Temporal dynamics and variation in the alfalfa root nodule and rhizosphere microbial communities of coastal sand and lawn soil

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ABSTRACT
Medicago sativa L. (alfalfa), a leguminous crop, is vital globally for forage, crop rotation, and soil fertility. Though, the microbiome of alfalfa grown in uncolonized land has not been studied yet. Here, we employed the high-throughput 16S rRNA and nifH gene amplicon sequencing to unravel the rhizosphere microbiome and nodules endosymbionts of alfalfa grown in poor lawn and coastal sandy soils. The root nodule of alfalfa of coastal sands and poor lawn soil, Sinorhizobia/Ensifer accounted for 80.599% and 67.727% of the total microbiome, respectively. The class Actinobacteria (except Frankiales) and order Rhizobiales, Burkholderiales, Pseudomonas, and Cytophaga were found significantly enriched. Culturable studies displayed the dominance of Bacillus followed by Arthrobacter, Microbacterium, and Streptomyces. Hydroponics studies revealed the efficiency of cultured isolates for nodulation and plant growth attributes. Overall studies decipher the soil indigenous microbiome associated with root nodule and rhizosphere of Alfalfa grown in uncolonized coastal and sandy land.

Introduction
The excessive use of chemical fertilizers causes soil degradation and salinization and negatively affects soil microbial ecology (Owamah et al. 2014; Sall et al. 2015; Singh et al. 2020b). Over-reliance on fertilizers also leads to nutrient exploitation and nutrient deficiency, thus serving as a limiting factor for improving food productivity, quality, and sustainability (Li et al. 2013). Soil microorganisms act as a biofertilizer, being a vital part of the soil ecosystem and acting as a soil conditioner by decomposing organic matter and cycling nutrient elements (Singh et al. 2016; Fierer 2017). In addition, they play a critical role in maintaining the productivity, function, and stability of ecosystems and are important indicators of soil quality and productivity (Gouda et al. 2018; De Andrade Barbosa et al. 2019).

Plant roots are naturally colonized by bacteria, which are beneficial to plant growth and tolerance to biotic and abiotic stresses (Kumar and Verma 2018; Kehr et al. 2019; Hansen et al. 2020; Kumar and Dubey 2020). These bacteria can be used as inoculants to improve crop productivity and plant resilience against abiotic stresses, such as soil salinization–alkalization (Kusumastuti et al. 2017; Kehr et al. 2019; Gupta et al. 2020). These microbial communities in the root zone can affect host and stem phenotypes in various ways such as growth promotion, drought tolerance, disease resistance etc. (Finkel et al. 2020). Moreover, the plant nutrition is also based on the microorganisms that successfully colonized the roots and helps in the soil nutrients such as nitrogen fixation, siderophore production, mineral solubilization, and secretion of phytohormones to promote growth have been in focus in plant nutrition (Olanrewaju et al. 2017; Asiloglu et al. 2020; Bononi et al. 2020). Furthermore, rhizosphere microorganisms provide a variety of trace elements essential for plant growth by degrading macromolecular substances and transforming inorganic substances (Horstmann et al. 2020).

Medicago sativa L. (alfalfa) is an important legume crop that is cultivated worldwide in temperate areas as a forage source. It is also used in crop rotation to supply the soil with nitrogen via its associated symbiotic nitrogen (N)-fixing bacteria (Andrews and Andrews 2017). In addition, alfalfa is a perennial herb with a deep tap root system and has high-resistance to drought and salt (Kaminsky et al. 2018), owing to the acidic substance secreted around the root system that regulates soil alkalinity to be close to the neutral pH 7.0 (Kumar and Dubey 2020). Therefore, planting alfalfa can improve soil pH balance; hence, it is one of the most appropriate plant species for land restoration. The alphaproteobacterium Sinorhizobium sp. meliloti (S. meliloti) is abundant in temperate soils and forms specialized structures in the roots of alfalfa plants called ‘nodules’ (Andrews and Andrews 2017).
Biological N-fixation by legume crops plays a substantial role in the global N cycle. It is roughly estimated that 200 Million tonne of N is fixed by legumes per year globally (Mahmud et al. 2020). In addition, non-symbiotic root zone microbes often play an important role in nitrogen fixation. Some non-symbiotic diazotrophs such as Azospirillum, Azotobacter, Beijerinckia, Bacillus, Enterobacter, Herbaspirillum, Klebsiella, and Pseudomonas have already been reported (Dos Santos et al. 2012). In bacteria, nitrogen fixation is regulated by nitrogenase, which provides positive advantages in competitive environments and in environments with depletion of bio-available nitrogen that directly affects plant growth promotion activity (Dos Santos et al. 2012; Korir et al. 2017; Singh et al. 2019). The nifH gene is used as a marker for N-fixing microbial identification and mapping (Fan et al. 2019; Zhang et al. 2020) and the identification and categorization of N-fixing Rhizobiales in the root nodules of legume crops (Menon et al. 2019; Singh et al. 2020a).

Here, we aimed to identify the bacterial community composition in the alfalfa rhizosphere and nodule and their functional attributes. To this end, we applied the next-generation Illumina sequencing of the 16S rRNA and nifH amplicons from metagenomic DNA (mtDNA) extracted from root nodules and rhizosphere soils of alfalfa grown in coastal sand and poor lawn soil. Overall, our findings provide theoretical guidance for future systematic and functional studies of the plant root microbiome as a novel biofertilizer.

