Article

Linkages of Soil Nutrients and Diazotrophic Microbiome under Sugarcane-Legume Intercropping

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Abstract: Intercropping significantly improves land use efficiency and soil fertility. This study examines the impact of three cultivation systems (monoculture sugarcane, peanut-sugarcane and soybean-sugarcane intercropping) on soil properties and diazotrophs. Sugarcane rhizosphere soil was sampled from the farmers' field. Soil properties and *nifH* gene abundance were analyzed by high throughput sequencing. Moreover, a total of 436,458 *nifH* gene sequences were obtained and classified into the 3,201 unique operational taxonomic units (OTUs). Maximum unique OTUs resulted with soybean-sugarcane intercropping (<375). The dominant groups across all cultivation were *Alpha-proteobacteria* and *Beta-proteobacteria*. On the basis of microbial community structure, intercropping systems were more diverse than monoculture sugarcane. In the genus level, *Bradyrhizobium*, *Burkholderia*, *Pelomonas*, and *Sphingomonas* were predominant in the intercropping systems. Moreover, diazotrophic bacterial communities of these cultivation systems were positively correlated to the soil pH and soil enzyme protease. Moreover, low available P recovered from intercropping system showed a strong correlation with higher nutrient uptake activity of soil microbes. Based on the results, our investigation concluded that intercropping system caused a positive effect on the growth of diazotrophic bacterial communities and it might boost the soil fertility and this kind of study helps to develop an eco-friendly technology for sustainable sugarcane production.

Keywords: intercropping; microbial community; high throughput sequencing; *nifH* gene; sugarcane

1. Introduction

Sugarcane (*Saccharum* spp.) is a long perennial true grass, cultivated in tropical and subtropical countries all over the world for the production of sugar and Bioenergy [1,2]. Sugarcane requires substantial amounts of nitrogen-containing fertilizers for optimum growth and crop production [3,4]. However, overuse of N fertilizer raises production costs, the microbial degradation activity for biomass recycling. Moreover, it can be dangerous to the environment through the emission of N-

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containing gases and soluble N compounds polluted the groundwater, which can lead to the eutrophication [5]. It is widely accepted that diazotrophs play an indispensable role to fix the atmospheric nitrogen gas as biologically available ammonium and provides numerous advantages for the plants [6]. Several leguminous plants like soybean, peanut, pea, chickpea, etc. are well known for N fixation and recently, rhizospheric and endophytic diazotrophic bacteria have been also isolated from various nonleguminous plants, including grasses [7,8], coffee [9], and sugarcane [10–13]. Among different soil management practices, intercropping has also played significant role in soil mineralization and two crops utilized all natural resources better than a single crop [14].

Sugarcane–legume intercrops permit proper utilization of soil and atmospheric N to cope up high production and quality with less fertilizer application to protect the environment [4,15] and several reports showed the intercrops caused significant changes in soil properties and microbial population [14,16,17]. Sugarcane plants are also fixing the atmospheric nitrogen through N2-fixation allied with diazotrophic bacteria like *Acetobacter* [18], *Barkholderia* [19], *Rhizobium* [20,21], *Enterobacter* [11], *Gluconacetobacter* [22], *Klebsiella* [11], *Microbacteriaum* [10] and *Herbasprillum seropedicae* [23]. Maximum diazotrophs that accomplish biological nitrogen fixation (BNF) with the nitrogen fixation gene (*nifH*) cluster, which is a highly conserved functional gene encodes the iron protein of nitrogenase [24] and has been thoroughly studied from environmental DNA samples [25,26]. It is a convenient marker in culture-independent studies, to provide evidence for significant N fixation in diverse environments [25,27]. To date, culture-independent methods have investigated N fixation in different habitats, including soils [16,28–31], plant parts [30,31] and water resources[32,33]. By focusing on the sequences of the *nifH* genes, large numbers of sequence data have been also obtained by independent culture methods [34,35]. Since it is claimed that some of these databases are also suitable for molecular analysis, it is imaginable that they might be used to study the diazotrophic community by means of Next-generation sequencing. Diazotrophic bacterial community can be affected by the numerous factors. Hamelin et al., [8] stated that certain plants facilitate favorable niches for diazotrophic bacteria. Moreover, plant biomass, chemical composition of the litter and root exudates may be playing the role as an intermediate for the composition of diazotrophic bacteria. Soil physicochemical factors, like pH and water holding capacity, microbial biomass carbon and nitrogen, total nitrogen, potassium, phosphorus and available phosphorus, electrical conductivity and soil nutrient availability, have also been identified as drivers of *nifH* gene diversity and abundance in different environments [28,31–33].

However, to the best of our knowledge, no study has been explored in the sugarcane-legume intercropping rhizospheric soil to characterize the diazotrophic community by using high throughput sequencing. Therefore, economic importance of diazotrophic microbes under intercropping systems remains unexplored. Microbial mapping and importance of diazotrophic microbes would provide essential information to our understanding about significance of diazotrophs in rhizospheric N pool. In our previous study, we already determined that intercropping system improved the diazotrophic population in soil by identification of cultivable microbes [36]. Therefore, in the present study, we aimed to (1) explore the uncultivable diazotrophic bacteria in intercropping system via nest generation sequencing and (2) their relation with the soil properties.

2. Materials and Methods

2.1. Soil sampling

Sugarcane rhizosphere soil samples were taken in April 2014, during the vegetative stage from the farmer fields and research stations of Guangxi Province, China (Table 1). Soil samples were collected from the sugarcane monoculture (M), sugarcane + peanut (SP) and sugarcane + soybean (SS) intercropped fields, up to the depth of 30 cm by an auger (3.5 cm in diameter) in close proximity of the roots. Three plots of monoculture and intercropping were evaluated at each location and five plants were selected from different rows of each plot and sampled, then mixed together to give a composite soil sample. All soil samples stored in the icebox and sent immediately to the lab within the following hours, and stored at 4°C prior to analysis. Air-dried composite samples were sieved

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through a 2.0 mm mesh and plant and roots residues were removed by hand earlier to chemical analysis.

