and biochar amended (BA) fields. Different letters over the bars in a single size fraction indicate significant differences ($p < 0.05$) between the two fields.

fertility attributes in BA over NB field, by 14.0% ($p < 0.01$) for available N, by 10.4% ($p < 0.05$) for MBC and by 30.4% ($p < 0.05$) for MBN. While soil pH unchanged ($p = 0.36$), soil bulk density was lower by 0.08 g cm^{-3} ($p < 0.05$), indicating an increase by 5% in soil porosity 6 years after a single BSA in BA field. Related to these changes, root volume and root biomass in soil was significantly larger in BA field compared to in NB field (Fig. S4), root biomass was increased by 22% in BA field over NB field.

Data of mass proportion of size fractions of water stable aggregates (WSAs) to bulk soil is presented in Fig. 1 while that of mean weight diameter (MWD) in Table 2. Sand-sized macroaggregates (2000–250 μm) and fine sand microaggregates (250–53 μm) possessed 15.1% and 9.2% in NB field while 19.2% and 13.1% in BA field, respectively. Coincidentally, there was a lower proportion of coarse silt (fine) micro-aggregates (53–20 μm) and of fine silt particles (20–2 μm fine silt) in BA field compared to NB field. Whereas, there was no difference between the two fields in proportion of clay fraction (<2 μm). Resultantly, MWD of water stable aggregates was 197.3 μm in NB but 247.4 μm in BA, being higher by 25.4% ($p < 0.05$). As given in Table S2, isotopic abundance ($\delta^{13}\text{C}$ ‰ PDB) of soil organic carbon for bulk soil and aggregate fractions was all relatively lower in BA field compared to NB field, being significant ($p < 0.05$) for macroaggregate fraction of 2000–250 μm and fine sit fraction of 20–2 μm . Overall, SOC was lighter in BA field by $0.85 \delta^{13}\text{C}$ ‰ PDB for bulk soil and $0.76 \delta^{13}\text{C}$ ‰ PDB for soil aggregates when accounted for the mass proportions.

As seen in Fig. S1(f), the biochar used for amendment was light in powder in size smaller than 300 μm , being highly porous with micron- and nano-pores inside. Soil morphology and structure of topsoil from the NB and BA fields is shown in Fig. S5. While the rooted topsoil was a little bit deeper in BA field than in NB field, there were distinct differences observed in soil structure between the two fields. In NB field, soil structure looked rigidly blocky and compacted, and root less consistently rooted, often with horizontal and vertical cracks, across the soil clod. In clear contrast, that from BA field appeared soft and porous with loosely coherent soil crumbs and rooted consistently throughout the clod. In addition, some rusty veins or spots could be discernible in/along fissures close to tiny roots in BA field (Fig. S5, upper and bottom), supporting a higher porosity for water conducting therein. In Fig. 2 was shown an image of a sample randomly selected from the separated macroaggregate fraction of topsoil respectively taken in NB and BA field. Hereby, the macroaggregate from NB field was seen rigid and angular with plain surface, with tightly cohered mineral particles inside. This macroaggregates was aggregated of silt or clay minerals hardly coated with organic matter and had mainly regular pores in size

of dozens microns in-between the aggregated mineral particles. Like a sponge, however, the macroaggregate from BA field looked clearly crumby and soft with rough surface, with smaller aggregates loosely coherent resulting in irregular pores in nano- and micron-size. And, the BA macro-aggregates had plenty of particulate OM embedded in-between or covering smaller aggregates. Interestingly, a portion of residual wheat biochar was clearly seen adhering to the minerals/smaller aggregates (Fig. 2, bottom right).

3.2. Soil extracellular enzyme activities

Data of extracellular enzyme activities (EEAs) measurement is provided in Table 3. Hereby, all measured EEAs were seen significantly increased in BA field compared to NB field though the extent varied with the different EEAs. In BA field compared to NB field, activity of β -cellobiosidase, N-Acetyl-glucosaminidase and sulfatase was shown elevated by over 50% while that of α -glucosidase, β -glucosidase and β -xylosidase very significantly increased by ca 30%. Comparatively, a smaller increase (by 16–17%) was observed for activity of phosphatase, phenol oxidase and peroxidase in BA field compared to NB field. Overall, normalized enzyme activity estimated with the Equation (2) was very significantly ($p < 0.01$) higher by 33% in BA than in NB field.

3.3. Gene abundance of soil microbial communities and composition at phylum level

Microbial gene abundance at DNA and RNA level of topsoil from BA and NB fields are listed in Table 4. Gene abundance for bacterial and fungi of the paddy topsoil samples was in an order of 10^{10} copies g^{-1} and 10^9 copies g^{-1} at DNA level while of 10^{12} copies g^{-1} and 10^{10} copies g^{-1} at RNA level, respectively. Bacterial gene abundance at DNA level was significantly ($p < 0.05$) higher by 32.4% in BA field compared to NB field although no difference at RNA level or for fungi at both levels. At the phylum level (Fig. 3), however, there were very significant differences in bacterial but not in fungal community, between BA and NB fields. Of the bacterial communities, the relative abundance of *Chlorobi* at DNA level was increased by 34.7% ($p < 0.01$) while that of *Actinobacteria* decreased by 25.2% ($p < 0.01$), in BA field over NB field. At RNA level, whereas, the relative abundance of *Acidobacteria*, *Cyanobacteria* and *Planctomycetes* was seen reduced respectively by 11.5% ($p < 0.01$), 66.5% ($p < 0.05$) and 16.1% ($p < 0.01$) in BA field over NB field. Fungal communities at phylum level were dominated by *Basidiomycota* and *Zygomycota* in the soil, which was no difference between BA and NB fields both at DNA and RNA level.

3.4. Soil microbial community structure and predicted functions

As shown in Table S3, microbial α diversity was similar both at DNA and RNA levels ($p > 0.05$) between the two fields. Whereas, significant difference occurred in microbial β diversity between NB and BA fields, at degrees different between bacteria and fungi. The results of weighted UNIFRAC PCoA analysis were exhibited in Fig. 4. The PC1, explaining 87.8% and 74.7% for the difference between DNA and RNA levels while PC2 with a much smaller explanatory of 4.7% and 7.7% for the difference between the two fields, respectively for bacterial and fungal community structure. In detail, the microbial communities of a single field both of bacteria and fungi at DNA level were separated from those at RNA level by PC1. For bacteria, however, the triplicated samples from a field were coherent to each other and separated by PC2 between BA and NB fields both at DNA and RNA level. But for fungi, the triplicated samples from a field were not separated between BA and NB fields at RNA level, but were separated from each other by PC2 at DNA level.