**Materials and methods**

**Sample collection and characteristics**

Samples were obtained from coastal sand in Qingdao (36.083426N; 120.453969E Shilaoren Beach towards Huanghai sea coastline) and roadside lawn from uncultivated sand (36.138304N; 120.443623E Shangdong Peninsula South, China) (Figure 1). The distance between the two sites was no more than 10 km, but the soil texture was different. Three plants in each site were uprooted gently to a 20 cm-depth by shovel and transported to the laboratory, where roots were washed carefully under running tap water and sterilized distilled water. Fifty nodules (about 200 mg) from each plant were picked and washed with 75% ethanol in a clean bend; then, total mtDNA was extracted in triplicate. Salinity, pH, and electrical conductivity (EC) of the soil solution (1:2.5 soil: water (w/w)) were measured. Physico-chemical characterization (soil type, pH, salinity, total organic matters, total N, and EC) of the sampled soil was performed according to the Food and Agriculture Organization of the United Nations (FAO) World Reference Base for Soil Resources (Table S1). Because rhizospheric soil could not be readily obtained from the sandy soil samples, we used the soil or sand at the root zone deeper than 1–10 cm where the plants had been uprooted for further experiments, as described previously (Yang et al. 2020).

**Isolation and identification of root-nodule bacteria**

To sample the nodules, plants were uprooted gently and transported to the laboratory, where roots were washed carefully under running tap water. Root surfaces were sterilized in 3% sodium hypochlorite solution, followed by 0.02% Tween 20 for 3 min. They were rinsed thrice in sterilized distilled water, blotted onto sterilized filter paper to dry, and ground using a sterilized mortar. Ground nodules were streaked onto plates separately containing Luria–Bertani (LB) agar, yeast-extract malt glucose agar (ISP2), and yeast-extract mannitol agar (YMA) and incubated at 28°C for 4 days.

Isolates were identified as described previously (Yang et al. 2020; Zheng et al. 2020). Briefly, genomic DNA was isolated and the 16S rRNA gene was amplified using PCR with the universal primers 27f (5′-AGAGTTTGATCCTTGTTACGACTT-3′) and 1492r (5′-GGTTACCTTGTAGACCT-3′). Amplified PCR products were subjected to 1.2% agarose gel electrophoresis for quality control and purification. Further, the purified PCR products were sequenced (Qingke Biotech Qingdao, China). The obtained sequences were curated and compared with the EzBioCloud database (Yoon et al. 2017) for gene annotation and similarity check (Yoon et al. 2017).

**Metagenomic sequencing and bioinformatic analysis**

Total mtDNA was isolated from root–zone soil and alfalfa nodule samples using a soil FastDNA spin kit (MP Biomedicals, LLC, Santa Ana, CA, USA) (Yang et al. 2020). Quality check was performed using 0.7% agarose gel electrophoresis. Then, mtDNA was amplified using the universal primer set 799F (5′-AACCAGTTAGTATACCCKG-3′) and 1193R (5′-ACGTCATCCACGGTAC-3′) targeting the V5–V7 regions of the bacterial 16S rRNA genes. nifH1 460–476 (5′-ADNGGCATCATYTCC-3′) and nifH2 115–131 (5′-TGYGAYCCNARGNGA-3′) primers were used for nifH genes (Zehr and Mcreynolds 1989). The PCR products were sequenced on an Illumina (San Diego, CA, USA) MiSeq PE300 platform by Allwegene Genomics (Beijing, China). The raw data are deposited in NCBI Bio Project under the accession number PRJNA666530.

Bioinformatic analysis was performed as previously published (Yang et al. 2020). Briefly, raw data were analyzed using UCHIME (http://drive5.com/uchime) (Edgar et al. 2011) and Ribosomal Database Project classifier (RDP) (Cole et al. 2014). Next, α-diversity indices (Shannon and Simpson diversity indices and Chao1 richness estimator) were calculated based on OTUs (Operational Taxonomic Units) using Mothur (https://www.mothur.org/) (Schloss et al. 2009). Venn diagrams were constructed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html), and community sequencing data were subjected to taxonomic diversity analysis using QIIME (Quantitative Insights Into Microbial Ecology)softare (Caporaso et al. 2010). The major components affecting the differences between samples (β-diversity) were determined using Principal Component Analysis (PCA) based on the OTU, diff-OTU, and Nonmetric multidimensional scaling (NMDS) analysis of the OTU.

**Construction of an artificial culture system for isolate nodulation**

Alfalfa seeds (variety WL323) were surface-sterilized as described above, washed with sterile water, and germinated on 1.0% water–agar plates at 25°C in the dark. Once rooting and germination had occurred, the seedlings were transferred to 500 mL of sterile N-free liquid medium containing...
0.46 g CaSO₄, 0.075 g KCl, 0.06 g MgSO₄·7H₂O, 0.136 g K₂HPO₄·2H₂O, 0.075 g iron citrate, and 1 mL of trace-element solution (2.86 g H₃BO₃, 1.81 g MnSO₄, 0.22 g ZnSO₄, 0.80 g CuSO₄·5H₂O, and 0.02 g H₂MoO₄ in 1000 mL ddH₂O) in 1000 mL of ddH₂O (Chen and Wang 2011) placed in a plastic lunch box (200 × 100 × 50 mm) scaffolded with a 96-well plate whose bottoms were cut to allow root expansion. After 2 days, the seedlings were single inoculated into 1 mL of nitrogen-free liquid medium with logarithmic phase (10⁹ cells mL⁻¹) Sinorhizobium sp., grown on YEM (Yeast extract mannitol broth) liquid medium, inside a controlled chamber with the following conditions: 16/8 h light/dark cycle, 23/18°C day/night temperature, and 55%–65% relative humidity. Plant growth was measured after 4 weeks.

Statistical analysis
Data are presented as the mean ± standard deviation (SD) of three independent experiments. Data were analyzed using one-way analysis of variance (One Way ANOVA), followed by Tukey’s test (SPSS v17.0, IBM, Armonk, NY, USA). P < 0.05 was considered significant (Singh et al. 2016).

Results
Bacterial community composition of the root zone of alfalfa grown in barren soils
There was no significant difference in the number of final tags between the root zones and nodules (Table S2). However, the number of OTUs diverged significantly (P < 0.001), with the nodules having approximately half of the number of OTUs of the root zone. Overall, no significant differences were found within the samples from root zones or nodules.

