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Negative impacts of excessive nitrogen fertilization on the abundance and diversity of diazotrophs in black soil with monocropping maize

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Abstract

Background: Excessive nitrogen fertilizer input and low nitrogen fertilizer use efficiency in maize in China are serious ecological and economic problems, which might affect the procedures in the nitrogen cycle. To reveal the effects of long-term excessive nitrogen fertilization on diazotrophs in maize rhizosphere and bulk soil, we performed a long-term (five-year) N-input experiment (N rates from 0 to 300 kg N ha -1) in black soil maize in northeast China. The effect of N fertilizer application rates on the abundance, structure and compositions of diazotrophic community in both the bulk soil and rhizosphere of maize were investigated by Real-time quantitative PCR and high-throughput sequencing, and a structural equation model was constructed based on this study.

Results: 1) Excessive N fertilization significantly reduced the abundance and diversity of diazotrophs. 2) The accumulation of Sphingobium was correlated positively with soil nitrate concentration and soil EC, and negatively with soil pH. The contrast correlation was found in Burkholderia . 3) Diazotrophs were enriched in maize rhizosphere, but the diversity and compositions of diazotrophic community were less affected by maize rhizosphere effect. 4) The enriched Bradyrhizobium and Methylobacterium in maize rhizosphere showed a significant positive correlation with of maize plant biomass.

Conclusions: Our results suggest that through affecting soil pH, nitrate and EC values, long-term excessive N input increase Sphingobium accumulation and reduce the abundance of beneficial diazotrophs such as Bradyrhizobium and Burkholderia which contribute to the decreased nitrogen use efficiency.

Background

The basis for sustainable agricultural development depends on health soil, in which soil microbial composition and abundance play an important role [1]. Diazotrophic bacteria, as the main microflora involved in biological nitrogen fixation, occupy a dominant ecological role in the soil-atmosphere N cycle [2–5]; and the abundance, diversity and compositions of diazotrophic community have been identified as key biological factors determining soil nitrogen fixation capacity [6, 7]. With the development of molecular biology technology, the *nifH* gene (a structural gene of nitrogenase reductase) is widely used as a molecular biomarker to detect the diazotrophic microbes in soil. It has been reported that diazotrophs are affected by various factors (abiotic/biotic factors), such as different kinds of fertilizer treatments [8–11], soil nutrients and pH [12, 13], geographical scale [14], application of Mo and biochar [15, 16] and crop types and cultivation history [17] and so on.

The Black Soil Region of Northeast China is one of the remaining “three big black soil regions” in the world and is a main grain production area in China, where about 14% of the national grain supply is produced [18]. Since the 1980s, the per unit area N fertilizer input has been increasing continually, and current nitrogen application in maize is as high as 270 kg ha^{-1}, much higher than the recommended nitrogen application of 168 kg ha^{-1} [19]. The input of excessive N-fertilizer not only failed to increase the

yield, but also reduced the utilization rate of nitrogen fertilizer and caused environmental pollution [20–24].

The decrease of nitrogen utilization rate of crops may be related to the soil microbes and affect the diazotrophs. Long-term fertilization in a typical lime concretion black soil of Anhui province, dramatically suppressed N fixation rates and the relative abundance of keystone and phylogenetically clustered N fixers [25]. Similar results occured in the acidic soils in Southern China [10] and a typical Plinthudult soil type in Southeast China [26]. While the characteristics of diazotrophic bacteria in the black soil area of Northeast China has not been reported. In addition, most related studies were mainly based on one-year field treatment or potted plant tests in laboratories [15, 16, 27, 28]. Long-term positioning of fertilization conditions was regarded to achieve stabile soil nutrients, enzyme activity and microbial community structure [17, 29]. On the other hand, the diazotrophs in unique ecological niche of rhizosphere soil is more closely related to the supply of N-nutrient to the plants, and can directly affect plant growth through positive and negative feedback [30]. In addition, little information about the N-fertilizer application rate is available in previous studies, which is essential to evaluate the quantitative relationship among nitrogen application, soil proprieties, plant biomass and diazotrophic community. Also, the structures of diazotrophic community have been rarely compared between the rhizosphere and bulk soil with long-term N fertilization [8, 9, 11].

Based on the information mentioned above, we performed this study in maize field at a long-term positioning experimental site with history of quantitative fertilization for 5 years in the black soil area of Northern China. The objectives were: 1) to investigate the dynamic change of diazotrophic community in both rhizosphere and bulk soil of maize field; 2) to evaluate the quantitative relationship among N fertilization rates, soil proprieties, plant biomass and diazotrophic community; and 3) to explore the beneficial diazotrophic community in the black soil area of northeast China.

Results

Maize biomass and soil properties

Different N fertilizer treatments significantly affected the shoot and root dry weight of maize at jointing stage (V8). With increasing N application, both shoot and root biomass increased and reached the maximum under the treatment of N180 ($P < 0.05$), and then decreased with further increase of N application (Fig. 1).

The properties of soil were shown in Table 1. A two-way ANOVA showed that both N application and rhizosphere effect significantly affected soil characteristics, and soil OM, AP, NO_3^--N content and EC values were affected by the interaction between N fertilizer treatments and rhizosphere effect (Table S1). With increasing N application, the contents of TN, NH_4^+-N (except bulk soil samples) and NO_3^--N showed increasing tendency, while AK and pH showed a trend of decrease, indicating that N fertilizer application contributed to the increase of available N in soil, and led to the decrease of soil AK and pH. In both

rhizosphere soil and bulk soil, the content of AP and AK were higher at the low nitrogen level than that in other treatments, while the pH was significantly decreased, and the EC values and NO_3^--N were significantly increased under the condition of excessive N fertilization. In addition, compared with bulk soil, the contents of TN (except N300) and AK in the rhizosphere soil at all nitrogen levels were significantly higher, and the EC values and NO_3^--N were significantly lower (except N0). It indicates that rhizosphere soil samples were beneficial to the enrichment of TN and AK and the consumption of mineral nutrients (EC values) and NO_3^--N.

Table 1

Soil physicochemical properties in the rhizosphere and bulk soils of maize cultured for the long-term different N fertilizer application rates.

Soil properties	Sampled site	N0	N60	N180	N300
TN(g kg^{-1})	Rhizosphere	**1.59 ± 0.04abc**	**1.68 ± 0.01a**	**1.60 ± 0.02abc**	1.64 ± 0.02ab
	Bulk soil	**1.38 ± 0.06d**	**1.53 ± 0.01c**	**1.42 ± 0.03d**	1.56 ± 0.04bc
OM(g kg^{-1})	Rhizosphere	**20.27 ± 1.43c**	23.17 ± 1.03bc	**24.10 ± 0.38b**	**27.60 ± 1.47a**
	Bulk soil	**24.10 ± 0.55b**	24.40 ± 0.68b	**20.57 ± 0.64c**	**23.23 ± 0.95bc**
AP(mg kg^{-1})	Rhizosphere	101.07 ± 2.02b	92.90 ± 1.80b	70.80 ± 1.76 cd	66.57 ± 4.49d
	Bulk soil	94.63 ± 2.44b	123.33 ± 2.85a	78.07 ± 3.41c	75.50 ± 6.01 cd
AK(mg kg^{-1})	Rhizosphere	**191.33 ± 4.81a**	**192.67 ± 2.91a**	**168.00 ± 2.89b**	**159.67 ± 1.33bc**
	Bulk soil	**169.67 ± 1.86b**	**145.67 ± 5.36 cd**	**134.33 ± 7.54d**	**133.67 ± 7.26d**
EC(mS m^{-1})	Rhizosphere	8.01 ± 0.05d	**9.04 ± 0.36d**	**8.91 ± 0.32d**	**19.37 ± 0.81b**
	Bulk soil	8.54 ± 0.81d	**17.53 ± 1.29b**	**12.36 ± 0.78c**	**22.77 ± 1.09a**
pH	Rhizosphere	**5.54 ± 0.04a**	5.20 ± 0.02c	5.13 ± 0.05c	**4.60 ± 0.01d**
	Bulk soil	**5.40 ± 0.05b**	5.07 ± 0.03c	5.12 ± 0.06c	**4.46 ± 0.06e**
NH_4^+-N(mg kg^{-1})	Rhizosphere	0.44 ± 0.10bc	0.47 ± 0.04abc	**0.52 ± 0.05ab**	**0.62 ± 0.06a**
	Bulk soil	0.40 ± 0.03bc	0.46 ± 0.04abc	**0.29 ± 0.03c**	**0.37 ± 0.04bc**
NO_3^--N(mg kg^{-1})	Rhizosphere	0.08 ± 0.01d	**0.11 ± 0.01d**	**0.26 ± 0.02d**	**1.73 ± 0.13b**
	Bulk soil	0.26 ± 0.07d	**1.17 ± 0.19c**	**1.59 ± 0.16b**	**2.25 ± 0.06a**

