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Diazotroph abundance and community structure are reshaped by rice straw incorporation and inorganic fertilization in rice-rice-green manure rotation

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Abstract:

Soil microbial community can be radically changed by the addition of inorganic or organic nutrients; however, the response of the diazotroph population to nutrient and residue management in wetland rice rotations is poorly understood. Here, we investigated the diazotrophic community to inputs of a leguminous green manure Chinese milk vetch (Mv, *Astragalus sinicus*) using quantitative PCR and Illumina Miseq sequencing of the *nifH* gene. Five treatments were compared in a Milk vetch–early rice–late rice rotation: 1) Control, no fertilization and straw return (CK); 2) rice straw incorporation alone (Rs); 3) inorganic fertilizer application alone (F); 4) F plus Rs (FRs); 5) similar with FRs but high-stubble (~35-40 cm) of late rice straw was remained (FRh). The results showed that cultivation practice affected the magnitude of soil available nutrient pools (N, P and K), but not the soil organic matter and total N pools. The rice straw significantly repressed the *nifH* gene abundance compared to the control, and also increased the number of diazotrophic bacteria species, Chao 1 values, Shannon and Simpson indexes more than other fertilization treatments. Multivariate regression tree analysis revealed that the community diversity and structure of diazotrophs were primarily shaped by soil nitrate and available P status, as well as C/N ratio. The most abundant genus *Bradyrhizobium* (21%-32%) tended to decrease in rice straw soil in comparison with the control but was significantly enhanced in the FRs treatment at seedling stage and in FRh treatment at flowering stage. Spearman correlation analysis showed that the dominant diazotrophic genera were positively related with soil available phosphorus, but responded differentially to soil total N, nitrate, and pH between the seedling and flowering stages. Overall, the planting and incorporation of vetch under different practices of residue and fertilizer management reshaped the diazotropic community during the green manure

season, highlighting the crucial roles of soil C, N, and P status or their ratios in shaping the population and diversity of diazotrophs.

Key words: Chinese milk vetch; diazotrophic community; inorganic fertilizer; *nifH*, rice; straw return.

1. Introduction

The excessive application of inorganic fertilizer nitrogen (N) in agriculture has caused severe environmental issues (Le et al., 2010; Liu et al., 2013; Gao et al., 2018). A major challenge for the sustainable production of rice is to maintain grain yield but with lower environmental impacts (Chen et al., 2014). Substitution of the inorganic fertilizer by organic substrates, such as straw and green manure is considered a promising way of reducing the chemical fertilizer input (YadvinderSingh et al., 2004; Xie et al., 2016). Legumes are favored crops cultivated in the rice-green manure rotation systems because of their ability to fix N by biological fixation, and enhance N use efficiency and rice productivity (Gao et al., 2011; Xie et al., 2016). It is estimated that the annual global amount of biological N fixation is 40-70 Tg in agricultural systems (Galloway et al., 2008; Herridge et al., 2008). Soil microbes are the key players involved in these processes, and have been well investigated during rice growth (Wang et al., 2017; Zhang et al., 2017), but little is known about their response to residue additions in green manure season.

The diazotrophs are the major microbial group involved in biological N fixation in rice paddies, and are highly sensitive to environmental conditions (Jangid et al., 2008), for instance, the changes of soil physicochemical properties and bio-environment induced by fertilization and straw incorporation (Bandyopadhyay et al., 2010; Wang et al., 2017).

However, there is considerable debate as to how the diazotrophs respond to varied fertilization practices. Some studies found that the long-term inorganic fertilization affected both diazotrophic abundance and community composition (Wang et al., 2017), while others showed marginal effects of fertilization on the abundance of *nifH*-containing microbes (Mårtensson et al., 2009). This suggests that the abundance and community structure of diazotrophs could be strongly but differentially affected by inorganic fertilization. The addition of organic substrates into soil generally has positive influences on diazotrophs. Since many of the microorganisms participating in N_2 fixation are heterotrophic or mixotrophic, the addition of external organic matter provides a source of energy and nutrients to support growth (Rahav et al., 2016). For example, the abundance of the *nifH* gene is enhanced by stubble retention or straw incorporation from rice plants (Wakelin et al., 2009; Tang et al., 2017). Moreover, different substrate quality can change soil physicochemical properties, such as pH, nutrient availability, and the quantity and quality of carbon, which would impose positive or negative influences on soil microbial populations (Geisseler and Scow, 2014; Levy-Booth et al., 2014). Besides the exogenous application of inorganic and organic substrates, plants could be another factor affecting the functions and activities of the soil microbiome, especially in the rhizosphere (Berendsen et al., 2012). The *nifH* gene abundance differed between soybean season and wheat season (Sun et al., 2015), implying that the microbial community may vary with the cultivated crops (Zhang et al., 2017). Thus, it is important to know how the diazotrophic community changes during the green manure season within rice-green manure rotations.

Chinese milk vetch (Mv) (*Astragalus sinicus* L.) is commonly grown within double-rice rotations in southern China, where the Mv is planted as winter green manure instead of a bare

fallow, and is then incorporated into soil together with rice straw or alone. The returned leguminous Mv residue is a rich source of N and could stimulate the utilization of rice straw by the microbial community. Previous studies have shown that the co-incorporation of Mv and rice straw could change microbial community composition and structure in a paddy soil (Xie et al., 2017; Gao et al., 2018) or benefit carbon sequestration and improvements in soil fertility (Poeplau, 2015). It is likely that microbial utilization of C and N within residues is influenced by the straw return strategy, which includes conventional return (straw crushed and directly incorporated into soil), straw mulching (straw cover on the field), or high stubble retention. In practice, straw management is currently changing from conventional return to high stubble retention because of the popularity of mechanized agricultural operations, which favor reduced tillage, and labor-saving approaches. To date, many studies have focused on the influences of green manure incorporation on rice productivity, and microbial or diazotrophic community changes during the rice growing seasons (Wang et al., 2017; Xie et al., 2017; Zhang et al., 2017), but little is known about changes in the green manure season, how the different straw return strategies and their combination with inorganic fertilizer N application affect the diazotrophic community composition and structure.

The study was carried out in a rice-rice-green manure rotation, in which the objectives were to investigate the influence of legumes, rice straw, and inorganic fertilization on microbial community composition and structure. Specifically, the two aims were, (i) to investigate whether different combinations of rice straw incorporation strategies and inorganic fertilizer application could shift the diazotrophic community composition and structure in the green manure season; (ii) to identify the key soil physicochemical factors shaping diazotroph diversity and community composition as affected by the diverse patterns

of straw incorporation and inorganic fertilization.

2. Materials and methods

2.1 Site description and experimental design

The field experiment was carried out from 2011 to 2017 at the Nan County, Hunan Province, China (29°13'N, 112°28'E) and was conducted on an Entisols, Fluvents, soil derived from lake sediment with a silt loam texture (USDA soil taxonomy). The basic physicochemical properties of 0-20 cm soil layer in 2011 were as follows: pH (H_2O) 7.70, soil organic matter 47.5 g kg^{-1}, total N 3.28 g kg^{-1}, total P 1.28 g kg^{-1}, total K 22.2 g kg^{-1}, alkali-hydrolyzale N 261.0 mg kg^{-1}, available phosphorus 15.6 mg kg^{-1} and available potassium 98.0 mg kg^{-1}.

