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Research article

# Split application of phosphorus fertilizer in Chinese milk vetch-rice rotation enhanced rice yield by reshaping soil diazotrophic community

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## ABSTRACT

Chinese milk vetch (CMV) is widely recognized as the leading leguminous green manure utilized in the rice-green manure rotation system throughout southern China. While bacteria that form symbiotic relationships with CMV are responsible for fixing a significant portion of nitrogen (N) within agroecosystems. diazotrophic organisms play an essential role in the N cycle and enhance the pool of N readily accessible to plants. The goals of the current study were to investigate the effects of shifting partial phosphorus (P) fertilizer application from the rice season to the CMV season within a CMV-rice rotation system on soil nutrient levels, activity of soil enzymes and stoichiometric ratios, as well as diazotrophic community structure. The treatments consisted of a control group, a winter fallow-rice rotation without fertilizer application, and the treatments P0, P1, P2, and P3, representing 0, 1/3, 2/3, and the full dose, respectively, of phosphorus fertilizer (60 kg ha$^{-1}$ $P_2O_5$) added in a single rotation system during the CMV season, while combined with 60 % of regular N application rate during the rice season. In comparison to P0, the application of treatments P1, P2, and P3 resulted in higher CMV dry biomass and rice production across the seasons from 2018 to 2021 and the P2 treatment significantly increased the contents of total N (TN), soil organic matter (OM), and available P (AP) by 49 %, 48 %, and 110 %, respectively. The activities of alkaline phosphatase and L-leucine aminopeptidase showed a significant decrease when subjected to the P1 and P2 treatments. The P2 treatment enhanced the relative abundance of *Frankia* and *Skermanella* by 2.6 % and 1.6 %, respectively, comparing with P0 treatment. Furthermore, correlation analysis revealed a positive relationship between *Skermanella* and *Mesorhizobium* with the contents of TN, OM, AP, ammonium-N, and nitrate-N. In conclusion, the application of 1/3 to 2/3 of the full dose P fertilizer in CMV season reshaped soil diazotrophic community, improved soil N content, and thereby increased rice yield with 40 % N fertilizer reduction.

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## 1. Introduction

Nitrogen (N) and phosphorus (P) are two of the critical limiting plant nutrients that are essential for rice production [1]. Throughout the past several decades, high amount of chemical fertilizers addition, primarily N-based fertilizers, has enhanced soil nutrient quality and agricultural yield [2,3]. Meanwhile, some studies have reported major soil N losses via denitrification, ammonia volatilization, leaching, and runoff processes through intensive agricultural management activities [4,5]. Furthermore, a considerable a portion of P is rendered immobile in soils, transforming into relatively unavailable inorganic and organic forms; therefore, P use efficiency is reduced in a season [6]. Excessive application of mineral fertilizers in agriculture is a widespread challenge. Therefore, partial replacement of mineral fertilizers with green manure will be effective in lowering fertilizer input [7]. Legumes are the most popular crop in a green manure-rice rotation system due to their capacity for biological N fixation, thus they can be used to reduce mineral fertilizer N dependence and environmental risks [8]. Moreover, they could increase the N fixing efficiency of soil microorganisms, but there are few studies focusing on the activity of soil N fixing bacteria in leguminous green manure-rice rotation system.

Biological N fixation (BNF) is the main natural mechanism by which atmospheric $N_2$ is converted into bioavailable ammonium-N ($NH_4^+$-N) [9]. BNF entails two main types of N fixation. One is symbiotic N fixation, which is mainly mediated by N-fixing microbes in symbiotic association with their plant hosts [10] The other is asymbiotic N fixation, which is mainly mediated by soil heterotrophic, autotrophic, or some N-fixing archaea [11]. The diazotrophs in soils are highly diverse, widely distributed in agricultural and natural soil ecosystems, and essential for N fixation [12]. However, the N fixation capacity and role of these organisms is controversial in different soil ecosystems [13]. Considering asymbiotic $N_2$-fixation is an energy-intensive process dependent on soil carbon (C) effectiveness, an increase in available C sources can reportedly enhance $N_2$ fixation rates [14]. Similarly, increases in N effectiveness have been reported to have inhibitory or stimulatory effects, whereas P application is commonly reported to enhance $N_2$ fixation of diazotroph in soils [15]. Although such nutrient factors could influence soil diazotrophs activity, our understanding regarding the impacts of fertilizer application on diazotrophic structure remains poor. The *nifH* gene, which is present in all diazotrophs and closely resembles the phylogeny of the 16S rRNA gene, serves as an excellent candidate for ecological research on community structure and composition [16]. The *nifH* gene encodes a subunit of the nitrogenase enzyme and serves as a marker for investigating the distribution and diversity of diazotrophs without the need for cultivation [17]. Therefore, the *nifH* gene associated with nitrogenase has been widely utilized to examine the abundance and community composition of diazotrophs [18].

Studying the abundance and community structure of soil diazotroph could facilitate sustainable agricultural development. Previous studies have found that the relative abundance, diversity and community results of diazotrophic groups can significantly affect N fixation efficiency and be sensitive to the long-term fertilization process [19]. From moderate to high N input significantly reduced the abundance of diazotrophic and their diversity, while P addition increased the abundance and association of diazotroph. At the same time, soil diazotrophic communities can cope with P limitation by changing their life history strategies (oligotrophic to symbiotic nutrients) [20]. Thus, understanding how the response of the diazotrophic community to P input during the green manure season in a green manure-rice rotation is essential.

Soil enzymes primarily act as catalysts generated by microorganisms, facilitating the cycling of C, N, and P in soil [21]. Soil enzyme activity stoichiometry can illustrate soil enzyme activity associated with nutrient cycling in an ecosystem, and the magnitude of the activity can characterize soil microbial nutrient requirements and soil nutrient limitation [22]. Soil enzymes are also essential mediators linking soil microbial and soil chemical processes, and enzyme activity can reflect the intensity of nutrient metabolism [23]. On a global scale, the stoichiometric ratio of enzymes for soil C, N and P is roughly 1: 1: 1 [24]. However, in different ecosystems, soil microbes change the stoichiometric the ratio of extracellular enzymes to fulfill their nutrient requirements in their environment [25]. In order to highlight the traits of microbial metabolism, this study involves calculating the ’lengths’ and ’angles’ of vectors on a plot representing the proportional activities of enzyme C:N versus C:P acquisition. This approach quantifies the relative investments in C vs nutrient acquisition (vector lengths) or P vs N acquisition (vector angles) [26,27]. Furthermore, translating these ratios into vector lengths and angles derived from enzyme activity quantify the nutrients prioritized for acquisition, and such analyses are used widely to assess microbial resource requirements versus soil nutrient limitations [28]. Calculated by length and angle can facilitate the quantification of microbial enzyme activity and relative nutrient requirements [26]. It presents explicit signs of relative limitations for C, as well as those for P and N [21]. Therefore, soil enzyme activity measurements and enzyme stoichiometry ratios can facilitate the elucidation of the response of soil microbes to C, N, and P nutrients and the impact on soil diazotrophic communities.

Although the role of green manure in N fixation and its potential application as a partial N fertilizer have been studied extensively [29], the effects of long-term P application on soil microbial nutrient requirements and diazotrophic community structure are unclear. The present study was conducted at a long-term experimental site to investigate the functional significance of diazotrophic community structure and soil nutrient relationships in Chinese milk vetch (CMV) flower bloom stage soils under CMV-rice rotations. The primary goals of this study were to explore (1) the effect of P split application on the crop yield and soil nutrient content; (2) the responses of diazotrophic community structure and diazotroph relative abundance to P fertilizer application; and (3) the synergistic relationships between the increases of soil N nutrients and altered soil diazobacterial community structure.

