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Effects of C and N application on *Azotobacter* and nitrogen cycle in farmland soils of central Guizhou, China

Zhiwei Qiao[1,2,3†], Chao Liu[2†], Ru Yan[2*] and Shihua Qin[1]

Abstract

Carbon sources and nitrogen sources are two important groups of substances in analyses of the effects of exogenous additives on soil microorganisms. Glucose (present in soil) and urea (extensively used in agricultural production) are substances often used to study the specific effects of carbon and nitrogen addition on microorganisms. *Azotobacter*, a non-symbiotic nitrogen-fixing bacterium, has been characterized in the laboratory and applied to soil in numerous studies. However, soil microorganisms display considerable diversity, and the effects of in exogenous substances stimulation on various microorganisms are uncertain. The potential effects of forced exogenous substance stimulation on *Azotobacter* are not well understood. Here, the effects of C and N application on *Azotobacter* growth and nitrogen cycle metabolism in farmland soil in central Guizhou, China, were studied through analysis of four treatment groups: control (CK), glucose treatment (C), urea treatment (N), and glucose + urea treatment (CN). The results showed that the relative abundances of the *Azotobacter* genus and relevant species were increased in group C, indicating promotion of *Azotobacter* growth ($P < 0.001$). The relative abundances of the *Azotobacter* genus and relevant species in group CN were significantly different from the abundances in group N and CK ($P < 0.05$). Furthermore, the relative abundances of *nif* genes (i.e., *nifH*, *nifD*, and *nifK*) and *nirD* were significantly increased in group C. However, the relative abundances of the aforementioned four nitrogen cycle-related genes did not significantly differ between group CN and groups CK and N. The main source species of the aforementioned four nitrogen cycle-related genes were *Azotobacter* species. The total nitrogen and alkali-hydrolyzed nitrogen contents in soil did not significantly differ in a comparison between group C and groups N and CN. Piecewise structural equation modeling analysis revealed that bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes had significant direct effects on the alkali-hydrolyzed nitrogen content in soil and had negligible direct effects on the total nitrogen content in soil. These findings improve the broader understanding of *Azotobacter* and provide theoretical support for reduced *Azotobacter* utilization in soil.

Keywords *Azotobacter*, Nitrogen cycle, Total nitrogen in soil, Alkali-hydrolyzed nitrogen, Piecewise SEM

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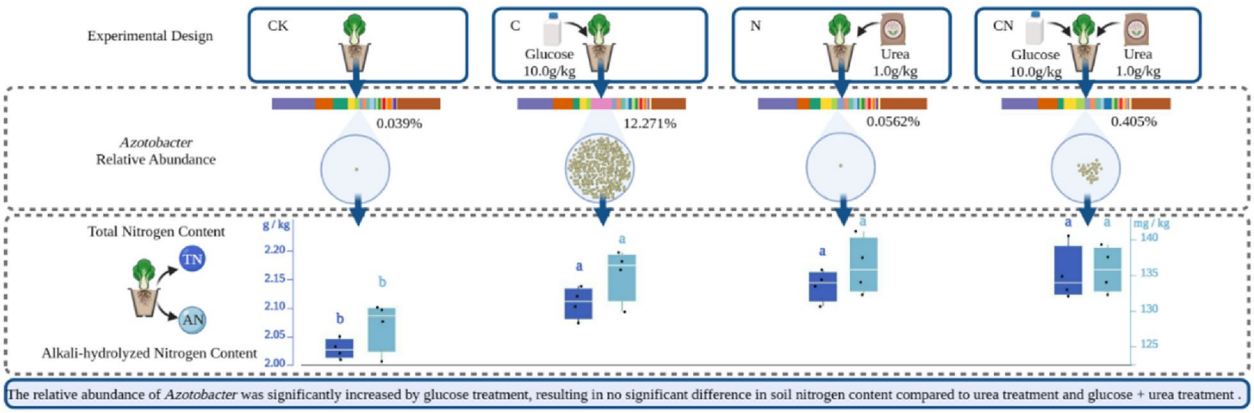
Graphical Abstract



Introduction

Nitrogen is a critical element for plant growth [1, 2] and a major limiting factor for ecosystem productivity [3]. However, the use of chemical nitrogen fertilizers is often inefficient [4]. Large amounts of nitrogen fertilizer are often used in agricultural production to meet crop nitrogen demands. Unfortunately, excessive use of nitrogen fertilizer can cause serious environmental problems such as soil compaction, acidification, increased greenhouse gas emissions, and loss of soil biodiversity [5, 6]. Annual nitrogen fertilizer consumption in China represents 23% of the total worldwide; its mean application rate of 191 kg/hm^2 in farmland is 2.6-fold greater than the global mean [7]. The Guizhou region is characterized by a typical karst geological terrain mainly composed of yellow soil, which plays a crucial role in agricultural production. However, this region exhibits low nitrogen fertilizer utilization rates [8], as well as significant soil and water loss; these factors adversely affect agricultural production and the ecological environment. Therefore, reducing nitrogen fertilizer application and improving nitrogen fertilizer utilization are key areas of nitrogen research for agricultural production in this region [9].

Biological nitrogen fixation is an important source of nitrogen within ecosystems [10, 11]; Studies have investigated the mechanism of biological nitrogen fixation using *Azotobacter* as a model organism [12]. *Azotobacter* can convert atmospheric N_2 into forms of nitrogen that are available to plants [12]. Promotion of the nitrogen fixation capabilities of soil microorganisms can substantially reduce the reliance on inorganic nitrogen fertilizers in agricultural ecosystems [13]. Microorganisms in the *Azotobacter* genus possess the ability to fix nitrogen [14]; they are characterized by independence from plant species and the capacity for autonomous nitrogen fixation in a non-symbiotic manner. Plate isolation experiments helped to identify strains within the *Azotobacter* genus; these strains were subsequently deployed on crops and soils. The application of *Azotobacter* led to greater nitrogen fixation within soil and a corresponding increase in plant nitrogen content, indicating a positive effect on soil nitrogen fixation [15–17]. However, microbial screening and application efficacy can be hindered by factors such as the inherent limitations of soil microorganisms [18]; the potential degradation of strain function after isolation, purification, and preservation [19, 20]; and the competitive nature of indigenous microorganisms after their application to soil [21]. Accordingly, the promotion of nitrogen fixation may be significantly improved by stimulating *Azotobacter* growth through the addition of exogenous substances to soil; this approach may have practical implications.