The popular KEGG Orthology (KO) classification scheme was used to show the differences between the fields for functional traits of the bacterial community at RNA level (Fig. 5). Hereby, a few functional

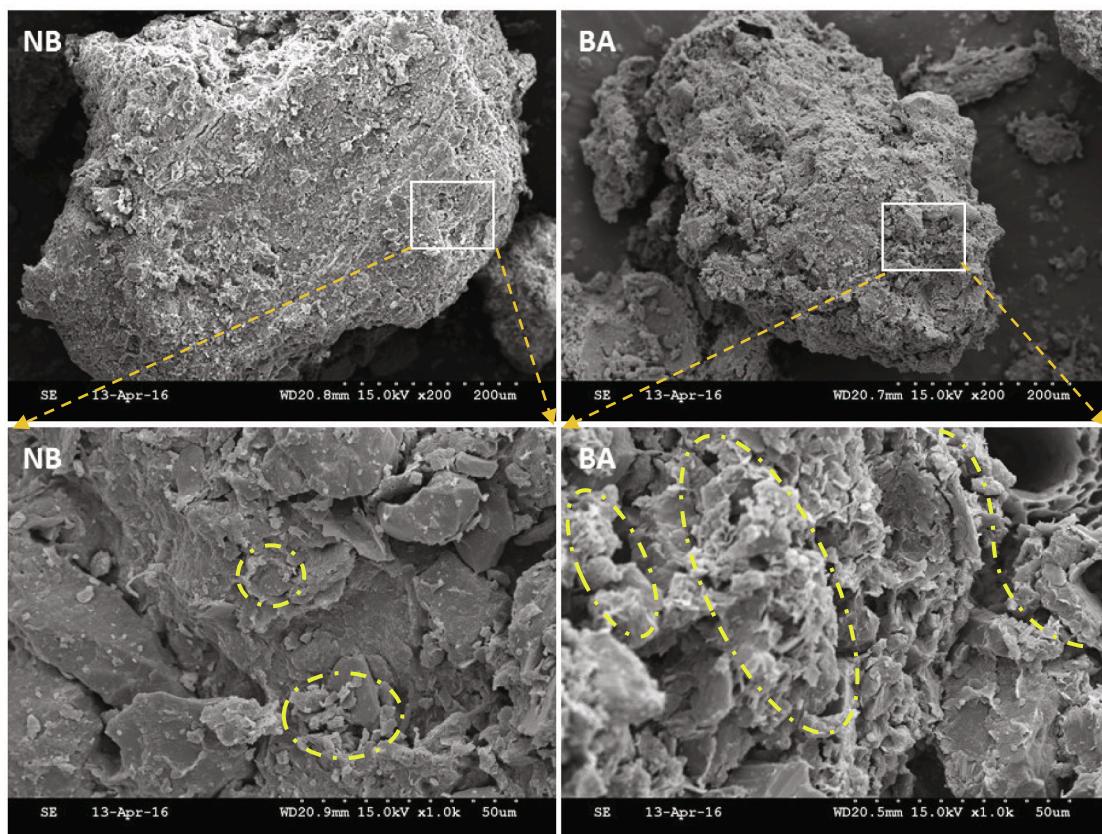


Fig. 2. Scanning electron microscopy image of a randomly selected macro-aggregate sample (x200, upper) and the structure inside the highlighted part in a white area (x1000, bottom) of topsoil respectively from no biochar (NB) and biochar amended (BA) field. The yellow dashed circles demonstrated the existence of particulate organic matter on the surface of finer/smaller aggregates within in the macroaggregate. Observation conditions refer to Section 2.3 in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Soil extracellular enzyme activities of topsoil from no biochar (NB) and biochar amended (BA) fields sampled and measured in 2015.

Field	α -glucosidase (nmol h ⁻¹ g ⁻¹)	β -glucosidase (nmol h ⁻¹ g ⁻¹)	β -xylosidase (nmol h ⁻¹ g ⁻¹)	β -cellobiosidase (nmol h ⁻¹ g ⁻¹)	N-Acetyl-glucosaminidase (nmol h ⁻¹ g ⁻¹)
NB	8.79 ± 0.09b	118.78 ± 10.30b	15.43 ± 0.52b	35.03 ± 1.84b	35.35 ± 0.46b
BA	11.61 ± 1.05 a	151.55 ± 6.64 a	20.64 ± 0.84 a	54.39 ± 7.71 a	53.14 ± 10.34 a
Field	Phosphatase (nmol h ⁻¹ g ⁻¹)	Sulfatase (nmol h ⁻¹ g ⁻¹)	Phenol oxidase (μ mol h ⁻¹ g ⁻¹)	Peroxidase (μ mol h ⁻¹ g ⁻¹)	Normalized enzyme activity
NB	172.33 ± 6.35b	12.37 ± 1.03b	0.94 ± 0.05b	10.14 ± 0.42b	0.14 ± 0.00b
BA	200.54 ± 18.43 a	18.87 ± 2.25 a	1.10 ± 0.06 a	11.78 ± 0.23 a	0.19 ± 0.01 a

Different letters in a single column indicate significant differences ($p < 0.05$) between the two fields.

Table 4

Microbial gene abundance of topsoil from no biochar (NB) and biochar amended (BA) fields sampled and measured in 2015.

Field	Bacteria (10 ¹⁰ copies g ⁻¹)		Fungi (10 ⁸ copies g ⁻¹)	
	DNA	RNA	DNA	RNA
NB	3.18 ± 0.38b	342.92 ± 31.27a	1.89 ± 0.69a	306.38 ± 167.7a
BA	4.21 ± 0.28a	285.83 ± 76.94a	2.32 ± 0.69a	238.90 ± 22.29a

Different letters in a single column indicate significant differences ($p < 0.05$) between the two fields.

groups (KO tier 2) exerted changes in BA field compared to NB field at RNA level but not at DNA level. Among second-tier functional categories, the abundance of those functional traits with “metabolism of other amino acids”, “amino acid metabolism” and “xenobiotics

biodegradation and metabolism” were seen significantly lower while those with “translation” and “transcription” higher, in BA field than NB field. In addition, significantly but slightly higher relative abundance of functions with “drug resistance” while significantly but slightly lower relative abundance of functions with “infectious diseases: parasitic”, “immune diseases” and “endocrine system” were observed in BA field than in NB field. Of course, these functions were known associated to human diseases or organismal systems but poorly addressed to environmental impacts.