## 2. Materials and methods

### 2.1. Overview of the research area and experimental design

The study took place in a specified region located at the long-term positioning rice experimental station of Xinfeng Brigade (30°53′N, 112°48′E), in Taihu farm, Jingzhou city, Hubei province, China. The traditional cropping system is middle-season rice followed by a winter fallow period or a cover crop. Three replicates of a randomized full block design were used to set up the study. The experimental plot areas were 32 $m^2$, 8 m long and 4 m wide. There were ridge furrows in every plot, with ridge widths of 50 cm and ridge heights of 15 cm.

The first CMV season began in Oct 2017 and rotated every year. The experimental treatments consisted of:1) CK, application of fertilizers is not utilized in the winter fallow-rice rotation; 2) P0, no P split application and the complete amount of P fertilizer (60 kg $ha^{-1}$ $P_2O_5$) were all applied in rice season; 3) P1, 1/3 of the full dose P fertilizer were moved to the CMV growing period, while the remainder was applied during the rice season; 4) P2, 2/3 of the complete dose P fertilizer were utilized during the CMV season; and 5) P3, the full dose P fertilizer addition in one rotation system all applications occurred in the CMV season. In P1, P2 and P3 treatments, P fertilizers were applied when sowing CMV in October of each year. During the rice season, the annual application rate for N fertilizer was 99 kg $ha^{-1}$, representing 60 % of the suggested amount, while the application rate for $K_2O$ fertilizer was 90 kg $ha^{-1}$. Among them, 70 % of the N fertilizer was used as base fertilizer and 30 % for top dressing, all P and K fertilizers were applied as base fertilizers. Urea (N 46 %), calcium super-phosphate ($P_2O_5$ 12 %), and potassium chloride ($K_2O$ 60 %) were used as N, $P_2O_5$, and $K_2O$ fertilizers, respectively. Additional management practices followed local agricultural guidelines.

## *2.2. Plant and soil sampling*

Chinese milk vetch was harvested and incorporated on the spot at the full blooming stage (around Apr. 15). The aboveground fresh weight was measured and subsamples were randomly from each plot to evaluate the dry weight along with N and P concentrations. At the rice maturity stage, rice grains in the whole plot were harvested and the yield was assessed following air drying. The data of CMV and rice yield were collected from 2018 to 2021 and the rest of the data were from 2021.

On Apr. 15, 2021, soil samples from the upper 0–20 cm were taken. In every plot, five soil cores were collected in succession following an “S” pattern at consistent intervals and merged to form a composite sample. Each composite sample was broken up into three pieces after the roots, litter, debris, and stones were removed for using in later tests. The two sub-samples were carried to the lab in an ice chest. The initial portion was put into storage for DNA extraction right away at −80 °C. Within two weeks, the second portion was used to conduct an analysis of the extracellular enzyme activity after being passed through a 2.0-mm sieve and kept at 4 °C. To be used in investigations of nutrient content, the third component was air-dried.

## *2.3. Plant and soil chemical analysis*

All plant samples were initially dried in an oven at 105 °C for 30 min, and then dried at 65 °C until they attained a constant weight. After drying, they were ground to pass a 0.84-mm sieve, digested by $H_2SO_4$-$H_2O_2$. The N and P concentrations were determined using the semi-micro Kjeldahl method [30] and the molybdenum blue method [31], respectively. To calculate plant nutrient uptake, the concentration was multiplied by the corresponding dry weight. Soil organic matter (OM) was analyzed using a titration method following oxidation with $K_2Cr_2O_7$ [32]. The total nitrogen (TN) content was assessed using the Kjeldahl method. Fresh soil samples were used to extract ammonium-N ($NH_4^+$-N) and nitrate-N ($NO_3^-$-N), which were measured through continuous flow analysis with 100 mL of 0.01 mol $L^{-1}$ $CaCl_2$ (Seal AA3, Norderstedt, Germany). The microbial biomass nitrogen (MBN) in the soil was estimated using the chloroform fumigation-extraction technique [33]. For extracting available phosphorus (AP), a solution of 0.5 mol $L^{-1}$ $NaHCO_3$ was utilized, and its concentration was measured using the molybdenum blue method.

## *2.4. Extracellular enzymatic activity and the level of microbial metabolic restriction measurement*

Detection kits were utilized to evaluate the activities of alkaline phosphatase (ALP), β-1,4-glucosidase (BG), β-D-cellobiosidase (CBH), and L-leucine aminopeptidase (LAP), (Jining, Shanghai, China), following the guidelines provided by the manufacturer. Ultimately, these enzyme activities were quantified as nanomoles of substrate released per gram of dry soil (U $g^{-1}$). Eq. (1) was used to determine vector lengths, while Eq. (2) was used to calculate vector angles. With longer vectors, microbial C restriction becomes stronger [26]. Microbial phosphorus limitation is indicated by vector angles exceeding 45°, while microbial nitrogen limitation is identified by vector angles below 45°. The calculations for vector length (Vector L) and vector angle (Vector A) are performed using the following equations [26].

$$\text{Vector L} = \text{SQRT}[((x)^2+(y)^2] \tag{1}$$

$$\text{Vector A} = \text{Degrees}[\text{atan2}(x, y) \tag{2}$$

“BG + CBH)/ALP” means “x”, “BG + CBH)/LAP” means “y”SQRT means “open square function”, Degrees for “angle conversion”, and atan2 for “arctangent function”. atan2((x, y) = arctan(y/x).

## *2.5. Extraction of DNA and PCR amplification*

DNA extraction was performed with the Fast DNA®SPIN Kit (MOBIO Power Soil DNA Isolation Kit). The quantity and quality of the extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA extracted from the soil was placed in a −20 °C refrigerator for further analysis. The primers *nifH*-F (5′ - AAAGGYGGWATCGGYAARTCCACCAC - 3′) and *nifH*-R (5′ - TTGTTSGCSGCRTACATSGCCATCAT - 3′) for *nifH* gene of nitrogen-fixing bacteria were determined by fluorescent

**Table 1**
Effect of phosphorus (P) fertilizer split application in Chinese milk vetch (CMV)-rice rotation system on CMV and rice yields (t/ha) in 2018–2021 seasons.

| Treatment | CMV dry biomass | | | | Rice grain yield | | | |
|---|---|---|---|---|---|---|---|---|
| | 2018 | 2019 | 2020 | 2021 | 2018 | 2019 | 2020 | 2021 |
| CK | | | | | 5.3 ± 0.0c | 6.9 ± 0.3c | 7.4 ± 0.4b | 6.1 ± 0.2c |
| P0 | 0.6 ± 0.1c | 1.7 ± 0.1d | 2.3 ± 0.1b | 3.4 ± 0.5c | 6.4 ± 0.1b | 8.2 ± 0.3b | 8.3 ± 0.2a | 7.7 ± 0.2b |
| P1 | 1.6 ± 0.2b | 3.4 ± 0.1c | 4.7 ± 0.2a | 5.4 ± 0.3a | 6.9 ± 0.1a | 9.9 ± 0.3a | 8.5 ± 0.4a | 8.6 ± 0.1a |
| P2 | 2.0 ± 0.1b | 4.2 ± 0.0b | 4.5 ± 0.7a | 5.6 ± 0.6a | 7.0 ± 0.1a | 10.2 ± 0.2a | 8.8 ± 0.4a | 8.7 ± 0.1a |
| P3 | 2.5 ± 0.1a | 5.0 ± 0.1a | 3.8 ± 0.1a | 4.1 ± 0.2b | 6.9 ± 0.1a | 9.9 ± 0.1a | 8.5 ± 0.2a | 8.5 ± 0.1a |

Note: The values in the table are “mean ± standard error”. Different lowercase letters indicate significant differences between treatments ($P < 0.05$).