The cropping system was early rice, followed by late rice, followed by a winter green manure within an annual rotation. The early rice (cv. Yuanzao 1) and late rice (cv. Huanghuazhan) were transplanted in mid-to-late April and mid-to-late July each year, respectively. The winter green manure Mv was planted at a seed rate of 30 kg ha^{-1} in early October each year and grew through the interval between the late and early rice. Five treatments were compared in the present study, and Mv crops were incorporated. Beyond that, each treatment included either an inorganic fertilizer application or rice straw incorporation, and could be summarized as follows: (i) CK, no fertilizer application and straw incorporation during the rice-rice-Mv rotation; (ii) Rs, rice straw incorporation alone (both the early and late rice straw were conventionally returned to the soil after harvest); (iii) F, inorganic fertilizer application alone (the rates of N, P, and K were 120, 26 and 60 kg ha^{-1} for early rice, and 144, 16 and 80 kg ha^{-1} for late rice, respectively); (iv) FRs, F plus Rs; (v) FRh, the early rice straw was conventionally returned to soil, while high stubble (about 35 cm)

from late rice straw was retained after harvest, and returned together with Mv incorporation before next early rice transplanting. Treatments were arranged in a randomized complete block design with three replicates. The plot size was 20 m^2 (4 m width by 5 m length).

In the treatments without rice straw incorporation, the above-ground parts of rice straw were removed from the plots. For the inorganic fertilization, half amount of the N (urea) and all P and K (superphosphate and potassium chloride, respectively) were applied as a base fertilizer to 5 cm soil depth, and the remaining N was top-dressed at the tillering stage of each rice season. The nutrients added by inorganic fertilizer or biological N fixation of Mv plants in each year are listed in Table A1. No fertilizer was applied during Mv's growth.

2.2 Soil sampling and analysis

Soil samples in the 0-20 cm layer were collected at the Mv seedling and flowering stages during the green manure season in 2017. Five randomized auger points in each plot were pooled and mixed thoroughly to provide one sample which was then divided into two sub-samples and transported to the laboratory on ice. One group was stored at -80℃ for DNA extraction, and the other one was used for soil chemical analysis. The soil ammonium (NH_4^+) and nitrate (NO_3^-) were extracted from fresh soils in 100 ml 0.01 M $CaCl_2$, and determined by continuous flow analysis (Seal AA3, Norderstedt, Germany). The air-dried soil was used for analysis of other properties as follows. Soil pH was determined with a glass electrode pH meter (soil:water=1:1, w:v); total N was analyzed by Kjeldahl digestion (Bremner, 1960); soil organic matter (SOM) was measured using a titration method after oxidation with $K_2Cr_2O_7$ (Yeomans and Bremner, 1988); soil available phosphorus (AP) was extracted with 0.5 mol L^{-1} $NaHCO_3$ and analyzed colorimetrically (Murphy and Riley, 1962); soil available

159 potassium (AK) was analyzed by flame photometry following extraction with 1 M
160 ammonium acetate (Walker and Barber, 1962).
161
162 *2.3 DNA extraction, PCR-amplification, and Illumina Miseq sequencing*
163 The genomic DNA was extracted using a PowerSoil® DNA Isolation kit (MO BIO
164 Laboratories, Inc., CA, USA) following the manufacturer's instructions. The DNA quantity
165 and quality were assessed using agarose gel electrophoretic analysis and a Nanodrop ND-
166 1000 spectrophotometer (Nano-Drop Technologies Inc., Wilmington, DE).
167 The primers used for PCR amplification were *nifH* Pol F (5'-TGC GAI CCS AAI GCI
168 GAC TC-3') and *nifH* AQER (5'-GAC GAT GTA GAT YTC CTG-3') (Poly et al., 2001;
169 Wartiainen et al., 2008). A unique barcode was added at the 5'-end of the reverse primer for
170 each sample. The PCR amplification program included initial denaturation at 95°C for 3 min,
171 followed by 30 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 60 s, and a final
172 extension at 72 °C for 10 min. Each sample had two PCR reactions and they were combined
173 together after amplification. The correct band was excised and purified using a SanPrep DNA
174 Gel Extraction Kit (Cat# SK8132, Sangon Biotech, Shanghai, China), and was quantified
175 with a Nanodrop ND-1000 Spectrophotometer. All samples were mixed together in equal
176 molar amounts from each sample for library construction using a TruSeq DNA kit according
177 to manufacturer's instructions, and then sequenced by an Illumina Miseq system.
178
179 *2.4 Bioinformatic analysis*
180 A total of 28141 sequences were obtained from the 30 samples. The raw sequences were
181 processed with QIIME pipeline (Caporaso et al., 2010). Low quality sequences were

removed, and the barcode was examined to assign sequences to each sample. The chimera sequences were removed by USEARCH v9.2, and frame shifts were corrected with FrameBot at default settings. The remaining sequences after quality control were translated into amino acid sequences for further analysis.

The sequences were clustered into operational taxonomic units (OTUs) at the 94% identity level (Tu et al., 2016). The representative sequences of OTUs were obtained with the most frequently ones and excluded singletons. Annotation for *nifH* OTUs at an 80% cutoff value was achieved with reference to the ARB database (http://wwwzehr.pmc.ucsc.edu/nifH_Database_Public/) (Zehr et al., 2003).

2.5 Statistical analysis

Data were subjected to analysis of variance using Proc ANOVA with SAS package 9.1 (SAS Institute, Cary, NC, USA). The cultivation practice (referring to the straw return strategy and inorganic fertilization) and sampling stage were treated as fixed effects and replication as a random effect for the data of soil physicochemical properties, gene copy numbers, alpha-diversity, and relative abundance of different genera. The least significant difference was used to determine treatment differences at a $P<0.05$ level of probability. Spearman correlation coefficients were employed to test the relationships between soil variables and relative abundance of genera in R. The principal coordinate analysis (PCoA) was performed based on the Bray-Curtis distance matrices, and the hierarchy clustering analysis was based on the weighted unifrac distance. A redundancy analysis (RDA) was conducted to investigate the correlation between the diazotrophic community composition and soil variables. PCoA, RDA, and clustering analysis were performed using the 'vegan' and 'GUniFrac' packages in

R. A multivariate regression tree analysis was performed to identify the most important soil factors for diazotroph diversity and community composition using 'mvpart' package (De'Ath, 2002).

3. Results

3.1 Effects of straw return and inorganic fertilization on soil properties

The sampling stage and/or cultivation practice had significant influences on the contents of soil available nutrients (NH_4^+-N, NO_3^--N, available P, and available K), soil pH, and C/N ratio, but not on the SOM and total N contents. No obvious interactions were found between sampling stage and cultivation practice (Table 1 and Table A2).

At the time of Mv planting and incorporation, the Rs treatment led to a marked increase of soil available K at both seedling and flowering stages of Mv growth, together with an obvious decease of NO_3^--N content at the flowering stage (P<0.05; Table 1). Compared to the Rs treatment, the inorganic fertilizer treatment F resulted in 0.6- to 1.5-fold increase in soil available P content, but 11-13% less available K, at the two sampling stages. In the combination treatments (FRs and FRh), there was a further increase of soil available P and K relative to the Rs and F treatments (P<0.05). The soil C/N ratio in these combined treatments decreased by comparison with the CK at flowering stage, while the pH values increased (P<0.05) (Table 1). There were no significant differences in the soil properties of the FRs and FRh treatments, except for the lower available K in FRh at the seedling stage (Table 1).

