Evaluation of Microbial Diversity, Community Composition and Function in Mixed Cropping Systems Using Three Legume Species Under the Application of Biochar or Chemical Fertiliser

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Abstract

Mixed cropping systems involve utilising multiple crop species on the field and diversifying aboveground plants. However, several contradicting results have been reported regarding their effects on soil microbial diversity. Therefore, to evaluate the effects of different leguminous species used in mixed cropping systems and the types of fertiliser on the diversity of soil microbes, a pot study was performed under maize/legume mixed cropping systems with one of three legumes, including cowpea [*Vigna unguiculata* (L.) Walp.], velvet bean [*Mucuna pruriens* (L.) DC.] and common bean (*Phaseolus vulgaris* L.), and one of three types of fertiliser treatments, namely chemical fertiliser (CF), carbonised chicken manure (CM) or the lack of fertiliser (Ctr). 16S rRNA analyses were conducted using the soils sampled from each pot for soil bacterial diversity assessment, and Tax4Fun2 was used for bacterial functional prediction analysis. A decrease in microbial diversity after CM application was observed in the soil with velvet bean + maize (MM) compared to the Ctr treatment, whereas an increase in microbial diversity was observed in the soil with common bean + maize (PM) in the same condition. With CM application, the abundance of treatment-unique bacteria increased with PM treatment, whereas their decrease was observed with MM treatment. In contrast, the abundance of dominant microbes, including Thaumarchaeota, Chloroflexi, Planctomycetes and Verrucomicrobia, was significantly lower in PM but higher in MM after CM application. Functional prediction analysis indicated that the dominant bacteria were involved in CM decomposition processes and nitrification in MM treatment. Legume species-dependent factors, including nutrient absorption and root exudate composition, might be important concerning soil bacterial diversities.

Introduction

The expansion of agricultural lands has been the primary cause of biodiversity loss in terrestrial ecosystems (Bossio et al., 2005; Kehoe et al., 2017; Zabel et al., 2019). Particularly, modern agricultural practices, including monoculture cropping systems, the intensive use of inorganic fertilisers and pesticides, lead to soil degradation and loss of genetic diversity (Díaz et al., 2006; Rohr et al., 2019). Therefore, establishing efficient agricultural crop production systems compatible with biodiversity conservation is a global challenge for future food security.

Among different scales of biodiversity, the diversity of soil microorganisms is especially important for the stability of agricultural ecosystems. They can be considered the main drivers of biogeochemical reactions beneficial for soil health and crop productivity (Chaparro et al., 2012; Singh, 2015). For example, their involvement is notable in biogeochemical processes essential for plant health and growth, including nutrient absorption, immune function, pathogen prevention and stress tolerance (Loreau et al., 2001; Nannipieri et al., 2003). Thus, agricultural management systems to maintain or increase soil microbial diversity must be established.

Among various agricultural practices that can potentially diversify soil microbes, the use of mixed cropping systems is receiving heightened attention. Legume-based intercropping systems have been reported to enhance soil microbial diversity and bacterial functions, including the mineralisation of available phosphorus (P) and nitrogen (N) (Gao et al., 2010; Li et al., 2013; Lian et al., 2019; Lingling Yu et al., 2020). Moreover, mixed cropping systems with field pea varieties have been demonstrated by Horner et al. (2019) to build stronger and larger cooccurrence networks in rhizosphere bacterial communities. These studies have suggested that crop diversification, including mixed cropping systems, improves soil microbial diversity and nutrient availability in agricultural soil. However, besides successful cases, no significant difference in bacterial diversity or functional availability in multiple cropping systems has been observed in previous studies (Granzow et al., 2017; Li et al., 2010; Zhang et al., 2010). These conflicting results indicate more complex mechanisms of microbial diversification in mixtures of plant species because soil microbial communities in mixed cropping systems become complex, reflecting synergistic and antagonistic interactions while plants grow in mixed cultures (Li et al., 2010). These interactions are influenced not only by mixed crop species but also by nutrient availability in the soil. However, it is still unclear how plant species-specific interaction in mixed cropping systems changes soil microbial community under varied nutrient availabilities, influenced by agricultural management factors, such as inorganic or organic fertiliser application. Therefore, information about the response of microbial diversity to the use of different fertilisers should be accumulated using variable legume species.

