Comparison of Paenibacillus polymyxa wild-type and Nif− mutant in colonization, plant-growth promotion and nitrogen fixation contribution

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Abstract

Aims

This study aimed to compare the effect on colonization, plant-growth promotion and nitrogen fixation contribution by inoculation with *Paenibacillus polymyxa* wild-type and Nif\(^{-}\)mutant.

Methods

*Paenibacillus polymyxa* wild-type and Nif\(^{-}\) mutant was labeled with GFP and then the GFP-labeled bacteria were used to inoculate cucumber. The colonization patterns of *P. polymyxa* WLY78 in these plants were observed under the 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

Observation by laser confocal microscopy revealed that both *P. polymyxa* WLY78 and Δ*nifB-V* mutant can effectively colonize cucumber root, stem and leaf tissues. Greenhouse experiments showed that inoculation with *P. polymyxa* WLY78 can significantly enhance the lengths and fresh weights of cucumber roots and shoots, but inoculation with Δ*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 Δ*nifB-V* mutant nearly can not provide nitrogen for plant.

Conclusions

This present study demonstrates that nitrogen fixation plays an important role in promoting plant growth.

Introduction

Nitrogen is the most important nutrient 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 it plays an important role in the sustainable development of agriculture (Raymond et al. 2004). Nitrogen-fixing microorganisms include symbiotic nitrogen-fixing bacteria, autogenous nitrogen-fixing bacteria and associative nitrogen-fixing bacteria. Associative nitrogen-fixing bacteria can colonize root surface cells, invade plant roots, and form close contact with plants, thereby promoting plant growth (Baldani et al. 1997). Associative nitrogen-fixing bacteria promote the absorption of nitrogen by non-legume plants (Geddes et al. 2015).
nitrogen fixation can 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). $^{15}$N isotope and N balance studies have shown that several sugarcane varieties obtain over 60% of their nitrogen ($< 150$ kg N ha$^{-1}$ year$^{-1}$) from biological nitrogen fixation performed mainly by *Acetobacter diazotrophicus* and *Herbaspirillum* spp. (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 provides nitrogen for wheat, maize and cucumber plants and promotes 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 furaricidins 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 is of great usage 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**

**Bacteria strains and culture conditions**

*Paenibacillus polymyxa* WLY78, isolated from the rhizosphere of bamboo in Beijing (Wang et al., 2013). This bacterium has multiple antagonistic activities against plant pathogens and produces IAA (Xie et al., 2016). The nitrogen-fixing gene cluster deletion mutant $\Delta$nifB-V of *P. polymyxa* WLY78 was constructed...
by a homologous recombination method. Primers 5’
CGGCCACGATGCCTCCGCGTAGAGGATCCGCGTGGTGGATGTGGA CG 3’ and 5’
AACGCTTTTTCCGTTATCATTCCTTCCACATCTATTCTTCGTC 3’ were used to amplify a 950 bp-long DNA
sequence located upstream of \textit{nifB}. Primers 5’ GAAGGAATGATAACCGAAAAACGTTCCCGT
C 3’ and 5’ GACTGCGAAAAGACATAATCGATAAGCTTCCAGCACAGGCTC 3’ were used to
amplify a 1107 bp-long sequence located downstream of \textit{nifV}. The two fragments were then fused with
\textit{BamH}I/\textit{Hind}III digested pRN5101 vector using Gibson assembly master mix (New England Biolabs),
generating the four recombinant plasmids. Then, the recombinant plasmid was transformed into \textit{P.
polymyxa} WLY78 as described by Wang et al., (2018), and the marker-free deletion mutant (the double-
crossover transformant) \textit{ΔnifB-V} was selected for from the initial erythromycin resistance (Em’)
transformants after several rounds of nonselective growth at 39˚C and confirmed by PCR amplification
and sequencing analysis.

\textit{P. polymyxa} WLY78 and \textit{ΔnifB-V} were inoculated into Luria-Bertani (LB) liquid medium, cultured at 30˚C
and 180 rpm to logarithmic growth phase, and then centrifuged to collect the bacterial cells and suspend
the bacterial cells with physiological saline. The cell concentration was set to 10^8 cells mL^-1.

