Organic manure rather than phosphorus fertilization primarily determined asymbiotic nitrogen fixation rate and the stability of diazotrophic community in an upland red soil

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Abstract：

Biological nitrogen fixation (BNF) plays a vital role in nitrogen supply in agricultural ecosystem but is generally impaired by agricultural fertilization. Understanding the trade-off between fertilization and BNF, and the underlying mechanisms are essential to optimize fertilization management for sustainable agriculture production. In this study, we examined the potential rate of asymbiotic N_2 fixation, *nif*H gene abundance, the composition and co-occurrence network of diazotrophic community in an acidic red soil received different organic and inorganic fertilization regimes for more than 40 years. Our results showed that long-term chemical fertilizer application drastically decreased soil microbial biomass, nitrogenase activity and *nif*H gene abundance in comparison to the unfertilized control. Organic manure application showed significantly positive effect on soil nitrogenase activity and crop yield via regulating soil pH and the key ecological cluster of diazotrophic community (module #2 which was mainly represented by *Bradyrhizobium*, *Pseudacidovorax* and *Azospirillum*), while phosphorus fertilization showed no obvious promotion effect. Organic manure amendment significantly increased the diversity of diazotrophs and enriched some diazotrophic taxa, particularly *Pseudacidovorax* and *Rhodopseudomonas* which were likely responsible for the high N_2-fixation potential. The control and organic manure amendment treatments possessed a more complex and stable diazotrophic network than the chemical fertilizer treatments did, which greatly facilitated the resistance of diazotrophic community to environmental stress and thus sustained a high N_2 fixation potential. Together, our study demonstrated that organic manure application can effectively alleviate the inhibitory effect of nitrogen fertilization on N_2 fixation via regulating soil property and shaping a more

stable diazotrophic network.

Keywords: Acidic red soil; Long-term fertilization; Nitrogenase activity; Diazotrophs; Co-occurrence network

1. Introduction

Both nitrogen (N) and phosphorus (P) are the two principal nutrient elements constraining the productivity in natural and agro-ecosystems (Camenzind et al., 2018; Du et al., 2020; Hou et al., 2020). P deficiency is more serious in acidic red soils as released P by weathering or fertilized P is apt to be co-precipitated with iron and manganese oxyhydroxides, and iron aluminides, or absorbed onto their surfaces (Vitousek et al., 2010). To meet an ever increasing demand for food, large amounts of chemical fertilizers are applied to agro-ecosystems to alleviate limitation of N and P in agriculture ecosystems, which have substantially changed N and P cycles and resulted in negative impact on the environment, such as eutrophication, soil acidification, greenhouse gas emission (N_2O) and biodiversity decreasing, etc. (Guo et al., 2010; Hu et al., 2015; Penuelas et al., 2013; Zhong et al., 2015). Unlike N fertilizer which can be chemically synthesized, P is mainly found in minerals with low bioavailability and is in short supply around the world. As the demand for P fertilizer in agriculture is ever increasing, confronting with the shortage of P-bearing minerals on a world scale, P is described as a “disappearing nutrient” (Gilbert, 2009). Furthermore, numerous studies have suggested that anthropogenic N inputs is distinctly increasing imbalance of N:P ratio and P limitation in tropical or subtropical regions, and consequently limit plant productivity and further impair ecological functions including biological N fixation, nitrification, litter decomposition, N mineralization and carbon storage, etc. in natural terrestrial ecosystems (Camenzind et al., 2018; Dai et al., 2020; Dynarski and Houlton 2018; Hou et al., 2020; Penuelas et al., 2013; Yao et al., 2018). However, the effect of imbalanced N:P ratio on soil ecological functions in agricultural ecosystems and the underlying

65 mechanisms were less known though P limitation resultant crop yield reduce is well known.

66 Biological nitrogen fixation (BNF), air dinitrogen reduction to ammonium catalyzed by
67 nitrogenase (Canfield et al., 2010), plays an important role in ecosystem nitrogen input and
68 contributes 40 - 100 Tg N to terrestrial ecosystem per year (Vitousek et al., 2013). According
69 to whether diazotrophs form the symbiotic structure with plants, diazotrophs are recognized
70 as symbiotic diazotrophs (e.g., *rhizobia* and *Frankia*, etc.) and non-symbiotic diazotrophs
71 (e.g., *Azotobacter* and *Azospirillum*, etc.). Although symbiotic diazotrophs dominantly
72 contribute ~60% of BNF, non-symbiotic diazotrophs are nearly ubiquitous in soil, litter, stalk,
73 leaves and even endophyte of plants, and thus are the important contributor to the N budges in
74 terrestrial ecosystems (Gupta et al., 2006; Reed et al., 2011). The results based on mass
75 balance and mathematic modeling suggested that the annual N input via asymbiotic nitrogen
76 fixation was more than 10 $kg \cdot ha^{-1} \cdot year^{-1}$, which almost balanced the N budget of mature
77 tropical forest in Amazonia rainforest after deducting atmospheric N deposition (Cleveland et
78 al., 2010). This estimate was corroborated by more findings from other tropical forests (Hedin
79 et al., 2009). Moreover, large-scale mathematic modeling study showed that asymbiotic
80 nitrogen fixation contributed a large proportion of N inputs than symbiotic nitrogen fixation
81 on the global scale in terrestrial ecosystems (Reed et al., 2011). Some studies suggested that
82 BNF are also important sources of N supply in fertilized agricultural ecosystems. For example,
83 it was found that asymbiotic nitrogen fixation contributed 24% of biomass nitrogen in cereals
84 (including maize, rice and wheat) on a global scale during a 50-year period (1961 to 2010)
85 (Ladha et al., 2016). Similarly, it was estimated that 29% - 82% of N for maize (*Zea mays L.*)
86 was derived from atmospheric nitrogen in arid and low nitrogen Sierra Mixe area, Mexico

87 (Van Deynze et al., 2018). Together, these studies suggest that BNF has the great potential to
88 partially replace chemical nitrogen fertilizer and alleviate the negative environmental effects
89 of N fertilization on agro-ecosystems (Reed et al., 2011; Wang et al., 2019). However, a
90 number of studies also reported that the intensive use of chemical nitrogen fertilization in
91 agricultural ecosystem usually exhibited an inhibitory effect on biological N fixation (Chao et
92 al., 2017; Fan et al., 2019; Tang et al., 2017). Understanding the trade-off between
93 fertilization and BNF and the underlying mechanisms in agricultural ecosystem is therefore
94 critical to maximize BNF in agricultural ecosystem. Furthermore, non-symbiotic diazotrophs
95 are highly diverse and functionally redundant in different soil environments (Chao et al., 2017;
96 Angel et al., 2018; Fan et al., 2019). To identify the keystone diazotrophs and their main
97 influential factors in different soils are prerequisite for field management optimizing and
98 manipulation of diazotrophs for sustainable agriculture production.

99 Converse to the inhibitory effect of chemical N fertilizer input on N_2 fixation, it was
100 found that phosphorus addition can significantly promote N_2-fixation activity in P-limited
101 forest and marine ecosystem (Camenzind et al., 2018; Dynarski and Houlton 2018; Mills et al.,
102 2004; Sanudo-Wilhelmy et al., 2001; Zheng et al., 2016), and significantly change
103 diazotrophic community in karst grassland soils and a Chinese fir plantation (Wang et al.,
104 2018; Xiao et al., 2020). Significant improvement of N_2-fixation activity and elevated *nif*H
105 gene expression under phosphorus fertilization were also observed in agriculture field (Tang
106 et al., 2017; Xun et al., 2018). However, these views were challenged by other studies. For
107 example, some studies showed that phosphorus fertilization alone had promotive effect on
108 N_2-fixation and diazotrophic abundance, but such an impact diminished when phosphorus

109 fertilizer was applied together with nitrogen fertilizer (Chao et al., 2017; Wang et al., 2018).
110 Similarly, some studies showed that organic mature fertilizer application had significantly
111 positive effect on the N_2-fixation and diazotrophic abundance in a rice paddy soil (Liao et al.,
112 2017). However, it was also reported that organic manure fertilizer alone or organic manure
113 combined with low-level chemical nitrogen fertilizer (262.5 kg-N ha^{-1}·$year^{-1}$) promoted the N_2
114 fixation, but the positive effect disappeared when both organic manure and high-level
115 nitrogen fertilizer (420 kg-N ha^{-1}·$year^{-1}$) were applied in a vertisol (Chen et al., 2020). These
116 contradictory results indicated that N_2-fixation and diazotrophic community vary largely in
117 different spatial scale and soil conditions and are synergistically determined by multiple
118 factors. A holistic understanding of how different fertilization practices (e.g., nitrogen,
119 phosphorus or organic manure fertilizations) interactively influence the N_2 fixation and
120 diazotrophs in different soils are therefore greatly needed to accurately estimate the
121 contribution and potential of asymbiotic N_2 fixation to N input in fertilized agricultural
122 ecosystem. Furthermore, most previous studies on the estimation of asymbiotic N_2 fixation
123 under different fertilization regimes were mainly based on the short-term trial (Fierer et al.,
124 2007; Rodríguez-Blanco et al., 2015). As such, our knowledge on the long-term effects of
125 inorganic and organic fertilization on N fixation rates and their associated N_2 fixers in
126 agroecosystems were still relatively scarce (Fan et al., 2019), particularly in acidic red soils in
127 which P could be a main limitation factor on N_2 fixation.
128 Therefore, in this study, nitrogenase activity and diazotrophic communities in an upland
129 red soil received 41-year fertilization practices including non-fertilization control (Control),
130 chemical N fertilizer only (N), chemical N and P fertilizers (NP), N fertilizer plus organic

manure (NM), NP plus organic manure (NPM) and chemical N, P and potassium fertilizers (NPK) were examined. We aimed to (1) assess the singular and combined effect of long-term application of N fertilizer, P fertilizer and organic manure on asymbiotic N_2 fixation rate and diazotrophic community in the acidic red soil, and (2) to characterize the key diazotrophic taxa responding to different fertilization regimes and estimate the possible regulation way to stimulate N_2 fixation in agricultural ecosystem. We hypothesized that the suppressive effect of N fertilization on N_2 fixation could be alleviated by P addition in P-limited acidic red soil, and that some key diazotrophic taxa might be sensitive to fertilization and fertilization-induced environment change, thus can be up-regulated via proper practice.

2. Materials and methods

2.1. Field trial description and soil sample collection

The field fertilization trials was established in 1978 at Qujing City, Yunnan province, Southwest China (25º18′6.8"N，103º53′55.4"E). The site has a subtropical monsoon climate with an annual mean temperature of 15 °C, and annual mean precipitation of 1000 mm, and the soil is classified as chromic cambisol according to the FAO soil classification system. The soil contained 20.6 g·kg^{-1} soil organic matter, 1.0 g·kg^{-1} total nitrogen, 67.0 mg·kg^{-1} available potassium (AK) and trace amounts of available phosphorus (AP) before the experiment started (Dai et al., 2009). The fertilization trials included six treatments: (1) non-fertilizer control (Control); (2) nitrogen fertilizer only (N); (3) inorganic nitrogen and phosphorus fertilizers (NP); (4) inorganic N fertilizer plus organic manure (NM); (5) inorganic nitrogen, phosphorus fertilizers plus organic manure (NPM) and (6) inorganic nitrogen, phosphorus and potassium fertilizers (NPK). The urea (N), superphosphate (P) and potassium sulfate or

potassium chloride were applied annually at a rate of 276 kg N hm^{-2}, 120 kg-P_2O_5·hm^{-2} and 112.5 kg-K_2O·hm^{-2}, respectively. Farmyard pig manure was applied as organic manure at a rate of 30 t hm^{-2}·$year^{-1}$ and contained ~ 83% organic matter, ~ 2% TN, ~ 0.60% AP and ~0.45% AK. Each treatment had three replicate plots, and each plot was about 33 m^2. All plots were randomly arranged and managed according to local habits, with maize (*Zea mays* cv Qidan 7) sown in summer and fallowed in winter.

Soil samples were collected in July (maize tasseling stage) and October (after harvest) 2019. After the litters were removed, five subsamples of topsoil (0 – 20cm) were collected between two maize plants and mixed as a biological sample for each plot. A total of 36 samples were collected. Soil samples were transported to the laboratory on dry ice. Subsequently, the soil was mixed and sieved (< 2mm) to remove the roots and stones. Then the soil samples were separated into two parts, with one stored at 4℃ for soil physicochemical properties and microbial enzyme activity analyses, and another one at -80 ℃ for DNA extraction.