External C and N sources have significant impacts on *Azotobacter* growth and nitrogen fixation; such effects have been extensively investigated [22–26]. External sources of carbon can promote *Azotobacter* growth, whereas external sources of nitrogen or high nitrogen content within soil can have an inhibitory effect [27, 28]. Because of variations in carbon and nitrogen sources, the promotion or inhibition of *Azotobacter* may yield considerably different results [29]. Previous studies have revealed that exogenous nitrogen does not have significant effects on non-rhizosphere nitrogen fixation or non-symbiotic nitrogen fixation [30]. Furthermore, highly efficient microbial nitrogen fixation can be achieved in nitrogen-rich environments [31]. These

discrepancies may be attributed to the competition between promotive and inhibitory effects. Thus far, there have been few studies concerning the effects of various additive substances on *Azotobacter*. Additionally, the significance of the impacts of changes in *Azotobacter* on nitrogen cycle genes (i.e., whether there is a threshold at which changes in *Azotobacter* influence nitrogen cycle genes) has not been elucidated; no studies have explored the pathways by which changes in *Azotobacter* and nitrogen cycle genes affect nitrogen in soil under C and N application.

The effects of external C (glucose, a sugar commonly used in laboratory screening of nitrogen-fixing microorganisms) and N (urea, a chemical nitrogen fertilizer widely used in agricultural production) sources on *Azotobacter* in soil were examined through a pot experiment in the field. Analyses of the effects of *Azotobacter* on nitrogen cycle genes and nitrogen in soil will improve the overall understanding of *Azotobacter*. We hypothesized that externally added carbon and nitrogen may have a significant effect on soil *Azotobacter* growth, and that this effect will lead to changes in biological nitrogen fixation gene abundance, and that changes in *Azotobacter* growth and nitrogen fixation gene abundance will have direct or indirect effects on soil nitrogen nutrients. Additionally, these analyses will provide theoretical support to reduce the utilization of *Azotobacter* in soil while enhancing nitrogen fixation in soil and improving the use of chemical nitrogen fertilizer.

Materials and methods

Field site

This study was conducted in Tongxin Village (26°18′36"N, 106°21′36"E, 1340 m a.s.l.), Daxiqiao Town, Xixiu District, Anshun City, Guizhou Province. The area has a plateau-type subtropical humid monsoon climate with 1334.6 mm mean annual precipitation and 14 °C annual mean temperature. The highest historical temperature recorded was 34.3 °C, and the lowest temperature was −7.6 °C. The land use type of the study area is arable land, with the soil type being yellow soil. The main crops grown are corn and rape, and the primary fertilizer used is urea. The basic physical and chemical properties of the soil were as follows: pH 7.18, total nitrogen content 2.08 g/kg, total phosphorus content 1.09 g/kg, total potassium content 36.36 g/kg, available phosphorus content 52.37 mg/kg, and organic matter 71.87 g/kg.

Experimental design

Considering the effects of the external environment on experimental conditions, an in-situ pot experiment was performed in this study. Four 4 m × 4 m plots were established in the study area, and twelve 0.5 m × 0.5 m sampling points were randomly selected in each plot. Surface soil (0–10 cm) was collected with a spade. Soil samples from sets of three sampling sites were thoroughly mixed, weighed in 5-kg quantities, and loaded into plastic pots. Four pots of soil were obtained from each plot, then divided into four treatment groups: blank (CK), glucose addition (C), urea addition (N), and combined glucose and urea addition (CN). The four plots consisted of four replicates, for a total of 16 pots. The amounts of glucose and urea added in all treatments are shown in Table 1; all treatment substances were fully mixed with soil samples before use. Oilseed rape, the main crop planted in the study area, was selected for pot experiments. Three oilseed rape seedlings were transplanted into each pot; after 15 days, only 1 plant was left in each pot, consistent with the growth pattern of oilseed rape seedlings. The experimental period extended from October 2020 to May 2021; this 7-month duration comprised a complete growing season for rape. After the rapeseed had matured, rapeseed samples were collected and weighed. Soil samples of ~ 500 g were also collected from the root surface layer (0–20 cm). After the removal of impurities, each soil sample was divided into two parts. Approximately 100 g of one part were placed on dry ice and promptly sent to Shanghai BioEngineering Co., Ltd. (China) for metagenomic sequencing. The remaining soil samples were air-dried and passed through a 1-mm soil sieve, then used for analyses of soil physical and chemical properties. Figure 1 shows the experimental design.

Test methods

Indicators in soil and plant samples

The total nitrogen content in soil was analyzed using the Kjeldahl method; organic matter was determined by potassium dichromate external heating. The alkali-hydrolyzed nitrogen content in soil was evaluated by alkaline hydrolysis diffusion. Soil pH was determined using a pHS-3C pH meter (Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China), with a water to soil ratio of 1:1. Plant dry weight was determined by direct measurement; the total nitrogen content in plants was quantified via sulfuric acid–perchloric acid elimination

Table 1 Bacterial community α-diversity metrics for each group

Group	Ace	Chao1	Shannon	Simpson
CK	2209.38 ± 45.09a	2227.20 ± 40.62a	6.434 ± 0.120b	0.933 ± 0.011a
C	2250.05 ± 80.23a	2272.68 ± 85.72a	6.320 ± 0.214b	0.937 ± 0.013a
N	2309.83 ± 92.29a	2345.43 ± 109.1a	6.557 ± 0.122ab	0.946 ± 0.009a
CN	2320.08 ± 90.55a	2342.87 ± 92.06a	6.649 ± 0.089a	0.947 ± 0.010a

Different lowercase letters in the same column indicate differences at the 0.05 level

