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RESEARCH ARTICLE

Isolation of rhizobacteria from the *Cenchrus fungigraminus* rhizosphere and characterization of their nitrogen-fixing performance and potential role in plant growth promotion

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Abstract

Aims To understand the species composition, abundance and diversity of nitrogen-fixing bacteria in the *Cenchrus fungigraminus* rhizosphere and to screen nitrogen-fixing bacteria to study their potential role in plant growth promotion.

Methods Soil were collected from 4 depths (G1, G2, G3 and G4) of the *C. fungigraminus* rhizosphere, and their physical and chemical properties were determined. The diversity and abundance of nitrogen-fixing bacteria and the *nif*H gene was analysed. Nitrogen-fixing bacteria were screened and selected to promote growth of *C. fungigraminus* seedlings.

Results The highest diversity and abundance of nitrogen-fixing bacteria was observed in the G2 samples collected from the *C. fungigraminus* rhizosphere. These bacteria mainly included Proteobacteria (93.91%), Actinobacteria (0.42%), and Firmicutes (0.18%) and were significantly affected by total nitrogen, available nitrogen and soil depth. The *nif*H gene copy number was highest ($1.56 \pm 0.17 \times 10^7$ *copies/g*) in G2. *Rhizobium pusense* No. 8 and No. 28 were isolated from G1 and G2, with nitrogenase activities of 145.06 ± 4.10 and 199.78 ± 7.50 *U/L*. The promotion experiment revealed that the plant height, root length, and leaf length of *C. fungigraminus* seedlings treated with both strains significantly increased by 56.79%, 76.99% and 55.71%, and the soil moisture and total nitrogen were significantly increased compared to the control ($P < 0.05$). The available nitrogen, organic matter and organic carbon in soil with strain No. 28 significantly increased compared to CK.

Conclusion Rhizobacteria in the *C. fungigraminus* rhizosphere play an important role as plant growth promoting rhizobacteria (PGPR). Two strains showed potential for the development of biological fertilizers.

Keywords *Cenchrus fungigraminus* · Rhizosphere · Nitrogen-fixing microorganisms · Plant growth-promoting rhizobacteria · *Rhizobium pusense*

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Introduction

Nitrogen is one of the most important elements affecting plant growth and development. Plants associate with microorganisms that are able to obtain molecular nitrogen from the atmosphere and convert it into ammonium nitrogen for direct use. Nitrogen-fixing bacteria are usually classified into symbiotic and nonsymbiotic bacteria (Tchan 1952). Symbiotic nitrogen-fixing microorganisms that form a symbiosis with legumes are the most efficient system for nitrogen fixation in nature and were first reported by Frank (1889) in stem- and root-nodulating bacteria. Furthermore, many rhizobial species, such as *Rhizobium oryziradicis*, *Rhizobium rhizoryzae*, *Rhizobium pseudoryzae*, *Rhizobium oryzicola*, and *Rhizobium taibaishanense*, have a nonsymbiotic association with crops (Zhang et al. 2015; Zhao et al. 2017). Currently, the problems regarding environmental contamination, energy shortages, food safety, etc., threaten sustainable agriculture. There is a need to reduce the use of chemical fertilizers. Recent studies have demonstrated that the host plant and its developmental stages play an important role in shaping the rhizosphere microbial community structure (Chaparro et al. 2014; Hou and Babalola 2013). The rhizosphere microbiome is mainly derived from plant roots and has been shown to enhance plant growth and plant stress tolerance under certain environmental conditions, such as soil type, soil nutrients, plant cultivar, climate change and anthropogenic activities (Igiehon and Babalola 2018; Vejan et al. 2016). Such bacteria are generally referred to as plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009). PGPR have been shown to enhance plant growth by direct or indirect mechanisms such as biological nitrogen fixation, phosphate solubilization, siderophore production, rhizosphere engineering, and phytohormone production (Chabot et al. 1996). According to Nakkeeran et al. (2005) and Swarnalakshmi et al. (2020), ideal PGPR should possess high rhizosphere competence, enhance plant growth capabilities, display a broad spectrum of action, be safe for the environment, be compatible with other rhizobacteria, and be tolerant to heat, UV radiation, and oxidizing agents. PGPR have been shown to promote the growth of many crops, including maize (Ferrarezi et al. 2022), wheat (Majeed et al. 2015) and rice (Barraquio et al. 1997). Rhizobia are employed as PGPR to increase plant growth through phosphate solubilization, the fixation of nitrogen, and the production of ACC deaminase, siderophores and indole-3-acetic acid (Cavite et al. 2021).

Nitrogen-fixing genes (*nif*) encode nitrogen-fixing enzymes that catalyse nitrogen fixation. Approximately 149 nitrogen-fixing genes, including *nif*H, *nif*D, *nif*K, *nif*E, *nif*N and *nif*B, have been identified (Santos et al. 2012). The *nif*H gene encodes a key enzyme involved in nitrogen fixation, i.e., dinitrogenase, which is highly conserved and widely distributed among nitrogen-fixing bacteria. The *nif*H gene expression in plants at different growth stages was consistent with changes in the composition of nitrogen-fixing bacteria (Lin et al. 2021). Average annual rainfall and temperature were reported to be the main factors affecting changes in endophytic nitrogen-fixing bacteria in different regions (Jia et al. 2020). High-throughput sequencing technology is able to overcome the limitation of microbiological studies based on traditional pure culture and can be used to quantitatively analyse samples of noncultivable, dominant, and rare bacteria and can comprehensively and accurately reflect flora composition and abundance (Monteiro et al. 2016).

Cenchrus fungigraminus Z. X. Lin & D. M. Lin & S. R. Lan sp. Nov. (*Pennisetum giganteum* Z. X. Lin or Giant grass has been used before) is a new species and perennial C4 plant of the family Gramineae with high yield and arid adaptability (Lin et al. 2022). It was collected in South Africa and has been widely used in environmental management (Zhou et al. 2021), in the production of edible and medicinal mushrooms (Su et al. 2022), and as fodder (Liu et al. 2018), yielding a good economic benefit for rural areas. Currently, *C. fungigramin*us is grown in 31 provinces in China, including Inner Mongolia, Qinghai, Hainan, Ningxia, Guizhou, Sichuan and Fujian, and in 106 countries worldwide (Lin and Lin 2018). The total nitrogen content of *C. fungigraminus* may exceed its total nitrogen uptake from soil and fertilizer, presumably due to the presence of the endophytic nitrogen-fixing bacteria. *Klebsiella* and *Bradyrhizobium* that were shown to be the main nitrogen-fixing bacteria present in the roots and stems of *C. fungigraminus* in Hainan Province (Lin et al. 2019). In this study, we evaluated the diversity and abundance of nitrogen-fixing bacteria in the rhizosphere of *C. fungigraminus* in

Hainan and screened the main PGPR to explore their potential effect on *C. fungigraminus* seedlings and soil. We hypothesized that the diversity and abundance of nitrogen-fixing bacteria in the *C. fungigraminus* rhizosphere would be significantly affected by soil depth and some strains may have the ability to promote plant growth as PGPR. Our results provide a theoretical basis for the screening and development of PGPR from the *C. fungigraminus* rhizosphere as biological fertilizers.