We found 1119 ± 79 and 1038 ± 56 OTUs from the filtered sequences from the soil of the roadside lawn (SR) and the soil from the Shilaoren coastal region (SS) root zones, respectively (Table S2), whereas only 664 ± 157 and 566 ± 83 OTUs were obtained from alfalfa nodules grown in the roadside lawn and coastal region, respectively. There was no significant difference between the OTU numbers of the two soil types. Furthermore, there were 1159 shared OTUs and 283 and 179 unique OTUs in SR and SS, respectively. The Shannon and Simpson diversity indices and Chao1 richness estimator for the 16S V5–V7 repicon sequencing of these samples are shown in Figure 2 and Table S3, whereas the Venn diagram is shown in Fig. S1.

Overall, in SS and SR, 99.05% ± 0.83% and 98.82% ± 0.98% of the sequences were respectively assigned to a bacterial taxonomy; in NS and NR, 98.09% ± 1.83% and 97.87% ± 1.42%. The nine most abundant phyla (>1% sequences) comprised 95.49% ± 0.35% and 96.84% ± 0.29% of the root zone community in SS and SR, respectively (Table S4), of which 40.65% ± 9.05% and 28.88% ± 9.47% belonged to the phylum Actinobacteria (Table S5, Fig.S2A).

The second most abundant phylum was Proteobacteria (30.56% ± 5.16%, SS; 27.25% ± 1.16%, SR), followed by Bacteroidetes (11.47% ± 6.26%; 9.79% ± 1.24%), Acidobacteria (3.92% ± 0.61%; 14.18% ± 3.02%), Gemmatimonadetes
(2.62% ± 0.91%; 2.93% ± 0.50%), Chloroflexi (2.61% ± 0.61%; 2.93% ± 0.50%), Saccharibacteria (2.49% ± 1.51%; 1.31% ± 0.72%), Planctomycetes (0.96% ± 0.40%; 3.21% ± 0.22%), Firmicutes (0.92% ± 0.53%; 0.61% ± 0.77%), and Verrucomicrobia (0.85% ± 0.21%; 3.30% ± 1.91%).

At the genus level, Sinorhizobium/Ensifer, which was the most dominant genus in the nodules, accounted for only 2.42% and 2.43% of the species in SS and SR, respectively. The majority of the common enriched genera in the two different soil types were different (Table S7).

The 16S rRNA amplicons from the uncultured root nodules were assigned to 10 phyla in nodule samples (NS) and 11 in roadside lawn nodule samples (NR) (>0.1% relative abundance), and an overview of the dominant phyla and genera is provided in Tables S5 and S6 and Figure 3. The most common phyla (>0.1%) in the Shilaoren coastal NS and NR were Proteobacteria (87.496% ± 6.233%, NS; 78.874% ± 7.352%, NR), Actinobacteria (6.846% ± 2.781%, 8.556% ± 3.385%), Bacteroidetes (1.280% ± 0.650%, 1.633% ± 0.594%), Acidobacteria (0.325% ± 0.027%, 1.368% ± 0.783%), Gemmatimonadetes (0.138% ± 0.034%, 0.204% ± 0.143%), Chloroflexi (0.195% ± 0.089%, 0.448 ± 0.265%), Saccharibacteria (0.205% ± 0.121%, 0.482% ± 0.211%) and Planctomycetes (0.105% ± 0.001%, 0.342% ± 0.268%), Firmicutes (0.125% ± 0.054%, 0.155% ± 0.062%), and Verrucomicrobia (0.078% ± 0.001%, 0.324% ± 0.178%). As predicted, the most prominent Proteobacteria genus was Sinorhizobium/Ensifer (80.599%; 67.727%).

The phylum Proteobacteria (Non-Ensifer), which accounted for 6.898% and 11.147% in NS and NR, was the most abundant, followed by Actinobacteria (6.846%; 8.556%). The ratio (NS/SS, NR/SR) of the relative abundance of the phylum Proteobacteria (Non-Ensifer) were 1.080 and 1.236, respectively, suggesting that bacterial species from Proteobacteria, except those from genus Ensifer, play an important role in the rhizosphere. The ratio of phylum Saccharibacteria, which was found to be another phylum enriched in rhizosphere or nodule samples NR/SR, was 1.115. Those of phylum Actinobacteria were 0.806 and 0.895, whereas those of Firmicutes were only 0.647 and 0.769, indicating that they are not enriched. Furthermore, the classes Alphaproteobacteria (excluding Ensifer), Betaproteobacteria, and Gammaproteobacteria were enriched in all four samples, but not Deltaproteobacteria (Table S6).

At the genus level, the top 10 genera in SS were as follows: Nocardioides (4.287%), Sphingomonas (4.185%), Arthrobacter (3.182%), Streptomyces (2.435%), Ensifer (2.416%), Patulibacter (2.150%), Solirubrobacter (1.821%), Microbacterium (1.702%), Flavisolibacter (1.217%), and Rhizobium (1.185%). The top 10 genera in SR were Ensifer (2.426%), Terrimonas (2.417%), Nocardioides (1.940%), Solirubrobacter (1.747%), Arthrobacter (1.647%), Patulibacter (1.615%), Sphingomonas (1.602%), Streptomyces (1.317%), Gaiella (1.311%), and Mycobacterium (0.877%). The relative abundance of the genus Terrimonas was significantly different between the lawn and coastal samples (P < 0.001). Therefore, these findings clearly indicate the root zone microbiomes of the different soil types were different (Table S7). In addition, the following genera accounted for more than 0.3% in NR: Microbacterium (1.040%), Arthrobacter (1.012%), Kineosporia (0.974%), Rhizobium (0.900%), Nocardioides (0.743%), Actinoplanes (0.583%), Streptomyces (0.496%), Sphingomonas (0.442%), Pseudonocardia (0.404%), Massilia (0.403%), and Mycobacterium (0.351%).