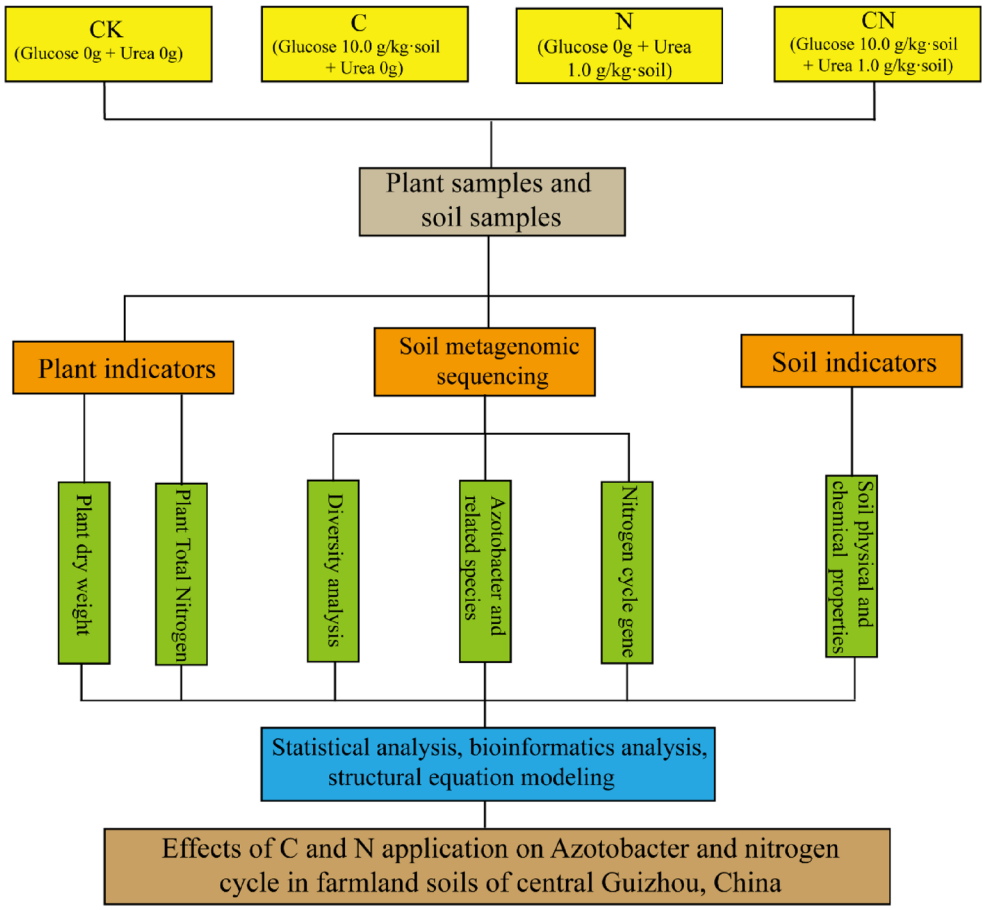


Fig. 1 Experimental design

and vanadium-molybdenum yellow colorimetric methods [32].

Soil metagenomic sequencing

(1) Soil DNA extraction and detection

Total community genomic DNA was extracted from soil samples using the cetyltrimethylammonium bromide (CTAB) method [33]. DNA concentration, integrity, and purity were assessed using an Agilent 5400 (Agilent Technologies Co. Ltd., USA).

(2) Library construction, detection, and sequencing

Libraries were constructed using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, USA). Polymerase chain reaction products were then purified using the AMPure XP system (Beverly, USA); library insert sizes were assessed using an Agilent 2100 (Agilent Technologies Co. Ltd., USA). Library concentrations were quantified by real-time polymerase chain reaction. The whole DNA libraries were sequenced using the Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) high-throughput sequencing platform.

Bioinformatics analysis

(1) Data quality control

To ensure data validity, raw sequencing data were preprocessed with Kneaddata software. Raw data were filtered using Trimmomatic (Version 0.36), with the default parameter value set to Q20. Quality sequences with scores below Q20 were removed. Quality control regarding the consistency and efficacy of preprocessing were assessed using FastQC (Version 0.11.2) [34].

(2) Metagenomic sequence analysis methods

DBA_UD (version 1.1.2), a splicing software based on the De Bruijn graph principle, was used to assemble

high-quality reads and obtain contigs. Open reading frames (ORFs) were predicted by Prodigal software (version 2.60), and genes longer than 100 bp were selected for protein sequence translation. All predicted gene sequences were clustered by CD-HIT software (version 4.6) (parameters set to 95% identification and 90% coverage) to construct a set of non-redundant genes [35].

(3) Species annotation

The gene sequence data used for protein translation were compared with the National Center for Biotechnology Information (NCBI) NT nucleic acid database and RefSeq whole-genome database using DIAMOND software (version 0.8.20); blastp homology alignment was conducted to obtain functional annotations and homologous species information. The screening criteria were E-value < 1e−5 and score > 60. Additionally, the species classification and annotation information were obtained for all genes. The relative abundances of microorganisms at multiple taxonomic levels (kingdom, phylum, class, order, family, genus, and species) were determined using the NCBI database containing microbial taxonomic information [36]. The soil metagenomic sequencing data have been submitted to the NCBI database under the biological number PRJNA1026382.

(4) KEGG functional annotations

GhostKOALA software (version 1.0) was used to compare the gene sequence data used for protein translation with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This analysis revealed the corresponding KO number for each sequence. Pathway and Module annotation information for each sequence were obtained on the basis of connections between KO numbers, Pathways, and Modules. Abundances at all functional levels in the KEGG database were determined for each sample [37]. Based on grouping information, LEfSe biomarker and Dunn test analyses were conducted to more comprehensively explore differences in species composition and functional composition among the samples [38].

(5) Nitrogen cycling functional genes and relative abundance

Soil turnover process includse nitrogen fixation, nitrification, denitrification, dissimilatory nitrite reduction to ammonium and assimilatory nitrate reduction to ammonium. The functional genes of coding key enzymes in the N turnover and corresponding KO numbers are as follows: nitrogen fixation (*nifH*, K02588; *nifD*, K02586; *nifK*, K02591), nitrification (*amoA*, K10944; *amoB*, K10945; *amoC*, K10946; *hao*, K10535), denitrification (*narG*, K00370; *narH*, K00371; *narI*, K00374; *nirK*, K00368; *nirS*, K15864; *norB*, K04561; *norC*, K02305; *nosZ*, K00376), dissimilatory nitrite reduction to ammonium (*napA*, K02567; *nirB*, K00362; *nirD*, K00363), assimilatory nitrate reduction to ammonium (*nasA*, K00372, *nasB*, K00367). The relative abundance of the number of KO corresponding to each gene in KEGG is the relative abundance of genes. This method can provide information about the functional potential of soil microbes, but to determine if the function of soil microbes is being expressed, we need to further study the soil microbial community through metatranscriptomics.

Data processing

Raw data were preliminarily sorted and calculated using Excel 2007. α-diversity analysis was performed using Micronas through the Au cloud website (https://www.bioincloud.tech/task-meta). Principal coordinates analysis and Venn diagram preparation were conducted based on the Bray–Curtis distance algorithm. The effects of different treatments on the *Azotobacter* genus and relevant species in soil were compared via Statistical Analysis of Metagenomic Profiles (STAMP) software, with the significance threshold set at $P < 0.05$. Comparisons among groups in terms of three *Azotobacter* species, soil texture, and rape were conducted by one-way analysis of variance and least significant difference multiple comparison in R software (v4.2.2), with the significance threshold set at $P < 0.05$. Piecewise structural equation modeling (SEM) was used to evaluate the direct and indirect associations of key *Azotobacter* species, bacterial α-diversity, and nitrogen cycle genes with nitrogen content in soil. Data visualization was performed using the ggplot2 and heatmap packages in R software.