Materials and methods

Sample collection and processing

C. fungigraminus was planted in 2017 at the Danzhou Demonstration Base (22 m × 37 m) of the National Engineering Research Center of Juncao Technology in Hainan Province, People's Republic of China (109°53' E and 19°50' N). In total, 4 groups of soil samples were collected in 2021 from the *C. fungigraminus* surface soil (G1), rhizosphere soil (1–5 mm from the plant roots; these samples were carefully shaken to collect the soil attached to the roots) (G2), 10 cm depth from the main root of *C. fungigraminus* (G3) and 20 cm depth from the main root of *C. fungigraminus* (G4). According to Li et al. (2020) and Xomphoutheb et al. (2020), G2 is rhizosphere soil, while G1, G3 and G4 are nonrhizosphere soil samples. Six natural soil replicates of each group were randomly collected from the demonstration base using a clean soil auger and placed in clean, labelled sample bags. Each sample was divided into two parts; one part was stored at 4 °C until physical and chemical analyses were performed. The other part was stored at -80 °C until further analysis. The collected soil samples were ground and sieved through a 2 mm sieve. DNA extraction from the soil samples was performed by a Soil DNA Isolation Kit (Invitrogen, USA) following the manufacturer's instructions. The integrity of the extracted DNA was determined by 1% (w/v) agarose gel electrophoresis with SYBR Safe (Invitrogen, USA). The concentration of DNA was estimated by a Nanodrop spectrophotometer (Thermo, USA). The soil used for the plant promotion experiment was obtained from the National Engineering Research Center of Juncao Technology.

High-throughput sequencing and diversity analysis

The phylogeny of the *nif*H gene is significantly consistent with the presence of specific16S rRNA, with highly conserved sequences, abundant data information, and variable regions; this gene can be used to indicated the existence of nitrogen-fixing bacteria in samples and to reveal the relationship between the community structure of nitrogen-fixing bacteria and the environment (Cantera et al. 2004; Terakado-Tonooka et al. 2008). An amplified fragment of *nif*H, a functional gene for nitrogen fixation, was selected for subsequent high-throughput sequencing. The primer sequences were *nif*H-F: 5′-TGCGAY CCSAARGCBGACTC-3′ and *nif*H-R: 5′-ATSGCC ATCATYTCRCCGGA-3′. The polymerase chain reaction (PCR) conditions were predenaturation at 98 °C for 5 min, denaturation at 98 °C for 30 s, and annealing at 55 °C for 30 s, for a total of 30 cycles, and then extension at 72 °C for 5 min. The target fragments were approximately 360 bp in size and were recovered using a gel extraction kit after 1% agarose gel electrophoresis of the amplified products for high-throughput sequencing by Shanghai Personalbio Technology Co., Ltd. (Lin et al. 2021).

The *nif*H fragments were sequenced and then analysed using the QIIME2 (v2.13.4) analysis platform. The quality of the reads and the effect of splicing were filtered by quality control, and the samples were effectively distinguished according to the sequence at the ends of the fore and aft bar-code and primer sequence to calibrate the sequence direction, namely, to optimize the data. Sequences for annotation were based on the Greengenes database (Release 13.8, http://greengenes.secondgenome.com/) (DeSantis et al. 2006). The alpha diversity was calculated at the operational taxonomic unit (OTU) level of each sample and evaluated according to the distribution of OTUs in different samples, and the appropriateness of the sequencing depth was reflected by the rarefaction curve. The matrix distance was calculated for each sample at the OTU level. Canonical correspondence analysis (CCA) is a multivariate technique used to relate the composition of a species to environmental gradients when species have a bell-shaped response curve (Molnar 1998). The CCA in this work was performed by Genescloud tools, an online platform for data analysis (https://www.genescloud.cn). At the species taxonomic composition level, the differences

in species abundance and composition between different samples (groups) were further analysed by various unsupervised and supervised sorting, clustering and modelling means, combined with the corresponding statistical test methods, and attempts were made to identify marker species by linear discriminant analysis (LDA) and linear effect size (LEfSe) analysis (Segata et al. 2011; Chang et al. 2022).

Determination of the *nif*H gene copy number by AQ-PCR

The *nif*H gene copy number of soil nitrogen-fixing bacteria was measured by absolute quantification polymerase chain reaction (AQ-PCR), which generates a standard curve by using a standard with a known copy number and determines the Ct value of the unknown sample, combining the standard curve to obtain the copy number (Walker 2001). The reaction system volume was 20 μL, which included 10 μL of 2 × SYBR real-time PCR premixture, 1 μL of *nif*H-F (10 *μmmol/L*) and 1 μL of *nif*H-R (10 *μmmol/L*) as primers, 1 μL DNA template, and 7 μL of ddH_2O. The reaction conditions were denaturation at 95 °C for 15 s followed by extension at 60 °C for 30 s, for a total of 40 cycles, and then collection of the fluorescence signal starting at 60 °C. AQ-PCR was performed using *nif*H plasmids with a concentration gradient from 10^{-1} to 10^{-6} as templates and a standard curve, and ddH_2O was used as a negative control. The gene copy number was calculated by the following formula.

$$y = -k \log x + b$$

where y is the Ct value; k is the slope of the standard curve; b is the intercept of the standard curve.

$$\text{Gene copy} = \text{x} \times \text{eluting volume (mL)}/\text{sample weight (g)}$$

Determination of soil physical and chemical characteristics

Research has demonstrated that PGPR play key roles in nutrient acquisition and assimilation and improving soil texture, nutrition, and pH, all leading to the enhancement of plant growth (Backer et al. 2018). The physical and chemical properties of the 4 groups of soil were analysed. The moisture content (WC) of the soil was determined by mass change after oven-drying at 105 °C until constant weight. The pH value was measured by a pH meter (PB-10, Sartorius, Germany) (Yang et al. 2012). The total nitrogen (TN), available nitrogen (AN), organic matter (OM) and organic carbon (OC) contents of the soil were determined following Baeumer (1974) and Schumacher et al. (2009).