In NS, the following genera accounted for more than 0.3%: Arthrobacter (1.314%), Nocardioides (0.831%), Microbacterium (0.776%), Rhizobium (0.691%), Streptomyces (0.581%), Sphingomonas (0.500%), and Massilia (0.365%).

The majority of the common enriched genera in the two samples belong to the phylum Actinobacteria and include Arthrobacter, Nocardioides, Microbacterium, and Streptomyces. In addition, Rhizobium and Sphingomonas belonging to α-proteobacteria and Massilia belonging to β-proteobacteria were enriched in both soil types. Kineospora, Actinoplanes, Pseudonocardia, and Mycobacterium, belonging to the phylum Actinobacteria, together accounted for more than 0.3% in roadside lawn samples. However, these same genera were also among the 50 most abundant in the sand nodule NS samples (0.238%, 0.240%, 0.082%, and 0.097%, respectively). Despite the vast differences in soil type, salinity, and texture, the
enriched genera in the nodule and rhizosphere of alfalfa were similar, indicating that the plant host mainly determined the root microbiome.

After excluding the dominant genus *Ensifer*, which resides in the nodule to fix nitrogen, the proportion of some genera in the samples became relatively higher. By comparing their relative abundances in SS and SR, we identified those that were dominantly enriched in the nodule or rhizosphere using the NS/SS and NR/SR ratios. We found several genera in both soil samples whose relative abundance in the nodule was ≥3 times greater than that in soil ($P < 0.001$), suggesting that they are abundantly residing inside the nodules or on the root surface. These include *Arthrobacter* (NS/SS = 3.53; NR/SR = 4.07), *Microbacterium* (3.90; 8.59), *Rhizobium* (4.98; 22.39), *Novosphingobium* (6.93; 13.36), *Kineosporia* (16.17; 31.77), *Shinella* (10.80; 43.67), *Bosea* (3.10; 4.39), *Ohtaekwangia* (3.43; 3.16), *Pseudomonas* (4.00; 4.71), *Mesorhizobium* (4.13; 6.25), and *Aquabacterium* (3.40; 12.42).

An NR/SR ratio greater than 3 was achieved in roadside lawn samples only ($P < 0.001$), including *Massilia* (4.35), *Actinoplanes* (6.26), *Devisia* (4.81), *Nistella* (3.91), *Bradyrhizobium* (5.77), *Pseudonocardia* (4.91), *Planosporangium* (3.86), *Pseudoxanthomonas* (7.12), *Dyadobacter* (30.38), and *Methyllobacterium* (6.00). In coastal sand samples, the NS/SS ratios of *Cellulomonas* (5.04) and *Agromyces* (4.57) were more than 3 (Table S7).

Among the top 50 most abundant genera, after excluding *Ensifer*, the seven whose proportion in both nodule samples was lower than that in the root zone (NS/SS = 0.38–0.89, NR/SR = 0.48–0.85) include *Patulibacter* (Actinobacteria, 0.64, 0.62), *Terrimonas* (Bacteroidetes, 0.89, 0.71), *Gaiella* (Actinobacteria, 0.74, 0.48), *Blastococcus* (Actinobacteria, 0.70, 0.85), *Iamia* (Actinobacteria, 0.74, 0.58), *Rubellimicrobium* (α-proteobacteria, 0.76, 0.70), and *Flavisolibacter* (Bacteroidetes, 0.38, 0.55), indicating they were not enriched in the alfalfa root (Table S7).

These enriched genera mainly belonged to the class Actinobacteria, and those with a lower proportion in the nodule belonged to the class Thermoleophilia (including *Patulibacter*, *Solirubrobacter*, and *Gaiella*), class Acidimicrobiia and order Acidimicrobiales (including *Iamia*), and class Actinobacteria and order Frankiales (including *Blastococcus*).

The enriched genera in the phylum Actinobacteria mainly belonged to the following orders: Micrococcales (*Arthrobacter*, *Microbacterium*, *Agromyces*, *Knoellia*, and *Cellulomonas*), Propionibacteriales (*Nocardioides*, *Aeromicrobium*), Kineosporiales (*Kineosporia*), Micromonosporales (*Actinoplanes*, *Planosporangium*), Streptomycetales (*Streptomyces*), and Pseudonocardiales (*Pseudonocardia*, *Actinosynnema*, and *Mycobacterium*).

Another common genus in the nodule belonging to the phylum Proteobacteria mainly belonged to the order Rhizobiales (*Rubellimicrobium*). In the class α-proteobacterium, the highly enriched genera (more than 3-fold) included *Novosphingobium* belonging to the order Sphingomonadales, and *Rhizobium*, *Ensifer*, *Shinella*, *Devisia*, *Rhi- doplanes*, *Microvirga*, *Methyllobacterium*, *Bosea*, and *Bradyrhizobium* belonging to Rhizobiales. *Variibacter* and *Mesorhizobium* in Rhizobiales were moderately enriched in both nodule samples. In β-proteobacterium, the highly enriched genera *Massilia* and *Aquabacterium* are under the Burkholderiales order. Meanwhile, in γ-proteobacteria, *Pseudomonas* and *Pseudoxanthomonas* were highly enriched in...
both samples; however, the ratios of Steroidobacter and Acidibacter were not changed.

The ratios of Flavisolibacter and Terrimonas (order Sphingobacterales, phylum Bacteroidetes) were decreased in both nodule samples, whereas those of Niastella (Sphingobacterales), Ohtaekwangia, Dyadobacter (both in Cytophagia), and Flavobacterium (Flavobacteriales) were enriched.