Table 1: Geographical locations and details of soil samples collected from sugarcane monoculture and sugarcane legume (peanut and soybean) intercropped fields in Guangxi, China

Information	Site 1	Site 2	Site 3	Site 4
Geographical locations	N22°34′6.51 E108°13′53,19	N22°36′43.73 E108°10′3.80	N23°11′50.76 E108°10′10.59	N22°49′51.76 E108°38′51.95
Details of location	GXAAS experiment Station, Mingyang Town, District Jiangnan ("MI")	Farmer field, Mingyang Farm, Wuxu Town, District Jiangnan ("MY")	GXAAS experiment station, Lijian Town, District Wuming ("W")	Farmer field, Wenshuipo Village, Changtang Town, District Qingxiu ("CT")
Soil type	Red sandy clay loam	Red sandy clay loam	Red loamy soils with high clay content	Red loamy soils with high clay content
Sampling date	20 April 2014	20 April 2014	22 April 2014	22 April 2014
Temperature (ºC)	28	30	24	23
Cropping system/sample codes	Sugarcane + Soybean (MISS)	Sugarcane + Soybean (MYSS)	-	Sugarcane + Soybean (CTSS)
	Sugarcane + Peanut (MISP)	Sugarcane (MYM1)*	Sugarcane + Peanut (WSP)	-
	Sugarcane (MIM)	Sugarcane + Peanut (MYSP)	Sugarcane (WS)	Sugarcane (CTM)
		Sugarcane (MYM2)*		
Past history (5 year)	Mostly monoculture crop was used like maize and sugarcane	Crop rotation and intercropping with water melon, peanut and soybean were used	Mostly monoculture crop was used like maize and sugarcane	Mostly monoculture sugarcane and other details not available

*Samples were taken from different fields, in respect to the intercropping crops

2.2 Soil analysis

Soil biological properties (chemical and enzymes) were examined. Soil pH analysis (soil: water = 1:1) performed by pH meter and soil organic carbon was assessed by dichromate oxidation [37]. Total N was detected through Semimicro-Kjeldahl method [38]. Nitrate nitrogen (NO_3^--N) and exchangeable ammonium nitrogen (NH_4^+-N) was estimated by $FeSO_4$/Zn reduction method of Carter [39]. Total phosphorus (P) was measured via Sodium carbonate fusion defined by Carter [39]. Available P was estimated through samples extraction method 0.5 M $NaHCO_3$, and determining P calorimetrically via molybdate [40]. Total K was estimated using a Flame photometry method [40]. Available K was estimated using the flame photometry method of 1 N ammonium acetate extraction [40]. Urease activity was assessed via phenol-sodium hypochlorite calorimetry and expressed as mg NH_3-N Kg^{-1} d^{-1} [41]. Nitrite reductase activity was examined using the Gerry reagent method and

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expressed as mg NO_2-N g^{-1} d^{-1} [42]. Protease activity was determined and expressed as mg Kg^{-1} d^{-1} [43]. Soil dehydrogenase activity was measured by the method of Singh and Singh [44]. Nitrogen fixation activity was assessed using the acetylene reduction assay (ARA) [45]. All soil samples were incubated individually with 10% acetylene for 3 h. Autoclave distilled water incubated with 10% acetylene used as control. The measurements were performed via a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan) furnished with a flame ionization detector and a column filled with DB-1701(Agilent, Santa Clara, USA).

2.3 DNA extraction, gene amplification and Illumina-sequencing

Genomic DNA was extracted from three independent soil samples and pooled together. After pooling, purification was performed using the Ezup Column Soil DNA Purification Kit (Sangon Biotech, Shanghai, China). DNA quality and concentration were estimated using a NanoDrop ND-2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), based on the ratios of 260 nm/280 nm and 260 nm/230 nm. Amplification of partial *nifH* sequences was performed with the degenerate Z-primers [46] by the Nested polymerase chain reaction. Ready-to-use 2×concentrated PCR masters (0.1 U μL^{-1} TaqDNA polymerase, 0.2 mM dNTP, 3 mM $MgCl_2$, 2×PCR buffer) obtained by Tiangen (Tiangen Biotech, Beijing, China) was used for PCR reaction. PCR amplification was performed with a Peltier Thermal Cycler (Bio- Rad, Hercules, USA). First step PCR was performed with outer primer (*nifH*3 and *nifH*4). The amplification protocol consisted of an initial denaturing step at 94°C for 4 min, then 30 cycles of 1 min each at 94°C, 55°C, and 72°C, followed by a final extension at 72°C for 7 min. The second step PCR entailed the same concentration of reagents as the initial PCR reaction, using first PCR-product as a template with inner primers (*nifH*1 and *nifH*2). A touchdown PCR strategy was used for *nifH* amplification to improve amplification specificity. In the first 20 touchdown cycles, the annealing temperature declined by 0.5°C per cycle from 67 to 57°C. Further fifteen cycles were performed at the annealing temperature of 57°C. PCR products were electrophoresed on agarose gels, stained with ethidium bromide, visualized to check the quality. The expected amplicons were excised from agarose gels, purified by TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China). The amplicons were used to construct libraries after qualified. The jagged ends of the fragment would be changed into blunt ends by using T_4 DNA polymerase, Klenow Fragment and T_4 Polynucleotide Kinase. Then an 'A' base was added to each 3' end to make it easier to add adapters. After all that, fragments too short would be removed by AMPure beads, only the qualified library was used for sequencing by Genome Analyzer/HiSeq (Illumina-Solexa, San Diego, USA).

2.4 Bioinformatics

To enhance the accuracy and reliability of results through Bioinformatics analysis, the raw sequence data were processed by the method described by Fadrosh et al. [47]. The high-quality paired-end reads were combined to tags based on overlaps by FLASH software [48]. The tags were clustered to OTUs (Operational Taxonomic Unit) by scripts of software USEARCH with a 97% similarity threshold [49], Chimeras were filtered out by using UCHIME [50], then the OTU unique representative sequences were obtained. All tags were mapped to each OTU representative sequences using USEARCH GLOBAL [51], and then the tags number of each OTU in each sample will be summarized to OTU abundance table. Taxonomic ranks were allocated to OTU representative sequence using Ribosomal Database Project (RDP) [52] trained on ARB *nifH* database [53]. At last, alpha diversity, beta diversity and the species distribution were analyzed based on OTU and taxonomic ranks. Meta-sequence data were deposited with the NCBI under Bio-Project accession Number PRJNA310619.

2.5 Statistical analysis

Box plot of soil properties performed by using Origin 2017SR2 (Northampton, MA, USA). Based on the OTU abundance, Venn diagram, PCA (package-ade4) and rank curve were drawn by software

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R (v3.0.3). The indices are calculated through Mothur (v1.31.2), and the corresponding rarefaction curves were drawn by Origin 2017SR2. Beta diversity heat map was drawn by 'aheatmap' in package 'NMF' of software R (v3.0.3). The tags number of each taxonomic rank (Phylum, Class, Order, Family, Genus, and Species) or OTU in different samples were summarized with the software R (v3.0.3). Species heat map analysis was done based on the relative abundance of each species in each sample. To minimize the differences degree of the relative abundance value, the values were all log transformed. If the relative abundance of certain species is 0, the half of the minimum abundance value will substitute for it. Heat maps were generated using the package 'gplots' of software R (v3.0.3) and the distance algorithm is 'euclidean', the clustering method is 'complete'. A representative OTU phylogenetic tree was constructed using the QIIME (v1.80) built-in scripts, including the fast tree method for tree construction and phylogeny tree was imaged by software R (v3.0.3) at last. Correlation and Canonical correspondence analysis (CCA) were performed to link microbial community and soil variables by using Past3 software [54].

