Nitrogen Fixation by *Paenibacillus Polymyxa* WLY78 is Responsible for Cucumber Growth Promotion

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Research Article

Keywords: Plant growth-promoting bacteria, *Paenibacillus polymyxa* WLY78, cucumber, nitrogen fixation, ¹⁵N isotope dilution

Posted Date: August 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-758683/v1>

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Version of Record: A version of this preprint was published at Plant and Soil on January 31st, 2022. See the published version at <https://doi.org/10.1007/s11104-022-05307-6>.

Abstract

Aims To study nitrogen contribution to cucumber derived from nitrogen fixation of *Paenibacillus polymyxa* WLY78.

Methods The *nif* gene cluster deletion mutant ($\Delta nifB-V$) of *P. polymyxa* WLY78 is constructed by a homologous recombination method. The GFP-labeled $\Delta nifB-V$ mutant was used to inoculate cucumber and to study colonization by confocal laser scanning microscope. The effects of plant-growth promotion were investigated by greenhouse experiments. The nitrogen fixation contribution was estimated by ^{15}N isotope dilution experiments.

Results Deletion of *nif* gene cluster of *P. polymyxa* WLY78 resulted in complete loss of nitrogenase activity. Observation by laser confocal microscopy revealed $\Delta nifB-V$ mutant can effectively colonize cucumber root, stem and leaf tissues, like wild-type *P. polymyxa* WLY78. Greenhouse experiments showed that inoculation with *P. polymyxa* WLY78 can significantly enhance the lengths and dry weights of cucumber roots and shoots, but inoculation with $\Delta nifB-V$ mutant can not. ^{15}N isotope dilution experiments showed that cucumber plants derive 25.93% nitrogen from nitrogen fixation performed by *P. polymyxa* WLY78, but the $\Delta nifB-V$ mutant nearly can not provide nitrogen for plant growth.

Conclusions This present study demonstrates that nitrogen fixation performed by *P. polymyxa* WLY78 is responsible for cucumber growth promotion.

Introduction

Nitrogen is one of the most important nutrients in plant growth, but plants can not directly use nitrogen in the atmosphere. Biological nitrogen fixation (BNF) is a process in which nitrogen-fixing microorganisms reduce nitrogen in the air to ammonia through nitrogenases. Biological nitrogen fixation is an important part of the natural nitrogen cycle (Dart 1986) and plays an important role in the sustainable development of agriculture (Raymond et al. 2004). Nitrogen-fixing microorganisms include symbiotic nitrogen-fixing bacteria, free-living nitrogen-fixing bacteria and associative nitrogen-fixing bacteria (Xu et al. 2017). Associative nitrogen-fixing bacteria can colonize root surface cells, invade plant roots, and form close contact with plants, thereby promote plant growth (Baldani et al. 1997). Associative nitrogen-fixing bacteria promote the absorption of nitrogen by non-legume plants (Geddes et al. 2015). Biological nitrogen fixation not only reduce the use of nitrogen fertilizer, but also improve soil fertility and the absorption of nutrients by crops (Farrar et al. 2014).

It has been reported that the associative nitrogen-fixing bacteria play an important role in promoting growth of non-legumes by fixing nitrogen and producing phytohormone (Chalk 1991). In earlier research, ^{15}N isotope and N balance studies have shown that several sugarcane varieties obtain over 60% of their nitrogen ($>150 \text{ kg N ha}^{-1} \text{ year}^{-1}$) from biological nitrogen fixation performed by diazotrophs (Boddey et al. 1995). Diazotrophic bacteria present in the mucilage of aerial roots

contribute 29–82% of the N nutrition of Sierra Mixe maize (Van Deynze et al. 2018). Inoculation with nitrogen-fixing *Klebsiella pneumoniae* 342 (Kp342) increased total N and N concentration in the wheat plant (Iniguez et al. 2004). Inoculation of the rhizobacteria including *Azospirillum brasilense* and *Azospirillum lipoferum* contributed up to 20-50% of the total nitrogen requirement of the oil palm seedlings through nitrogen fixation (Amir et al. 2003). Diazotrophic *Paenibacillus beijingensis* BJ-18 provided nitrogen for wheat, maize and cucumber plants and promoted plant growth, nitrogen uptake and metabolism (Li et al. 2019). A recombinant nitrogen-fixing *Pseudomonas protegens* Pf-5 X940 was constructed by introducing the *nif* genes of *Pseudomonas stutzeri* A1501 via the X940 cosmid to the beneficial rhizobacterium *Pseudomonas protegens* Pf-5, and inoculation of *Arabidopsis*, alfalfa, tall fescue and maize with Pf-5 X940 increased the ammonium concentration in soil and plant productivity under nitrogen-deficient conditions (Fox et al. 2016; Setten et al. 2013). Inoculation with *Azospirillum brasilense* Ab-V5 cells enriched with exopolysaccharides and polyhydroxybutyrate enhances the productivity of maize under low N fertilizer input (Oliveira et al. 2017)