2.2. Soil physicochemical property analyses

The soil physicochemical characteristics were determined according to established protocols (Zhao et al., 2019 ; Xiong et al., 2021). Briefly, soil pH was determined in deionized water (soil: water, 1 : 2.5) using a pH meter. Soil water content was determined by the oven-drying method at 105 °C to constant weight. Soil ammonium (NH_4^+-N) and nitrate (NO_3^--N) were extracted with 1 M KCl and determined with a continuous flow analyzer (AA3, Bran +Luebbe, Germany). Soil available phosphorus (AP) was determined by the molybdenum blue colorimetric method (Murphy and Riley 1962). Total carbon (TC) and

175 nitrogen (TN) were determined by the Dumas method with Element Analyser (Vario EL
176 III-Elementar, Germany). Total phosphorus (TP) was digested with H_2SO_4 and $HClO_4$, and
177 determined according to the molybdenum blue colorimetric method (Murphy and Riley
178 1962).

179 **2.3. Soil respiration, microbial biomass carbon and nitrogenase activity analysis**

180 Soil basal respiration (SR) was determined by the microcosm method (Franz 1995).
181 Briefly, 10g of fresh soil was placed in a sterile 120 ml serum bottle, and the soil water
182 content was adjusted to 60% of field water capacity. Then the bottles were sealed with rubber
183 stopper and aluminum cover and incubated at 25 °C for 24 h, and the CO_2 concentration in
184 the headspace of bottles was determined using a gas chromatograph (Agilent 7890A, USA).

185 Soil microbial biomass carbon (MBC) was determined by the chloroform fumigation
186 method (Franz 1995). Briefly, 10g fresh soil was placed in glass culture dish in duplicate,
187 with one fumigated with chloroform at 25 °C in the dark for 24 h and another one incubated
188 under the same condition without fumigation as the control. Then, all the soil samples were
189 extracted with 0.5 M K_2SO_4 and measured by a TOC/TON analyzer (Multi N/C 3100,
190 Analytik Jena, Germany).

191 Nitrogenase activity was evaluated using the acetylene reduction method as described
192 previously (Han et al., 2019). Briefly, 10 g fresh soil were weighted into sterile 120 ml serum
193 bottles, and glucose (1 mg C g^{-1} dry soil) and a citric acid (1 mg C g^{-1} dry soil) solution were
194 added to the bottles to ensure non-limitation of carbon source for diazotroph growth. The
195 bottles were sealed with rubber stoppers and 10% (v/v) acetylene (C_2H_2) was injected into
196 each bottle, which were incubated at 28 °C for 48 h. The ethylene (C_2H_4) concentration in the

197 headspace of bottles were determined with gas chromatograph (Agilent 7890A, USA).

198 **2.4. Soil DNA extraction and quantitative PCR of *nif*H gene**

199 Soil total DNA was extracted from 0.5 g soil using the PowerSoil DNA Isolation Kit
200 (MO BIO laboratories, Carlsbad, USA) according to the manufacturer's protocol. The
201 concentration and purity of the extracted DNA were determined using a Nanodrop ND-2000c
202 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All the DNA
203 samples were stored at -40 ℃ for downstream experiment.

204 Quantitative PCR (qPCR) analysis of *nif*H gene was conducted on a Roche
205 LightCycler® 480 system (Roche Life Science, USA) using the SYBR Green method, with
206 the primer set PolF/PolR (Poly et al., 2001). The amplification was carried on using the
207 thermal cycle program: 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C
208 for 30 s and plate read at 86 °C. The qPCR reactions (25 μL) contained 12.5 μL 2× SYBR
209 Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), 2 μL of DNA template (1–10 ng), 0.5 μL (10
210 μM) of each primer and 2 μl of Acetylated Bovine Serum Albumin (10 mg/ml, BSA^{-C},
211 Promega, USA). Standard curves were generated using 10-fold serial dilutions of plasmid
212 containing *nif*H gene fragment. Amplifications efficiencies for *nif*H gene were estimated at
213 100.4% ($R^2 > 0.99$).

214 **2.5. High-throughput sequencing and bioinformatics analysis**

215 PCR amplification of the *nif*H gene was conducted with barcoded primer sets
216 PolyF-PolyR (Poly et al., 2001). The PCR reactions were conducted in three parallels in 25
217 μL mixtures containing 12.5 μL 2 × Premix (TaKaRa Bio Inc., Shiga, Japan), 0.5 μL (10 μM)
218 of each barcode primer, 2 μL of $10mg \cdot ml^{-1}$ Acetylated Bovine Serum Albumin (BSA, Promega,

219 USA) and 2 μL of DNA template (1-10 ng). Negative control was also included to ensure no
220 contamination. The thermocycling parameters for PCR were the same as above mentioned.
221 The PCR products were purified using the Agarose Gel DNA purification kit (TaKaRa Bio)
222 following the manufacturer's instructions. Purified amplicons were pooled in equimolar and
223 then sequenced on the platform of Illumina NovaSeq PE250 sequencer (Personalbio,
224 Shanghai, China).

225 The *nif*H sequences were analyzed with the QIIME-1.9.1 pipeline (Caporaso et al., 2010)
226 and USEARCH v10 (Edgar, 2010). Firstly, primer sequences and low-quality sequences with
227 score less than 20 or containing ambiguous nucleotides were removed. Then the paired-end
228 reads of each sample were merged to a single sequence. The remaining sequences were
229 converted to amino acid sequences using the FunGene Pipeline of the Ribosome Database
230 Project (Wang et al., 2013). The sequences that do not match the *nif*H protein sequences or
231 contain termination codons were discarded. The remaining sequences were compared against
232 the *nif*H gene database (Gaby and Buckley 2014). The remaining high-quality sequences were
233 clustered into operational taxonomic units (OTUs) at 95% similarity using UCLUST (Edgar
234 2010) algorithm, and all singleton OTUs were discarded.

235 **2.6. Co-occurrence network analysis and structural equation model construction**

236 The co-occurrence network of diazotrophic community was constructed according to
237 previous protocols (Hartman et al., 2018; Huang et al., 2019). Firstly, the OTUs occurring in
238 less than 1/3 of the samples were removed and the sensitive OTUs were identified using the R
239 package 'indicspecies' and 'edgeR' (Hartman et al., 2018). Then, Spearman correlations
240 between diazotrophic species were calculated with the R package 'Hmisc', and the adjusted

241 *P*-values were calculated using the method of Benjamini-Hochberg (BH) (Csardi and Nepusz
242 2006). The network modules were identified using the method of fast greedy algorith, with
243 the adjusted *P*-values and *r*_values cutoff at 0.01 and 0.6, respectively (Clauset et al., 2004).
244 Finally, the network images were visualized with the Fruchterman-Reingold layout using R
245 package 'igraph'. The co-occurrence patterns of diazotrophic communitiy between chemical
246 fertilizer group (N, NP and NPK treatments) and non-chemical fertilizer group (Control, NM
247 and NPM treatments) were further constructed by combining sequence data from each three
248 treatments and the two seasons following the above-mentioned method, and were visualized
249 with Gephi (https://gephi.org/). We defined the nodes as network hubs (degree > 130;
250 closeness centrality > 0.4) referring to their roles in network structure (Agler et al., 2016;
251 Xiong et al., 2021). The robustness of microbial network was evaluated using the index of
252 'proportion of the giant component' by removing nodes in the network and assessing the
253 degradation rate of network robustness using both deliberate attacks and random attacks
254 methods. The deliberate attack indicated that the pre-specifies nodes were removed from
255 static network based on the importance of nodes (degree), and random attacks indicated that
256 the nodes were randomly removed from static network regardless of their importance (Fan et
257 al., 2018).

258 The structural equation model (SEM) was conducted using IBM SPSS Amos 22 to
259 quantify the direct and indirect effects of the soil physicochemical properties (e.g., pH, TN,
260 TC/TN and AP) and the relative abundance of the main modules on the nitrogenase activity.
261 All variables were standardized by Z transformation (mean = 0, standard deviation = 1) using
262 the function of 'scale' in R package. Maximum likelihood method was used to fit the model

263 (Zhao et al., 2019). The standardized total effects (STEs) of the soil properties and
264 fertilization treatments on the nitrogenase activity were calculated.

265 **2.7. Statistical analysis**

266 The raw data of gene abundance were log-transformed in order to ensure the normality.
267 One-way and two-ways variance analysis (ANOVA) based on Duncan test ($P < 0.05$) were
268 performed to test the difference between treatments with SPSS 22.0 (IBM, USA). The
269 beta-diversity of diazotrophic community was assessed by computing weighted UniFrac
270 distance matrices and then ordinated using nonmetric multidimensional scaling (NMDS). The
271 significance of community dissimilarity was tested with PERMANOVA using the 'adonis'
272 function (permutations = 999) of the 'vegan' package in R (Dixon 2003). Redundancy
273 analysis (RDA) was conducted to test the influence of environmental parameters on
274 community structure with R software in 'vegan' package (Dixon 2003). The alpha-diversity
275 of *nif*H gene was calculated using Faith's phylogenetic diversity (PD) (Faith 1992).

276 **2.8 Accession numbers**

277 The raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA)
278 under the accession number PRJNA694102.

279 **3. Results**

280 **3.1 Soil properties and crop yields**

281 Soil properties varied greatly after 41-years fertilization treatments (Table 1). For both
282 seasons, the Control treatment had the highest pH value ranging from 6.57 to 6.6, followed by
283 manure addition treatments (NM and NPM, pH at 5.80 - 5.98), with the lowest pH recorded in
284 three chemical fertilizer treatments (N, NP and NPK, pH at 4.52 - 4.71) ($P < 0.001$, Table 1).

In summer, NH_4^+-N concentration ranged between 9.9 - 24.55 mg·kg^{-1} and was the highest in the NPK treatment, and then in the N treatment (P < 0.001, Table 1). In autumn, NH_4^+-N concentration was significantly decreased and showed no significant difference among six treatments (0.40 – 1.63 mg·kg^{-1}) (P < 0.001, Table 1). Similarly, NO_3^--N concentration decreased across the seasons, and the Control treatment had the lowest NO_3^--N concentration for both seasons. (P < 0.001, Table 1). Phosphorus fertilization significantly increased the AP and TP contents in the NP, NPM and NPK treatments compared to non-phosphorus addition treatments (Control, N and NM) (P < 0.001, Table 1), but showed no visible effect on the TC and TN contents. The N fertilization significantly decreased the TC and TN contents by 24.9% and 14.3%, (P < 0.001, Table 1), while organic manure application significantly increased the TC and TN contents by 21.2% and 55.3%, respectively, compared to the Control treatment (P < 0.001, Table 1).

For maize yield, long-term application of single nitrogen fertilization (N treatment) drastically reduced maize yield to total crop failure since 1985, while there was no significant difference among other five treatments, with a mean yield of recent three years (2017-2019) ranging between 7230.0 and 8677.5 kg·hm^{-2} (P < 0.05, Fig. 1a).

3.2 Soil respiration and microbial biomass carbon

Soil respiration (SR), soil respiration quotient (SQ) and microbial biomass carbon (MBC) showed significant difference among treatments (Fig. 1b-c). SR was significantly lower in the N treatment (0.31 ug C d^{-1}·g^{-1} dry soil) than any other treatments (0.47 - 0.57 ug C d^{-1}·g^{-1} dry soil) in summer (P < 0.05, Fig. 1b), but showed no significant difference among treatments in autumn (P > 0.05, Fig. 1b). MBCs in three chemical fertilizer treatments (N, NP and NPK)

were significantly lower than that in Control and organic manure treatments (NM, NPM), with lowest value observed in the N treatment ($P < 0.05$, Fig. 1c). Consequently, SQ showed an opposite trend to SR, with a significantly higher SQ in the N treatment than any other treatments ($P < 0.05$, Fig. 1d), indicating a suppression of microbial metabolism by unbalanced N fertilizer application.

3.3 Soil nitrogenase activity and *nif*H gene abundance

Nitrogenase activity, evaluated with the acetylene reduction method, ranged from 0.36 to 310.89 nmol·d^{-1}·g^{-1} dry soil among all treatments (Fig. 2a). The highest nitrogenase activity was found in the Control treatment for both seasons (310.89 and 120.43 nmol·d^{-1}·g-^{1} dry soil in summer and autumn, respectively), followed by NM and NPM treatments ($P < 0.05$, Fig. 2a). The nitrogenase activity was almost completely inhibited in N fertilizer alone application treatment (N) ($P < 0.05$, Fig. 2a). Compared to N treatment, P fertilization treatments (NP and NPK) showed slight promotion on nitrogenase activity ($P > 0.05$ in NP treatment and $P < 0.05$ in NPK treatment), while organic manure fertilization (NM and NPM) significantly enhanced nitrogenase activity about 35 times ($P < 0.05$, Fig. 2a). The *nif*H gene abundance showed similar pattern as nitrogenase activity, and was significantly higher in Control, NM and NPM treatments (8.44×10^6 - 1.68×10^7 copies g^{-1} dry soil) than in three chemical fertilizer treatments (N, NP and NPK, 4.86 - 6.77×10^6 copies g^{-1} dry soil) ($P < 0.05$, Fig.2b).

