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Journal

Land Degradation and Development, 29(5)

ISSN

1085-3278

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Publication Date

2018-05-01

DOI

10.1002/ldr.2904

Peer reviewed

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Received: 30 May 2017 | Revised: 4 December 2017 | Accepted: 4 February 2018
DOI: 10.1002/ldr.2904

RESEARCH ARTICLE

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Root growth improvement of mesquite seedlings and bacterial rhizosphere and soil community changes are induced by inoculation with plant growth-promoting bacteria and promote restoration of eroded desert soil

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Funding information
(CONACYT); UC Mexus-CONACYT; Bashan Institute of Science, USA; Shanbrom Family Foundation; CONACYT graduation scholarship, Grant/Award Number: 364014

Abstract

Soil degradation is an ecological disturbance, usually human-caused, that negatively affects the vegetation and climate of an ecosystem, particularly arid and semiarid environments. These degraded soils can be restored by using native perennial plants inoculated with specific microorganisms. We studied the changes in root growth and the rhizosphere bacterial community of mesquite seedlings (*Prosopis articulata*) after inoculation with the endophytic bacteria *Bacillus pumilus* ES4, over 3 cycles of growth in the same soil under desert climatic conditions, and found that inoculation significantly enhanced root biomass during the growth cycles but not shoot biomass or root and shoot lengths. Fluorescent in situ hybridization analysis demonstrated that *B. pumilus* colonized the root cap, apical meristem, and elongation zone, forming small colonies, on roots from soil-grown mesquite. Inoculation also significantly changed the bacterial community structure of rhizophere and nonrhizosphere (without plants) soils based on denaturing gradient gel electrophoresis profiles. The changes were highly stable, and the bacterial community structure was maintained throughout the experimental period and not affected by plant replacement. The 16S rRNA pyrosequencing confirmed the changes on structure of bacterial community and revealed an impact on the top taxonomic levels analyzed. The rhizospheres of inoculated plants showed a significant increase in the abundance of Proteobacteria and Acidobacteria coupled with a concomitant decrease in Actinobacteria, whereas an opposite response was observed in nonrhizospheric degraded soils. Overall, inoculation with *B. pumilus* reduced bacterial diversity but increased the *Rhizobium* population in the soil. The class Bacilli, despite *B. pumilus* inoculum, showed minimal variation.

KEYWORDS
Bacillus, desert plants, mesquite, plant growth-promoting bacteria, restoration of arid lands, rhizosphere bacteria community

1 | INTRODUCTION

Mesquite amargo (*Prosopis articulata S. Watson*), one of approximately 45 species of mesquite trees and shrubs, is a thorny legume shrub or small tree (2–15 m tall). It is a common, climax vegetation inhabitant of the southern Sonoran Desert and other parts of Mexico and the southern USA (León de la Luz, Pérez-Navarro, & Breceda, 2000). This tree, when mature, serves as the dominant tree in many resource islands and therefore directly contributes to the natural vegetation of these deserts (Bashan, Davis, Carrillo-Garcia, & Linderman, 2000; Carrillo-Garcia, Leon de la Luz, Bashan, & Bethlenfalvay, 1999; Roberts, 1989).

The soil of these resource islands, in addition to supplying nutrients, water, and shade, is rich with microorganisms, which as a community maintain high soil fertility and support new desert vegetation (Bashan & de-Bashan, 2010). In contrast, the barren area between the resource islands as well as degraded areas where vegetation has been removed is poorly populated by microorganisms and barely supports plant growth even after rainfall. Only on rare occasions are these barren desert areas

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colonized by pioneer plants (Bashan, Vierheilig, Salazar, & de-Bashan, 2006) or invasive grasses (Bashan, Salazar, Moreno, Lopez, & Linderman, 2012). The eroded soils of arid areas are often the targets of restoration efforts, but because they are usually planted with native or exotic plants, but without including microorganisms in the restoration process, more often than not these restoration attempts fail (Bainbridge, 2007; Cowie et al., 2011; Gao et al., 2002).

Many studies show that if native plants are inoculated with plant growth-promoting bacteria (PGPB) and/or mycorrhizae fungi, revegetation of degraded arid soils accelerates and soil health indicators improve (de-Bashan, Hernandez, & Bashan, 2012; Kim, Glick, Bashan, & Ryu, 2012; Medina & Azcón, 2012). Efforts to restore degraded arid lands with plants inoculated with microorganisms were carried out previously in different countries around the world. In semiarid areas of southern Spain, a combination of arbuscular mycorrhizal (AM) fungi, rhizobia, PGPB, and yeasts was used to promote the growth of plants and enhance soil fertility (Armada, Portela, Roldán, & Azcón, 2014; Armada, Roldán, & Azcón, 2014; Benabdellah, Abbas, Abourouh, Aroca, & Azcón, 2011; Herrera, Salamanca, & Barea, 1993; Marulanda, Barea, & Azcón, 2009; Medina, Vassilev, Alguacil, Roldán, & Azcón, 2004; Medina, Vassileva, Caravaca, Roldán, & Azcón, 2004; Mengual, Roldán, Caravaca, & Schoebitz, 2014; Mengual, Schoebitz, Azcón, & Roldán, 2014; Ortiz, Armada, Duque, Roldán, & Azcón, 2015; Pérez-Fernández, Calvo-Magro, & Valentine, 2016; Requena, Jimenez, Toro, & Barea, 1997; Requena, Perez-Solis, Azcon-Aguilar, Jefferies, & Barea, 2001; Valdenegro, Barea, & Azcón, 2001). In southwestern USA, the PGPB *Azospirillum brasilense* and *Bacillus pumilus* significantly promoted the growth and development of *Atriplex lentiformis* growing on extremely nutritionally poor mine tailings (de-Bashan, Hernandez, Bashan, & Maier, 2010; de-Bashan, Hernandez, Nelson, Bashan, & Maier, 2010), whereas in southern Senegal, number of different species of PGPB promoted the ectomycorrhizal symbiosis of *Acacia holosericea* (Duponnois & Plenchette, 2003; Founoune et al., 2002). Other examples include India, where native trees inoculated with a consortium of PGPB performed well in degraded parkland soil (Ramachandran & Radhapriya, 2016), and China, where rock-weathering bacteria supported plant growth (Wu, Zhang, & Guo, 2017). In the southern Sonoran Desert of Mexico and in Argentina, inoculation with the agricultural PGPB species *A. brasilense* significantly promoted the growth, establishment, and survival of several legume trees and cacti (Bacilio, Hernandez, & Bashan, 2006; Bashan, Rojas, & Puente, 1999; Bashan et al., 2012; Bashan, Salazar, & Puente, 2009; Bashan, Salazar, Puente, Bacilio, & Linderman, 2009; Carrillo, Li, & Bashan, 2002; Carrillo-Gracia, Bashan, Diaz-Rivera, & Bethlenfalvay, 2000; Felker, Medina, Soulier, & Velicce, 2005; Leyva & Bashan, 2008; Puente & Bashan, 1993). Other desert PGPB, both rhizospheric and endophytic, promoted the growth of cacti of varying sizes (Lopez, Tinoco-Ojanguren, Bacilio, Mendoza, & Bashan, 2012; Puente, Li, & Bashan, 2004, 2009).

In degraded desert soil regions, water alone does not restore fertility because the topsoil, which contains microorganisms essential for plant growth, has been lost. Applying large amounts of compost is both impractical and expensive, leading us to ask whether reintroducing native PGPB could help restore soil fertility. To test this hypothesis, we inoculated degraded soil with native PGPB, potentially capable of promoting the growth of mesquite, to learn whether the tree + PGPB combination would modify the soil microbial community over time and restore soil fertility. We also evaluated the bacterial community in the rhizosphere of mesquite seedlings and the surrounding rhizosphere soil after inoculation with *B. pumilus* ES4 by employing three molecular-based analyses: denaturing gradient gel electrophoresis (DGGE), fluorescent in situ hybridization (FISH), and 16S RNA-pyrosequencing.