Data are means ± standard error (n = 3). Different letters indicated the significant difference of soil single characteristics under long-term different N fertilizer application rates in all samples (including rhizosphere and bulk soils); Values in bold indicate significant differences between bulk and rhizosphere soil (Duncan multiple-range test, $P < 0.05$). TN: total nitrogen; OM: organic matter; AP: available phosphorus; AK: available potassium; EC: electrolyte; NH_4^+-N: ammonium nitrogen; NO_3^--N: Nitrate nitrogen.

The copy numbers of nifH **gene and their correlation with soil properties**

The two-way ANOVA analysis showed that both N fertilization and rhizosphere effect had significant effects on the copy numbers of *nifH* gene ($P < 0.05$), but there was no significant interaction ($P = 0.952$) (Table S2). The range of *nifH* gene copy numbers were $4.13–8.91 \times 10^5$ per g dry soil in rhizosphere soil, and $1.41–6.93 \times 10^5$ per g dry soil in bulk soil (Fig. 2). It can be seen that the copy numbers of *nifH* gene in rhizosphere soil was higher than that in bulk soil, and with the increase of N application, the copy numbers of *nifH* gene gradually decreases, and the excessive N application significantly reduced the *nifH* gene copy numbers ($P < 0.05$).

Pearson's correlation coefficients showed that *nifH* gene copy numbers in rhizosphere and bulk soil were positively correlated with AK and pH ($P < 0.05$), while negatively correlated with EC values and NO_3^--N ($P < 0.05$) (Table 2). In addition, AP in rhizosphere soil was positively correlated with *nifH* gene copy numbers ($P < 0.05$).

Table 2
Pearson's correlation coefficients between total *nifH* gene copy numbers and soil physicochemical properties in the rhizosphere and bulk soils.

Compartment	TN	OM	AP	AK	EC	pH	NH_4^+-N	NO_3^--N
Rhizosphere	0.093	-0.487	**0.768****	**0.713****	**-0.631***	**0.688***	-0.511	**-0.641***
Bulk soil	-0.388	0.282	0.538	**0.688***	**-0.779****	**0.780****	0.155	**-0.859****
Values in bold indicate significant correlations, *$0.01 < P \leq 0.05$, **$P \leq 0.01$.								

Diazotrophic α-diversity and its correlation with soil properties

After quality filtering and screening of amino acid sequences, a total of 375,772 high-quality *nifH* sequences were obtained from 32 soil samples. Among them, one sample with too few sequences (bulk soil, N04) was removed, and the remaining sample sequences were between 1882 and 39,170 (Table S3). Therefore, each sample was randomly sampled from 1882 sequences for subsequent analysis. According to 97% similarity, a total of 845 OTUs were kept and each sample contained about 88–240 OTUs with the coverage of 94.3–98.1% (Table S3). The rarefaction curves (Figs. S1a and b) indicated that the obtained sequences could objectively and accurately reflect the abundance and diversity of diazotrophs.

The *α*-diversity of diazotrophs shown in Fig. 3 demonstrated that all the *α*-diversity indices were similar between the bulk soils and the rhizosphere soils. While significant decrease of *α*-diversity was found at excessive N fertilization (N300). The two-way ANOVA analysis (Table S4) further confirmed the results in Fig. 3 that the abundance and diversity of soil diazotrophs were mainly affected by N fertilization rates ($P < 0.05$), while the rhizosphere had no significant effect ($P > 0.05$), and there was no significant

interaction between the N levels and rhizosphere effect. The one-way ANOVA analysis evidenced no significant difference between the rhizosphere and bulk soil at all the N fertilization rates, and among the N0, N60 and N180 treatments, but the excessive N fertilization (N300) significantly ($P < 0.05$) decreased the *α*-diversity of diazotrophs.

Pearson's correlation coefficients showed that OTU number, Chao1 index and Shannon index of diazotrophic in all samples (rhizosphere and bulk soil) were positively correlated with pH, negatively correlated with EC values and NO_3^--N contence ($P < 0.05$), while Simpson diversity index was contrary. In addition, chao1 index and shannon index in rhizosphere soil were positively correlated with AP, negatively correlated with OM, and OTU number was positively correlated with AK and negatively correlated with OM (Table S5).

Community Structure Analyses And Relative Abundance Of Diazotrophs

To assess the effects of N fertilization application and rhizosphere effect on the compositions of diazotrophic community, hierarchical cluster analysis (UPGMA), PCoA, NMDS, ANOSIM and PERMANOVA were performed based on OTU classification level. The results of hierarchical cluster analysis (Fig. 4a) showed that all the samples were divided into four clusters according to different N fertilizer application rates. The rhizosphere and bulk soil samples of the same N treatments were clustered together. Similar to the hierarchical cluster analysis, PCoA analysis based on bray-curtis distance also showed that all the samples were divided into four groups corresponding to the N fertilization rates in the first principal axis (42.24%) and the second principal axis (12.85%), and N300 was the treatment distantly separated from the other treatments, while the samples of rhizosphere and bulk soil were significantly separated in the fourth principal axis (6.33%) (Figs. 4b and S2a). The Stress value of NMDS analysis was 0.08, and the grouping situation was consistent with the PCoA result (Fig. S2b). ANOSIM and PERMANOVA analysis showed the same results (Table S6) and demonstrated significant differences in the compositions of diazotrophic community among different N fertilizer application rates in all samples ($P < 0.05$), but no significant difference between rhizosphere and bulk soil ($P > 0.05$). Therefore, the differences of community compositions were mainly affected by N fertilizer application rates, especially by the excessive N fertilization.

The OTUs obtained from each sample were classified into different genera, and the 10 most abundant genera with relative abundance more than 2% were shown in Fig. 5a. *Pseudomonas, Burkholderia, Azospirillum* and *Bradyrhizobium* were the four most abundant dominant genera, accounting for 20.75–70.34% of the total *nifH* gene sequences. The results of heatmap analysis of these dominant genera showed that community compositions were mainly affected by N fertilizer application rates, and the relative abundance of the dominant genera at the N300 was far different from that in the other treatments (Fig. 5b).