**Beta-diversity analysis of 16S rRNA gene sequences**

The microbiome of the SS and NS was similar to that of the SR and NR; however, some genera had different relative abundances. To clarify the difference between the microbiomes of alfalfa grown in two different soil types, we analyzed the differences between samples (β-diversity) using PCA based on diff-OTU (Figure 3B) and NMDS analysis of the 16S OTU (Figure 3C). A clear separation along axis 1 explaining 77.87% of the overall variation was observed, confirming that root zones and nodules harbor distinct microbiomes. Axis 2 explained 10.34% of the overall variation and mainly separated the root-zone samples, with obvious clustering between alfalfa grown in the two soil types. This suggests that growth conditions affect β-diversity. Results of the NMDS analysis indicated that the two root zones and the two nodules had different microbiomes and formed four distinct circles (Figure 3C). The triplicate samples in the same group were all within a circle, which means that the difference within the group is not obvious, whereas there is no intersection among the groups, indicating distinct differences.

**Composition analysis of nifH genes in root zones and nodules**

A total of 29, 146 ± 2, 131 and 28, 678 ± 5, 287 clean sequences were obtained from NS and NR samples, and 27, 898 ± 7, 488 and 32, 115 ± 645 from the SS and SR (Table S8). Therefore, the number of sequences was found to be similar in the four samples. However, OTU identification results revealed that there were approximately six times more OTUs in the root zones than in the nodules, indicating that root-zone diversity was richer (Table S8, Figure 2B).

The Venn diagram of nifH OTUs identified 37 shared OTUs in the four different alfalfa root-related microbiomes (Fig. S3), whereas the associated α-diversity indices for nifH replicon sequencing are shown in Table S9. In the nodules, 98.331% ± 0.425% and 99.786% ± 0.013% sequences were identified as Proteobacteria (Table S10). In the root zones, the relative abundances (>1%) of the nifH genes at the phylum level in the SS and SR samples were respectively as follows: Proteobacteria, unidentified, Cyanobacteria; Verrucomicrobia, Actinobacteria, Firmicutes (Table S10, Figure 4). Proteobacteria was the most dominant phylum. This is different from the data we had previously obtained, wherein Cyanobacteria was the most abundant in the root zone of wild soybean grown in coastal sand (Yang et al. 2020).

At the genus level, Azohydromonas was the most abundant in the coastal sand SS samples, followed by Bradyrhizobium, Pelomonas, Azotobacter, Nostoc, Ideonella, Pseudacidovorax, Desulfomicrobium, Rhodobacter, Mycobacterium, and Skermanella (Table S10). Meanwhile in the roadside lawn soil, Bradyrhizobium was the most dominant, followed by Ruminiclostridium, Skermanella, Rhodobacter, Azohydromonas, Nostoc, Rhodospirillum, Pseudacidovorax, Ideonella, Pelomonas, and Rhodoplanes (Table S11).

The class β-proteobacteria, order Burkholderiales, genera Azohydromonas, Pelomonas, Ideonella, and Pseudacidovorax in sand samples accounted for more than 26.4%. However, in roadside poor lawn soil samples, they accounted for only 8.0% (P < 0.001). The relative abundance of the nifH gene fragments at the class, order, and family levels are illustrated in detail in Fig. S4.

We found a significant difference in the relative abundance of Firmicutes between SS and SR (1.161% and 11.711%, respectively) (P < 0.001) (Table S10). Conversely, no significant difference was observed in the nodule samples, which may be a result of host selection in different environments. After excluding the dominant nifH-fixing bacteria, the relative abundance of Rhizobium was 90.940% and 33.706% in NS and NR, respectively, and the respective NS/SS and NR/SR ratios reached 1377.9 and 842.6 (Table S11).

After excluding Ensifer, the following genera were enriched in NS (P < 0.001): Rhodoplanes (NS/SS = 85.7), Desmonostoc (17.5), Nitrospirillum (3.9), and Geodermatophilus (2.4) (Table S10); and in NR (P < 0.001), Azohydromonas (NR/SS = 2.5), Xanthobacter (4.6), Azotobacter (4.3), Lyso bacter (30.6), Mesorhizobium (17.1), Azorhizobium (6.9), Leptolyngbya (57.4), Klebsiella (20.7), Burkholderia (5.5), Sphingomonas (4.7), Marinobacterium (29.5), Chroococcidiopsis (4.4), Pseudomonas (9.1), Desulfarculus (14.8), and Mycobacterium (12.7). Thus, these results revealed that nifH was highly enriched in the rhizosphere of alfalfa grown in grassy lawn (Table S11).

**Beta-diversity analysis of nifH sequences**

nifH sequence diversity was significantly different between NS and NR, and SS and SR (Figure 4). Based on the results of NMDS analysis, NS, NR, SS, and SR formed one line (Fig. S5). We found a clear separation between the nodule and root-zone samples along PC1 and confirmed a general pattern in root zones and nodules harboring distinct nifH genes during the PCA analysis (Figure 4B and C). Along PC2, the root zone samples were also separated, but there was no separation between the two nodules.

**Nodule bacteria**

Nodule bacteria were cultured by using the set of growth media for enumeration of all the possible cultivar. Overall, 112 isolates were purified and characterized from NS and NR samples. Similarity analysis of 16S rRNA gene sequence displayed that the YMA agar plates isolates were identified as Ensifer meliloti. In the ISP2 agar plate, which is often used to isolate Actinobacteria, 5 colonies were screened from NS and NR samples and identified as Massilia, Bacillus was the most dominant bacterial genus in the LB agar plate, with 20 NS and 25 NR isolates. Genera Paenibacillus and Lysinibacillus were also isolated in the LB agar plate, as well as Enterobacter (8 isolates) and Pantoea (7 isolates). Furthermore, three Actinobacteria genera (23 isolates) with more than one representative isolate, including Arthrobacter (10 isolates), Streptomyces (8 isolates), and Microbacterium (5 isolates), were identified (Sequence data not shown).
To investigate the effect of the plant–bacteria interactions on alfalfa growth and nodule formation, we developed a hydroponics cultivation method using nitrogen-free medium and evaluated its potential to promote plant growth (data not shown). Due to the higher dominance of *Sinorhizobium*, we have inoculated it to alfalfa seedlings for the nodule formation and was observed it within four weeks of cultivation. The form and size of nodules were different between samples obtained from coastal sand and lawn soil. The nodules collected from the fields were ginger-shaped or Y-shaped, but the shape and size of nodules in the artificial culture was spherical and bigger (Figure 5).