2 | MATERIALS AND METHODS

2.1 | Plants

Mesquite amargo seeds were collected from the El Comitan area (24°07′36″N, 110°25′48″W), 15 km west of La Paz, Baja California Sur, Mexico in July–August 2012. We used only the seeds that had dried on the tree inside the pods because they exhibited a high germination rate (>80%). Perforated pods infected with the beetle larvae *Acanthoscelides obtectus* (Say) were discarded. Seeds were extracted manually from the pods and stored at 4 °C in hermetically sealed, glass containers with silica gel to reduce humidity (Leyva & Bashan, 2008).

2.2 | Bacteria

B. pumilus ES4 (GenBank, accession number FJ032017), isolated from the rhizoplane of a cactus from the same area where mesquite grow, is a PGPB that solubilizes rock phosphate. It promotes the growth of the giant cardon cactus, the desert shrub quailbush (*A. lentiformis*), and the microalga *Chlorella vulgaris* (de-Bashan, Hernandez, Bashan, & Maier, 2010; Hernandez, de-Bashan, Rodriguez, Rodriguez, & Bashan, 2009; Puente et al., 2004).

2.3 | Soil and growth conditions

Soil that had lost its topsoil due to housing development and wind erosion was collected from the El Comitan area. The soil is classified as an alluvial haplic yermosol (Bashan et al., 2000; Carrillo-Gracia et al., 2000). The soil characteristics are low total carbon levels (400 mg/kg), that is, mostly inorganic carbon, low total nitrogen (20 mg/kg), and a reduced microbial biomass (Bashan et al., 2000; Trejo et al., 2012). Most annual plants cannot grow in this soil even after irrigation or rainfall. The samples came from a site that has been unproductive for over three decades. Soil was collected in buckets from several barren patches to a depth of 30 cm and sieved to 2 mm. Seeds were planted in 120 g of soil in black, plastic, conical pots (2.5 cm inner diameter and 15.5 cm long) assembled in prefabricated trays commercially used for production of trees in nurseries (Polietilenos del Sur, Mexico City). This pot size allows for 30 days of uninterrupted growth of mesquite seedlings.

Two repetitions of the experiment were conducted in a shade house exposed to ambient conditions of temperature and humidity with protection against herbivores, insects, gusts of wind, and reduced solar irradiation. Because of the length of the outdoors experiments (~4 months long each, gross time), the temperature, humidity, and solar irradiation varied. The temperature was an average of 30 °C and ranged between 18 and 32 °C, reaching >40 °C in the middle of summer (Carrillo-Gracia et al., 2000). The relative humidity in the dry

season is 57% (February to July) and 64% in the wet season (August to January). Solar radiation was constant at ~1,000 μmol photons m^2 s^{-1}.

2.4 | Disinfection and germination of mesquite seeds

Batches of 150 intact mesquite seeds were used per each growth cycle from seedling transplant to harvest. Seeds were slowly and constantly agitated at room temperature (25–28 °C) in ~100 ml of 2% Tween 20 (Sigma-Aldrich) for 10 min, which was decanted and then treated with 4% sodium hypochlorite for 5 min. After decanting, the disinfected seeds were thoroughly washed five times in distilled water for 1 min each until the chlorine odor was eliminated. The seeds were imbibed in 200 ml of sterile distilled water under constant agitation at room temperature for 7 hr. Seeds were later germinated in Petri dishes on wet paper towels, in the dark, at 35 ± 1 °C for 3 days. This procedure was repeated for each of the six cycles of growth (three cycles per experiment).

2.5 | Bacterial cultivation and production of inoculant

B. pumilus ES4 was cultivated on TYG (Tryptone, Yeast, Glucose) medium (Bashan, Trejo, & de-Bashan, 2011) at 120 rpm and 35 ± 1 °C for 24 hr. Five milliliters of this inoculum were transferred into 50 ml of fresh TYG and grown for 16–18 hr under the same conditions. The purity of the final culture was checked by colony morphology on nutrient agar (#N9405, Sigma-Aldrich) incubated at the same temperature overnight.

The inoculant was produced as alginate beads (2–3 mm in diameter; de-Bashan & Bashan, 2010). During the polymerization process, the bacterial population is normally reduced (Bashan, Hernandez, Leyva, & Bacilio, 2002), so the processed beads were incubated in TYG medium for an additional ~16 hr at the conditions listed above. The medium was decanted, and the beads were washed three times with 0.85% sterile saline solution. This procedure assured that a large number of live bacteria were immobilized in the beads. Because the dry alginate bead inoculant can be maintained without losing effectiveness for extended periods of time (Bashan & Gonzalez, 1999), the beads were completely dried. This was done by placing a single uniform layer of beads in a sterile metal tray in a laminar flow hood for 24 hr. Once dried, the inoculant was placed in small flasks for inoculation of plants.

Quality control of the inoculant was performed by dissolving 100 mg of dry beads in 50-ml sterile Corning tubes containing 30 ml of citrate buffer (55-mM Na-citrate, 30-mM anhydrous EDTA, and 150-mM NaCl-pH 8) and stirred for 1 hr until the beads completely dissolved. The suspension was centrifuged at 12,800 *g* for 10 min, the supernatant was discharged, and the cells were suspended in 1-ml saline solution and serially diluted. The colony-forming units (CFU) were determined after cultivation on 2% nutrient agar plates for 72 hr at 35 ± 1 °C. The same inoculant was added to the plants in all cycles of growth. No decline in CFU number (2.3×10^8 CFU/ml) was observed during the duration of the study.

2.6 | Planting and inoculation

Uniform 72-hr-old germinated seedlings were sown, one per pot, together with 0.2 g of inoculant corresponding to 1.6×10^8 CFU/pot. Each seedling was irrigated with 40-ml distilled water every 3 days to maintain field capacity (Trejo et al., 2012).

2.7 | Measurements of plant parameters

At every end of a growth cycle (30 days), the lengths of the main roots and the shoots of 10 plants were measured. The plant parts were dried in an oven at 65 °C for 48 hr until a constant dry weight was achieved. Dry weight was determined on an analytical scale (Ohaus Pioneer, NJ).

2.8 | Extraction of total DNA

Rhizosphere soil samples (~3 g), free of roots, were collected from five plants per treatment, and each was frozen and stored in 2-ml tubes (Neptune, San Diego, CA) at −80 °C. Analysis by polymerase chain reaction (PCR)-DGGE is not affected by cold storage (Campbell, Clark, & Zak, 2009). Extraction of DNA followed the methods of Lopez, Bashan, Trejo, and de-Bashan (2013) in triplicate (0.6 g each) per treatment.

2.9 | PCR and DGGE analyses

The V5–V8 region of the 16S rRNA gene was PCR amplified using the following bacterial primers: F984GC (5′GC-AACGCGAAGAACCTTAC-3′) and R1378 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′; Heuer, Krsek, Baker, Smalla, & Wellington, 1997) at a concentration of 5 μM of each primer. The rest of the PCR procedure and DGGE analysis were performed according to Lopez et al. (2013). Gel images were recorded with a gel documentation system (Gel Doc XR, Bio-Rad Laboratories) and the pattern of bands analyzed by Quantity One 4.6.7 software (Bio-Rad Laboratories).

2.10 | Environmental DNA isolation

Environmental DNA was extracted from the collected soil samples, treated and untreated, using 0.25 g of sample per isolation. The PowerSoil DNA Isolation kit (MoBio Labs/Qiagen, Carlsbad, CA) was used for the extraction according to manufacturer's instructions. The quality of extracted DNA was assessed by visualization on 1% agarose gel with a high molecular mass ladder (Invitrogen, ThermoFisher Scientific, Waltham, MA).