Results and analysis

Effects of external C and N sources on bacterial community structure in soil

We investigated the impact of external C and N sources on the bacterial community structure and α-diversity (Table 1 and Fig. 2). Regarding soil bacterial α-diversity, the Ace, Chao1, and Simpson indices of the groups were in the order of CN > N > C > CK; there were no significant differences among the groups. The bacterial Shannon indices for groups CK, C, N, and CN were 6.434, 6.320, 6.557, and 6.649, respectively. Although group C had the lowest Shannon index value, the values did not significantly differ between group CK and group C. These results indicated that the exogenous carbon and nitrogen additions in this experiment had a small effect on soil microbial diversity.

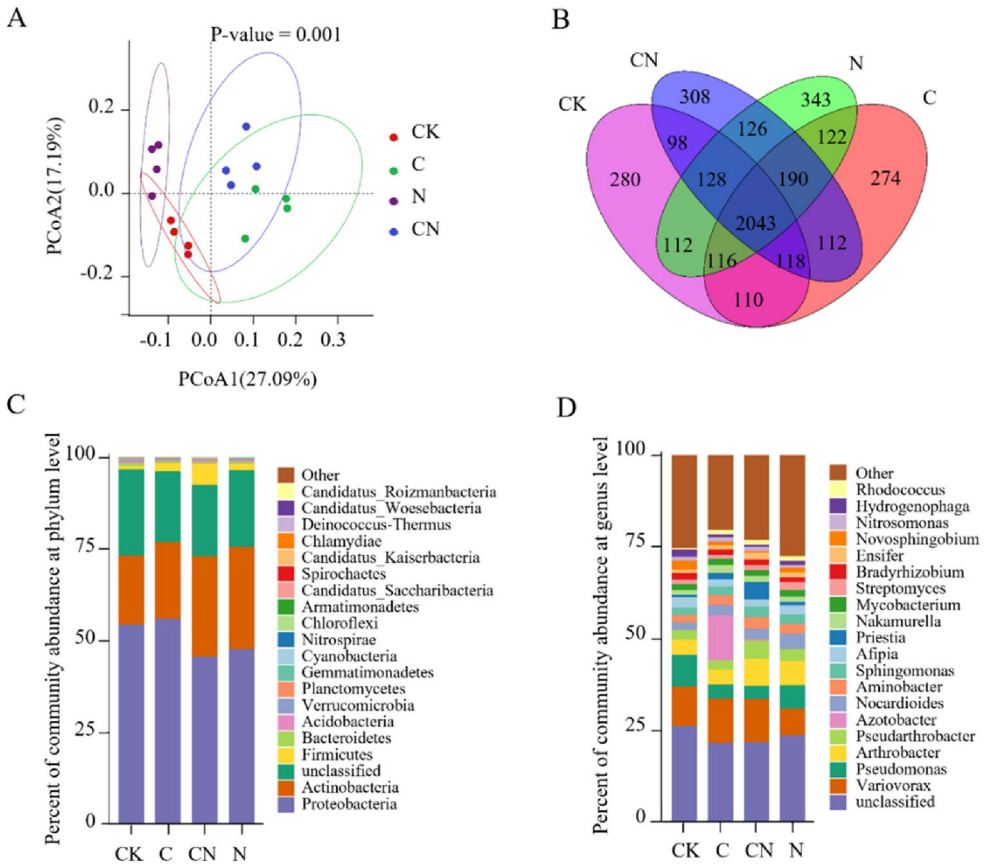


Fig. 2 Effects of C and N application on soil bacterial communities: **A** principal coordinates analysis map of bacterial communities based on Bray–Curtis distance; **B** Venn diagram of bacterial communities; **C** relative abundances of major bacterial phyla; **D** relative abundances of major bacterial genera

However, principal coordinates analysis of bacterial community structure based on Bray–Curtis distance showed that microorganisms in the group CK, C, N, and CN soils formed four distinct clusters, which were significantly separated along the first axis ($P < 0.001$). Combined with PerMANOVA test, the soil bacterial community structure composition was significantly different ($P < 0.05$) among the four treatments, indicating that the application of C and N significantly affected soil bacterial community structure (Table 2). The Venn diagram of bacterial community structure showed that groups CK, C, N, and CN had 2043 shared species; their unique species were 280, 274, 343, and 308, respectively (Fig. 2B). Overall, the four groups had a higher number of shared species, compared with the number of unique species; this finding indicated that species differences were minimal.

In this study, bacterial community composition was analyzed based on relative abundances at the phyla and genera levels (Fig. 2C and D, respectively). *Proteobacteria*, *Actinomycetes*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* were the most abundant phyla in soil, together representing > 70% of the community. *Proteobacteria* had the highest relative abundance in group C (56.04%), which was significantly higher than the abundances in groups N and CN (47.65% and 45.71%, respectively, ($P < 0.05$). This finding suggests that external carbon sources have a stimulatory effect on

Table 2 PerMANOVA test of the differences in bacterial community structure based on Bray–Curtis distance measures

Group	R^2	P
CK-vs-C	0.398222502	0.028
CK-vs-CN	0.419906381	0.026
CK-vs-N	0.342048447	0.022
C-vs-CN	0.363717393	0.033
C-vs-N	0.468969347	0.036
CN-vs-N	0.438665934	0.025

$R^2 > 0$ reflecting a difference between groups; $P < 0.05$ reflecting statistical significance

Proteobacteria growth, while external nitrogen sources have an inhibitory effect on *Proteobacteria* growth. In particular, the relative abundance of *Azotobacter*, which belongs to the *Proteobacteria*, displayed a significant change between group CK and group C ($P<0.05$). Treatment with glucose increased the relative abundance of *Azotobacter* by 310.30-fold to 12.27%, making it the dominant genus in group C. The *Azotobacter* abundance in group N did not significantly differ from the abundance in group CK; Group CN displayed a significant difference in *Azotobacter* abundance compared with groups CK and N. The above results indicated that the exogenous carbon stimulation of the farmland soil in central Guizhou could stimulate the growth potential of the nitrogen-fixing bacteria *Azotobacter* to become a dominant bacterium, and then change the structure of soil microbial community.