3.2 nifH gene copy number

The *nifH* gene copy number ranged from 4.5×10^6 to 25.0×10^6 g^{-1} soil across treatments at the

Mv seedling stage, which was higher than that at Mv flowering stage (11.2×10^6 to 15.3×10^6 g^{-1} soil) (Fig. 1). At the seedling stage, the gene copy number in the Rs treatment decreased strongly compared to the CK. The reverse impacts were observed in the inorganic fertilizer application or by the combinations of FRs and FRh (Fig. 1). At the flowering stage, the cultivation practice had limited effects on *nifH* gene copy number compared with the CK. Similar to the pattern at the seedling stage, the gene copy number tended to decrease in the straw return treatment (Rs), but increased in the combination treatment (FRs), and significant differences were detected between the Rs and FRs (Fig. 1). The relative influence of soil variables on *nifH* gene copy number was evaluated by relative weight analysis (Fig. 2). Nitrate and available P explained most of the variation of *nifH* gene abundance at the seedling stage (37% and 24%, respectively), while available K and available P were the most two variables at the flowering stage (27% and 25%, respectively) (Fig. 2)

3.3 The richness and diversity of diazotrophic community

On the basis of OTUs at 94% similarity, the alpha-diversity of the diazotrophic community was mainly affected by the cultivation practice, rather than sampling stage, but interactions were pronounced (Table 2). Greater OTU richness (Chao1 and Observed species) was observed in the CK and Rs treatments than that in other F-containing treatments where the inorganic fertilizer tended to repress it. The trends of diazotroph diversity (Shannon diversity and Simpson index) were consistent with the OTU richness, which showed that the diazotrophic bacteria in the Rs were more diverse than that in the treatments applied with inorganic fertilizer (Table 2). Multivariate regression tree analysis indicated that diazotroph diversity and richness were mainly shaped by soil available P at the seedling stage and by

NO_3^- at the flowering stage (Fig. 3).

3.4 Diazotrophic community structure

For community composition discrimination, a PCoA was performed based on the Bray-Curtis distance of OTU compositions (Fig. 4a and b). At the seedling stage, PCoA analysis showed that Rs was separated from other groups at both seedling and flowering stages of Mv growth. The hierarchy clustering analysis further confirmed these results, and the biggest differences in diazotroph structure occurred between Rs and FRs at seedling and between Rs and FRh at flowering stage, respectively (Fig. 4 c and d). In addition, the diazotrophic communities formed four clusters at the Mv flowering: CK, Rs, FRh, and others, showing that diazotroph communities differed between treatments (Fig. 4 b and d).

The redundancy analysis showed that the examined soil variables explained 50.9% of the variation of diazotroph community composition at the Mv seedling stage, and the first two axes attributed to 19.6% and 9.3%, respectively (Fig. 5a). According to the vectors, the diazotrophic communities of the Rs treatment were positively correlated with soil C/N ratio and pH, whereas in the FRs and FRh treatments, the communities were associated with soil NO_3^-, available P, total N, and SOM contents (Fig. 5a). At the Mv flowering stage, soil variables explained 74.7% of the variation, which was attributed to soil TN ($F=2.71$, $P=0.049$), SOM ($F=2.93$, $P=0.029$), available P ($F=3.34$, $P=0.007$), and available K ($F=2.36$, $P=0.023$) (Fig. 5b). The diazotrophic community of the Rs treatment was associated with SOM, soil total N, and the C:N ratio, while it was affected more by soil available K in FRs treatment, and by soil pH, available P and NH_4^+ in the FRh treatment (Fig. 5b).

3.5 Relative abundance of the diazotrophic taxa

OTUS of all treatment samples were taxonomically classified into 14 different genera which accounted for about 70% of the total sequences, with the other unclassified genera attributed to the remaining 30%. The seven most abundant genera with relative abundance more than 1% are presented in Fig. 6. The genus *Bradyrhizobium* was by far the most dominant group in all treatments, containing 21.8%-29.1% and 21.4%-32.2% of the total *nifH* gene sequences in soils at the Mv seedling and flowering stages, respectively (Fig. 6). The relative abundance of *Bradyrhizobium* was slightly reduced in the Rs soil by comparison with the CK, but was significantly increased (P<0.05) by FRs at seedling stage and by FRh at flowering stage. The *Anaeromyxobacter* and *Burkholderia* abundances were significantly increased in the Rs treatment by comparison with the CK at the flowering stage. There were no differences in the genus abundance between the FRs and FRh treatments, except for greater abundance of *Anaeromyxobacter* in FRh at seedling stage (Fig. 6). Multivariate regression tree analysis showed that the diazotroph community composition was mainly shaped by available P and available K at the Mv seedling stage and by C/N ratio at the flowering stage, respectively (Fig. 7).

A Spearman analysis indicated that soil variables were closely correlated, but differentially, with most of the abundant genera (Fig. 8). As to the most abundant two genera, the relative abundance of *Bradyrhizobium* at the Mv flowering stage was closely related to soil available P and negatively to the C/N ratio. The *Azospirillum* abundance was positively related to soil NO_3^- and available P contents (Fig. 8). By contrast with *Bradyrhizobium* and *Azospirillum*, the relative abundance of *Rhodobacter* was closely associated with SOM, TN, available P and negatively correlated with pH at the seedling stage. In addition, the relative

297 abundance of *Anaeromyxobacter* and *Burkholderia* genera were negatively correlated with
298 soil NO_3^- content at seedling stage and total N at flowering stage, respectively.
299
300 **4. Discussion**
301
302 *4.1 Successive straw return and inorganic fertilizer application changed the diazotrophic*
303 *abundance and richness in green manure season*
304 Soil microbes are highly sensitive to the environmental changes, such as soil C, N, and P
305 availability or their ratios. It has been reported that long-term inorganic fertilizer application
306 generally decreases soil microbial abundance (Wang et al., 2017), while the frequent return of
307 rice straw could increase the diazotrophic diversity and richness, but reduce the *nifH* gene
308 expression (Tang et al., 2017). Similar results were found in the present study where the *nifH*
309 gene copy number was increased (relative to the control) in the treatments with inorganic
310 fertilizer application but decreased in the treatment of straw incorporation alone (Fig. 1). The
311 diazotrophic community was more diverse in the straw treatment, and less so in the
312 treatments receiving inorganic fertilizer (Table 2). The results indicated that the diazotroph
313 communities were altered by the straw and fertilization practices.
314 Nitrogen and P are essential for the growth and metabolism of organisms and play a
315 pivotal role in biological N fixation (Wurzburger et al., 2012). Depending on the various
316 environmental factors in different ecosystems, N availability may have contrasting effects on
317 the abundance of *nifH* gene, where enhanced and suppressed impacts have been both
318 documented (Gonzalez Perez et al., 2014; Reardon et al., 2014; Zhalnina et al., 2015; Wang
319 et al., 2017). Nonetheless, high soil NO_3^--N and NH_4^+-N content generally inhibit the

members of the diazotrophic community, while lower N status could stimulate the biological N fixation (Coelho et al., 2008; Wang et al., 2017). Rice straw has a relatively low N concentration, but is rich in C. In the Rs treatment, rice straw incorporation might be expected to stimulate microbial growth and enhance the immobilization of soil available N (Rao and Mikkelsen, 1976). The lower soil NO_3^--N content was observed in Rs treatment is consistent with this expectation (Table 1). Furthermore, the significantly lower soil available P content in the Rs treatment compared with that in the F or combination treatments might further exacerbate such impacts. In agreement with that, soil nitrate and available P were identified as the primary predictor of *nifH* gene abundance (Fig. 2). In addition, the increased *nifH* gene abundance in the Rs treatment might be partly attributed to the increased relative abundance of dominant diazotroph genera *Anaeromyxobacter* and *Burkholderia* (Fig. 6).