2.11 | Pyrosequencing

A commercial service (Molecular Research LP, Shallowater, TX) using a Roche 454 FLX Titanium sequencer was utilized. The sample was a composite sample of the three cycles of the first repetition of the experiment. Briefly, the universal primer 454-16S for Eubacteria were amplified by a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix kit (Qiagen, Valencia, CA) and the following conditions: 3 min at 94 °C, 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, followed by a final elongation of 72 °C for 1 min. All the PCR products were mixed and purified using Agencourt Ampure beads (Agencourt Bioscience, MA) and then sequenced. The sequencing service included quality control, operational taxonomic units (OTUS) at 97% similarity, and removal of chimeras. The final OTUs were determined by BLASTn, a curated database derived from

GreenGenes (DeSantis et al., 2006), the Ribosomal Database Project (http://rdp.cme.msu.edu), and National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Frequencies of OTUs were collected into databases at each taxonomic level.

2.12 | FISH of mesquite roots

Ten days after planting, five plants of mesquite seedlings per treatment were removed from the pots and the roots carefully washed with saline solution (0.85% NaCl). Main and lateral roots approximately 3 cm long were excised from each sample with a sterile scalpel, transferred to 2-ml tubes containing 96% ethanol, and stored at −20 °C until analysis. Because mesquite roots are thick, which can interfere with FISH microscopy, they were sectioned. First, the roots were washed for 1 hr with 96% ethanol, followed by three washes (1 hr each) with 100% ethanol, and finally, washed for 1 hr with 99.5% acetone (Sigma-Aldrich), all at 4 °C. The fixed roots were infiltrated with resin (Technovit 9100, Hatfiled, PA, and Electron Microscopy Science, Hatfiled, PA) according to the manufacturer's instructions. After infiltration, they were cut by hand on a sterile surface under a binocular microscope into main root, secondary roots, root tip, and root hair zone. The segments were then embedded in the resin and polymerized according to the manufacturer's instructions. Sections 2 μm thick were obtained using a microtome (Leica RM 2255, Biosystems, Nussloch, Germany), and the sections were mounted on glass slides at room temperature (~25–28 °C) and dried overnight at the same temperature.

B. pumilus was specifically detected on root sections by FISH according to the procedure of Trejo et al. (2012) using two probes for hybridization. The first was 9 μl of a mixture of three probes specific for the domain bacteria: EUB-338-I (5′-GCT GCC TCC CGT AGG AGT-3′; Amann et al., 1990), EUB-338-II (5′-GCA GCC ACC CGT AGG TGT-3′), and EUB-338-III (5′-GCT GCC ACC CGT AGG TGT-3′; Daims, Brühl, Amann, Schleifer, & Wagner, 1999). The second consisted of 18 μl of a specifically designed probe (FITCBPUM, this study) for *B. pumilus* (5′-CTC TCG CAC TTG TTC CC-3′) labeled with the fluorochrome 56-FAM (IDT echnologies, IA).

For visualization, the slides were mounted in AF1 antifading reagent (Citifluor, London, UK) and observed with an epifluorescent microscopy (Olympus BX41, Tokyo, Japan) using Cy3 (552 nm, red fluorescence) and FITC (495 nm, green fluorescence) filters (Olympus America, Melville, NY) after a 3-min incubation as described by Covarrubias, de-Bashan, Moreno, and Bashan (2012). Briefly, separate images of each photomicrograph were recorded by a digital camera (Evolution VF Cooled Color, Media Cybernetics, Silver Spring, MD) and processed with the Image Analyzer ProPlus 6.3.1.542 (Media Cybernetics). All the bacterial cells fluoresced red, whereas *B. pumilus* cells were green. The two signals were digitally superimposed yielding high specific identification of *B. pumilus* as yellow cells.

2.13 | Experimental design and statistical analyses

Two identical experiments were conducted. Each experiment consisted of three cycles of 30 days (total of 90 days per experiment). One cycle covered the growth period of mesquite seedlings from planting and inoculation with *B. pumilus* ES4 to the harvesting of the entire plant. After harvesting, the soil in the pot was retained and was reused as substrate for the next cycle of growth (Trejo et al., 2012) to measure the increase in soil fertility over time. Each experiment consisted of four treatments of which three were controls: (a) [BP] rhizosphere soil containing one mesquite seedling and inoculated with *B. pumilus* ES4; (b) [BO] *B. pumilus* ES4 inoculated directly to the soil, without mesquite seedling; (c) [PO] rhizosphere soil of mesquite seedling; and (d) [IS] barren soil without mesquite or *B. pumilus* ES4. Each treatment consisted of 20 replicates where a single pot served as a replicate. Plant growth data per experiment were analyzed using Student's *t* test and analysis of variance and Tukey's post hoc analysis at $p \leq .05$ using Statistica v.10 (Tibco Software, Palo Alto, CA) and are presented as an average with standard error.

Bacterial community analysis of all treatments was done by comparing the densitometric profiles of the bands in the DGGE gel images. The profiles obtained from DGGE gels were analyzed for similarity using the Dice coefficient, and dendrograms were built from the unweighted pair group, matching the band average using the Quantity One 4.6.7 package (Bio-Rad Laboratories). Similarity varied from 0 to 1, where 1 indicates 100% similarity. Additionally, similarities among profiles for all treatments at each time frame were evaluated by nonmetric multidimensional scaling analysis (NMDS; Venables & Ripley, 2002) using Statistica v.8.

Ecological estimators of the rhizosphere bacterial communities were obtained from the OTUs defined by DGGE and 16S rRNA gene pyrosequencing. For DGGE, richness, diversity, and evenness were obtained by Multivariate Statistical Package 3.22 (Kovach Computing Services, Anglesey, Wales), using the intensity of a band (OTU) as the measure of abundance within the densitometric profile of each sample (Iwamoto et al., 2000). Changes in DGGE bacterial diversity among treatments were analyzed by one-way analysis of variance in Minitab (Minitab, Coventry, UK). For the pyrosequencing data, the estimators of rarefaction curves, richness, and diversity were obtained from OTUs at 97% of similarity and performed in EstimateS 9.1.0 (RK Colwell, http://purl.oclc.org/estimates). Patterns of correlation between the abundance of bacterial groups and inoculation treatments were analyzed by principal component analysis (PCA). PCA was applied to the standardized matrix of abundance of phylotypes (231 genus and 6 candidate divisions of bacteria).

3 | RESULTS

3.1 | Effect of inoculation with *B. pumilus* ES4 on mesquite parameters above and below ground during three cycles of growth in the same soil

In repetitions of the experiment, mesquite seedlings inoculated with *B. pumilus* ES4 had a larger root system based on dry weight than inoculated plants in the same degraded soil (Figure 1a,b, lower case letters). The ES4-inoculated plants had a larger root system following growth in successive cycles of the same soil: Cycle 1 < Cycle 2 < Cycle 3 (Figure 1 a,b, capital letters). In Experiment 2, the uninoculated mesquite plants grew better in Cycle 2 than in Cycle 1, but a smaller root system developed when compared with an inoculated plant (Figure 1b).