Effects of external C and N sources on *Azotobacter* genus and relevant species in soil

Examination of the species with significant differences in relative abundance among the groups (Fig. 3) revealed significant increases ($P<0.001$) in *Azotobacter chroococcum*, *Azotobacter salinestris*, and *Azotobacter vinelandii* in group C, compared with the other groups. As shown in Fig. 4, the relative abundance of *A. chroococcum* in group C represented 9.11% of all species; this was 219.23-, 42.95-, and 298.52-fold higher than the corresponding relative abundances in groups N, CN, and CK (Fig. 4A). Furthermore, the relative abundance of *A. salinestris* in group C was 2.61%; this was 197.79-, 14.06-, and 388.21-fold higher than the corresponding relative abundances in groups N, CN, and CK (Fig. 4B). Finally, the relative abundance of *A. vinelandii* in group C was 0.54%; this was 317.68-, 21.79, and 237.81-fold higher

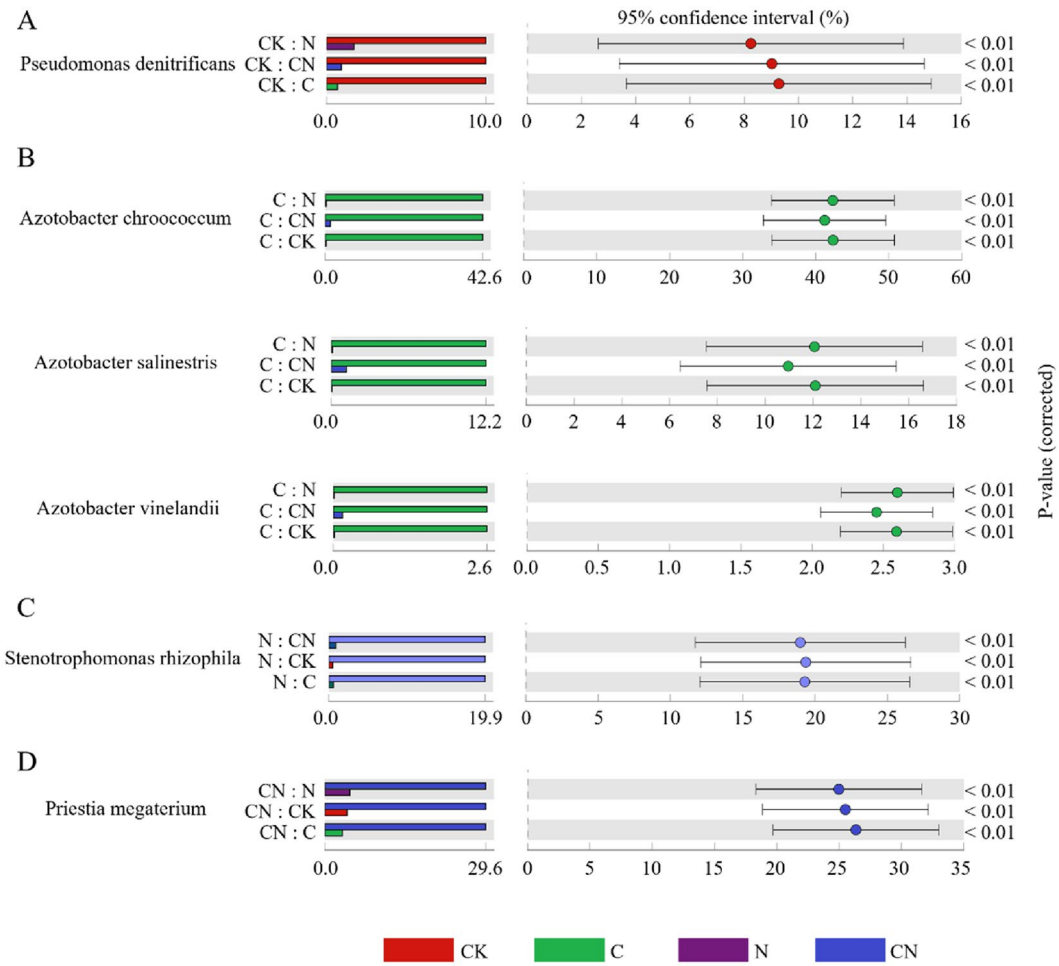


Fig. 3 Species with significant differences in relative abundance among groups; extended error bars are shown. **A** Significantly different species between group CK and other groups ($P<0.01$, mean ratio $n=3$; identical values for comparisons in other panels); **B** significantly different species between group C and other groups; **C** significantly different species between group N and other groups; **D** significantly different species between group CN and other groups

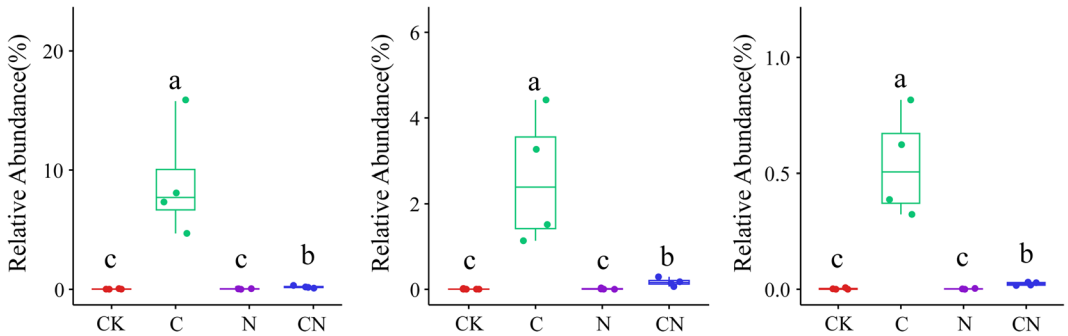


Fig. 4 Relative abundances in different groups: *Azotobacter chroococcum* (**A**), *Azotobacter salinestris* (**B**), and *Azotobacter vinelandii* (**C**)

than the corresponding relative abundances in groups N, CN, and CK (Fig. 4C). Notably, the relative abundances of *A. chroococcum*, *A. salinestris*, and *A. vinelandii* did not significantly differ between group N and group CK. This result further demonstrated that soil nitrogen-fixing bacteria *Azotobacter* in central Guizhou could increase their relative abundance to form dominant bacteria under the stimulation of exogenous carbon.

Effects of variations in *Azotobacter* genus and relevant species on nitrogen cycle genes

The *Azotobacter* genus and its corresponding species exhibited significant changes in group C. In particular, during the nitrogen cycle, the nitrogen fixation and nitrate dissimilation reduction pathways were significantly enhanced in group C. The relative abundances of the *nif* genes *nifD*, *nifH*, and *nirD* were significantly higher in group C than in other groups; the relative abundance of the nitrate dissimilation-related gene *nifK* was significantly higher in group C than in group CK. However, there were no significant differences in the relative abundances of genes associated with these pathways among groups N, CN, and CK, as shown in Fig. 5A and B. The main source species of the differential genes *nifD*, *nifH*, *nifK*, and *nirD* in group C were *Azotobacter* species (Fig. 5C).