The results also showed that diversity was closely related to soil C/N ratios. Higher C/N values could result in a competitive advantage for free living diazotrophs (Mirza et al., 2014). Due to the relatively less N input, the CK and Rs treatments had greater C/N ratios by comparison with the F, FRs, and FRh treatments at the Mv flowering stage, which might also explain the higher diversity in the Rs treatment than others. Consistently, the diazotroph diversity at this stage was mainly shaped by soil NO_3^- content and C/N ratios (Fig. 3b). Taken together, the gene copy numbers and diazotrophic diversity had relatively moderate values in the combinations of fertilization and rice straw incorporation, suggesting that both the N and P nutrients and C supply are important factors in regulating the shifts of diazotrophic diversity and richness.

Soil pH has been considered as a key factor affecting soil microbial richness and diversity (Levy-Booth et al., 2014; Wang et al., 2017). It is often due to the negative feedback

from intensified soil acidification caused by long-term inorganic fertilization (Guo et al., 2010). This could also partly explain the higher diversity in Rs treatment relative to those receiving fertilizer additions since higher pH values were observed in the straw treatment than the control. However, the reduction in the diversity index was not been fully offset by the combinations of fertilization and straw incorporation, which might imply the strong impacts of long-term inorganic fertilizer application on the diversity of the diazotrophic community.

4.2 Straw return and inorganic fertilizer application reshaped the diazotrophic community structure during the growth of Chinese milk vetch

Straw mulching and return, especially when combined with inorganic fertilization, could significantly reshape the soil diazotrophic community structure (Tang et al., 2017). Many studies have found that the community structure of soil ammonia-oxidizing bacteria, ammonia-oxidizing archaea, fungi, and diazotrophic bacteria could be easily affected by the long-term fertilization (Geisseler and Scow, 2014). In this study, the shifts of diazotrophic community structure were consistently observed in response to straw mulching and fertilizer application (Fig. 4). Compared to the control, the diazotrophic community structure under the Rs treatment was separated from others during the Mv growth, and the biggest differences in diazotroph structure occurred between Rs and FRs at seedling and between Rs and FRh at flowering stage, respectively (Fig. 4), which showed that cultivation practices indeed affected the soil diazotrophic community structure.

Soil is a very complex environment and the formation of microbial community's structure is often regulated by various interacting soil variables (Young, 1998; Geisseler and

366 Scow, 2014). The availability of soil N, P, and K are the important factors shaping
367 diazotrophic community composition (Tang et al., 2017; Wang et al., 2017). Varied
368 responses of the diazotrophic community structure and populations to soil nutrient status
369 were observed, which might have resulted from the greater availability of soil NO_3^- and
370 available P in the treatments with inorganic fertilizer application relative to the Rs treatment.
371 In accordance with that, the diazotrophic communities in the FRs and FRh treatments were
372 associated with soil NO_3^-, available P, total N, and SOM contents at the Mv seedling stage,
373 and affected more by soil available K in FRs treatment, and by soil pH, available P and NH_4^+
374 in the FRh treatment (Fig. 5). The results indicated that soil nutrient availability, which is
375 highly responsive to fertilizer input, is crucial for the establishment of soil diazotrophic
376 community structure.

377 Likewise, our results also showed that the soil C/N ratio and soil organic matter
378 concentration played important roles in regulating soil diazotrophic community composition.
379 The quality and quantity of added straw could influence the response to different fertilization
380 practices by altering microbial mineralization and immobilization turnover (Curtin and Fraser,
381 2003; Cusack et al., 2011). However, the practice of straw incorporation, i.e. conventionally
382 returned to soil and high stubble retention, under the present conditions, had marginal
383 differences in shaping the diazotrophic community and structure.

384

385 *4.3 Straw return and inorganic fertilizer application influenced the dominant diazotrophic*
386 *genera during the growth of Chinese milk vetch*

387 The diazotrophic genera may respond differently to the various soil physicochemical
388 properties, due to their individual sensitivities to changes of the soil environment (Wakelin et

al., 2009; Wang et al., 2017). There were clearly various responses or sensitivities of the different dominant diazotrophic genera to soil conditions in this study (Figs. 6, 7 and 8). Cultivation practices such as fertilization and straw incorporation are common ways of altering environmental conditions of soil microbes, and thus could change the composition of diazotrophic genera depending on their competitiveness and external environment. The *Bradyrhizobium* genus is ubiquitous in soil and is a commonly known N_2-fixing bacterium, which includes symbiotic N-fixing bacteria and free-living soil diazotrophs (Kahindi et al., 1997). As expected, the genus *Bradyrhizobium* was the most dominant group in all treatments, containing 21%-32% of the total *nifH* gene sequences in soils at the seedling and flowering stages of Chinese milk vetch (Fig. 6). The relative abundance of *Bradyrhizobium* was slightly reduced in Rs soil in comparison with CK, but significantly increased in FRs at seedling stage and in FRh at flowering stage. This was likely to have caused by the lower level of soil available P and higher C/N ratio in Rs treatment, since the relative abundance of *Badyrhizobium* was positively related to the soil's available P content, but negatively correlated with soil C/N ratios (Fig. 8). These results were in agreement with the concept that the dominant genera abundance was mainly shaped by the soil available P and C/N ratio (Fig. 7). The significant correlations between the relative abundance of *Azospirillum* and *Rhodobacter* and the content of soil available N and P implied that these two diazotrophic genera were very sensitive to soil N and P nutrients status. Interestingly, the Rs treatment selectively increased the relative abundance of *Anaeromyxobacter* and *Burkholderia*, especially at the flowering stage. They were negatively associated with either soil nitrate or total N content, but positively related to soil C/N and pH. The lower soil nitrate content in Rs treatment further indicated that these two dominant genera were more sensitive to soil C and

412 N status than others.

413 Changes in soil quality occur slowly and need time for the soil microbial community to
414 adapt to the altered environments and then stabilize (Geisseler and Scow, 2014; Wang et al.,
415 2017). In the present study, we found that the relative abundance of the dominant genera
416 *Bradyrhizobium* was generally lower than that reported by other studies during the rice
417 growing seasons (Wang et al., 2017). This might be due to the different soil texture and
418 different sampling seasons. The soil samples were collected during the winter season of the
419 Chinese milk vetch, which had relatively lower temperatures than the rice seasons, thus
420 leading to lower overall microbial activity. In addition, the differences might also be
421 associated with the cultivated plants, since different *nifH* gene abundances between soybean
422 and wheat seasons have been reported (Sun et al., 2015). Nitrogen fixing bacteria strongly
423 interact with cultivated plants (Leguminosae vs. Poaceae), which could change the sensitivity
424 and responses of diazotrophic genera to various soil conditions (Tan et al., 2003).

425 Taken together, at the time of Chinese milk vetch planting and incorporation, straw
426 incorporation alone had relatively sufficient C inputs, but was short of N and P, which
427 repressed the *nifH* gene abundance and the relative abundance of dominant diazotroph
428 genera. The results highlighted the importance of straw incorporation coupled with N and P
429 inputs to maintain microbial activity and diversity in the rice-rice-green manure rotations in
430 subtropical regions.

431

432 **5. Conclusion**

433 In conclusion, this study has demonstrated that straw incorporation and inorganic fertilizer
434 application lead to significant changes in soil diazotrophic community structure and

populations during the growth of Chinese milk vetch in a paddy soil. The straw alone could supply enough C to support microbial activity, but it contained relatively little N and P, thus reducing the *nifH* gene abundance and tending to decrease the relative abundance of the diazotrophic genera. The high C inputs with lower nutrient availability in straw enhanced diazotrophic diversity, while successive inorganic fertilizer applications decreased it. Although the combination of Mv, straw, and fertilization did not fully reverse the decline, the co-utilization practice helped stimulate the dominant diazotroph abundance by comparison with straw alone. Overall, the results suggest that both the C sources introduced by the rice straw and the N and P supplied by fertilizer application were crucial for improving soil quality and the population and diversity of soil diazotrophic community.