For fungi, the relative abundance of sequences assigned to functional guilds with ecological significance were observed in this study. When compared by trophic modes (Fig. 6), the proportion of “pathotrophic” fungal group to the total fungal community at DNA level was significantly ($p < 0.01$) decreased in BA field compared to NB field. However, no difference was detected between the two fields in proportion of fungal groups with other two trophic modes at DNA level or

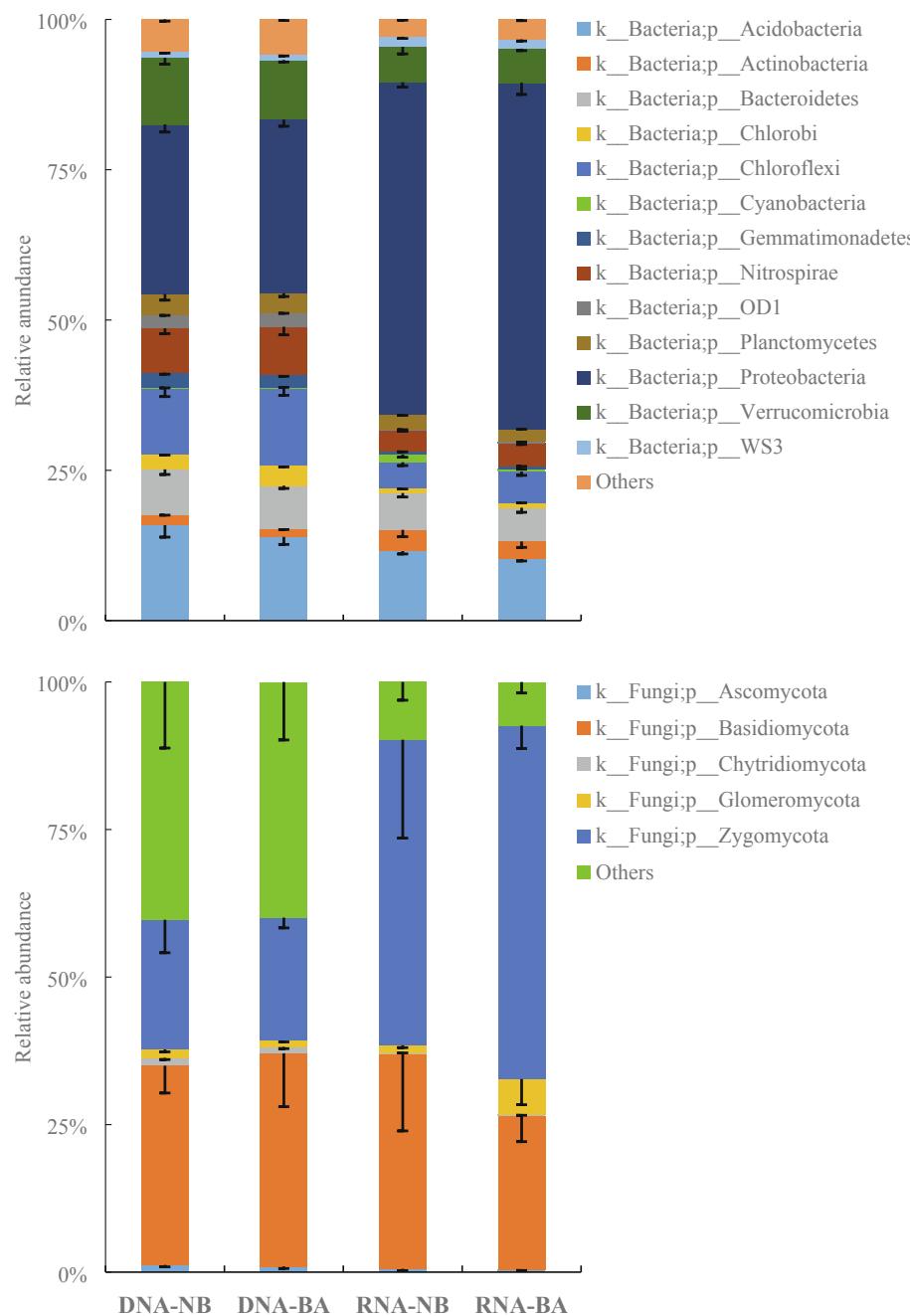


Fig. 3. Taxonomic breakdown of proportional change in bacteria (top) and fungi (bottom) at the phylum level of topsoil sample from no biochar (NB) and biochar amended (BA) fields. Phyla with relative abundances less than < 1% are not shown.

any trophic mode at RNA level. Furthermore, the changes of nine guilds involved in the three trophic categories were listed in Table 5. In BA field over NB field, the proportion of “bryophyte parasite” fungi was very significantly ($p < 0.01$) and that of “plant pathogen” fungi significantly ($p < 0.05$) reduced at DNA level both by 2 folds. Whereas, there were no significant ($p > 0.05$) difference in proportion of animal Endosymbiont and Arbuscular Mycorrhizal in BA field compared to NB field.

4. Discussions

4.1. Soil carbon sequestration and aggregation boosted in biochar field

For the last decade, intensified high yielding rice production in this area had been challenged with excess N and P input (Wang et al., 2009)

and by climate change of winter-spring warming and spring and autumn drying (Huang and Xu, 2009). With an overall trend of soil acidification, topsoil SOC under conventional farming management was mostly unchanged or even in decline (Liu and Jiang, 2009; Ma et al., 2009). In this study, topsoil SOC content in the field without biochar was almost unchanged compared to that before trial (Table 1). Whereas, topsoil (0–15 cm) SOC storage calculated with data in Table 2 was 44.9 t ha^{-1} in BA field compared to 34.0 t ha^{-1} in NB field. SOC storage was 35.1 t ha^{-1} under BSA of 10 t ha^{-1} in 2009 (Zhang et al., 2012) and 36.9 t ha^{-1} before the experiment. Accordingly, there occurred an apparent SOC increase of $8\text{--}10 \text{ t ha}^{-1}$ in BA field, or of 10.9 t ha^{-1} relative to NB field, 6 years following a single biochar addition. Assuming the preservation in soils of biochar OC (4.7 t ha^{-1} at a dose of 10 t ha^{-1}), a potentially acute SOC sequestration in BA field could be $3.3\text{--}5.1 \text{ t ha}^{-1}$, at a rate of $0.5\text{--}0.7 \text{ t C ha}^{-1}$ per annum,