**Table 2**
Effect of phosphorus (P) fertilizer split application on nitrogen (N) and P uptakes (kg/ha) by Chinese milk vetch (CMV) and rice in 2021 season.

| Treatment | CMV | | Rice | |
|---|---|---|---|---|
| | N uptake | $P_2O_5$ uptake | N uptake | $P_2O_5$ uptake |
| CK | | | 65.0 ± 6.80c | 33.7 ± 0.94b |
| P0 | 90.9 ± 4.63c | 11.7 ± 0.66c | 90.9 ± 4.72bc | 48.3 ± 0.97a |
| P1 | 130.8 ± 2.56b | 18.3 ± 2.17b | 125.5 ± 9.70a | 57.8 ± 1.04a |
| P2 | 156.3 ± 2.60a | 26.4 ± 1.61a | 119.6 ± 5.65 ab | 45.7 ± 1.97 ab |
| P3 | 118.2 ± 2.35b | 15.8 ± 0.43b | 127.7 ± 9.79a | 47.5 ± 2.03a |

Note: The values in the table are “mean ± standard error”. Different lowercase letters indicate significant differences between treatments ($P < 0.05$).

quantitative PCR [34]. The specific amplification procedure and PCR reaction system were Denaturation: 95 °C for 10 min, 40 cycles; annealing: 50 °C for 30 s; extension: 72 °C for 40 s. The PCR mixture included 15 μL of 1 × SYBR premix Ex Taq along with 0.25 μL of each primer. Additionally, it included four μL of dNTPs (at a concentration of 2.5 mmol) and one μL of DNA template (50 ng). Electrophoresis was performed on the PCR products obtained, using a 2.0 % agarose gel. From each sample, the amplicons were combined, cleaned, measured, and then analyzed with the MiSeq PE300 platform commissioned by Shanghai Majorbio Bio-Tec (Majorbio Bio-TecCo. Ltd) for high-throughput sequencing.

Following demultiplexing, the resulting sequences were integrated using Flash [35] and subsequently underwent quality filtering with fastp (version 0.19.6) [36]. The Dada2 plugin [37] in the Qiime2 pipeline (version 2020.2) was used to denoise the high-quality sequences [38], utilizing suggested parameters to attain single nucleotide resolution based on the error profiles derived from the samples. The sequences that are denoised by Dada2 are typically known as amplicon sequence variants (ASVs). Using the Silva database and the Naive Bayes consensus taxonomy classifier available in Qiime2, taxonomic classification of ASVs was performed (https://www.arb-silva.de/). The free Majorbio Cloud Platform was utilized to perform the sequencing data analyses (cloud.majorbio.com). Using the Usearch11-uparse algorithm for clustering, sequences were categorized into operational taxonomic units (OTUs) at a 97 % similarity threshold (http://www.drive5.com/uparse/). OTUs were identified by their frequency, with singletons being excluded from the analysis. The nifH OTUs were annotated based on a 70 % cutoff value, utilizing references from the ARB database (http://wwwzehr.pmc.ucsc.edu/nifH_Database_Public/).

### *2.6. Statistical analysis*

Data analysis was conducted using SPSS software, version 20.0. (IBM Ltd., Chicago, IL, USA). The difference in soil nutrient content, enzyme activity, and alpha-diversity were tested employing one-way analysis of variance (ANOVA). The 0.05 probability level was adopted to compare the significance of differences through Duncan’s Multiple Range Test. The associations between soil variables and the relative abundance of genera were examined using R with Spearman correlation coefficients. Principal coordinate analysis (PCoA) was conducted using Bray-Curtis distance matrices, while hierarchical clustering was conducted by employing the weighted unifrac distance. The correlation between the activities of soil enzymes and soil variables was tested by a redundancy analysis (RDA). The “vegan” and “GUniFrac” packages in R were used to perform PCoA and RDA.

## 3. Results

### *3.1. Effect of annual P fertilizer split application on CMV and rice yields*

CMV yield increased in 2018 and 2019 along with a rise in the ratio of P fertilizer applied in the CMV season (Table 1). In 2020, the CMV yield did not significantly differ among the P1, P2 and P3 treatments, but those yields increased by 105 %, 97 % and 64 %, respectively, in comparison to the P0 treatment in the same year. P fertilizer split application stimulated grain yield for the succeeding rice compared with P0 treatment except 2020 season. When compared to P0 treatment, rice yields of the P1, P2, and P3 treatments increased by 7%–9%, 17%–25 %, and 10%–12 % in 2018, 2019, and 2021, respectively. However, in the same year, there was no

**Table 3**
Effect of phosphorus (P) fertilizer split application on soil nutrient content and extracellular enzyme activity.

| Treatment | CK | P0 | P1 | P2 | P3 |
|---|---|---|---|---|---|
| $NH_4$-N (mg/kg) | 1.34 ± 0.18b | 4.12 ± 0.98a | 4.90 ± 0.27a | 3.94 ± 0.10a | 4.47 ± 1.12a |
| $NO_3$-N (mg/kg) | 1.63 ± 0.44c | 6.36 ± 0.29b | 10.65 ± 2.15a | 9.22 ± 1.58a | 7.28 ± 0.85b |
| TN (g/kg) | 0.72 ± 0.01c | 0.82 ± 0.01b | 1.11 ± 0.11a | 1.22 ± 0.03a | 1.10 ± 0.11a |
| MBN (mg/kg ) | 17.38 ± 2.70c | 22.92 ± 2.36b | 34.91 ± 6.90b | 43.83 ± 1.84a | 48.34 ± 2.28a |
| OM (g/kg) | 14.44 ± 1.46b | 14.20 ± 0.04b | 20.37 ± 1.13a | 21.01 ± 0.88a | 22.05 ± 1.61a |
| AP (mg/kg) | 8.13 ± 1.26b | 10.63 ± 0.58b | 23.29 ± 0.89a | 22.29 ± 0.77a | 24.87 ± 0.36a |
| C/N | 11.56 ± 1.14a | 10.09 ± 0.09a | 10.95 ± 1.11a | 9.99 ± 0.63a | 12.24 ± 1.87a |
| ALP (U/g) | 3.06 ± 0.36a | 2.66 ± 0.28 ab | 1.85 ± 0.25c | 2.10 ± 0.17c | 2.21 ± 0.10bc |
| LAP (U/g) | 2.50 ± 0.34 ab | 1.91 ± 0.31bc | 1.61 ± 0.17c | 2.01 ± 0.26bc | 3.05 ± 0.16a |
| BG + CBH (U/g) | 0.54 ± 0.06a | 0.61 ± 0.02a | 0.62 ± 0.02a | 0.62 ± 0.04a | 0.69 ± 0.02a |
| vector length | 0.30 ± 0.04c | 0.45 ± 0.06 ab | 0.58 ± 0.06a | 0.47 ± 0.05a | 0.39 ± 0.02b |
| vector angle | 51.31 ± 4.14a | 55.02 ± 3.09a | 48.06 ± 4.92a | 47.09 ± 4.66a | 36.08 ± 1.74b |

Note: OM, TN, $NH_4^+$, $NO_3^-$, MBN, AP, ALP, LAP, and BG + CBH represent organic matter, total nitrogen, ammonium nitrogen, nitrate nitrogen, microbial biomass nitrogen, available phosphorus, alkaline phosphatase, L-leucine aminopeptidase, and β-1,4-glucosidase+ β-D-cellobiosidase, respectively. The values in the table are "mean ± standard error". Different lowercase letters indicate significant differences between treatments ($P <$ 0.05).