Among variable fertilisation treatments, biochar, an organic fertiliser, has been reported by many previous studies to have positive effects on soil microbial diversity and functionality in mixed cropping systems (Chen et al., 2016; Francioli et al., 2016; Gong et al.,

2009; Gul et al., 2015; He et al., 2008). For example, the combined use of biochar composed of plant residues and legume-based intercropping systems could enhance the soil microbial functionality important for nutrient absorption by plants, such as N-fixation and P-solubilisation in the rhizosphere (Duchene et al., 2017; Liao et al., 2019; Liu et al., 2017). Also, biochar application increases available N to plant because biochar increases soil pH and stimulates the access of ammonia-oxidising bacteria to NH_4^+ and reduces gaseous N loss from soil by reducing the denitrification potential (Lu Yu et al., 2020). Furthermore, in mixed cropping systems, N mineralisation of biochar, the process of transformation of organic N to mineral N through soil microorganism activities, can be different by legume species because individual plant species can stimulate different microorganisms involved in the N cycle through their root activities, such as root exudation and fine root turnover (Acosta-Mercado and Lynn, 2006; Cardarelli et al., 2020; Marschner et al., 2004; Sanchez-Contreras et al., 2007). However, still few studies have examined the effects of biochar on bacterial functionality and diversity among variable legume species.

Studies focusing on 'rare' microbial taxa are notably important to evaluate the mechanisms of soil microbial diversification and functionality. Recent studies have suggested that rare microbial taxa (~1%–3% within the relative abundance), appearing only under certain fertilisation management conditions, have a critical role in soil multifunctionality in agricultural soils (Chen et al., 2020; Hol et al., 2010; Kurm et al., 2017). This can be due to the overproportional roles played by these rare taxa in soil nutrient cycles, including N and C cycles and the degradation of complex chemicals (Jousset et al., 2017). Therefore, the ecology of rare microbes should be considered together to examine bacterial functional variability with the microbial diversification process.

This study aimed to elucidate plant species-specific effects of cereal/legume mixed cropping on soil microbial community structures, focusing on the interaction among legume species and fertiliser types. It was hypothesised that the combination of legume species and fertiliser types is important for bacterial diversity and community structure and the abundance of rare bacterial species. Further, the growth of a rare microbial community in diversified treatment was assumed to have an important role in the degradation process of complex chemicals, especially focusing on organic N degradation. Thus, a greenhouse experiment of legume-maize mixed cropping was performed using three legume varieties and chemical or biochar [carbonised chicken manure (CM)] to measure bacterial diversity and community structure and their functionality based on 16S rRNA analysis.

Materials And Methods

2.1. Soil sampling

The soil used in this experiment was sampled from abandoned land for 30 years at the university farm located at the Field Science Centre for Northern Biosphere, Hokkaido University, Japan (43°04'N, 141°20'E). The properties of the soil are given in Table 1. The soil type was clay loam with 44.6% sand, 21.5% silt and 33.9% clay.

2.2. Experimental design

A pot experiment was performed in a greenhouse at the Graduate School of Hokkaido University. The sampled soil was air-dried and sieved with 2 mm mesh and subsequently filled into Wagner pots (surface area = 1/5000 a). Each pot contained 1.8 kg air-dried soil. The experimental design was completely randomised, included three fertiliser treatments × four mixed cropping treatments, and conducted in triplicates. The pots received one of the three types of fertiliser treatments, namely control ('Ctr'), chemical fertiliser containing P and K ('CF') or biochar made from CM (50 g pot⁻¹ carbonised CM; 'CM'). The application rate for CF was 30 kg P ha⁻¹ and 50 kg K ha⁻¹. The soil and the CM chemical property are described in Table 1.

Each pot then received one of the four types of plant treatment: (1) single maize (*Zea mays* L.; SM), (2) mixture of cowpea [*Vigna unguiculata* (L.) Walp.] and maize (VM), (3) mixture of velvet bean [*Mucuna pruriens* (L.) DC.] and maize (MM) and (4) mixture of common bean (*Phaseolus vulgaris* L.) and maize (PM). Three replications were included in each treatment. During these treatments, maize and three legume seeds, including cowpea, velvet bean and common bean, were sprouted for 2 weeks in small pots filled with vermiculite before transplantation to Wagner pots. During the experiment, the temperature was maintained at 25°C to 30°C, and the plants were grown for 50 days after transplantation.

