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Effect of inoculation with nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 on maize plant growth and the microbiome indigenous to the rhizosphere

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ABSTRACT

Plant growth promoting diazotrophs with the ability to associate with plant roots are in common use as inoculants to benefit crop yield and to mitigate chemical nitrogen fertilization. However, limited information is available in understanding to what extent the plant growth-promoting effect of the inoculum has on the plant's nitrogen acquisition as well as on the impact of inoculation on the indigenous rhizosphere microbial population. Here we reported on experiments that assessed how endophytic *Pseudomonas stutzeri* A1501 inoculated on maize improved plant growth and plant nitrogen content using a ^{15}N dilution technique under two water regime conditions. The effects of inoculation and different water regimes were also assessed for the maize rhizospheric and surface soil communities by MiSeq community sequencing combined with qPCR of functional genes and transcripts (*nifH* and *amoA*) related to nitrogen cycling. Results support maize inoculated with *P. stutzeri* A1501 grew better and accumulated more nitrogen with a lower $\delta^{15}N$ signature after 60 days than did plants inoculated with *nifH*-mutant and sterilized A1501 cells (non N_2-fixing controls). Inoculant contribution to the plant was estimated to range from 0.30 to 0.82 g N/plant, depending on water conditions. Inoculation with *P. stutzeri* A1501 significantly altered the composition of the diazotrophic community that *P. stutzeri* became dominant in the rhizosphere, and also increased the population of indigenous diazotrophs and ammonia oxidizers and functional genes transcripts. Redundancy analysis revealed that soil compartment and A1501 inoculation treatments were the main factors affecting the distribution of the diazotrophic community.

Introduction

The amount of terrestrial biologically fixed N produced is in excess of 150 Tg/year, which is equivalent to industrial N production from anthropogenic sources [8]. Besides true symbiotic associations with the formation of root nodules, a wide variety of soil nitrogen-fixing bacterial species have the ability to colonize the roots of non-leguminous plants without formation of differentiated structures. These types of associations, including bacterial colonization of root surfaces or endophytic interactions within root tissues, are often referred to as associative symbiosis [4,17,36]. Inoculation of cereal crops with associative nitrogen-fixing bacteria such as *Azospirillum*, *Herbaspirillum*, or *Pseudomonas* spp. has been shown to enhance crop yields [16,27,28,30]. Nitrogen fixation quantified using the ^{15}N isotope approach, by comparing the performance of wild-type and *nif*-mutant strains, was used to obtain quantitative estimates of the proportion of plant nitrogen (N) obtained from atmospheric N_2 [7].

The N_2-fixing bacterium, *Pseudomonas stutzeri* A1501 (formerly identified as *Alcaligenes faecalis*) was originally isolated from the rice rhizosphere [11,45,50]. Analysis of the complete nucleotide sequence of the genome revealed a genomic island containing the nitrogen fixation genes as well as the presence of genes required for the interaction with plants [48]. Inoculation of plant callus, and rice plantlets showed that the nitrogen-fixing *P. stutzeri* strain A15 and A1501 (re-isolated from rice seedlings inoculated with A15) were endophytic bacteria that could enhance rice plant growth and may provide rice plants with fixed nitrogen [30,34,49,50]. To what extent N_2-fixation rates could be affected by environmental stresses such as water regime conditions, remain unknown as well

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Table 1
Properties of the soil used.

	Nature soil	Sterilized soil (after γ-irradiation)
pH	6.5	6.8
NH_4^+–N (mg/kg)	1.90	4.23
NO_3^-N (mg/kg)	3.45	2.34
Total N (%)	0.12	0.22
Organic C (mg/kg)	8.86	9.94
Abundance of bacteria (16S rRNA gene copy numbers per g dry soil)	8.39×10^8	n.d.
Abundance of archaea (16S rRNA gene copy numbers per g dry soil)	1.02×10^7	n.d.
Abundance of nitrogen fixing organisms (*nifH* gene copy numbers per g dry soil)	9.58×10^5	n.d.
Abundance of ammonia-oxidizing bacteria (*amoA* gene copy numbers per g dry soil)	1.13×10^6	n.d.
Abundance of ammonia-oxidizing archaea (*amoA* gene copy numbers per g dry soil)	3.56×10^6	n.d.

n.d., not detected.

as the influence of the inoculation on the indigenous rhizosphere microbial population.

Maize (*Zea mays* L.) has become the major grain crop in the world in terms of total production [15]. The rhizosphere is defined as a biologically active zone in which plant roots and microorganisms interact [35]. The importance of the plant microbiome has been recently recognized and it has been proposed as acting like the plants second genome which plays a critical role for plant health [5]. Previous studies mainly using low-resolution methods demonstrated that two different outcomes on indigenous rhizosphere bacterial population could occurred as a result of inoculation, i.e. significant perturbation by altering the composition of the indigenous microbial community [24,37] or no effect on the resident community [10,20]. However, only a few studies have focused on the effects inoculation has on root-associated N cycling microbes (i.e. N_2-fixers or nitrifiers). Babic et al. [3] found an increased alfalfa yield when inoculated with diazotrophic strains, which could be attributed to increased nitrogen content and the abundances of *nifH* and *amoA* genes. Thus, it is important to understand the responses of these taxonomic groups which may be related to their contribution to plant nutrition and biomass [6,21]. There is still limited information on the influence of inoculation performed under greenhouse and field conditions on the communities and activities of indigenous taxonomic groups present in the rhizosphere.

To date there are no reports on how *P. stutzeri* A1501 can promote maize growth. Therefore, the first objective of this study was to establish to which extent *P. stutzeri* could contribute to plant growth and plant total N. Performances of the wild-type strain and a *nifH*-mutant strain on maize growth were assessed under different water regime conditions (water stress and well-watered) using the ^{15}N isotopic dilution technique. Then, the effects of inoculation and different water regimes were also assessed for the maize rhizospheric and surface soil communities by MiSeq (16S rRNA and *nifH* genes) sequencing combined with qPCR of functional genes related to nitrogen cycling (*nifH* and *amoA*).

Materials and methods

Plant, soil, and inoculant preparation

Soil samples were collected from a moderate climate (annual average temperature 12.2 °C) cropland (Beijing, China). Soil characteristics are described in Table 1. Soil was sterilized by γ-irradiation (60 kGy; ^{60}Co; 72 h) using the facility at the Institute of Atomic Energy, China Academy of Agriculture Science (CAAS, Beijing). The sterilization efficiency was tested by CO_2/CH_4 production measurement and PCR amplification. When the sterilized soil samples were incubated anaerobically at 25 °C for two weeks, there was no CO_2 or CH_4 production. 16S rRNA and *amoA* genes copy numbers were not detected by qPCR, indicating the effectiveness of the sterilization process (Table 1). No amplification inhibition was observed for the γ-irradiation treated soil samples.

Maize (*Zea mays* L., genotype hybrid Zhengdan 958) seeds were surface disinfected with 2% NaClO for 1 min followed by 70% ethanol for 10 min, and rinsed with sterile water three times. After sterilization, seeds underwent accelerating germination in a climate chamber at 25 °C. After germination, uniform seedlings were selected for continued growth in the greenhouse. The wild type *P. stutzeri* A1501 and the *nifH* mutant strain 1502 were used in this study. A1501/1502 were grown in LB medium to yield approx. 8×10^8 cells mL^{-1} as determined by spectrophotometry (OD_{620} = 1.0). After centrifugation at 5000 rpm for 30 min, the supernatant was discarded and cells were washed with a sterile NaCl solution (0.8%). The 1502 *nifH* mutant strain was constructed by insertion of a Km cassette into the *Bgl*II site within the *nifH* coding sequence from plasmid pUC4H described before [11]. Correct insertion was confirmed by PCR and sequencing. The nitrogenase activity was measured following the acetylene reduction assay [11].

Greenhouse experiment setup

Pot experiments were carried out in a greenhouse (25–30 °C with a 16 h light/8 h dark cycle) at the CAAS (Beijing). Soil microcosms were prepared in polyethylene containers, each with 10 kg of dry soil. Different microcosms were prepared using non-sterilized soil matrix or soil matrix sterilized by γ-irradiation as indicated above. Two water treatments conditions were used: water-filled pore space values of 20 (simulated water stress condition) and 80 (simulated well-watered condition) were achieved by adding appropriate volumes of water to 10 kg of dry soil (bulk density of 1.6 g cm^{-3}), mixing thoroughly in the pots. Before planting, KCl and Na_2HPO_4 were applied to soil as basal fertilizers at amounts of 17 mg K and 50 mg P per kg soil, respectively. After maize inoculation and planting, during the maize growth, sterilized water was added to maintain the water table levels. Soil moisture was monitored by a water content detector (Meacon, China) according to the manufacturer's instructions.