The one-way ANOVA (Table S7) showed that excessive N fertilizer application significantly reduced the relative abundance of *Burkholderia* and *Rhodobacter* ($P < 0.05$). The relative abundance of *Burkholderia* was significantly higher under extreme nitrogen deficiency treatment (N0), *Rhodobacter* was significantly higher under moderate nitrogen deficiency treatment (N60), and *Bradyrhizobium* was significantly higher under normal fertilizer treatment (N180). In addition, the relative abundance of *Bradyrhizobium* in rhizosphere was significantly higher than that in bulk soil under normal fertilization treatment, while no difference was recorded between the rhizosphere and bulk soil among the other dominant genera ($P < 0.05$).

Analysis Of Driving Factors Of Diazotrophs In Black Soil

According to the above results, the structures of diazotrophic communities were variable under different N fertilizer application rates, and the following Mantel test and CCA analysis on soil characteristics and diazotrophic structure in rhizosphere and bulk soil samples respectively revealed the environmental variables correlating to the shifting in diazotrophic communities.

Mantel test showed that there were different driving factors for diazotrophic communities between rhizosphere and bulk soil, and the diazotrophs in rhizosphere soil was significantly positively correlated with OM, AP, AK, NO_3^--N, pH and EC values, while in the bulk soil was only significantly positively correlated with AK, pH and EC values ($P < 0.05$). However, the diazotrophs in both rhizosphere and bulk soil showed the strongest correlation with pH and EC values (Table 3). The effects of soil properties on the structures of diazotrophic community were further analyzed by CCA for the significantly correlated soil variables (Figs. S3a and b). In rhizosphere soil samples, the significantly correlated soil variables represented 71.94% of the total variations, which explained 31.48% and 11.9% variations in the first and second axis, respectively. In bulk soil samples, the correlated soil variables represented 45.38% of the total variations, which explained 27.17% and 10.1% variations in the first and second axis, respectively. Multiple regression tree analysis of enriched OTUs from all samples (Fig. S4) showed that the compositions of diazotrophic community were mainly mediated by pH, while AP and AK contents also contributed to some extent.

Table 3
Mantel test correlations between diazotrophic community structure and soil physicochemical properties.

Variable	Sampled site	R	P	Permutation number
TN	Rhizosphere	0.00821	0.952	999
	Bulk soil	0.10756	0.600	999
OM	Rhizosphere	0.48317	0.002	999
	Bulk soil	-0.11813	0.522	999
AP	Rhizosphere	0.52865	0.001	999
	Bulk soil	0.14870	0.257	999
AK	Rhizosphere	0.62707	0.001	999
	Bulk soil	0.27146	0.040	999
EC	Rhizosphere	0.88613	0.002	999
	Bulk soil	0.34367	0.029	999
pH	Rhizosphere	0.86795	0.001	999
	Bulk soil	0.81881	0.001	999
NH_4^--N	Rhizosphere	0.12294	0.465	999
	Bulk soil	-0.17892	0.250	999
NO_3^--N	Rhizosphere	0.90852	0.001	999
	Bulk soil	0.19171	0.199	999
Values in bold indicate significant effects, $p < 0.05$.				

Pearson's correlation coefficients were conducted among the 10 dominant diazotrophic genera enriched in rhizosphere soil, soil properties and plant biomass (sum of shoot and root dry weights). The results (Fig. S5, Table S8) showed that the tested soil variables were closely related to the most enriched diazotrophs. The NO_3^--N, OM and EC values were significantly positively correlated with the relative abundance of *Sphingobium* ($P < 0.05$), while significantly negatively correlated with that of *Burkholderia*; while AP, AK and pH showed reverse correlations with these two genera. In addition, NH_4^+-N was negatively correlated with the relative abundance of *Burkholderia*. *Azospirillum* was negatively correlated with EC and OM and positively correlated with pH. *Pseudomonas* was negatively correlated with EC, NO_3^--N and positively correlated with AK. *Rhodobacter* was positively correlated with AK. However, the relative abundance of *Bradyrhizobium*, *Methylobacterium*, *Klebsiella* and *Paraburkholderia* were not

significantly correlated with the soil characteristics, indicating that they were less affected by the environmental variables. Interestingly, a significant positive correlation between plant biomass and the relative abundances of *Bradyrhizobium* and *Methylobacterium* ($P < 0.05$) was found.

Based on the above results and relevant ecological knowledges, a SEM was constructed to further analyze the direct or indirect effects of N application and rhizosphere effect on the soil properties, diazotrophic absolute abundance and α-/β-diversities (Fig. 6). From the results of standardization, N application and the rhizosphere effect can affect the soil properties and the absolute abundance of diazotrophs directly. However, the α-diversity of diazotrophs was affected by soil properties indirectly mediated by different N fertilizer application rates, and the β-diversity of diazotrophs was mainly affected by N fertilizer application rates directly.

Discussion

According to the plant biomass (Fig. 1) in this study, 180 kg N ha^{-1} was confirmed as the reasonable N fertilization for maize grown in the test soil [31], and excessive N fertilizer application (300 kg ha^{-1}) resulted in a significant decrease in plant biomass and in the diazotroph diversity (Fig. 3). This result was consistent with previous studies that plant biomass was not directly proportional to N fertilizer application rates [32, 33]. However, the decreased biomass of maize under the excessive N fertilizer application is an interesting phenomenon, which was also reported in other plants [34, 35]. According to the Leibig Law of minimum, the plant growth (biomass accumulation) is determined by the nutrient with the lowest concentration, so the addition of excessive N in the present study could not help the plant growth. The negative effects of excessive N on maize growth may be due to that the excessive N fertilizer is toxic for maize that fit the Shelford Law of tolerance. The toxic effect of excessive N fertilizer application might be from the modification of plant metabolism, or from modification of soil environments, such as the degradation of soil properties [34, 35] and the alternation of soil microbial community that in turn could decrease the growth of maize. The present study offered evidence of alternation in soil properties and soil microbial communities caused by excessive N application, as well as the correlation among them.

Firstly, the results in the present study clearly evidenced the alternation in soil properties under the excessive N fertilization, which is similar to those in some other studies. Previous studies have shown that excessive N fertilizer input can lead to soil acidification and increase of soluble salt concentration [20]. Soil pH has been considered to be the main factor affecting microbial abundance and diversity [13, 36–39], and high EC values may increase the incidence of some crop diseases (such as maize root rot) [40–42]. Similar to the previous studies, excessive N fertilizer input led to a decrease in soil pH and an increase in EC values in our present study. No doubt that the low pH (4.60 in rhizosphere and 4.46 in bulk soil) and high salinity (EC 19.37 mS m^{-1} in rhizosphere and 22.77 mS m^{-1} in bulk soil) (Table 1) could affect the metabolism and nutrient absorption of maize, which presented optimal growth at pH 5.5-7.0 or pH 4.9–7.3 [43], and then decrease the growth of plant [34, 35]. In addition, the acidic condition could enhance the solubility of Fe, Mn, Bo, Cu and Zn and decreased the solubility of Mo, Mg, Ca, K and S,

which may cause the toxic effects of the heavy metal and reduce the availability of some mineral nutrients [44].