Paenibacillus polymyxa WLY78 is a nitrogen-fixing bacterium containing a compact *nif* gene cluster consisting of 9 genes (*nifBHDKENXhesAnifV*) (Wang et al. 2013; Xie et al. 2014). In addition to nitrogen fixation, this bacterium has the ability of phosphate solubilization and IAA production (Xie et al. 2016). Also, this bacterium can produce fusaricidins that are a class of cyclic lipopeptide antibiotics to inhibit plant pathogenic fungi (Li et al. 2019). These specific traits suggest that *P. polymyxa* WLY78 is a member of plant growth-promoting bacteria (PGPB) and has great potential as an inoculant in agriculture. However, the nitrogen contribution to plants derived from nitrogen fixation of *P. polymyxa* WLY78 is unclear. In this study, the *nif* gene cluster deletion mutant ($\Delta nifB-V$) of *P. polymyxa* WLY78 is constructed. Comparisons of *P. polymyxa* wild-type and $\Delta nifB-V$ mutant in colonization, plant-growth promotion and nitrogen fixation contribution rate are investigated. Our study will provide foundation for application of *P. polymyxa* WLY78 as a biofertilizer in agriculture.

Materials And Methods

Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are summarized in Table 1. *Paenibacillus polymyxa* WLY78 was isolated from the rhizosphere of bamboo in Beijing (Wang et al. 2013). *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant were routinely grown in LB medium (per liter contains: 10 g NaCl, 5 g yeast and 10 g tryptone) at 30°C with shaking. Nitrogen-limited medium (per liter contains: 10.4 g Na₂HPO₄, 3.4 g KH₂PO₄, 26 mg CaCl₂·2H₂O, 30 mg MgSO₄, 0.3 mg MnSO₄, 36 mg Ferric citrate, 7.6 mg Na₂MoO₄·2H₂O, 10 mg p-aminobenzoic acid, 10 mg biotin, 4 g glucose as carbon source and 0.3 g glutamic acid as nitrogen source) (Wang et al. 2013) was used for assay of nitrogenase activity (Wang et al. 2013).

Escherichia coli JM109 was used as routine cloning. Thermo-sensitive vector pRN5101 (Zhang et al. 2013) was used for gene disruption in *P. polymyxa* WLY78. The recombinant plasmid pGFP300 (Hao and Chen 2017) carrying the *gfp* gene was used for labeling *P. polymyxa* strains. When appropriate,

antibiotics were added in the following concentrations: 100 µg/ml ampicillin, 12.5 µg/ml tetracycline and 5µg/ml erythromycin for maintenance of plasmids.

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype and/or relevant characteristics	Source or reference
strains		
<i>Paenibacillus polymyxa</i>		
WLY78	Wild-type strain	(Wang et al. 2013)
$\Delta nifB-V$	<i>nif</i> gene cluster deletion mutant	This study
GFP-tagged <i>P. polymyxa</i> WLY78	<i>P. polymyxa</i> WLY78 carrying plasmid pGFP300; Tet ^r	(Hao and Chen 2017)
GFP-tagged $\Delta nifB-V$	$\Delta nifB-V$ carrying plasmid pGFP300; Tet ^r	This study
<i>E. coli</i>		
JM109	General cloning host; <i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, $\Delta(lac-proAB)$/F'[traD36, proAB⁺, lacIq, lacZΔM15]</i>	Sangon Biotech Co.
Plasmids		
pRN5101	Temperature-sensitive <i>E. coli-Bacillus</i> shuttle vector, Em ^r	(Zhang et al. 2013)
pRDnifB-V	<i>nif</i> gene cluster deletion vector based on pRN5101	This study
pGFP300	<i>gfp</i> gene in pHY300PLK	(Hao and Chen 2017)

Construction of $\Delta nifB-V$ mutant of *P. polymyxa* WLY78

The nitrogen-fixing gene cluster deletion mutant $\Delta nifB-V$ of *P. polymyxa* WLY78 was constructed by a homologous recombination method. For doing this, the upstream fragment and downstream fragment flanking *nif* gene cluster were amplified by PCR using Phanta[®]Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China), respectively. Primer 1 (5' CGGCCACGATGCGTCCGGCGTAGAGGATCCGCGTGGTG