Isolation and identification of nitrogen-fixing strains

The screened strains were tested for nitrogenase activity, phosphorus-dissolving capacity, ammonia production capacity and siderophores production capacity. In addition, indole tests (tryptophan in peptone was decomposed to produce indole), catalase tests (toxic hydrogen peroxide was decomposed to H_2O and O_2), and NO_3^- reduction tests (nitrate was reduced to nitrite and ammonia) were used to screen the PGPR. Each fresh soil sample (2 g) from the 4 groups was added to 18 mL ddH_2O, mixed well and then diluted to 10^{-5}. Then, 0.1 mL of the diluted solution was inoculated into Ashby's nitrogen-free medium and incubated at 30 °C for 24–48 h under aerobic conditions (Lin et al. 2020). The colonies were picked randomly and inoculated in Ashby's nitrogen-free medium for screening and preservation. The nitrogenase activity of the isolated bacteria was determined by a soil nitrogenase double antibody enzyme-linked immunosorbent assay (ELISA) kit (Thermo, USA). The solution changes colour from blue to yellow, and the intensity of the colour is measured at 450 nm using a spectrophotometer. The ammonia production capacity, nitrate reduction capacity (Marschner 1995), and catalase activity (Kuscu 2019) of the representative strains were measured. The selected representative strains were inoculated into Chrome Azurol S (CAS) test medium and incubated at 30 °C for 24–48 h to observe the siderophores around the colonies. The selected representative strains were inoculated into inorganic phosphorus medium (glucose 10.0,

$(NH_4)_2SO_4$ 0.5, yeast extract 0.5, NaCl 0.3, KCl 0.3, $MgSO_4$ 0.3, $FeSO_4$ 0.03, $MnSO_4$ 0.03, $Ca_3(PO_4)_2$ 5.0, agar 15.0, pH 7.0-7.5, *g/L*) and organic phosphorus medium (glucose 10.0, $(NH_4)_2SO_4$ 0.5, yeast extract 0.5, NaCl 0.3, KCl 0.3, $MgSO_4$ 0.3, $FeSO_4$ 0.03, $MnSO_4$ 0.03, lecithin 0.2, $CaCO_3$ 1.0, agar 15.0, pH 7.0-7.5, *g/L*) at 30 °C for 24–48 h to observe the phosphorus-solubilized circles around the colonies (Madigan and Martinko 1997).

Whole-cell protein pattern analysis by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) offers a fairly fast and easy method of identifying a large number of strains, and it has an adequate level of taxonomic resolution at the species and subspecies levels. SDS-PAGE protein pattern analysis has been successfully used to identify various types of bacteria (Kim et al. 2010). The isolated bacteria were inoculated into Ashby's nitrogen-free liquid medium (with 30 °C, 150 rpm shake cultured) and grown to the logarithmic phase with an OD_{600} value of 1.0, and the bacterial colonies were clustered by SDS-PAGE whole-cell protein electrophoresis to determine the homology of the strains (Lajudie et al. 1994). The Tanon GIS series digital gel image processing system and image analysis were used to analyse the homology of the diversity of strains.

The 16S rDNA sequence PCRs were performed as a total reaction system of 25 μL, which contained 2 × PCR TaqMix 12.5 μL, template DNA 1.0 μL, primer 27F (10 *μmmol/L*) 1 μL, primer 1492R (10 *μmmol/L*) 1 μL, and ddH_2O 9.5 μL. The 16S rDNA universal primers were 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. The PCR thermal cycling conditions were predenaturation at 94 °C for 5 min, denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s, for a total of 30 cycles, followed by extension at 72 °C for 5 min and detection by 1% agar gel electrophoresis. Samples were recovered and sent to Fuzhou Boshang Biotechnology Co., Ltd. for DNA sequencing. The 16S rDNA sequences of each strain were blasted from nucleotide to nucleotide on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison, and homology was established using the neighbour-joining algorithm of MEGA (v5.1) software (Saitou and Nei 1987; Tamura et al. 2004; Kumar et al. 2016).

Promotional properties of PGPR

To assay growth promotion under different PGPR solutions, three treatments were further selected to test the ability of the bacteria to promote *C. fungigraminus* seedling growth in pot experiments at 28–30 °C in a greenhouse (natural light). During the experiment, 15 mL of selected bacterial solution (Ashby's nitrogen-free liquid medium, 30℃ shake cultured at 150 rpm) with a concentration of 2×10^9 *cfu/mL* was poured onto the *C. fungigraminus* seedlings near the roots. Experimental treatment-1 (T1) and experimental treatment-2 (T2) were treated with a single strain solution, and experimental treatment-3 (T3) was treated with a combined suspension with both strains (1:1, v/v). A control group (CK) was treated with the same volume of medium solution. There were 20 replicates in each group. The *C. fungigraminus* growth status (including the root length, root number, plant height, leaf length, and leaf number) and soil physical and chemical characteristics were observed and recorded after 5 weeks of treatment.

Data analysis

Statistical analysis of data was carried out by SPSS (v9.1) software, and the results were expressed as the means ± standard deviations (SDs). The data were statistically evaluated using a one-way analysis of variance (ANOVA) test, and values presented with different lowercase letters are significantly different at $P<0.05$.

Results

Soil physiochemical properties

The physical and chemical properties of the samples collected from different locations of *C. fungiraminus* soil in Hainan Province are shown in Table 1. The soil pH value and contents of TN, AN, OM and OC gradually decreased with soil depth, and the contents of each parameter were lowest in G4.

Table 1 Physical and chemical characteristics of *C. fungigraminus*' soil collected from different locations ($n=6$, means ± SDs)

Sources	Moisture content (WC, %)	pH	Total nitrogen (TN, *g/kg*)	Available nitrogen (AN, *mg/kg*)	Organic matter (OM, %)	Organic carbon (OC, %)
G1	8.80 ± 0.04^{a}	6.08 ± 0.02^{a}	0.20 ± 0.12^{a}	88.08 ± 3.64^{a}	7.18 ± 0.39^{a}	4.12 ± 0.22^{a}
G2	8.28 ± 0.12^{b}	6.18 ± 0.05^{b}	0.12 ± 0.01^{b}	53.67 ± 2.67^{b}	4.61 ± 0.46^{b}	2.65 ± 0.27^{b}
G3	8.46 ± 0.18^{b}	5.92 ± 0.01^{c}	0.07 ± 0.02^{c}	35.58 ± 1.01^{c}	2.37 ± 0.11^{c}	1.36 ± 0.06^{c}
G4	8.92 ± 0.07^{a}	5.84 ± 0.02^{d}	0.06 ± 0.06^{c}	32.08 ± 1.01^{d}	1.93 ± 0.22^{d}	1.11 ± 0.13^{d}

Values presented with different lowercase letters are significantly different at $P<0.05$

However, the water content increased gradually and was highest in G4.

Determination of the *nif*H gene copy number

The results of the genomic DNA extracted from the 24 samples and the electrophoretic amplification of *nif*H were detected by 1% agar gel electrophoresis (Fig. S1). The Ct value was obtained by AQ-PCR amplification of the *nif*H gene and was plotted as the ordinate. The standard curve equation was $Y = -3.59X + 39.89$, $R^2 = 0.999$, and the amplification efficiency was 89.91% (Fig. S2). The melting curve had a single peak, indicating that there was no nonspecific amplification or primer dimer. The copy number of the *nif*H gene was highest in G2 ($1.56 \pm 0.17 \times 10^7$ *copies/g*) (Fig. S3).