Microcosms inoculation

The identical protocol was applied to sterile soil microcosms and the natural soil microcosms. A dual water regime included well-watered condition and water stress condition. Inoculation were performed by dipping the maize seedling roots in NaCl solution containing 8×10^8 bacterial cells/ml before planting in soil. Three inoculation conditions were used with nine repetitions per condition: (i) inoculation with *P. stutzeri* A1501 (ii) inoculation with *nifH* mutant 1502 (iii) inoculation with sterilized suspension of *P. stutzeri* A1501.

Plant harvest and sample collection

After 60 days of growth, the maize plants were harvested to determine shoot/root weight. Surface soil and rhizospheric soil were collected for future molecular and soil chemical analyses (including total N, C, organic C, and ammonium and nitrate concentrations measurements). First, the surface soil was collected from the upper 1–2 cm of the soil layer. The rhizospheric soil was defined as the soil closely adhering to the roots [23,26]. In brief, after removing the loosely attached soil by hands, the soil closely associated with the roots was washed with sterile water and collected by centrifugation. After sampling, each soil fraction was homogenized and frozen in liquid N_2. Soils were stored at −80 °C for future analyses.

^{15}N dilution assay

Soil microcosms planted with maize using both nature soil and sterilized soil prepared as described above were treated with approximately 50 mg/kg soil ^{15}N-ammonium sulfate (10.18% atom, Shanghai Research Institute of Chemical Industry, China). The substrate was added to the soil and homogenized before the experiment started. After a 60-day maize growth period, maize leaf and stem tissue were collected for ^{15}N measurements by mass spectrometry. Analysis of foliage N concentration and $^{15}N/^{14}N$ ratio was conducted by mass spectrometry at the Institute of Environment and Sustainable Development in Agriculture, CAAS. The changes in $^{15}NH_4^+/^{14}NH_4^+$ ratio were determined and used to quantify the contribution of nitrogen derived from the diazotrophic strain to the plant. The percentage of N derived from atmospheric N_2 (% Ndfa) via BNF for individual maize plants was calculated by the equation:

$$\%\,\mathrm{Ndfa} = 1 - \delta^{15}N_{\mathrm{inoculated}}/\delta^{15}N_{\mathrm{control}}$$

in which $\delta^{15}N_{\mathrm{inoculated}}$ is the average per mil ^{15}N excess of the maize with *P. stutzeri* A1501 inoculation at day 60 of the growing period; and $\delta^{15}N_{\mathrm{control}}$ is the per mil ^{15}N excess of maize with *nifH* mutant 1502 or sterilized inoculum at day 60 of the growing period.

Total N fixed (kg/plant) was calculated by the equation:

$$N_{\mathrm{fixed}} = N_t \times \%\,\mathrm{Ndfa} \times \mathrm{Biomass}$$

N_t is total N concentration. Biomass (kg/plant) was determined by destructive sampling each plant for both above and below ground dry mass at day 60 of the growing period.

DNA/RNA extraction and cDNA synthesis

For molecular analysis, 3 soil samples were chosen randomly from 9 replicate samples. Genomic DNA was extracted from 0.5 g (wet weight) of soil using a DNeasy PowerSoil Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentration was determined with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA was extracted from 1.5 g (wet weight) soil samples with RNA PowerSoil® Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). RNA quality and concentration were determined using a Nanodrop spectrophotometer by measuring the absorbance ratio at 260/280 nm, which was consistently found to be >1.80. An additional determination by agarose gel electrophoresis was done for RNA integrity measurements and generally showed three clear bands corresponding to the 23S, 18S, and 5S ribosomal subunits. After DNA was entirely removed by gDNA Eraser (checked by PCR), cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (TAKARA, Japan) according to the manufacturer's instructions, in which the extracted RNA is reverse-transcribed to cDNA using random primers. All DNA/cDNA samples were stored at −20 °C for future analyses.

Illumina MiSeq and data analysis

Using the Illumina MiSeq PE300 sequencing platform (Illumina, Inc., CA, USA), the diversity and composition of total microbial and diazotrophic communities were assessed by sequencing analysis of V3-V4 hypervariable regions of 16S rRNA and the *nifH* gene, respectively [29,31]. Before experiment, several sets of primers of 16S rRNA (515F/806R, 336F/806R and 515F/907R) and *nifH* (nifHF/nifHR and PolF/PolR) [23,25,29] have been tested for the soil used in this study. In general, primer sets cover a broad range of taxa and diversity were chosen. For *nifH*, *Proteobacteria* were the predominant diazotrophs in this soil, which was consistent with previous studies that describe the major diazotrophs in agricultural soils [22,51]. Therefore, 515F/806R and PolF-PolR-degenerated universal primers were used for further PCR amplification of an internal fragment of the 16S rRNA and *nifH* gene, respectively. These primers each contained a set of an 8-nucleotide barcode sequence unique to each sample. The MiSeq-sequencing data analysis primarily followed the protocol described by Collavino et al. [9]. Quality filtering of the sequencing reads were initially performed with the QIIME package (v1.2.1), and then checked for chimeras using UCHIME. Raw sequences were selected based on sequence length, quality, primer, and tag according to several principles: (i) the reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (ii) exact barcode matching, two nucleotide mismatches in primer matching, and reads containing ambiguous characters were removed; and (iii) only overlapping sequences longer than 10 bp were assembled according to their overlap sequence. Amplicon libraries of the 16S rRNA and *nifH* genes were examined using the FunGene Pipeline of RDP server (http://fungene.cme.msu/edu/FunGenePipeline) with the default settings. Short and low quality sequences were further removed by non-specific blast hits compared with the reference database. Following that process, the nucleotide sequences were classified into operational taxonomic units (OTUs) under the threshold of 97% identity using UCLUST. Chimeric sequences were identified and removed using Usearch (version 8.0.1623). All analyses were implemented using the R version 3.0.1 [33].

The 16S rRNA and *nifH* sequences obtained from this study were deposited in the NCBI-SRA (Sequence Read Archive) database under the following accession numbers: SRP102049.

qPCR and RT-qPCR

Quantitative PCR of bacterial and archaeal 16S rRNA genes, bacterial and archaeal *amoA* genes, and *nifH* genes were performed according to published protocols [9,23]. Template DNA was tested in a dilution series, and higher amplification efficiencies were always obtained after 10-fold dilution. Inhibition was observed without dilution. All PCR standards were prepared from a plasmid DNA mixture of clones obtained from soil samples or the *P. stutzeri* A1501 strain. Real-time PCR (qPCR) conditions were optimized using a standard dilution series over seven orders of magnitude covering 1×10^2–1×10^8 copies of template per assay. A control was always run using template water instead of soil DNA/cDNA extract. All thermal protocols are described in Table S1. qPCR was performed in triplicate. The amplification efficiencies were 90%–110%. Specific amplification of bacterial and archaeal 16S rRNA genes, *nifH*, and *amoA* genes were checked by melting curve analysis (resulting in a single peak) and by agarose gel electrophoresis. Thus, the numbers of A1501 cells associated with roots were determined by multiplying the copy numbers of *nifH* genes (by qPCR) with the relative abundance of reads of *nifH* genes representing these strains (by MiSeq).

Rhizosphere colonization efficiency of inoculant

Before this experiment, to test if *P. stutzeri* A1501 can colonize the surface or endosphere of maize root, maize inoculated with A1501 strain was planted under soil culture condition for 20 days. When sampling, the rhizospheric soil samples were collected from the surface of root. After root surface was cleaned by sodium hypochlorite (2%, 1 min) and sonication (30 s at 50–60 Hz, 3 times), the endosphere fraction was pre-homogenized before the DNA extraction by sterile glass beads beating (at a speed of 6 m/s for 30 s) to excrete cells. DNA-based qPCR results showed that the amounts of A1501 cells in the endosphere were significantly higher than the control of non-inoculation, albeit much lower than that in the rhizospheric soil. This result suggested that A1501 could colonize in the inside of maize roots as endophytic but may be mostly at the surface of roots in this case. Thus in the present study, the

colonization of *P. stutzeri* wild type and mutant derivative in the surface of maize roots was mainly considered.