GATGTGGA CG 3') and Primer 2 (5' AACGCTTTTTTCGGTTATCATTCCTTCACATCTATTTTCGTC 3') were

used to amplify a 950 bp-long sequence located upstream of *nifB*. Primer3 (5' GAAGGAATGATAACCGAAAA

AGCGTTCCCGTC 3') and Primer 4 (5' GACTGCGCAAAAGACATAATCGATAAGCTTCCTGATAAGGCAG

ACAAGGCTC 3') were used to amplify a 1107 bp-long sequence located downstream of *nifV*. The two fragments were then fused with *Bam*H^I/*Hind* III digested pRN5101 vector using Gibson assembly master mix (New England Biolabs), generating the recombinant plasmid pRDnifB-V. Then, the recombinant plasmid pRDnifB-V was transformed into *P. polymyxa* WLY78 as described by Wang et al., (2018), and the marker-free deletion mutant (the double-crossover transformant) $\Delta nifB-V$ was selected from the initial erythromycin resistance (Em^r) transformants after several rounds of nonselective growth at 39°C and confirmed by PCR using the primer 5 (5' GCATAAATTGTACACGTTGA 3') and primer 6 (5' AGGCTCATAAACACCGTATC 3').

Growth and nitrogenase activity of the wild type and mutant strains

To measure growth, *P. polymyxa* WLY78 and $\Delta nifB-V$ were grown in 20 mL of LB media in 50 mL flasks shaken at 200 rpm at 30°C to logarithmic growth phase. The cultures were collected by centrifugation, washed three times with sterilized water and then resuspended in sufficient nitrogen medium (nitrogen-limited medium supplemented with 100 mM NH₄Cl) to a start OD₆₀₀ of 0.02. Every 2 h, the growth of *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant strains were determined by absorbancy at 600 nm.

Acetylene reduction assays were performed as described previously to measure nitrogenase activity (Wang et al. 2013). *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant strains were grown in 50 mL of LB medium overnight. The cultures were collected by centrifugation, washed three times with sterilized water, and then resuspended in a nitrogen-limited medium to a final OD₆₀₀ of 0.4. Then, 4 mL of the culture was transferred to a 26-mL test tube and the test tube was sealed with a rubber stopper. The headspace in the tube was then evacuated and replaced with argon gas. Then, approximately 2.2 mL of C₂H₂ (10% of the headspace volume) was injected into the test tubes. After incubating the cultures at 30 °C for the corresponding time, a 100 μ L gas sample was taken out and injected into gas chromatography to quantify ethylene (C₂H₄) production. The nitrogenase activity was expressed in nmol C₂H₄/mg protein/h.

Preparation of soil, seeds and bacterial suspension

The soil (0-20 cm deep topsoil) was taken from the Shangzhuang Experimental Station of China Agricultural University. The soil was low nitrogen (7.8 mg kg⁻¹) sandy soil. After the soil was air-dried and crushed, the debris were removed with a 2 mm sieve to reduce heterogeneity, and then packed into plastic pots with a diameter of 20 cm and a height of 14 cm. Each pot was filled with 2 kg of soil to grow cucumbers. No trace elements were applied during plant growth.

Cucumber seeds ("Zhongnong 8" of Beijing Shengfeng Garden Agricultural Technology Co., Ltd.) were first disinfected with 10% sodium hypochlorite for 10 minutes, then were rinsed with sterile water three

times, and finally were distributed in a sterile petri dish with damp filter paper at room temperature (25°C) for 3-5 days in the dark.

The bacterial suspension of *P. polymyxa* WLY78 and $\Delta nifB-V$ used in inoculation was prepared as follows. *P. polymyxa* WLY78 and $\Delta nifB-V$ were inoculated into LB liquid medium, cultured at 30°C and 180 rpm to logarithmic growth phase, and then harvested by centrifugation and finally suspended with physiological saline (0.89% w/v NaCl in deionized water). The cell concentration was set to 10^8 cells mL⁻¹.