Nitrogen-fixing bacterial population diversity

Through the prepossessing of the sequences and the removal of low-quality and ambiguous sequences, a total of 3.28 million raw sequences were obtained from the 24 samples. After initial quality filtering, noise removal and correction, 3.07 million valid sequences remained with sequence lengths ranging from 33 to 444 bp; the average sequence length was 319 bp. Based on the principle of similarity greater than 97%, the total OTU numbers of the G1, G2, G3 and G4 samples were 1650, 1821, 1704 and 1729, respectively. The richness of nitrogen-fixing bacteria in rhizosphere soil (G2) was higher than that in non-rhizosphere soil (G1, G3, G4) (Fig. S4a). Four groups of sample dilution curves with Observed_species were stable as the soil depth deepened; the sequences were constructed by QIIME2 (v2.13.4). Nonmetric multidimensional scaling (NMDS) revealed that 6 samples in each group were well clustered and indicated that the amount of sequenced data was reliable and reflected the diversity of the nitrogen-fixing bacterial communities (Fig. S4b). Alpha diversity analysis revealed the richness and diversity of the microbial communities in each group of samples. Table 2 shows that the Chao 1 index, the number of observed_species, and the Shannon index of nitrogen-fixing bacterial species were highest in G2. The diversity and abundance of the nitrogen-fixing bacterial groups were the highest in the *C. fungigraminus* rhizosphere soil (Table 2).

Species abundance and community structure

The overall structure of the nitrogen-fixing bacteria is shown in a circle packing chart (Carrión et al. 2019)

Table 2 Alpha diversity index of the *nif*H gene in *C. fungigraminus* soil from different locations ($n=6$, means ± SDs)

Sources	Chao1	Goods_coverage (%)	Observed_species	Pielou_e	Shannon	Simpson
G1	2028.63 ± 237.27^{b}	99.56 ± 0.05^{a}	1647.05 ± 210.33^{a}	0.72 ± 0.01^{c}	7.69 ± 0.21^{c}	0.98 ± 0.00^{b}
G2	2366.97 ± 142.22^{a}	99.46 ± 0.04^{b}	1825.12 ± 152.10^{a}	0.77 ± 0.02^{a}	8.37 ± 0.12^{a}	0.99 ± 0.00^{a}
G3	2294.24 ± 148.45^{a}	99.43 ± 0.03^{b}	1706.62 ± 111.97^{a}	0.74 ± 0.02^{b}	7.98 ± 0.25^{b}	0.99 ± 0.01^{b}
G4	2307.77 ± 148.12^{a}	99.44 ± 0.03^{b}	1726.88 ± 134.63^{a}	0.77 ± 0.01^{a}	8.31 ± 0.19^{a}	0.99 ± 0.00^{a}

There was a significant difference between G1 and G2 and between G3 and G4 in the Chao1 index and the Goods_coverage percentage ($P<0.05$). There were significant differences in the Pielou_e index and the Shannon index among the groups. There were no significant differences in the Simpson index between G2 and G3 and no significant differences in the Observed_species index among the groups ($P>0.05$)

in which the different domain, phylum, order, family, genus and species levels have different microbiota. At the phylum level, 90.93–95.98% of the bacteria belonged to Proteobacteria, with the greatest abundance occurring in G1 and the least abundance in G4. Actinobacteria accounted for 0.12–0.42% of the bacteria and had the greatest abundance in G2. Firmicutes, Cyanobacteria and Verrucomicrobia were present in small amounts in the 4 groups, with abundances of 0.09–0.34%, 0.05–0.29% and 0.06–0.14%, respectively. Spirochaetes and Chlorobi were present in G2, with abundances of 0.02% and 0.01%, respectively, but did not appear in the other groups. Unclassified clades were also present in the 4 groups with abundances of 3.66–5.35% (Fig. 1a).

At the genus level, the community composition and relative abundance of nitrogen-fixing bacteria in the *C. fungigraminus* rhizosphere soils were significantly different between different locations. The genera with the high abundance in the 4 groups included *Bradyrhizobium* (29.64–39.71%), *Geobacter* (9.35–28.99%), *Azospirillum* (1.75–15.15%), *Desulfovium* (1.75–15.15%), *Desulfovibrio* (2.67–5.58%), *Burkholderia* (1.42–6.76%), *Halorhodospira* (0.68–2.73%), *Methylosinus* (0.51–4.30%), *Azotobacter* (1.07–2.45%), *Paraburkholderia* (0.30–1.70%), *Frankia* (0.11–0.34%), *Rhizobium* (0.0023-0.01%) and unidentified bacteria (23.26–31.81%). Phyla with high abundance in G1 included Proteobacteria (95.97%), Actinobacteria (1.16%), Verrucomicrobia (0.98%), Firmicutes (0.89%), Cyanobacteria (0.61%) and other unidentified genera (3.65%). In G2, bacterial genera present in high abundance included *Proteobacteria* (93.91%), *Actinobacteria* (0.42%), *Firmicutes* (0.18%), *Verrucomicrobia* (0.06%), *Cyanobacteria* (0.04%), *Rhizobium* (0.0054%) and unidentified genera (5.35%). In group 3, *Proteobacteria* (90.93%), *Actinobacteria* (3.10%), *Firmicutes* (0.34%), *Cyanobacteria* (0.29%), and *Verrucomicrobia* (0.14%) were the main community components. In G4, genera with high abundance included *Proteobacteria* (94.10%), *Actinobacteria* (0.38%), *Firmicutes* (0.32%), *Cyanobacteria* (0.16%), and *Verrucomicrobia* (0.09%) (Fig. 1b).

As shown in Fig. 2a, the numbers of OTUs in G1, G2, G3 and G4 were 1437, 1600, 1489 and 1447, respectively, with 445 common OTUs among all groups. The numbers of unique OTUs in each group were 534 (G1), 399 (G2), 327 (G3) and 326 (G4). The overall classification of samples based on R language and pheatmap software was plotted as a heatmap, and the top 50 classification units in terms of relative abundance are shown in Fig. 2b. The colour

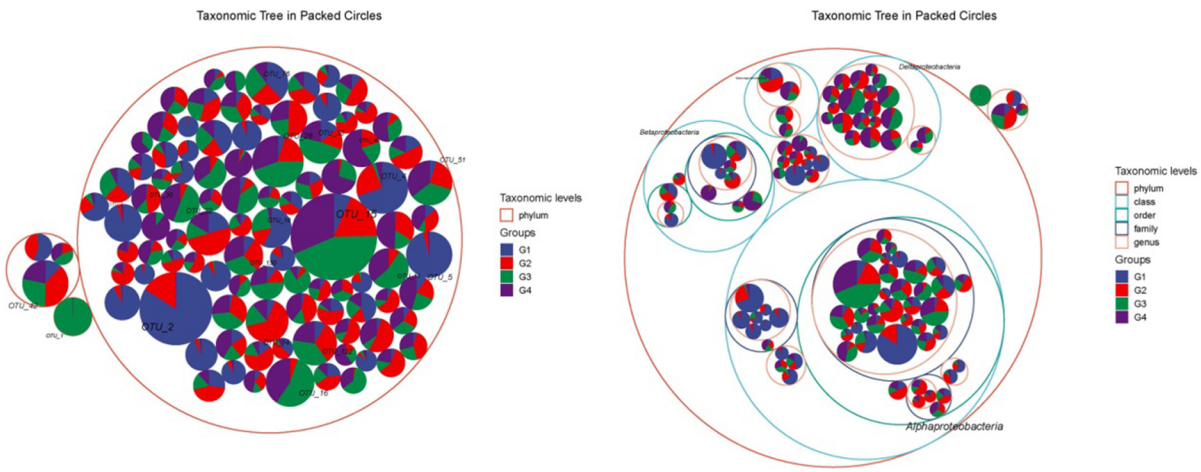

Fig. 1 Composition of the nitrogen-fixing bacterial community at different taxonomic levels in a circle packing chart. **a** shows the composition at the phylum level, and **b** shows the composition at the genus level. Each dot represents the specific taxonomic attribution of the OTU at the phylum level. The largest circle represents the level of the phyla, and the circles with different colours and sizes represent classes, orders, families, genera and species. The dots represent the top 100 most abundant OTUs, and their area is proportional to the abundance of the OTUs. Therefore, the abundance of taxa corresponding to the circle are also indicated by the area of the origin in the circle. The larger the sector area of the OTU in each group, the higher the abundance of the taxon in each group