2.3. Chemical property analysis

After plant growth soil was sampled from each pot, soils were sampled and measured for pH and extractable NH_4^+ and NO_3^- concentrations 50 days after transplantation. For soil pH, 6 g soil was shaken for 30 min with 30 mL Milli-Q water, and pH was subsequently measured by a pH sensor (AS800; ASONE Co., Japan). For extractable NH_4^+ and NO_3^- , the samples were extracted with a KCl solution (2 mol L^{-1}), followed by colorimetric analysis using a flow injection analyser system (ACLA-700; Aqualab Co., Ltd., Japan). A two-way analysis of variance (ANOVA) was then performed to investigate the interaction between environmental factors and experimental treatments (Table 2).

2.4. DNA extraction and 16S rRNA sequencing

Using the same sampled soils, DNA was extracted with NucleoSpin® Soil (Takara Bio, Inc., Japan) according to the manufacturer's instructions. The extracted DNA was subsequently amplified by polymerase chain reaction (PCR) targeting the V4 region of 16S rRNA (amplicon size ~250 bp; forward primer 515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and reverse primer 806R: 5'-GGACTACHVGGGTWTCTAAT-3'). To perform PCR, 10 μL AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 μL of the forward primer, 0.4 μL of the reverse primer, 7.2 μL nuclease-free water and 1 μL DNA extract were mixed. The first PCR cycle was set to 95°C for 10 min and then 20 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by 72°C for 7 min. The PCR products were subsequently purified with Agencourt AMPure XP (Beckman Coulter) according to the given protocol. Then, the purified products were quantified with the QuantiFluor® ONE dsDNA System by a Quantus® Fluorometer E6150 (Promega, Madison, WI, USA).

Another PCR was performed with the utilisation of amplicon-obtained products to make them Ion Torrent sequence sample-specific. To achieve this, the 515F forward primer with the Ion Xpress Barcode Adapters Kit sequence and the 806R reverse primer attached to the Ion Xpress sequence of the Ion P1 adaptor were used (Thermo Fisher Scientific K.K.). The first PCR products were diluted to 2000 ng mL^{-1} , and 1 μL of each product was subsequently mixed with 10 μL AmpliTaq Gold® 360 Master Mix, 0.4 μL of the forward primer, 0.4 μL of the reverse primer and 7.2 μL nuclease-free water. The second PCR cycle was set to 95°C for 10 min and then 5 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by 72°C for 7 min. The second PCR products were purified in accordance with the same method outlined above. The final length and concentration of the amplicons were confirmed using a Bioanalyzer DNA 1000 Kit (Agilent Technologies, USA). The library was subsequently diluted to 50 pM and loaded into the Ion 318 chip using Ion Chef Instruments with an Ion PGM Hi-Q Chef Solutions. The samples were sequenced on an Ion PGM Sequencer with Ion PGM Hi-Q View Sequence Solutions (Ion Torrent Life Technologies, USA). Sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under accession number PRJNA743765.

2.5. Sequence processing

The barcoded 16S rRNA gene sequences were denoised, quality-filtered and assessed using the DADA2 algorithm implemented in Quantitative Insights Into Microbial Ecology (QIIME2) and with its workflow (Bolyen et al., 2019). Rarefaction was performed with minimal reads among all samples, and sequence data were subsampled to 41,095 sequences per sample. The R package Vegan (version 2.5.6) was used to access sample depth and generate the a-rarefaction curve plot (Fig. S1). The rarefaction curve was then evaluated using the interval of a step sample size of 1000.

2.6. Measurement of bacterial abundance

To measure bacterial abundance, quantitative PCR (qPCR) was performed using the extracted DNA, diluted 50 times with nuclease-free water. The 515F/806R primer pairs described above were used to amplify the V4 region of the 16S rRNA. For the standard curve, the PCR products from the DNA extracted from the Ctr pots were used, which were purified with AMPure XP and further diluted to five stages of different concentrations. The samples were prepared with 10.4 μL KAPA SYBR Fast qPCR kit (Kapa Biosystems, USA), 0.08 μL of the forward primer, 0.08 μL of the reverse primer and 2 μL diluted DNA extract. Nuclease-free water was added to achieve the final volume of 20 μL . CFX96 Touch® Real (Bio-Rad Laboratories, Inc., USA) was used, and the cycling

condition was set to 95°C for 30 s and then 35 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, followed by 95°C for 1 min and subsequently 55°C to 95°C by 1°C increment for 10 s. Ct values (threshold cycle) were calculated after quantifying the amplification results using qpcR R package version 1.4.1.