Colonization of *P. polymyxa* and $\Delta nifB-V$ on cucumber

The recombinant plasmid pGFP300 carrying the *gfp* gene was transferred into $\Delta nifB-V$ by electrotransformation as described in *P. polymyxa* WLY78 (Hao and Chen 2017), yielding GFP-tagged $\Delta nifB-V$ mutant. The GFP-tagged $\Delta nifB-V$ mutant and GFP-tagged *P. polymyxa* WLY78 suspension were obtained as described above to final concentration at 10^8 cells mL⁻¹. The sterilized cucumber seeds were sown in sterile glass bottles containing 100 mL of 1/2 MS semi-solid agar medium, and one seed was placed in each bottle. After dark treatment for about a week, the seeds were grown into seedlings, which were transferred to a light incubator (27°C, 70% humidity and 16 h day/8 h night, with light at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for cultivation. The seedlings were regularly watered (tap water) to 40% relative soil moisture by weighing method every 5 days. After the seedlings grow 2-3 young leaves, inoculate the cell suspension of GFP-tagged strains at the root of cucumber. Three days later, the colonization of GFP-labeled strains in cucumber tissues were placed on a slide and visualized with an excitation wavelength of 488 nm with a laser confocal scanning microscope (CLSM, Olympus FluoViewTM FV1000 confocal microscope). The emitted light was collected in the 500-565 nm range for GFP, and the images were collected with FV10-ASW software (03.01.02.02, Olympus Europa Holding GmbH, Hamburg Germany).

Greenhouse pot experiment

The research was conducted in the greenhouse of China Agricultural University using greenhouse potting. The experimental design was arranged by random factors, with three inoculation treatments and two nitrogen level treatments. Each treatment was repeated three times, for a total of 18 pots of cucumber plants. Nitrogen level treatment included high nitrogen and low nitrogen levels. Nitrogen fertilizer was applied in the form of ¹⁵N labeled (NH₄)₂SO₄ (10.16% ¹⁵N atom, Shanghai Research Institute of Chemical Industry, China). The high nitrogen level was 250 mg N kg⁻¹ soil, and the low nitrogen level was 83 mg N kg⁻¹ soil. Nitrogen fertilizer was applied in three times, one-third each time, and the first time was applied as a base fertilizer, followed by 7 and 14 days after transplantation.

The inoculation treatment included three treatments: inoculation of *P. polymyxa* WLY78 (WT), $\Delta nifB-V$, and equal amount of physiological saline (as a control). The germinated cucumber seeds with robust and consistent growth were picked and immersed in the bacterial suspension for 20 minutes. The seeds were immersed in physiological saline for 20 minutes as a control group, and then transplanted into plastic

pots. Four seeds were planted in each pot, and three repetitions were set for each treatment. In the first and second weeks after planting, the *P. polymyxa* WLY78 and $\Delta nifB-V$ bacterial suspensions were re-inoculated into the roots of the plants, and the control group was added with the same amount of physiological saline. Place the flower pots under the best conditions in the greenhouse (15 h light/9 h dark cycle, 25–30/15–20°C day/night temperature and 40% day/60% night humidity). The seedlings were regularly watered every 3 days until the relative humidity of the soil reached 40%.

Plant sample collection

On 30th day of cucumber planting, the plants were collected by destructive sampling. The whole seedling was first uprooted, and rinsed with deionized water to remove the soil attached to the root system, then the root and shoot samples were separated, and the length of the root and shoot were measured. The root and shoot samples were killed in an oven at 105°C for 30 minutes, and then dried at 65°C until constant weight for dry weight analysis. Then the dried samples were immediately frozen in liquid nitrogen for subsequent analysis.

Contribution of nitrogen by biological nitrogen fixation

The ^{15}N isotope dilution technique was used to quantitatively determine the contribution of inoculated bacteria to cucumber biological nitrogen fixation. The above dried sample was ground, sieved with a 1 mm sieve and placed in a bag, and the cucumber N content and ^{15}N enrichment determination were performed by DELTA V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Inc., United States). The cucumber without inoculation were used as references to calculate the BNF contribution. After inoculation with *P. polymyxa* WLY78 and $\Delta nifB-V$, the percentage of nitrogen from cucumber biological nitrogen fixation to the nitrogen content in cucumber (% Ndfa) was calculated by the following formula:

$$\% \text{Ndfa} = \left(1 - \frac{\% \text{NdfF}}{\% \text{NdfNF}} \right) \times 100$$

Among them, %NdfF is the ^{15}N enrichment of cucumber stems and leaves of inoculation treatment, and %NdfNF is the ^{15}N enrichment of cucumber stems and leaves of uninoculated treatment (control group).

Statistical Analysis

Graphs were prepared using GraphPad Prism software v. 8.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed using SPSS software version 20 (SPSS Inc., Chicago, IL, United States). One-way analysis of variance (ANOVA) was employed to check the significant differences between treatments. Means of different treatments were compared using the least significant difference (LSD) at the 0.05 or 0.01 level of probability.