Secondly, the application of excessive N fertilizer decreased the α-diversity (Fig. 2) and changed the community composition (Figs. 4 and S2b) of diazotrophs in both rhizosphere and bulk soils. These results were similar to those obtained in several previous studies. A long-term addition of NPK fertilizer in a Calcic Kastanozems soil with wheat-soybean rotation lead to decreased abundance and diversity of diazotrophs [8]; while N fertilizer application at dose of 300 kg ha^{-1} in Red Soil of wheat-maize rotation also lead to decreased abundance and diversity of diazotrophic [10]. And our study was performed with different levels of N fertilizer (N0, N60, N180, N300) in black soil with mono-cropping maize. Therefore, the decrease of abundance and alternation of the structure composition of diazotrophs in response to the long-term excessive N fertilization is common in different soil types (including rhizosphere soil) and in different cropping systems. The shifting of diazotrophs community composition might be explained by their great genomic and biochemical diversity, which is determinant for their adaptation to the environmental factors.

The application of different levels of N fertilizer in the present study made it possible to horizontally compare the influence of different N fertilizer application rates on the diazotrophs, which was not reported up to date. Interestingly, no significant difference was found in diversity of diazotrophs among the low N (N0, N60) and normal fertilization (N180), and only the long-term excessive N fertilization significantly decreased the abundance and diversity of diazotrophs (Figs. 2 and 3). These phenomena were consistent to the biomass increase and decrease, implying the possible relationships between the diazotrophs and the plant growth.

The different compositions of diazotrophic community were clearly demonstrated by the clustering analysis, in which the diazotrophic communities were separated according to the N fertilizer application rates; moreover, the communities from excessive N fertilization formed a cluster far distant with those from the other treatments (Figs. 4 and 5). So, the structure of diazotrophic community would be seriously changed by the N fertilizer application dose that exceeded a certain threshold. Among the ten most abundant genera (Fig. 5, Table S7), the stability of relative abundance in both rhizosphere and bulk soils against the N levels for *Azospirillum, Herbaspirillum, Klebsiella, Methylobacterium, Paraburkholderia* and *Pseudomonas* demonstrated that they might live as saprophytes by using the combined N as their N source. This estimation is consistent to the TN contents in the tested soils, in which the background N content is very high (1.38–1.68 g kg^{-1}, Table 1) [45] and is sufficient to inhibit the biological N fixation, e.g. the diazotrophs can not fix N_2 in these soils.

Different from the six genera mentioned above, the relative abundances of *Bradyrhizobium, Burkholderia, Rhodobacter* and *Sphingobium* were significantly changed sequentially (Fig. 5, Table S7): addition of N fertilizer decreased the relative abundance of *Burkholderia*, so it was most abundant in N0 treatment; while the abundances of *Rhodobacter, Bradyrhizobium* and *Sphingobium* were significantly enhanced in the treatments of N60, N180 and N300, respectively (Fig. 5, Table S7). These changes demonstrated that

different levels of N fertilizer application enriched distinct diazotrophic bacteria, which might be related to their adaption to the decreased pH values and increased EC values in soils caused by the fertilization (Table 1).

It is well known that the roots of plant can enrich the some microbes via secretion of secondary metabolites, casts of roots and modification the rhizosphere microenvironments [46–48]. Previous studies had found that the diazotrophs in the rhizosphere soil of sorghum, soybean, maize was more abundant than that in the bulk soil [10, 27, 49, 50]. Our study also showed that maize rhizosphere had a significant effect on the copy numbers of *nifH* gene, and the diazotrophs in rhizosphere soil were also more abundant than that that in bulk soil (Fig. 2, Table S2). In addition, NO_3^--N in rhizosphere soil was lower than that in bulk soil (Table 1), which was due to the selective absorption of soil nutrients by plants [51]. The selectivity may be one of the reasons for the enrichment of diazotrophic in rhizosphere. The proliferation of more diazotrophic in the rhizosphere should help provide more nitrogen nutrients or other beneficial effects to the plant. However, there was no significant difference in the compositions of diazotrophic communities in rhizosphere and bulk soil, indicating that rhizosphere effect had little influence on the diazotrophic community in the tested soils, which was consistent with previous studies [10, 17].

Based on our research, through the construction of structural equation model (SEM), it seems that the N fertilizer application rates and rhizosphere effect could directly lead to the variation of soil characteristics and diazotrophic abundance. Then, the variation of α-diversity of diazotrophic mediated by soil characteristics. Finally, α-diversity and N fertilizer application rates mediated the separation of β-diversity of diazotrophs in the tested soils (Fig. 6). The results revealed the influencing factors of diazotrophic community structure variation and the causal relationship among each of the variables, which provided a good idea for the study of subsequent variation mechanisms of microbial community structure.

In our study, excessive N fertilizer input led to a decrease in soil pH and an increase in EC values, which were significantly related to the abundance and diversity of diazotrophs (Tables 2 and S5). Among them, pH was negatively correlated with *Sphingobium*, while positively correlated with *Burkholderia* (Fig. S5), indicating that the low soil pH under excessive N application led to the accumulation of *Sphingobium*, while reducing the relative abundance of *Burkholderia*. *Sphingobium* were reported can degrade many kinds of polycyclic aromatic hydrocarbons (PAHs) and their derives [52], and *Burkholderia* was famous for its bioremediation and antifungal properties [53], suggested that excess N fertilizer despite increased the degradation ability of soil pollutants, but its resistance to pathogen was reduced, thus increasing the probability of occurrence of soil spread diseases.

Long-term excessive N fertilizer input also had a profound impact on soil nutrient content (Table 1). The variation of soil properties also had a significant impact on the compositions of diazotrophic community (Figs. S3 and S4, Table 3). In particular, the NO_3^--N and EC values in soil were positively correlated with *Sphingobium* and negatively correlated with *Burkholderia*, which were agreed with the relationship between soil pH and these two diazotrophic bacteria, indicating the synergistic effect on the structure of

diazotrophic community of high nitrate content, EC values and low pH caused by excessive N fertilizer input. On the contrary, the soil AP and AK negative correlation with *Sphingobium* and positive correlation with *Burkholderia* imply that the proportion of nitrogen, phosphorus and potassium in soil was also crucial for maintaining a normal diazotrophic community, which proved the importance of balanced application of NPK from the perspective of soil microbes [54].

The variation of soil microbial community structure has a feedback regulation effect on plant [1]. However, little is known about the feedback of plant from the change of diazotrophic community. Based on our study, it was found that plant biomass was positively correlated with the relative abundances of *Bradyrhizobium* and *Methylobacterium* ($P < 0.05$, Fig. S5). *Bradyrhizobium*, which is widely distributed in soil, has important biological functions, including photosynthesis, symbiotic and free-living nitrogen fixation, denitrification and aromatic compound degradation, and plays an important role in the global nitrogen cycle. The multiple functions make it important for agricultural production [55–57]. In addition, *Methylobacterium* is also widely existed in the environment. It is a kind of diazotrophic bacteria that can produce plant growth hormone and can be classified as plant growth-promoting bacteria [59, 60]. This indicated that the increase of biomass under normal N fertilization (N180) was more likely related to the enrichment of these two diazotroph. Based on the above analysis, *Bradyrhizobium* and *Methylobacterium* may be utilized to develop efficient microbial fertilizers.

Conclusions

This study argues that excess N fertilizer input led to soil acidification, increased soluble salt concentration, and excess nitrate nitrogen, which directly led to a significant decrease in abundance and diversity of diazotrophs. These changes lead to the accumulation of *Sphingobium* and the decrease of *Burkholderia*, which may be one of the important reasons for the decrease in N fertilizer utilization efficiency of maize. Reasonable N fertilization (N180) and reduction of N fertilizer (N0, N60) input had little influence on the abundance, diversity and compositions of diazotrophic community, and contributed to the enrichment of beneficial diazotrophs (*Bradyrhizobium, Methylobacterium*, etc.) in the tested soils. The nitrogenase activity and the relationship between the increase of *Bradyrhizobium/Methylobacterium* relative abundance and enhanced biomass of maize needs further study.