Colonization efficiency of *P. stutzeri* A1501 and 1502 (*nifH*-mutant strain) in maize rhizosphere was assayed under hydroponic or soil culture conditions. All experiments under each condition were performed in nine replications. Hydroponics experiments were done in 120 ml test tubes with sterilized Hoagland's nutrient solution. A1501 and 1502 strains associated with maize plants and were incubated for 20 days in a growth chamber (28 °C, 10-h light). Seedlings were pre-germinated and then transferred into sterile rubber-stoppered glass tubes containing 30 ml sterile nutrient solution. When the bacterial cultures reached OD_{620} = 1.0 (8×10^8 cells ml^{-1}), the bacteria were centrifuged at a speed of 5000 g for 15 min, washed with a sterile NaCl solution (0.8%), and diluted 100 and 10000 times fold. Treatments consisted of three dilutions (8×10^4, 8×10^6 and 8×10^8) and a control of a sterilized A1501 cell inoculation. Seeds were incubated with different concentrations of bacterial suspension for 10 min and transferred to the tubes. Root samples were collected at days 10 and 20. Bacteria were enriched by vortexing root samples for 30 min in PBS solution, and the number of A1502 (*nifH*-mutant) microorganisms were then counted on minimal lactate medium (medium K) [11]. Antibiotics were used at the following concentrations: 100 μg/mL ampicillin (Amp), 50 μg/mL kanamycin (Km), 10 μg/mL tetracycline (Tc), and 34 μg/mL spectinomycin (Spc). Colony-forming units (CFUs) on these plates were counted after incubating at 30 °C for 24 h. The absolute numbers of A1501 cells were determined by qPCR combined with MiSeq approach as described above. At the end of the incubation period, roots from the 10^6 dilution treatment were chosen, washed carefully, and cut into 0.5 cm pieces and then placed in 5% glutaraldehyde in 0.03 M pipes buffer for 2 h at room temperature under a vacuum. Fixation, staining, dehydration, air-drying, and coating were conducted as described by Viret et al. [46]. The specimens were examined with a scanning electron microscope (SEM, SU8010, Hitachi, Japan). For the rhizosphere soil culture composition experiments, sterilized and maize growth microcosms were sampled after 20 days, which had been inoculated with bacterial preparation (8×10^8 cells/ml *P. stutzeri* A1501 or 1502 strains) or sterilized inoculum. Bacteria were re-isolated from root surfaces and counted following the same procedure as described above.

Statistical analyses

For qPCR results, all DNA and cDNA abundance data were initially tested for normality using the Shapiro–Wilk normality test. The data were then log-transformed (using the natural logarithm method). An ANOVA design was employed to test for the effect of soil compartment, water regime condition, and A1501 inoculation on the gene abundance and functional gene transcripts. In addition, we also tested for the effects of interactions between two or more factors (such as soil compartment × water condition × A1501 inoculation).

Diversity index such as observed species, richness (Chao1), Shannon diversity, and rarefaction curve were calculated in R using the diversity, estimate, rarecurve, and specnumber functions. Between-group variation was tested by pairwise analysis of similarity (ANOSIM). For beta-diversity, ordinations were produced using permutated non-metric multidimensional scaling (NMDS). In addition, redundancy analysis (RDA) or canonical correspondence analysis (CCA) was chosen to examine the relationship between the microbial community and environmental variables at OTU level using CANOCO V4.5. To tease apart the relative importance of soil compartment, A1501 inoculation and water condition on microbial community similarity, VPA (variance partitioning canonical correspondence analysis) was performed to attribute the variation observed in the microbial communities to the environmental vari-

Table 2
Evaluation of the nitrogen-fixing capacity of maize in a pot experiment using ^{15}N isotope-dilution method.

		Shoot weight (g)		Root weight (g)		Shoot N content (%)		Shoot ^{15}N abundance (%)		Shoot $\delta^{15}N$	
		WS	WW	WS	WW	WS	WW	WS	WW	WS	WW
Sterilized soil	A1501 inoculation	35.2 b (1.0)	82.7 b (3.9)	8.2 b (0.4)	16.4 b (1.6)	3.97 b (0.13)	3.28 a (0.08)	2.73 a (0.19)	3.72 a (0.05)	6242.9 a (346.6)	9497.9 a (146.3)
	1502 inoculation (*nifH*-mutant)	30.3 a (1.5)	65.8 a (2.7)	7.8 ab (0.6)	14.9 ab (1.3)	3.47 a (0.11)	3.17 a (0.16)	2.89 ab (0.18)	4.64 b (0.10)	7571.3 b (390.8)	13161.1 b (222.8)
	No inoculation	28.9 a (0.5)	62.4 a (3.4)	7.2 a (0.2)	13.1 a (1.1)	3.38 a (0.08)	3.09 a (0.12)	3.14 b (0.15)	5.20 c (0.13)	7812.7 b (429.3)	13910.5 c (391.3)
Nature soil	A1501 inoculation	29.3 b (0.5)	74.2 b (2.2)	8.9 b (0.8)	17.0 b (1.6)	3.45 a (0.10)	3.36 a (0.08)	2.48 a (0.11)	3.65 a (0.10)	5956.1 a (295.2)	7659.5 a (139.1)
	1502 inoculation (*nifH*-mutant)	25.6 a (0.9)	62.8 a (3.0)	7.0 a (0.5)	15.7 ab (2.0)	3.37 a (0.09)	3.21 a (0.16)	2.99 b (0.16)	4.33 b (0.08)	6935.6 b (264.3)	10035.1 b (236.0)
	No inoculation	23.8 a (0.4)	59.0 a (2.5)	6.7 a (0.4)	14.6 a (1.1)	3.28 a (0.13)	3.29 a (0.12)	3.72 c (0.29)	4.92 c (0.18)	7123.7 b (392.0)	11253.1 b (101.2)

WS, water stress; WW, well-watered. Values are means of n = 9 each and standard deviations are given in brackets. Different letters indicate a significant difference ($P < 0.05$) by ANOVA analysis.

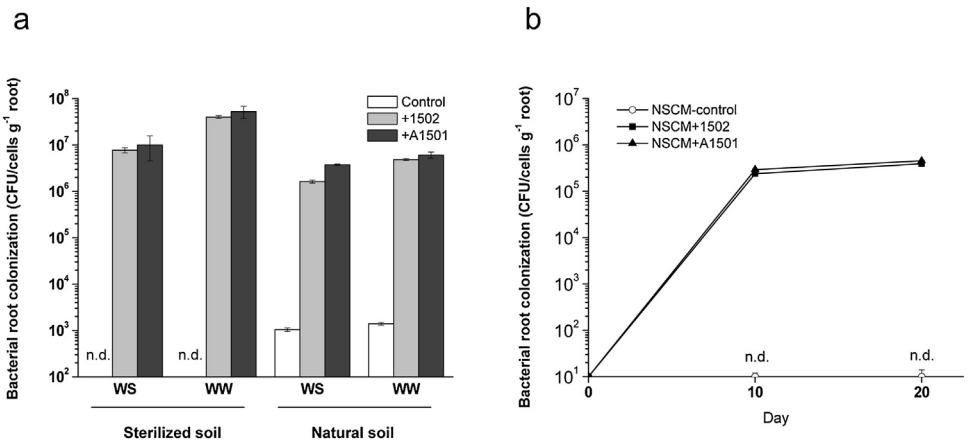

Fig. 1. Colonization of nitrogen-fixing bacterium *P. stutzeri* A1501 and 1502 (*nifH* mutant) strains adhere to maize plant root in soil microcosms at day 20 (a) and sterilized Hoagland's nutrient solution culture medium (b). The number of bacteria in root samples was determined by plate counting method or molecular analysis (qPCR/MiSeq sequencing). All values are mean ± standard deviation (n = 9). Control, sterilized A1501 inoculation; +1502, with *nifH*-mutant strain inoculation; +A1501, with wild-type strain inoculation. NSCM-control, control of no A1501 inoculation in sterile nutrient solution culture medium; NSCM + 1502, *nifH*-mutant strain inoculation in sterile nutrient solution culture medium; NSCM + A1501, with A1501 inoculation in sterile nutrient solution culture medium. n.d., not detected.

ables. All soil characteristics data were $\log_2(x+1)$ transformed for standardization. Data was analyzed using R [33] and by Minitab 16 Statistical Software.