2.7. Statistical analysis

To quantify the diversity of soil microbial communities, the Shannon index (Shannon, 1948) and the Simpson index (Simpson, 1949), an estimation of community α -diversity, were calculated. For each diversity index, two-way ANOVA was performed using fertiliser treatments and plant species as factors with the emmeans R package version 1.4.7 (Lenth et al., 2020). Multiple comparisons were subsequently performed using the Tukey-Kramer method. Also, permutational ANOVA (PERMANOVA; permutation = 9999) was conducted, and significant phyla ($p < 0.001$) were subsequently identified for the interactions between fertiliser treatments and plant types using two-way ANOVA.

The numbers of operational taxonomy units (OTUs) for each plant treatment within the same fertiliser treatment were illustrated using Venn diagrams. VennDiagram R package version 1.6.20 was applied using the cooccurred OTUs among three replications for each treatment. OTUs that specifically appeared by plants species within each fertilisation were defined as unique OTUs and classified to the phylum. The relative abundance of unique OTUs within the total abundance was plotted for PM and MM treatments that were significantly different in diversity indices within plant species.

Functional profiling of the prokaryotic community was conducted with the Tax4Fun2 R package (Wemheuer et al., 2020). The rarefied OTU table was used for Tax4Fun2 searches, and metagenome functional profiles were predicted to generate a table of Kyoto Encyclopedia of Gene and Genomes orthologs (Kanehisa and Goto, 2000). To evaluate biogeochemical reactions, 36 gene-coding enzymes related to the organic N degradation and N metabolism pathway were selected (Table S1; Harter et al., 2014; He et al., 2021; Sulieman and Tran, 2014; J. Wang et al., 2020) and visualised with 'ComplexHeatmap' R package.

Results

3.1. Diversity indices and abundance of bacterial communities

The interaction effects between plant species and fertilisation were shown by soil microbial diversity indices. Within PM treatment, a significantly lower Simpson's diversity indicator was observed with no fertilisation (Ctr) compared to CM treatment (Fig. 1). In contrast, a relatively lower diversity was shown by MM treatment with CM application compared to Ctr treatment.

Bacterial absolute abundance, based on qPCR analyses, showed a significant effect of plant treatments when averaged across fertiliser types (Table S2). The bacterial abundance of SM and VM treatments was significantly different. However, there was no correlation between bacterial absolute abundance and diversity indicators.

The analysis of the soil 16S rRNA gene sequence provided 738 to 1196 OTUs per sample. Venn diagrams showed that soils under Ctr, CF and CM treatments had 297, 293 and 283 core OTUs (the overlapped area across plant treatments), respectively (Fig. 2) and increasing numbers of unique OTUs with increasing diversity indices, for example, unique OTU numbers increased from 28 to 84 for PM compared to Ctr and CM, whereas it decreased from 52 to 35 in MM. The relative abundance of these unique bacteria within the whole bacterial abundance increased from 1.9% (Ctr) to 5.2% (CM) in PM treatment but decreased from 4.3% (Ctr) to 1.9% (CM) in MM treatment (Fig. 3). The abundance of Acidobacteria and Verrucomicrobia was suggested by the community structures of these unique bacterial taxa to dominate within unique microbial communities and was correlated to the increase of unique OTU numbers during comparison of different treatments. In contrast, the abundance of Gemmatimonadetes, Chloroflexi and Planctomycetes was not correlated to the increase in unique OTU numbers, although they were considered to be the dominant phyla within unique OTUs.

3.2. Environmental factors and dominant OTU composition

The relative abundance of Thaumarchaeota, Armatimonadetes, Chloroflexi, Planctomycetes, Verrucomicrobia and Proteobacteria contributed to the changes in community structures, and their abundance was influenced by the interactions between plant species

and fertiliser treatments (Table 3; Fig. S2). Within CM treatment, Thaumarchaeota, Chloroflexi, Planctomycetes and Verrucomicrobia were significantly higher in MM treatment than PM treatment ($p < 0.05$; Table 4).