Effects of external C and N sources on rape yield, soil nitrogen contents, and soil physicochemical properties

The dry weight of rape was significantly greater in groups C and N than in group CK (Fig. 6A). There was no significant difference in the dry weight of rape between group C and group N. As depicted in Fig. 6B, total nitrogen content in plants was significantly greater in group C (13.89%) than in group CK. Furthermore, group N exhibited significantly greater total nitrogen content in plants, compared with group C. Soil pH did not significantly differ among groups, as shown in Fig. 6C. Additionally, total nitrogen content in soil was significantly greater in groups C, N, and CN, compared with group CK (Fig. 6D). However, the three groups did not exhibit significant differences in total nitrogen content. Figure 6E shows that the four groups did not exhibit significant differences in alkali-hydrolyzed nitrogen content. Moreover, groups C and CN had a higher organic carbon content in soil compared with group N, but the three groups did not display significant differences in organic carbon content in soil (Fig. 6F).

Effects of bacterial α-diversity, *Azotobacter,* and nitrogen cycle genes on nitrogen content in soil

Piecewise SEM analysis (Fig. 7) further illustrated the direct and indirect effects of bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes on soil nitrogen levels. The findings showed that bacterial α-diversity (Ace, Chao1, Shannon index, and Simpson index), *Azotobacter* in soil (*A. chroococcum*, *A. vinelandii*, and *A. salinestris*), and nitrogen cycle genes (*nifK*, *nifD*, *nifH*, and *nirD*) had direct effects on alkali-hydrolyzed nitrogen content in soil, with respective path coefficients of 0.39, 0.68, and 0.82, as well as significance levels of 0.05, 0.01, and 0.001. *Azotobacter* also had indirect effects on alkali-hydrolyzed nitrogen content in soil through direct effects on nitrogen cycle genes, with a path coefficient of 0.73 and a significance level of 0.01. Bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes had negligible direct effects on total nitrogen content in soil, as shown in Fig. 7B.

Discussion

Effects of external C and N sources on *Azotobacter* genus and relevant species

As anticipated, all the groups exhibited the identical dominant bacterial phyla, namely *Proteobacteria*, *Actinomycetes*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*, which is consistent with the composition of most agricultural soils. However, the relative abundance of the *Proteobacteria* in group C was significantly higher in this experiment because the relative abundance of the *Azotobacter* genus was 310.30-fold higher in group C

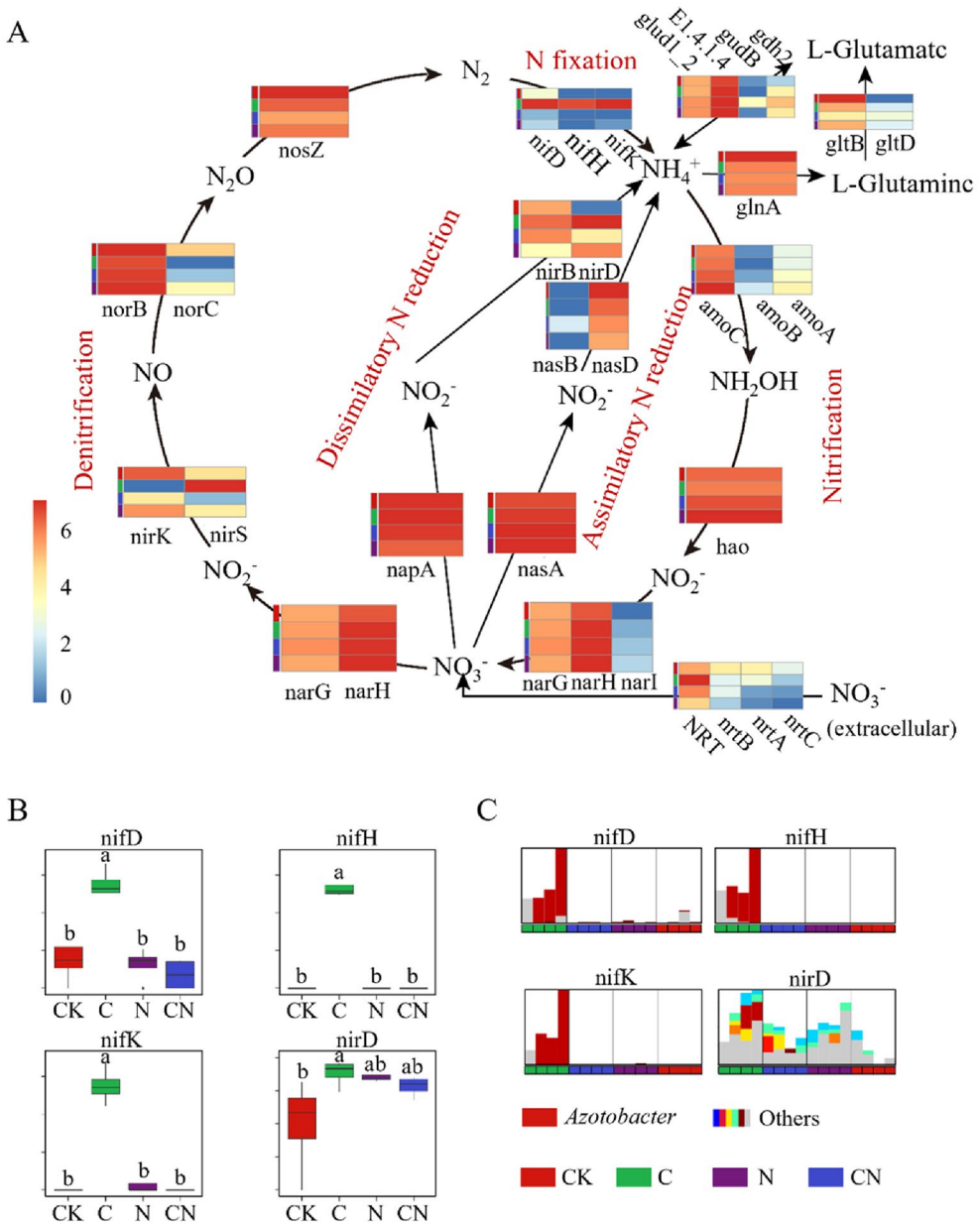


Fig. 5 Main functional genes involved in nitrogen cycle pathways and their species sources. **A** Nitrogen metabolic pathways and relative abundances of functional genes; **B** significant differences in relative abundance of functional genes ($P < 0.05$); **C** species origin of significantly different genes in group C

than in group CK ($P < 0.001$). Moreover, the respective relative abundances of *A. chroococcum*, *A. salinestris*, and *A. vinelandii* were 298.52-, 388.21-, and 237.82-fold higher in group C than in group CK; the respective relative abundances of *A. chroococcum*, *A. salinestris*, and *A. vinelandii* were 6.95-, 27.70-, and 10.78-fold higher in group CN than in group CK; There were no significant differences between the N and CK groups. These results validated our hypothesis that the simultaneous addition of carbon and nitrogen significantly promotes the growth of *Azotobacter*. However, differently from the hypothesis, sole carbon addition significantly stimulated the growth of *Azotobacter*, while sole nitrogen addition did not have a significant effect on the growth of *Azotobacter*. There could be several reasons for these results. Firstly, *Azotobacter* species are chemoheterotrophic microorganisms found in soil. They require external sources of carbon to meet their energy demands and achieve growth [39].