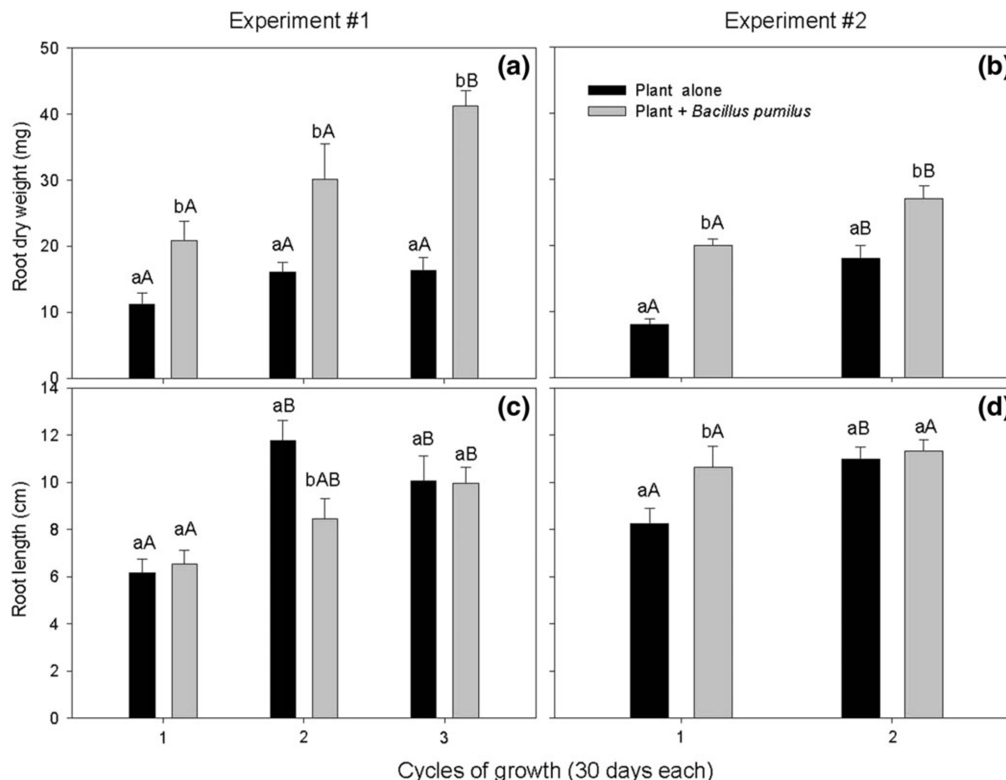

FIGURE 1 Effect of inoculation with *Bacillus pumilus* ES4 on root parameters (DW, a,b; root length, c,d) of mesquite seedlings during three cycles of growth in the same soil. Pair of columns, in each subfigure separately, denoted by a different lower case letter differ significantly by Student's *t* test at $p < .05$. Columns, in each subfigure separately, denoted by different capital letters differ significantly by one-way analysis of variance and Tukey's post hoc analysis at $p \leq .05$. Whiskers indicate standard error

Generally, no positive effect on root length was recorded in Experiments 1 and 2 except for an enhancement of length in the first cycle of the second experiment (Figure 1c,d). Similar effects were recorded on shoot length and shoot dry weight (Figure S1). Plants of the third cycle of the second experiment were destroyed by Hurricane Odile (Level 3) in 2015, which demolished the shade house and dispersed its entire contents.

3.2 | Changes in bacterial community structure of mesquite rhizosphere after inoculation with *B. pumilus* ES4 during three cycles of growth in the same soil by DGGE analysis

Similarity dendrograms and 3D NMDS analyses, both derived from DGGE gels and performed twice in every growth cycle (10 and 30 days), showed a similar pattern regardless of the cycle or date of sampling (Figure 2). Two distinctive groups emerged in all the analyses: (a) soil treatments that were inoculated with *B. pumilus* with or without a mesquite seedling and (b) soils with no inoculation (the controls, with or without plants). Inoculation significantly changed the bacterial community structure of the rhizosphere in presence of a plant or without one, with a high (60%) similarity (Figure 2a-f). These changes were very stable, and the bacterial community structure was maintained throughout the 90-day experiment and not affected by two plant replacements made during the experimental period. Only small variations of the same patterns were shown by NMDS (Figure 2a-f). The identical analyses performed in Experiment #2 were very similar confirming the patterns listed above. Bacterial richness and Shannon diversity index calculated from the DGGE study showed lower values in rhizosphere soil of *B. pumilus*-inoculated mesquite. However, when calculating differences among the diversity of soil samples, no significant difference was found (Table 1).

3.3 | Effect of inoculation with *B. pumilus* ES4 on the soil bacterial metagenome during three cycles of mesquite growth

The pyrosequencing results yielded 11,970 sequences (OTUs) of which 3,970 originated from the plant rhizosphere soil inoculated with *B. pumilus*, 3,568 from rhizosphere soil alone, 2,709 from soil without mesquite or *B. pumilus*, and 1,723 from soil inoculated with *B. pumilus*. Rarefaction curves (accumulation of OTUs) indicated that sequencing depth adequately represented the estimated species richness (Figure 3a). This result was supported by Good's coverage (above 98%), approaching the saturation of phylotype coverage and corroborated by richness estimators with values within the confidence intervals or relatively close to the estimated ACE, Jack 1, and Chao 1 values (Table 2). Interestingly, the rhizosphere soil of mesquite seedlings inoculated with *B. pumilus* (BP) exhibited the lowest bacterial diversity (by Shannon or Simpson indices) and consistent lower evenness (E), thus less equitable (Table 2). Differences in bacterial composition were also revealed by cluster analysis (Figure 3b), where the rhizosphere of inoculated plants (BP) had the lowest similarity regarding other soil samples (soil without mesquite or *B. pumilus* [IS], or soil inoculated with *B. pumilus* [BO]).