The significant increase in soil pH in CM treatment was due to the content of salt-based ions, such as potassium, sodium and calcium, which can reduce the exchangeable hydrogen ions in the soil (Table 2). Also, CM application significantly increased NO_3^- -N concentration ($p < 0.001$), but NH_4^+ -N concentration had no significant difference ($p = 0.90$).

3.3. Functional prediction by Tax4Fun2

Two-way ANOVA analysis indicated that gene abundance coding ammonium oxidation, carbamate kinase, glutamate dehydrogenase, nitrate reductase, nitrite oxidoreductase and nitrite reductase (NO-forming) was significantly influenced by the interaction effects of plant type and fertiliser (Table S1). In Ctr treatment, gene abundance coding ammonium oxidation and nitrite reductase (NO-forming) were higher in mixtures of legume and maize (Fig. 4). In contrast, in CF, there was no difference in gene abundance coding ammonium oxidation and nitrite reductase (NO-forming) between single and mixed cropping treatments. CM treatment most altered gene abundance among other fertilisation treatments. Especially, with CM application, MM treatment showed the highest abundance in glutamate dehydrogenase, carbamate kinase, ammonium oxidation and nitrate reductase.

Discussion

4.1. Characteristics of unique bacteria

The diversity index and unique bacterial abundance in PM were significantly higher in CM treatment, whereas CM negatively affected the diversity and unique bacterial abundance in MM (Fig. 1 and 2). There was no universal effect of mixed legume species and fertiliser types on soil bacterial diversities. There was also no consistent statement about microbial diversity in multiple cropping systems using cereals and legumes in previous studies. For example, Fu et al. (2019) and Kihara et al. (2012) found that an intercropping system with maize and soybean increased microbial diversity, whereas Yu et al. (2019) showed that there was no significant effect of the intercropping system on microbial diversity. This study demonstrated both plant species and fertiliser types were related to the mechanisms behind the diversification of soil microbes. Especially, this study highlighted the importance of the use of CM, compared to CF, in association with the diversification of soil microbes. Liao et al. (2019) indicated that different microbial communities could utilise plant exudates depending on the presence or absence of biochar in the intercropping system using the ^{13}C tracer method. Biochar application changes the influence of plant exudates on microbial community and diversity in the intercropping system due to the absorption of root exudates on its surface area or its porosity characteristics.

Also, plant species were identified as an important factor controlling the presence of unique bacteria when CM was applied as different plants have different abilities to recruit specific bacterial communities (Zhang et al., 2019). Regarding unique OTUs, the abundance of Acidobacteria and Verrucomicrobia was positively correlated with the percentage of unique OTUs and the diversity indices (Fig. 3). In rhizosphere soils, these bacteria often dominate the present bacterial communities due to their relatively higher metabolic potential (Barns et al., 2007; Bnger et al., 2020; Lee et al., 2008). Whole-genome sequence analyses of Acidobacteria strains revealed that they are involved in carbohydrate metabolism to utilise plant root exudates (de Chaves et al., 2019; Kielak et al., 2016; Nielsen et al., 2014). Also, Verrucomicrobia were reported to hold diverse communities in the rhizosphere (Bnger et al., 2020), and their role in polysaccharide degradation processes was identified (Dunfield et al., 2007; He et al., 2017; Pold et al., 2018). It is possible that the amount and quality of root exudates affected soil microbiomes differently when PM and MM were compared, as soil microbes showed a preference in association with the exudate (substrates) supplied from the plant roots (Duchene et al., 2017; Zhahlnina et al., 2018). This study indicated that biochar application on mixed cropping systems diversifies the soil bacterial community in a short period only with certain legumes, although further studies are needed to investigate the chemical composition of root exudates and their effect on unique bacterial community and their interaction with the use of biochar.