**Discussion**

Analysis of the bacterial rhizosphere community as well as with the nodule can give us a insightful assessment of microbial mediated plant development. Therefore, studying the abundantly enriched bacteria in the nodule samples and those in the root zone of the soil samples is necessary to address this. Although the relative abundance of bacteria was relatively small in both NS and NR nodules, it could correctly reflect the enrichment compared with that in the soil after the dominant bacteria *Ensifer* in the nodules was excluded. Furthermore, bacteria in the root zone can fix nitrogen via non-symbiotic bacteria, thus transforming biologically inactive nitrogen to be readily used by organisms (Singh et al. 2019; Soares et al. 2020). Therefore, we analyzed the root zone microbiome not only based on the 16Sr RNA amplicons but also on the nifH repions.

The composition of the bacterial communities associated with alfalfa root revealed diverse bacterial taxa in the nodules, except for the most represented α-proteobacterium *Ensifer meliloti*. These include members of Actinobacteria, Flavobacteria, γ-proteobacteria, and β-proteobacteria, which might support activities related to plant growth promotion (Pini et al. 2012). This paper is the first to explore the total bacterial community at the class to single species levels associated with *M. sativa* plants grown in mesocosms conditions. Within α-proteobacteria, the families Sphingomonadaceae and Methylobacteriaceae were abundant inside plant tissues, whereas in soil, α-proteobacteria, Hyphomicrobiaceae, Methylocystaceae, Bradyrhizobiaceae, and Caulobacteraceae were dominant (Pini et al. 2012). However, the difference at the family level was difficult to identify. Thus, we analyzed the data at the genera level, as there were different trends of the genera affiliated with one family. We found that *Navosphingobium* belonging to Sphingomonadaceae was higher in both NS and NR root nodules, but *Sphingomonas* belonging to Sphingomonadaceae was not significantly changed. Non-*Ensifer* α-, β-, γ-proteobacteria at the class level were significantly enriched, which was consistent with a previous study (Pini et al. 2012).

Actinobacteria are highly tolerant to various stresses, including salinity, low and high pH, low water availability, extreme temperatures, radiation, and pressure, and thrive in extreme ecosystems (Alsharif et al. 2020). Hence, desert plants harbor diverse bacterial communities mainly dominated by the phylum Actinobacteria, similar to our analysis (Alsharif et al. 2020). Class Actinobacteria was highly enriched, especially at the genus level. Among the top 11 enriched genera, seven belong to Actinobacteria, including *Arthrobacter*, *Nocardiooides*, *Microbacterium*, and *Streptomyces*. Thus,
Actinobacteria may be involved in salinity tolerance in coastal regions. A previous study showed that, during nodule development, the relative abundances of some bacteria may change (Hansen et al. 2020). For instance, *Ensifer* accounted for 94–96% in young and active nodules but decreased to 86% in senescent nodules. The genus *Pseudomonas* had an average relative abundance of 2.45% in young nodules; however, its level decreased to 0.03% and 0.05% in active and senescent nodules, respectively. Here, the relative abundances of *Pseudomonas* were 0.137% and 0.300% in NS and NR, respectively, which was within this range of data (0.03%–2.45%). In contrast, those of *Streptomyces* and *Actinoplanes* from Actinobacteria increased from 0.1% in young nodules to 1.60% in senescent nodules (Hansen et al. 2020). Here, the relative abundances of *Streptomyces* (0.581%, 0.496%) and *Actinoplanes* (0.240%, 0.583%) did not vary during nodule development. The root nodule microbiome during nodule development is dynamic.

In the SS alfalfa root zone, the relative abundances of phyla Actinobacteria (40.65%), Proteobacteria (30.56%), Bacteroidetes (11.47%), and Acidobacteria (3.92%) were partially different from those of Proteobacteria (39.67%), Actinobacteria (26.00%), Bacteroidetes (10.67%), Chloroflexi (6.00%), Acidobacteria (3.67%), and Planctomycetes (3.00%) in the root zone of wild soybean grown in the same coastal sand region (Yang et al. 2020). On the other hand, in the nodules, *Sinorhizobium/Ensifer* accounted for 80.60% in alfalfa NS, whereas it accounted for 96.66% in wild soybean grown in the same coastal region. These differences were attributed to the difference in nodule size between the two legumes (Yang et al. 2020). At the genus level, *Sphingomonas* (0.086%), *Microbacterium* (0.066%), *Arthrobacter* (0.060%), *Nocardoides* (0.059%), *Flavobacterium* (0.042%), *Streptomyces* (0.037%), and *Pseudomonas* (0.032%) were most significantly enriched in wild soybean nodules, particularly *Sphingomonas* (Yang et al. 2020). In alfalfa nodules, *Arthrobacter, Nocardoides, and Microbacterium* belonging to the phylum Actinobacteria were the top enriched genera. This difference in the most abundant genera might be due to the root exudates of the two legumes, although both were grown in the same environment.