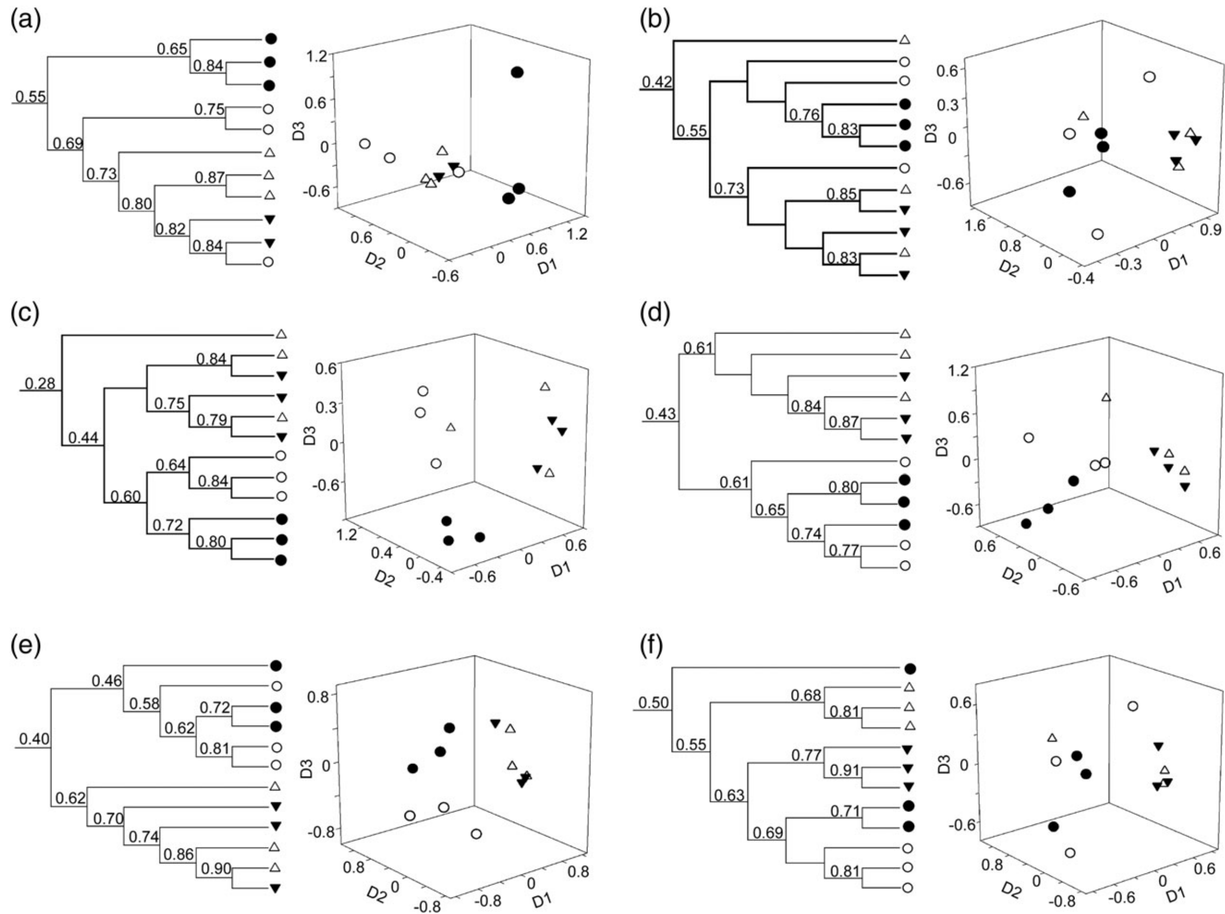

FIGURE 2 Comparison of the bacterial community structure of mesquite seedlings growing in three independent cycles of growth after inoculation with plant growth-promoting bacteria and evaluated after 10 (a,c,e) and 30 (b,d,e) days of cultivation: ▼ = rhizosphere soil of mesquite; ● = rhizosphere soil of mesquite inoculated with *Bacillus pumilus* ES4; △ = soil without mesquite or *B. pumilus* ES4; and ○ = soil inoculated with *B. pumilus* ES4; (a,b,c,d,e,f, left side) dendrograms from cluster analyses of denaturing gel electrophoresis of bands by Dice coefficient; (a,b,c,d,e,f, right side) 3D configuration derived from nonmetric multidimensional scaling analysis. Data with the same symbols represent the replicates within each treatment

TABLE 1 Ecological attributes for soil bacterial communities analyzed by denaturing gradient gel electrophoresis

Treatments	*N*	OTU richness (S)[a]	Shannon index (H)[a]	Evenness (E)[a]
[BP] Rhizosphere soil of mesquite seedling inoculated with *B. pumilus* ES4	18	10	0.96 ± 0.15 NS	0.99
[IS] Barren soil without mesquite or *B. pumilus* ES4	18	11	1.00 ± 0.10 NS	0.99
[BO] *B. pumilus* ES4 inoculated directly to the soil without a plant	18	11	1.01 ± 0.19 NS	0.99
[PO] Rhizosphere soil of mesquite seedling	17	12	1.05 ± 0.13 NS	0.99

Note. *B. pumilus* = *Bacillus pumilus*; NS = No significant differences among bacterial diversity of soil samples by one-way analysis of variance (F = 1.03, α = 0.372).

[a]S, H, and E were performed in Multivariate Statistical Package considering each band as an operational taxonomic unit (OTU), whose abundance is given by the band intensity within the corresponding densitometric profile (Iwamoto et al., 2000).

Almost 26% of total OTUs were shared by the four samples of soils (mesquite inoculated with *B. pumilus* [BP], plant without inoculation [PO], soil inoculated with *B. pumilus* [BO], and soil without mesquite or *B. pumilus* [IS]). A similar proportion of OTUs was found exclusively in the initial degraded soil or the rhizosphere soil of inoculated mesquite (Figure 3c).

Overall changes in the structure of bacterial communities among the treatments were observed at the phylum level and further examined at lower taxonomic levels. In Figure 4a, the distribution of the abundance of phyla indicated that *Proteobacteria* increased from ~32% in the initial soil without mesquite and inoculum to ~70% in the inoculated plant soil, concomitant with the change in *Actinobacteria* from ~45% to 3% in the same soils. Phylum *Acidobacteria* was primarily detected in soils with plants and *B. pumilus* ES4-inoculated plants. No *Acidobacteria* were detected in soil lacking plant or microbe additions. Bacteroidetes generally increased in soils with added bacteria or plants, and interestingly, the candidate division Tm7 (Saccharibacteria) was detected only in nonrhizosphere soils that were inoculated with *B. pumilus*.

Figure 4b shows the analysis of the classes after the treatments. The distribution of abundance among classes was consistent with the

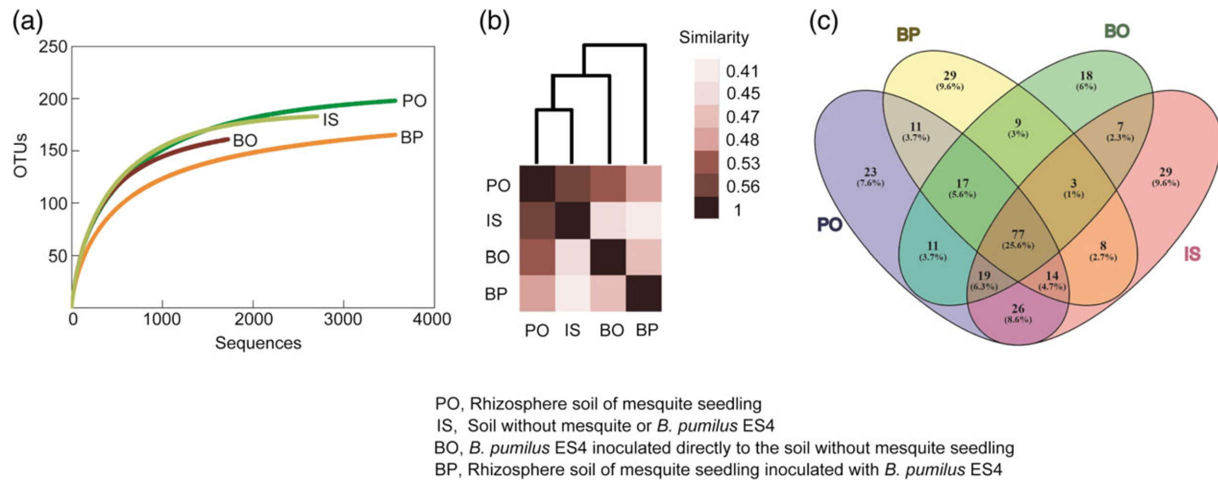

FIGURE 3 (a) Rarefaction curves, (b) cluster analysis, and (c) Venn diagram of soil bacterial communities with 301 operational taxonomic units (OTUs) defined at 97% similarity. Rarefaction curves were calculated by 100 randomizations without extrapolation in EstimateS 9.1.0. Asymptotic curves indicate that sequencing depth adequately represents the estimated richness. Cluster analysis by Jaccard's index shows the similarity in the composition of OTUs among samples. Venn diagram was constructed with Venny 1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) and represents the number of OTUs, unique and shared (overlapping areas) between combinations of samples. Integer values or percentages in parenthesis accumulate 301 OTUs (100%) analyzed [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Coverage, richness, and diversity estimates for soil bacterial communities analyzed by 16S rRNA pyrosequencing

	[BP] Rhizosphere soil of mesquite seedling inoculated with *B. pumilus* ES4	[IS] Soil without mesquite or *B. pumilus* ES4	[BO] *B. pumilus* ES4 inoculated directly into soil without plants	[PO] Rhizosphere soil of mesquite seedling
Sequences (*N*)	3,970	2,709	1,723	3,568
OTUs[a]	168	183	161	198
Good[b]	99.37	99.63	98.43	99.41
ACE[d]	183	185	183	209
Chao 1[d,f]	183 (174–208)	185 (183–193)	183 (170–217)	209 (202–228)
Jack 1[d]	168	183	161	198
Shannon (H)[d]	3.78	4.15	4.16	4.22
Evenness (E)[c]	0.4562	0.5250	0.5583	0.5159
Simpson (1/D)[d,e]	19.54	24.6	29.7	33.14

Note. *B. pumilus* = *Bacillus pumilus*.