3. Results

3.1 Soil analysis

Box plots for the soil pH, organic matter (OM), soil nutrient (Total N, P, K, N (NO_3), N (NH_4), available P and K) and enzymes (nitrogenase, nitrite reductase, urease, protease and dehydrogenase) contents of the samples representing the tree cropping system were displayed in Figure 1. Lower pH was found in monoculture than both intercropping. Higher OM content was found in peanut intercropping than monoculture and soybean intercropping. Similar, Intercropping system showed higher average of total N, total K, N (NH_4), available K, nitrite reductase, protease, dehydrogenase and nitrogenase (Figure 1). The differences in soil chemical properties between cropping systems, encouraged us to carry out separate analyses for diazotrophic microbial communities.

3.2 Distribution, diversity and richness of operational taxonomic units

After sequencing, 522,223 raw sequenced reads were obtained and after trimming their 436,458 effective reads were recovered from the all soil samples with an average 83.66% read utilization ratio (Table S1). The high quality paired-end reads were combined to tags based on overlaps, 435,439 tags were obtained in total with 39,585 tags per sample on average, and the average length is 358-366 bp with approx. 99% connecting ratio. Chimera filtered tags were assembled into Operational Taxonomic Units (OTU) at 97% similarity and a total of 3,201 OTUs was acquired. Outs rank curved showed maximum OTUs recovered with MISS and less with MIM (Figure S1). Cultivation systems wise maximum OTUs resulted with soybean-sugarcane intercropping (<376) and followed by monoculture (<263) and peanut-sugarcane intercropping showed less OTUs (<254) (Table S2). In order to display the differences of OTU composition of different rhizospheric samples, principal component analysis (PCA) was used and a scattered PCA plot showed similarity between soil samples of all locations except MIM and minor difference on the basis of cultivation system except MIM (Figure S2). In order to find out the apparent uniformity recommended by the PCA, we calculated alpha diversity to recognize the differences among bacterial communities from different cultivation systems (Tables S2). In soybean-sugarcane intercropping, the analyses of different richness and diversity indices revealed a slight increase in richness in communities from monoculture, and lower community diversity in peanut-sugarcane intercropping (Tables S2) and cultivation systems based box plot on observed species and Shannon's index also showed the similar results (Figure 2a, b). Similarly, the rarefaction curves showed richness with soybean intercropping (Figure S3). To conclude similarities and differences in community structure among three cultivation systems, pairwise weighted UniFrac, unweighted UniFrac and Bray-Curtis, dissimilarities were calculated and ordinated in scattered plot and diversity heat map (Figure 3a; Figure S4). The PCA plot of Bray-Curtis dissimilarity showed higher diversity among cultivations at different locations MI and MY. However, W and CT showed less diversity and similar trends also followed by heat map based on unweighted pair-wise, UniFrac distances and Bray-Curtis matrix (Figure 3a; Figure S4).

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Moreover, based on the OTU abundance of different cultivation systems, OTU of each group was listed and a Venn diagram was drawn. Venn diagram showed maximum unique OTUs were found in soybean-sugarcane intercropping and followed by monoculture and peanut-sugarcane intercropping. Maximum overlapped OTUs (284) identified among soybean-sugarcane intercropping and monoculture and followed by monoculture and peanut-sugarcane intercropping and 266 OTUs were overlapped in all three cultivation systems (Figure 3b).

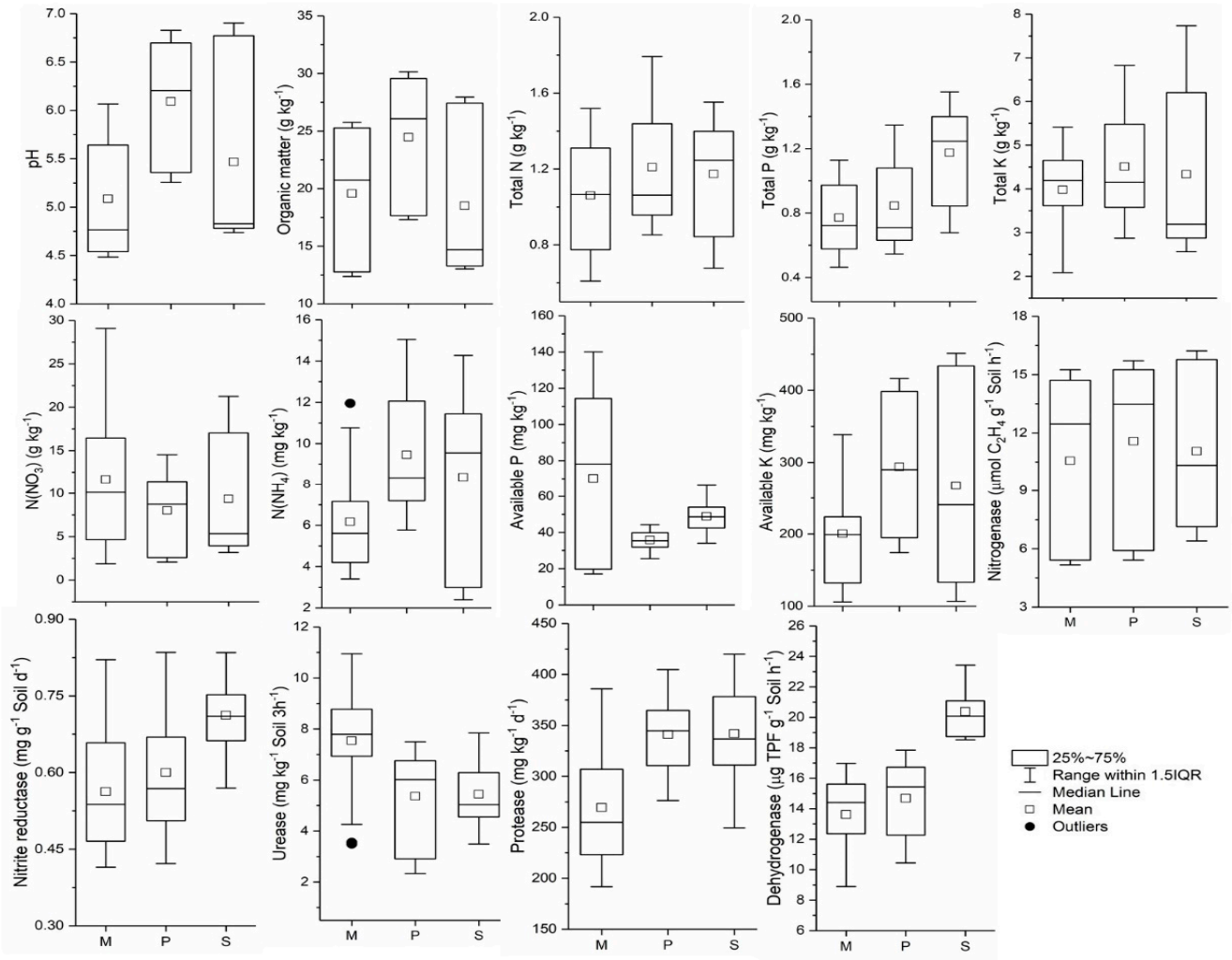

Figure. 1: The distribution of soil chemical and biological properties among three cultivation systems. M-sugarcane monoculture (n=15), P-peanut-sugarcane intercropping (n=9) and S-soybean-sugarcane intercropping (n=9)