Results

Effect of P. stutzeri *inoculation on shoot and root yields*

The first set of experiments aimed at comparing the effect of inoculation of *P. stutzeri* on maize planted in sterile or natural soil, using water stress or well-watered conditions. In both cases, non-inoculated controls and controls with a *nifH* mutant strain (1502) devoid of nitrogenase activity were included. After 60-days growth in sterile and natural soil (Table 2) significant differences in the plant production were observed between the two water treatments. Greater maize growth was always detected under well-watered condition. When maize was planted in the sterilized soil, inoculation of maize with *P. stutzeri* A1501 caused a significant increase in shoot and root weight (20.2% and 31.2%) compared with the control without A1501 inoculation under water stress and well-watered conditions ($p < 0.05$), respectively. When maize was planted in the natural soil, significant inoculation effect of A1501 on plant growth promotion was also observed, 25.2% and 23.9% promotion were found compared with the non-inoculated control under water stress and well-watered conditions, respectively (Table 2). However, the treatment using 1502 (*nifH* mutant strain) only provided a potential increase (6.2% and 6.7% on average) in plant biomass in sterilized soil and natural soil matrix, respectively (Table 2). The concentration of N in plant shoots all increased with A1501 compared with *nifH*-mutant inoculation and non-inoculation controls.

Contribution of inoculated P. stutzeri *A1501 strains to maize plant N*

The contribution of BNF to maize plant N by inoculated *P. stutzeri* A1501 wild type was evaluated using a *nifH* mutant as a control inoculum in pot experiments with natural and sterilized soil matrix under water stress and well-watered conditions, respectively. Significant differences ($p < 0.05$) were observed in shoot ^{15}N abundance and $\delta^{15}N$ in all treatments (Table 2). For both natural and sterilized soil with inoculum, 60 days after inoculation, the $\delta^{15}N$ value was significantly lower in leaf and stem tissues of A1501-treated maize plants than that of *nifH* mutant treated plants, suggesting that nitrogen fixation was active during the growing period (Table 2). In the sterilized soil, average % Ndfa measured in maize plants was approximately 27.8% under well-watered condition as compared to 17.5% under water stress condition. Correspondingly, the amount of N fixed in maize was 0.30 g/plant when water was deficient and 0.90 g/plant when well-watered. When in the natural soil, the A1501-inoculated plants received 23.6 and 14.1% of their nitrogen from N_2 under well-watered and water stress conditions, respectively. Correspondingly, the amount of N fixed in maize was 0.18 g/plant when water was deficient and 0.72 g/plant when well-watered.

Extent of maize rhizosphere colonization by P. stutzeri

Extent of the colonization of root tissue under different conditions was evaluated by CFUs counting or qPCR/MiSeq quantification. Twenty days after inoculation in sterilized or natural soil conditions, it was observed that the colonization rate of the *nifH* mutant was comparable to that of the wild type (Fig. 1a). For hydroponics, the population size of A1501 on maize root surfaces reached an average of 2.9×10^5 CFUs g^{-1} and 4.5×10^5 CFUs g^{-1} from roots at days 10 and 20, respectively; the population size of 1502 on maize root surfaces was similar with that of A1501; as expected, CFU count from the control treatment was negligible (Fig. 1b). Localization of the bacteria on the root surface was examined by SEM 20 days after inoculation. Abundant numbers of bacilliform A1501-like cells were visualized on the root surface and root hair compared with the control (data not shown).

Abundance of nitrogen fixers and ammonia oxidizers

For the natural soil microcosms planted with maize, the abundance of nitrogen-fixing and ammonia-oxidizing microorganisms were determined by quantifying the copy numbers of *nifH* and *amoA* genes, respectively (Fig. 2). In addition, the abundance of bacteria and archaea were determined by quantifying the copy numbers of 16S rRNA genes, respectively (Fig. S1). Statistical analyses (ANOVA) were shown in Tables S2–S4. Overall, 16S rRNA genes of bacteria and archaea, *amoA* genes of AOB and AOA, and *nifH* gene

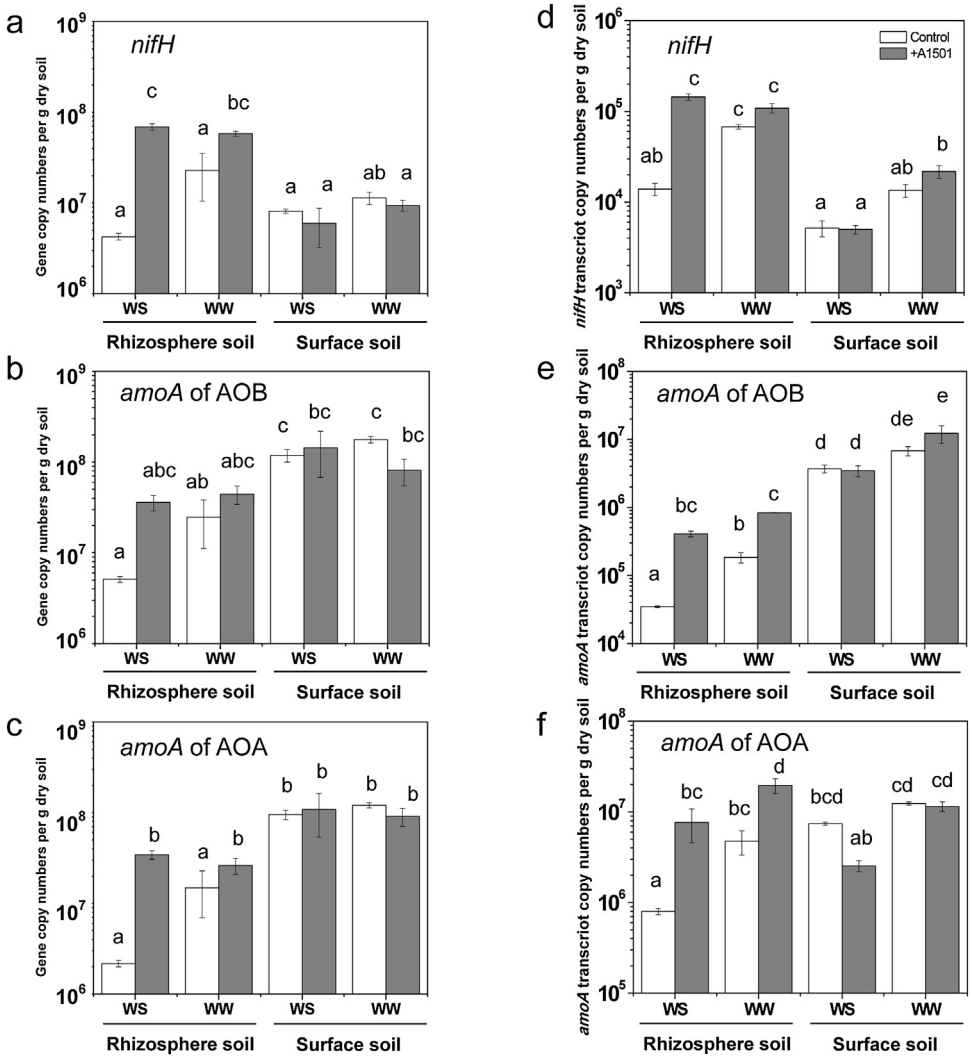

Fig. 2. Copy numbers of genes and transcripts of *nifH* of diazotrophs, *amoA* of AOB, and *amoA* of AOA in two compartments of soil (rhizospheric soil and surface soil) in soil microcosm planted with maize at day 60 determined by qPCR. WS, water stress; WW, well-watered. Control, sterilized A1501 suspension sample; +A1501, with A1501 inoculation. The values are mean ± standard deviation (n = 3).
The statistical analysis was carried out with ANOVA followed by Duncan test. Different letters indicate a significant difference ($P < 0.05$).

copy numbers varied during the 60 day maize planting period (Fig. 2 and Fig. S1).

ANOVA analysis showed significant differences between *nifH* gene copy numbers depending on soil compartments (surface soil and rhizospheric soil) (Fig. 2a, Table S2). This was also noted for *amoA* gene copy numbers of AOB and AOA (Fig. 2b and c, Table S3) and accounted for most of the variance in the data. The *nifH* gene abundance was significantly higher in rhizospheric soil than in surface soil ($p < 0.05$). In contrast, the abundance of *amoA* genes of AOB and AOA was significantly higher in surface soil than in rhizospheric soil ($p < 0.01$). For the rhizospheric soil, significantly higher *nifH* gene copy numbers were found in the inoculated plant under water stress and well-watered conditions ($p < 0.01$; Fig. 2a). *amoA* genes of AOB and AOA followed similar patterns as *nifH* abundance (Fig. 2b and c).