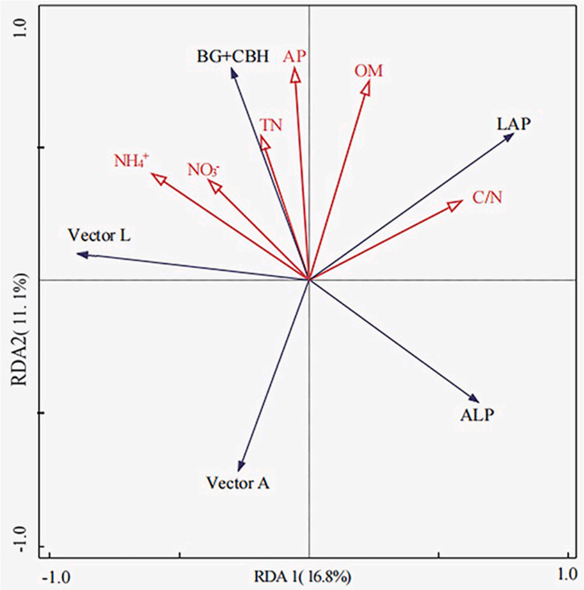

**Fig. 1.** Redundancy analysis of extracellular enzyme activity and soil nutrient content as affected by phosphorus (P) fertilizer split application. The positions and lengths of the arrows indicate the directions and strengths, respectively, of the effects of variables on the diazotrophic communities. The black arrows represent soil enzyme activities and the red arrows represent soil nutrient content. The samples were analyzed in triplicate plots.

discernible change in rice production among P1, P2, and P3 treatments. Therefore, partial or full dose of P fertilizer application in CMV season was the direct factor influencing soil nutrients contents that were essential for maintaining CMV and rice yields. The P2 treatment significantly increased the accumulations of N and P in CMV comparing with other treatments (Table 2). There were no significant differences in the rice P uptakes among P fertilizer applied treatments, which meant different P application methods did not change the P absorption by rice crops.

### *3.2. Soil nutrient content and extracellular enzyme activity at CMV full blooming stage*

The P2 treatment significantly increased soil $NO_3^-$-N, TN, MBN, OM, and AP contents by 45 %, 49 %, 91 %, 48 %, and 110 %, respectively, comparing with those in P0 treatment (Table 3). Soil ALP activities were considerably lower in the P1 and P2 treatments than those in the CK and P0 treatments. Furthermore, the LAP activity of P3 treatment was significantly higher than those of P0, P1, and P2 treatments. The activities of C acquisition enzymes (BG + CBH) were not significantly different for all the treatments.

Through the calculations of vector length and angle, microbial relative C and P limitations were evaluated. Vector length and angle varied significantly based on the amount of P applied in the CMV season. Both the vector length and angle of the P1 and P2 treatments were substantially larger than that of P3 treatment (Table 3). In addition, RDA revealed that soil AP and $NH_4^+$-N contents exerted

**Table 4**
Effect of phosphorus (P) fertilizer split application on diazotroph alpha-diversity in a Chinese milk vetch-rice rotation system.

| Treatment | Observed species | Shannon index | Simpson index | ACE value | Chao1 value |
|---|---|---|---|---|---|
| CK | 1926 ± 23.4a | 6.18 ± 0.03a | 0.0066 ± 0.0011a | 3167 ± 96a | 2782 ± 59 ab |
| P0 | 1943 ± 45.7a | 6.17 ± 0.08a | 0.0059 ± 0.0009a | 2808 ± 67b | 2747 ± 73 ab |
| P1 | 1893 ± 31.4a | 6.24 ± 0.04a | 0.0058 ± 0.0010a | 2828 ± 56b | 2698 ± 33b |
| P2 | 1941 ± 43.7a | 6.19 ± 0.08a | 0.0058 ± 0.0007a | 2904 ± 58 ab | 2904 ± 37a |
| P3 | 1810 ± 41.3a | 6.02 ± 0.09a | 0.0068 ± 0.0004a | 2917 ± 117 ab | 2632 ± 42b |

Note: Values indicate mean ± standard error (n = 3). Different letters in the columns represent significant differences between treatments ($P < 0.05$).

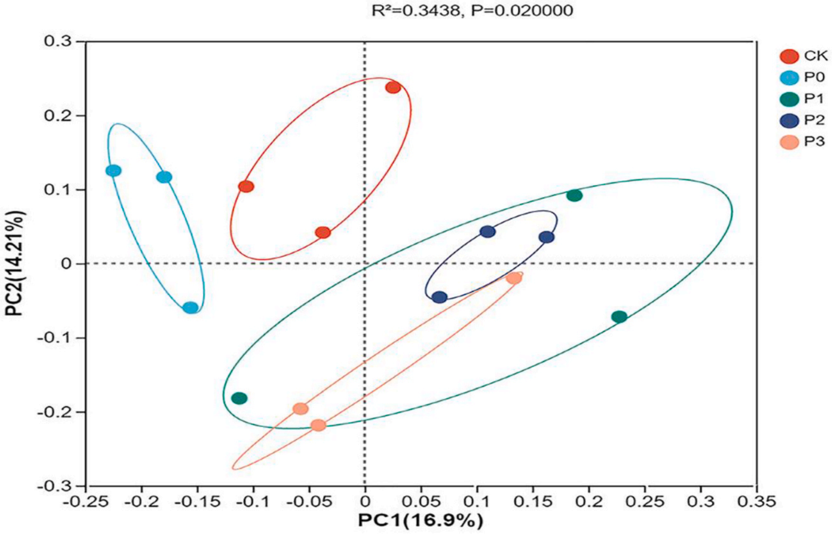

**Fig. 2.** Principal Coordinates Analysis of diazotrophic community structure as affected by phosphorus (P) fertilizer split application. Bray-curtis was used for this analysis and different group elliptical indicate a significant difference between treatments. R2 indicates the degree of correlation between the projected values of the sample points on the principal components and the raw data ($p < 0.05$). The samples were analyzed in triplicate plots.

considerable influence on soil enzyme activities and vector length and angle (Fig. 1). AP and $NH_4^+$-N had significantly positive correlations with vector length and C acquisition enzyme activity.

### 3.3. Diazotroph community structure at CMV full blooming stage

The high-throughput sequencing of the 16S rRNA gene was used to evaluate the diversity of the microbial population. The total number of qualified reads was 963,653 after deleting the ambiguous bases and chimera, cutting the barcodes and primers, and filtering the low-quality reads. In order to execute downstream studies at the same sequencing depth, each sample was standardized to 32,122 reads. P fertilizer split application has a major impact on the alpha-diversity of diazotrophic communities, according to 97 % Operational Taxonomic Unit (OTU) similarity (Table 4). Compared with the P0 and P1 treatments, P2 treatment had a higher OTU richness (Chao1 indices), which indicated that diazotrophic abundance was reduced in the P1 and P3 treatments. In contrast, soil diazotrophic diversities (Shannon and Simpson indices) were not significantly different among treatments. Principal Coordinates Analysis (PCoA), which relies on the Bray-Curtis distance of OTU composition, was employed for community composition discrimination (Fig. 2). Diazotrophic community structures in the P2 and P3 treatments were consistent with those in P1 treatment, but the community composition in the P0 treatment was significantly different from that in P1 treatment.

All the samples' OTUs were taxonomically divided into 11 separate genera, which together accounted for almost 90 % of the total sequences and the remaining 10 % being unclassified genera. The majority of the *nifH* gene sequences in the soil belonged to the genus *Anaeromyxobacter*, which accounted for 20.3%–24.1 % of all the treatments (Fig. 3). The P2 and P3 treatments enhanced the relative abundance of *Frankia* by 2.6 % and 3.6 %, comparing with P0 treatment. *Azotobacter* abundance was lower in the P1, P2 and P3 treatments than that in the P0 treatment; however, in comparison to the P0 treatment, the relative abundance was 9.8 % lower in the P1 treatment. In addition, compared to the P0 treatment, the relative abundance of the *Skermanella* genus was considerably increased by 1.6 % in the P2 treatment. The relative abundance of *Mesorhizobium* was greater in the P1, P2 and P3 treatments than that in the P0 treatment, while *Bradyrhizobium* had the highest relative abundance (5.1 %) in the P0 treatment. The findings of the Spearman analysis revealed a substantial correlation between the majority of the abundant diazotrophic genera and soil nutrients (Fig. 4). Most soil nutrients contents showed a substantial positive correlation with *Mesorhizobium* and *Skermanella*, including TN, AP, MBN, OM, and

$NH_4^+$-N. The genus *Frankia* had significant positive correlations with TN and vector length. In addition, *Bradyrhizobium* and *Azotobacter* abundance exhibited significant and positive correlations with vector angle. Therefore, the increased relative abundance of diazotrophic genus exhibited significantly correlation with soil N, OM, and AP contents.