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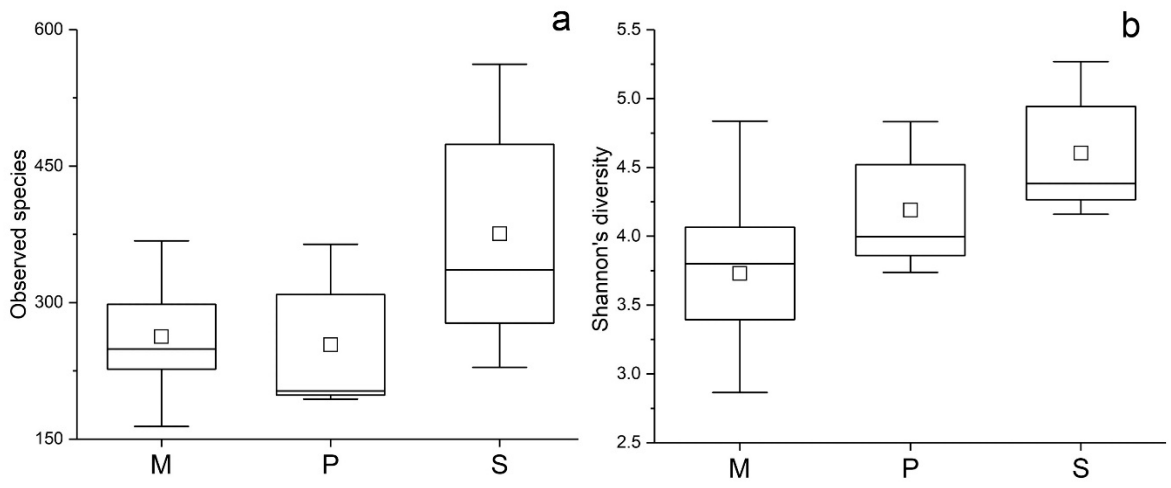

Figure 2: Alpha diversity box-plot among three cultivation systems. **a**) Observed species and **b**) Shannon's Index, M-sugarcane monoculture (n=5), P-peanut-sugarcane intercropping (n=3) and S-soybean-sugarcane intercropping (n=3)

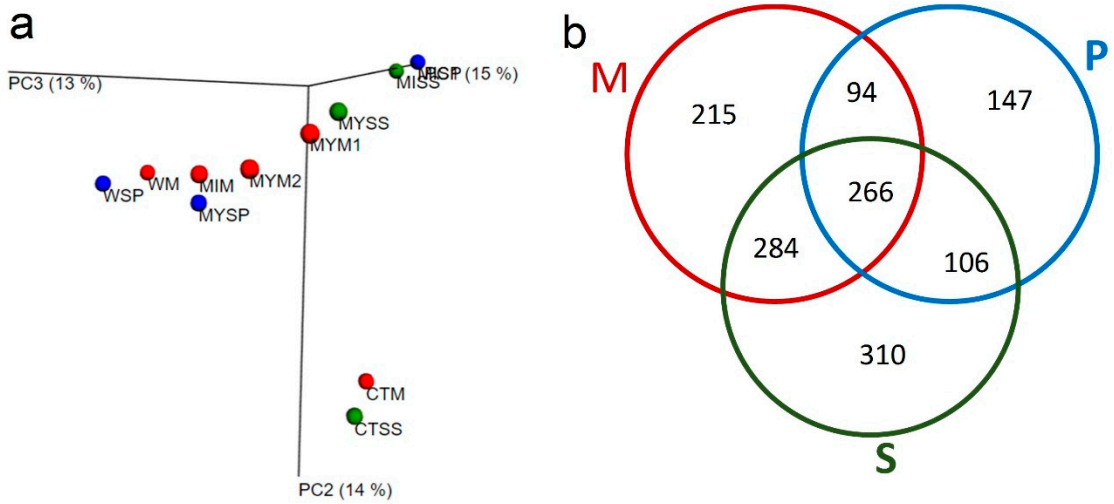

Figure 3: (a) Beta diversity metrics among three cultivation systems, (**b**) Venn diagrams showing the overall overlap of OTUs between three cultivation systems (M, P and S) obtained in four different locations. M-sugarcane monoculture (n=5), P-peanut-sugarcane intercropping (n=3) and S-soybean-sugarcane intercropping (n=3)

3.3 Diversity of bacterial communities

A total of 436,458 high-quality sequences of *nifH* gene with an average length of 360 bp were obtained from the all samples, and all sequences belonging to *Proteobacteria* phyla, two classes (*Alpha-proteobacteria* and *Beta-proteobacteria*), three orders (*Burkholderiales, Rhizobiales* and *Sphingomonadales*), seven families (*Alcaligenaceae*, *Bradyrhizobiaceae*, *Burkholderiaceae*, *Comamonadaceae*, *Methylocystaceae*, *Rhizobiaceae* and *Sphingomonadaceae*), seven genera (*Azohydromonas*, *Bradyrhizobium*, *Burkholderia*, *Pelomonas*, *Pseudacidovorax*, *Rhizobium*/*Agrobacterium* group and *Sphingomonas*), and five species (*Burkholderia silvatlantica*, *Pelomonas saccharophila*, *Pseudacidovorax* sp. ptl-2, *Rhizobium* sp. W3 and *Sphingomonas azotifigens*) were identified among all cultivation systems of four locations, and a major percentage of sequence data were unclassified. Next-generation sequencing results were clearly distinguished the differences among all sites on the basis of their bacterial community compositions. Changes in rhizospheric diazotrophic bacterial community compositions were also noticed among

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individual samples of three cultivations, showing some variation of the abundance of each group by the locations. Cultivation wise maximum average abundance of *Proteobacteria* was identified with peanut-sugarcane intercropping and followed by soybean-sugarcane intercropping and monoculture. However, maximum abundance of *Alpha-proteobacteria* was recovered with monoculture and *Beta-proteobacteria* with peanut-sugarcane intercropping. On the basis of order, maximum abundance of *Burkholderiales* was resulted in peanut-sugarcane intercropping, *Rhizobiales* with monoculture and *Sphingomonadales* with soybean-sugarcane intercropping, respectively. Different patterns of community structure were observed at the family and genera levels across all sites (Figure 4; Figure S5). Among all the samples, the highest abundance was identified in family *Rhizobiaceae* and followed by *Alcaligenaceae*, *Burkholderiaceae*, *Comamonadaceae*, *Bradyrhizobiaceae*, *Sphingomonadaceae* and *Methylocystaceae*, respectively. Maximum abundance of family *Alcaligenaceae*, *Bradyrhizobiaceae* and *Comamonadaceae* were resulted with peanut-sugarcane intercropping, and family *Burkholderiaceae* and *Sphingomonadaceae* were identified with soybean-sugarcane intercropping. However, family *Rhizobiaceae* and *Methylocystaceae* resulted with monoculture (Figure 4). Similar to the family, higher abundance resulted with genus *Rhizobium/Agrobacterium* group and followed by *Azohydromonas*, *Burkholderia*, *Bradyrhizobium*, *Pelomonas*, *Sphingomona* and *Pseudacidovorax*, respectively (Figure S5).