Methods

Experimental site and sampling

The long-term nitrogen fertilizer application experiment was started in 2014 in Lishu County, Jilin Province, China (43.34°N, 124.11°E), which is located in the hinterland of Songliao Plain. The soil type is typical black soil belonging to Udolls in the USDA Soil Taxonomy System [61], and the field is subject to continuous maize cropping, one season a year. This experiment was designed as a randomized block with 6 nitrogen treatments and 4 repetitions for each treatment. Treatment includes: N0: no N-fertilizer; N60: 60 kg N ha^{-1}; N120: 120 kg N ha^{-1}; N180: 180 kg N ha^{-1}; N240: 240 kg N ha^{-1}; N300: 300 kg N ha^{-1}. N-fertilizer in urea was supplied together with the phosphorus fertilizer (P_2O_5 100 kg ha^{-1}) and

potassium fertilizer (K_2O 120 kg ha^{-1}) in consistent, including N0. Each plot was 10 m long and 6 m wide, with a total of 24 plots. Unplanted area of 2 m·2 m was left in each plot for sampling bulk soil. Maize variety was Zheng Dan 958, which is the most used cultivar in the local region. The planting density of maize was 65,000 plants ha^{-1}, with 60 cm row spacing and 25.6 cm plant spacing.

The seeds were planted on May 1st, 2018 and the grain were harvested on October 3rd, 2018. According to the growth characteristics of maize, four N fertilization rates were selected for soil sampling: extreme N deficiency (N0), moderate N deficiency (N60), normal N fertilization (N180), and excessive N fertilization (N300). Plant and soil samples were collected on June 27 (V8 stage when the plant had 8 fully expanded leaves). Three plants with uniform growth were randomly selected in the corresponding treatment plot. After cutting off the aerial part of the plant, the soil volume (including the roots) of 30 cm · 12.8 cm · 20 cm (long · wide · depth) surrounding the plant was taken. The roots were separated from soil by vigorously shaking and the soil tightly attached to the roots was collected as rhizosphere soil [10]. The rhizosphere soils from the three plants in the same plot were mixed into a sample. Bulk soil (0–20 cm) was collected in the unplanted area. Each collected sample was thoroughly homogenized through 2 mm mesh and divided into two parts: one was stored at 4 ºC for soil property analysis, and the other was stored immediately at -80 °C for metagenomic DNA extraction. The shoots and roots of each maize were harvested simultaneously and were washed three times with water to completely remove the adsorbed soil particles, then they were placed in envelopes separately and dried at 80°C for dry weight measurement.

Soil Property Analysis

The ammonium N content (NH_4^+-N) and nitrate N content (NO_3^--N) were measured using fresh soil samples, and then the remaining soil samples were air-dried to determine the total N (TN), organic matter (OM), available P (AP), available K (AK), pH and EC values. Soil pH was measured by an acidity meter (Sartorius PB-10); EC values were measured by a conductivity meter (METTLER TOLEDO); TN was determined by Kjeldahl method. The soil OM content was determined by the potassium dichromate volumetric method; and 0.5 M $NaHCO_3$ extraction-molybdenum antimony anti-Spectrophotometry method (Olsen method) was used to measure the AP. AK was determined by 1 M NH_4OAc extraction-flame photometric method. Soil NH_4^+-N and NO_3^--N were extracted with 2 M KCl and measured using a continuous flow analyzer (TRAACS 2000, Bran and Luebbe, Norderstedt, Germany).

Soil DNA extraction and Real-time quantitative PCR of nifH gene

Soil DNA was extracted from 0.5 g of soil using a Fast®DNA SPIN Kit (MP Biomedical, Solon, OH, USA) and was purified subsequently by PowerClean®DNA Clean-up Kit (MoBio Laboratories Inc., Carlsbad, CA,

USA). DNA extraction quality was determined by electrophoresis in 1% (w/v) agarose gel, and DNA concentration and purity were determined by Nanophotometer (Implen, Munich, Germany).

The copy numbers of *nifH* gene were measured by ChamQ™ SYBR® Color qPCR Master Mix (2·) (Vazyme Biotech Co., Ltd, Nanjing, China) on the LineGene 9600 Plus real-time PCR system. The *nifH* gene-specific primers were *nifH*-F (5'-AAAGGYGGWATCGGYAARTCCACCAC-3') and *nifH*-R (5'-TTGTTSGCSGCRTACATSGCCATCAT-3') (Rösch et al., 2002). Thermal cycling conditions for the assays consisted of 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 40 s. Amplification was carried out in a total volume of 20 μL containing 10 μL of ChamQ™ SYBR® Color qPCR Master Mix (2·), 2 μL of template DNA, 0.4 μL of 5 μM Primer F; 0.4 μL of 5 μM Primer R; 7.2 μL nuclease-free water. Melting curves were used to analyze the specificity of the amplified products. A standard curve was obtained from serial 10-fold dilutions of linearized plasmids containing the clone *nifH* gene. Each set of qPCR reaction was performed in triplicate. Reaction efficiency was 85.49% and R^2 was 0.999.

The sequencing of nifH gene and bioinformatics analysis

The diazotroph community compositions were determined using the Illumina MiSeq platform. Primers of *nifH* gene were used as the primers in qPCR step, and barcode was added to identify each sample. Thermal cycling conditions for the assays consisted of 95 °C for 3 min, followed by 37 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min. The 20 μL reaction mixture contained 4 μL TransStart FastPfu buffer (5×), 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM Primer F, 0.8 μL 5 μM Primer R, 0.4 μL TransStart FastPfu DNA polymerase, 10 ng template DNA, and complement nuclease-free water to 20 μL. Three technical replicates were conducted for each sample, and the technical replicates were subsequently pooled into one tube. The obtained PCR products were checked by electrophoresis in 2% (w/v) agarose gel,then AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) was used to purify the recovered products, and QuantusTM Fluorometer (Promega, USA) was used for quantitative detection of the recovered products. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP253214).

The raw *nifH* gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH [62], in which forward and reverse reads had the overlapping base length at least 10 bp, and the maximum mismatch ratio of overlap region was 0.2. Then the sequencing reads were analyzed using QIIME platform [63] to remove the low-quality sequences, including the quality score < 20, ambiguous nucleotides, or mismatched primer and barcode. After sorting the sequences of samples according to barcodes, barcode and primer sequences were also removed. The remaining sequences were translated into amino acid using the FunGene Pipeline [64]. The chimeras and translated protein sequences that did

not match the *nifH* protein were then discarded [65], while the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity cutoff by UPARSE [66]. Representative sequences from each OTU were taxonomically classified by the BLAST algorithm-based search against the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis

The software mothur [67] was used to count α-diversity of diazotrophs (OTU number, Chao1 estimator, Shannon diversity index, Simpson diversity index and coverage) based on the filtered sequences, and the rarefaction curves were constructed with R software. Statistical analyses were performed in SPSS 21.0 for Windows. Differences in soil properties, maize shoot and root biomass, *nifH* gene copy numbers, and α-diversity of diazotrophs among all N fertilization treatments were determined by one-way ANOVA followed by Duncan's test ($p < 0.05$). A two-way ANOVA was employed to evaluate the interaction effects of N fertilizer application rates and rhizosphere effect on soil properties and *nifH* gene copy numbers. Pearson's correlation coefficients were applied to measure the relationship among *nifH* gene copy numbers, α-diversity and relative abundances of dominant diazotrophs and soil characteristics.