## 4. Discussion

### 4.1. Effect of P fertilizer split application on crop yield

Considering soil quality changes gradually, soil microbial community structure stabilizes over time [39]. Therefore, the responses of the diazotrophic community in agroecosystems to long-term fertilizer application must be measured under stable crop yield over many years. Numerous studies indicate that efficient BNF depends on a sufficient availability of other nutrients, especially P [40]. It is widely accepted that P application meets legume requirements and enhances nodulation and N-fixation capacity, in turn enhancing N nutrition [41]. Furthermore, although we observed that P utilization was low in the current season, P fixed in the soil would be available for use by subsequent crops [42]. In the present study, the improvement of symbiotic N fixation by P application in CMV can increase soil AP and N contents simultaneously. According to the results, the annual P fertilizer split application increased the yields of both CMV and rice, providing a theoretical basis for the annual regulation of P fertilizer in the mid-season rice area of the Jianghan Plain (Table 1). In addition to yield increase, CMV incorporation is more likely to provide a higher N and P return, suggesting a possibility for replacing more N fertilizer during rice seasons.

### 4.2. Effect of P fertilizer split application on soil nutrient content and soil enzyme activity

The present study showed that long-term cover crop cultivation and P fertilizer application could increase soil OM content when compared with that in the P0 treatment (Table 3), because a high yield of cover crops returned could facilitate soil C sequestration. And OM is a key indicator of soil quality, and an increase in OM content improves soil fertility [43]. Soil N is also critical for plant growth and development and growth of soil microorganisms [44]. TN, $NH_4^+$-N, $NO_3^-$-N, and MBN were significantly higher in the P2 and P3 treatments than in the P0 treatment (Table 3), mainly because P split application increased the N fixation capacity of CMV root nodules and potentially increased soil microbial N-fixation capacity. In addition, P application increased soil AP content; however, there were no significant differences in AP content among the P1, P2 and P3 treatments (Table 3). Such results indicate that when the P fertilizers were absorbed by crops with AP, the excess AP was fixed in the soil or utilized by microbes. Therefore, increasing soil OM, N, and AP contents not only support the growth and development, but also regulate soil microbial activity [44]. Soil nutrients can indirectly act on soil enzymes by influencing microbial growth, resulting in varying degrees of correlation between soil nutrient content and soil enzyme activity [45]. In the present study, P fertilizer application and soil nutrient influenced soil enzyme activity. The results showed that P fertilizer split application reduced soil ALP activity and the P1 and P2 treatments resulted in the lower activities (Table 3). Moreover, soil LAP activity was affected significantly by P application, with the lowest soil LAP activity in the P1 treatment and the most elevated in the P3 treatment (Table 3). The findings align with earlier research that the P application increased the AP content so that the microbes reduced organic P mineralization, but the higher P input could potentially increase organic N mineralization [46].

Split application of P fertilizer further reduced the C and P limitations by soil microbes because of the insufficient organic C and low AP in the basic soil , however, full dose of P application in CMV season increased the N limitation (Table 3). In contrast, partial P application and CMV incorporation replenished the soil C and P pools. This is inconsistent with the findings of most studies where fertilizer application reduced soil microbial activity and affected soil nutrient cycling [47]. Conversely, it is consistent with the results that organic fertilizer and P fertilizer application increased the activity of diazotroph and provided adequate soil nutrients [48]. In addition, according to the RDA results (Fig. 1), soil AP and OM had the most significant effects on soil enzyme activity, and the higher the AP and OM contents, the lower the ALP activity. In contrast, the relationships between AP and LAP activity were opposite with those between AP and ALP. The greatest impact on soil enzyme activity was caused by AP and there was a substantial negative relationship between AP and vector angle (Fig. 1). Based on the above results, the soil enzyme stoichiometric ratios observed in the present study could be used as indicators of the nutrients limiting soil microbial activity. Whereas the ratios among C, N, P and other elements required for the growth and physiological, It plays a crucial role in shaping the composition of soil microbial community [49]. As a soil beneficial bacteria, the community changes of diazotrophs are highly responsive to the balance of soil nutrients [48]. However, soil enzyme activities and stoichiometric ratios in response to diazotrophic microbial activity and the major underlying factors require further studies.

### 4.3. Effect of P fertilizer split application on the soil diazotrophic community structure and dominant genera

Numerous studies have reported that the composition of the soil diazotrophic community could be easily impacted by long-term fertilizer application [50]. In the research conducted here, P1, P2 and P3 treatments did not alter soil diazotrophic diversity but reduced diazotrophic richness (Table 4). Additional studies have similarly shown that P and N are essential factors affecting diazotrophic communities, with P being one of the critical factors limiting BNF [51]. The results of the present study also showed that P fertilizer addition enhanced soil N content, which often led to a decrease in soil diazotrophic abundance [21]. In addition, this study also showed that P application resulted in significant differences in diazotrophic community composition, but not among the P1 and P2 treatments (Fig. 2). Similarly, other studies have reported that N-related soil properties (including $NO_3^-$-N, $NH_4^+$-N, and TN) are the major factors influencing diazotrophic community structure [51]. Therefore, it is consistent with the results of previous studies that

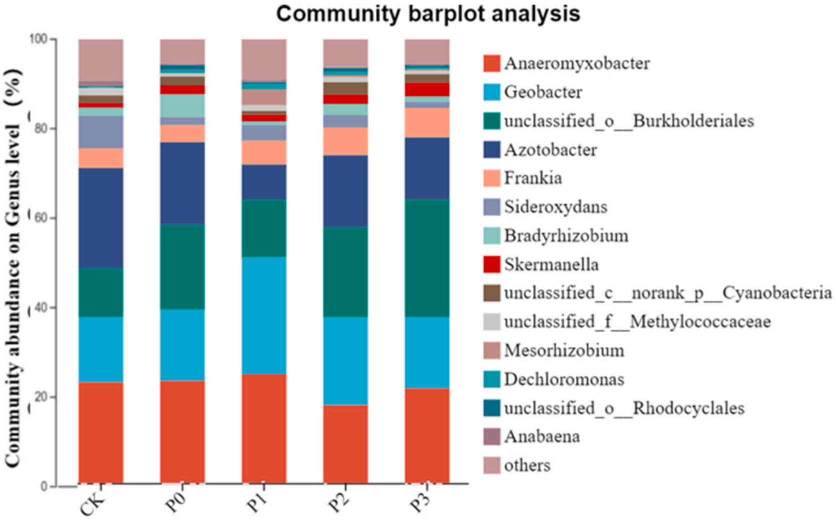

**Fig. 3.** Relative abundance of the 11 most abundant genera (>1 %) as affected by phosphorus (P) fertilizer split application. The samples were analyzed in triplicate plots and the average of triplicates is displayed for each treatment in this figure.

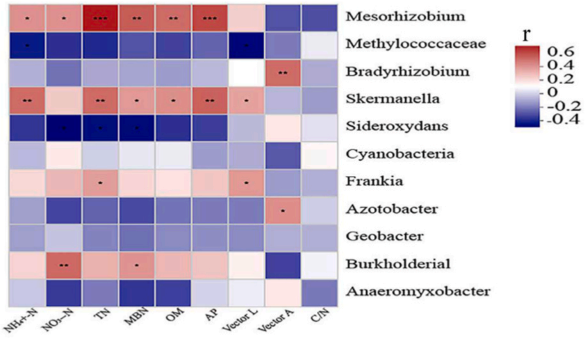

**Fig. 4.** Spearman correlation analysis between the relative abundance of dominant diazotroph genera and soil nutrient content. r indicates the correlation coefficient; *** Significant at the 0.001 level ($P < 0.001$), ** Significant at the 0.01 level ($P < 0.01$), * Significant at the 0.05 level ($P < 0.05$). Abbreviations: $NH_4^+$, $NO_3^-$, TN, MBN, OM, AP, Vector L, Vector A, and C/N represent ammonium nitrogen, nitrate nitrogen, total nitrogen, microbial biomass nitrogen, organic matter, available phosphorus, vector length, vector angle, and carbon to nitrogen ratio, respectively.

soil nutrient content influences diazotrophic community structure.