Transcriptional activity of nitrogen-fixing organisms, ammonia-oxidizing bacteria, and archaea

Quantification of the copy numbers of *nifH* mRNA of diazotrophs and *amoA* mRNA of ammonia oxidizers was determined (Fig. 2d, e, and f). ANOVA analysis showed significant differences between *nifH* transcript copy numbers depending on soil compartments ($p < 0.01$; Fig. 2d, Table S2). Comparing the different soil compartments, higher *nifH* transcript numbers were found in rhizospheric soil, lower in surface soil. Water treatment was also found to significantly affect *nifH* transcript numbers, especially in the surface soil, and was in most cases the second important factor explaining the variance ($p < 0.01$). Moreover, a significant increase of *nifH* transcripts was noted in the rhizospheric soil ($p = 0.019$, Fig. 2d) after inoculation of A1501.

Significant difference in AOB *amoA* transcript copy numbers was observed in different soil compartments ($p < 0.01$; Table S3). Higher *amoA* transcript numbers were observed in the surface soil. Water treatment also significantly influenced AOB and AOA *amoA* transcript numbers ($p < 0.01$; Fig. 2e). Both bacterial and archaeal *amoA* transcript copy numbers were significantly higher under well-watered condition (Fig. 2f, Table S3). Interestingly, significant increase of bacterial and archaeal *amoA* transcripts were observed in the rhizospheric soil ($p < 0.01$) after inoculating with A1501 (Fig. 2e and f).

The ratios of diazotroph *nifH* transcript to gene abundance were significantly higher in rhizospheric soil than in surface soil ($p = 0.031$, Fig. S2a). The ratios of *amoA* transcript to gene abundance of AOB were significantly higher in surface soil than in rhizospheric soil ($p = 0.025$). The ratios of *amoA* transcript to gene abundance of AOA were significantly higher in rhizospheric soil than in surface soil ($p < 0.01$) and were greater than those of AOB and diazotrophs (Fig. S2a). On the other hand, changes in the ratios of *nifH* transcript to gene abundance of diazotrophs and those of AOB and AOA were not sensitive to the water conditions or A1501 inoculation treatments (Fig. S2b and S2c).

Illumina MiSeq sequencing-based assessment of 16S rRNA gene and nifH *gene diversity*

The influences of diazotroph *P. stutzeri* A1501 inoculation and water treatment on the composition of the microbial community in rhizospheric and surface soil were analyzed by using Illumina MiSeq in the natural soil microcosm. A total of 482,539 and 264,385 high-quality bacterial and archaeal 16S rRNA sequence reads were obtained, respectively (Table 3). The reads clustered into a sum of 57,516 and 2861 OTUs at 3% distance sequence dissimilarity within the bacteria and archaea. In total, 19 (of a total of 82) major bacterial groups at the class level were identified from all of the samples (Fig. 3a; Table S5).

For *nifH*, a total of 553,816 *nifH* sequence reads comprising 1198 unique sequences were obtained from soil samples that had been subject to different treatments (Table 3). The valid sequences distributed into five phyla, 10 classes, 40 families, and 67 genera (Table S5). Considering that *nifH* genes are often more conserved than 16S rRNA genes, thus a cut-off value of 97% may represent strain level differences. OTUs 1–3 accounted for 10.3%, 7.9%, and 7.3%, respectively of the total OTUs and showed high similarity with the *Nostocaceae* family, *P. stutzeri*, and *Anabaena* sp. L-31, respectively (Table S6). When referring to the *nifH* gene-containing community structure, 11 (of a total of 28) major phylotypes at the order level were identified (Fig. 3b; Table S5). *Nostocales*, *Rhizobiales*, and *Rhodospirillales* orders were predominant in all soil samples. Within the α-*Proteobacteria* diazotrophs, higher relative abundance of *Rhizobiales* was found in rhizosphere soil, whereas higher relative abundance of *Rhodospirillales* was found in surface soil. Moreover, relative abundance of *Pseudomonadales* was greater after A1501 inoculation than the control in all soil samples, especially in rhizosphere soil (Fig. 3b).

Bacterial, archaeal, and diazotrophic diversity measures based on OTUs, observed species, Chao1 richness, and Shannon's diversity index revealed no significant enrichment as affected by water conditions, A1501 inoculation, and soil compartments (Table 3). The rarefaction curve showed no significant diazotrophic richness among all treatments (Fig. S4). Meanwhile, we visualized the Bray–Curtis distances between samples using NMDS to determine how dissimilar the soil microbial communities were at day 60 of maize growth. The different soil compartments showed the largest differences in both overall bacterial and diazotrophic communities. Rhizospheric soil samples were tightly grouped with a contrasting, large dispersion of surface soil samples (Fig. 4a and b). In addition, obvious separation between A1501-inoculated and non-inoculated samples were observed in rhizospheric soil for diazotrophic community (Fig. 4b). However, no significant influence on total archaeal community structure was identified without regard to treatment (Fig. S3).

Both numbers of OTUs and relative abundance of sequences assigned to *P. stutzeri* species were higher in samples from A1501 inoculated soil (especially in rhizospheric soil) than those of non-inoculated soil (Table 4). The copy numbers of *P. stutzeri* strains were calculated by multiplying the copy numbers of *nifH* genes

Table 3
Sample summary of operational taxonomic unit (OTU, at 97% sequence similarity) and alpha diversity indices: Observed species, Shannon and Chao1 index of *nifH* gene, bacterial- and archaeal- 16S rRNA gene sequences. Coverage of all samples ranged from 95–99%. Values represent average ± standard deviation of three samples per treatment.

Target gene	Water treatment	A1501 inoculation	Rhizospheric soil					Surface soil				
			High quality reads (10^4)	Number of OTUs	Observed species	Chao1	Shannon	High quality reads (10^4)	Number of OTUs	Observed species	Chao1	Shannon
16S rRNA gene of Bacteria	WS	−	1.98 ± 0.16	5413 ± 59	4228 ± 21	19030 ± 172	10.8 ± 0.03	2.03 ± 0.02	5228 ± 179	4252 ± 72	20438 ± 546	10.7 ± 0.06
	WS	+	2.08 ± 0.14	4901 ± 39	4512 ± 46	20642 ± 254	10.9 ± 0.05	1.84 ± 0.01	5381 ± 59	4066 ± 87	18450 ± 756	10.5 ± 0.05
	WW	−	2.23 ± 0.12	5149 ± 128	4448 ± 5	20662 ± 97	10.9 ± 0.02	1.91 ± 0.03	5447 ± 115	3683 ± 111	16311 ± 815	10.1 ± 0.14
	WW	+	2.10 ± 0.13	5237 ± 62	4522 ± 4	20954 ± 687	10.9 ± 0.01	1.90 ± 0.01	5285 ± 163	3903 ± 47	18212 ± 553	10.1 ± 0.16
16S rRNA gene of Archaea	WS	−	1.12 ± 0.01	444 ± 11	59 ± 3	229 ± 33	4.3 ± 0.10	1.23 ± 0.02	345 ± 12	71 ± 1	300 ± 29	4.8 ± 0.03
	WS	+	1.12 ± 0.01	563 ± 9	62 ± 1	163 ± 3	4.5 ± 0.03	1.30 ± 0.12	306 ± 8	65 ± 3	253 ± 19	4.7 ± 0.11
	WW	−	1.17 ± 0.01	369 ± 27	67 ± 1	272 ± 26	4.5 ± 0.05	1.16 ± 0.21	345 ± 22	61 ± 1	186 ± 16	4.5 ± 0.05
	WW	+	1.21 ± 0.01	467 ± 14	71 ± 3	270 ± 42	4.9 ± 0.07	1.18 ± 0.12	405 ± 8	59 ± 1	333 ± 31	4.2 ± 0.01
nifH gene	WS	−	1.96 ± 0.26	447 ± 19	355 ± 7	560 ± 13	5.47 ± 0.06	2.52 ± 0.22	521 ± 19	376 ± 5	584 ± 27	5.77 ± 0.02
	WS	+	2.83 ± 0.28	511 ± 19	346 ± 4	555 ± 18	5.26 ± 0.03	1.82 ± 0.27	426 ± 23	347 ± 1	529 ± 4	5.48 ± 0.06
	WW	−	1.89 ± 0.28	447 ± 19	372 ± 12	517 ± 11	5.53 ± 0.05	2.37 ± 0.33	465 ± 27	346 ± 6	531 ± 13	5.60 ± 0.02
	WW	+	2.52 ± 0.22	484 ± 18	342 ± 2	520 ± 15	5.27 ± 0.04	2.55 ± 0.18	501 ± 9	341 ± 3	552 ± 18	5.57 ± 0.01