The OTU-based hierarchical cluster analysis (Unweighted Pair-group Method with Arithmetic Mean), Principal coordinate analysis (PCoA), nonmetric multidimensional scaling analysis (NMDS), analysis of similarity (ANOSIM) and PERMANOVA based on the Bray–Curtis distance were employed to calculate the community similarities among the samples. Then the dominant diazotrophic bacteria and corresponding heatmap were visualized using R at genus level.

A Mantel test was carried out to determine the correlation between the community composition of rhizosphere and bulk soil and soil properties. Only variables that had significant effects ($p < 0.05$) by the Mantel test were further used to perform a CCA analysis using Cannoco for Windows [68]. In addition, the mvpart package [69] of the R was used to construct a multivariate regression tree (MRT) to comprehensively explore main soil variables that best shaped the diazotrophic community composition.

Structural Equation Models

In order to quantify the importance of N fertilizcation rates and rhizosphere effect on soil properties and diazotrophic community, we constructed a structural equation model based on the current ecological knowledges. And theoretical model hypothesised that: 1) N fertilization rates and rhizosphere effect directly affect diazotrophs, respectively; 2) N fertilization rates and rhizosphere effect indirectly affect diazotrophs by changing soil properties; 3) β-diversity of diazotrophs was affected by the change of α-diversity. The variable of rhizosphere effect was created by assigning the value 1 to the rhizosphere soil and 0 to bulk soil. All the measured soil properties and β-diversity of the diazotrophs (based on bray-curtis distance matrix) were dimensionally reduced through NMDS, and the first axis of NMDS was used to represent the variance matrix of soil characteristics and β-diversity of the diazotrophs. Shannon index was used to represent α-diversity. The abundance of diazotrophs was expressed by *nifH* gene copy

numbers. All variables were normalized by Z transformation (mean = 0, standard deviation = 1), and a covariance matrix of these variables was inserted into AMOS 24.0 (SPSS, Chicago, IL, USA) for SEM construction and analysis. Maximum likelihood estimation was used to fit the covariance matrix into the model [70]. Chi-square ($P > 0.05$), goodness of fit index ($GFI > 0.90$), and root mean square error of approximation ($RMSEA < 0.05$) were used to ensure the model adequately fit [71].

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The obtained sequences were submitted to the NCBI Sequence Read Archive (SRA) with accession number SRP253214.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
XS and GM designed research. LC, KL and WS performed the research. LC and XW analyzed data. LC, EW, XS, and GM wrote the paper. CT and WC Provided resources. All authors read and approved the final manuscript.

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Figures

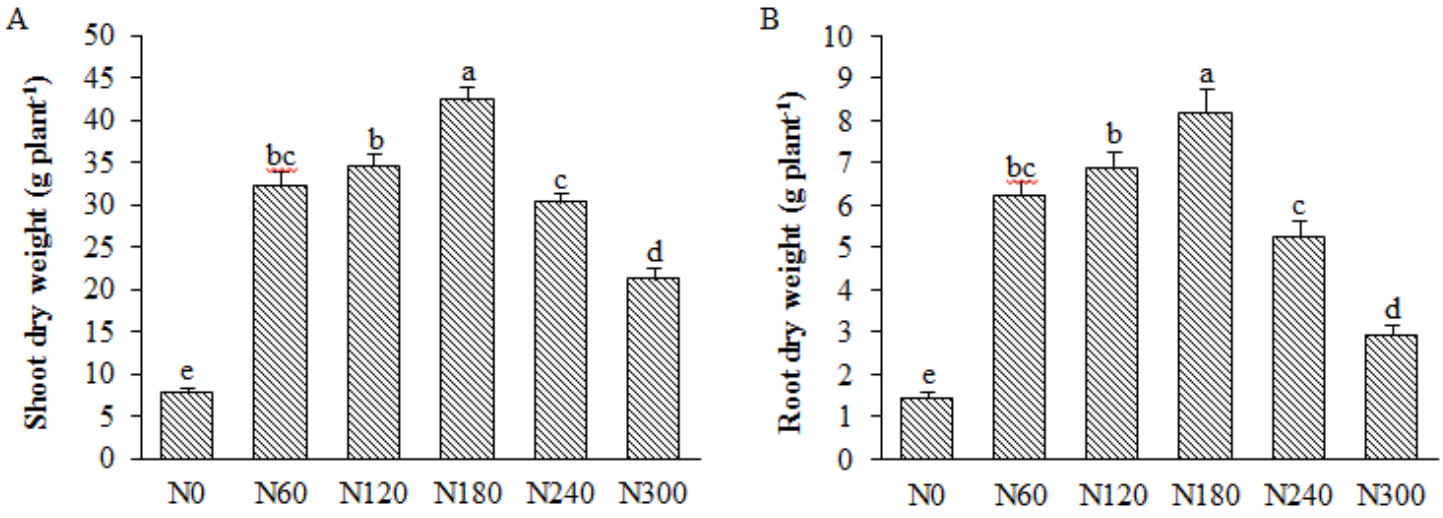

Figure 1

Biomass of maize under long-term different N fertilizer application rates. (A) Shoot dry weight; (B) Root dry weight. Data are means ± standard error (n=12). Different letters on the bars indicate significant differences among the long-term different N fertilizer application rates (Duncan multiple-range test, P<0.05).

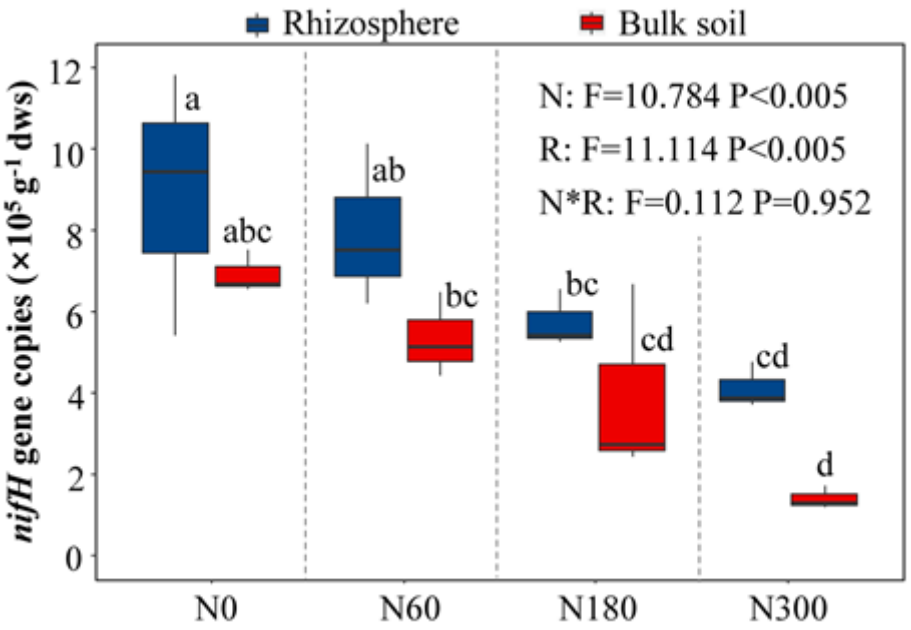

Figure 2

Abundance of the nifH gene in maize rhizosphere and bulk soils under long-term different N fertilizer application rates. Data are means ± standard error (n=3). All of the data were analyzed using a two-way analysis of variance, N: N fertilizer application rates; R: rhizosphere effect (bulk and rhizosphere). Different letters on the bars represent significant differences between all of the data by one-way anova (Duncan multiple-range test, P<0.05).