In addition to symbiotic nitrogen fixation, autotrophic nitrogen-fixing microorganisms can fix nitrogen independently in soil. For instance, *Azotobacter* possesses a strong nitrogen fixation capacity and can secrete auxin to promote plant growth and fruit development. In the root zone of wild soybean grown in coastal sand, the relative abundances of *nifH* of Cyanobacteria (49.00%), Proteobacteria (32.32%), Delta/epsilon (10.00%), and Firmicutes (0.67%) were significantly different from those of Proteobacteria (68.06%), Cyanobacteria (6.96%), and Verrucomicrobia (2.119%) in the root zone of alfalfa. The relative abundances of *nifH* of *Nos-toc* (30.56%), *Geobacter* (7.55%), *Bradyrhizobium* (7.87%), and *Azohydromonas* (3.34%) in the wild soybean root zone was also different from those of *Sinorhizobium* (15.76%), *Azohydromonas* (11.136%), *Bradyrhizobium* (10.522%), *Pelomonas* (8.947%), and *Rhizobium* (0.066%) in the alfalfa root zone. This indicates that their *nifH* genes are phylogenetically diverse and different.

Legume-rhizobium mutualism has vital protagonism in the history of life, but its compatibility and evolution are poorly described. We have developed a set of media to isolate the maximum number of nodule endophytes of the alfalfa plant from distinct habitats. During isolation, *Ensifer meliloti* was procured only on YMA media, while only 5 colonies of *Massilia* were procured. These isolates were previously reported to play an important role in dissolving phosphorus.
and potassium in soil (Zheng et al. 2017). Interestingly, they were isolated and identified from NS and NR samples which endorsed their plant growth promotion characteristics.

As reported previously, the genus Bacillus was reported as the predominant bacterial species on LB Agar (20 colonies in NS and 25 in NR). Bacilli possess versatile traits that protect plants against abiotic stresses, including heat, cold, and freezing (Tiwari et al. 2017; PrajaktA et al. 2019). The genera Paenibacillus and Lysinibacillus, which provide their host with multiple benefits, including nitrogen fixation, phosphate solubilization, and biocontrol activities (Grady et al. 2016), were also isolated from our samples. Isolates from the genera Bacillus, Paenibacillus, Arthrobacter, and Microbacterium have been reported in the seeds of at least three different varieties of alfalfa (López et al. 2018), indicating that these genera are quite prevalent in alfalfa.

For further investigation of the nodulation ability of cultured strains, enumerated from sampled alfalfa plants, the hydroponics cultivation method was adopted. Nodulation results showed that isolates have the potential to fix N and promote plant growth (Figure 5).

Conclusions

Plants recruit microorganisms through root exudates, and these microorganisms promote the circulation of rhizosphere material through their physiological metabolism. In addition, the metabolites produced by these microorganisms influence plant growth. Here, we focused on alfalfa, an important legume in agriculture and animal husbandry, grown in coastal sands and barren soil without human intervention. The root nodule and rhizosphere microbiota of alfalfa were analyzed using Illumina sequencing to identify the microorganisms that affect plant growth, which had not yet been elucidated and analyzed. We found that the classes Actinobacteria except the orders Frankiales (e.g. Arthrobacter, Microbacterium, and Kineosporia), Rhizobiales in α-proteobacterium (e.g. Rhizobium, Shinella, Bosea, and Mesorhizobium), Sphingomonadales in α-proteobacterium (e.g. Novosphingobium), Burkholderiales in β-proteobacterium (e.g. Aquabacterium), γ-proteobacteria (Pseudomonas and Pseudoxanthomonas), and Cytophaga in the phylum Bacteroidetes (e.g. Ohtaekwangia and Dyadobacter) were significantly enriched in the nodules of alfalfa. In addition, the diversity of the nifH gene, including in symbiotic nitrogen-fixing bacteria in the nodule and autotrophic nitrogen fixation in the root-zone soil, was analyzed. We showed that the nifH gene from Rhizobium was highly enriched in nodules, which enriched the diversity of nitrogen fixation in alfalfa nodules and shows it can be applied as a biofertilizer. Bacterial isolation and assessment of nodulation efficiency should be conducted in future studies to better understand plant–microbe interactions and their applications for agriculture and soil nourishment.

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Disclosure statement

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References

Alsharif W, Saad MM, Hirt H. 2020. Desert microbes for boosting sustainable agriculture in extreme environments. Front Microbiol. 11:1666.

Andrews M, Andrews ME. 2017. Specificity in legume-Rhizobia symbioses. Int J Molec Sci. 18:705.

Asiloglu R, Shiroishi K, Suzuki K, Turgay OC, Murase J, Harada N. 2020. Protist-enhanced survival of a plant growth promoting rhizobacteria, Azospirillum sp. B510, and the growth of rice (Oryza sativa L.) plants. Appl Soil Ecol. 154:103599.

Bononi I, Chiaramonte JB, Pansa CC, Moitinho MA, Melo IS. 2020. Phosphorus-solubilizing Trichoderma spp. from Amazon soils improve soybean plant growth. Sci. Report. 10.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Method. 7:335–336.

Chen WX, Wang ET. 2011. Root nodule bacteria in China. Beijing, China: China Science Press.

Cole JR, Wang Q, Fish JA, Chai B, Mccarrell DM, Sun Y, et al. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633–D642.

De Andrade Barbosa M, De Sousa Ferraz RL, Coutinho ELM, Coutinho Neto AM, Da Silva MS, Fernandes C, et al. 2019. Multivariate analysis and modeling of soil quality indicators in long-term management systems. Sci Total Environ. 657:457–465.

Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R. 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics. 13:162.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27:2194–2200.

Fan K, Delgado-Baquerizo M, Guo X, Wang D, Wu Y, Zhu M, et al. 2019. Suppressed N fixation and diazotrophs after four decades of fertilization. Microbiom. 7:143.

Fierer N. 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. Nat Rev Microbiol. 15:579–590.

Finkel OM, Salas-Gonzalez I, Castrillo G, Conway JM, Lawrence TF, Teixeira P, Wilson ED, Fitzpatrick CR, Jones CD, Dangl JL. 2020. A single bacterial genus maintains root growth in a complex microbiome. Nature. 587:103–108. DOI: 10.1038/s41586-020-2778-7.