Acknowledgements

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Declarations of interest: none.

Appendix A. Supplementary data

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590

Tables

Table 1. Soil physicochemical properties at seedling and flowering stages of Chinese milk vetch.

Stage	Treatment	Soil organic matter (g/kg)	Total N (g/kg)	NO_3^- (mg/kg)	NH_4^+ (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	C/N ratio	pH
Seedling	CK	45.9±0.4 a	2.8±0.0 a	25.7±2.3 b	5.5±1.4 a	11.5±0.8 c	88.2±0.8 d	9.5±0.0 a	7.67±0.01 b
	Rs	44.5±0.4 a	2.7±0.1 a	25.8±0.9 b	5.6±1.3 a	10.1±0.3 c	106.0±1.5 c	9.7±0.1 a	7.73±0.01 a
	F	46.2±1.0 a	2.8±0.0 a	30.0±4.9 ab	4.3±1.6 a	16.3±0.6 b	94.4±0.8 cd	9.7±0.1 a	7.69±0.02 ab
	FRs	44.3±1.0 a	2.8±0.1 a	35.9±2.6 a	3.8±1.7 a	19.7±2.2 ab	139.3±4.7 a	9.3±0.2 a	7.68±0.02 b
	FRh	46.0±0.9 a	2.8±0.0 a	34.2±4.0 ab	5.2±1.3 a	21.2±1.2 a	121.2±6.6 b	9.7±0.2 a	7.67±0.01 b
Flowering	CK	45.7±0.2 a	2.7±0.0 a	4.7±0.8 a	1.4±0.8 a	5.7±0.4 bc	78.9±1.6 c	9.9±0.1 a	7.73±0.02 b
	Rs	46.0±3.3 a	2.7±0.2 a	2.6±0.3 b	1.2±0.6 a	2.8±1.1 c	93.1±3.3 b	10.1±0.1 a	7.79±0.05 ab
	F	43.6±0.3 a	2.6±0.0 a	3.4±0.2 ab	2.7±0.0 a	7.1±0.3 b	81.0±1.3 c	9.7±0.1 ab	7.77±0.04 ab
	FRs	46.8±2.1 a	2.9±0.1 a	3.3±0.3 ab	2.0±0.5 a	12.4±1.4 a	109.7±3.4 a	9.5±0.1 b	7.85±0.03 a
	FRh	43.0±1.2 a	2.6±0.0 a	4.3±0.5 ab	2.4±0.1 a	11.8±1.4 a	105.8±2.4 a	9.5±0.1 b	7.87±0.02 a

Values indicate mean±SE (n=3). Different letters in the columns represent significant differences between treatments within each stage ($P < 0.05$).

Table 2. The diazotrophic alpha-diversity as influenced by varied straw return and fertilization practices in a Chinese milk vetch-based system.

Stage	Treatment	Observed species	Chao1 index	Shannon diversity	Simpson index
Seedling	CK	1623±103 a	2746.0±62.4 a	7.5±0.1 a	0.982±0.002 ab
	Rs	1539±31 ab	2564.0±43.1 ab	7.6±0.1 a	0.982±0.003 ab
	F	1332±32 cd	2312.0±101.5 c	7.2±0.0 bc	0.978±0.001 bc
	FRs	1341±37 cd	2205.0±5.5 c	7.1±0.1 bc	0.978±0.001 bc
	FRh	1448±82 bc	2368.0±137.3 bc	7.4±0.1 ab	0.981±0.001 ab
Flowering	CK	1399±51 bc	2356.3±50.4 bc	7.2±0.1 bc	0.979±0.002 bc
	Rs	1550±56 ab	2672.3±99.9 a	7.6±0.2 a	0.984±0.003 a
	F	1384±34 cd	2299.7±93.4 c	7.2±0.0 bc	0.978±0.000 bc
	FRs	1354±20 cd	2232.3±41.9 c	7.3±0.0 ab	0.981±0.001 ab
	FRh	1244±42 d	2160.7±75.1 c	7.0±0.1 c	0.975±0.001 c
Stage (S)		0.0424	0.0870	0.0597	0.3249
Cultivation practice (C)		0.0010	0.0034	<0.0001	0.0271
S × C		0.0317	0.0320	0.0238	0.0607

Values indicate mean±SE (n=3). Different letters in the columns represent significant differences between treatments across stages ($P < 0.05$).

600 **Figure legend**

601

602 **Figure 1. *nifH* gene copy number in soils sampled at the seedling and flowering stages of**
603 **Chinse milk vetch as affected by the continuous straw return or fertilization practices.**
604 The data were subjected to a two-way analysis of variance. Abbreviations: S, stage; C,
605 cultivation practice; Different capital letters above the gray columns and lowercase letters
606 above the black columns indicate significant differences among cultivation practices at
607 seedling and flowering stages by LSD test, respectively ($P < 0.05$). The asterisks within each
608 cultivation practice indicate significant differences between two stages (*, $P < 0.05$; **, $P <$
609 0.01). Vertical bars represent the standard error of four replicates.

610

611 **Figure 2. Relative influence of soil physicochemical properties on abundance of *nifH***
612 **genes at the seedling and flowering stage of Chinese milk vetch evaluated using relative**
613 **weight analysis.** Abbreviations: AK, available potassium; AP, available phosphorus; C/N,
614 carbon to nitrogen ratio; SOM, soil organic matter; TN, total nitrogen. Values are means of
615 three replicates.

616

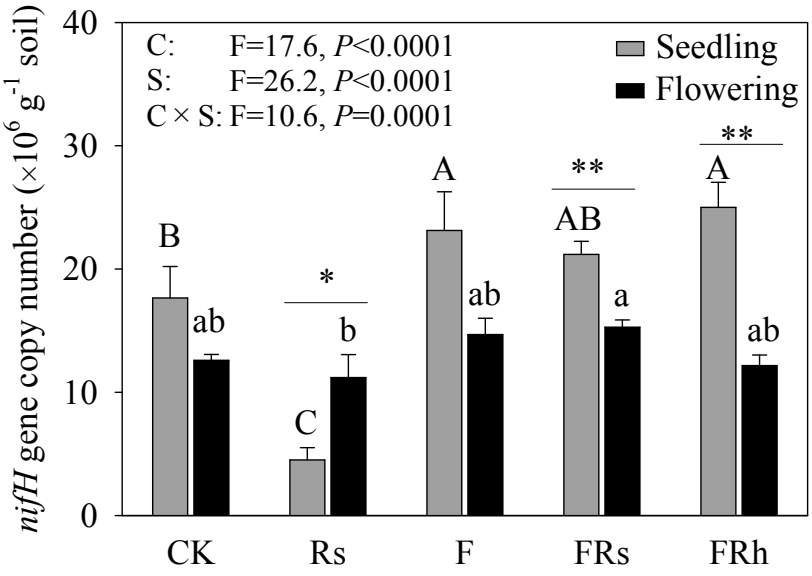
617 **Figure 3. Multivariate regression tree analysis of alpha diversity (observed species,**
618 **Chao 1, Shannon and Simpson indexes) of diazotrophs and soil physicochemical**
619 **variables.** Treatments and the number of samples included in the analysis are shown at the
620 bottom. Abbreviations: AK, available potassium; AP, available phosphorus; C/N, carbon to
621 nitrogen ratio; SOM, soil organic matter; TN, total nitrogen.