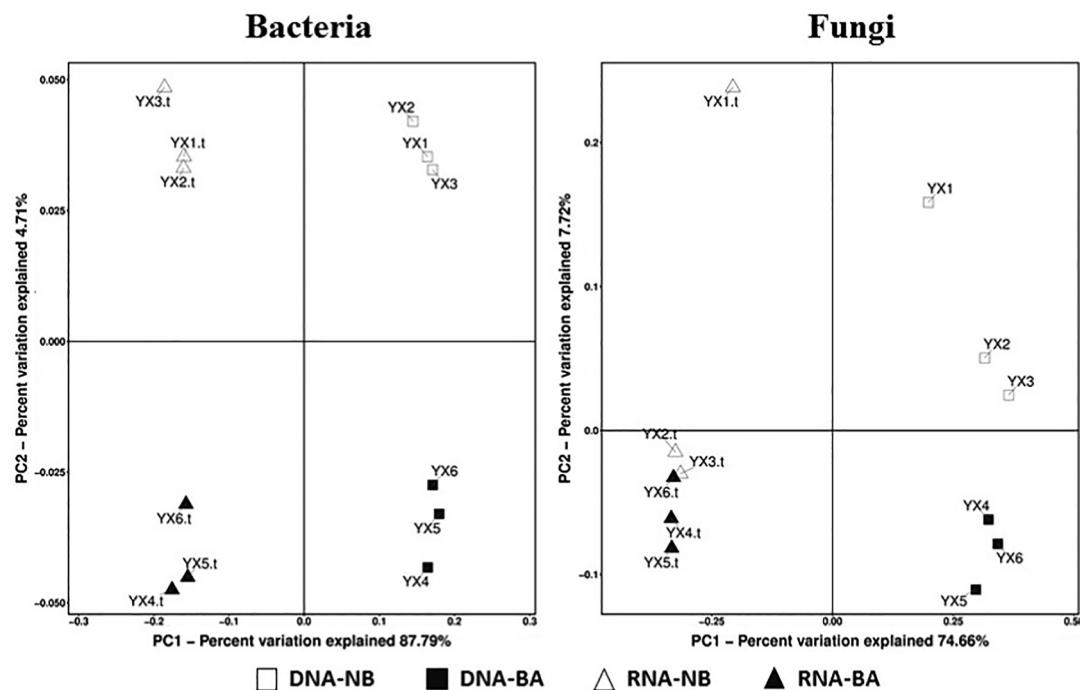


Fig. 4. Principle coordinate analysis of bacterial (left) and fungal (right) OTU weighted UNIFRAC distances at DNA and RNA level of topsoil in no biochar (NB) and biochar amended (BA) fields, respectively.

throughout 2009–2015. This rate could be relevant to an increase per annum by 1.3–2.0 % in topsoil SOC storage over that before amendment, being over three folds as that the “4 per 1000 Initiative” demanded (Rumpel et al., 2018). Lu (2015) reported a high increase in SOC storage (by 15% per annum grand mean) with plowed incorporation of whole crop residue in rice–wheat system in short term over 5 years. Even so, the SOC increase in BA field was to the high end of SOC sequestration rates of 0.2–0.8 t ha⁻¹ and of 0.3–0.6 t ha⁻¹ at 95% interval under improved management practices found respectively for China and global (Minasny et al., 2017). Therefore, increase in SOC in the rice paddy was boosted with biochar amendment.

This greater increase in topsoil SOC storage could be explained by a biochar-promoted SOC sequestration of crop derived OC via increased crop production and root biomass input to soil. Using rice yield data in Table S1 and wheat yield in Wang et al. (2018), the grain to straw ratio of 1.0 (Lu, 2015) and an average straw OC content of 40% for rice and wheat from the area (Sun et al., 2018), an overall crop residue C input per annum could amount to 6.4 t C ha⁻¹ in BA field compared to 5.2 t C ha⁻¹ in NB field since 2011. Being regarded as the major source for SOC accumulation in soils (Rasse et al., 2005), root biomass was

0.92 t ha⁻¹ in BA field and 30% higher than NB field, when measured in 2015 (Fig. S4). There was an overall lower ¹³C abundance (Table S2) of bulk soil and soil aggregates (weighted of their mass proportions) by ca 30% in BA field than in NB field, similar to their difference in root biomass. As both rice and wheat were plants with C3 metabolism pathway, the plant derived OC should be lighter than biochar, which was a solid residue via high temperature thermal decomposition with much light carbon volatilized (Lehmann and Joseph, 2015). Light carbon, often in form of particulate OM, could be physically protected intra- and inter-macroaggregates (seen in Fig. 2) contributing mainly to SOC sequestration (Six et al., 2000; Le Guillou et al., 2012; Six and Paustian 2014). In our previous works (Zhou et al., 2008; Song et al., 2012), recent SOC sequestration in rice paddies from southern China had been elucidated for physically protection as POM and for chemical binding to iron hydroxides as mineral associated pool (MAOM, Cotrufo et al., 2019), the latter was seen with lighter carbon also in silt and clay fractions from BA field (Table S2). Hereby, the high SOC sequestration rate with biochar could be related to soil pertinent reactivity with input carbon molecules as per the clayey texture and mixed clay mineralogy with iron oxyhydrates, often in reduced condition for rice paddy

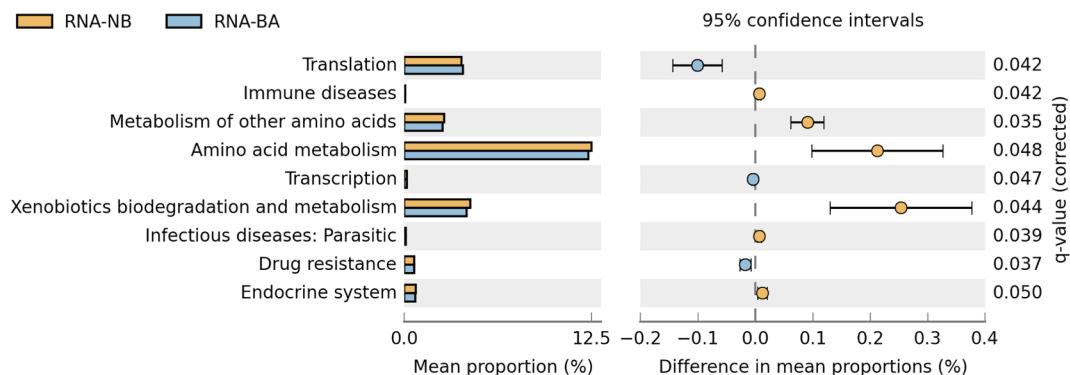


Fig. 5. Functional traits of bacterial community on KEGG pathway (KO tier 2) at RNA level of topsoil in biochar amended field (BA) relative to no biochar (NB) field. The left part shows the relative abundance of different functions of both fields while the right part the size of the proportion differences in the BA field from the NB field at 95% confidence interval, with the significance at the $p < 0.05$.

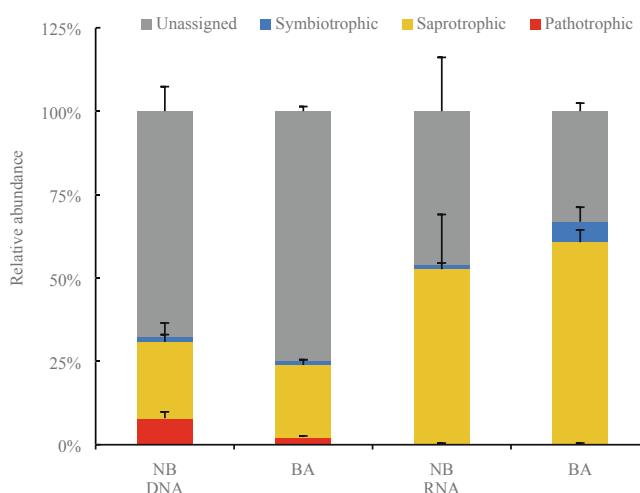


Fig. 6. Mean relative abundance of reads assigned to different fungal guilds of topsoil in biochar amended field (BA) relative to no biochar (NB) field.

management in farm level (Wiesmeier et al., 2019).