Although diazotrophic taxa under long-term fertilizer application have been studied extensively, the responses of specific diazotrophic genera to long-term organic and inorganic fertilizer application vary widely [52]. Soil microbial community assembly is a complex process influenced by multiple interacting variables [53]. Analysis of the genus level abundance of diazotrophs indicated that P1 and P3 treatments reduced the relative abundance of autotrophic N-fixing bacteria (*Azotobacter*) and symbiotic N-fixing bacteria (*Bradyrhizobium*) compared with other treatments (Fig. 3). The relative abundance of symbiotic N-fixing bacteria (*Frankia* and *Mesorhizobium*) increased with P2 treatment. Since different diazotrophic genera are distinctly adapted to substantial AP content, P application influences their competitiveness. Therefore, the P application in the current study changed the proportion of dominant diazotrophic groups and reshaped soil diazotrophic genera structure.

Heatmap analysis of the correlation between diazotrophic genera and soil nutrient content showed that soil TN, $NH_4^+$-N, $NO_3^-$-N, AP, and OM were significantly and positively correlated with the genera *Skermanella* and *Mesorhizobium* (Fig. 4). Moreover, the genus *Frankia* was also positively correlated with TN, and the genera *Azotobacter* and *Bradyrhizobium* exhibited significant positive correlations with vector angles, with larger vector angles associated with greater P soil nutrient constraints (Fig. 4). The findings showed that the treatments P1, P2, and P3 enhanced the relative abundance of *Skermanella* and *Mesorhizobium* within the diazotrophic community while also increasing the N content in the soil, in particular, the soil with P2 treatment had the highest N nutrient content. However, previous study showed N application decreased diazotrophic genera relative abundance under high soil N content [54], thus, different treatment could elicit different diversification for the diazotrophic genera and such responses directly influence diazotrophic community structure. Therefore, studying the covariation among crop yield, soil nutrient content, and soil microbial properties can enhance our understanding of plant-soil-microbe responses under different fertilizer application practices.

## 5. Conclusion

The present study has demonstrated that P fertilizer application lead to significant increase in soil AP, thus affecting soil enzyme activity and reducing the P limitation to soil microorganisms during the CMV season. Also, The application of P reduced the relative abundance of self-sufficient diazotrophs while enhancing the presence of symbiotic diazotroph to the diazotrophic community structure, which is conducive to the fixation of soil N. Therefore, the split application of P in CMV-rice rotation increased the soil TN, $NH_4^+$-N, $NO_3^-$-N and MBN contents, which were closely associated with increased N and P uptakes by CMV and rice yield. Overall, appropriate P fertilizer application in CMV season can improve the community structure of soil diazotrophic community, increase the soil N content, and thus potentially reduce the input of N chemical fertilizer in rice season.

## CRediT authorship contribution statement

**Qiaoli Leng:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Yanan Sun:** Visualization, Investigation. **Jianan Cao:** Investigation. **Donghui Li:** Investigation. **Mingjian Geng:** Resources, Funding acquisition, Conceptualization. **Zaihua Guo:** Conceptualization. **Weidong Cao:** Resources, Funding acquisition. **Qiang Zhu:** Writing – review & editing, Supervision, Methodology.

## Ethics statement

We ensure that we have written entirely original works, and the work and/or words of others have been appropriately cited or quoted and permission has been obtained where necessary. We did not publish or submit manuscripts describing essentially the same research in more than one journal of primary publication. The corresponding author ensure that all appropriate co-authors and no inappropriate co-authors are included on the paper, and that all co-authors have seen and approved the final version of the paper and have agreed to its submission for publication.

## Data availability statement

Supplementary data have been deposited at National Center for Biotechnology Information with accession numbers https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1025149.

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## 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.

## References

[1] J.A. Bhat, M.C. Kundu, G.C. Hazra, G.H. Santra, B. Mandal, Rehabilitating acid soils for increasing crop productivity through low-cost liming material, Sci. Total Environ. 408 (20) (2010) 8, https://doi.org/10.1016/j.scitotenv.2010.07.011.

[2] A. Chen, W. Zhang, R. Sheng, Y. Liu, H. Hou, F. Liu, G. Ma, W. Wei, H. Qin, Long-term partial replacement of mineral fertilizer with in situ crop residues ensures continued rice yields and soil fertility: a case study of a 27-year field experiment in subtropical China, Sci. Total Environ. 787 (2021) 9, https://doi.org/10.1016/j.scitotenv.2021.147523.

[3] K. Ren, M. Xu, R. Li, L. Zheng, H. Wang, S. Liu, W. Zhang, Y. Duan, C. Lu, Achieving high yield and nitrogen agronomic efficiency by coupling wheat varieties with soil fertility, Sci. Total Environ. 881 (2023) 10, https://doi.org/10.1016/j.scitotenv.2023.163531.

[4] M. Chen, J. Xu, Z. Li, D. Li, Q. Wang, Y. Zhou, W. Guo, D. Ma, J. Zhang, B. Zhao, Long-term nitrogen fertilization-induced enhancements of acid hydrolyzable nitrogen are mainly regulated by the most vital microbial taxa of keystone species and enzyme activities, Sci. Total Environ. 874 (2023) 13, https://doi.org/10.1016/j.scitotenv.2023.162463.

[5] X. Zhao, Y. Zhou, J. Min, S. Wang, W. Shi, G. Xing, Nitrogen runoff dominates water nitrogen pollution from rice-wheat rotation in the taihu lake region of China, Agric. Ecosyst. Environ. 156 (2012) 11, https://doi.org/10.1016/j.agee.2012.04.024.

[6] K. Tian, Z. Xing, Y.K. Kalkhajeh, T. Zhao, W. Hu, B. Huang, Y. Zhao, Excessive phosphorus inputs dominate soil legacy phosphorus accumulation and its potential loss under intensive greenhouse vegetable production system, J. Environ. Manag. 303 (2022) 10, https://doi.org/10.1016/j.jenvman.2021.114149.

[7] H. Liang, S. Li, L. Zhang, C. Xu, Y. Lv, S. Gao, W. Cao, Long-term green manuring enhances crop n uptake and reduces n losses in rice production system, Soil Till. Res. 220 (2022) 10, https://doi.org/10.1016/j.still.2022.105369.

[8] J.K. Ladha, M.B. Peoples, P.M. Reddy, J.C. Biswas, A. Bennett, M.L. Jat, T.J. Krupnik, Biological nitrogen fixation and prospects for ecological intensification in cereal-based cropping systems, Field Crops Res. 283 (2022) 34, https://doi.org/10.1016/j.fcr.2022.108541.

[9] D. Xiao, X. Liu, R. Yang, Y. Tan, W. Zhang, X. He, Z. Xu, K. Wang, Nitrogen fertilizer and amorpha fruticosa leguminous shrub diversely affect the diazotroph communities in an artificial forage grassland, Sci. Total Environ. 711 (2020) 10, https://doi.org/10.1016/j.scitotenv.2019.134967.

[10] H. Chen, C. Zheng, Y. Qiao, S. Du, W. Li, X. Zhang, Z. Zhao, C. Cao, W. Zhang, Long-term organic and inorganic fertilization alters the diazotrophic abundance, community structure, and co-occurrence patterns in a vertisol, Sci. Total Environ. 766 (2021) 9, https://doi.org/10.1016/j.scitotenv.2020.142441.