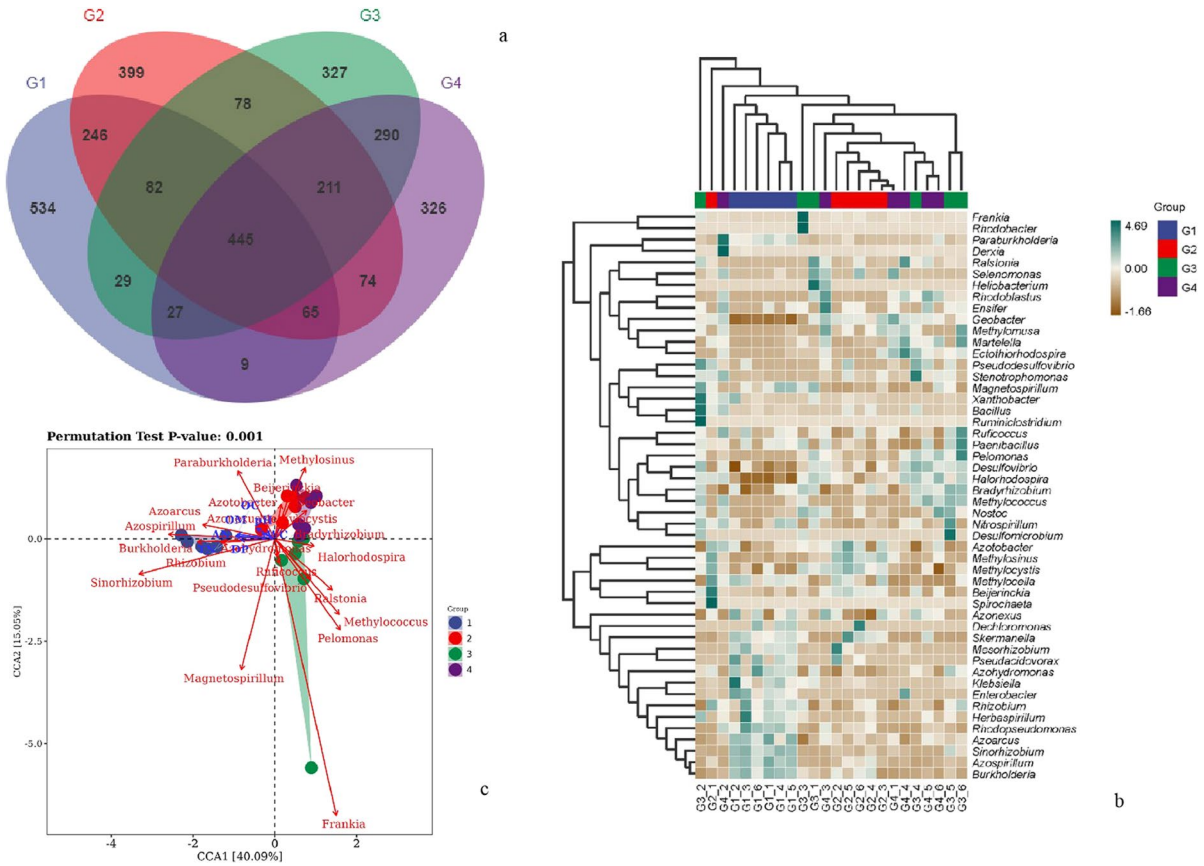

Fig. 2 **a** is a Venn diagram of the OTU distribution of all samples, and the number in the Venn diagram indicates the number of OTUs that were shared or unique between samples from different locations. **b** Heatmap of the top 50 most abundant genera (darker green indicates higher abundance, lighter yellow indicates lower abundance). **c** Canonical correspondence analysis (CCA) based on the nitrogen-fixing bacterial community composition and physicochemical variables

change on the heatmap shows that the nitrogen-fixing bacterial communities of G1 and G2 had analogous compositions, while the nitrogen-fixing bacterial communities of G3 and G4 clustered together. The same result is also shown in Fig. S1b. The relative abundances of *Paraburkholderia*, *Ruficoccus*, *Azonexus*, *Azohydromonas*, *Azoarcus*, *Azospirillum*, *Burkholderia* and *Rhizobium* were higher in G1 than in the other groups, while the relative abundances of *Azotobacter*, *Methylococcus*, *Rhizobium* and *Burkholderia* were highest in G2. *Azotobacter*, *Methylocystis*, *Methylosinus*, *Beijerinckia*, and *Geobacter* had relatively high abundances in G3, while in G4, *Ralstonia*, *Pelomonas*, *Desulfovibrio*, *Halorhodospira*, *Magnetospirillum*, *Frankia*, and *Bradyrhizobium* had higher relative abundances.

TN, AN and depth (DP) explained more than 80% of the bacterial community variation. In *C. fungigraminus* surface soil (G1), TN, AN, OM and OC were the main factors and were positively affected by *Azospirillum*, *Burkholderia* and *Rhizobium*. Compared to G1, DP was the only significant main factor and contributed nearly 40% of the total nitrogen-fixing bacterial community variation in G2, G3 and G4 (Fig. 2c). LEfSe analysis showed an abundance that was plotted based on the overall classification of the samples. With an LDA value of 3 (species diversity and richness, $P < 0.001$), Actinobacteria and Proteobacteria had higher abundance at the phylum level, and at the genus level, *Bejerinckia*, *Bradyrhizobium*, *Methylocystis*, *Rhizobium* and *Azospirillum* had higher

abundance (Fig. S5). The distributions of *Bejerinckia* and *Methylocystis* in G2 were significantly higher than those of other genera, and these genera were the most abundant in this group and can be used as marker species. These results are consistent with the composition taxonomic statistics in Fig. 1.

Isolation, purification, identification and performance of PGPR

The bacterial suspension was spread on Ashby's solid medium, and bacterial morphotypes with high colony number and vigorous growth were selected. Through microscopic examination and nitrogenase activity determination, 28 bacterial morphotypes were selected for further physiological and biochemical tests (Table 3). Of these, 7 representative strain classes were divided by SDS-PAGE electrophoresis analysis (Fig. 3a).