Moreover, heat maps were determined to get a graphical colored matrix of individual sample sequence data. Heat map displayed abundance based genus/species clustering of each genus/species, longitudinal clustering specifies the similarity of all genus/species between the samples, and the horizontal clustering directs the similarity of certain genus/species among the samples, the closer the distance and the shorter the branch length is, the more similar the genus/species composition between the samples (Figure 5). Heat map showed higher abundance via dark spots with the four genus *Rhizobium/Agrobacterium* group and *Azohydromonas*, *Burkholderia* and *Bradyrhizobium* and they are clustered with each other (Figure 5a). Genus heat map was clustered the individual soil samples into the two major groups (WSP, MYM2, MYSS, MIM, WM, MYM1 and MYSP) and (MISP, CTM, CTSS and MISS) and four minor groups (Figure 5a). In two locations CT and MY, soil samples of intercropping and monoculture were clustered together and MI and W showed differences with monoculture (Figure 5a). Next to this, species abundance based heat map also showed two major clusters among species and three major clusters among individual soil samples (Figure 5b). *Rhizobium* sp. W3 and *Burkholderia silvatlantica* showed higher abundance *via* dark spots and *Sphingomonas azotifigens* showed least abundance (Figure 5b).

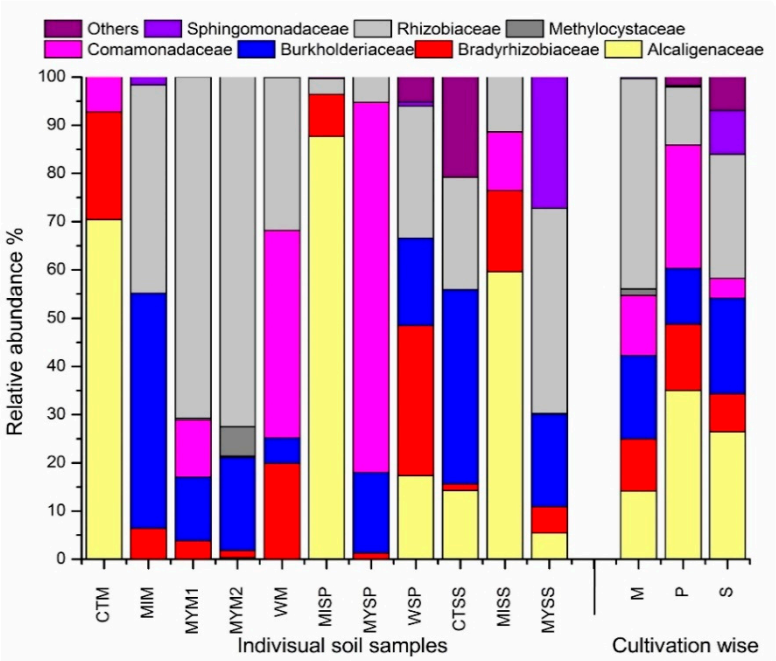

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Figure 4: Relative distribution of diazotrophic bacterial taxa at family level among three cultivation systems samples from four different locations of Guangxi, China, M-sugarcane monoculture (n=5), P-peanut-sugarcane intercropping (n=3) and S-soybean-sugarcane intercropping (n=3)

3.4 Linking between soil properties and microbial community

Soil pH positively related with protease. Total N-P-K, N-NO_3, available K and nitrogenease were positively related to soil organic matter (Table 2; Figure 6). On the contrary, soil organic matter showed a significant negative correlation with nitrite reductase. Among soil minerals, total N showed a significant positive correlation with total P and available K, and total K with N-NO_3 and N-NH_4, whereas strong negative correlation was found with total P and nitrite reductase. Moreover, nitrogenase positively correlated with total N, total K and N-NO_3. However, urease showed strong negative correlation with N-NH_4 (Table 2).

Soil pH positively related with Azohydromonas and OM positively related with *Bradyrhizobium*, whereas soil pH negative correlation was found with *Burkholderia* (Table 3). For soil minerals, total N-P showed a significant positive correlation with *Bradyrhizobium* and total K showed positive correlation with *Sphingomonas*. N-NH_4 showed positive correlation with *Pelomonas* and a negative correlation found with Shannon diversity index, whereas available P positively correlated with *Rhizobium/Agrobacterium* and available K showed positive correlation with *Bradyrhizobium* and *Sphingomonas.* Among soil enzymes, protease positively correlated with *Azohydromonas*. On the contrary, urease, nitrite reductase and protease negatively correlated with *Pelomonas, Bradyrhizobium, Burkholderia* and *Rhizobium/Agrobacterium,* respectively (Table 3).

To further discover these relations, we performed a canonical correlation analysis (CCA) based on the microbial abundance and soil variables (Figure 6). CCA ordination showed that soil properties positively influenced the microbial composition. The results of the CCA showed positive correlation of soil pH, available K, total N, N-NO_3 and protease with CCA axis 1, and based on magnitude and position they were important for explaining the variations between the microbial communities. The relatively small magnitude of the OM, total P, total K, N-NH_4, nitrite reductase, nitrogenase, urease and dehydrogenase vectors indicates that these parameters were not as strongly correlated to community composition than other (higher magnitude vectors). However, higher magnitude of available P showed a negative correlation as compaired to other soil variables. Moreover, some bacterial genera, *Azohydromonas*, *Bradyrhizobium*, *Burkholderia*, *Pseudacidovorax*, *Rhizobium* and *Sphingomonas* positively correlated with soil chemical and biological properties. *Azohydromonas* and *Rhizobium* were most distinguished genus resulted by CCA. On the contrary, *Pelomonas* showed negative correlation with all soil properties (Figure 6).