[a]The operational taxonomic units (OTUs) were defined at 97% or similarity.

[b]Good's coverage was obtained by the formula $G = 1 - n/N$, where n is the number of singletons and N is the total number of sequences in the sample (Kemp & Aller, 2004).

[c]Shannon evenness was calculated as $E = H/\ln(S)$, where H is the Shannon index and S is the total number of sequences in the sample (Ling et al., 2010).

[d]Richness (ACE, Jack 1, and Chao1) and diversity estimators (Shannon and Simpson's reciprocal index) were computed in EstimateS 9.1.0.

[e]Simpson is computed as the reciprocal of the Simpson's index of diversity where the lowest value is 1.

[f]Chao mean ± 95% confidence intervals in parentheses.

abundance of phyla across treatments. Actinobacteria dominated the bacterial communities of treatments with bacteria only or plants added and lacked representatives of γ-proteobacteria and Sphingobacteria, whereas α- and β-proteobacteria increased in every treatment with plants or inoculated with *B. pumilus*, with the highest in the rhizosphere of inoculated plants (31%). In soils where mesquite or the bacterial inoculant had been introduced into the soil, the percentage of Actinobacteria significantly decreased, especially in the inoculated soil with mesquite. In the Bacilli class (Figure 4c), the families *Paenibacillaceae* and *Bacillaceae* were dominant in all treatments, but few distinguishing differences among the soils were observed.

PCA corroborated differences in the abundance of phylotypes from mesquite and/or *B. pumilus* ES4 soil samples compared with the untreated soils. The ordination pattern of soil samples by PCA was supported by an accumulated variance of 86% in the first two axes; *Rubrobacter* (loading value = 0.63) and *Flavisolibacter* (loading value = 0.39) had the highest correlation to Axis 1. Tm7 (loading value = 0.55) and *Rhizobium* (loading value = 0.53) had the highest correlation to Axis 2, thus separating rhizosphere soil of mesquite inoculated with *B. pumilus* from the other treatments. Further analysis indicated that the abundance of certain phylotypes was correlated with specific soil samples (Figure 5a). Therefore, *Rubrobacter* (class Actinobacteria) dominated in soils without mesquite or *B. pumilus* ES4 but not in the other treatments. In contrast, *Flavisolibacter* (class Bacteroidetes) and *Massilia* (class α-proteobacteria) and *Chloracidobacterium* (Acidobacteria) were more abundant in inoculated

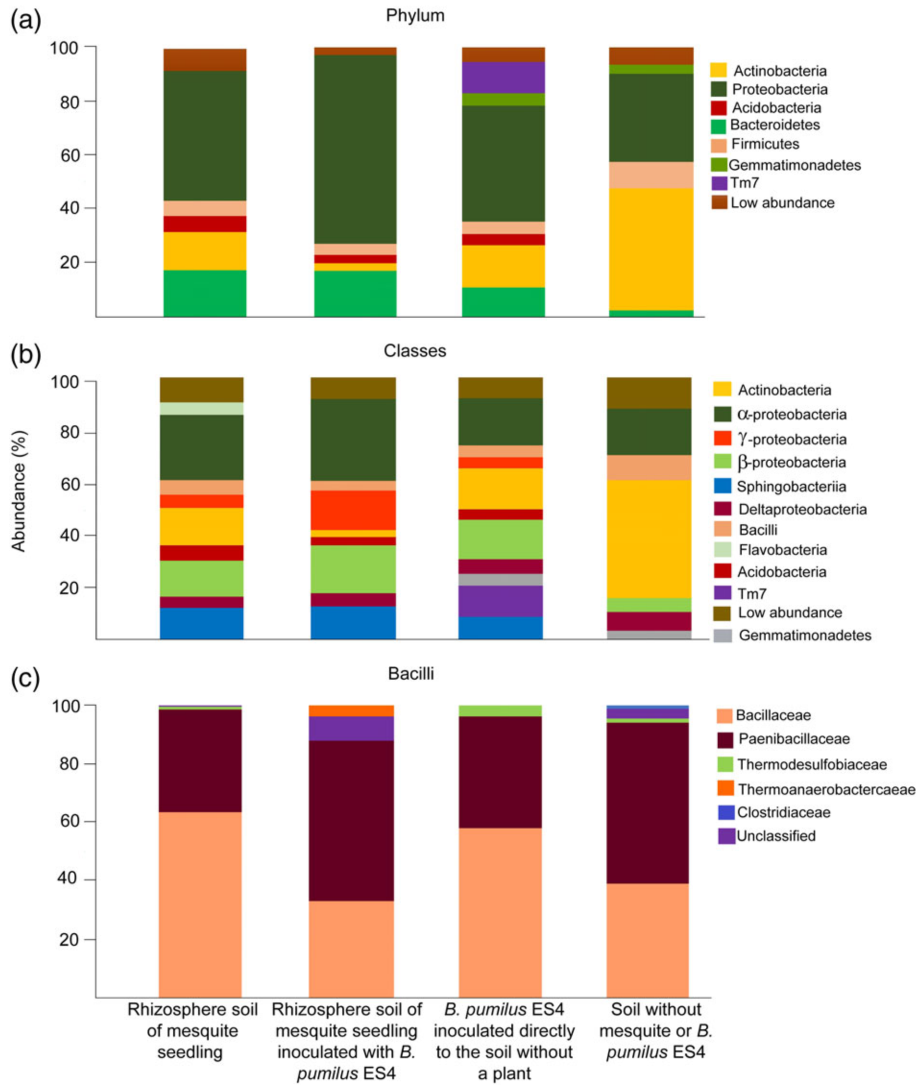

FIGURE 4 Distribution of bacterial abundance across (a) phyla, (b) classes, and (c) bacilli by 16S rRNA pyrosequencing of a composite sample of soil from all cycles and sampling time [Colour figure can be viewed at wileyonlinelibrary.com]

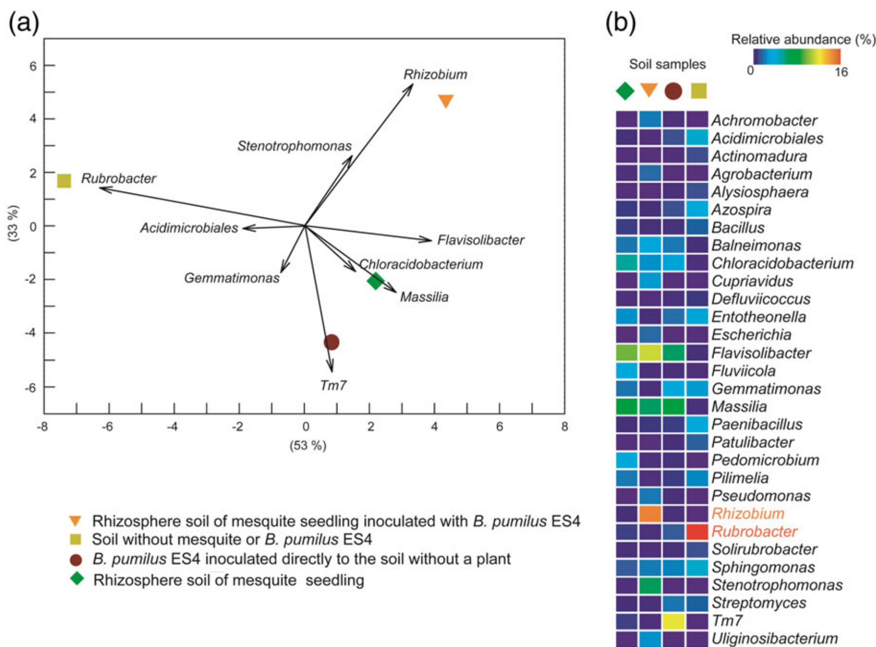

FIGURE 5 (a) Biplot of principal component analysis (PCA) of soil samples based on the abundance of 237 phylotypes and (b) heat map of relative abundance for the Top 30 most significant phylotypes obtained from PCA. Short vectors are not displayed in the PCA biplot [Colour figure can be viewed at wileyonlinelibrary.com]

soils and when plants were grown. *Rhizobium* spp. (α-proteobacteria) were abundant in rhizosphere soil of mesquite seedlings inoculated with *B. pumilus* ES4, whereas Tm7 (Saccharibacteria) was abundant in soils inoculated only with the *B. pumilus* strain (Figure 5a,b).