Eleven representative strain determination experiments included the indole test, catalase test, methyl red test, and NO_3^- reduction test. The ammonia production capacity, phosphorus solubility activity and iron-producing carrier ability were also measured to determine the basic functions of the representative strains. Table 4 shows that none of the 11 selected strains had the ability to decompose glucose and tryptophan, which indicated the absence of intestinal pathogens. Strains No. 8 and No. 28 had the ability to convert the fixed nitrogen into ammonia. Both strains also displayed a positive ability to catalyse hydrogen peroxide, produce H_2O and O^{2-}, reduce NO_3^- to NO_2^- and even produce nitrogen compounds for plant growth. Strains No. 8 and No. 28 produced siderophores, and it was inferred that they may have the

Table 3 Colony characteristics and nitrogenase activity ($n=3$, means ± SDs) of bacterial morphotypes in *C. fungigraminus* soil from different locations

Sources	Stain number	Colony characteristic	Nitrogenase activity (*U/L*)	Stain number	Colony characteristic	Nitrogenase activity (*U/L*)	Stain number	Colony characteristic	Nitrogenase activity (*U/L*)
G1	1	Purple, rod shaped	143.74 ± 4.20	2	Purple, rod shaped	156.31 ± 5.80	3	Purple, rod shaped	143.52 ± 6.20
	4	Purple, rod shaped	157.64 ± 1.80	5	Purple, rod shaped	129.62 ± 7.10	6	Red, rod shaped	173.97 ± 2.20
	7	Red, rod shaped	140.87 ± 5.40	8	Red, rod shaped	145.06 ± 4.10	9	Red, rod shaped	117.04 ± 3.30
G2	10	Purple, rod shaped	119.25 ± 11.60	11	Purple, rod shaped	107.55 ± 9.50	12	Red, rod shaped	141.31 ± 6.10
	13	Purple, rod shaped	140.65 ± 5.80	14	Purple, short rod-shaped	100.27 ± 6.40	15	Red, rod shaped	105.35 ± 5.40
	16	Purple, short rod-shaped	122.11 ± 3.80	17	Purple, short rod-shaped	94.75 ± 7.70	18	Purple, globular	146.39 ± 7.50
	19	Red, rod shaped	134.25 ± 1.50	20	Red, short rod-shaped	141.53 ± 1.60			
G3	21	Red, short rod-shaped	163.82 ± 6.30	22	Red, long rod	164.26 ± 8.10	23	Purple, rod shaped	200.67 ± 6.20
	24	Purple, rod shaped	187.65 ± 3.80	25	Purple, short rod-shaped	171.76 ± 3.90			
G4	26	Purple, short rod-shaped	179.92 ± 4.30	27	Red, rod shaped	192.28 ± 5.80	28	Red, rod shaped	199.78 ± 7.50

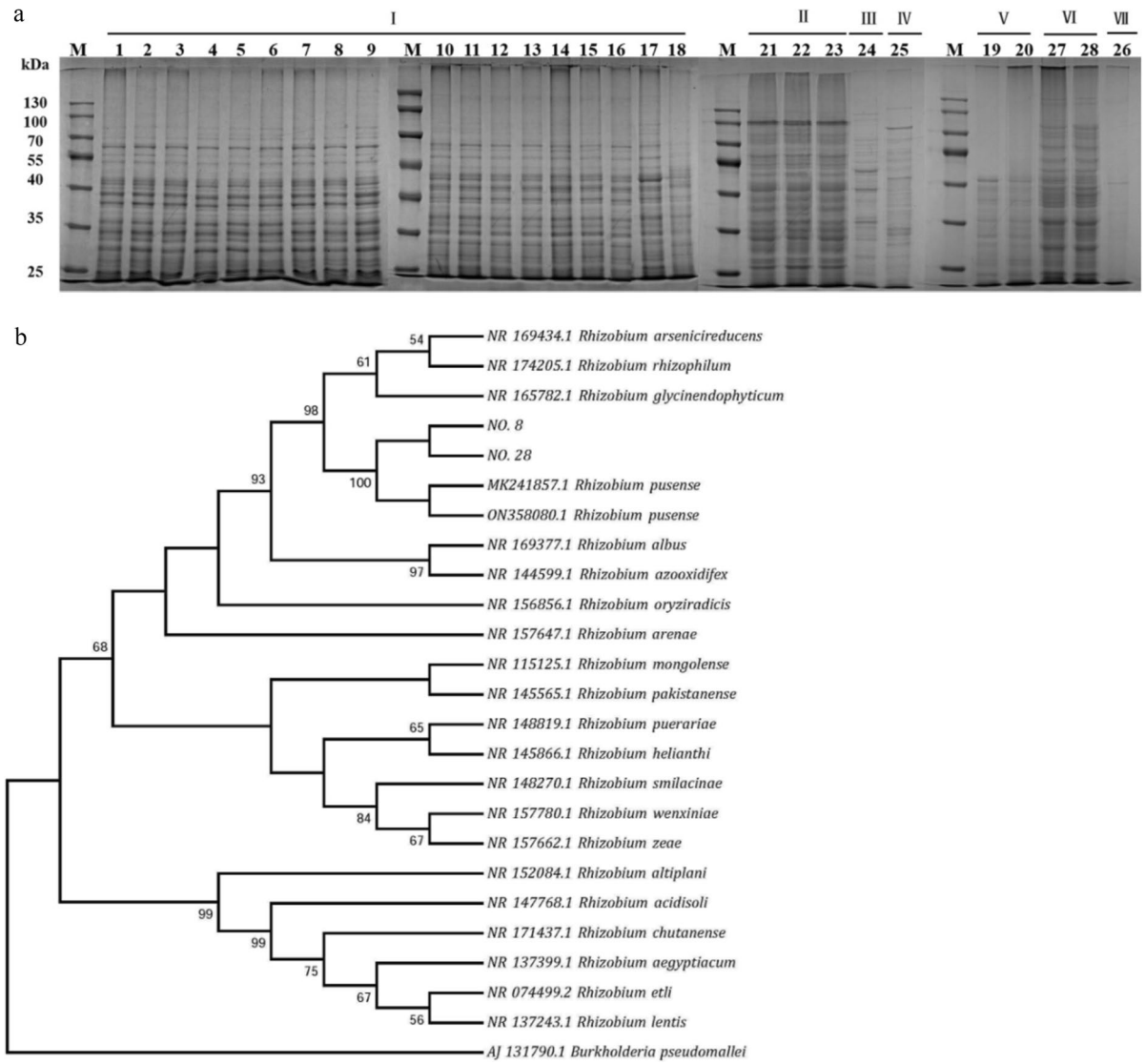

Fig. 3 **a** SDS-PAGE electrophoretic clustering analysis of whole-cell proteins of 28 bacterial morphotypes into 7 classes; **b** evolutionary tree constructed by the neighbour-joining procedure in MEGA (v5.1) software. The length and bootstrap confidence values of each branch are above or below the sequence branch

ability to dissolve unavailable calcium phosphate salts into bioavailable phosphate salts for plant absorption. In all, these capabilities suggest that strains No. 8 and No. 28 may have good promotion potential for plant growth as PGPR.