3.4 Diversity and community composition of diazotrophic community

A total of 3,228,024 high quality *nif*H gene sequence reads were obtained from 36 soil samples, with a mean of 89,667 reads per sample. To equalize the sequencing effort, all samples were then rarefied to 66,774 sequences, and the resulting 2597 OTUs at 95%

329 similarity were subjected to alpha- and beta-diversity analyses. The Chao 1 diversity and
330 Phylogenetic diversity ranged between 350.72-1077.71 and between 45.12 -88.62 in all
331 treatments, respectively, with significantly higher values in NM and NPM treatments than any
332 other treatments ($P < 0.05$, Table S1).

333 The Non-metric multidimensional scaling (NMDS) analysis clearly showed that the
334 diazotrophic communities were clearly separated among NM-NPM, Control and N-NP-NPK
335 treatments but showed no visible separation between two seasons (Fig. 3a). The significant
336 differences of diazotrophic community among different treatments were also confirmed by
337 Adonis test (Treatment: $R^2 = 0.743$, $P < 0.001$, Table S2). Redundancy analysis (RDA) further
338 showed that the first two axes explained 29.28% and 11.65% of variation for diazotrophic
339 community. The ANOVA test showed that pH, TC, TC/TN and NO_3^--N were the most
340 important factor influencing the diazotrophic community ($P < 0.05$, Fig. 3b).

341 Taxonomic analysis showed that the diazotrophic communities mainly consisted of
342 *Bradyrhizobium* (30.27%), *Rhodopseudomonas* (11.95%), *Pseudacidovorax* (5.42%),
343 *Azospirillum* (4.92%), *Rhizobium* (3.82%), *Burkholderia* (3.46%) and *Methylobacterium*
344 (2.35%) within Proteobacteria (Fig. 4). *Bradyrhizobium* (14.32% - 50.38%), *Pseudacidovorax*
345 (0.69% - 16.18%) and *Azospirillum* (0.47% - 19.65%) were widely present in all soil samples
346 while the relative abundance varied largely among six treatments. Particularly, the relative
347 abundance of *Pseudacidovorax* was significantly higher in the Control, NM and NPM
348 treatments (2.75% - 16.18%) than in three chemical fertilizer treatments (N, NP and NPK)
349 (0.69% - 4.54%, $P < 0.05$, Fig. 4). Intriguingly, *Ectothiorhodospira* was exclusively present
350 in three chemical fertilizers treatments (N, NP and NPK) with a proportion of 2.19% - 23.18%

while *Rhodopseudomonas* was significantly enriched in NM and NPM treatments with a proportion of 19.98% - 39.53%, ($P < 0.05$, Fig. 4).

3.5 The co-occurrence network and keystone taxa of diazotrophic community

Correlation network analysis was further conducted to evaluate the effect of long-term fertilization on the association of diazotrophs. It showed that diazotrophs network was clearly grouped into three major modules (modules #1, #2 and #5) and long-term fertilization drastically changed the cumulative relative abundance of major genera in three modules (Fig. 5a, c). Each module was composed of different genera, with the module 1 mainly represented by *Rhodopseudomonas* and *Rhizobium*, the module 2 by *Bradyrhizobium*, *Azospirillum* and *Pseudacidovorax* and the module 5 by *Bradyrhizobium* and *Azotobacter* (Fig. 5b). *Bradyrhizobium* and *Pseudacidovorax* dominated module 2 with a significantly higher proportion than other modules (Fig. 5b). The cumulative relative abundance of module 1 was highest in NM and NPM treatments while the values of module 5 was highest in N, NP and NPK treatments (Fig. 5c). Interestingly, the cumulative relative abundance of module 2 significantly decreased in N, NP and NPK treatments in comparison to the Control, NM and NPM treatments, showing similar trend as nitrogenase activity (Fig. 2a and Fig. 5c). More intriguingly, a significantly positive correlation was observed between cumulative relative abundance of module 2 and nitrogenase activity ($R^2 = 0.46$, $P < 0.001$, Fig. 5d), but not between the cumulative relative abundance of module 1, module 5 and the nitrogenase activity (Fig. 5d). Coincidently, significantly positive correlation was observed between the cumulative relative abundance of dominant genera in module 2 (e.g., *Pseudacidovorax*, *Bradyrhizobium* and *Azospirillum*) and soil nitrogenase activity ($P < 0.001$, Fig. S1), but not

found for the dominant genera in module #1 and #5 (Fig. S1).

As the most distinct difference in soil physiochemical parameters, nitrogenase activity, *nifH* gene abundance, and the dissimilarity of diazotrophic community were observed between three chemical fertilizer (N, NP and NPK) and three non-chemical fertilizer (Control, NM and NPM) treatments. We further assessed the co-occurrence patterns of diazotrophic communities between N-NP-NPK treatments and Control-NM-NPM treatments by combining sequence data from each three treatments and the two seasons. The result showed that three non-chemical fertilizer treatments (Control-NM-NPM group) had higher network nodes (1105), edges (26512) and average degree (47.986) than three chemical fertilizer treatments (N-NP-NPK group) did (nodes: 392, edges: 1042, average degree: 5.316; a higher average degree representing a greater network complexity) (Fig. 6a). A higher proportion of negative correlation was also observed in the network of Control-NM-NPM group (10.19%) than in N-NP-NPK group (0.96%), and a typical power-law degree distribution pattern was observed for both groups (Fig. 6c). We also estimated network stability by removing nodes randomly or deliberately in static network, and found that the network structure in Control-NM-NPM group was more stable than those of N-NP-NPK group (Fig. 6d). All these suggested that non-chemical fertilizer treatments had a more complex and stable diazotrophic network association than chemical fertilizer treatments (Fig. 6a, d). Furthermore, the number of 'hub nodes' (nodes with high values of degree (> 130) and closeness centrality (> 0.4) was also higher in the network of Control-NM-NPM group than that of N-NP-NPK group, and these hub nodes were mainly affiliating within the genera of *Bradyrhizobium*, *Pseudacidovorax*, and *Azospirillum* (Fig. 6b).

Structural equation model (SEM) was used to quantify the effect of long-term fertilization treatments on soil properties, diazotrophic keystone taxa and nitrogenase activity and crop yield. The result showed that 83% and 71% of variation in nitrogenase activity and maize yield were directly or indirectly explained by different fertilization practices and fertilization-induced change in soil properties and diazotrophs attributes, respectively (Fig. 7a). Nitrogen fertilizer application alone (N treatment) significantly decreased soil pH and TC: TN value and thus negatively affected soil nitrogenase activity ($P < 0.001$, Fig. 7a), and accounted for the strongest negative effect on nitrogenase activity and crop yield (with a STE value of -0.913 for nitrogenase activity and -0.86 for crop yield) (Fig. 7b). Organic mature application (NM and NPM treatments) had significantly positive effect on soil pH and the cumulative relative abundance of module 2, and then on soil nitrogenase activity ($P < 0.05$, Fig. 7a). Organic manure application (with a STE value of 0.841) also positively affected maize yield synergistically with increased pH (with a STE value of 0.875) (Fig. 7b). Phosphorus fertilization showed no significant effect on nitrogenase activity ($P > 0.1$), but positively influenced crop yield via increasing AP content ($P < 0.05$, Fig. 7a).

4. Discussion

Our result showed that long-term inorganic chemical fertilizer application (N, NP and NPK treatments) and organic mature application (NM and NPM treatments) in the acidic red soil had opposite effects on nitrogenase activity, *nif*H gene abundance, diazotrophic community composition and co-occurrence pattern. Compared to the Control, inorganic chemical fertilizer application significantly decreased the nitrogenase activity and *nif*H gene

abundance, and N fertilizer application alone (N treatment) almost completely inhibit the nitrogenase activity. This was consistent with previous observations that chemical fertilizer (N) addition significantly inhibited the N_2-fixation activity and *nif*H gene abundance in a Chinese fir forest and red soil (Tang et al., 2017; Wang et al., 2018). Some studies based on long-term fertilization trial also showed that nitrogen fertilization significantly decreased N_2-fixation activity and diazotrophs abundance in both upland and paddy soils (Chao et al., 2017; Tang et al., 2017). As N_2 fixation is a metabolically costly process, diazotrophs preferentially use available nitrogen in the environment, and the nitrogenase enzyme is highly sensitive to ammonia (Chao et al., 2017; Masepohl and Hallenbeck 2010; Shi et al., 2016). It is therefore reasonable to see the suppressive effect of N fertilization on soil N_2-fixation activity in these studies.

Our results further suggested that organic manure application (NM and NPM) significantly improved nitrogenase activity and *nif*H gene abundance in comparison to three chemical fertilizer treatments, and that soil nitrogenase activities were positively associated with pH, TC, TN and TC/TN ratio but negatively with inorganic nitrogen. Similarly, Huan and colleagues (2020) found that the combination of chemical and organic fertilizers significantly increased biological N_2-fixation rate and *nif*H gene abundance in vertisol soil, and attributed it to the increased soil nutrient status and pH (Chen et al., 2020). In addition to nitrogen, soil pH and carbon were generally recognized as the crucial factors affecting N_2 fixation activity and diazotrophic abundance (Chao et al., 2017; Chen et al., 2020; Fan et al., 2018; Li et al., 2019; Wakelin et al., 2010; Wang et al., 2018). It has been suggested that low pH is not conducive to the activity of nitrogenase and the optimal pH for diazotrophs is

between 7.5 and 8.0 (Roper and Gupta 2016). A study based on field $^{15}N_2$ labeling technique also suggested that the amount of BNF in four types of paddy soils is positively correlated with soil pH, and that the aluminum toxicity is another limiting factor for BNF in acidic red soil (Wang et al., 2019). It was also found that lime addition can alleviate the inhibition effect of nitrogen application on N_2-fixation activity and *nif*H gene abundance in acidic red soil (Chao et al., 2017). In our study, inorganic chemical fertilization drastically decreased soil pH by ~ 2 units (pH at 4.5 - 4.7 in N, NP and NPK) compared with the Control (pH at 6.6). In contrast, organic manure application significantly improved soil pH by 1.1 – 1.5 units in comparison to inorganic chemical fertilization treatments. All these suggested that soil pH is also a determinative factor for BNF and soil acidification caused by nitrogen fertilization can greatly exacerbate the inhibitory effect of N fertilization on N_2-fixation in acidic red soils. Moreover, diazotrophs in soil and rhizosphere are mainly heterotrophic and highly dependent on the carbon source from plant and organic matter as they metabolically maintain higher respiration to avoid the inhibition effect of O_2 on nitrogenase (Hill 1988; Li et al., 2021; Reed et al., 2011). The increased soil pH and high organic carbon input in NM and NPM treatments greatly facilitate the growth of diazotrophs, and thus enhance the nitrogenase activity and *nif*H gene abundance. The similar favorable soil condition in the Control (e.g., the highest pH and higher TC/TN ratio induced by rare N input condition) well explained the reason that it possessed the highest nitrogenase activity and *nif*H gene abundance among all treatments. This could be further corroborated by the lowest soil respiratory quotient and the highest microbial biomass carbon in the Control, NM and NPM treatments. The high N_2-fixation potential in the Control also partially explained why the crop yield in this treatment did not

460 markedly reduce after long-term “zero” fertilization, while the real N budget and nutrient
461 balance in the Control need to be further explored.

462 As nitrogen fixation is an energy-intensive process, BNF is supposed to be highly
463 dependent on the availability of phosphorus. Coincidently, significant promotion of
464 N_2-fixation activity by phosphorus addition was commonly observed in P-limited forest soils
465 (Wang et al., 2018; Zheng et al., 2016), marine water (Garcia et al., 2015; Mills et al., 2004;
466 Sanudo-Wilhelmy et al., 2001), and upland red soil without fertilization for long-term
467 (Dynarski and Houlton 2018). Similarly, significant promotion in N_2-fixation activity and
468 *nif*H gene expression by long-term phosphorus fertilization were also observed in a paddy soil
469 (Tang et al., 2017). Acidic red soils are particularly prevalent in arable lands in Southern
470 China, and P deficiency is much more pronounced as P is readily co-precipitated with iron
471 and manganese oxyhyduoxides, iron aluminides, or directly absorbed onto their surfaces in
472 red soils (Vitousek et al., 2010). Thus we hypothesized that P addition would promote N_2
473 fixation and alleviate the suppressive effect of N fertilization on it in the acidic red soil.
474 Surprisingly, we found that phosphorus fertilizer application (NP, NPK, NPM) showed slight
475 or no obvious promotion effect on nitrogenase activity and *nif*H gene abundance in this study.
476 The inconsistency between findings in our study and others could potentially be attributed to
477 two factors. One is that the phosphorus might not reach the limitation threshold for
478 nitrogenase activity in our study site as this area is one of the most important phosphate
479 producing area in China, and the AP content (9-10 $mg \cdot kg^{-1}$ in the Control and N treatments) in
480 our study site is relatively higher than those in P-limited natural ecosystems and the paddy
481 soil in Tang’s study (2017) in which AP in the Control was less than 4 $mg \cdot kg^{-1}$. Secondly, the

482 inhibitory effect of long-term nitrogen fertilizer input and the resulting soil acidification on
483 nitrogenase activity may greatly weaken the promoting effect of phosphorus fertilization in
484 NP and NPK treatments. Similarly, the strong promotion effect of organic manure also may
485 conceal the impact of phosphorus fertilization in NM and NPM treatments. Together, all these
486 suggested that long-term application of organic manure, rather than phosphorus fertilization,
487 effectively alleviated the inhibitory effect of nitrogen fertilization on N_2 fixation process by
488 regulating soil property in this acidic red soil.