Following, POM as fresh carbon particularly those of fine roots and root exudates had been known as the key drivers for soil aggregation (Six et al., 2004; Six and Paustian, 2014). With SOC sequestration, a great promotion of soil aggregation was seen in the clayey rice paddy following biochar amendment. With a 25% increase in MWD of soil aggregate fractions (Table 2), proportion of sand sized macroaggregates and fine sand sized microaggregates was increased by 32% averaged on their mass weight in BA field over NB field (Fig. 1). Following a three class hierarchy of macroaggregates (2000–250 µm), microaggregates (250–53 µm) and fine aggregates (or fine particles) (<53 µm) (Bach et al., 2018), soil aggregation degree characterized by the proportions of macro- and micro- aggregates to that of fine particles was increased by 48.3% in BA field over NB field. Changes of all these aggregation parameters appeared much greater than the average increase in soil aggregate stability by 8% with biochar in a meta-analysis (Omondi et al., 2016). The increase hereby was similar to that in a US silt loamy maize soil, under conservation tillage or manure application over 10 years (Mikha and Rice, 2004). Possibly due to the biochar in size of 0.3 mm, the increase in proportion both of macro- and micro-aggregate fraction was much higher than those of rice soils observed with prolonged rice cultivation over centuries (Liu et al., 2016), with straw or manure amendment (Li et al., 2007) and with conservation tillage or combined organic/mineral fertilization (Zhou et al., 2009) over decades. Unlike a great increase in soil aggregate MWD in a sandy soil under no till (Khademalrasoul et al., 2014), biochar amendment at doses similar to this study generally led to short term insignificant (Liu et al., 2014b) or small (<10%) (Fungo et al., 2017) increase in water stable macroaggregates in SOC-poor soils. Biochar particles, often embedded inside the macro-aggregates as tiny particles (Yu et al., 2016), could act as a persistent core to bridge or connect organic/mineral complexes (seen in Fig. 2), promoting the development of water stable macroaggregates. As a result of aggregation greatly developed, the soil in the BA field looked soft and spongy with pores of wide range of sizes compared to rigid and compact clod in NB field (Fig. 2; Fig. S5). As a result, soil porosity as a key function provided by soil aggregates (Rabot et al., 2018) could be greatly improved, as seen in Fig. 2 and Fig. S5. Due partly to this, deeper roots and larger root volume were found in BA topsoil than in NB (Fig. S4), with a 30% difference in total root biomass between them as mentioned above. As addressed by Lehmann et al. (2015), with mediating organic-mineral and microbial interactions in soil-plant interface, biochar addition to soil did bring about soil carbon sequestration in addition to biochar carbon input. This could be linked to a legacy of enhanced soil aggregation over years following biochar

Table 5
Percentages of reads assigned to different fungal guilds at DNA and RNA levels of topsoil in no biochar (NB) and biochar amended (BA) fields sampled and measured in 2105.

Field	Animal pathogen	Bryophyte parasite	Plant pathogen	Undefined saprotroph	Wood saprotroph	Animal endosymbiont	Arbuscular mycorrhizal	Ecto-mycorrhizal	Lichenized
<i>DNA level</i>									
NB	0.02 ± 0.01a	7.32 ± 1.75a	0.61 ± 0.21a	22.83 ± 2.67a	0.04 ± 0.02a	0.03 ± 0.01a	1.45 ± 0.42a	0.12 ± 0.03a	0.10 ± 0.06a
BA	0.00 ± 0.00a	2.08 ± 0.03b	0.20 ± 0.07b	21.58 ± 1.59a	0.04 ± 0.01a	0.02 ± 0.01a	1.05 ± 0.19a	0.23 ± 0.30a	0.09 ± 0.04a
<i>RNA level</i>									
NB	0.00 ± 0.00a	0.40 ± 0.06a	0.03 ± 0.02a	52.09 ± 16.45a	0.10 ± 0.05a	0.00 ± 0.00a	1.28 ± 0.41a	0.09 ± 0.02a	0.02 ± 0.03a
BA	0.01 ± 0.00a	0.38 ± 0.10a	0.06 ± 0.05a	60.21 ± 3.67a	0.07 ± 0.02a	0.00 ± 0.01a	6.04 ± 4.35a	0.07 ± 0.03a	0.00 ± 0.01a

Different letters in a single column of a pair of NB and BA indicate a significant difference ($p < 0.05$) between the two fields.

amendment, potentially driving positive changes in soil physical and microbial conditions and in turn soil productivity and health via biological responses, specifically of root (Gregory, 2007).

4.2. Microbial growth and enzyme activities largely promoted in biochar field

Improved soil aggregation provided better protection of microbial community from predators and thus could drive positive changes in bioactivity and ecosystem services (Six and Paustian, 2014), particularly in rice paddies (Pan et al., 2015b). In BA field over NB field in this study, MBC was higher by 11% but MBN and normalized extracellular enzyme activity was greater by ca 30% (Tables 2 and 3) despite of a sharp difference in SOC accumulation by 45% and also in nutrient input following 2011 (Table S1). Hardy et al., (2019) argued that the long-term effect of charcoal was related to a modification of soil ecological niche related to nutrient availability and pH rather than to an alteration of the carbon source available to biota. The finding here indicated soil microbial communities were not constrained by less nutrient input in BA field, probably due to microbial community with better N and P use efficiency in biochar amended than no amended soils (Anderson et al., 2011). This was supported with enhanced microbial biomass carbon by 26% but decreased the metabolic quotient by 17% seen in field BSA studies (Zhou et al., 2017). Again, slight to great increase in MBN was reported in short term BSA experiments using same biochar at 20 t ha⁻¹ in various rice paddies from southern China (Chen et al., 2013, 2015). Moreover, the increase in normalized EEA (Table 3) was comparable to that in a fertilized rice paddy high in SOC under rice straw amendment at 4.5 t ha⁻¹ for 25 yr (Li et al., 2019) and to that in an Indian soil poor in SOC under continuous compost amendment at 5 t ha⁻¹ for 32 yr (Nayak et al., 2007). In rice paddies under continuous rice cultivation over a time span of 300 yr, normalized EEA was increased even by 50% compared to the initial soil (Wang et al., 2015). Therefore, the short term legacy of an overall enhancement of microbial metabolic activity was prominent in BA soils following a single biochar application. And, soil aggregation in BA soil was influenced by SOC change, which led to but not driven by, microbial growth and activity change (Hardy et al., 2019; Demenois et al., 2020).