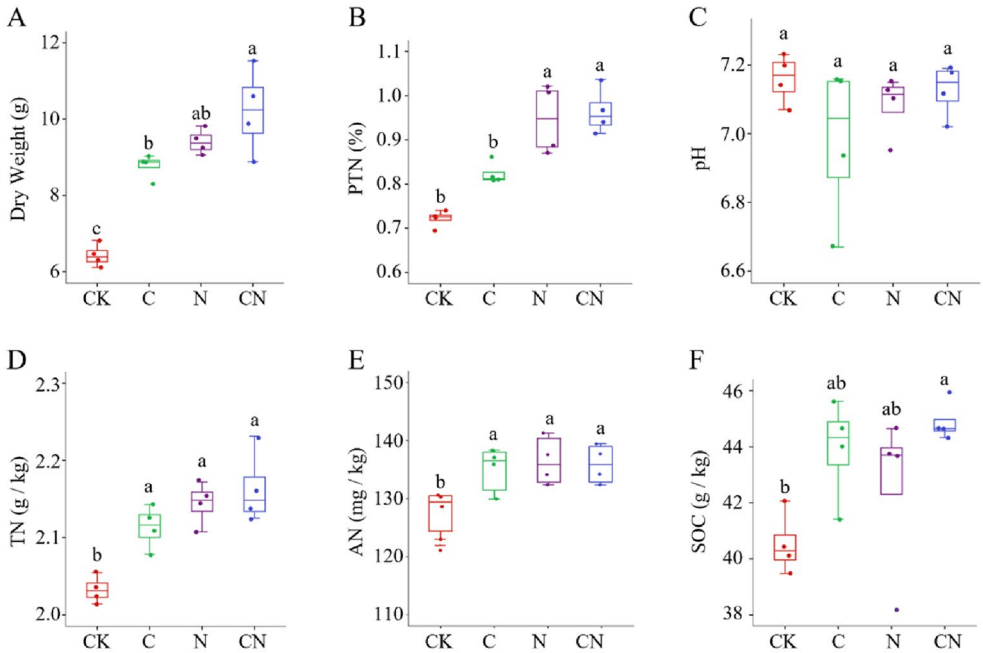


Fig. 6 Groupwise comparisons of **A** dry weight of rape, **B** total nitrogen content in plants (PTN), **C** soil pH, **D** total nitrogen content (TN) in soil, **E** alkali-hydrolyzed nitrogen (AN) content in soil, and **F** soil organic carbon (SOC) content. Lowercase letters in each graph denote significant differences ($P < 0.05$)

Because of the high energy consumption involved in biological nitrogen fixation, these microorganisms prefer easily accessible carbon sources [40]. Therefore, none of the microbial genera except *Azotobacter* in this study responded significantly to exogenous carbon addition. Secondly, external nitrogen sources increase the nitrogen content in soil, particularly with respect to nitrogen availability, which has a considerable effect on *Azotobacter* community structure in soil [39]. Previous studies demonstrated that nitrogen addition has a negative effect on *Azotobacter* levels in soil [41, 42]. In the present study, the overall relative abundance of the *Azotobacter* genus and the relative abundances of *A. chroococcum*, *A. salinestris*, and *A. vinelandii* in group N were not statistically different from those in group CK. The sole addition of nitrogen did not exert a negative effect on the relative abundance of *Azotobacter*, which could be due to the fact that *Azotobacter* was in a dormant state under unfavorable conditions [43, 44] and did not show a significant response to short-term nitrogen stimulation [45]. Thirdly, a meta-analysis showed that high organic carbon content in soil could mitigate the negative effect of nitrogen addition on *Azotobacter* [13]. The relative abundances of the *Azotobacter* genus and its relevant species were significantly higher in group CN than in groups CK and N, indicating that the simultaneous addition of exogenous carbon and nitrogen sources could significantly promote the growth effect of *Azotobacter*, while the promotion effect of carbon sources on *Azotobacter* is inhibited by nitrogen. When exogenous carbon sources were added or the intrinsic carbon content of the soil was maintained at a high level and the nitrogen level was low, the extent to which *Azotobacter* was carbon-stimulated was unaffected by nitrogen inhibition. In the present study, these amounts of increase in *Azotobacter* growth by the addition of carbon alone have never been reported in previous studies, indicating significant potential for stimulation of Azotobacter growth in farmland soil through the addition of an appropriate carbon source.

Effects of *Azotobacter* genus and relevant species on *nif* genes

The results demonstrated that microbial nitrogen fixation in soil was enhanced in group C, consistent with the findings in previous studies [40]. Indeed, the input of exogenous carbon provides energy for microorganism metabolism in soil, thereby enhancing nitrogen fixation activity in soil. Nevertheless, groups CN and N displayed negligible differences in microbial nitrogen fixation in soil compared with group CK, which may be attributed to two factors. First, *Azotobacter* uses exogenous nitrogen, rather than nitrogen fixation, for growth [46]. Second, differential genes between group C and other groups, including *nifD*, *nifH*, and *nifK*, mainly originated