3.4 | Colonization of mesquite roots by *B. pumilus* ES4 evaluated by FISH

B. pumilus colonized several parts of the root system of mesquite and established very small colonies on the tissues. Figure 6 shows representative images of bacterial colonization of the root cap (Figure 6d, e), apical meristem (Figure 6c), and root elongation zone (Figure 6a,b). This pattern was observed in all three cycles.

4 | DISCUSSION

Restoration of degraded arid and semiarid soils is very slow and frequently not successful. Yet these soils increase in area worldwide, causing poverty, land abandonment, and migration of rural populations to urban areas. Studies of degraded soils have shown that quantitative measurement of the soil bacterial community is correlated with the level of degradation and human-inflicted changes. Composition, size, and function of these soil microbial communities differentiate ecosystems and man-made impacts imposed on them (Harris, 2003). The most favorable way to return these soils to productivity, that is, supporting healthy vegetation, is by rehabilitating the structure and functioning of organisms both aboveground and belowground (Cowie et al., 2011).

One of the proposed fundamental theories about the recurrent failure of natural revegetation in eroded desert areas and the difficulty with establishing the native plant population is that the top soil has lost its beneficial plant-associated microorganisms. This happens even during occasional years of plentiful rainfall (Drezner, 2006). However, water alone does not restore soil fertility/health or its microbial communities. Bashan and de-Bashan (2010) proposed that essential plant growth-promoting microorganisms should be artificially reintroduced, and this approach has been taken in several experimental cases (see Section 1 for specific cases).

Both PGPB and AM fungi are beneficial for plants in harsh and limiting environments as alleviators of plant stress (de-Bashan et al., 2012; Sylvia & Williams, 1992). Although AM fungi are commonly recognized as essential components of plant–soil systems in arid lands (Bashan et al., 2000; Carrillo-Garcia et al., 1999; Nobel, 1996; Requena et al., 2001), PGPB are not yet given the same recognition

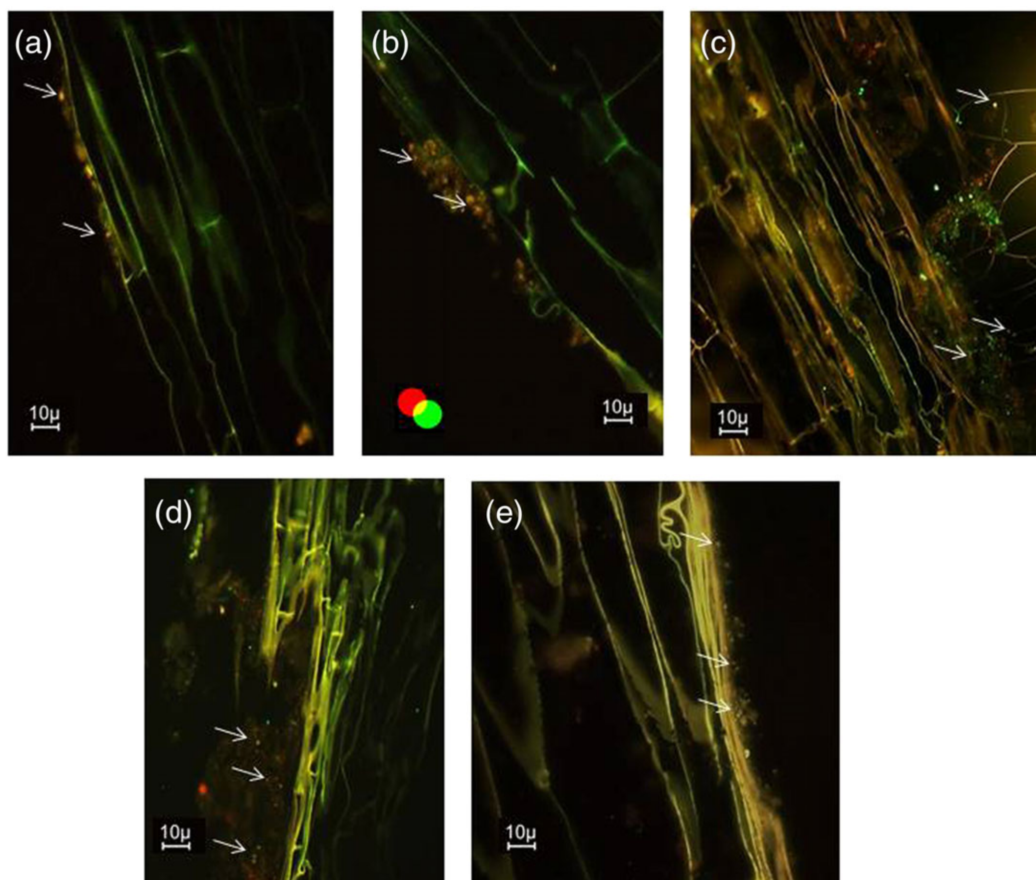

FIGURE 6 Root colonization of mesquite seedlings inoculated by *Bacillus pumilus* monitored 10 days after inoculation by fluorescent in situ hybridization. (a) Elongation zone (Cycle 1); (b) elongation zone (Cycle 2); (c) apical meristem; (Cycle 2); (d) root cap (Cycle 2); (e) root cap (Cycle 3). Two types of probes were used: an equimolar mixture of probes EUB-338 I, II, and II, which covers the domain of bacteria. A *B. pumilus*-specific probe (FITCBPUM) was used. The probe FITCBPUM were labeled with the fluorochrome 56-FAM (green). The mix of EUB I, II, and III was labeled with the fluorochrome Cy3 (red). Positive fluorescent signals that identify the bacteria are therefore a combination of red and green that yields a green-yellow-orange tone, depending on the intensities of the individual color channels. Arrows indicated location of yellow observed colonies of *B. pumilus* [Colour figure can be viewed at wileyonlinelibrary.com]

even though using them for arid land restoration provides three major benefits: (a) cost reduction by decreasing the amount of fertilizer and compost usually needed; (b) easier reestablishment of native plants in eroded soils where they had previously grown; and (c) enhancement of initial poor plant performance by improving drought and salinity tolerance (de-Bashan et al., 2012). Appropriate native PGPB for restoration can be isolated directly from soil or from plants already growing there (Felker et al., 2005; Grandlic, Mendez, Chorover, Machado, & Maier, 2008; Lopez et al., 2012; Puente et al., 2004, 2009). Another approach is to evaluate isolated strains from the large number of proven agricultural PGPB strains (Bacilio et al., 2006; Bashan et al., 1999; Bashan et al., 2012; Carrillo et al., 2002; Carrillo-Garcia et al., 2000; Lopez-Lozano, Carcaño-Montiel, & Bashan, 2016). This study selected the native *B. pumilus* strain ES4, which was isolated from roots of cardon cactus (Puente et al., 2009). Most cardon cacti initiate their life cycle as nurslings of mesquite (Carrillo-Garcia et al., 1999). As demonstrated previously for *B. pumilus* ES4 (de-Bashan, Hernandez, Bashan, & Maier, 2010) and for other bacteria (Leyva & Bashan, 2008), inoculation significantly increases the root biomass of mesquite, a plant known for developing an extensive root system but meager foliage at the beginning of its growth (Felker, 2009). Increased root biomass improves the chance of survival of the plant in an arid environment.