Soil EEA could be proxies for organic matter decay and thus the availability of substrates for microbial or plant uptake, potentially related to biological diversity and ecosystem functioning as well as soil fertility (Kandeler et al., 1999; Marx et al., 2001; Caldwell, 2005). As result of biochar-enhanced macroaggregate build-up, where labile or relatively young OC often occluded intra-macroaggregates or inter-microaggregates (Fig. 2), soil microorganisms and their associated EEA could be micro-spatially separated from decomposable organic materials (Navarro-García et al., 2012; O'Brien and Jastrow, 2013). Coping with the increased growth of microbial communities, biochemical activities could be greatly enhanced in micro-pores in-between micro-aggregates within macroaggregates, which acted as functional microbial hotspots in soil (Li et al., 2019). While the carbon degrading enzyme activities measured with MUB protocol were shown prompt responses to SOC accumulation derived likely of plant residues as labile POM (Wang et al., 2015), those measured using L-DOPA were relatively insensitive to SOC dynamics (Allison and Jastrow, 2006). Dorodnikov et al. (2009) reported an increase in carbon-degrading enzyme activities under elevated air CO₂ following addition of easily decomposable substrates, potentially accelerating C turnover in the wheat soil. Hereby, the increase in BA field over NB field in normalized EEA (by 33%) and in individual carbon-degrading EEA (by 30–50%) was higher than that in oxidizing EEA as well as phosphatase (by 16–17%) (Table 3). In a fertilized rice paddy from an adjacent area, increase in activities of invertase (by 85.5%) was much greater than in those of urease and phosphatase (by 30–40%), under 20-yr rice straw amendment at 4.5 t ha⁻¹ (Wang et al., 2008). Of course, the smaller increase in phenol oxidase and peroxidase could be explained, in part, with

recalcitrant SOC pools rich in lignin derived carbon substrates (Guggenberger et al., 1994; Sinsabaugh, 2010).

When scaled by SOC value, there was an insignificant difference in overall EEA between BA and NB field. As though biochar OC inherently inert for microbial bio-degradation (Wang et al., 2016), the accumulated OC in BA soil seemed much biologically active for physical protection instead of chemical recalcitrance responsible for SOC sequestration of plant derived young carbon (Six and Paustian, 2014). Such legacy was comparable to a clay loam maize field from Sweden under cattle manure at 8 t ha⁻¹ per annum over 60 years (Enwall et al., 2007) and to paddy soils under rice cultivation over centuries (Wang et al., 2015; Liu et al., 2016). When scaled by MBC, however, the individual EEA were seen significantly increased in BA over NB field. This could suggest an increased production capacity of EEA by soil microbes to improve biological cycling of C and N, and their use efficiency (Dorodnikov et al., 2009; Anderson et al., 2011). In particular, MBC or MBN scaled activities of chitinase and of sulfatase for degrading N-containing carbon substrates, together with β-celllobiosidase, were seen highly increased in BA over NB field. These changes could contribute to biological transformation and availability of N in soil, leading to increase in fertilizer N use efficiency by over 50% in the biochar treated field (Zhang et al., 2010). Crop residue biochar could promote microbial N retention and biological cycling in amended soil rich in SOC (Ameloot et al., 2015) though pyrolysis conditions could have tradeoffs between carbon stability/sequestration potential and nutrient availability in the produced biochars (Crombie et al., 2015). The finding here supported the biological manipulation of soil N fertility and agronomic performance in a contaminated soil amended with same biochar (Zhou et al., 2018). Similar to the finding of improved N and P transformation with altered microbial community in a grass soil in a pot treatment with BSA (Anderson, et al., 2011), this study pointed to a soil functioning legacy for crop production with improved nutrient supply via enhanced carbon sequestration and microbial growth under BSA. The markedly positive changes in microbial biomass and enzyme activities for nutrient transformation could be important components for short term legacy for soil fertility following biochar amendment.

4.3. Microbial diversity unaffected but community structure altered in biochar field

Changes in microbial abundance and community structure with biochar over long run were poorly addressed (Gul et al., 2015; Zhou et al., 2017; Hardy et al., 2019). Alteration with biochar of microbial community structure and diversity had been noticed widely in short term lab incubation or greenhouse pot experiments (Anderson, et al., 2011; Xu et al., 2014; Gul et al., 2015; Kolton et al., 2017; Zhou et al., 2017). Yet, such alteration was seen often inconsistent or even contrasting either between bacterial and fungi, or between total and active communities as well as between soils with distinct properties in field rice soils (Chen et al., 2013, 2015, 2016). Prominently, no difference was visible in α diversity of bacterial or fungi both in DNA and RNA levels between the BA field and NB field as sampled and measured in 2015 (Table S3), 6 years following a biochar addition in BA field. Microbial diversity could be hardly affected with management practices in agricultural soils even for long run (Frey et al., 1999) or related to soil aggregation even promoted with SOC enhancement (Demeois et al., 2020). Change in microbial diversity of rice soil at DNA level was notably small across a chronosequence of rice cultivation over centuries (Wang et al., 2015) or insignificant following a wetland shifting to rice cultivation for 30 years (Jin et al., 2012) and under combined organic/mineral fertilization for 15 years (Zhang et al., 2004). With biochar addition, α-diversity of fungi in rice paddy was not affected (Chen et al., 2015; Tian et al., 2016; Yao et al., 2017) while that of bacterial could be modified slightly but inconsistently either across sites (Chen et al., 2015) or between addition rates (Zheng et al., 2016). Therefore, as a management tool, biochar application did not exert a significant

modification on microbial community diversity in the rice soil.

Whereas, the changes between BA field over NB field in relative abundance and thus community composition could be of notice in this study. Relatively higher and lower microbial gene abundance was observed respectively at DNA and RNA level and insignificantly higher bacterial-to-fungal ratio (F/B ratio) were seen in BA field over NB field. Hereby, a 32% increase in bacterial gene abundance at DNA level was parallel to a 30% increase in MBN, in BA field compared to NB field (Table 4). This seemed different from the decreased B/F ratio in population number in a similar rice soil following SOC accumulation under long term combined organic/mineral fertilization (Liu et al., 2011). The present finding is similar to the increasing bacterial dominance with prolonged rice cultivation in a rice soil chronosequence derived from salt marsh in a neighboring area of China (Wang et al., 2015). Thereby, changes in microbial diversity and fungal abundance were neither remarkable nor consistent across the chrono-sequence soils despite of a very sharp bacterial gene abundance response to a consistently growing accumulation of SOC and particularly of POM under prolonged rice cultivation over 50–700 years. A study using PLFA (Dong et al., 2014) showed highly increased bacteria and actinomycetes abundance but unchanged fungi abundance under long term organic/mineral fertilization in rice paddies from subtropical China. Whereas, short term biochar addition increased microbial abundance towards a bacteria-dominated community in temperate soils from Europa (Gomez et al., 2014). Similarly, short term amendment of rape seed biochar sharply increased bacterial gene abundance and promoted organic pollutant decomposition in a submerged rice soil, with proportional response to addition rates up to 7% (Tong et al., 2014). In a long term field experiment, biochar strongly influenced microbial PLFA community composition and functions in a rice soil (Tian et al., 2016). In paddies with varying soil conditions in our previous studies, short term wheat biochar addition consistently increased bacterial gene abundance while decreased (Chen et al., 2013) or unchanged (Chen et al., 2015) that of fungi, with community structure inconsistently modified (Chen et al., 2015, 2016). As abiotic properties of soil readily respondent to biochar (Gul et al., 2015), amendment of wheat biochar hereby strengthened a bacterial dominated microbial community with the community structure modified in the rice paddy.