622

623 **Figure 4. Principal coordinate analysis of diazotrophic community composition in soils**
624 **sampled at the seedling and flowering stages of Chinese milk vetch as affected by the**
625 **continuous straw return or fertilization practices.** The samples were analyzed in triplicate
626 plots.
627
628 **Figure 5. Redundancy analysis of the diazotrophic community structure in soils**
629 **sampled at the seedling and flowering stages of Chinese milk vetch as affected by the**
630 **continuous straw return or fertilization practices.** The positions and lengths of the arrows
631 indicate the directions and strengths, respectively, of the effects of variables on the
632 diazotrophic communities. The samples were analyzed in triplicate plots. Abbreviations: AK,
633 available potassium; AP, available phosphorus; C/N, carbon to nitrogen ratio; SOM, soil
634 organic matter; TN, total nitrogen.
635
636 **Figure 6. Relative abundances (%) of the seven most abundant genera (>1%) in soils**
637 **sampled at the seedling and flowering stages of Chinese milk vetch as affected by the**
638 **continuous straw return or fertilization practices.** The samples were analyzed in triplicate
639 plots. Different letters above columns in each genus indicate significant differences among
640 treatments at $P < 0.05$.
641
642 **Figure 7. Multivariate regression tree analysis of the dominant genera abundance and**
643 **soil physicochemical variables.** Treatments and the number of samples included in the
644 analysis are shown at the bottom. Abbreviations: AK, available potassium; AP, available
645 phosphorus; C/N, carbon to nitrogen ratio; SOM, soil organic matter; TN, total nitrogen.

Figure 8. Spearman correlation analysis between the relative abundances of dominant diazotrophic genera and soil physicochemical variables at the seedling and flowering stages of Chinese milk vetch. r indicates the correlation coefficient; *, $P < 0.05$. Abbreviations: AK, available potassium; AP, available phosphorus; C/N, carbon to nitrogen ratio; SOM, soil organic matter; TN, total nitrogen.

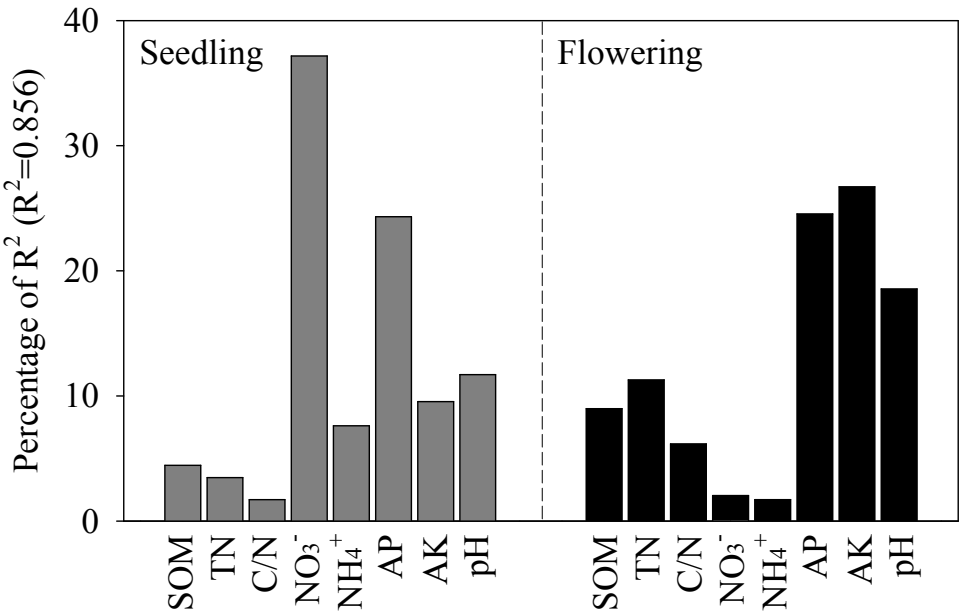
654 **Figures**



655

656 **Figure 1**

657



658
659 **Figure 2**
660

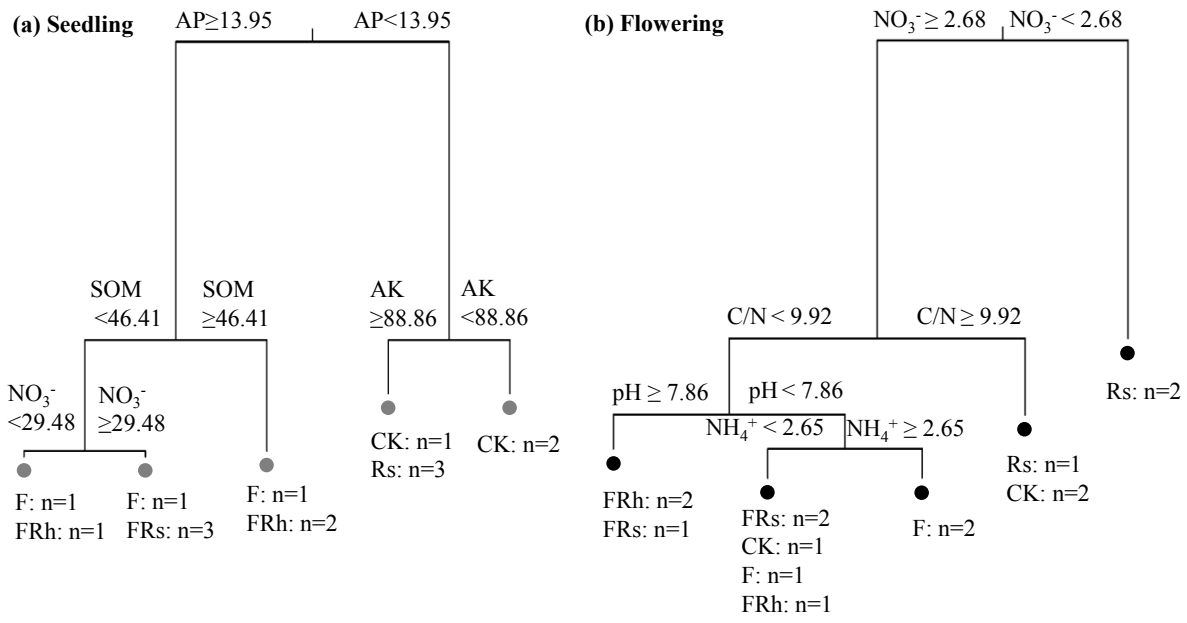

(a) Seedling
AP≥13.95
AP<13.95
SOM <46.41
SOM ≥46.41
AK ≥88.86
AK <88.86
NO3- <29.48
NO3- ≥29.48
F: n=1 FRh: n=1
F: n=1 FRs: n=3
F: n=1 FRh: n=2
CK: n=1 Rs: n=3
CK: n=2
(b) Flowering
NO3- ≥ 2.68
NO3- < 2.68
C/N < 9.92
C/N ≥ 9.92
Rs: n=2
pH ≥ 7.86
pH < 7.86
NH4+ < 2.65
NH4+ ≥ 2.65
Rs: n=1 CK: n=2
FRh: n=2 FRs: n=1
FRs: n=2 CK: n=1 F: n=1 FRh: n=1
F: n=2