4.2. Enzyme activity related to the N cycle

Functional prediction analysis with Tax4Fun2 indicated that without fertiliser (Ctr) the presence of legume facilitated nitrification and denitrification with higher NO_3^- concentrations in the soil (Fig. 4; Table 2), as shown by higher gene abundance coding ammonia oxidation and nitrite reductase (NO-forming). In contrast, mixed cropping systems in CF treatment did not show positive effects on ammonia oxidation and nitrite reductase (NO-forming) activities with low NO_3^- concentrations in the soil. In the early stage of mixed cropping systems, P is often a limitation of plant growth (Ghosh et al., 2009), whereas N becomes a limitation during sufficient P supply (Isaac et al., 2012). Although fertilisers containing P and K facilitated N-fixation by legume (Cadisch et al., 1993; Collins et al., 1986; Sangakkara et al., 1996), and this study showed more nodulation in MM and PM with CF treatment (Table S3), the low gene abundance in the mixed treatment indicated that N deficiency occurred between maize and legume through CF application. Moreover, compared to Ctr and CF, CM treatment dynamically affected N cycling in each cropping system because of their organic N contents. In MM with CM treatment, the abundance of ammonia oxidation and nitrite reductase (NO-forming) was higher. The abundance of carbamate kinase and glutamate dehydrogenase, which mediate amino acid metabolism, was higher than other plant treatments. A higher abundance of those genes might be related to the fast decomposition of organic N in CM and the supply of more N to plants and the greater growth of coplanted maize in MM (Table S3). In contrast, the lower abundance of those enzymes in PM might be related to the slower growth of maize. In previous research, intercropped maize (*Z. mays* L.) with wheat (*Triticum aestivum* L.) or faba bean (*Vicia faba* L.) showed maize growth suppression in the initial cultivation period to avoid nutritional competition with neighbour species (Li et al., 2011). In this study, a similar pattern appeared in PM, whereas little suppression was observed in MM. It is also consistent with a previous study with intercropping using velvet bean, which showed fewer maize growth suppressions or even better growth than monocropping (Akobundu et al., 2000; Correia et al., 2014). These studies on weed reduction by cover crop effects of velvet bean were significant with maize growth, but they lacked focus on the mechanism to avoid N competition between velvet bean and maize. Thus, this study provided a better understanding of how velvet bean maintains greater growth of maize than other legumes in terms of the bacterial community driving N cycling.

While comparing community structures at the phylum level within CM-applied soils, the abundance of Thaumarchaeota, Chloroflexi, Planctomycetes and Verrucomicrobia was significantly higher in MM compared to PM (Table 4). Especially, Thaumarchaeota, an archaea ubiquitously present in a wide variety of ecosystems, would contribute to the increase of ammonium-oxidising function (Brochier-Armanet et al., 2012; Cardarelli et al., 2020; Pester et al., 2011; Spang et al., 2010). Also, members of Planctomycetes can perform anaerobic oxidation of ammonium to dinitrogen via the annamox pathway, which might be related to higher denitrification functionality in MM (Andrei et al., 2019; Fuerst and Sagulenko, 2011). Moreover, Verrucomicrobia and Chloroflexi are often associated with carbohydrate and amino acid degradation (Bayer et al., 2018; Herlemann et al., 2013; Martinez-Garcia et al., 2012; Yamada et al., 2005). The results of this study indicated that MM with CM enhanced dominant bacterial growth and facilitated organic N decomposition and nitrification, producing plant deliverable N. However, Tax4Fun2 is a prediction of bacterial functionality from 16S amplicon sequences, so further efforts are required to determine bacterial functional DNA sequencing in relation to the nutrient cycle.

Conclusion

Mixed cropping systems diversify soil bacterial communities only under a specific combination of legume species and fertilisers. Soil bacterial diversity was increased when biochar was used for the common bean-based mixed cropping system with an increase in the number of unique OTUs (treatment-specific OTUs). Especially, plant root associated bacteria such as Verrucomicrobia and Actinobacteria were increasing in diversified treatment.

With biochar application, bacterial functionalities, such as ammonia oxidation and denitrification, were higher in the velvet bean-based mixed cropping system with a low diversified bacterial community. Some dominant bacterial phyla, such as Thaumarchaeota, Planctomycetes, Verrucomicrobia and Chloroflexi, contributed higher enzyme abundance related to organic N decomposition in the mixed cropping system with velvet bean and biochar.

Further efforts are needed to confirm the effects of plant root exudates on soil microbial community and diversity in association with biochar application. Also, as a limitation of the pot study, only bacterial diversity and functionality were evaluated early on cultivation. Therefore, field research to evaluate the long-term effects of mixed cropping systems on the bacterial diversification process and functionality associated with the variability of legume species and fertiliser types is required.