For microbial community composition at phylum level, there were very significant differences in bacterial but not in fungal community, between BA and NB fields (Figs. 3 and 4). At DNA level was found a moderate (by 25–35%) but contrasting difference between BA and NB fields (Fig. 3) in relative abundance of *Actinobacteria* phylum, known as Gram positive, mostly aerobic and favored with high soil N (Dai et al. 2018), and in that of *Chlorobi*, an anaerobic Gram negative major bacterial phylum capable of photosynthesis in paddy (Asakawa and Kimura, 2008). At RNA level, lower relative abundance of *Acidobacteria*, *Cyanobacteria* and *Planctomycetes*, though in minor proportions, were seen in BA field than in NB field. Particularly, *Cyanobacteria* as a typical autotrophic aquatic bacterial phylotype was sensitive to N and often causes water blooms for serious environmental risks (Huisman et al., 2018). Similarly in a pot study, Anderson et al (2011) could show significantly increased bacterial families involved in N transformation and cycling for the observed N supply improvement in biochar added grass soil. Though not measured in this study, soil solution concentration of inorganic N was lower under biochar than without biochar, generally by 10% in slightly acid medium textured soil under crop residue biochar (Nguyen et al., 2017), reducing the potential environmental risk for water blooming and nitrous oxide emission (Cayuela et al., 2014).

The increased bacterial (rather than fungal) gene abundance with community structure altered, with divergent phyla composition changes, had been increasingly reported in many short term biochar amended soils (Liao et al., 2014; Lu et al., 2015; Jenkins et al., 2017). In a salt-stressed maize soil 2 years following a wheat biochar addition, bacterial community changed with sharp increase in abundance and a

shifted community structure while fungal community with slight increase in abundance but community structure unmodified (Lu et al., 2015). In a short term soil incubation with biochar addition, shift of bacteria community structure occurred at the genus and phylum levels, while that of fungi only at the genus level (Liao et al., 2014). Jenkins et al. (2017) noted much greater differences in taxa of bacteria than those of fungi from STAMP differential abundance testing of sequencing data following biochar amendment to soils from UK and Italy. Of course, bacterial community structure change in biochar amended paddies could vary with soil types/conditions (Chen et al., 2015).

4.4. Soil health improvement beyond SOC sequestration in biochar field

Improvement of soil and plant health could be observed, following the microbial community changes, in biochar fields. Together with the EEAs change, bacterial community composition shifted towards better nutrient transformation and utilization as well as carbon use efficiency in biochar field six years following a single BSA, providing benefits for crop production (Wang et al., 2018) besides greenhouse gas emission mitigation (Zhang et al., 2010, 2012). When field-measured in 2010, the ratio of net primary production to heterotrophic respiration was 8.2 and 5.7 (Zhang et al., 2013), and emission factor of N fertilizer was 0.43 and 0.21 kg N₂O-N kg⁻¹N fertilized (Zhang et al., 2010), for the rice paddy respectively under no biochar and biochar at 10 ha⁻¹. In coincident with the changes in microbial growth and enzyme activities mentioned above, all these suggested an improved microbial carbon/nitrogen use efficiency in the biochar amended paddy, whereby N fertilization reduced. This corresponded to the general reduction of microbial metabolic quotient, greatly in heavy textured soils (Zhou et al., 2017). Such feature could help understand the legacy of soil biological functions subsequently modified following biochar amendment, potentially by microbial adapting to a nutrient-rich and high pH (micro-) environment (Jenkins et al., 2017).

Following the marked increase in microbial biomass and EEAs, there was slight increases in gene abundance of “translation” “transcription” and “drug resistance” bacterial in BA field over NB field. This could be related to a potentially improved metabolic activity of bacterial community (Fig. 5). However, the abundance of functional groups related to amino acids metabolism and xenobiotics biodegradation was seen to decrease significantly in BA field relative to NB field. This could suggest bacterial community in BA field was less stressed by N availability (Anderson, et al., 2011) compared to in NB field, for less N lost via leaching and emission (Borchard et al., 2019). This is parallel to the marked increase in soil available N and MBN in BA field over NB field (Table 2). Build-up of SOC from crop residue incorporated could be limited with N availability (Kirkby et al., 2014) and enhanced excavation of N with amino-acid metabolic bacterial could lead to loss of SOC at cost of organic matter decomposition in NB field, partly explaining its much lower SOC under same crop residue management. Such change further contributed to SOC sequestration in biochar-treated soil following application (Zheng et al., 2016). While biochar could have a significant role in protecting N (Clough et al., 2013), there could be higher abundance of bacterial involved in N cycling for modulating N retention and improving N supply in biochar added soil (Anderson et al., 2011). In the farm (Table S1), N nutrition to rice plant and thus N use efficiency was seen significantly improved in BA field than in NB field (2.5 kg more grain per kg of N fertilized, on average). Such improvement of agronomic use efficiency was already addressed for an adjacent rice soil under combined organic/inorganic fertilization (Pan et al., 2009). Unlike the findings by Anderson et al. (2011), Chen et al. (2016) and Kolton et al. (2017), a stimulation of bacterial growth capable of degrading recalcitrant carbon substrates was not clearly seen in the BA soil in this study, where were significantly increased input of young carbon from biomass production as mentioned in Section 4.1. In addition, the decrease in groups with functions of “xenobiotics biodegradation and metabolism” could be in adaptation to lesser amount of

pesticides used in BA field (Wang et al., 2018).

More interesting was the decrease in the proportion of “pathotrophs” to total fungal community at DNA level, in line with the decrease in guilds “bryophyte parasite” and “plant pathogen” observed, in BA field compared to NB field (Table 5). As plant diseases from pathotrophic fungi was common in humid subtropical area, less amount of pesticides used in the BA field (Wang et al., 2018) could point to improved plant defense potentially via decrease in fungal pathotrophs, in the biochar treated field with less N input. This was similarly shown with a decrease in potential plant pathogens in a non-rice soil 3 years following a single BSA (Yao et al., 2017). Biochar treatment could help to suppress plant disease with the increased diversity and metabolic activity of the rhizosphere microbiome (Kolton et al., 2017).