489 NMDS and RDA analyses suggested that the diazotrophic communities largely varied
490 among different fertilization practices but not between two seasons, suggesting strong impacts
491 of long-term fertilization on diazotrophic community. Diazotrophic community was clearly
492 separated among NM-NPM, Control, N-NP-NPK treatments, with pH, TC, TC/TN and NO_3^-
493 as the main influencing factor. These findings were consistent with a number of previous
494 observations showing that long-term fertilization significantly changed diazotrophic
495 community composition (Chao et al., 2017; Fan et al., 2019; Fan et al., 2018; Tang et al.,
496 2017), and further confirmed the vital role of soil pH, N fertilization and organic carbon in
497 determining the community composition, *nif*H gene abundance and N_2 fixation rate.

498 Our network and SEM analyses further suggested that organic manure application
499 positively influenced nitrogenase activity by regulating soil pH and the relative abundance of
500 module #2 (which was mainly represented by *Bradyrhizobium*, *Pseudacidovorax* and
501 *Azospirillum*). Except for nitrogen fixation with leguminous plants, *Bradyrhizobium* also acts
502 as the important plant growth promoting rhizobacteria on non-legume plants (e.g. secreting
503 phytohormones and siderophores, and exhibiting antagonistic effects towards many plant

504 pathogenic fungi) (Antoun et al., 1998; Shin et al., 2016). It is therefore frequently identified
505 as the most abundant diazotrophic taxa in different soil types across fertilized agriculture field
506 to natural forest (Fan et al., 2018; Han et al., 2019; Meng et al., 2019). *Bradyrhizobium* and
507 *Azospirillum* were also identified as the putative keystones potentially responsible for the
508 observed active N_2 fixation rate in a semi-arid desert based on $^{15}N_2$ labelling technology
509 (Miao et al., 2020). *Pseudacidovorax* is a newly discovered genus (Kämpfer et al., 2008), and
510 the members of *Pseudacidovorax* have been isolated from soil, plant endophyte and marine
511 water with high N_2 fixation capability being confirmed (Wedage et al., 2019; Zhang and Chen
512 2012). The module #2 was positively correlated with soil nitrogenase activity and
513 *Azospirillum*, *Bradyrhizobium* and *Pseudacidovorax* were present in all treatments with
514 varied proportion. All these suggested that *Azospirillum*, *Bradyrhizobium* and
515 *Pseudacidovorax* may act as the keystone taxa in diazotrophic community and play a vital
516 role in N_2 fixation in diverse habitats. In laboratory condition, the nitrogenase activity of
517 isolate *Pseudacidovorax austerolens* is sensitive to inorganic nitrogen addition and the
518 optimal pH is at 7.0 for both nitrogenase activity and its growth (Tyagi and Singh 2015).
519 Coincidingly, *Pseudacidovorax* were much more abundant in three non-chemical fertilization
520 treatment than in the chemical fertilizer treatments, suggesting that *Pseudacidovorax* is highly
521 sensitive to the chemical nitrogen input and soil acidification, but can be greatly enriched in
522 unfertilized soil and soils amended with organic manure. Furthermore, *Rhodopseudomonas*
523 was significantly enriched in NM and NPM treatments with a proportion of 19.98% ~39.53%.
524 *Rhodopseudomonas* has been identified as the active soil free-living diazotrophs based on
525 ^{15}N-RNA-SIP technique (Angel et al., 2018). Moreover, it was showed that

526 *Rhodopseudomonas palustris* could use multiple carbon compounds (including aromatic
527 compounds) as carbon source and electron donors (Sasika et al., 1994), and possessed three
528 types of nitrogenases and multiple pathways for energy metabolism (Larimer et al., 2004).
529 These may help to explain why *Pseudacidovorax* and *Rhodopseudomonas* were markedly
530 enriched in the NM and NPM treatments. Together with the significantly improved
531 nitrogenase activity in the NM and NPM treatments, it suggested that long-term organic
532 manure amendment had great potential to enrich some active diazotrophic group like
533 *Pseudacidovorax* and *Rhodopseudomonas* to drive high N_2 fixation and offset the inhibitory
534 effect of N fertilization in agriculture soil.

535 Interaction among species, rather than the presence or absence of species strongly
536 affected ecosystem function through directly regulating pathways of energy and material
537 flows or indirectly regulating abundance of functional microbial groups (Faust and Raes
538 2012). Considering the high similarity in soil properties and diazotrophic community traits
539 within N-NP-NPK treatments and within Control-NM-NPM treatments, we evaluated the
540 co-occurrence patterns of diazotrophic communities for the two groups. Our results showed
541 that the diazotrophic community had higher network complexity (more nodes and edges, and
542 higher average degree), stability (higher network robustness and negative correlation) and
543 'hub nodes' in three non-chemical fertilization treatments (Control-NM-NPM group) than in
544 three chemical fertilization treatments (N-NP-NPK group). The results were consistent with
545 previous studies, showing that organic fertilizer application significantly increased the
546 network complexity and stability than chemical fertilizer treatments for bacterial and
547 diazotrophic communities (Chen et al., 2020; Schmid et al., 2018). It has been suggested that

548 the interactions of species were generic and representative features of ecosystem function, and
549 more stronger interactions among species represented more active ecosystem process and
550 function (Faust and Raes 2012). Large-scale ecological surveys across the major
551 grain-producing areas of China also indicated that organic amendment strongly shaped soil
552 microbial community and co-occurrence pattern, and generated a more complex microbial
553 network with more functional modules and higher abundant functional groups involving in
554 carbon, nitrogen and phosphorus recycling in comparison to chemical fertilizer application
555 (Ling et al., 2016). In addition to the complexity of network, the types of interaction (e.g.,
556 cooperative and mutual exclusion) among species are also important facets of ecosystem
557 processes and functions (Röttjers and Faust 2018). It was suggested that the stronger
558 cooperative interaction in microbial community result in more coupling and positive feedback
559 and thus decrease the stability of community, while the higher proportion of negative
560 interaction is beneficial to ecosystem network stability and multifunctionality maintenance in
561 response to environmental perturbation over time (Coyte et al., 2015). We further found that
562 the diazotrophic network in the Control-NM-NPM group possessed a much higher proportion
563 of negative correlation and was more stable compared with the N-NP-NPK group. This could
564 be largely attributed to the mitigation of soil acidification in these treatments, as some
565 previous studies also suggested that soil pH is a major driver of diazotrophic community
566 assembly, and the network in neutral pH range (6.6 - 7.5) has a higher complexity and higher
567 proportion of negative correlations than aciddic or alkaline soils (Feng et al., 2018, Fan et al.,
568 2018, Wang et al., 2017). All these, together with the higher N_2-fixing potential in the Control
569 and two organic manure treatments, further confirmed that higher microbial network

570 complexity is beneficial to soil ecosystem functions such as nutrient cycling (Wagg et al.,
571 2019), and implied that the increased network complexity and stability induced by long-term
572 organic manure amendment greatly facilitate the resistance and adaptation of diazotrophic
573 community to the environmental stress caused by the large amount of chemical N fertilizer
574 input, and thus sustain the high N_2-fixing potential in the acidic red soil.

575 **5. Conclusion**

576 Overall, our study based a long-term fertilization field trial in an acidic red soil
577 suggested that inorganic chemical fertilizer containing N fertilizer application significantly
578 decreased soil nitrogenase activity and *nif*H gene abundance as a result of soil acidification
579 and inhibition by ammonia, while organic manure application greatly mitigated the inhibitory
580 effects of nitrogen fertilization on nitrogenase activity and *nif*H gene abundance. Contrary to
581 our hypothesis, phosphorus fertilizer application showed no obvious promotion effect on
582 nitrogenase activity and *nif*H gene abundance, which may be attributed to the dominant effect
583 of nitrogen fertilizer and organic mature and the lower phosphorus limitation threshold in this
584 soil. We further found that different long-term fertilization regimes largely changed
585 diazotrophic community composition due to the variations in soil properties such as soil pH,
586 TC and TC/TN ratio. Moreover, SEM analysis further revealed that organic manure
587 application positively influenced nitrogenase activity and crop yield by regulating soil pH and
588 keystone taxa which was mainly represented by *Bradyrhizobium*, *Pseudacidovorax* and
589 *Azospirillum*. Organic manure amendment significantly increased the diversity of diazotrophs
590 and specifically enriched diazotrophic taxa like *Pseudacidovorax* and *Rhodopseudomonas*
591 which is likely responsible for the high N_2-fixation potential. The co-occurrence network

analysis further suggested that the control and organic manure amendment treatments shaped a more complex and stable diazotrophic network, which potentially improved the resistance and adaptation ability to the environmental stress caused by the nitrogen fertilization over time. Collectively, our study provides strong field evidences showing that the application of organic manure combined with chemical fertilizers could effectively alleviate the inhibitory effect of nitrogen fertilization on N_2 fixation by regulating soil property, diazotrophic keystone, and shaping a more stable diazotrophic network. Our work shed new insight into the mechanisms of long-term fertilization affecting asymbiotic nitrogen fixation and provided a theoretical basis for manipulating soil microbiome for agricultural sustainability.

Acknowledgement

This work is funded by a joint Royal Society -National Natural Science Foundation of China (RS-NSFC) Newton Advanced Fellowship award (NAF/R1/180191, 41861130357). We would like to thank Gui-Bao Wang for assistance in field sampling, and Chao Xiong, An-Hui Ge for their generous help in data analyses.

Reference

Agler, M.T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S.T., Weigel, D., Kemen, E.M., 2016. Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLOS Biol. 14, e1002352. https://doi.org/10.1371/journal.pbio.1002352

Angel, R., Panhölzl, C., Gabriel, R., Herbold, C., Wanek, W., Richter, A., Eichorst, S.A., Woebken, D., 2018. Application of stable-isotope labelling techniques for the detection of active diazotrophs. Environ. Microbial. 20, 44-61. https://doi.org/10.1111/1462-2920.13954

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R., Lalande, R., 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). Plant Soil 204, 57-67. https://doi.org/10.1023/A:1004326910584

Camenzind, T., Hättenschwiler, S., Treseder, K., Lehmann, A., Rillig, M.C., 2018. Nutrient limitation of soil microbial processes in tropical forests. Ecol. Monogr. 88, 4-21. https://doi.org/10.1002/ecm.1279

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of earth's nitrogen cycle. Science 330, 192-196. http://dx.doi.org/10.1126/science.1186120

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335-336. https://doi.org/10.1038/nmeth.f.303

Chao, W., Zheng, M., Song, W., Wen, S., Wang, B., Zhu, C., Shen, R., 2017. Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China. Soil Biol. Biochem. 113, 240-249. https://doi.org/10.1016/j.soilbio.2017.06.019

Chen, H., Zheng, C.Y., Qiao, Y.Q., Du, S.Z., Li, W., Zhang, X.Q., Zhao, Z., Cao, C.F., Zhang, W.J., 2020. Long-term organic and inorganic fertilization alters the diazotrophic abundance, community structure, and co-occurrence patterns in a vertisol. Sci. Total Environ. in press. https://doi.org/10.1016/j.scitotenv.2020.142441

Dai, C.H., Wang, J.S., Dai, P., 2009. Soil fertility evolution and its effects on maize production in a long-term experiment on red soil. Plant Nutr. Fertil. Sci. 15, 1051-1056. http://dx.chinadoi.cn/10.3321/j.issn:1008-505X.2009.05.010

Clauset, A., Newman, M.E.J., Moore, C., 2004. Finding community structure in very large networks. Phys. Rev. E 70, 066111. https://doi.org/10.1103/PhysRevE.70.066111

Cleveland, C.C., Houlton, B.Z., Neill, C., Reed, S.C., Townsend, A.R., Wang, Y., 2010. Using indirect methods to constrain symbiotic nitrogen fixation rates: a case study from an Amazonian rain forest. Biogeochemistry 99, 1-13. https://doi.org/10.1007/s10533-009-9392-y

Coyte, K.Z., Schluter, J., Foster, K.R., 2015. The ecology of the microbiome: networks, competition, and stability. Science 350, 663-666.