PGPR strains No. 8 (GenBank Accession No.: OQ119905) and No. 28 (GenBank Accession No.: OQ119906) homologous sequences were downloaded from NCBI and compared; the likelihood with known strains exceeded 97%, and the sequences belonged to *Rhizobium*. The 2 typical strains clustered with MK241857.1, ON358080.1, NR169377.1, and NR144599.1, and the bootstrap value exceeded 97%, which indicated that the 2 strains belonged to *Rhizobium pusense* (Fig. 3b). *Rhizobium pusense* No. 8 and *Rhizobium pusense*

Table 4 Physiological and biochemical characteristics of representative strains

Class	Representative strain No.	Indole test	Catalase test	Methyl red test	NO_3^-reduction test	Ammonia production test	Iron carrier capacity	Phosphorus-dissolving capacity	
								Inorganic phosphorus	Organic phosphorus
I	1	-	+	-	+	+	-	-	-
	6	-	-	-	-	+	-	-	+
	8	-	+	-	+	+	+	-	+
	13	-	-	-	+	+	-	-	+
	18	-	+	-	+	+	+	-	+
II	23	-	+	-	-	-	-	+	-
III	24	-	-	-	+	+	-	+	-
IV	25	-	-	-	-	+	-	+	-
V	20	-	-	-	+	+	+	-	+
VI	28	-	+	-	+	+	+	-	+
VII	26	-	+	-	+	+	-	-	-

"+" indicates a positive reaction, "-" indicates a negative reaction

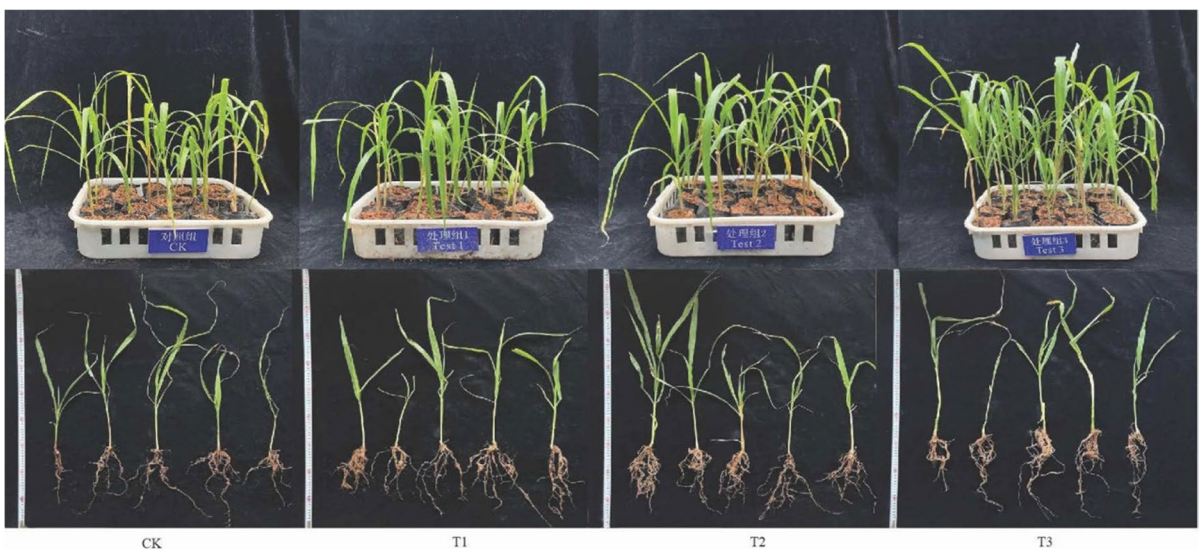

Fig. 4 Effects of *R. pusense* solutions on the soil and growth of *C. fungigraminus* seedlings

No. 28 clustered together and were screened in G1 and G2, respectively (Fig. 3b, Table 3).

C. fungigraminus seedling promotion test of PGPR

The plant height, leaf length, number of leaves, root length, and tiller number of *C. fungigraminus* seedlings after watering with *R. pusense* No. 8 bacterial solution (T1), No. 28 bacterial solution (T2), and the mixed bacterial solution (T3) are shown in Table 5; Fig. 4. Compared to the CK, the root length, height, and leaf length of *C. fungigraminus* in T3 significantly increased by 56.79%, 76.99% and 55.71% ($P < 0.05$), respectively. The AN, OM

Table 5 Effects of *R. pusense* No. 8 (T1), *R. pusense* No. 28 (T2), and the mixture (T3) on *C. fungigraminus* seedlings and soil ($n = 5$, means ± SDs)

Groups	*C. fungigraminus* seedlings					*C. fungigraminus* seedlings' soil					
	Root length (cm)	Number of roots	Plant height (cm)	Leaf length (cm)	Number of leaves	WC (%)	pH	TN (g/kg)	AN (mg/kg)	OM (%)	OC (%)
CK	537.55 ± 55.12^{a}	8^{a}	22.25 ± 4.29^{a}	18.31 ± 2.60^{a}	3^{a}	15.59 ± 0.26^{a}	5.93 ± 0.27^{a}	0.26 ± 0.18^{a}	38.97 ± 17.62^{a}	0.48 ± 0.27^{a}	2.78 ± 1.57^{a}
T1	786.74 ± 57.83^{b}	14^{bc}	37.35 ± 8.61^{b}	24.12 ± 4.82^{b}	4^{b}	25.02 ± 0.94^{b}	6.22 ± 0.17^{a}	0.69 ± 0.52^{b}	120.63 ± 8.08^{b}	2.77 ± 0.99^{b}	16.05 ± 5.76^{b}
T2	801.57 ± 59.48^{c}	13^{b}	34.09 ± 7.51^{b}	22.29 ± 2.42^{b}	4^{b}	26.91 ± 0.82^{b}	7.04 ± 0.07^{b}	0.38 ± 0.03^{c}	92.63 ± 16.16^{b}	0.94 ± 0.28^{c}	5.47 ± 1.66^{c}
T3	842.80 ± 66.54^{d}	15^{c}	39.38 ± 4.72^{c}	28.51 ± 2.79^{c}	4^{b}	21.48 ± 0.66^{b}	6.91 ± 0.18^{b}	0.86 ± 0.60^{d}	108.97 ± 26.50^{b}	1.85 ± 0.79^{c}	10.73 ± 4.60^{c}

Values presented with different lowercase letters are significantly different at $P < 0.05$

and OC of *C. fungigraminus* soil in T2 significantly increased by 3.09 times, 5.77 times and 5.77 times, respectively. T1, T2 and T3 significantly increased the WC and TN in the soil.