Inoculation of PGPB such as *Azospirillum* spp. into rich agricultural soil had only marginal effect on the bacterial community structure of maize and tomato rhizospheres (Felici et al., 2008; Herschkovitz et al., 2005), mainly due to the massive population of other rhizosphere bacteria, that is, 10^8–10^9·CFU cultivable bacteria/g. Yet, in highly degraded soils such as unstructured toxic mine tailings, inoculation with *A. brasilense* and *B. pumilus* made a detectable difference in bacterial community structure (de-Bashan, Hernandez, Bashan, & Maier, 2010; de-Bashan, Hernandez, Nelson, et al., 2010). A likely explanation for the difference in results is that mine tailings contain an extremely low population of bacteria, for example, 10^3–10^5 CFU/g (de-Bashan, Hernandez, Bashan, & Maier, 2010; Iverson & Maier, 2009). As shown here, *B. pumilus* ES4 alone helped restore a degraded desert soil. Furthermore, the current study showed that inoculation of degraded soil with *B. pumilus*, even in the absence of the host plant, changed the bacterial community structure. In contrast to the mine tailings study, our pyrosequencing results show that the collected soils contained a diversity of heterotrophic bacteria. Inoculation of *B. pumilus* ES4 at 10^6 CFU/g onto a slow-growing plant or even into soil without a plant created a large shift in the bacterial population, which was detected by molecular techniques.

Studies of natural bacterial community structure in several arid zones were documented in Mexico (Aguirre-Garrido et al., 2012), the Negev Desert (Ben-David, Zaady, Sher, & Nejidat, 2011; Berg, Unc, & Steinberger, 2015; Kaplan et al., 2013), and the Mojave Desert, USA (Ewing, Southard, Macalady, Hartshorn, & Johnson, 2007). However, restoration attempts often alter bacterial populations and reveal completely different patterns (Chen et al., 2015), which had happened in degraded soil or mine tailings in southeast Spain (Carrasco et al., 2010; García, Roldán, & Hernández, 2005), in Brazil (Araújo, Borges, Tsai, Cesarz, & Eisenhauer, 2014), and in China (Zhang, Cao, Han, & Jiang, 2013). Using PGPB for restoration may shift the populations further (de-Bashan, Hernandez, Bashan, & Maier, 2010; de-Bashan, Hernandez, Nelson, et al., 2010).

By using two complementary approaches, DGGE and 454-pyrosequencing, we confirmed that the inoculation of mesquite with *B. pumilus* produced successional changes in the rhizosphere bacterial community after three cycles of cultivation. Pyrosequencing suggested changes in bacterial diversity with lower values in the rhizosphere soil of the inoculated mesquite. In the uninoculated or soils without plants, Proteobacteria, Actinobacteria, Acidobacteria, Bacterioidetes, and Saccharibacteria were the most distinctive phyla, with a considerable increase in Proteobacteria and Acidobacteria abundance and a decrease in Actinobacteria in the inoculated treatments. These results support previously observed patterns in biological soil crusts of the Sonoran Desert (Nagy, Perez, & Garcia-Pichel, 2005) and global patterns seen previously (Fierer, Bradford, & Jackson, 2007; Trivedi, Delgado-Baquerizo, Anderson, & Singh, 2016). Recently, Trivedi et al. (2016) conducted a meta-analysis of 120 publications to analyze soil microbial composition covering temperate, tropical, and arid biomes around the world. They found an overall pattern revealing that Proteobacteria and Acidobacteria are strongly correlated to natural ecosystems whereas Actinobacteria are more related to systems involving agricultural practices and suggested that bacterial composition by itself may be an indicator of degraded soils. Acidobacteria are r-strategists that typically decrease under soil management (Fierer et al., 2007). However, in our study, the presence of plants and/or soil inoculated with *B. pumilus* ES4 appeared to reverse this pattern from the initial soil. The explanation of the abundance of Acidobacteria may be related to an increase of soil carbon and lower pH (Trivedi et al., 2016).

One explanation for the abundance of Actinobacteria in the degraded soils (without plants) is that this phylum is very abundant in diverse ecological niches, especially in extreme environments such as hot deserts (Fierer et al., 2012). This group has a complex life cycle with a characteristic tolerance to low soil moisture and lives both as filamentous and dormant forms (Shivlata & Satyanarayana, 2015).

In our study, the differential increase in abundance of Rhizobiales in the rhizosphere of inoculated or uninoculated mesquite suggested that these bacteria were revived or reactivated. This was revealed by the abundance of *Rhizobium* in the rhizosphere of soil of inoculated seedlings. This finding supports the well-known role of rhizobia on the growth of native legumes of arid lands (Jorquera et al., 2016) and the documented interactions of rhizobia with many species of PGPB in agriculture (Martínez-Hidalgo & Hirsch, 2017). Inoculation with *B. pumilus* ES4 also seems to have slightly reduced the abundance of several members of its own phylum (Firmicutes). However, *Paenibacillaceae* and *Bacillaceae* remain in the rhizosphere bacterial community, thus contributing directly or indirectly to plant growth promotion.

Although this study was conducted under desert climatic conditions, by default, it was more protected than plants directly transplanted into desert soil having high herbivore pressure, local pests, and humane activity (Bashan et al., 2012; Bashan, Salazar, & Puente, 2009). Moreover, the size of the pots may be a limiting factor. Therefore, the results presented here should be considered only as an indicator to what might happen during real desert restoration.

In summary, this study showed that inoculation of both barren soils and mesquite seedlings growing in these soils significantly changed the bacterial community structure and composition of the soil over time. In particular, we detected taxa with functional roles in the

nitrogen cycle. The inoculated *B. pumilus* ES4 colonized young areas of the roots and gradually enhanced root biomass in successive growth cycles in the same soil indicating its potential improvement in soil health indicators and soil biodiversity.

ACKNOWLEDGMENTS

At CIBNOR, we thank Manuel Moreno for technical help, Alfonso Medel for help in heat map design, Juan-Pablo Hernandez for laboratory assistance, and Salvador Reyes for greenhouse assistance. This study was supported by 2013 UC Mexus-CONACYT Collaborative Research Grant "Searching for bacteria living in the rhizosphere of native desert plants in the Sonoran Desert that restore soil fertility to degraded land in Baja California," and writing time was provided by The Bashan Institute of Science, USA. M. C. G. is a recipient of a CONACYT graduation scholarship (364014). Support to A. M. H. and M. M. came from the Shanbrom Family Foundation. This is a contribution 2017-023 of the Bashan Institute of Science, USA.

AUTHORS' CONTRIBUTIONS

Cristina Galaviz acquired samples and performed most analyses and data presentation. Blanca R. Lopez executed the metagenomic analysis, data presentation, and writing of the paper. Luz E. de-Bashan performed data analyses and interpretations and helped in writing the paper. Ann M. Hirsch advised during the project and revised the manuscript. Maskit Maymon advised during the project, executed the metagenomic analysis, and revised the manuscript. Yoav Bashan managed the entire project and wrote the paper including the final version.

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How to cite this article: Galaviz C, Lopez BR, de-Bashan LE, Hirsch AM, Maymon M, Bashan Y. Root growth improvement of mesquite seedlings and bacterial rhizosphere and soil community changes are induced by inoculation with plant growth-promoting bacteria and promote restoration of eroded desert soil. *Land Degrad Dev*. 2018;1–14. https://doi.org/10.1002/ldr.2904