Preparation of soil and seeds

The soil was taken from the Shangzhuang Experimental Station of China Agricultural University. They
were all 0–20 cm deep topsoil. The soil was low nitrogen 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 35 cm and a height of 25 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 rinsed with sterile water three times,
and spread the seeds in a sterile petri dish with damp filter paper. Leave it in the dark at room temperature
(25˚C) for 3–5 days until the seeds germinate. During the period, 1–2 mL of sterile water was added
dropwise with a pipette to keep the filter paper moist.

Colonization of \textit{P. polymyxa} and \textit{ΔnifB-V} on cucumber

The recombinant plasmid pGFP300 carrying the \textit{gfp} gene (Hao and Chen, 2017) was transferred into \textit{P.
polymyxa} WLY78 and \textit{ΔnifB-V} to prepare cell suspension of the GFP-tagged strains so that the cell
concentration was 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 will grow into seedlings, which will be transferred to a light
incubator for cultivation. 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
plant tissues were observed with a laser confocal scanning microscope (CLSM, Olympus FluoViewTM FV1000 confocal microscope), and images were collected with FV10-ASW software.

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 $^{15}$N labeled (NH$_4$)$_2$SO$_4$ (10.16% $^{15}$N 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 deionized water (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 deionized water 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 deionized water. Place the flower pots under the best conditions in the greenhouse to obtain suitable light conditions. 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 fresh weight and length of the root and shoot were weighed. 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. The dried sample was ground, sieved with a 1 mm sieve and placed in a bag, and the plant N content and $^{15}$N enrichment determination were performed by an isotope mass spectrometer. The remaining 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 plant biological nitrogen fixation. 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:
\[
%\text{Ndfa} = \left(1 - \frac{\%\text{Ndff}}{\%\text{Ndff}}\right) \times 100
\]

Among them, %Ndff is the $^{15}$N enrichment of cucumber stems and leaves of inoculation treatment, and %Ndff 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**

Colonization of *P. polymyxa* WLY78 and ΔnifB-V mutant in cucumber

*P. polymyxa* WLY78 contains a compact *nif* gene cluster consisting of 9 genes (*nifB nifH nifD nifK nifE nifN nifX hesA nifV*) located in a 10.5 kb. A *nif* gene cluster deletion mutant (ΔnifB-V) was constructed by recombination as described in Materials and Methods. The ΔnifB-V mutant does not have nitrogenase activity, no matter this mutant is cultivated in medium containing ammonium or no ammonium.

*P. polymyxa* WLY78 and the ΔnifB-V mutant were individually labelled with GFP and then the GFP-labelled bacteria were used to inoculate cucumber. After 3 days of inoculation, the samples of the cucumber roots, stems and leaves were observed under laser confocal microscopy. The cells of *P. polymyxa* WLY78 not only colonized on the surface of cucumber root, but also colonized interior of root, stem and leaf (Fig. 1a-c). The presence of bacterial cells was observed in the vascular bundle of the stem and the leaves. Similarly, cells of the ΔnifB-V mutant were also observed in the primary root cortex of cucumber (Fig. 1d), stem (Fig. 1e) and leaf vein (Fig. 1f). The results indicated that *P. polymyxa* WLY78 and ΔnifB-V can colonize outside and inside of cucumber tissues. Deletion of the *nif* gene cluster did not affect the colonization of *P. polymyxa* WLY78.

Colonization pattern of *P. polymyxa* WLY78 in root (a), stem (b) and leaf (c). Colonization pattern of ΔnifB-V mutant in root (d), stem (e) and leaf (f). Scale bars (a–f) = 100 µm.

Effects of *P. polymyxa* WLY78 and ΔnifB-V on the growth of cucumber

Cucumber samples were collected on the 30th day after plantation, and the length and fresh weight of plant shoots and roots were measured to evaluate the effects of inoculation with *P. polymyxa* WLY78 and ΔnifB-V mutant on plant growth under low and high nitrogen conditions. The un-inoculated plants served
as a control. Compared to the un-inoculated control group, cucumber plants inoculated with \( P. \text{polymyxa WLY78} \) under low nitrogen conditions showed increase of 63.03\% in shoots fresh weight, of 71.20\% in root fresh weight, of 53.31\% in shoot length and of 97.51\% in root length, but they showed a little increase in lengths and weights under high nitrogen conditions (Fig. 2a-d). Compared with the un-inoculated control group, the cucumber plants inoculated with the \( \Delta \text{nifB-V} \) mutant showed a little increase in the fresh weight and length of the shoots and roots under both low and high nitrogen conditions (Fig. 2a-d). Notably, the fresh weight and length of the shoots and roots of cucumbers inoculated with \( \Delta \text{nifB-V} \) mutant under both conditions are similar to those of cucumbers inoculated with \( P. \text{polymyxa WLY78} \) under high nitrogen conditions. Figure 3 is an experimental diagram of the greenhouse cultivation. The data indicate that the diazotrophic \( P. \text{polymyxa WLY78} \) can effectively promote plant growth under low nitrogen conditions and disruption of \( \text{nif} \) genes encoding nitrogenase results in almost loss of the ability of promoting plant growth.