Discussion

Microorganisms have been shown to be able to form many biological communities in soil ecosystems. The formation and function of soil ecosystems at different depths are greatly influenced by microbial communities (Amundson et al. 2015). Soil depth significantly affected the abundance of dominant groups such as Actinomycetes and Proteobacteria (Li et al. 2014). The soil microbial community composition was shown to be closely correlated to the soil physical and chemical properties (Araújo et al. 2013; Wei et al. 2013) and is greatly affected by the vertical distribution of the soil texture. An important objective of research on rhizosphere microbes that fix nitrogen is to extend biological nitrogen fixation as a significant source of nitrogen for plants (Singh et al. 2020). Soil microorganisms are essential for biogeochemical cycles, colonizing plant roots, improving soil fertility and plant health and increasing crop production (Hayat et al. 2010). In the present study, the physical and chemical properties of the soil and the diversity of nitrogen-fixing bacteria from different soil locations of *C. fungigraminus* were compared. With increasing soil depth, the concentrations of soil TN, AN, OC and OM gradually decreased, reaching their lowest values at 20 cm depth from the rhizosphere soil. Analysis of the α diversity of nitrogen-fixing bacteria in the 4 groups showed that the Shannon index of rhizosphere soil was significantly higher than that of the other soil samples, while depth was the most significant factor, and the other groups were significantly different from surface soil in terms of the Chao1 index, Shannon index and Simpson index. Soil depth represents a strong physio-chemical gradient that greatly affects soil-dwelling microorganisms. More intensive interkingdom cooccurrence patterns were observed in the upper mineral layer (0–5 cm) than in the above organic and lower mineral soils, signifying a substantial influence of soil depth on biotic interactions (Mundra et al. 2021). Plant roots release a variety of secretions, including

sugars, organic acids, amino acids, hormones, and extracellular enzymes; these substances have been shown to provide sufficient nutrition and energy for microorganisms (Zhang et al. 2004). Highly abundant genera in the four soils included *Bradyrhizobium*, *Geobacter*, *Azospirillum*, *Desulfovibrio*, *Burkholderia*, *Halorhodospira*, *Methylosinus*, and *Azotobacter*. The abundances of *Paraburkholderia*, *Frankia* and *Rhizobium* in rhizosphere soil were higher than those in the other groups. We also found that the diversity of the *nif*H gene in the rhizosphere soil was higher than that in the nonrhizosphere soil of *C. fungigraminus*, which was consistent with the trend observed in high-throughput sequencing results. *nif*H is an ideal genetic marker to detect diversity and phylogeny (Hamelin et al. 2010).

There are many species of PGPR, including *Rhizobium*, *Bacillus, Klebsiella* and *Pseudomonas*. They are generally capable of fixing nitrogen, producing plant growth hormones and secreting antibiotics (Kloepper et al. 1989). According to Lin et al. (2019), *Klebsiella variicola* mainly colonizes the endothelial layer of *C. fungigraminus* roots as a plant endophyte. The contents of organic matter and total nitrogen in soil are the main criteria for evaluating soil fertility and quality. It has been widely reported that Rhizobia were able to promote the growth of crops and increase soil fertility (Shota et al. 2022). In our work, 11 representative strains were isolated, and their nitrogenase activity, ammonia production capacity, siderophore production capacity and phosphorus solubilization capacity were detected. Of these, 2 strains with high nitrogenase activity and soluble phosphorus activity were selected. Furthermore, TN, AN and OM had significant effects on the nitrogen-fixing bacterial community under different treatments and had positive effects on *Azospirillum*, *Burkholderia* and *Rhizobium.* The similar results of CCA between the environmental factors and distribution of rhizobial genospecies showed that soil pH and the contents of total phosphorus, total potassium and total organic carbon were the main determinants of the community structure of *S. davidii* rhizobia (Cao et al. 2021). Two strains clustered with *R. pusense*, and the bootstrap value exceeded 97%. Previous studies indicated 16S rDNA sequence analysis as an authenticated technique used to study bacterial isolates at the species level (Imran et al. 2010; Alam et al. 2011). *R. pusense* is a gram-negative bacterium belonging to the Rhizobiaceae family. It was able to degrade glycosylated phenols and flavonoids secreted by plant roots and can produce phytohormones (Badhai et al. 2017). *R. pusense* MB-17 A, a PGP rhizobium isolated from mung beans, showed significant siderophore production, ammonia production and phosphorus-solubilizing ability, and the fresh weight and nodule number of mung bean were significantly increased 60 days after inoculation (Chaudhary et al. 2020). Nitrogen-fixing bacteria with phosphorus solubilizing ability can dissolve some unavailable calcium phosphate salts in the soil and convert them into bioavalable phosphorus salts for absorption and utilization by plants; Phosphorus solubilizing bacteria aslo can improve the efficiency of phosphorus absorption and utilization by plants by releasing organic acids to dissolve inorganic phosphorus in the soil, or by secreting metabolites to degrade organic phosphorus (Chauhan et al. 2017; Mehta et al. 2013). However, according to Bashan et al. (2013), we need more work on phosphorus-solubilizing ability of *2 R. pusense* strains in future. Plant growth promotion could be the result of the beneficial functions of applied PGPR isolates, such as plant growth hormone production, nitrogen fixation, and P solubilization (Majeed et al. 2015). *R. pusense* No. 8 and *R. pusense* No. 28 were selected from the surface soil and rhizosphere soil in the present study, respectively, as potential soil improvers. Both strains promoted plant height, leaf length and root length growth of *C. fungigraminus*. Meanwhile, the growth promotion effect of the 2 strains solution was greater than that of the single strains. However, *R. pusense* No. 8 increased the concentrations of available nitrogen, organic matter and organic carbon in the soil more than *R. pusense* No. 28 and the mixture. The increase in the available nitrogen content is able to directly enhance the absorption of nitrogen by plants. Organic matter can provide nutrients for plants, promote plant growth, improve the soil structure, and improve the water retention ability of soil (Kiba and Krapp 2016). Furthermore, the inoculation of PGPR with multifunctional traits is better than inoculation of PGPR with single traits (Imran et al. 2014). The response of plants to different isolates was variable, which may be attributed to their individual traits and rhizospheric competencies, and most of the bacteria displayed good survival and plant promoting ability in the rhizosphere.

Conclusion

In summary, this foundational work examined the composition and diversity of nitrogen-fixing bacteria in *C. fungigraminus* rhizosphere soil, which were significantly affected by depth. *R. pusense* has plant growth-promoting ability as a PGPR. *C. fungigraminus* is becoming an important plant and is spreading worldwide. The use of effective PGPR is an opportunity for improving production in addition to maintaining soil structure and fertility. The study provides a theoretical basis for the development of plant growth-promoting rhizobacteria and the development of biological fertilizers. However, in the future, *R. pusense* should be applied so as to restore soil fertility, phosphorus solubilizing ability and safety to the environment, humans and animals, and the impact of long-term application on soil physical and chemical properties and microorganisms needs to be monitored and further studied.

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Author contributions Jing Li, Biaosheng Lin, Zhanxi Lin and Dongmei Lin conceived and designed the experiments; Jing Li, Yanglin Liu, Bingxin Zhou, Tingting Li and Hui Lin performed the experiments and analysed the data; Jing Li, Bingxin Zhou, Tingting Li, Biaosheng Lin and Guodong Lu performed the statistical analyses and wrote the manuscript. All authors reviewed and approved the final draft.

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Data availability All authors confirm that the data supporting the findings of this study are available within the article.

Declarations

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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