Declarations

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Conflicts of interest/Competing interests

To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

Availability of data and material

Sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under accession number PRJNA743765 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA743765>). The other datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Code availability

The code used in this study are available from the corresponding author on reasonable request.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1. Chemical properties of soil and carbonised chicken manure (CM). The number of replications was 3 for each property measurement.

Chemical properties	Soil	CM
Water content (%)	19.5 ± 0.1	19.1 ± 1.2
C/N	14.7 ± 0.2	10.0 ± 0.4
pH (1:10)	6.3 ± 0.0	10.4 ± 0.3
EC (µs/cm)	78.5 ± 3.5	12740 ± 793
TN (%)	0.25 ± 0.01	4.06 ± 0.45
TC (%)	—	40.5 ± 3.4
P g kg ⁻¹ soil	0.21 ± 0.01	36.8 ± 1.0
Ca g kg ⁻¹ soil	5.9 ± 0.3	134 ± 5
Mg g kg ⁻¹ soil	0.53 ± 0.01	15.1 ± 0.3
K g kg ⁻¹ soil	0.56 ± 0.01	45.3 ± 0.9

Table 2. Soil pH, nitrate and ammonium content after plant cultivation. The expressed plant species included single maize, SM; maize cropped with velvet bean [*Mucuna pruriens* (L.) DC.], MM; maize cropped with common bean (*Phaseolus vulgaris* L.), PM; maize cropped with cowpea [*Vigna unguiculata* (L.) Walp.], VM. Fertilisation was undertaken for the control, Ctr; chemical fertiliser, CF; and carbonised chicken manure, CM. Two-way ANOVA was performed to examine the effects of the interactions between plant species and fertiliser treatments. *p*-values are shown at the bottom of the table.

Treatment	pH (H ₂ O)	NO ₃ ⁻ -N (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)
SM			
Ctr	6.7 ± 0.03	8.2 ± 1.6	4.7 ± 0.30
CF	6.7 ± 0.04	7.1 ± 0.4	3.4 ± 0.75
CM	7.6 ± 0.09	15.9 ± 2.4	5.4 ± 1.22
MM			
Ctr	6.7 ± 0.02	10.9 ± 4.2	4.2 ± 2.64
CF	6.7 ± 0.02	6.3 ± 3.3	4.7 ± 1.37
CM	7.3 ± 0.10	19.7 ± 6.2	5.1 ± 2.48
PM			
Ctr	6.7 ± 0.02	12.8 ± 2.2	6.0 ± 2.80
CF	6.6 ± 0.02	7.9 ± 3.2	5.7 ± 0.98
CM	7.2 ± 0.12	35.0 ± 19.3	2.6 ± 0.37
VM			
Ctr	6.7 ± 0.02	10.6 ± 3.2	4.5 ± 1.35
CF	6.7 ± 0.03	6.7 ± 3.1	4.9 ± 0.68
CM	7.2 ± 0.08	30.8 ± 13.2	5.4 ± 2.22
Two-way ANOVA	<i>p</i>		
Plant	<0.001	0.45	0.92
Fertiliser	<0.001	<0.001	0.90
Plant × fertiliser	<0.01	0.91	0.39

Table 3. Bacteria phyla with permutational $p < 0.001$ (permutation = 9999). Two-way ANOVA with plant species and fertiliser treatments as the factors was performed. p -values are shown in the table.

Phylum	Two-way ANOVA		
	Fertiliser	Plant	Plant × fertiliser
Thaumarchaeota	0.018	0.19	<0.01
Acidobacteria	<0.001	<0.01	0.063
Actinobacteria	0.35	<0.01	0.074
Armatimonadetes	<0.001	0.0021	<0.01
Bacteroidetes	0.097	0.5	0.12
Chloroflexi	<0.001	<0.001	<0.001
Gemmatimonadetes	0.48	0.51	0.048
Latescibacteria	0.34	0.18	0.48
Nitrospirae	<0.001	<0.001	0.47
Planctomycetes	<0.001	0.096	<0.001
Proteobacteria	<0.001	0.16	<0.01
Verrucomicrobia	<0.001	<0.001	<0.001

Table 4. Relative abundance of the phyla showed a significant interaction between plant species and fertiliser treatments. The results from multiple pairwise comparisons are shown as different letters, indicating significant differences between treatments ($p < 0.05$).