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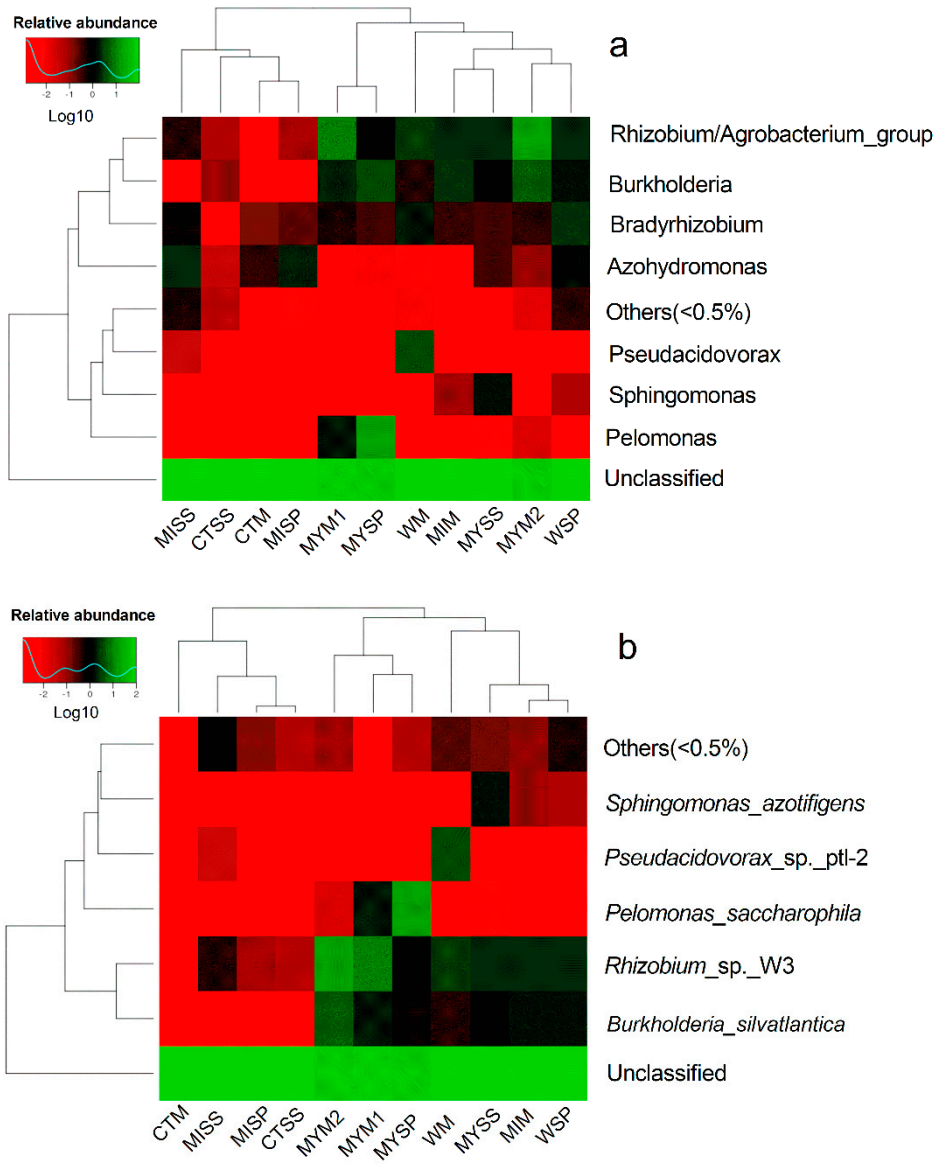

Figure 5: Relative abundance heatmap of diazotrophic bacterial taxa at genes (**a**) and species (**b**) levels among three cultivation samples from four different locations of Guangxi, China. Longitudinal clustering indicates the similarity of all species among different samples, and the horizontal clustering indicates the similarity of certain species among different samples. The closer the distance is and the shorter the branch length is, the more similar the species composition is between the samples. M-sugarcane monoculture (n=5), P-peanut-sugarcane intercropping (n=3) and S-soybean-sugarcane intercropping (n=3)

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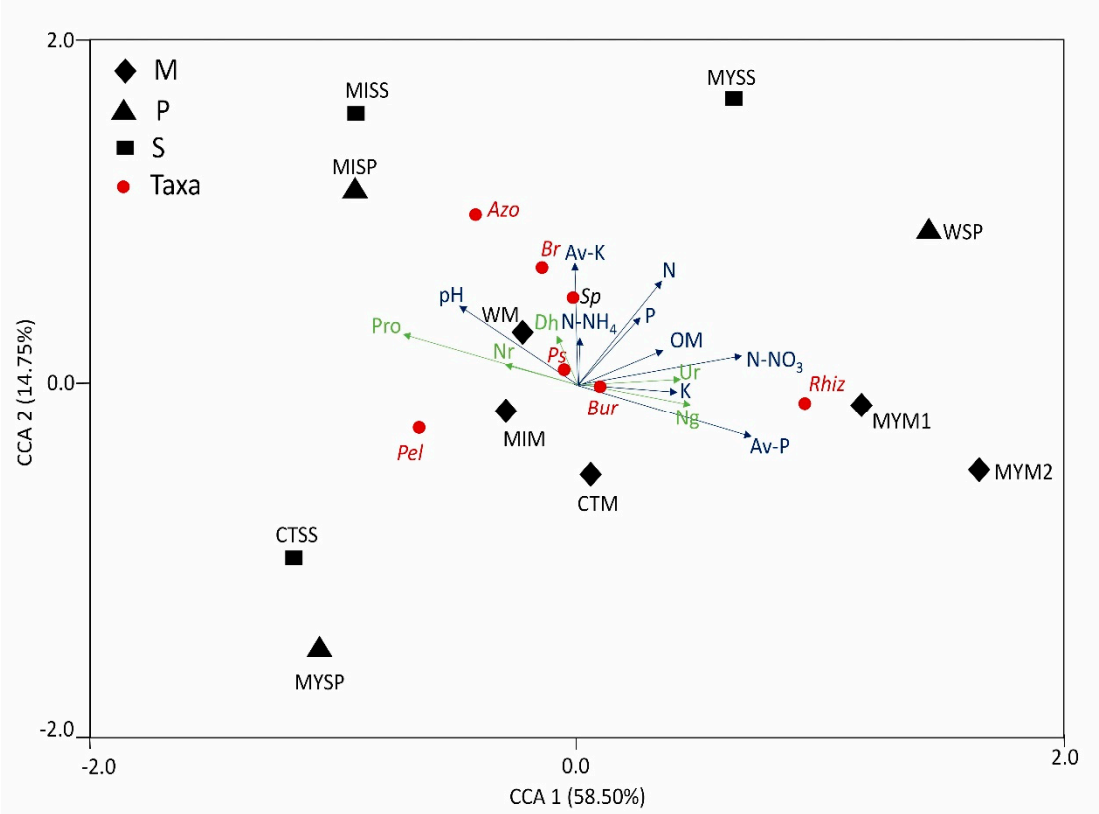

Figure 6: Ordination diagrams from canonical correspondence analysis (CCA) of microbial abundances and soil parameters. Blue and green arrows indicate the direction and magnitude of soil chemical and biological parameters associated with microbial abundance. Sugarcane monoculture (M-diamond), peanut-sugarcane intercropping (P-triangle) and soybean-sugarcane intercropping (S-square), red dot for the taxa. *Azo-Azohydromonas*. *Brad-Bradyrhizobium*, *Bur-Burkholderia*, *Pel- Pelomonas*, *Ps- Pseudacidovorax*, *Rhiz- Rhizobium/Agrobacterium* group and *Sp-Sphingomonas*, OM-organic matter, Av-available, Ur-Urease, Nr-Nitrite reductase, Pr-Protease, Dh-Dehydrogenase and Ng-Nitrogenase.