Results

The *nif* gene cluster deletion mutant ($\Delta nifB-V$) of *P. polymyxa* WLY78 leads to complete loss of nitrogenase activity

P. polymyxa WLY78 contains a compact *nif* gene cluster consisting of 9 genes (*nifB nifH nifD nifK nifE nifN nifX hesA nifV*) located within a 10.5 kb region. The *nif* gene cluster deletion mutant ($\Delta nifB-V$) was constructed by recombination as described in Fig. 1a. The disruption of *nif* gene cluster was confirmed by PCR (Fig. 1b). The $\Delta nifB-V$ and the wild-type strains exhibited similar growth phenotypes on nitrogen-limited medium with ammonium as nitrogen sources (Fig. 1c). However, the $\Delta nifB-V$ mutant did not have nitrogenase activity (Fig. 1d), indicating that the *nif* gene cluster is essentially required for nitrogen fixation under nitrogen limitation.

The colonization of cucumber by GFP-Tagged $\Delta nifB-V$ mutant

The cucumber colonization by *P. polymyxa* WLY78 has been previously described (Hao and Chen 2017). Here, the $\Delta nifB-V$ mutant was labeled with GFP and then the GFP-labeled bacteria were used to inoculate cucumber and examine colonization. After 3 days of inoculation at the root of cucumber, the samples of the cucumber roots, stems and leaves were observed under laser confocal microscopy. The cells of GFP-tagged $\Delta nifB-V$ mutant not only colonized on the surface of cucumber root, but also colonized interior of root, stem and leaf (Fig. 2d-f), as observed in the wild-type *P. polymyxa* WLY78 (Fig. 2a-c). The presence of bacterial cells was observed in the vascular bundle of the stem and the leaves. The results indicated that *P. polymyxa* WLY78 and $\Delta nifB-V$ can colonize outside and inside of cucumber tissues.

Effects of *P. polymyxa* WLY78 and $\Delta nifB-V$ on the growth of cucumber

Cucumber samples were collected on the 30th day after plantation, and the length and dry weight of cucumber shoots and roots were measured to evaluate the effects of inoculation with *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant on plant growth under low and high nitrogen conditions. The non-inoculated cucumber served as a control. Compared to the non-inoculated control group, cucumber inoculated with *P. polymyxa* WLY78 under low nitrogen conditions showed increase of 68.18% in shoots dry weight, of 59.15% in root dry weight, of 37.61% in shoot length and of 38.52% in root length, but they showed a little increase in lengths and weights under high nitrogen conditions (Fig. 3a-d). Compared with the non-inoculated control group, the cucumber inoculated with the $\Delta nifB-V$ mutant showed a little increase in the dry weight and length of the shoots and roots under both low and high nitrogen conditions (Fig. 3a-d). Fig. 4 is an experimental diagram of the greenhouse cultivation. The data indicate that the diazotrophic *P. polymyxa* WLY78 can effectively promote plant growth under low nitrogen conditions and disruption of *nif* genes encoding nitrogenase results in almost loss of the ability of promoting plant

Nitrogen fixation in cucumber provided by *P. polymyxa*

To estimate the contribution of BNF, ^{15}N isotope dilution technique was used to analyze the inoculated cucumber grown in soil which contains ^{15}N -labeled $(\text{NH}_4)_2\text{SO}_4$ as N fertilizer in greenhouse conditions (Table 2). The nitrogen derived from gaseous nitrogen (%Nd_{fa}) in the *P. polymyxa* WLY78 inoculated

cucumber and the $\Delta nifB-V$ strain inoculated cucumber under low N conditions is $25.93 \pm 2.32\%$ and $2.93 \pm 6.57\%$, respectively. Whereas, the nitrogen derived from gaseous nitrogen (%Ndfa) in the *P. polymyxa* WLY78 inoculated cucumber and the $\Delta nifB-V$ strain inoculated cucumber under high N conditions is $1.54 \pm 0.66\%$ and $-0.22 \pm 1.02\%$, respectively. These results indicate that the cucumber plant has incorporated the nitrogen provided by BNF of *P. polymyxa* WLY78 under low N conditions and BNF is inhibited by high concentration of available nitrogen in the environment. The $\Delta nifB-V$ strain-inoculated cucumbers nearly did not derive nitrogen from BNF, consistent with the $\Delta nifB-V$ strain has no nitrogenase. The data also indicate that *P. polymyxa* WLY78 can be used to provide nitrogen nutrition to plants and reduce the use of nitrogen fertilizers.