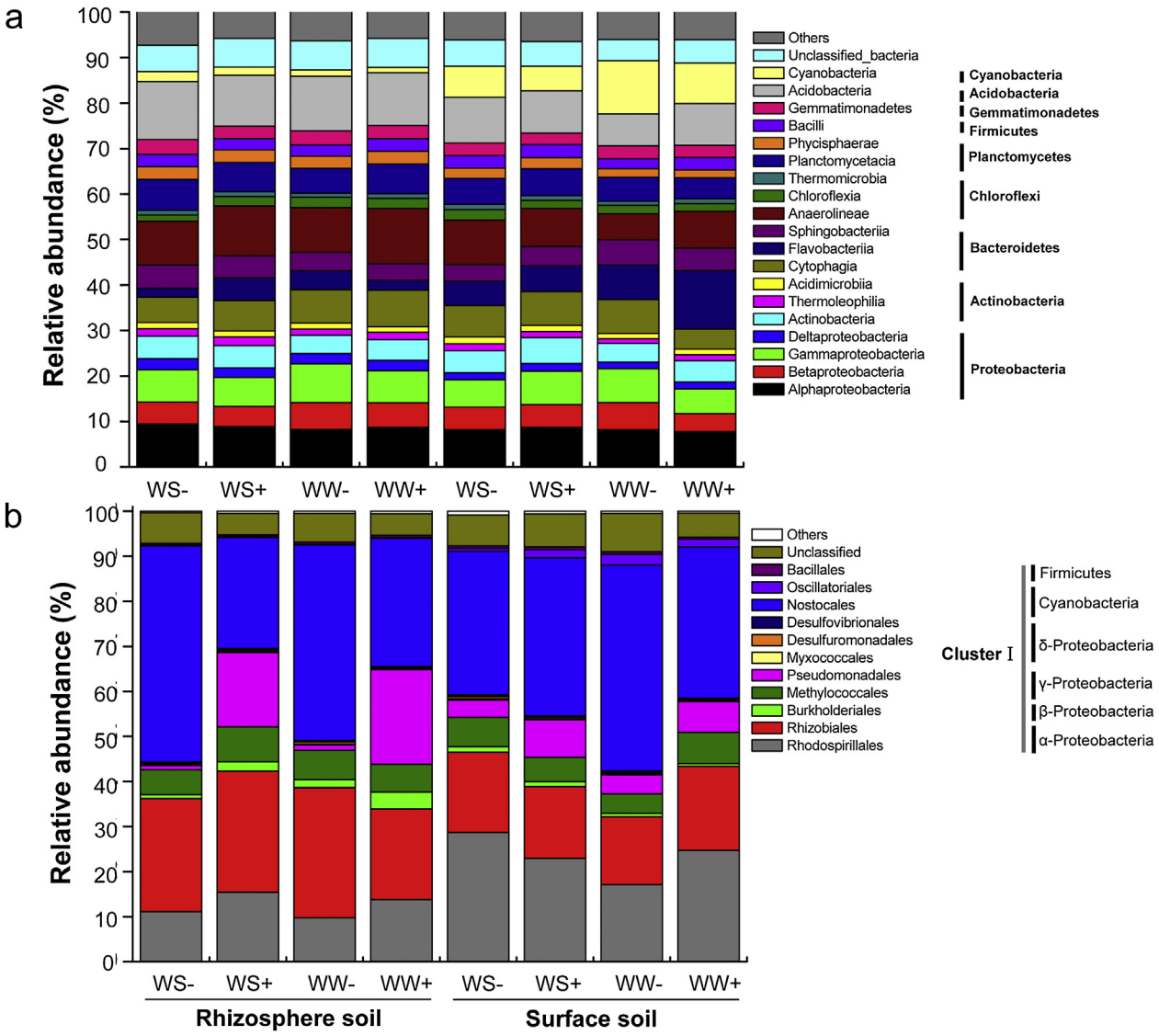

Fig. 3. Community composition of Bacteria targeting 16S rRNA gene at the class level (a), and of diazotrophs targeting *nifH* gene at the order level (b) for samples collected from two compartments of soil (rhizospheric soil and surface soil) in maize planted soil microcosm at day 60 determined by MiSeq sequencing. Phylogenetic groups accounting for <1.5% of the total community were treated together as the other group. WS-, with sterilized A1501 inoculation under water stress condition; WS+, with A1501 inoculation under water stress condition; WW−, with sterilized A1501 inoculation under well-watered condition; WW+, with A1501 inoculation under well-watered condition.

Table 4
Number of reads assigned to *Pseudomonas stutzeri* based on MiSeq community sequencing of *nifH* gene, relative abundance and quantification of *P. stutzeri* in the rhizospheric and surface of soil planted with maize. The values are mean ± standard deviations (n = 3). Different letters indicate a significant difference ($P < 0.05$) by ANOVA analysis. WS, water stress; WW, well-watered.

Soil compartment	Water treatment	A1501 inoculation	Number of reads assigned to *P. stutzeri*	Relative abundance of sequences assigned to *P. stutzeri* (%)	Quantification of *P. stutzeri* (gene copies per gram dry soil)
Rhizospheric soil	WS	–	177 ± 22 a	0.90 ± 0.05 a	$4.05 \times 10^4 \pm 5.06 \times 10^3$ a
	WS	+	4616 ± 157 c	16.31 ± 1.49 c	$1.14 \times 10^7 \pm 8.16 \times 10^4$ d
	WW	–	202 ± 38 a	1.06 ± 0.05 ab	$2.45 \times 10^5 \pm 1.34 \times 10^5$ ab
	WW	+	5258 ± 845 c	20.86 ± 1.48 c	$1.15 \times 10^7 \pm 7.47 \times 10^5$ d
Surface soil	WS	–	938 ± 139 ab	3.72 ± 0.23 a	$3.01 \times 10^5 \pm 3.71 \times 10^4$ abc
	WS	+	1516 ± 297 b	8.32 ± 3.05 b	$1.10 \times 10^6 \pm 6.04 \times 10^5$ abc
	WW	–	980 ± 157 ab	4.14 ± 0.31 ab	$4.94 \times 10^5 \pm 9.14 \times 10^4$ bc
	WW	+	1710 ± 259 b	6.71 ± 0.71 ab	$6.39 \times 10^5 \pm 1.46 \times 10^5$ c

with the relative abundance of reads of *nifH* genes representing these groups. The results (Table 4) showed that the *P. stutzeri* population was more abundant in the inoculated treatment than the control, especially in the rhizospheric soil.

Environmental influences the composition of diazotrophic and bacterial community

Redundancy analysis (RDA) was used to reveal what environmental factors shifted the composition of diazotrophic and

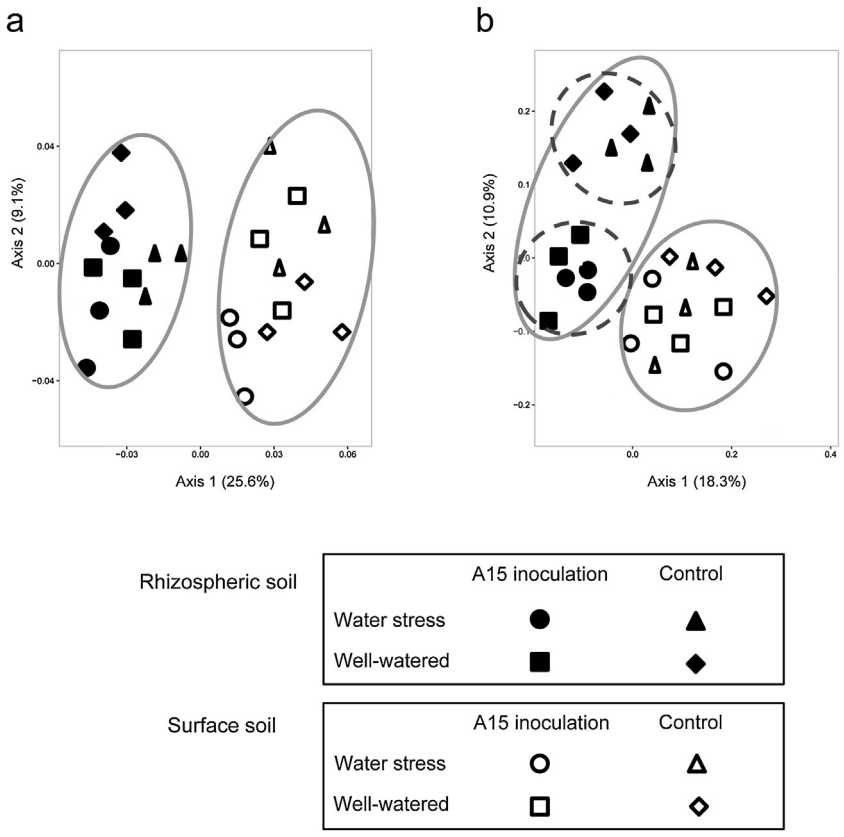

Fig. 4. Non-metric multidimensional scaling representation of the 16S rRNA gene of Bacteria (a) and *nifH* gene (b) sequence composition based on 97% DNA identity for two different soil compartments and water treatments in maize planted soil microcosm at day 60. For bacterial 16S rRNA gene, principal components 1 and 2 explained 25.6% and 9.1% of the variance, respectively. For *nifH* gene, principal components 1 and 2 explained 18.3% and 10.9% of the variance, respectively.