Table 2. Correlation between soil chemical and biological properties of sugarcane monoculture and sugarcane legume (peanut and soybean) intercropped samples

Variables	pH	OM	N	P	K	NO_3	NH_4	Av-P	Av-K	Ur	Nr	Pro	Dh
pH													
[a]OM	-0.19												
[a]N	0.14	0.72**											
[a]P	0.12	0.55*	0.66**										
[a]K	-0.31	0.69**	0.34	-0.14									
[b]NO_3	-0.33	0.58*	0.52	0.17	0.59*								
[b]NH_4	0.07	0.44	0.42	-0.2	0.7**	0.5							
[b]Av-P	-0.25	-0.16	-0.07	-0.14	0.16	0.13	-0.09						
[b]Av-K	0.24	0.7**	0.66**	0.5	0.39	0.3	0.33	-0.47					
Ur	-0.34	0.16	0.13	0.52	-0.32	0.22	-0.64**	0.02	0.05				
Nr	0.47	-0.67**	-0.36	-0.72**	-0.17	-0.32	0.08	0.16	-0.26	-0.5			
Pro	0.73**	-0.16	0.04	-0.1	-0.24	-0.27	-0.06	-0.46	0.25	-0.21	0.52		
Dh	0.00	0.17	0.36	-0.19	0.4	0.27	0.28	0.03	0.29	-0.27	0.33	0.43	
Ng	-0.48	0.81***	0.56*	0.14	0.8***	0.76**	0.5	0.1	0.33	0.1	-0.44	-0.21	0.44

*p<0.1, **p<0.05, ***p<0.01, [a]g Kg^{-1}, [b]mg Kg^{-1}, OM-organic matter, Av-available, Ur-Urease (mg kg^{-1} soil $3h^{-1}$), Nr-Nitrite reductase (mg g^{-1} soil d^{-1}), Pr-Protease (mg kg^{-1} d^{-1}), Dh-Dehydrogenase (µg TPF g^{-1} soil h^{-1}), Ng-Nitrogenase (µmol C_2H_4 g^{-1} soil h^{-1})

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329 **Table 3**. Correlation between soil properties and microbial population of sugarcane monoculture and sugarcane legume (peanut and soybean) intercropped samples
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Variables	*Azoh*	*Brad*	*Bur*	*Pel*	*Ps*	*R/A*	*Sp*	Obj	Shan
pH	0.88***	0.26	-0.58**	-0.09	0.05	-0.51	-0.27	-0.10	-0.05
[a]OM	-0.13	0.61**	0.48	0.28	0.24	0.33	0.37	0.16	-0.11
[a]N	0.36	0.63**	0.25	-0.08	0.06	0.36	0.27	-0.14	-0.22
[a]P	0.21	0.85***	0.25	-0.32	0.35	0.31	-0.14	0.28	0.28
[a]K	-0.34	-0.01	0.36	0.47	-0.01	0.22	0.63**	0.00	-0.19
[b]NO_3	-0.30	0.33	0.15	0.01	0.21	0.38	0.38	-0.15	-0.34
[b]NH_4	0.12	-0.06	0.18	0.56*	-0.35	-0.04	0.46	-0.37	-0.70**
[b]Av-P	-0.21	-0.32	0.31	-0.23	-0.33	0.66**	-0.09	-0.15	0.19
[b]Av-K	0.36	0.58*	-0.14	-0.13	0.24	-0.19	0.61**	-0.05	0.00
Ur	-0.25	0.37	0.11	-0.55**	0.52	0.46	-0.19	0.30	0.40
Nr	0.45	-0.57**	-0.63**	-0.13	-0.38	-0.44	0.04	-0.50	-0.20
Pro	0.61**	0.25	-0.70**	0.00	0.22	-0.66**	-0.16	-0.08	-0.09
Dh	0.07	0.08	-0.27	-0.04	-0.03	-0.15	0.48	-0.27	-0.11
Ng	-0.42	0.29	0.49	0.38	0.13	0.46	0.37	0.01	-0.30

*p<0.1, **p<0.05, ***p<0.01, [a]g Kg^{-1}, [b]mg Kg^{-1}, OM-organic matter, Av-available, Ur-Urease (mg kg^{-1} soil $3h^{-1}$), Nr-Nitrite reductase (mg g^{-1} soil d^{-1}), Pr-Protease (mg kg^{-1} d^{-1}), Dh-Dehydrogenase (µg TPF g^{-1} soil h^{-1}), Ng-Nitrogenase (µmol C_2H_4 g^{-1} soil h^{-1}), *Azo-Azohydromonas*. *Brad-Bradyrhizobium*, *Bur- Burkholderia*, *Pel- Pelomonas, Ps- Pseudacidovorax, Rhiz- Rhizobium*/*Agrobacterium* group and *Sp-Sphingomonas*

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4. Discussion

To understand the functional diversity, composition, structure and dynamics of diazotrophs communities under sugarcane-legume intercropping. We carried out this study to examine the effect of different cultivation systems on the soil properties and their relation to the diazotrophs. Previous studies have investigated that soil microbial diversity, soil enzyme activities, crop yield could be affected by land management practices, and all these soil properties were increased under intercropping systems than monoculture [4,6,17,27]. Moreover, our previous study also discussed that soil biochemical and enzymes have a direct effect by intercropping system and it enhanced the diazotrophic bacterial population [36,55]. Kent and Triplett, [56] have specified that root secretions and rhizodeposition of different plant root zones influenced the structural and functional diversity of rhizosphere. Furthermore, the soil type, cropping practices, plant growth stages, and ecological factors influence the rhizospheric microbial community [16]. Our results also correlated with these studies. Soil enzymes are essential for nutrient cycling. Soil enzyme activity is also an important bio-indicator of soil fertility and quality. Activities of soil urease, nitrite reductase, protease, dehydrogenase and nitrogenase are important indexes for evaluating biochemical processes related to soil N transformation. In our study, monoculture showed higher urease and intercropping samples were showed higher activity of nitrite reductase, protease, dehydrogenase and nitrogenase (Figure 1). One possibility is that in monoculture for higher urease activity, N-fertilizer rate was same in monoculture and intercrops, and intercrops utilized soil urea faster than monoculture. Nitrite reductase, protease and nitrogenase play significant roles in the nitrogen cycle and higher activities of these enzymes showed that vital microbial community enhanced in intercropping system. Reduction of atmospheric dinitrogen into the ammonia by nitrogenase enzyme is an energy consuming process, therefore, it's not easily regulated [57] and favorable environmental factors like acidic pH, less available N concentration and rich C sources will increase free-living N-fixation [58]. Positive correlation of pH with microbial population showed similarity with the past studies (Table 3; Figure 6) [27,58,59]. Recently, sequence-based community structure analysis is progressively accepted to study rhizospheric bacterial communities [34,35]. Through cultivation-independent molecular technique, we deliver an evaluation of reaction and recovery of rhizospheric diazotrophs communities influenced by different cultivation systems. Moreover, in the present study nested PCR was used to amplify the functional *nifH* gene from the rhizospheric soil samples. Nested PCR decreases the chance of multiple target site amplification s and enhances the detection consistency. The effectiveness of the nested PCR method has been verified several times in the previous *nifH* gene diversity studies [16,32,58,60]. Li et al. [16] reported that terrestrial ecosystems had less abundance of diazotrophic bacteria therefore, direct amplification of the *nifH* gene from soil samples is hard. In the present study, we have been using nested PCR to enhance the sensitivity and the specificity of *nifH* gene amplification, to investigate the genetic diversity from rhizospheric soil samples. Our results showed that, 436,458 high-quality sequences of *nifH* gene belonged to the *proteobacteria* phyla. More specifically, these sequences were affiliated with the genera *Rhizobium*/*Agrobacterium*, *Azohydromonas*, *Burkholderia*, *Bradyrhizobium*, *Pelomonas*, *Sphingomonas* and *Pseudacidovorax* (Figure 5a), all of which contain species that are known to fix nitrogen in symbioses with legumes [61–64]. The *nifH* gene sequences of the present study with the uncultivable diazotrophs of different rhizosphere, revealed common genera like *Azohydromonas* [25], *Burkholderia* [19], *Bradyrhizobium* [25,59], *Pelomonas* [28] and *Rhizobium* [25], and our result also showed similarity with past studies by different molecular tools [26,30,32,58]. The high abundance of *Alpha-proteobacteria* (especially *Burkholderia* and *Rhizobium*) *nifH* genes found in the monoculture samples in farmer field, Mingyang Farm, Wuxu Town, District Jiangnan, Nanning City, due to the crop rotation and intercropping with leguminous plants (soybean and peanut) or watermelon. Moreover, *Rhizobium* sp. is well notorious root colonizer of a wide range of nonlegume plants like sugarcane [6,19], sorghum [25], rice [65], maize [19] and switch grass [66], and *Burkholderia* sp. has been already detected as an active diazotrophs in sugarcane [19]. Regardless of diazotrophs, the most abundant taxa was *Alpha-proteobacteria*, and this outcome was usually consistent with past researchers who verified that *Alpha-proteobacteria* were the most abundant and