Table 2 Quantification of biological nitrogen fixation in the inoculated cucumber plants grown in soil containing high N and low N conditions.

Treatment	%Ndfa	
	Low nitrogen	High nitrogen
Control	-	-
<i>P.polymyxa</i> WLY78	25.93±2.32 ^a	1.54±0.66 ^a
$\Delta nifB-V$	2.93±6.57 ^b	-0.22 ± 1.02 ^a

The results came from three biological replicates, the error represents SD, lowercase letters a and b indicate that there is a significant difference between the groups (P <0.05), while the same letter indicates that there is no significant difference.

Discussion

In this study, both GFP-tagged *P. polymyxa* WLY78 and GFP-tagged $\Delta nifB-V$ mutant are able to colonize the roots, stems, and leaves of cucumber. This result shows that the deletion of the *nif* gene cluster did not affect the colonization. Similarly, both of wild-type *Klebsiella pneumoniae* 342 and the *nifH* mutant can colonize wheat (Iniguez et al. 2004) and both wild-type *Pseudomonas stutzeri* A1501 and its *nifH* mutant can colonize maize root (Ke et al. 2019). Wild-type *Acetobacter diazotrophicus* PAI5 and the *nifD* mutant have the same ability of colonization in sugarcane (Sevilla et al. 2001). A difference between our study with other's is that the $\Delta nifB-V$ mutant of *P. polymyxa* WLY78 has a deletion of a compact *nif* gene cluster comprising 9 genes (*nifBHDKENXhesAnifV*) and the *nifH* mutant or *nifK* mutant or *nifD* mutant has a deletion of a single *nif* gene.

The effects of *P. polymyxa* WLY78 and $\Delta nifB-V$ on cucumber growth under different nitrogen concentrations were further studied through greenhouse cultivation experiments. Compared to the uninoculated control and the inoculation with the $\Delta nifB-V$ mutant, inoculation with *P. polymyxa* WLY78 significantly increased the dry weights and lengths of cucumber shoots and roots under low nitrogen

conditions, but this effect was not found in the cucumbers inoculated with the $\Delta nifB-V$ mutant. These results have revealed that the nitrogen fixation of *P. polymyxa* WLY78 plays an important role in promoting plant growth. Phosphate solubilization and IAA production of *P. polymyxa* WLY78 may exhibit a minor role in promoting plant growth. Similarly, inoculation with *Pseudomonas stutzeri* A1501 strain can increase the root and shoot dry weight of maize, but this effect is not found in the maize inoculated with *nifH* mutant (Ke et al. 2019). In N_2 -deficient conditions, Kallar grass inoculated with *Azoarcus* sp. BH72 grew better and accumulated more nitrogen than plants inoculated with the *nifK* mutant strain (Hurek, et al. 2002.). Inoculation with *K. pneumoniae* 342 resulted in increased dry weight, chlorophyll content, total N, and N concentration of wheat in comparison with the uninoculated and *nifH* mutant-inoculated controls (Iniguez et al. 2004).

The ^{15}N isotope dilution technique is commonly used to determine the contribution of nitrogen-fixing bacteria to plant nitrogen. In this study, the ^{15}N isotope dilution technique was used to determine the biological nitrogen fixation of *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant. Cucumber plants derived $25.93 \pm 2.32\%$ from the nitrogen fixation of *P. polymyxa* WLY78 under low nitrogen fixation, but the $\Delta nifB-V$ mutant hardly fixed nitrogen. Similar reports are found that nitrogen fixation of *P. beijingensis* BJ-18 provided 27.8% nitrogen for cucumber under low nitrogen conditions (Li et al. 2019) and *P. polymyxa* P2b-2R provided 15% nitrogen for maize (Padma et al. 2017).

Declarations

Funding This work was supported by the National Key Research and Development Program of China (No. 2019YFA0904700).

Conflict of interest The authors have no conflicts of interest to declare.

Availability of data and material All data generated or analysed during this study are included in this published article.

Authors' contributions Conceptualization: Chen SF; Experiments: Liu S and Li Q; Methodology: Liu S, Li Q, Li YB and Hao TY; Writing: Liu S, Li Q and Chen SF; Funding acquisition: Chen SF. All authors read and approved the final manuscript.

Ethics approval

Not applicable

References

1. Amir HG., Shamsuddin ZH., Halimi MS., Ramlan MF, Marziah M (2003) N_2 fixation, nutrient accumulation and plant growth promotion by rhizobacteria in association with oil palm seedlings. Pak J Biol Sci 6, 1269-1272.