https://doi.org/10.1126/science.aad2602
Csardi, G., Nepusz, T., 2006. The igraph software package for complex network research. Interjournal Complex System 1695, 1-9.
Dai, Z., Liu, G., Chen, H., Chen, C., Wang, J., Ai, S., Wei, D., Li, D., Ma, B., Tang, C., Brookes, P.C., Xu, J., 2020. Long-term nutrient inputs shift soil microbial functional profiles of phosphorus cycling in diverse agroecosystems. ISME J. 14, 757-770. https://doi.org/10.1038/s41396-019-0567-9
Dixon, P., 2003. VEGAN., a package of R functions for community ecology. J. Veg. Sci. 14, 927-930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x
Du, E., Terrer, C., Pellegrini, A.F.A., Ahlström, A., Van Lissa, C.J., Zhao, X., Xia, N., Wu, X., Jackson, R.B., 2020. Global patterns of terrestrial nitrogen and phosphorus limitation. Nat. Geosci. 13, 221-226. https://doi.org/10.1038/s41561-019-0530-4
Dynarski, K.A., Houlton, B.Z., 2018. Nutrient limitation of terrestrial free-living nitrogen fixation. New Phytol. 217, 1050-1061. https://doi.org/10.1111/nph.14905
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461. https://doi.org/10.1093/bioinformatics/btq461
Faith, D.P., 1992. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61, 1-10. https://doi.org/10.1016/0006-3207(92.91201-3
Fan, K., Delgado-Baquerizo, M., Guo, X., Wang, D., Wu, Y., Zhu, M., Yu, W., Yao, H., Zhu, Y., Chu, H., 2019. Suppressed N fixation and diazotrophs after four decades of fertilization. Microbiome 7, 143. https://doi.org/10.1186/s40168-019-0757-8
Fan, K., Weisenhorn, P., Gilbert, J.A., Shi, Y., Bai, Y., Chu, H., 2018. Soil pH correlates with the co-occurrence and assemblage process of diazotrophic communities in rhizosphere and bulk soils of wheat fields. Soil Biol. Biochem. 121, 185-192. https://doi.org/10.1016/j.soilbio.2018.03.017
Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 10, 538-550. https://doi.org/10.1038/nrmicro2832
Feng M., Adams J.M., Fan K., Shi Y., Sun R., Wang D., Guo X., Chu H., 2018. Long-term fertilization influences community assembly processes of soil diazotrophs. Soil Biol. Biochem. 126, 151-158. http:// 10.1016/j.soilbio.2018.08.021
Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil bacteria. Ecology 88, 1354-1364. https://doi.org/10.1890/05-1839
Franz, S., Richard, O., Ellen, K., Rosa, M., 1996. Methods in soil biology. Springer-Verlag, Berlin Heidelberg New York.
Gaby, J.C., Buckley, D.H., 2014. A comprehensive aligned *nif*H gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. Database (Oxford) 2014, bau001. https://doi.org/10.1093/database/bau001
Garcia, N.S., Fu, F., Sedwick, P.N., Hutchins, D.A., 2015. Iron deficiency increases growth and nitrogen-fixation rates of phosphorus-deficient marine cyanobacteria. ISME J. 9, 238-245. https://doi.org/10.1038/ismej.2014.104
Gilbert, N., 2009. Environment: the disappearing nutrient. Nature 461, 716-718. https://doi.org/10.1038/461716a
Guo, J.H., Liu, X.J., Zhang, Y., Shen, J.L., Han, W.X., Zhang, W.F., Christie, P., Goulding, K.W.T., Vitousek, P.M., Zhang, F.S., 2010. Significant acidification in major chinese

croplands. Science 327, 1008-1010. https://doi.org/10.1126/science.1182570
Gupta, V.V.S.R., Roper, M.M., Roget, D.K., 2006. Potential for non-symbiotic N_2-fixation in different agroecological zones of southern Australia. Soil Res. 44, 343-354. https://doi.org/10.1071/SR05122
Han, L.L., Wang, Q., Shen, J.P., Di, H.J., Wang, J.T., Wei, W.X., Fang, Y.T., Zhang, L.M., He, J.Z., 2019. Multiple factors drive the abundance and diversity of the diazotrophic community in typical farmland soils of China. FEMS Microbiol. Ecol. 95, fiz113. https://doi.org/10.1093/femsec/fiz113
Hartman, K., van der Heijden, M.G.A., Wittwer, R.A., Banerjee, S., Walser, J.C., Schlaeppi, K., 2018. Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. Microbiome 6, 14. https://doi.org/10.1186/s40168-017-0389-9
Hedin, L.O., Brookshire, E.N.J., Menge, D.N.L., Barron, A.R., 2009. The Nitrogen paradox in tropical forest ecosystems. Annu. Rev. Ecol. Evol. Syst. 40, 613-635. https://doi.org/10.1146/annurev.ecolsys.37.091305.110246
Hill, S., 1988. How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. 54, 111-129. https://doi.org/10.1016/0378-1097(88.90172-3
Hou, E., Luo, Y., Kuang, Y., Chen, C., Lu, X., Jiang, L., Luo, X., Wen, D., 2020. Global meta-analysis shows pervasive phosphorus limitation of aboveground plant production in natural terrestrial ecosystems. Nat. Commun. 11, 637. https://doi.org/10.1038/s41467-020-14492-w
Hu, H.W., Chen, D., He, J.Z., 2015. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. FEMS microbiol. Rev. 39, 729-749. https://doi.org/10.1093/femsre/fuv021
Huang, X., Zhou, X., Zhang, J., Cai, Z., 2019. Highly connected taxa located in the microbial network are prevalent in the rhizosphere soil of healthy plant. Biol. Fertil. Soils 55, 299-312. https://doi.org/10.1007/s00374-019-01350-1
Kämpfer, P., Thummes, K., Chu, H.L., Tan, C.C., Arun, A.B., Chen, W.M., Lai, W.A., Shen, F.T., Rekha, P.D., Young, C.C., 2008. *Pseudacidovorax intermedius* gen. nov., sp. nov., a novel nitrogen-fixing betaproteobacterium isolated from soil. Int. J. Syst. Evol. Microbiol. 58, 491-495. https://doi.org/10.1099/ijs.0.65175-0
Ladha, J.K., Tirol-Padre, A., Reddy, C.K., Cassman, K.G., Verma, S., Powlson, D.S., van Kessel, C., Richter, D.B., Chakraborty, D., Pathak, H., 2016. Global nitrogen budgets in cereals: a 50-year assessment for maize, rice and wheat production systems. Sci. Rep. 6, 19355. http://doi.org/10.1038/srep19355
Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M.L., Pelletier, D.A., Beatty, J.T., Lang, A.S., Tabita, F.R., Gibson, J.L., Hanson, T.E., Bobst, C., Torres, J.L.T., Peres, C., Harrison, F.H., Gibson, J., Harwood, S.C., 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nat. Biotechnol. 22, 55-61. https://doi.org/10.1038/nbt923
Li, Y., Pan, F., Yao, H., 2019. Response of symbiotic and asymbiotic nitrogen-fixing microorganisms to nitrogen fertilizer application. J. Soils Sediments 19, 1948-1958. https://doi.org/10.1007/s11368-018-2192-z

741 Li, Y., Yuan, L., Xue, S., Liu, B., Jin, G., 2021. Artificial root exudates excite bacterial
742 nitrogen fixation in the subsurface of mine soils. Appl. Soil Ecol. 157, 103774.
743 https://doi.org/10.1016/j.apsoil.2020.103774
744 Liao, H., Li, Y., Yao, H., 2017. Fertilization with inorganic and organic nutrients changes
745 diazotroph community composition and N-fixation rates. J. Soil. Sediment. 18,
746 1076-1086. https://doi.org/10.1007/s11368-017-1836-8
747 Ling, N., Zhu, C., Xue, C., Chen, H., Duan, Y., Peng, C., Guo, S., Shen, Q., 2016. Insight into
748 how organic amendments can shape the soil microbiome in long-term field
749 experiments as revealed by network analysis. Soil Biol. Biochem. 99, 137-149.
750 https://doi.org/10.1016/j.soilbio.2016.05.005
751 Masepohl, B., Hallenbeck, P.C., 2010. Nitrogen and molybdenum control of nitrogen fixation
752 in the phototrophic bacterium *Rhodobacter capsulatus*. Recent Advances in
753 Phototrophic Prokaryotes 675, 49-70. https://doi.org/10.1007/978-1-4419-1528-3_4
754 Meng, H., Zhou, Z., Wu, R., Wang, Y., Gu, J.D., 2019. Diazotrophic microbial community
755 and abundance in acidic subtropical natural and re-vegetated forest soils revealed by
756 high-throughput sequencing of *nif*H gene. Appl. Microbiol. Biot. 103, 995-1005.
757 https://doi.org/10.1007/s00253-018-9466-7
758 Miao, L., Feng, W., Zhang, Y., Bai, Y., Sun, Y., She, W., Mao, H., Lai, Z., Qin, S., 2020.
759 Chemoheterotrophic diazotrophs contribute to nitrogen incorporation in a semiarid
760 desert. Biol. Fertil. Soils 56, 1165-1176. https://doi.org/10.1007/s00374-020-01492-7
761 Mills, M.M., Ridame, C., Davey, M., La, R.J., Geider, R.J., 2004. Iron and phosphorus
762 co-limit nitrogen fixation in the eastern tropical north Atlantic. Nature 429, 292-294.
763 https://doi.org/10.1038/nature02550
764 Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of
765 phosphate in natural waters. Anal. Chim. Acta 27, 31-36.
766 https://doi.org/10.1016/S0003-2670(00.88444-5
767 Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., Boucher, O.,
768 Godderis, Y., Hinsinger, P., Llusia, J., Nardin, E., Vicca, S., Obersteiner, M., Janssens,
769 I.A., 2013. Human-induced nitrogen-phosphorus imbalances alter natural and
770 managed ecosystems across the globe. Nat. Commun. 4, 2934.
771 https://doi.org/10.1038/ncomms3934
772 Poly, F., Monrozier, L.J., Bally, R., 2001. Improvement in the RFLP procedure for studying
773 the diversity of *nif*H genes in communities of nitrogen fixers in soil. Res. Microbiol.
774 152, 95-103. https://doi.org/10.1016/S0923-2508(00.01172-4
775 Reed, S.C., Cleveland, C.C., Townsend, A.R., 2011. Functional ecology of free-living
776 nitrogen fixation: a contemporary perspective. Annu. Rev. Ecol. Evol. Syst. 42,
777 489-512. https://doi.org/10.1146/annurev-ecolsys-102710-145034
778 Rodríguez-Blanco, A., Sicardi, M., Frioni, L., 2015. Plant genotype and nitrogen fertilization
779 effects on abundance and diversity of diazotrophic bacteria associated with maize
780 (*Zea mays* L.). Biol. Fertil. Soils 51, 391-402.
781 https://doi.org/10.1007/s00374-014-0986-8
782 Roper, M.M., Gupta, V.V.S.R., 2016. Enhancing non-symbiotic N_2 fixation in agriculture. The
783 Open Agriculture Journal 10, 7-27. http://dx.doi.org/10.2174/1874331501610010007
784 Röttjers, L., Faust, K., 2018. From hairballs to hypotheses-biological insights from microbial