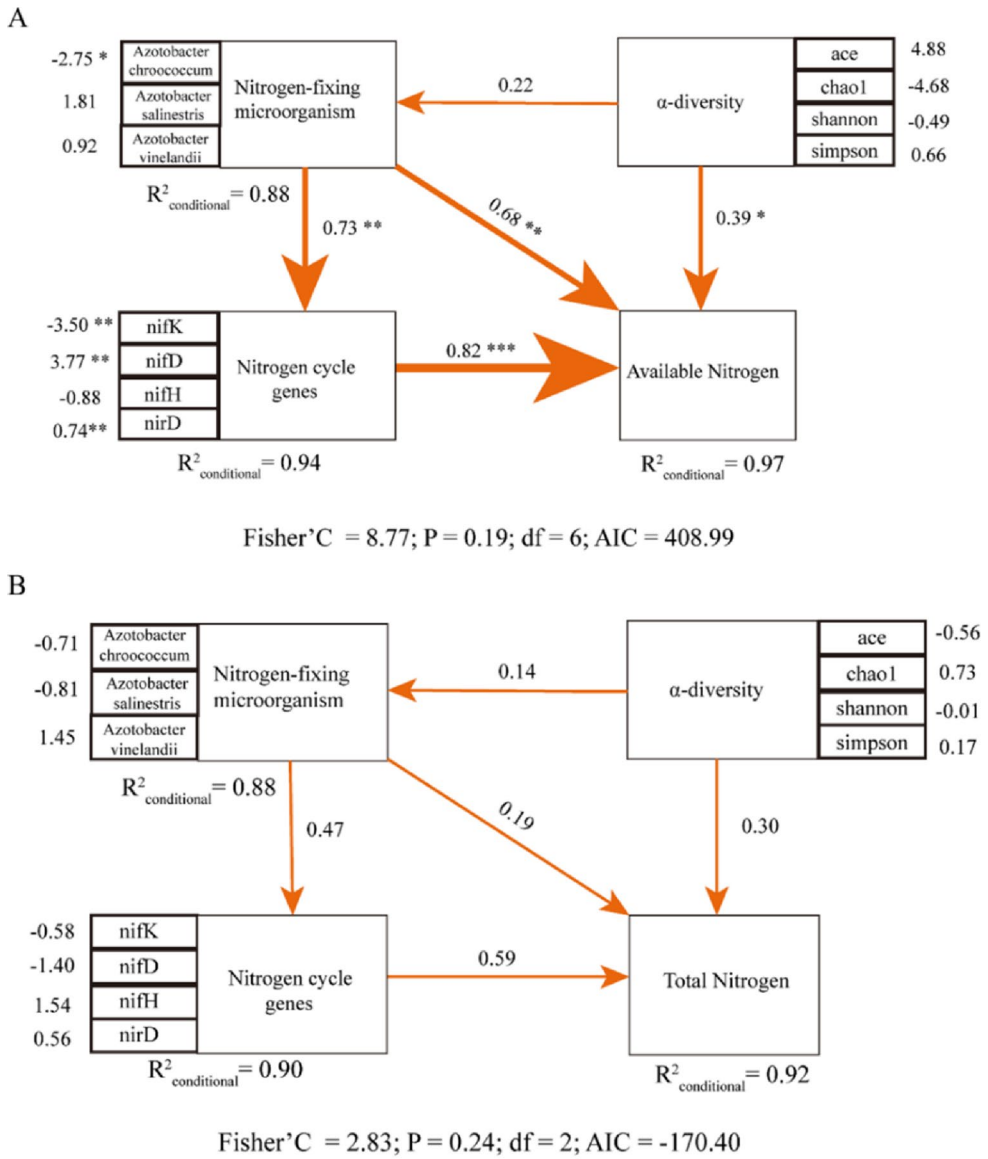


Fig. 7 Contribution paths of bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes to nitrogen contents in soil and plants. Piecewise SEM showed that bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes had direct and indirect effects on alkali-hydrolyzed nitrogen content in soil (**A**); they had direct effects on total nitrogen content in soil (**B**). Each composite variable contained several measurement variables. Numbers adjacent to the arrows are path coefficients; the arrow thickness indicates the strength of the relationship. The proportion of variance explained (i.e., R^2) appears below each composite variable in the model at the following significance levels: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

from *Azotobacter*; the expression levels of *nif* genes in *Azotobacter* are affected by the availability of N and C sources. Additionally, during exposure to excessive nitrogen, GlnK-NifL-NifA complexes are generated by *Azotobacter* to inhibit the activities of *nif* genes; excessive nitrogen can mask metabolic signals related to carbon status, leading to the inhibition of *nif* gene expression because of carbon deficiency [12]. Therefore, the relative abundance of the soil nitrogen-fixing bacteria *Azotobacter* could be adjusted by regulating the exogenous carbon input in central Guizhou yellow soil, which in turn could improve the capacity of soil biological nitrogen fixation.

Effects of bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes on nitrogen in soil

In the present study, *Azotobacter* species and nitrogen cycle genes were significantly affected by glucose treatment. Glucose has a positive effect on net nitrogen immobilization in soil [13]. Specifically, external glucose can increase the total nitrogen content in soil, consistent

with the present results. Moreover, bacterial α-diversity has been closely associated with nitrogen nutrients in soil [47, 48]. Therefore, bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes were selected as influencing factors in this experiment. The results showed that bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes had significant direct effects on alkali-hydrolyzed nitrogen content in soil; however, they had negligible direct effects on total nitrogen content in soil. Notably, variations in bacterial α-diversity and *Azotobacter* growth are directly associated with water-soluble organic nitrogen in soil [49]. Water-soluble organic nitrogen is regarded as a component of the alkali-hydrolyzed nitrogen content in soil. The significant direct effects of bacterial α-diversity and *Azotobacter* on alkali-hydrolyzed nitrogen content in soil may be attributed to this relationship. Both NO_3^- and NH_4^+ fixation pathways can occur concurrently in soil [50], as depicted in the nitrogen cycle in Fig. 5A. Glucose treatment (group C) significantly enhanced the nitrogen fixation and nitrate dissimilation reduction pathways, increasing NH_4^+ levels in soil and converting NO_3^- to NH_4^+ to avoid nitrogen loss. Alkali-hydrolyzed nitrogen consists of nitrate and ammonium nitrogen. Therefore, nitrogen cycle genes have a significant direct effect on alkali-hydrolyzed nitrogen content. Conversely, the total nitrogen content in soil, which includes more complex forms of nitrogen compared with alkali-hydrolyzed nitrogen, is affected by various factors. This complexity may explain why bacterial α-diversity, *Azotobacter*, and nitrogen cycle genes had a negligible direct effect on the total nitrogen content in soil.

Conclusions

In conclusion, this study showed that an additional carbon source (glucose) could significantly stimulate the growth of *Azotobacter* and increase soil nitrogen content by enhancing nitrogen fixation. Therefore, this study provides a theoretical basis for the use of exogenous carbon to increase soil nitrogen content, an idea for further in-depth analysis of the role of *Azotobacter* in regulating agricultural nitrogen, and a new perspective for the efficient use of nitrogen. The enhancement of nitrogen fixation by *Azotobacter* can facilitate nitrogen fertilizer reduction and efficiency, thereby supporting sustainable development of green agricultural production. Additionally, the energy requirements of *Azotobacter* during nitrogen fixation offer an opportunity to investigate efficient utilization of organic fertilizers or agricultural organic fertilizer wastes, which is an important direction for future scientific research in agricultural production.

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Author contributions
ZQ, CL, RY, and SQ designed the experiment. ZQ and CL prepared the samples, ZQ, CL, RY, and SQ performed the experiments, analyzed data, and wrote the paper. ZQ, CL, and RY reviewed and checked all the details. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This research has been confirmed for publication in the journal.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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