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common group in soil [25,59,67]. Based on the observed *nifH* OTUs richness and calculated estimates, the sugarcane soil from the formers field is the most diverse in the soil sites included in this study. The predominance of *nifH* OTUs related to *proteobacteria* and the nonappearance of *nifH* gene sequence related to Cyanobacteria, Firmicutes and Archaea has been also described from some previous studies [25,31,33,60,68]. Unexpectedly, there was no evidence of *Gamma-proteobacterial nifH* OTUs. The absence of these sequences is confirmed by the cultivation-dependent diazotrophs study from tropical soils where only *Alpha-proteobacteria* and *Beta-proteobacteria* have been found [23,69,70]. Next, we found *Burkholderia silvatlantica*, *Pelomonas saccharophila*, *Pseudacidovorax* sp., *Rhizobium* sp. W3 and *Sphingomonas azotifigens* species and they are well known plant and soil associated microbes (Figure 5b). Perin et al. [19] reported a cultivable diazotrophs *Burkholderia silvatlantica* strain isolated from soil in Brazil. *Pelomonas saccharophila* strain also characterized as diazotrophs and hydrogen-oxidizing bacteria previously from Japan [71]. Zhang and Chen [72] isolated and characterized an N-fixers strain of *Pseudacidovorax* sp. from seawater of China. Kaur et al. [73] reported an N-fixers *Rhizobium* sp. W3 strain isolated from hexachlorocyclohexane dumpsite of India. An N-fixer strain of *Sphingomonas azotifigens* was also isolated from rice plants in Japan [65]. All these findings concluded that these species played important roles in the soil for the plant growth promotion and mineral acquisition.

The results obtained by other studies indicated that monoculture cultivation reduced the soil fertility and microbial diversity (negatively affected the structure, function and stability of soil microflora), especially during the ratoon [74]. The Shannon's diversity index of the *nifH* gene ranged 2.85-5.27 in this study (Figure 2), which is higher than those in other studies [25,33,75–78]. In the present study, soybean-sugarcane intercropping enhanced the values of species richness and diversity (Table S2). These results concluded that the monoculture cultivation system might alter the microenvironment of rhizospheric diazotrophs, and thus initiating soil bacterial community reductions. In our study, Pearson correlation analysis discovered the impact of soil properties on soil microbial diversity and community composition, which recommended that the variation in bacterial community abundance and diversity could be partly due to changes in the soil properties. Pearson correlation analysis and CCA plot revealed that microbial community had positive links with the soil pH, nitrite reductase and protease. (Table 2; Figure 6). Thus, the observed reductions in the soil pH and the activities of soil enzymes nitrite reductase and protease in monoculture could explain the declining in the diversity of microbial communities. Moreover, soil enzymes showed higher activity of nitrate reductase in intercropped soil samples and this enzyme play essential role in denitrification. Denitrification is a process of nitrate reduction into a simple nitrogen molecule (nitric oxide) by diazotrophs and different bacterial species and enzymes have played important role in this process [79]. Likewise, intercropped fields showed higher protease activity, these results described the significant role of diazotrophic bacteria in N mineralization. Recently, Cole et al. [52] also testified significant role of soil proteases in N mineralization, and this process played an important role for available N of plant and plant growth.

Microbial diversity analyses suggest that microbial composition is largely affected by soil parameters but provide little evidence about the linkage. Therefore, CCA was used to discover the link between microbial composition and soil variables. Soil total N, total P, OM and N-NO_3 appeared to be important soil variable and correlate positively. On the contrary, less available P in intercropping and negative correlation with microbial abundance showed that due to nitrogen fixing microbes P uptake enhanced significantly and it reduced the soil available Li et al. [80] reported that intercropping reduced the Olsen P in the soil. Moreover, higher phosphate uptake enhanced the plant growth and diazotrophic microbes like *Bradyrhizobium*, *Burkholderia* and *Rhizobium* are well-known phosphate solubilizer [81–83].

5. Conclusion

In conclusion, this study presents a comparative study of the *nifH* gene biodiversity of sugarcane rhizosphere soil by next-generation sequencing in intercropped sugarcane. This study found that an

extensive variety of diazotrophic bacteria were strongly collaborating with soil variable and intercropping cultivation system significantly enhanced the microbial diversity. Most of the soil variables positively correlated with diazotrophs. Maximum number of OTUs recovered from soybean-sugarcane intercropping and less available P of soil has given an idea to utilization of intercropping system for nutrient mineralization, and we found that *Alpha-proteobacteria* abundantly recovered from the sugarcane rhizosphere. We concluded that soil physicochemical properties, especially nutrient elements, soil pH and soil enzymes positively influenced the bacterial community. However, these all result need to investigate further in regards to identify the important microbial candidates and need to understand their interaction with plant to find out the nature of mechanisms of N cycling and P mineralization.

Supplementary Materials: The following are available online, Figure S1, S2, S3, S4 and S5, and Table S1 and S2.

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