785 networks. FEMS Microbiol. Rev. 42, 761-780. https://doi.org/10.1093/femsre/fuy030
786 Sanudo-Wilhelmy, S.A., Kustka, A.B., Gobler, C.J., Hutchins, D.A.,Yang, M., Lwiza, K.,
787 Burns, J., Capone, D.G., Raven, J.A., Carpenter, E.J., 2001. Phosphorus limitation of
788 nitrogen fixation by *Trichodesmium* in the central Atlantic ocean. Nature 411, 66-69.
789 https://doi.org/10.1038/35075041
790 Sasika, C., Ramana, C.V., Raghuveer Rao, P., 1994. Nitrogen fixation by *Rhodopseudomonas*
791 *palustris* OU 11 with aromatic compounds as carbon source/electron donors. FEMS
792 Microbiol. Lett. 122, 75-78. https://doi.org/10.1111/j.1574-6968.1994.tb07146.x
793 Schmid, C.A.O., Schröder, P., Armbruster, M., Schloter, M., 2018. Organic amendments in a
794 long-term field trial—consequences for the bulk soil bacterial community as revealed
795 by network analysis. Microb. Ecol. 76, 226-239.
796 https://doi.org/10.1007/s00248-017-1110-z
797 Shi, H.W., Wang, L.Y., Li, X.X., Liu, X.M., Hao, T.Y., He, X.J., Chen, S.F., 2016.
798 Genome-wide transcriptome profiling of nitrogen fixation in *Paenibacillus* sp.
799 WLY78. BMC Microbiol. 16, 25. https://doi.org/10.1186/s12866-016-0642-6
800 Shin, W., Islam, R., Benson, A., Joe, M.M., Kim, K., Gopal, S., Samaddar, S., Banerjee, S., Sa,
801 T., 2016. Role of diazotrophic bacteria in biological nitrogen fixation and plant
802 growth improvement. Korean J Soil Sci. Fertil. 49, 17-29.
803 https://doi.org/10.7745/KJSSF.2016.49.1.017
804 Tang, Y., Zhang, M., Chen, A., Zhang, W., Wei, W., Rong, S., 2017. Impact of fertilization
805 regimes on diazotroph community compositions and N_2 -fixation activity in paddy
806 soil. Agric. Ecosyst. Environ. 247, 1-8. https://doi.org/10.1016/j.agee.2017.06.009
807 Tyagi, S., Singh, D.K., 2015. *Pseudacidovorax austerolens* sp. nov., a *nif*H bacterium isolated
808 from Himalayan valley soil, India. Ann. Microbiol. 65, 217-223.
809 https://doi.org/10.1007/s13213-014-0852-9
810 Van Deynze, A., Zamora, P., Delaux, P.M., Heitmann, C., Jayaraman, D., Rajasekar, S.,
811 Graham, D., Maeda, J., Gibson, D., Schwartz, K.D., Berry, A.M., Bhatnagar, S.,
812 Jospin, G., Darling, A., Jeannotte, R., Lopez, J., Weimer, B.C., Eisen, J.A., Shapiro,
813 H.Y., Ané, J.M., Bennett, A.B., 2018. Nitrogen fixation in a landrace of maize is
814 supported by a mucilage-associated diazotrophic microbiota. PLOS Biol. 16,
815 e2006352. https://doi.org/10.1371/journal.pbio.2006352
816 Vitousek, P.M., Menge, D.N.L., Reed, S.C., Cleveland, C.C., 2013. Biological nitrogen
817 fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philos. Trans.
818 R. Soc. Lond., B, Biol. Sci. 368, 20130119. https://doi.org/10.1098/rstb.2013.0119
819 Vitousek, P.M., Porder, S., Houlton, B.Z., Chadwick, O.A., 2010. Terrestrial phosphorus
820 limitation: mechanisms, implications, and nitrogen-phosphorus interactions. Ecol.
821 Appl. 20, 5-15. https://doi.org/10.1098/rstb.2013.0119
822 Wagg, C., Schlaeppi, K., Banerjee, S., Kuramae, E.E., van der Heijden, M.G.A., 2019.
823 Fungal-bacterial diversity and microbiome complexity predict ecosystem functioning.
824 Nat. Commun. 10, 4841. http://dx.doi.org/10.1038/s41467-019-12798-y
825 Wakelin, S.A., Gupta, V.V.S.R., Forrester, S.T., 2010. Regional and local factors affecting
826 diversity, abundance and activity of free-living, N_2-fixing bacteria in Australian
827 agricultural soils. Pedobiologia 53, 391-399.
828 https://doi.org/10.1016/j.pedobi.2010.08.001

Wang, Q., Quensen, J.F., Fish, J.A., Kwon Lee, T., Sun, Y., Tiedje, J.M., Cole, J.R., 2013. Ecological patterns of *nif*H genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. Mbio 4, e00592-13. https://doi.org/10.1128/mBio.00592-13

Wang Y., Li C., Kou Y., Wang J., Tu B., Li H., Li X., Wang C., Yao M., 2017. Soil pH is a major driver of soil diazotrophic community assembly in Qinghai-Tibet alpine meadows. Soil Biol. Biochem. 115, 457-555. https://doi.org/10.1016/j.soilbio.2017.09.024

Wang, Q., Wang, J., Li, Y., Chen, D., Ao, J., Zhou, W., Shen, D., Li, Q., Huang, Z., Jiang, Y., 2018. Influence of nitrogen and phosphorus additions on N_2-fixation activity, abundance, and composition of diazotrophic communities in a chinese fir plantation. Sci. Total Environ. 619, 1530-1537. https://doi.org/10.1016/j.scitotenv.2017.10.064

Wang, X., Liu, B., Ma, J., Zhang, Y., Hu, T., Zhang, H., Feng, Y., Pan, H., Xu, Z., Liu, G., Lin, X., Zhu, J., Bei, Q., Xie, Z., 2019. Soil aluminum oxides determine biological nitrogen fixation and diazotrophic communities across major types of paddy soils in China. Soil Biol. Biochem. 131, 81-89. https://doi.org/10.1016/j.soilbio.2018.12.028

Wedage, W.M.M., Aberathne, A.H.M.N.R., Harischandra, I.N., Gunawardana, D., 2019. A nodulation-proficient nonrhizobial inhabitant of *Pueraria phaseoloides*. Sci. World J. 2019, 2019. https://doi.org/10.1155/2019/9782684

Xiao, D., Xiao, L., Che, R., Tan, Y., Liu, X., Yang, R., Zhang, W., He, X., Wang, K., 2020. Phosphorus but not nitrogen addition significantly changes diazotroph diversity and community composition in typical karst grassland soil. Agric. Ecosyst. Environ. 301, 106987. https://doi.org/10.1016/j.agee.2020.106987

Xiong, C., He, J.Z., Singh, B.K., Zhu, Y.G., Wang, J.T., Li, P.P., Zhang, Q.B., Han, L.L., Shen, J.P., Ge, A.H., Wu, C.F., Zhang, L.M., 2021. Rare taxa maintain the stability of crop mycobiomes and ecosystem functions. Environ. Microbiol. in press. https://doi.org/10.1111/1462-2920.15262

Xiong, C., Zhu, Y.G., Wang, J.T., Singh, B., Han, L.L., Shen, J.P., Li, P.P., Wang, G.B., Wu, C.F., Ge, A.H., Zhang, L.M., He, J.Z., 2021. Host selection shapes crop microbiome assembly and network complexity. New Phytol. 229, 1091-1104. https://doi.org/10.1111/nph.16890

Xun, W., Wei, L., Huang, T., Yi, R., Wu, X., Miao, Y., Wei, R., Li, D., Shen, Q., Zhang, R., 2018. Long-term agronomic practices alter the composition of asymbiotic diazotrophic bacterial community and their nitrogen fixation genes in an acidic red soil. Biol. Fertil. Soils 54, 329–339. https://doi.org/10.1007/s00374-018-1264-y

Yao, Q., Li, Z., Song, Y., Wright, S.J., Guo, X., Tringe, S.G., Tfaily, M.M., Paša-Tolić, L., Hazen, T.C., Turner, B.L., Mayes, M.A., Pan, C., 2018. Community proteogenomics reveals the systemic impact of phosphorus availability on microbial functions in tropical soil. Nat. Ecol. Evol. 2, 499-509. https://doi.org/10.1038/s41559-017-0463-5

Zhang, L.H., Chen, S.F., 2012. *Pseudacidovorax intermedius* NH-1, a novel marine nitrogen-fixing bacterium isolated from the south China sea. World J. Microbiol. Biotechnol. 28, 2839-2847. https://doi.org/10.1007/s11274-012-1093-3

Zhao, Z.B., He, J.Z., Geisen, S., Han, L.L., Wang, J.T., Shen, J.P., Wei, W.X., Fang, Y.T., Li, P.P., Zhang, L.M., 2019. Protist communities are more sensitive to nitrogen

873 fertilization than other microorganisms in diverse agricultural soils. Microbiome 7, 33.
874 https://doi.org/10.1186/s40168-019-0647-0
875 Zheng, M., Li, D., Lu, X., Zhu, X., Zhang, W., Huang, J., Fu, S., Lu, X., Mo, J., 2016. Effects
876 of phosphorus addition with and without nitrogen addition on biological nitrogen
877 fixation in tropical legume and non-legume tree plantations. Biogeochemistry 131,
878 65-76. https://doi.org/10.1007/s10533-016-0265-x
879 Zhong, Y., Yan, W., Shangguan, Z., 2015. Impact of long-term N additions upon coupling
880 between soil microbial community structure and activity, and nutrient-use efficiencies.
881 Soil Biol. Biochem. 91, 151-159. https://doi.org/10.1016/j.soilbio.2015.08.030
882

Table 1. Soil physiochemical properties in different treatments and the significance difference analyses based on two-way ANOVA for the effect of fertilization and sampling season on soil properties

Treatments	SWC (%)	NH_4^+-N(mg/kg)	NO_3^--N (mg/kg)	AP (mg/kg)	pH	TC (%)	TN (%)	TP ($g \cdot kg^{-1}$)	TC/TN
				Summer					
Control	0.28±0.01(ab)	11.76±1.50(c)	31.25±5.92(d)	9.01±2.62(c)	6.60±0.04(a)	2.32±0.29(b)	0.14±0.01(c）	0.76±0.05(b)	16.04±1.79(a)
N	0.27±0.01(b)	16.26±0.61(b)	66.22±16.59(c)	10.29±5.47(c)	4.61±0.26(c)	1.67±0.129(c）	0.12±0.01(c）	0.59±0.02(c）	13.98±0.23(b)
NP	0.28±0.01(ab)	11.45±1.87(c)	55.76±30.90(cd)	154.85±35.91(a)	4.52±0.00(c)	1.89±0.09(c）	0.14±0.01(c）	1.26±0.06(a)	13.68±0.36(b)
NM	0.28±0.02(ab)	9.90±0.50(c)	135.40±12.95(b)	20.21±0.97(c)	5.80±0.26(b)	2.56±0.15(ab)	0.20±0.02(b)	0.76±0.08(b)	12.75±0.40(b)
NPM	0.29±0.01(a)	11.49±2.10(c)	203.27±3.11(a)	113.72±7.14(b)	5.95±0.18(b)	2.90±0.27(a)	0.23±0.03(a)	1.37±0.10(a)	12.47±0.52(b)
NPK	0.29±0.01(a)	24.55±3.51(a)	34.55±2.61(d)	126.53±30.29(ab)	4.70±0.05(c)	1.82±0.16(c）	0.13±0.01(c）	1.21±0.17(a)	13.61±0.41(b)
				Autumn					
Control	0.22±0.00(B)	1.28±0.79(A)	5.90±0.97(C)	9.14±2.51(E)	6.57±0.07(A)	2.15±0.24(B)	0.14±0.01(B)	0.54±0.05（C)	14.82±1.42(A)
N	0.24±0.01(AB)	1.42±0.18(A)	42.24±12.83(B)	5.41±1.15(E)	4.54±0.06(C)	1.68±0.14(C）	0.12±0.01(C）	0.46±0.01(BC)	14.23±0.47(AB)
NP	0.25±0.01(A)	1.63±0.94(A)	64.95±2.38(A)	114.69±6.56(A)	4.56±0.05(C)	1.86±0.12(BC)	0.14±0.01(BC)	1.25±0.10(A)	13.55±0.35(BC)
NM	0.24±0.02(AB)	0.61±0.06(A)	70.13±2.15(A)	27.28±1.90(D)	5.84±0.11(B)	2.67±0.04(A)	0.22±0.00(A)	0.71±0.03(B)	12.13±0.43(D)
NPM	0.25±0.01(A)	0.40±0.08(A)	67.38±1.53(A)	99.25±8.73(B)	5.98±0.13(B)	2.76±0.02(A)	0.22±0.00(A)	1.28±0.15(A)	12.46±0.26(CD)
NPK	0.25±0.01(A)	1.59±1.83(A)	49.16±12.66(B)	77.71±11.64(C)	4.71±0.05(C)	1.98±0.34(BC)	0.15±0.03(B)	1.17±0.19(A)	13.14±0.32(BCD)
Treatment(*P*)	0.057	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Season(*P*)	<0.001	<0.001	<0.001	0.002	0.692	0.863	0.469	0.014	0.154
Treatment × Season(*P*)	0.2	<0.001	<0.001	0.013	0.998	0.623	0.484	0.53	0.604

Note: Control: non-fertilizer control; N: inorganic nitrogen fertilizer only; NP: inorganic nitrogen and phosphorus fertilizers; NM: inorganic N fertilizer plus organic manure; NPM: inorganic nitrogen, phosphorus fertilizers plus organic manure; NPK: inorganic nitrogen, phosphorus and potassium fertilizers. SWC: soil water content; NH_4^+-N: ammonium nitrogen; NO_3^--N: nitrate nitrogen; AP: available phosphorus; TC: total carbon; TN: total nitrogen; TP: total phosphorus; TC/TN: carbon-nitrogen ratio. Data in the table were mean ± standard deviation (n = 3). Different lowercase and uppercase letters represent significant differences ($P < 0.05$) among treatments in the summer and autumn season, respectively. The same as below.