2. Baldani JI, Caruso L, Baldani VLD, Goi SR, Dobereiner J (1997) Recent advances in BNF with non-legume plants. *Soil Biol Biochem* 29: 911-922. [https://doi.org/10.1016/s0038-0717\(96\)00218-0](https://doi.org/10.1016/s0038-0717(96)00218-0).
3. Boddey RM, Deoliveira OC, Urquiaga S, Reis VM, Deolivares FL, Baldani VLD, Dobereiner J (1995) Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. *Plant Soil* 174: 195-209. <https://doi.org/10.1007/bf00032247>.
4. Chalk PM (1991) The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non-legumes. *Plant Soil* 132: 29-39. <https://doi.org/10.1007/bf00011009>.
5. Dart PJ (1986) Nitrogen fixation associated with non-legumes in agriculture. *Plant Soil* 90: 303-334. <https://doi.org/10.1007/bf02277405>.
6. Farrar K, Bryant D, Cope-Selby N (2014) Understanding and engineering beneficial plant-microbe interactions: plant growth promotion in energy crops. *Plant Biotechnol J* 12: 1193-1206. <https://doi.org/10.1111/pbi.12279>.
7. Fox AR, Soto G, Valverde C, Russo D, Lagares A, Zorreguieta A, Alleva K, Pascuan C, Frare R, Mercado-Blanco J, Dixon R, Ayub ND (2016) Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940. *Environ Microbiol* 18: 3522-3534. <https://doi.org/10.1111/1462-2920.13376>.
8. Geddes BA, Ryu MH, Mus F, Costas AG, Peters JW, Voigt CA, Poole P (2015) Use of plant colonizing bacteria as chassis for transfer of N₂-fixation to cereals. *Curr Opin Biotechnol* 32: 216-222. <https://doi.org/10.1016/j.copbio.2015.01.004>.
9. Hao TY, Chen SF (2017) Colonization of wheat, maize and cucumber by *Paenibacillus polymyxa* WLY78. *PLoS One* 12: e0169980. <https://doi.org/10.1371/journal.pone.0169980>.
10. Hurek T, Handley LL, Reinhold-Hurek B, Piche Y (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Mol Plant Microbe In* 15: 233-242. <https://doi.org/10.1094/mpmi.2002.15.3.233>.
11. Iniguez AL, Dong YM, Triplett EW (2004) Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. *Mol Plant Microbe In* 17: 1078-1085. <https://doi.org/10.1094/mpmi.2004.17.10.1078>.
12. Ke XB, Feng S, Wang J, Lu W, Zhang W, Chen M, Lin M (2019) Effect of inoculation with nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 on maize plant growth and the microbiome indigenous to the rhizosphere. *Syst Appl Microbiol* 42: 248-260. <https://doi.org/10.1016/j.syapm.2018.10.010>.
13. Li YB, Li YL, Zhang HW, Wang MY, Chen SF (2019) Diazotrophic *Paenibacillus beijingensis* BJ-18 provides nitrogen for plant and promotes plant growth, nitrogen uptake and metabolism. *Front Microbiol* 10: 1119. <https://doi.org/10.3389/fmicb.2019.01119>.
14. Li YL, Chen SS (2019) Fusaricidin produced by *Paenibacillus polymyxa* WLY78 induces systemic resistance against Fusarium wilt of cucumber. *Int J Mol Sci* 20: 5240. <https://doi.org/10.3390/ijms20205240>.