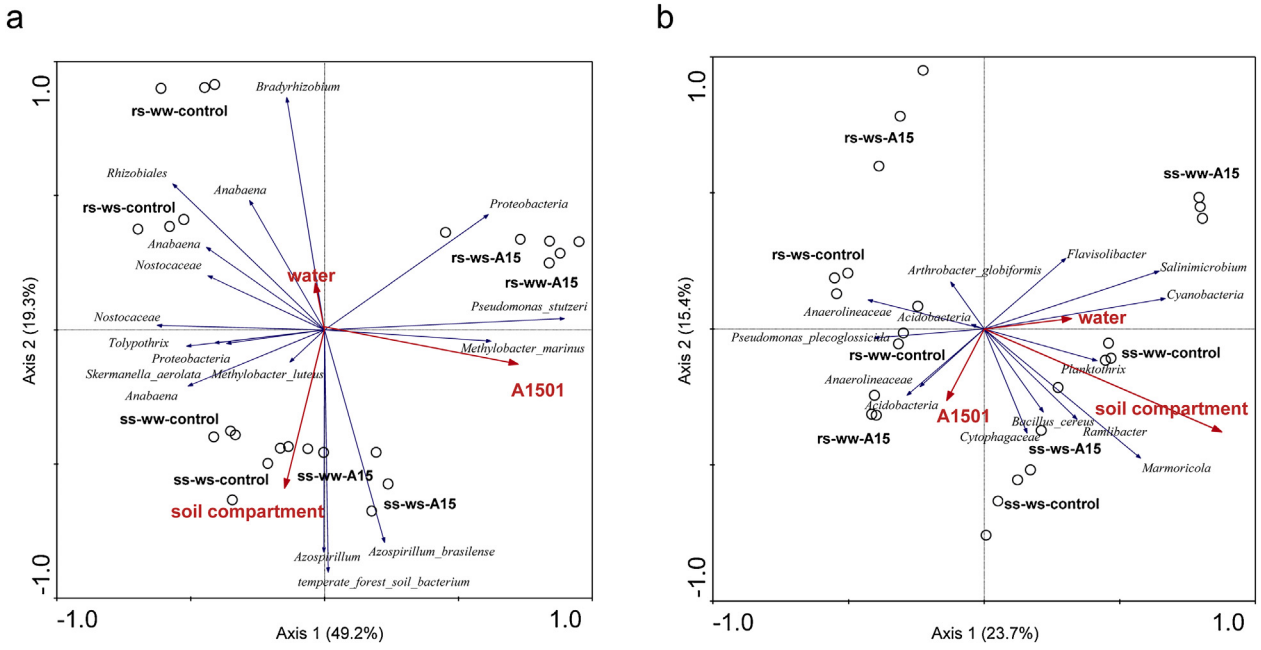

Fig. 5. Proportion of the top 20 OTUs of *nifH* gene (a) and bacterial 16S rRNA gene (b) related to soil compartment, A1501 inoculation and water regime condition. Each sample was labelled by soil compartment (rs, rhizospheric soil; ss, surface soil), water condition (ws, water stress; ww, well-watered), and diazotroph inoculation (control, sterilized A1501 inoculation; +A15, A1501 inoculation).

bacterial community in the natural soil. Environmental variables included soil compartment (rhizospheric and surface soil), A1501 inoculation and water condition (water stress and well-watered condition) (Fig. 5). For *nifH*, axis 1 and 2 of the RDA biplot contribute 49.2% and 19.3% to the overall pattern, respectively (Fig. 5a). For bacterial 16S rRNA, axis 1 and 2 of the RDA biplot contribute 23.7%

and 15.4% to the overall pattern, respectively (Fig. 5b). The RDA showed that A1501 inoculation (P = 0.012) and soil compartment (P = 0.034) were the main determinants of the structure changes in the diazotrophic community. While soil compartment (P = 0.01) was the strongest factors affecting the structure changes in the bacterial community. Meanwhile the relationship between environmental variables and bacterial composition revealed a similar trend as diazotrophic community (Fig. 5b).

Variance partitioning analyses (VPA) was further performed to assess the contributions of soil compartment and A1501 inoculation to the microbial community variance (Fig. 6). This result showed that 34.5% and 23.0% of the variance could be explained by these two components for diazotrophic and bacterial community, respectively. A1501 inoculation and soil compartment could independently explain 25.1% and 9.4% of the variation of diazotrophic communities, respectively (Fig. 6b). Soil compartment and A1501 inoculation could independently explain 20.4% and 2.6% of the variation of bacterial communities, respectively (Fig. 6c). Interactions among the three major components seemed to have less influence than did individual components.

Discussion

Inoculation effects of nitrogen-fixing P. stutzeri *A1501 on maize growth*

The design of our experiment was to test contribution of endophytic *P. stutzeri* A1501 to maize plant growth and plant N via BNF under two water regimes, and how indigenous microbial population and functional groups (N_2 fixers, AOB and AOA) behaved in rhizosphere of maize with respect to "A1501 inoculation" and "water condition". As inoculums, the rhizosphere colonization efficiencies of wild type and mutant derivative were tested in hydroponic, sterilized and nature soil culture independently. In all the conditions assayed, numberous inoculants had been reisolated from the root system compared with the control, combined with the observation that inoculation treatment significantly increased proportion of *P. stutzeri* in rhizosphere as revealed by MiSeq *nifH* community analysis, suggesting that 1501 strains have established a tight relationship with roots and may be favored by plant host. It is not surprising for the capacity of colony of A1501, since the directly evidence the endophytic nature of the colonization process has been shown previously in the case of rice [34]. Our previous work also indicated A1501 could colonize in the inside of maize roots as endophytic. *Pseudomonas* are considered as opportunist and can rapidly adapt to the environment according to variations in the nature and quantity of root exudates [12]. It has been well established that rhizosphere high C/N ratios promote the establishment of nitrogen-fixing bacteria [32]. Previous work also has confirmed that another of the recombinant nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 colonizes the rhizoplane of maize and wheat [16]. Notably, the wild-type A1501 and *nifH*-mutant colonized maize roots equally both in hydroponic and soil culture. Similar population sizes have been reisolated from root tissue suggesting that deficiency of *nifH* gene does not affect the ability of the nitrogen-fixing bacteria to colonize the maize plant. This observation is consistent with those previously reported in wheat and sugarcane systems, which have also shown equal colonization of wild-type and *nif*-mutant strains [16,39]. Hence, results of present study indicate that colonization of the maize root system by *P. stutzeri* A1501 is crucial for plant growth promotion as noted in significant increases in root and shoot dry weight. Indeed, significant increase in plants biomass (an average of 25.4%) and N concentration (an average of 7.8%), and lower $\delta^{15}N$ value in the plant treated with A1501 strain all have been observed. Meanwhile this effect has not been seen in plants inoculated with the *nifH*-mutant strain or non-inoculated plants. These results indicate that transfer of biologically fixed nitrogen is likely the main reason for maize growth promotion. Nevertheless, maize plants inoculated with 1502 *nifH*-mutant strains have a potential increase of 6.5% on average in biomass accumulation, and of 2.7% on average in N content in shoot tissue (Table 2), with respect to uninoculated plants. It is postulated that the minor growth-promotion of plants biomass may be attributed to other bacterial characteristics such as production of phytohormones [41]. This is in contrast to the results of Pham et al. [30] in which BNF by inoculated *P. stutzeri* strain A15 was responsible for a small contribution to rice plant nitrogen. The difference in response between rice and maize may reflects different physiological properties of rice and maize (C3 vs. C4 plants) that may result in changes in the nature of root exudates and rhizosphere soil characteristics (such as redox conditions and oxygen distribution), which could subsequently influence nitrogen fixation. Alternatively, it is known that different soil types (e.g. rice paddy vs. sandy soil) may influence the competence, survival rate and effectiveness of the inoculum [38].