883 **Figure legends**

884 Fig. 1.Effects of different fertilization practices on (a) maize yield (mean value of 2017-2019), (b) soil
885 respiration rate (SR), (c) microbial biomass carbon (MBC) and (d) soil respiration quotient (SQ). MBC
886 and SQ in samples from summer were not measured. Control: non-fertilizer control; N: inorganic
887 nitrogen fertilizer only; NP: inorganic nitrogen and phosphorus fertilizers; NM: inorganic N fertilizer
888 plus organic manure; NPM: inorganic nitrogen, phosphorus fertilizers plus organic manure; NPK:
889 inorganic nitrogen, phosphorus and potassium fertilizers. Different lowercase and uppercase letters
890 represent significant differences ($P < 0.05$) among treatments in the summer and autumn season,
891 respectively. Vertical bars indicate standard deviation of three replicates.

892 **Fig. 2.** Soil nitrogenase activity based on acetylene reduction method (a) and the *nif*H gene
893 abundances (b) among different fertilization treatments. Different lowercase and uppercase
894 letters represent significant differences ($P < 0.05$) among treatments in the summer and
895 autumn season, respectively. Vertical bars indicate standard deviation of three replicates. The
896 treatment denotations are the same as Fig. 1.

897 **Fig. 3.** Non-metric multidimensional scaling analysis (NMDS) (a) and redundancy analysis
898 (RDA) (b) of diazotrophs community among different fertilization treatments. The different
899 colors and shapes represent the different treatments and sampling stages, respectively. And the
900 "*" next to the arrows represent the variables significantly influencing diazotrophic
901 community. Significant levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The treatment
902 denotations are the same as Fig. 1.

903 **Fig. 4.** The relative abundances of top 10 *nif*H gene phylotypes at the genus level in different
904 treatments. Vertical bars indicate standard deviation of three replicates within each treatment.

905 The treatment denotations are the same as in Fig. 1.

906 **Fig. 5.** Network diagram with nodes colored according to different treatments (a). The nodes
907 were grouped into three ecological clusters (modules #1, #2 and #5). The relative abundance
908 of dominant diazotrophic genera in three main ecological clusters (b). The cumulative relative
909 abundance of three ecological clusters in different fertilization treatments (c). Regressions
910 relationship between the nitrogenase activity and the cumulative relative abundance of the
911 main diazotrophic ecological clusters (d).

912 **Fig. 6.** The co-occurrence network of diazotrophic community within non-chemical fertilizer
913 treatment group and chemical fertilizer treatment group (a), "networks hubs" of network
914 within each group (b), degree distribution pattern (c) and robust analysis of network (d). Note:
915 the non-chemical fertilizer group includs the N, NP and NPK treatments, and the chemical
916 fertilizer group includs the Control, NM and NPM treatments.

917 **Fig. 7.** Structural equation modeling (SEM) showing the effects of nitrogen, phosphorus and
918 organic manure fertilizer inputs on nitrogenase activity and crop yield (a), and the
919 standardized total effect (STE) derived from SEM (b). Note: the red and black arrows
920 represent the positive and negative impact, respectively. The dotted arrow indicates
921 non-significant effect between two variables. The thickness of the arrow and the value next to
922 the arrow represent the size of the path coefficient, and r^2 represents the variance that can be
923 explained by endogenous variables. Chi_Squre, *P*_value, NFI, IFI and RMSEA represent the
924 degree of model fitting. Significance level: $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

925

926

Supplementary tables and figures:

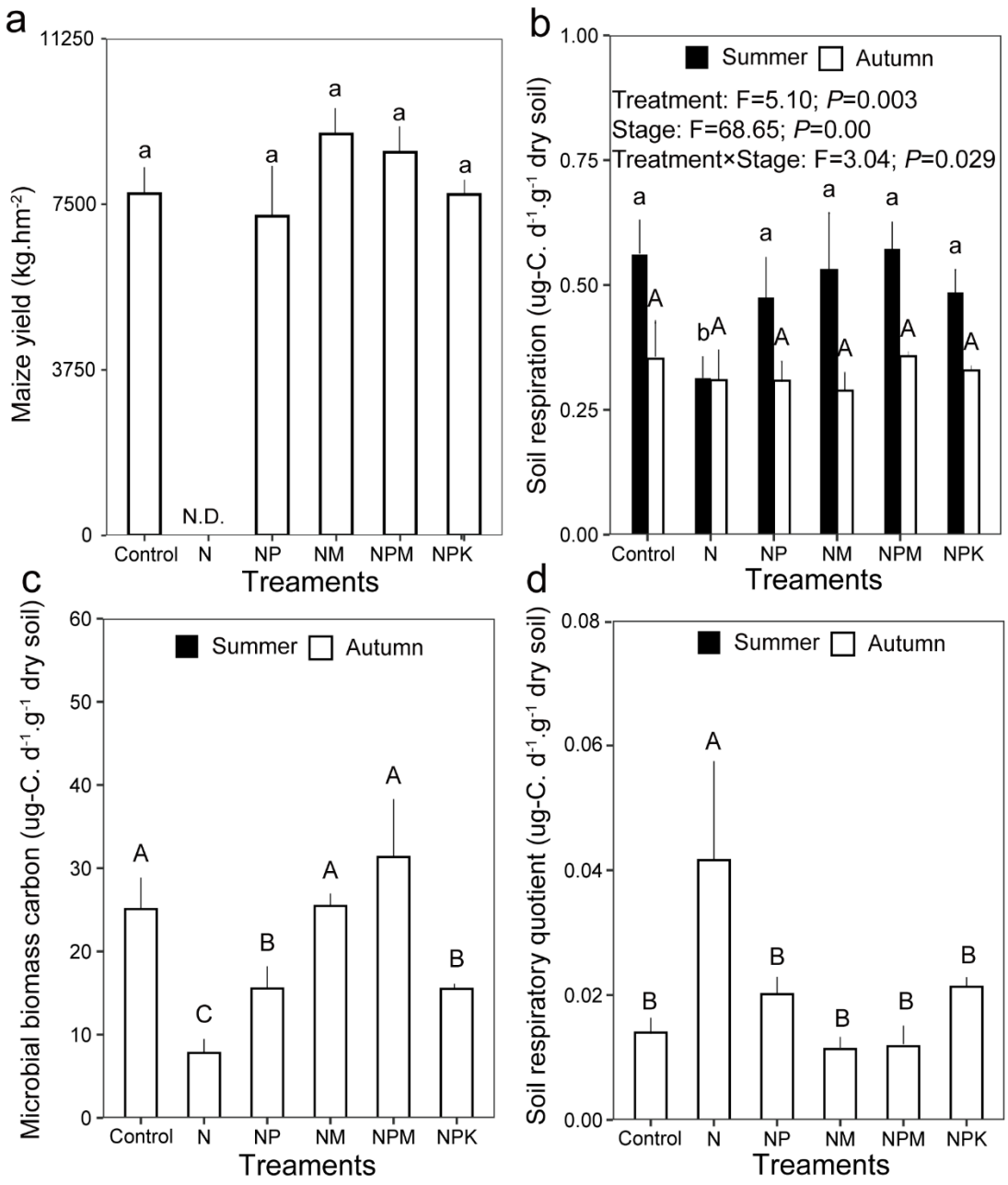
Table S1. The alpha- and phylogenetic diversity of *nif*H genes among different fertilization treatments

Table S2. ADONIS (PERMANOVA) analyses for the effect of fertilization practice and season on diazotrophic community

Table S3. Spearman correlation between nitrogenate activity, *nif*H gene abundance, PD, and the cumulative relative abundance of module 2 and soil physiochemical properties.

Fig. S1. Regression relationship between soil nitrogenase activity and the cumulative relative abundance of dominant genera and in module #1, #2 and #5.

937 Figure 1



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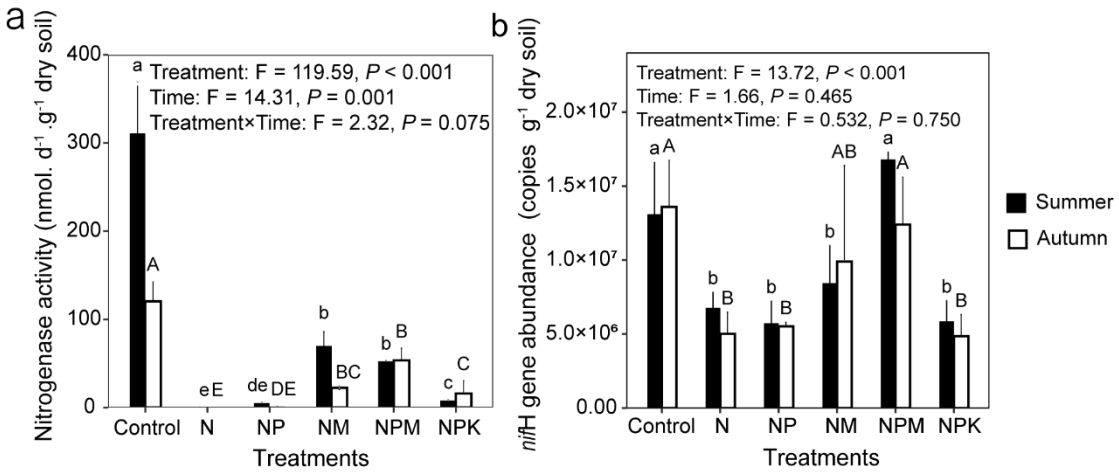
939 Figure 2



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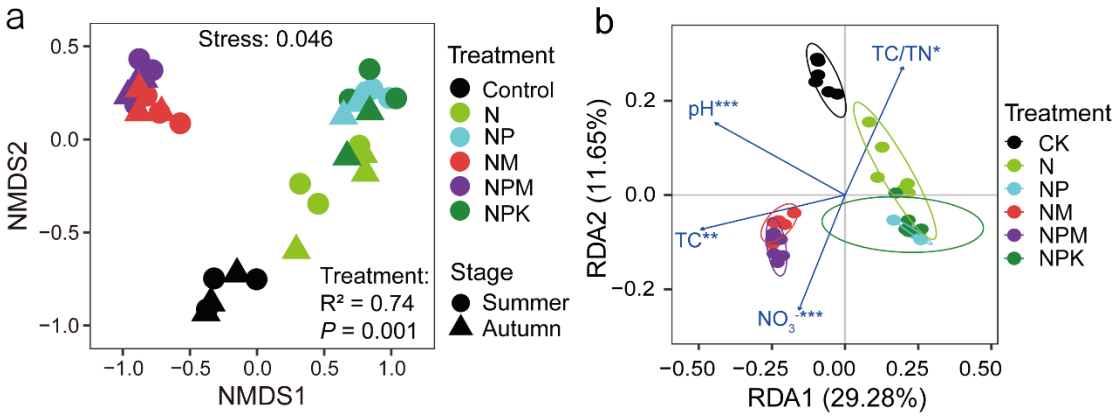

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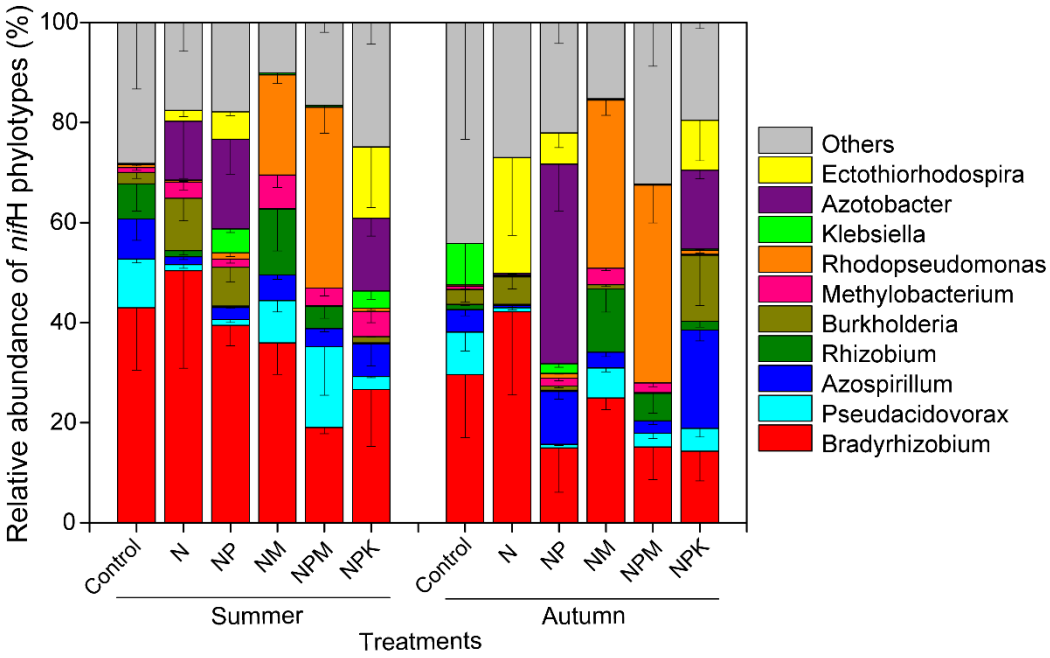