Indeed, the above mentioned positive biotic changes in BA field were witnessed by the farmer managing the land. Since he was well informed of the crop yield gain by over 10% and N agronomic use efficiency improved by over 50% shortly following biochar addition (Zhang et al., 2010, 2012), fertilizer application of N and also of P and K was largely reduced in the biochar field, in responding to the government call on reduction of fertilizer use in the Tai Lake region. Fortunately, crop yield in BA field was not adversely affected with the much low P and K fertilization for their initial richness the rice paddy (Liang, 2004) and partly for the biochar’s role in improving soil nutrient availability (Anderson et al., 2011; Biederman & Harpole 2013). Compared to NB field, the healthier soil in BA field could be characterized by a significantly higher rice production (on average 0.72 t ha⁻¹ more) and fertilizer yield return (on average 1.2 kg more per kg of total input of N, P and K including biochar added) throughout the 6 years following a single biochar addition (Table S1). Specifically, fertilizer N use efficiency was increased by 12% on average in BA field (28.6 kg grain kg⁻¹N) over NB field (25.5 kg grain kg⁻¹N), even similar to that (by 13%) reported for the beginning year in biochar added rice paddies from South China (Huang et al., 2013). More interestingly, rice plants in BA field turned relatively resistant to rice leaf feeder and rice plant-hoppers, and protective against false smut, with reduced use of pesticides, a few years following BSA in BA field (Fig. S3; Supplement Information 1). Particularly, a very low yearly variability of rice grain production (<5%) was achieved in BA field compared to NB field with the variability as high as 30% (Table S1). All these in-farm information could further evidence an improved soil health functioning, possibly mediated with the changes in microbial functioning groups mentioned above.

Noted by Elad et al., (2010), system acquainted resistance (SAR) with biochar was mainly attributed to the small DOC pool with redox potential in biochar amended soil/plant system (Harel et al. 2012; Mehari et al. 2015). On one hand, DOC in the wheat biochar was really rich (Table 1) but short-lived, and could be effective for rice growth only for the first rice season following addition (Korai et al., 2018). In fact, this pool was quickly used up by methanogenic bacterial, causing significant methane emission first season following its addition in BA field (Zhang et al., 2012). On the other hand, a legacy of plant protection functioning in the BA field here could be a short term response to the positive changes following BSA in bacterial and fungal community composition related to plant pathogens or antibiotic activity. The reduction of pathogenic fungi guilds observed here could represent the potential immobilization and deactivation of pathogenic enzymes and toxic metabolites by biochar (Jaiswal et al., 2018), in addition to reduction of disease risks without N in excess in BA field. Thus, with reduced N application, BSA could potentially promote plant defense than in enhancing production itself (Viger et al., 2015; Bonanomi et al., 2015), beyond nutrient cycling improvement (Anderson et al., 2011; Biederman and Harpole, 2013).

Overall, this study demonstrated a legacy for soil health improvement, in line with subsequent SOC sequestration and the promoted soil aggregation over years following one time biochar amendment in the slightly acid clayey rice paddy. Promoted soil aggregation through the

biochar-soil-root interaction (Lehmann et al., 2015) could likely contributed to soil resistance to physical constraints related to aeration or moisture retention (resistance to drought for example, Liang et al., 2014). Plant root as an indicator of plant growth health, controlled not only nutrient acquisition and physical support, but also carbon supply for microbes and subsequent carbon sequestration, thus root system could be a management tool for agriculture (Gregory, 2007). A great increase by 30% in measured root biomass in BA field over NB field could be a major part of soil health, not only with nutrient excavation and carbon input for the C increase but also with stress resistance for better yielding as often observed in field (Fig. S3; Supplement Information 1). However, both improved nutrient availability and disease resistance could be linked to microbial community change as respectively addressed by Anderson et al., (2011) and Kolton et al., (2017). Again, microbial community structure or composition was mediated necessarily by soil aggregates (Kögel-Knabner et al., 2008; Dorodnikov et al., 2009; Gul et al., 2015). Thus, an interaction between SOC sequestration, soil aggregation, root development and microbial community change could be a potentially and mechanistically linked driver for the legacy of soil health and/or crop productivity following biochar amendment in this rice paddy in clayey texture and rich in soil nutrients. In other words, the functioning could be mediated with the biochar reacted soil/plant system, not provided merely by biochar itself as increasingly aged (Hardy, et al., 2017b; Yi et al., 2020). However, the robust processes regarding SOC turnover, microbial selection/competition for C resource and nutrient transformation and how long the legacy could preserved still unraveled.

4.5. Perspective and recommendation for biochar in agriculture

Thanks to the legacy following biochar amendment, high and stable rice production at relatively low input of fertilizer (and/or pesticides) could be obtained for some years following a single biochar addition at a proper rate (10 t ha⁻¹ here), with synergic benefits of reduction of GHGs emission and of potential risks to waters and food. This study highlighted an added value of soil health improvement for high, stable and nutrient efficient rice production to SOC increase and emission reduction with biochar amendment in rice soils (Zhang et al., 2010, 2012, 2013; Liu et al., 2012; Mohammadi et al., 2016). We recommend that an action of using crop residue biochar in rice agriculture could be a strategic solution connecting global goals for sustainable development, food security and climate change mitigation (Chabbi, et al., 2017). This could be particularly important for rice agriculture of China, which had been challenged with climate variability (Pan et al., 2011) and excess use of nutrients related to issues of reactive N and waters eutrophication (Peng et al., 2009). Thus, soil carbon increase, particularly following amendment of crop residue biochar, could bring significant benefits for food production in world agriculture, as the focus of the “4 per 1000 Initiative” (Rumpel et al., 2018). Future research should look into the linkage between soil health and SOC enrichment, focusing the interactions between changes in abiotic and biotic players in soil.

5. Conclusions

In a farm trial, this study demonstrated a legacy of soil health improvement with soil SOC increase over years following one time biochar amendment in the rice paddy. In biochar field over no biochar field, a big soil carbon increase (by 45%) with biochar and plant residue promoted (by 30%) soil aggregation, which was accompanied by increased microbial growth and enzyme activities at similar extent. Although diversity both of bacterial and fungi unchanged, there were positive changes in microbial community structure with increased relative abundance of potentially beneficial functioning groups of bacterial but reduced abundance of fungi guilds related to soil-borne pathogens, in the biochar field 6 years following an addition compared to

no biochar field. Improved soil health could be tracked with the higher (by 10%) yield but lower inter-annual variability and higher nutrient efficient rice production, in the biochar field than in the no biochar. Biochar in the clayed nutrient rich paddy helped to build-up and sustain a coupling of soil aggregation, microbial community change and/or root development with SOC increase, leading to a legacy of soil health and/or crop productivity improvement, over years following biochar addition. As a particular case of biochar in rice agriculture, the study highlighted a great potential role of carbon increase as per required by the “4 per 1000 Initiative” to enhance soil fertility and improve soil-(plant) health for ensuring food production in world agriculture facing land degradation and climate change impacts. There are still knowledge gaps for the robust soil-plant-microbial interactions mediating such legacy following carbon increase with biochar in the long run.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2020.114567>.

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