15. Oliveira ALM, Santos OJAP, Marcelino PRF, Milani KML, Zuluaga MYA, Zucareli C, Goncalves LSA (2017) Maize inoculation with *Azospirillum brasilense* Ab-V5 cells enriched with exopolysaccharides and polyhydroxybutyrate results in high productivity under low N fertilizer input. *Front Microbiol* 8: 1873. <https://doi.org/10.3389/fmicb.2017.01873>.
16. Padda KP, Puri A, Zeng QW, Chanway CP, Wu XQ (2017) Effect of GFP-tagging on nitrogen fixation and plant growth promotion of an endophytic diazotrophic strain of *Paenibacillus polymyxa*. *Botany* 95: 933-942. <https://doi.org/10.1139/cjb-2017-0056>.
17. Raymond J, Siefert JL, Staples CR, Blankenship RE (2004) The natural history of nitrogen fixation. *Mol Biol Evol* 21: 541-554. <https://doi.org/10.1093/molbev/msh047>.
18. Setten L, Soto G, Mozzicafreddo M, Fox AR, Lisi C, Cuccioloni M, Angeletti M, Pagano E, Diaz-Paleo A, Ayub ND (2013) Engineering *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. *PLoS One* 8: e63666. <https://doi.org/10.1371/journal.pone.0063666>.
19. Sevilla M, Burris RH, Gunapala N, Kennedy C (2001) Comparison of benefit to sugarcane plant growth and $^{15}\text{N}_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif^- mutant strains. *Mol Plant Microbe In* 14: 358-366. <https://doi.org/10.1094/mpmi.2001.14.3.358>.
20. Van Deynze A, Zamora P, Delaux PM, Heitmann C, Jayaraman D, Rajasekar S, Graham D, Maeda J, Gibson D, Schwartz KD, Berry AM, Bhatnagar S, Jospin G, Darling A, Jeannotte R, Lopez J, Weimer BC, Eisen JA, Shapiro HY, Ane JM, Bennett AB (2018) Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. *PLoS Biol* 16: e2006352. <https://doi.org/10.1371/journal.pbio.2006352>.
21. Wang LY, Zhang LH, Liu ZZ, Zhao DH, Liu XM, Zhang B, Xie JB, Hong YY, Li PF, Chen SF, Dixon R, Li JL (2013) A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli*. *PLoS Genet* 9: e1003865. <https://doi.org/10.1371/journal.pgen.1003865>.
22. Wang TS, Zhao XY, Shi HW, Sun L, Li YB, Li Q, Zhang HW, Chen SF, Li JL (2018) Positive and negative regulation of transferred *nif* genes mediated by indigenous GlnR in Gram-positive *Paenibacillus polymyxa*. *PLoS Genet* 14: e1007629. <https://doi.org/10.1371/journal.pgen.1007629>.
23. Xie JB, Du ZL, Bai LQ, Tian CF, Zhang YZ, Xie JY, Wang TS, Liu XM, Chen X, Cheng Q, Chen SF, Li JL (2014) Comparative genomic analysis of N_2 -fixing and non- N_2 -fixing *Paenibacillus* spp.: organization, evolution and expression of the nitrogen fixation genes. *PLoS Genet* 10: e1004231. <https://doi.org/10.1371/journal.pgen.1004231>.
24. Xie JB, Shi HW, Du ZL, Wang TS, Liu XM, Chen SF (2016) Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in *Paenibacillus polymyxa* and its closely related species. *Sci Rep* 6: 21329. <https://doi.org/10.1038/srep21329>.
25. Xu PX, Han LL, He JZ, Luo F, Zhang LM (2017) Research advance on molecular ecology of symbiotic nitrogen fixation microbes. *J Appl Ecol* 28: 3440-3450. doi: 10.13287/j.1001-

Figures

Figure 1

Construction and characterization of *nif* gene cluster deletion mutant (Δ nifB-V) of *P. polymyxa* WLY78. a. General scheme of construction of *nif* gene cluster deletion mutant. b. The *nif* cluster disruption was confirmed by PCR using the primer 5 and primer 6. c. Comparison of growth of the WT (the wild-type) and Δ nifB-V (deletion mutant) strains. Growth of the WT and Δ nifB-V strains in nitrogen-limited medium supplemented with 100 mM NH₄Cl as the nitrogen source. d. Nitrogenase activities of WT and Δ nifB-V grown anaerobically in nitrogen-limited medium.

Figure 2

Colonization of GFP-labeled *P. polymyxa* WLY78 and GFP-labeled $\Delta nifB-V$ mutant in cucumber. Colonization pattern of *P. polymyxa* WLY78 in root (a), stem (b) and leaf (c). Colonization pattern of $\Delta nifB-V$ mutant in root (d), stem (e) and leaf (f). Scale bars (a–f) = 100 μ m.

Figure 3

Biomass of cucumber inoculated with *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant, with non-inoculated cucumber as control. Length of shoots (a) and roots (b). Dry weights of shoots (c) and roots (d). The error bars in the figure indicate SD values. Statistical analysis is performed using One-way ANOVA. * Indicates $P < 0.05$, ** indicates $P < 0.01$.

Figure 4

Cucumber cultivation in greenhouse with *P. polymyxa* WLY78 and $\Delta nifB-V$ mutant, with non-inoculated cucumber as control. The growth of cucumber seedlings under low (a) and high (c) nitrogen conditions. The shoot and root of cucumber under low (b) and high (d) nitrogen conditions.