Figure 3

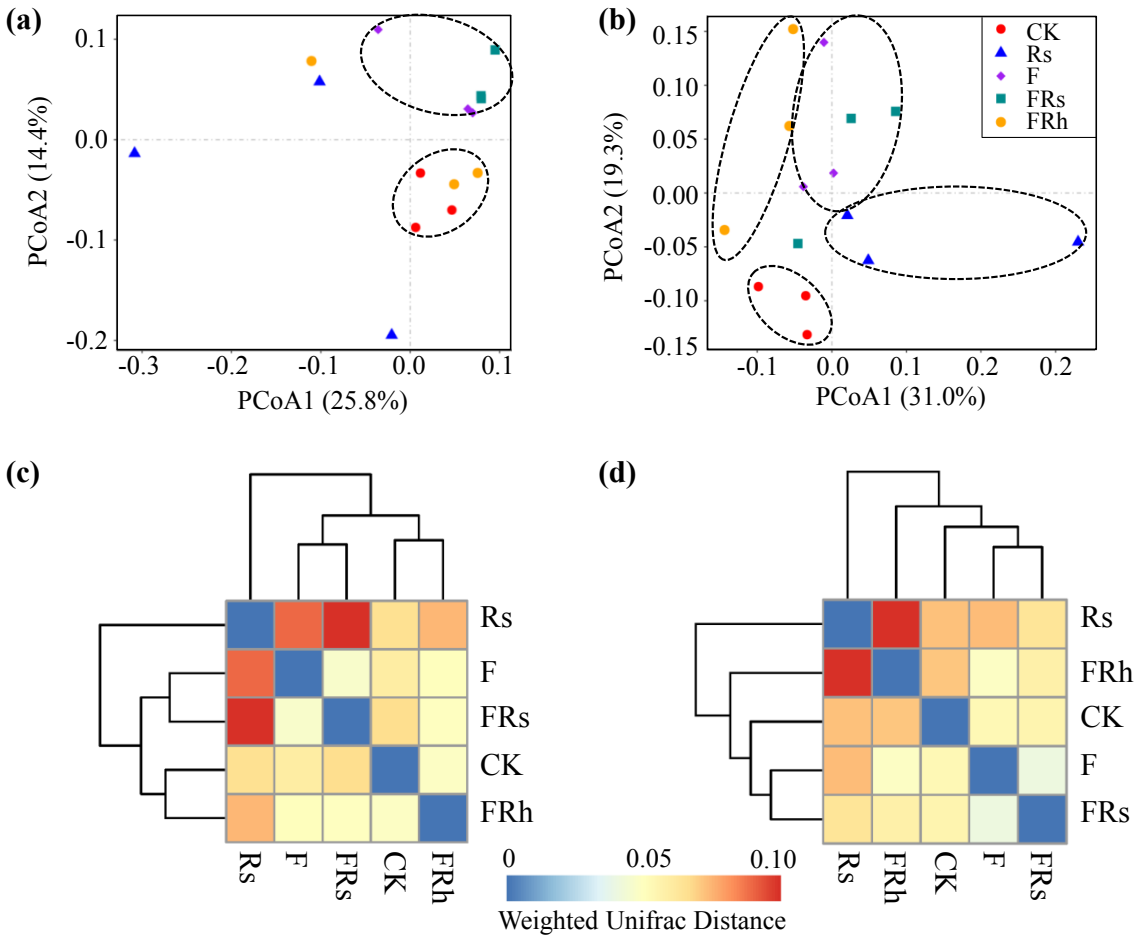
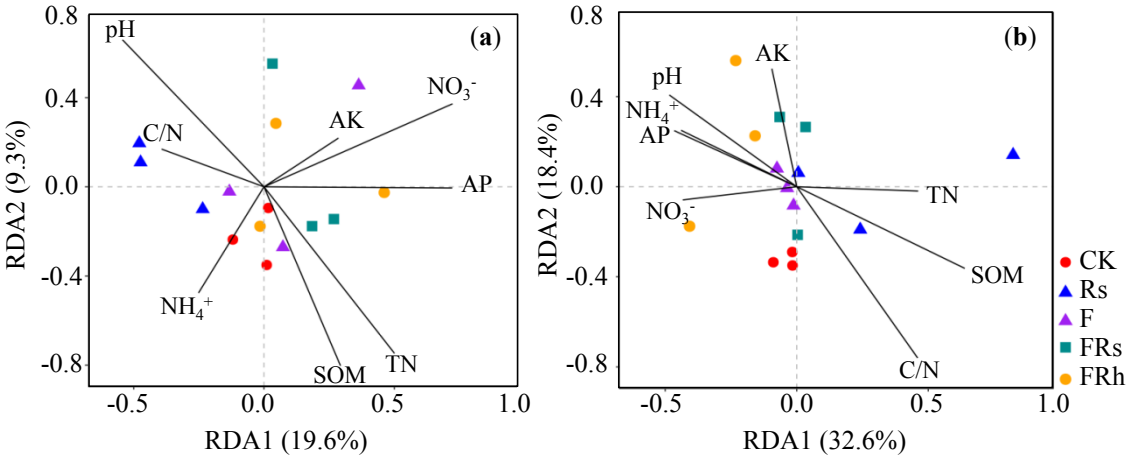

(a)
PCoA2 (14.4%)
PCoA1 (25.8%)
(b)
PCoA2 (19.3%)
PCoA1 (31.0%)
CK
Rs
F
FRs
FRh
(c)
Rs
F
FRs
CK
FRh
(d)
Rs
FRh
CK
F
FRs
0
0.05
0.10
Weighted Unifrac Distance