Treatment	Relative abundance (%)					
	Thaumarchaeota	Armatimonadetes	Chloroflexi	Planctomycetes	Verrucomicrobia	Proteobacteria
Ctr						
SM	3.23 ^a	0.58 ^a	7.84 ^{ab}	6.76 ^a	2.93 ^a	29.93 ^b
MM	4.70 ^{ab}	0.46 ^a	7.58 ^a	8.53 ^b	4.37 ^b	28.19 ^{ab}
PM	5.22 ^{ab}	0.42 ^a	7.84 ^{ab}	8.21 ^{ab}	4.20 ^b	27.92 ^{ab}
VM	5.66 ^b	0.53 ^a	8.74 ^b	10.74 ^c	4.57 ^b	25.13 ^a
CF						
SM	4.94 ^a	0.62 ^a	8.76 ^b	9.75 ^b	4.08 ^a	26.95 ^a
MM	4.21 ^a	0.60 ^a	8.03 ^{ab}	8.48 ^{ab}	4.43 ^a	26.49 ^a
PM	4.72 ^a	0.57 ^a	7.95 ^{ab}	8.70 ^{ab}	3.93 ^a	27.05 ^a
VM	4.23 ^a	0.53 ^a	7.71 ^a	7.63 ^a	3.78 ^a	28.31 ^a
CM						
SM	5.50 ^{ab}	0.83 ^b	12.10 ^c	10.94 ^b	5.89 ^b	22.79 ^{ab}
MM	6.67 ^b	0.72 ^b	12.27 ^c	11.19 ^b	6.19 ^b	20.04 ^a
PM	4.01 ^a	0.76 ^b	10.65 ^b	9.09 ^a	4.56 ^a	24.21 ^b
VM	6.19 ^b	0.48 ^a	8.70 ^a	7.94 ^a	4.50 ^a	25.14 ^b

Figures

Figure 1

Box-plot analysis of bacterial OTU α-diversity indicating (a) Shannon's index and (b) Simpson's index. Two-way ANOVA and Tukey–Kramer's pairwise comparison were performed on calculated α-diversity indices: · $p < 0.25$; * $p < 0.05$; ** $p < 0.01$. The abbreviations of plant species were as follows: single maize, SM; maize cropped with velvet bean, MM; maize cropped with common bean, MP; maize cropped with cowpea, VM. Fertiliser treatments were abbreviated as no fertiliser, Ctr; chemical fertiliser, CF; and carbonised chicken manure, CM.

Figure 2

Distribution of OTUs among different mixed cropping systems within fertiliser treatments. The numbers show the shared and unique OTUs in each treatment. Overlapping circles show the shared OTUs among treatments. Each circle includes OTUs that cooccurred among three replications.

Figure 3

Relative abundance of unique OTU phyla found in MM (a) and PM (b) treatments. Bar plots indicate the total relative abundance dimension with the averaged value across replications. Only phyla with a relative abundance of >0.1% in the averaged value are represented.

Figure 4

(a) Predicted bacterial genes coding the N metabolism pathway based on the KEGG database (*p < 0.05; **p < 0.01; ***p < 0.001). Individual KEGG codes are ammonium oxidation [EC: 1.14.99.39], carbamate kinase [EC: 2.7.2.2], carbonic anhydrase [EC: 4.2.1.1], cyanate lyase [EC: 4.2.1.104], formamidase [EC: 3.5.1.49], glutamate dehydrogenase [EC: 1.4.1.2; EC: 1.4.1.3; EC: 1.4.1.4], glutamate synthase [EC: 1.4.1.13, 1.4.1.14, EC: 1.4.7.1], glutamine synthetase [EC: 6.3.1.2], hydroxylamine dehydrogenase [EC: 1.7.2.6], nitrate reductase [EC: 1.7.99.-], nitric oxide reductase [EC: 1.7.2.5], nitrilase [EC: 3.5.5.1], nitrite oxidoreductase [EC: 1.7.5.1; EC: 1.7.99.-],

nitrite reductase [EC: 1.7.7.1; 1.7.1.15; 1.7.2.2], nitrite reductase (NO-forming) [EC: 1.7.2.1], nitrogenase [EC: 1.18.6.1], and nitrous oxide reductase [EC: 1.7.2.4]. (b) N metabolism pathway (ko00910) and enzymes based on the KEGG database.

Supplementary Files

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