Quantification of BNF in \( P. \text{polymyxa WLY78} \)- and \( \Delta \text{nifB-V} \) mutant-inoculated cucumbers

To estimate the contribution of BNF, \(^{15}\text{N}\) isotope dilution technique was used to analyze the inoculated cucumber grown in soil contain \(^{15}\text{N}\)-labeled (NH\(_4\))\(_2\)SO\(_4\) as N fertilizer in greenhouse conditions (Table 1). The nitrogen derived from gaseous nitrogen (%Ndfa) in the \( P. \text{polymyxa WLY78} \) inoculated cucumber and the \( \Delta \text{nifB-V} \) strain inoculated cucumber under low N conditions is 25.93\% ± 2.32\% and 2.93\% ± 6.57\%, respectively. Whereas, the nitrogen derived from gaseous nitrogen (%Ndfa) in the \( P. \text{polymyxa WLY78} \) inoculated cucumber and the \( \Delta \text{nifB-V} \) strain inoculated cucumber under high N conditions is 1.54 ± 0.66\% and −0.22 ± 1.02\%, respectively. These results indicate that the cucumber plant has incorporated the nitrogen provided by BNF of \( P. \text{polymyxa WLY78} \) under low N conditions and BNF is inhibited by high concentration of available nitrogen in the environment. The \( \Delta \text{nifB-V} \) strain-inoculated cucumbers nearly did not derive nitrogen from BNF, consistent with the \( \Delta \text{nifB-V} \) strain has no nitrogenase. The data also indicate that \( P. \text{polymyxa WLY78} \) can be used to provide nitrogen nutrition to plants and reduce the use of nitrogen fertilizers.

| Treatment         | %Ndfa               |
|-------------------|---------------------|
|                   | Low nitrogen | High nitrogen |
| Control           | -            | -             |
| \( P. \text{polymyxa WLY78} \) | 25.93 ± 2.32\(^a\)  | 1.54 ± 0.66\(^a\) |
| \( \Delta \text{nifB-V} \) | 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 Δ*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 does 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 Δ*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 Δ*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 Δ*nifB-V* mutant, inoculation with *P. polymyxa* WLY78 significantly increased the fresh weights and lengths of cucumber shoots and roots under low nitrogen conditions, but this effect is not found in the cucumbers inoculated with the Δ*nifB-V* mutant. Notably, inoculation with *P. polymyxa* WLY78 under low nitrogen conditions led to the increased levels of root length and weight were much higher than those of shoot length and weight, consistent with the recent results obtained by inoculation with diazotrophic *P. beijingensis* BJ-18 in maize, wheat and cucumber (Li et al. 2019). It was observed that copy numbers of *P. beijingensis* BJ-18 are much higher in roots than in shoot, suggesting that the densities of diazotrophs are positively correlated to plant growth traits (Li et al. 2019). 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 N₂-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 ¹⁵N isotope dilution technique is commonly used to determine the contribution of nitrogen-fixing bacteria to plant nitrogen. In this study, the ¹⁵N isotope dilution technique was used to determine the biological nitrogen fixation of *P. polymyxa* WLY78 and Δ*nifB-V* mutant. Cucumber plants derived 25.93% ± 2.32% from the nitrogen fixation of *P. polymyxa* WLY78 under low nitrogen fixation, but the Δ*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 *Paenibacillus polymyxa* P2b-2R provided 15% nitrogen in maize by nitrogen fixation (Padda et al. 2017).

**Declarations**

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**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, Hao TY and Zhang HW; Writing: Liu S, Li Q and Chen SF; Funding acquisition: Chen SF. All authors read and approved the final manuscript.

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