Gouda S, Kerry RG, Das G, Paramithiotis S, Shin HS, Patra JK. 2020. Desert microbes for boosting sustainability in extreme environments. Front Microbiol. 11:1666.

Grady EN, Macdonald J, Liu L, Richman A, Yuan ZC. 2016. Current knowledge and perspectives of Paenibacillus: a review. Microb Cell Fact. 15:203.

Gupta G, Dhar S, Dass A, Sharma VK, Shukla L, Singh R, et al. 2020. Assessment of bio-inoculants-mediated nutrient management in terms of productivity, profitability and nutrient harvest index of and soil nourishment.
pigeon pea–wheat cropping system in India. J Plant Nutr. 43 (19):2911–2928.

Hansen BL, Pessotti RDC, Fischer MS, Collins A, El-Hifnawi L, Liu MD, et al. 2020. Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. mBio. 11(4): e01917–1920.

Horstmann JL, Dias MP, Medina-Silva R, Astarita LV, Hansen BL, Pessotti RDC, Fischer MS, Collins A, El-Hifnawi L, Liu MD, et al. 2020. Streptomyces sp. GLV45 from fabaceae rhizosphere benefits growth of soybean plants. Brazilian J Microbiol. 51 (4):1861–1871.

Kaminsky LM, Thompson GL, Trexler RV, Bell TH, Kao-Kniffin J. 2018. Medicago sativa has reduced biomass and nodulation when grown with soil microorganisms conditioned to high Phosphorus inputs. PhytoBiomes. J 2:237–248.

Pearl JL, McNary C, Lowman JS, Mei C, Anderud ZT, Smith ST, et al. 2019. Salt-Tolerant halophyte rhizosphere bacteria stimulate growth of alfalfa in salty soil. Front Microbiol. 14(10):1849.

Korir H, Mungai NW, Thuita M, Hamby Y, Masso C. 2017. Co-inoculation effect of Rhizobia and plant growth promoting rhizobacteria on common bean growth in a Low Phosphorus soil. Front Plant Sci. 8:141.

Kumar A, Dubey A. 2020. Rhizosphere microbiome: engineering bacterial competitiveness for enhancing crop production. J Advanced Res. 24:373–352.

Kumar A, Verma JP. 2018. Does plant-microbe interaction confer stress tolerance in plants? A review? Microbiol Res. 207:41–52.

Kusumastuti L, Astuti A, Sarjiyah S. 2017. Contribution of rhizobium and superlative plant growth promoting activity of indigenous rhizobacterium isolated from a saline tolerant pokkali rice (Oryza sativa L. var. pokkali) grown in a long-term fertilized soil. Int J Syst Evol Microbiol. 67:2514–2519.

Li Y, Zhan W, Ma L, Huang G, Oenema O, Zhang F, Dou Z. 2013. A nondenitrifying, novel phytohormone-stimulating, and superlative plant growth promoting trait of Streptomyces sp. CLV45 from fabaceae rhizosphere. Appl Environ Microbiol. 79:7537–7541.

Singh RK, Singh P, Li HB, Song QQ, Guo DJ, Solanki MK, et al. 2020a. Diversity of nitrogen-fixing rhizobacteria associated with sugarcane: a comprehensive study of plant-microbe interactions for growth enhancement in saccharum spp. BMC Plant Biol. 20:220.

Singh RP, Manchanda G, Maurya I, Wei Y. eds. 2020b. Microbial versatility in varied environments. Singapore: Springer. https://doi.org/10.1007/978-981-15-3028-9.

Singh RP, Manchanda G, Singh RN, Srivastava AK, Dubey RC. 2016. Selection of alkali-tolerant and symbiotically efficient chickpea nodulating rhizobia from North-West Indo Gangetic plains. J Basic Microbiol. 56:14–25.

Singh RP, Manchanda G, Yang Y, Singh D, Srivastava AK, Dubey RC, Zhang C. 2019. Deciphering the factors for nodulation and symbiosis of mesorhizobium associated with cicer arietinum in northwest India. Sustain. 11:7216.

Soares R, Trejo J, Lorite MJ, Figueira E, Sanjuan J, Videira ECI. 2020. Diversity, phylogeny and plant growth promotion traits of nodule associated bacteria isolated from lotus parviflorus. Microorganism. 8(4):499.

Tiwari S, Prasad V, Chauhan PS, Lata C. 2017. Bacillus amyloliquefaciens confers tolerance to various abiotic stresses and modulates plant response to phytohormones through osmoprotection and gene expression regulation in rice. Front Plant Sci. 8:1510.

Yang Y, Liu L, Singh RP, Meng C, Ma S, Jing C. 2020. Nodule and root zone microbiota of salt-tolerant wild soybean in coastal sand and saline-alkali soil. Front Microbiol. 11:2178.

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J. 2017. Introducing ezbiocloud: a taxonomically unified database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol. 67:1613–1617.

Zehr JP, McReynolds LA. 1989. Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. Appl Environ Microbiol. 55:2522–2526.

Zhang J, Peng S, Shang Y, Brunel B, Li S, Zhao Y, et al. 2020. Genomic diversity of chickpea-nodulating rhizobia in Ningxia (north central China) and gene flow within symbiotic Mesorhizobium meliloti populations. Syst Appl Microbiol. 43:126089.

Zheng BX, Bi QF, Hao XL, Zhou GW, Yang XR. 2017. Massilia physiologica sp. nov., a phosphate solubilizing bacteria isolated from a long-term fertilized soil. Int J Syst Evol Microbiol. 67:2514–2519.

Zhang C. 2019. Deciphering the factors for nodulation and symbiosis of alfalfa in salty soil. Front Microbiol. 14(10):1849.