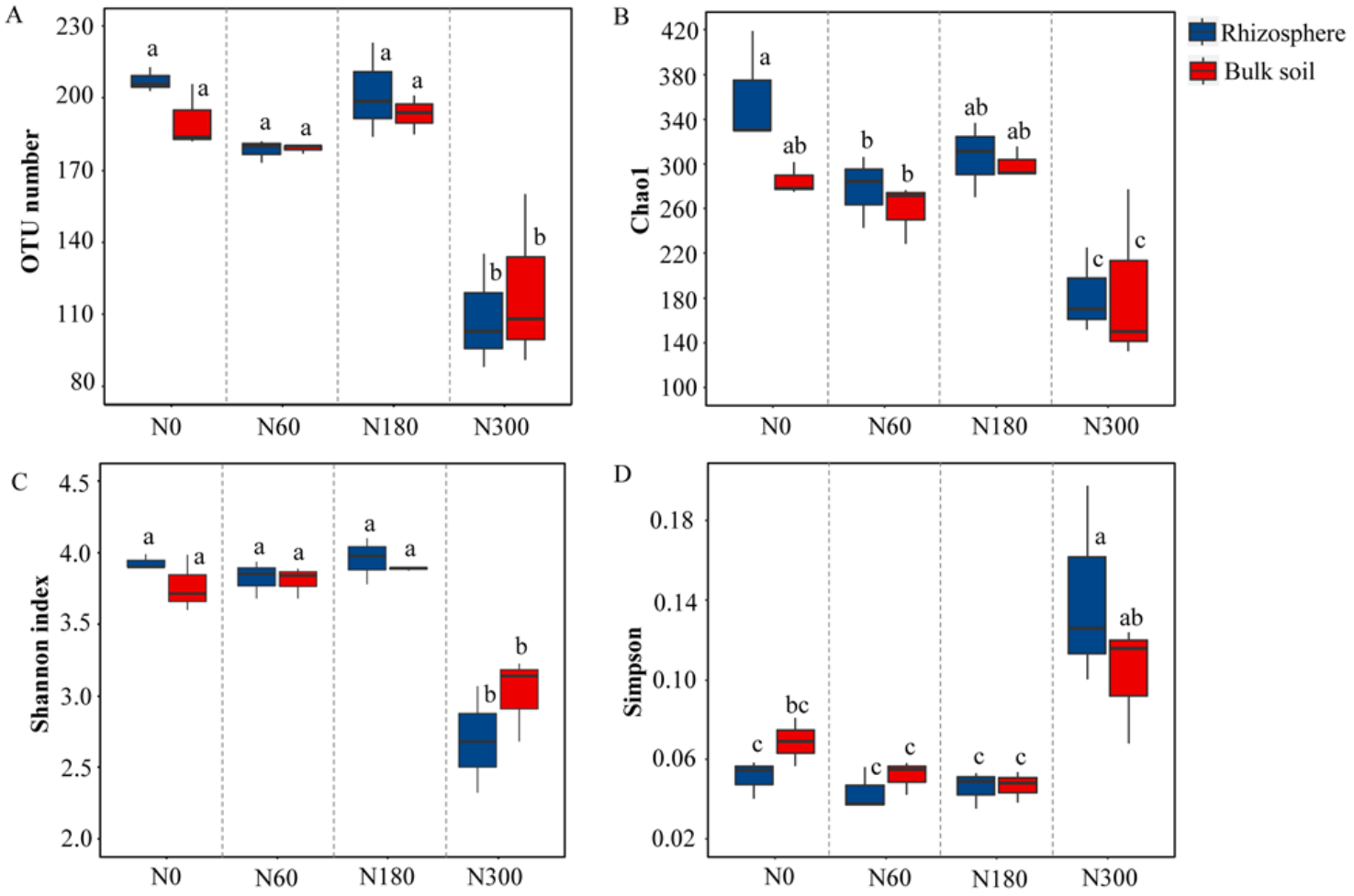

Figure 3

α-diversity of diazotrophs in maize rhizosphere and bulk soils under long-term different N fertilizer application rates. A: OTU number; B: the Chao1 estimator; C: the Shannon diversity index; D: the Simpson diversity index. Data are means ± standard error (n=3). Different letters on the bars indicate significant differences among the long-term different N fertilizer application rates (Duncan multiple-range test, $P<0.05$).

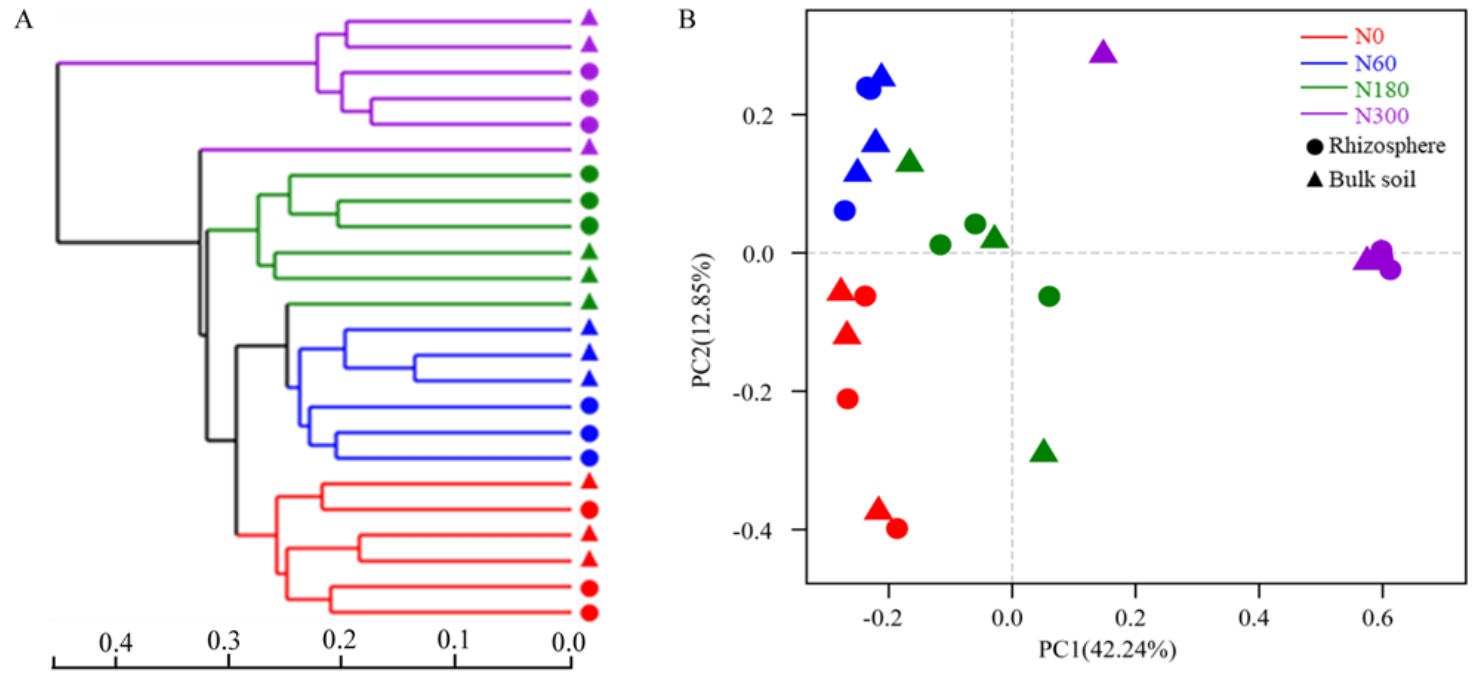

Figure 4

Hierarchical clustering analysis by UPGMA (A) and principal co-ordinates analysis (B) of diazotrophs in maize rhizosphere and bulk soils under long-term different N fertilizer application rates. The above analyses were based on the OTU-based bray-curtis distance matrix. The samples were analyzed in triplicate plots.