Figure 4



667

668 **Figure 5**

669

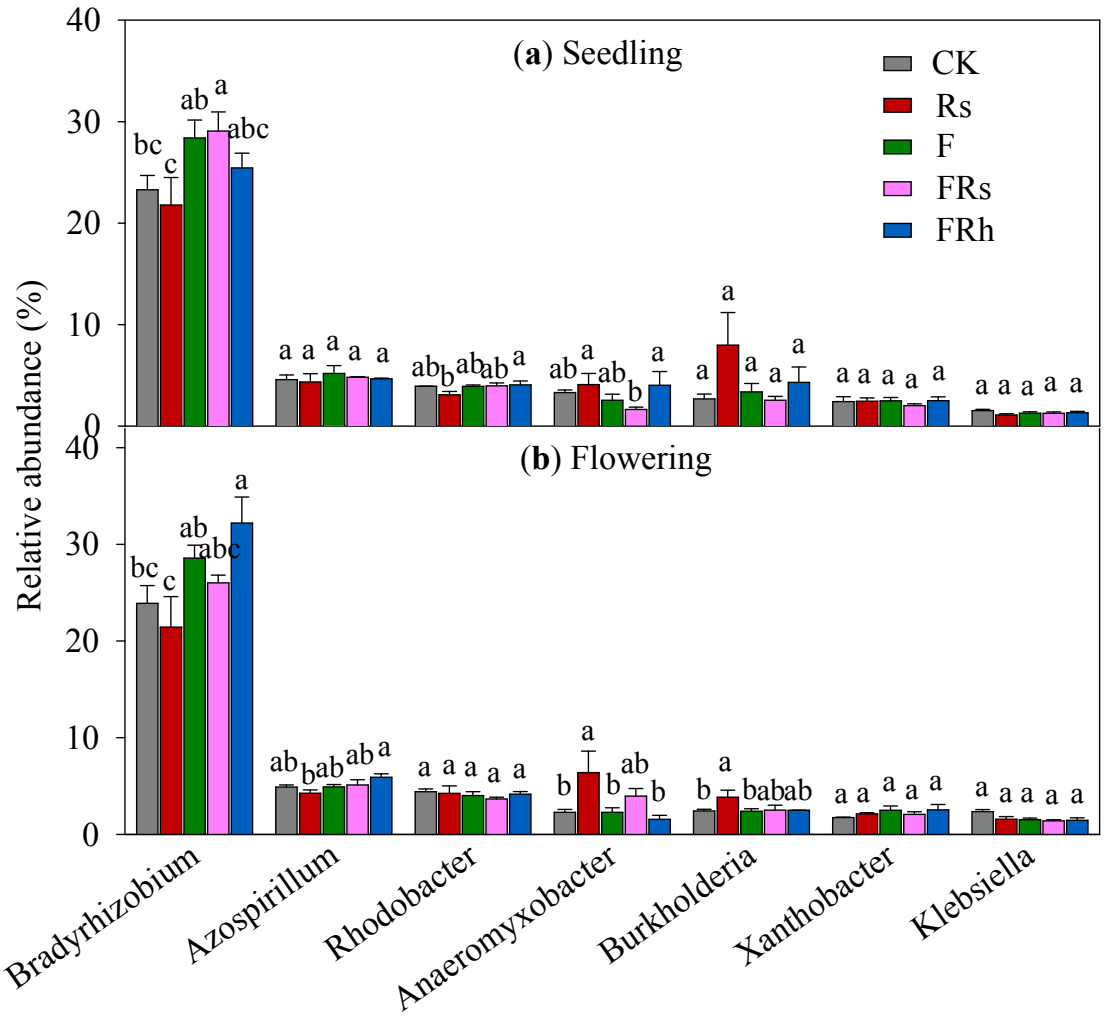
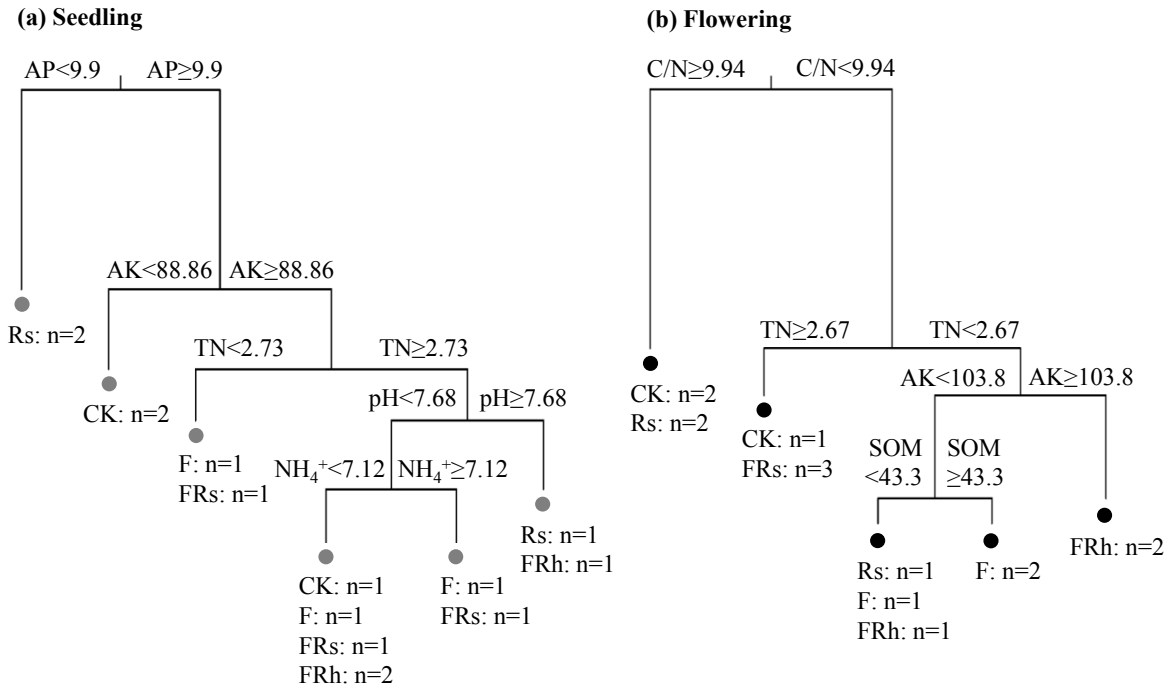

(a) Seedling
(b) Flowering
CK
Rs
F
FRs
FRh
Relative abundance (%)
0
10
20
30
40
Bradyrhizobium
Azospirillum
Rhodobacter
Anaeromyxobacter
Burkholderia
Xanthobacter
Klebsiella

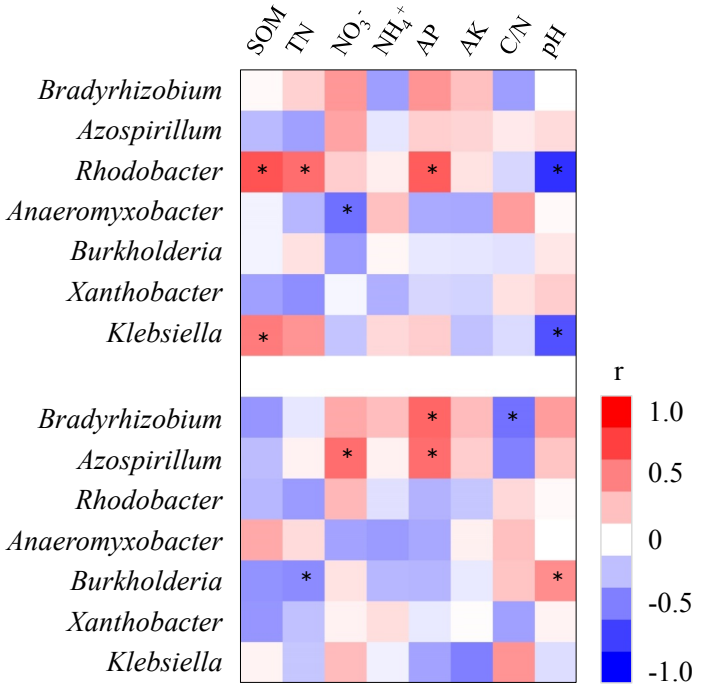
Figure 6



673

674 **Figure 7**

675



676

677 **Figure 8**

678

Appendix A. Supplementary data

Table A1

The estimated amount (kg ha^{-1}) of exogenous N, P and K inputs by chemical fertilizer application (N_{CF}, P_{CF}, and K_{CF}) and biological N fixation of Chinese milk vetch (N_{BF}) each year [#].

Treatment	Mv season	Early-rice season			Late-rice season			Annually total input		
	N_{BF}	N_{CF}	P_{CF}	K_{CF}	N_{CF}	P_{CF}	K_{CF}	N	P	K
CK	45	0	0	0	0	0	0	45	0	0
Rs	47	0	0	0	0	0	0	47	0	0
F	47	120	26	60	144	16	80	311	42	140
FRs	46	120	26	60	144	16	80	310	42	140
FRh	51	120	26	60	144	16	80	315	42	140

[#] The nutrient input by precipitation and irrigation was not included, and the NPK brought by straw return was considered as nutrient cycling within the double rice rotation system, not an exogenous input.

689 **Table A2**

690 The two-way analysis of variation for the soil properties influenced by stage and cultivation

691 practice in this Chinese milk vetch-based system

Soil properties	Stage (S)		Cultivation practice (C)		S × C	
	F	*P*	F	*P*	F	*P*
Soil organic matter	0.16	0.691	0.24	0.9118	1.37	0.285
Total N	1.61	0.220	1.01	0.427	0.87	0.499
NO_3^-	375.75	**<.0001**	2.44	0.0846	2.41	0.0868
NH_4^+	16.72	**<.0001**	0.15	0.959	0.60	0.668
Available P	116.11	**<.0001**	30.16	**<.0001**	0.89	0.491
Available K	61.18	**<.0001**	56.24	**<.0001**	2.92	0.050
C/N ratio	3.77	0.068	3.61	**0.025**	1.52	0.237
pH	42.63	**<.0001**	2.29	0.099	2.67	0.066

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