[11] X. Liu, C. Liu, W. Gao, C. Xue, Z. Guo, L. Jiang, F. Li, Y. Liu, Impact of biochar amendment on the abundance and structure of diazotrophic community in an alkaline soil, Sci. Total Environ. 688 (2019) 8, https://doi.org/10.1016/j.scitotenv.2019.06.293.

[12] Y. Guo, S. Cheng, H. Fang, Y. Yang, Y. Li, F. Shi, Y. Zhou, Copper and cadmium co-contamination affects soil bacterial taxonomic and functional attributes in paddy soils, Environ. Pollut. 329 (2023) 12, https://doi.org/10.1016/j.envpol.2023.121724.

[13] H. Yu, X. Liu, C. Yang, Y. Peng, X. Yu, H. Gu, X. Zheng, C. Wang, F. Xiao, L. Shu, Z. He, B. Wu, Q. Yan, Co-symbiosis of arbuscular mycorrhizal fungi (AMF) and diazotrophs promote biological nitrogen fixation in mangrove ecosystems, Soil Biol. Biochem. 161 (2021) 13, https://doi.org/10.1016/j.soilbio.2021.108382.

[14] W. Song, J. Wang, L. Hou, Effects of frequency and amount of stover mulching on soil nitrogen and the microbial functional guilds of the endosphere and rhizosphere, Front. Microbiol. 13 (2022) 9, https://doi.org/10.3389/fmicb.2022.976154.

[15] J. Zhou, M. Ma, D. Guan, X. Jiang, N. Zhang, F. Shu, Y. Kong, J. Li, Nitrogen has a greater influence than phosphorus on the diazotrophic community in two successive crop seasons in northeast China, Sci Rep-Uk 11 (1) (2021) 11, https://doi.org/10.1038/s41598-021-85829-8.

[16] H. Zhu, Y. Teng, X. Wang, L. Zhao, W. Ren, Y. Luo, P. Christie, Changes in clover rhizosphere microbial community and diazotrophs in mercury-contaminated soils, Sci. Total Environ. 767 (2021) 10, https://doi.org/10.1016/j.scitotenv.2021.145473.

[17] S. Li, C. Peng, T. Cheng, C. Wang, L. Guo, D. Li, Nitrogen-cycling microbial community functional potential and enzyme activities in cultured biofilms with response to inorganic nitrogen availability, J Environ. Sci. China 76 (2019) 11, https://doi.org/10.1016/j.jes.2018.03.029.

[18] V.V.S.R. Gupta, B. Zhang, C.R. Penton, J. Yu, J.M. Tiedje, Diazotroph diversity and nitrogen fixation in summer active perennial grasses in a mediterranean region agricultural soil, Front. Mol. Biosci. 6 (2019) 20, https://doi.org/10.3389/fmolb.2019.00115.

[19] Z. Ma, T. Liang, H. Fu, Q. Ma, D. Chang, J. Zhang, Z. Che, G. Zhou, W. Cao, Long-term green manuring increases soil carbon sequestration via decreasing $qCO_2$ caused by lower microbial phosphorus limitation in a dry land field, Agric. Ecosyst. Environ. 374 (2024) 109142, https://doi.org/10.1016/j.agee.2024.109142.

[20] R. Che, Y. Deng, F. Wang, W. Wang, Z. Xu, Y. Hao, K. Xue, B. Zhang, Li Tang, H. Zhou, X. Cui, Autotrophic and symbiotic diazotrophs dominate nitrogen-fixing communities in Tibetan grassland. soils, Sci. Total Environ. 639 (2018) 997–1006, https://doi.org/10.1016/j.scitotenv.2018.05.238.

[21] X. Wang, Y. Li, L. Wang, Y. Duan, B. Yao, Y. Chen, W. Cao, Soil extracellular enzyme stoichiometry reflects microbial metabolic limitations in different desert types of northwestern China, Sci. Total Environ. 874 (2023) 11, https://doi.org/10.1016/j.scitotenv.2023.162504.

[22] W. Xue, W. Zhang, Y. Chen, Heavy thinning temporally reduced soil carbon storage by intensifying soilmicrobial phosphorus limitation, Plant Soil 484 (1–2) (2023) 16, https://doi.org/10.1007/s11104-022-05782-x.

[23] C. Duan, Y. Wang, Q. Wang, W. Ju, Z. Zhang, Y. Cui, J. Beiyuan, Q. Fan, S. Wei, S. Li, L. Fang, Microbial metabolic limitation of rhizosphere under heavy metal stress: evidence from soil ecoenzymatic stoichiometry, Environ. Pollut. 300 (2022) 11, https://doi.org/10.1016/j.envpol.2022.118978.

[24] R.L. Sinsabaugh, B.H. Hill, J.J.F. Shah, Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment, Nature 462 (7274) (2009) 5, https://doi.org/10.1038/nature08632.

[25] M. Mooshammer, W. Wanek, S. Zechmeister-Boltenstern, A. Richter, Stoichiometric imbalances between terrestrial decomposer communities and their resources: mechanisms and implications of microbial adaptations to their resources, Front. Microbiol. 5 (2014) 10, https://doi.org/10.3389/fmicb.2014.00022.

[26] D.L. Moorhead, R.L. Sinsabaugh, B.H. Hill, M.N. Weintraub, Vector analysis of ecoenzyme activities reveal constraints on coupled c, n and p dynamics, Soil Biol. Biochem. 93 (2016) 7, https://doi.org/10.1016/j.soilbio.2015.10.019.

[27] X. Bai, M.A. Dippold, S. An, B. Wang, H. Zhang, S. Loeppmann, Extracellular enzymeactivity and stoichiometry: the effect of soil microbial element limitation during leaf litter decomposition, Ecol. Indicat. 121 (2021) 13, https://doi.org/10.1016/j.ecolind.2020.107200.

[28] H. Xu, Q. Qu, G. Li, G. Liu, V. Geissen, C.J. Ritsema, S. Xue, Impact of nitrogen addition on plant-soil-enzyme C-N-P stoichiometry and microbial nutrient limitation, Soil Biol. Biochem. 170 (2022) 9, https://doi.org/10.1016/j.soilbio.2022.108714.

[29] X. Meng, Y. Li, Y. Zhang, H. Yao, Green manure application improves rice growth and urea nitrogen use efficiency assessed using N-15 labeling, Soil Sci. Plant Nutr. 65 (5) (2019) 8, https://doi.org/10.1080/00380768.2019.1635872.

[30] S.D. Bao, Soil and Agricultural Chemistry Analysis, China Agricultural Press, Beijing, China, 2008.

[31] J. Murphy, J.P. Riley, A modified single solution method for the determination of phosphate in natural waters, Anal. Chim. Acta (1962), https://doi.org/10.1016/S0003-2670(00)88444-5.

[32] J.C. Yeomans, J.M. Bremner, A rapid and precise method for routine determination of organic-carbon in soil, Commun Soil Sci. Plan. 19 (13) (1988) 10, https://doi.org/10.1080/00103628809368027.

[33] P.C. Brookes, J.F. Kragt, D.S. Powlson, D.S. Jenkinson, Chloroform fumigation and the release of soil-nitrogen the effects of fumigation time and temperature, Soil Biol. Biochem. 17 (6) (1985) 5, https://doi.org/10.1016/0038-0717(85)90143-9.

[34] C. Roesch, A. Mergel, H. Bothe, Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil, Appl. Environ. Microbiol. 68 (8) (2002) 3818–3829, https://doi.org/10.1128/AEM.68.8.3818-3829.2002.

[35] T. Magoc, S.L. Salzberg, Flash: fast length adjustment of short reads to improve genome assemblies, Bioinformatics 27 (21) (2011) 2957–2963, https://doi.org/10.1093/bioinformatics/btr507.