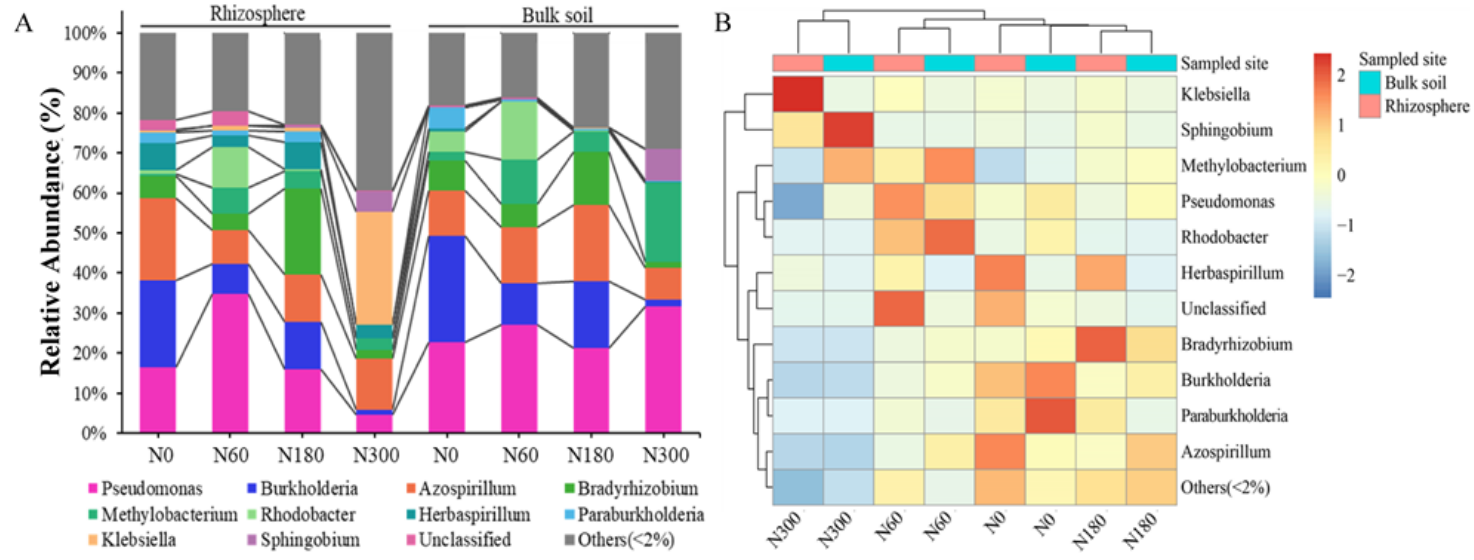

Figure 5

Taxonomic composition of diazotrophic communities at the genus level in maize rhizosphere and bulk soils under long-term different N fertilizer application rates. A: Relative abundances (%) of the most abundant genera (>2%); B: Heatmap showed the relative abundance of the most abundant genera (> 2%).

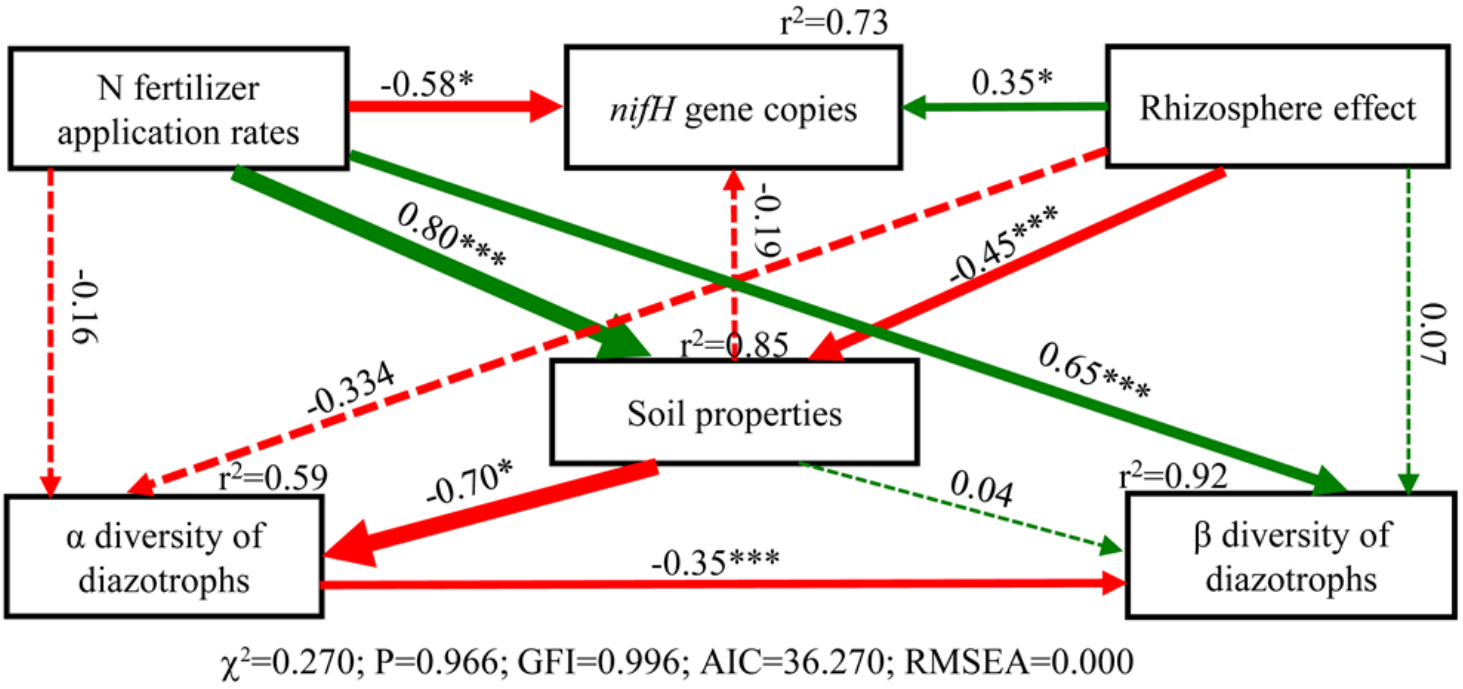

Figure 6

Structural equation model (SEM) illustrating the direct and indirect effects of N fertilizer application rates or rhizosphere effect on soil properties and alpha/beta diversity of diazotrophs. Continuous and dashed arrows represent the significant and nonsignificant correlations, respectively. Adjacent number that are labeled in the same direction as the arrow represents path coefficients, and the width of the arrow is in proportion to the degree of path coefficients. Green and red arrows indicate positive and negative relationships, respectively. r2 values indicate the proportion of variance explained by each variable. Significance levels are denoted with *P < 0.05, **P < 0.01, ***P < 0.001. Standardized total effects calculated by the SEM were displayed below the SEM. The low chi-square (χ2), nonsignificant probability level (P > 0.05), high goodness-of-fit index (GFI > 0.90), low Akaike information criteria (AIC), and low root-mean-square errors of approximation (RMSEA < 0.05) listed below the SEMs indicate that our data matches the hypothetical model.

Supplementary Files

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