The inoculation of plants with endophytic diazotrophs has the potential to improve productivity of major cereals although the mechanisms related to plant growth promotion are still debated as the explanation for the improved productivity [13]. One on hand, different ^{15}N-based methods (e.g. ^{15}N enrichment, $^{15}N_2$ exposure and ^{15}N natural abundance) have been employed to obtain quantitative estimates of the biologically fixed N. The ^{15}N diluted method has an advantage in glasshouse investigations, and can provide an assessment of endophytic BNF over plant life cycle. However, it should be mentioned that there will be error in the estimation of % Ndfa, because spatial and temporal uniformity of ^{15}N enrichment is not easily adequately achieved [44]. On the other hand, the instability and low efficiencies of crop inoculation in the field in terms of % Ndfa have been often observed because they were always related to various environmental factors [43]. For instance, BNF levels (may shift from 10% to 30% of the total plant N) have been found to vary among the plant cultivars, soil types, or the bacterial inoculants [1,27]. In this study, inoculation under water stress condition led to a decrease in soil moisture was accompanied with an 8–10% decrease of N contribution to maize plant N in two soil microcosms. This might be attributed to water limitation could alter the enzyme activity [40] and root exudation, which would subsequently influence surrounding organisms. In addition, our results also showed slight difference of % Ndfa between sterilized and natural soil microcosm. These results imply that the changes in microbial habitants or soil characteristics may relate to BNF contribution to the plant. Indeed, the effect of inoculation on plant growth has been diminished when transferred to natural soil, which is consistent with the study on rice plants [19]. In this light, *nifH*-mutant derivative as the control, its different response to environment and competitiveness due to genetic modification compared with wild type which may lead to subtle distinctions, should be considered. Furthermore, the mechanisms of distribution of N fixed by diazotrophic inoculants, e.g. when and how the available fixed N is utilized by host plants still need to be further documented.

Effect of diazotroph inoculant on microbial communities and activities in rhizosphere

In this study we hypothesized that the dynamics of functional guilds (N_2 fixers and ammonia oxidizers) in the rhizospheric soil would be positively affected by the addition of inoculants. Here surface soil is considered as a control, instead of bulk soil, which has likely presented a different chemistry and microbial composition than bulk soil (e.g. easier access to oxygen and substrate).

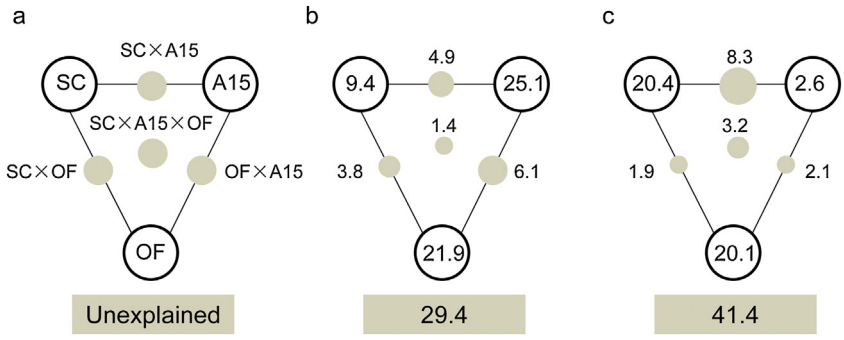

Fig. 6. Variation partitioning analysis of microbial community explained by soil compartment (SC), A1501 inoculation (A15), and other factors (OF) (S). (a) General outline; (b) diazotrophic communities; (c) bacterial communities. Each diagram represents the biological variation partitioned into the relative effects of each factor or a combination of factors, in which geometric areas were proportional to the percentages (%) of explained variation. The edges of the triangle represent the variation explained by each factor alone. The sides of the triangles represent interactions of any two factors, and the center of the triangles represent interactions of all factors.

Using the high-resolution sequencing combined with qPCR technique, we detected the dominant diazotrophic taxa enriched in the rhizospheric soil which were clearly separated from the surface ones. Most obviously, MiSeq *nifH* sequencing results indicated that inoculated with *P. stutzeri* A1501 had increase of 26 times-fold in the numbers of reads of *P. stutzeri* strains in the rhizospheric soil, and of 1.7 times-fold in the surface soil. The relative abundance of *P. stutzeri* ranged from 1.09% to 20.87% when treated with the inoculant. Correspondingly, the population of *P. stutzeri* increased 1–2.5 orders of magnitude compared with the non-inoculated control. Based on the regression analysis, the different compositions of diazotrophic community appear to be associated with A1501 inoculation. In most cases, treatment with the inoculant increases the proportion and population of the studied microorganisms suggests that *P. stutzeri* A1501 strains have become dominant in the rhizosphere. Additionally, *nifH* gene and gene transcript copy numbers were also rather high in the rhizospheric soil, especially after A1501 inoculation. This result matches well with previous research that inoculation with diazotrophic strain was found to be associated with the abundance of *nifH* genes in alfalfa rhizosphere [3]. Furthermore, the inoculation effect of A1501 on the *amoA* genes of AOB and AOA abundance and transcripts showed a tendency toward the *nifH* gene. The results presented here are in agreement with previous researches [3,18], in which higher numbers of *amoA* copies of AOB and AOA were observed when treated with diazotrophic inoculants, and the AOB and AOA *amoA* genes also followed a similar pattern as seen with *nifH* gene abundance. Interestingly, however, the *nifH* gene transcripts per gene copy numbers were very low (about 1/1000) at day 60 of plant growth and these ratios did not increase significantly after inoculation, although *nifH* genes and transcripts abundances had increased after inoculation. This suggests that presence of nitrogenase genes alone or even of their transcripts often does not reflect an accurate representation of the active diazotroph community and of their N_2 fixation rates [14]. It is tightly regulated on multiple levels in the cell, such as post-translational regulation by noncoding RNA [52]. Moreover, other possible interpretations should be considered as well, such as: (i) mRNA of *nifH* was more unstable than that of *amoA,* and (ii) transcriptional activity of *nifH* was depressed at the later growing stage of maize (day 60). Thus additional more time points of sampling or $^{15}N_2$ gas feeding coupled with stable isotope probing (SIP) and high-resolution imaging secondary ion mass spectrometry technique (NanoSIMS) are needed to get a whole picture of relationship between nitrogen fixation rate and active diazotrophic community.

To the best of our knowledge, it is the first study on application of high-resolution sequencing to the assessment of the effect of inoculation with nitrogen-fixing bacteria on microbial community in maize soil. The advantages of deep sequencing have been stated in other studies applied to trace biocontrol inoculants and microbial community [37,42]. In this case population dynamics of *P. stutzeri* A1501 strains have been effectively traced by deep-sequencing combined with quantitative PCR targeting *nifH* gene. However, direct means of measuring the target organisms (like using specific primers) is necessary and at least a fairly accurate method. Indeed, quantification of A1501 cells based on the designed specific-primers matched well with the present results (data not shown). Also note that the deep-sequencing results based on 16S rRNA gene have shown a relative lower-resolution on tracing target organisms and may underestimate the inoculation effect. Therefore, directly tracking functional genes can represent strain level differences and give a more accurate identification, and offer better insight into nitrogen fixation capacity in an environment than would be obtained by extrapolating from 16S rRNA data [47].

Previous studies based on deep-sequencing have suggested that microbial community composition is cooperatively affected by maize plant [29], as well as by the indigenous soil [26]. It is possible that effects of exogenous population, i.e. introduced by inoculation, on rhizospheric microbiota would be weakened in different planting conditions or soil types. To address this possibility, the inoculation effect on microbial community and activity should be explored across many more factors such as plant growing stages, plant cultivars and soil types in the future.

Conclusion

Our results demonstrated that inoculation with nitrogen-fixing bacterium *P. stutzeri* A1501 substantially improved the growth of maize under experimental greenhouse condition. This outcome could be attributed to BNF in which the maize plant obtains significant fixed N that varied with different water regimes. Therefore, inoculation of maize with *P. stutzeri* A1501 strains could potentially reduce the dosage of synthetic fertilizers in practice. Concurrently, inoculation with *P. stutzeri* A1501 had a positive effect on the population of the N_2-fixing and ammonia-oxidizing communities and functional genes transcripts in the rhizosphere. Moreover, community composition of diazotrophs showed obvious differentiation between rhizospheric and surface soil samples, which may have been significantly related to A1501 inoculation. Thus, our study emphasized the promotion of plant growth and clear disturbances on the rhizospheric microbiota due to inoculation with N_2-fixing bacterium *P. stutzeri* A1501. However, a better understanding of plant/rhizobacteria ecology and the relationship among rhizosphere microbial community, nitrogen-fixing rate and plant growth is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.10.010.

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