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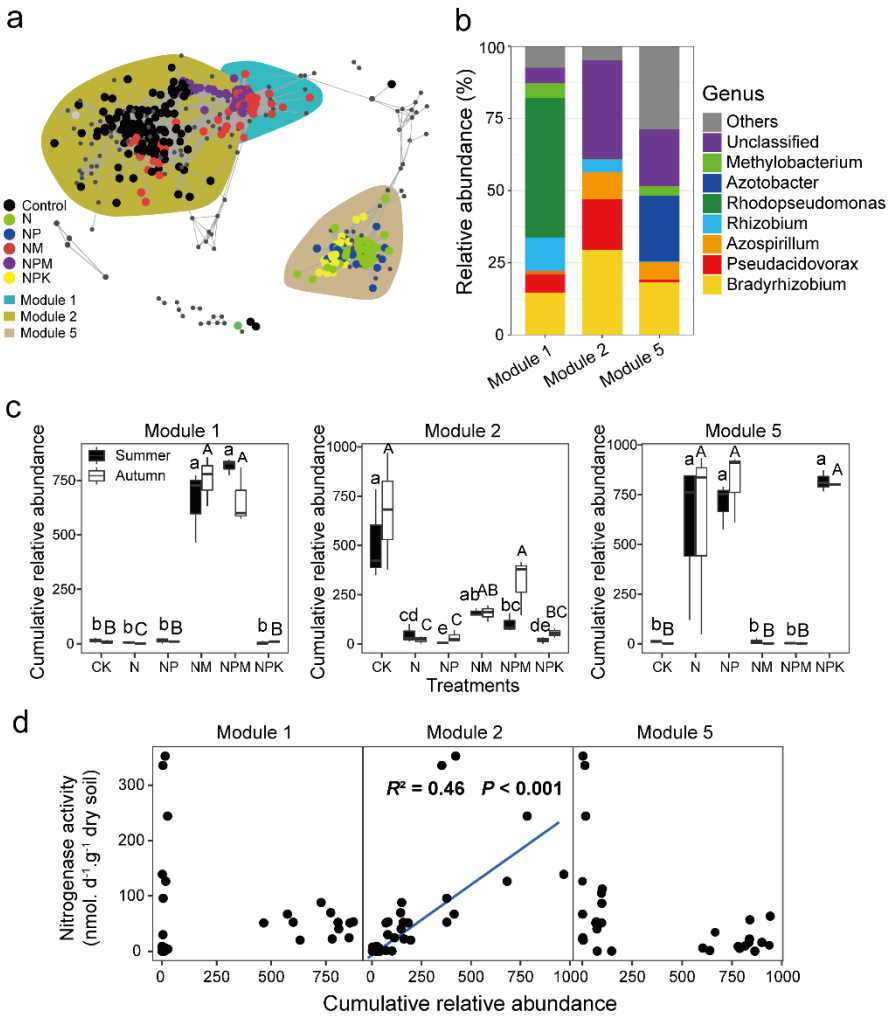
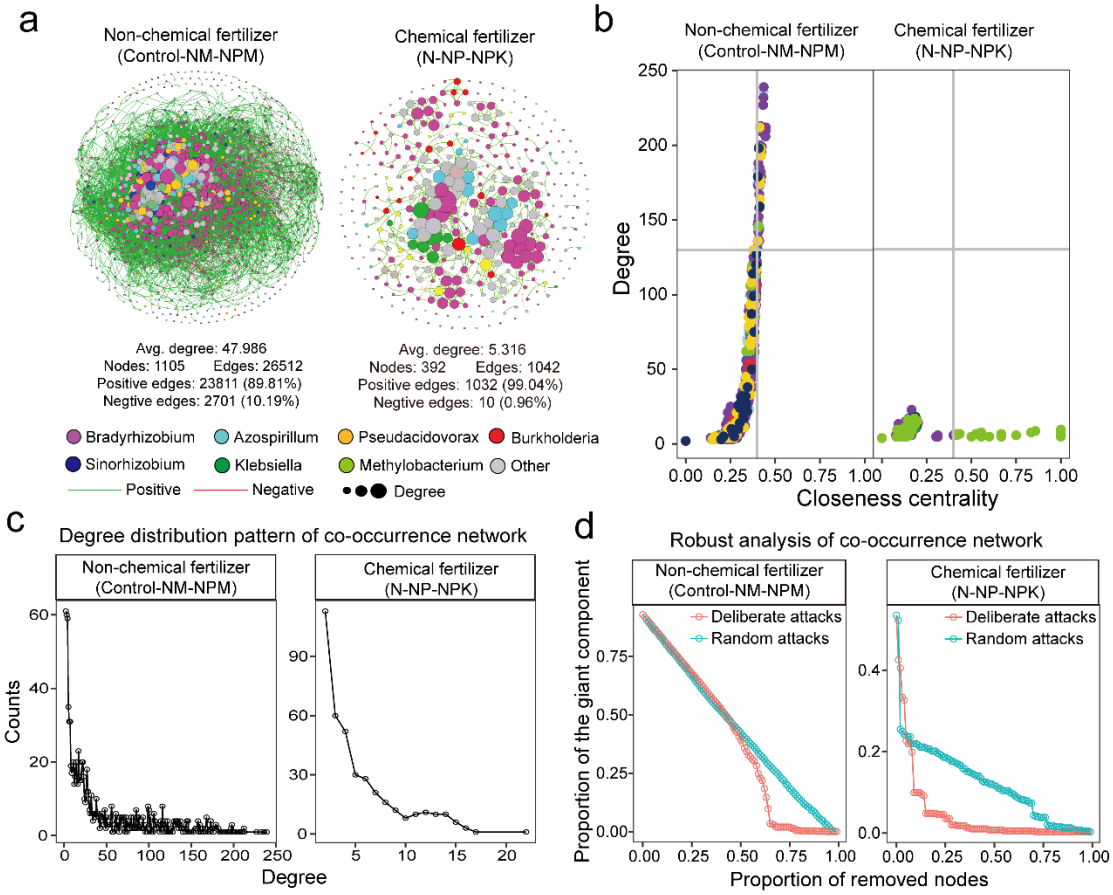
945 Figure 5



946

947 Figure 6



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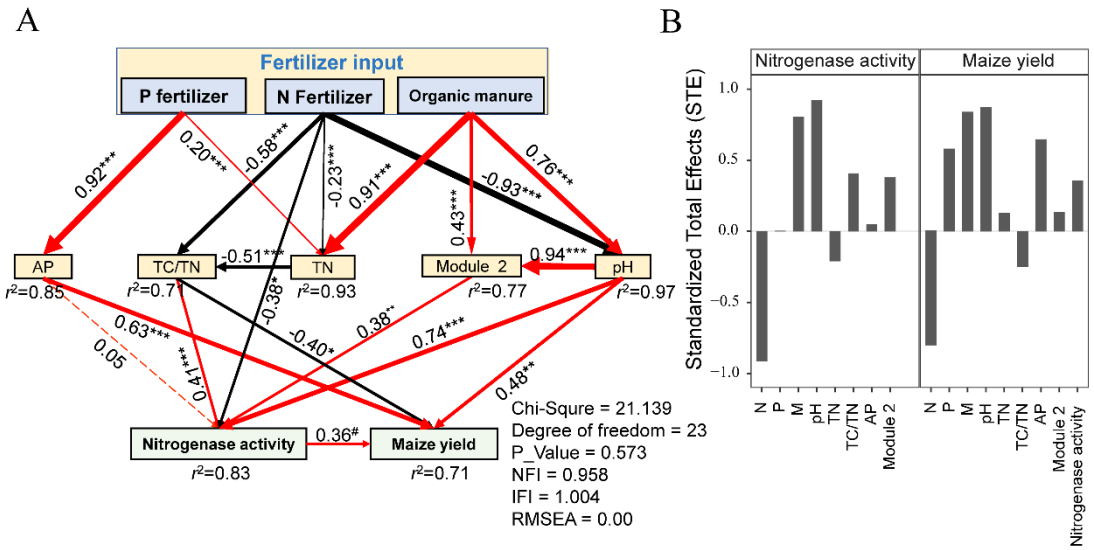
950 Figure 7



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952 Supplementary tables and figures

Table S1. The alpha diversity of diazotrophic community among different fertilization treatments.

	Summer			Autumn		
	Chao1	PD	Shannon	Chao1	PD	Shannon
Control	643.69(176.29) b	62.37(9.97) b	4.96(0.78) ab	607.92(64.35) b	59.64(5.40) b	3.92(2.10) a
N	418.35(150.07) b	51.63(10.55) b	3.96(0.33) c	341.74(96.83) c	45.12(9.09) b	3.15(0.68) a
NP	350.72(38.74) b	47.09(5.25) b	4.45(0.20) bc	357.98(25.41) c	49.14(5.67) b	3.62(0.84) a
NM	1077.71(256.42) a	88.62(13.49) a	5.71(0.57) a	1038.91(104.47) a	88.43(6.50) a	5.09(0.45) a
NPM	986.68(224.87) a	86.36(12.81) a	4.73(0.50) bc	946.53(194.27) a	82.58(16.95) a	3.96(1.26) a
NPK	356.12(95.88) b	47.14(10.91) b	4.01(0.34) c	398.77(86.91) c	50.09(5.27) b	4.39(0.03) a

Note: Values in the table are the means of three replicates. Different lower letters within a column represent significant difference among different treatments (Duncan's test, $P < 0.05$). Control: non-fertilizer control; N: nitrogen fertilizer only; NP: inorganic nitrogen and phosphorus fertilizers; NM: inorganic N fertilizer plus organic manure; NPM: inorganic nitrogen, phosphorus fertilizers plus organic manure; NPK: inorganic nitrogen, phosphorus and potassium fertilizers. PD: phylogenetic diversity.

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Table S2. ADONIS (PERMANOVA) analyses for the effect of fertilization practice and season on diazotrophic community

	F	R^2	*P*_value
Treatments	17.45	0.743	< 0.001***
Season	1.794	0.015	0.124
Treatments × Season	1.076	0.046	0.415
N_{effect}	17.712	0.159	< 0.001***
P_{effect}	7.798	0.069	< 0.001***
OM_{effect}	52.778	0.473	< 0.001***
P_{effect} × OM_{effect}	3.477	0.031	0.009**

Note: N_{effect}, P_{effect} and OM_{effect} represent the effect of nitrogen fertilizer, phosphorus fertilizer and organic manure on diazotrophic community. Significant levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Table S3. Spearman correlation between nitrogenate activity, *nif*H gene abundance, PD, and the cumulative relative abundance of module 2 and soil physiochemical properties

	pH	TC	TN	TC/TN	NO_3^--N	NH_4^+-N	AP
Nitrognase activity	0.888**	0.657**	0.598**	-0.113	-0.038	-0.090	-0.082
*nif*H abundance	0.744**	0.653**	0.550**	-0.079	0.174	-0.014	-0.087
Phylogenetic diversity (PD)	0.652**	0.712**	0.709**	-0.0539**	0.532**	-0.312	-0.025
Cumulative relative abundance of module 2	0.885**	0.587**	0.522**	-0.105	0.030	-0.328	-0.334*

Note: Significant levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Figure S1

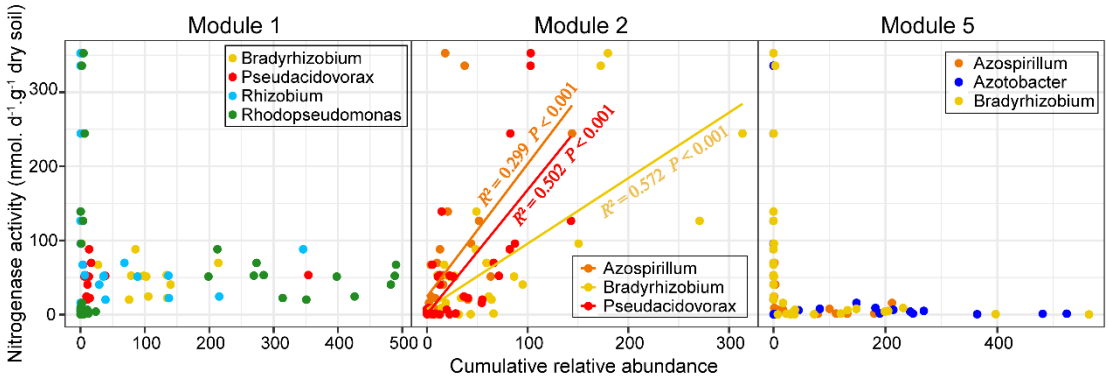

Fig. S1. Regression relationship between soil nitrogenase activity and the cumulative relative abundance of dominant genera and in module #1, #2 and #5