[36] S. Chen, Y. Zhou, Y. Chen, J. Gu, Fastp: an ultra-fast all-in-one fastq preprocessor, Bioinformatics 34 (17) (2018) 884–890, https://doi.org/10.1093/bioinformatics/bty560.

[37] B.J. Callahan, P.J. Mcmurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, Dada2: high-resolution sample inference from illumina amplicon data, Nat. Methods 13 (7) (2016) 581, https://doi.org/10.1038/NMETH.3869.

[38] E. Bolyen, J.R. Rideout, M.R. Dillon, N.A. Bokulich, C.C. Abnet, G.A. Al-Ghalith, H. Alexander, E.J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J.E. Bisanz, K. Bittinger, A. Brejnrod, C.J. Brislawn, C.T. Brown, B.J. Callahan, A.M. Caraballo-Rodriguez, J. Chase, E.K. Cope, R.D. Silva, C. Diener, P.C. Dorrestein, G. M. Douglas, D.M. Durall, C. Duvallet, C.F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J.M. Gauglitz, S.M. Gibbons, D.L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G.A. Huttley, S. Janssen, A.K. Jarmusch, L. Jiang, B.D. Kaehler, K.B. Kang, C.R. Keefe, P. Keim, S.T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M.G.I. Langille, J. Lee, R. Ley, Y. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B.D. Martin, D. Mcdonald, L. J. Mciver, A.V. Melnik, J.L. Metcalf, S.C. Morgan, J.T. Morton, A.T. Naimey, J.A. Navas-Molina, L.F. Nothias, S.B. Orchanian, T. Pearson, S.L. Peoples, D. Petras, M.L. Preuss, E. Pruesse, L.B. Rasmussen, A. Rivers, M.S. Robeson, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S.J. Song, J.R. Spear, A.D. Swafford, L. R. Thompson, P.J. Torres, P. Trinh, A. Tripathi, P.J. Turnbaugh, S. Ul-Hasan, J.J.J. van der Hooft, F. Vargas, Y. Vazquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, W. Walters, Y. Wan, M. Wang, J. Warren, K.C. Weber, C.H.D. Williamson, A.D. Willis, Z.Z. Xu, J.R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, J. G. Caporaso, Reproducible, interactive, scalable and extensible microbiome data science using qiime 2, Nat. Biotechnol. 37 (9) (2019) 1091, https://doi.org/10.1038/s41587-019-0252-6.

[39] C. Garcia-Delgado, V. Barba-Vicente, J.M. Marin-Benito, J.M. Igual, M.J. Sanchez-Martin, M.S. Rodriguez-Cruz, Influence of different agricultural management practices on soil microbial community over dissipation time of two herbicides, Sci. Total Environ. 646 (2019) 11, https://doi.org/10.1016/j.scitotenv.2018.07.395.

[40] H. Wang, Y. Qiu, K. Zhang, Y. Zhao, Y. Li, Y. Wang, Y. Bai, Y. Zhang, S. Hu, Alterations in substrate stoichiometry control the responses of soil diazotrophs to nutrient enrichment, Soil Biol. Biochem. 179 (2023) 10, https://doi.org/10.1016/j.soilbio.2023.108975.

[41] R.B. Kuang, H. Liao, X.L. Yan, Y.S. Dong, Phosphorus and nitrogen interactions in field-grown soybean as related to genetic attributes of root morphological and nodular traits, J. Integr. Plant Biol. 47 (5) (2005) 11, https://doi.org/10.1111/j.1744-7909.2005.00072.x.

[42] S. Li, Z. Wang, B.A. Stewart, Differences of some leguminous and non-leguminous crops in utilization of soil phosphorus and responses to phosphate fertilizers, Adv. Agron. 110 (110) (2011) 125, https://doi.org/10.1016/B978-0-12-385531-2.00003-7.

[43] Y.T. He, W.J. Zhang, M.G. Xu, X.G. Tong, F.X. Sun, J.Z. Wang, S.M. Huang, P. Zhu, X.H. He, Long-term combined chemical and manure fertilizations increase soil organic carbon and total nitrogen in aggregate fractions at three typical cropland soils in China, Sci. Total Environ. 532 (2015) 10, https://doi.org/10.1016/j.scitotenv.2015.06.011.

[44] Z. Zhou, M. Zheng, J. Xia, C. Wang, Nitrogen addition promotes soilmicrobial beta diversity and the stochastic assembly, Sci. Total Environ. 806 (2022) 9, https://doi.org/10.1016/j.scitotenv.2021.150569.

[45] T. Li, J. Gao, L. Bai, Y. Wang, J. Huang, M. Kumar, X. Zeng, Influence of green manure and rice straw management on soil organic carbon, enzyme activities, and rice yield in red paddy soil, Soil Till. Res. 195 (2019) 7, https://doi.org/10.1016/j.still.2019.104428.

[46] L. Luo, H. Meng, R. Wu, J. Gu, Impact of nitrogen pollution/deposition on extracellular enzymeactivity, microbial abundance and carbon storage in coastal mangrove sediment, Chemosphere 177 (2017) 9, https://doi.org/10.1016/j.chemosphere.2017.03.027.

[47] D. Song, J. Tang, X. Xi, S. Zhang, G. Liang, W. Zhou, X. Wang, Responses of soil nutrients and microbial activities to additions of maize straw biochar and chemical fertilization in a calcareous soil, Eur. J. Soil Biol. 84 (2018) 10, https://doi.org/10.1016/j.ejsobi.2017.11.003.

[48] L. Yang, J. Bai, N. Zeng, X. Zhou, Y. Liao, Y. Lu, R.M. Rees, J. Nie, W. Cao, Diazotroph abundance and community structure are reshaped by straw return and mineral fertilizer in rice-rice-green manure rotation, Appl. Soil Ecol. 136 (2019) 10, https://doi.org/10.1016/j.apsoil.2018.12.015.

[49] Z. Zhang, H. Li, J. Hu, X. Li, Q. He, G. Tian, H. Wang, S. Wang, B. Wang, Do microorganism stoichiometric alterations affect carbon sequestration in paddy soil subjected to phosphorus input? Ecol. Appl. 25 (3) (2015) 14, https://doi.org/10.1890/14-0189.1.

[50] M. Feng, J.M. Adams, K. Fan, Y. Shi, R. Sun, D. Wang, X. Guo, H. Chu, Long-term fertilization influences community assembly processes of soil diazotrophs, Soil Biol. Biochem. 126 (2018) 8, https://doi.org/10.1016/j.soilbio.2018.08.021.

[51] X. Dai, D. Song, Q. Guo, W. Zhou, G. Liu, R. Ma, G. Liang, P. He, G. Sun, F. Yuan, Z. Liu, Predicting the influence of fertilization regimes on potential N fixation through their effect on free-living diazotrophic community structure in double rice cropping systems, Soil Biol. Biochem. 156 (2021) 10. https://coi.org/10.1016/j.soilbio.2021.108220.

[52] C. Wang, M. Zheng, W. Song, S. Wen, B. Wang, C. Zhu, R. Shen, Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China, Soil Biol. Biochem. 113 (2017) 10, https://doi.org/10.1016/j.soilbio.2017.06.019.

[53] Y. Sun, Z. Liu, Y. Zhang, Z. Lai, W. She, Y. Bai, W. Feng, S. Qin, Microbial communities and their genetic repertoire mediate the decomposition of soil organic carbon pools in revegetation shrublands in a desert in northern China, Eur. J. Soil Sci. 71 (1) (2020) 13, https://doi.org/10.1111/ejss.12824.

[54] N.V.D.S. Ribeiro, M.S. Vidal, S.C.L. Barrios, V.L.D. Baldani, J.I. Baldani, Genetic diversity and growth promoting characteristics of diazotrophic bacteria isolated from 20 genotypes of brachiaria spp, Plant Soil 451 (1–2) (2020) 19, https://doi.org/10.1007/s11104-019-04263-y.