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REGULAR ARTICLE

Using native trees and cacti to improve soil potential nitrogen fixation during long-term restoration of arid lands

Nguyen E. Lopez-Lozano · Moisés G. Carcaño-Montiel · Yoav Bashan

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Abstract

Background and aims A major problem in all restoration and rehabilitation projects is to restore the function of the ecosystem. Nitrogen, next to water, is the most limiting factor for productivity of arid terrestrial ecosystems.

Methods We used a successful restored area, completed 10 years earlier, in comparison with an undisturbed scrubland area and a remaining, disturbed area in the southern Sonoran Desert in Baja California, Mexico. We compared the abundance of the *nif*H gene, estimated by qPCR, potential N_2 fixation activity by acetylene reduction assay, and diversity of diazotrophs by denaturing gradient gel electrophoresis in the rhizosphere of the most representative plant species, the cardon cactus and the mesquite used for restoration.

Results The abundance of N_2-fixing bacteria in the rhizosphere of cardon growing with mesquite had significantly higher abundance of *nif*H gene than the rhizosphere of cardon that grew separately. Across all samples, the potential N_2 fixation was significantly higher in soil samples from the restored site than samples from the undisturbed and disturbed sites.

Conclusions Successful long-term restoration improved the potential N_2 fixation to a level similar to undisturbed lands. Beneficial interactions between cardon and mesquite are a promising venue for desert reforestation by their contribution to improve N_2 fixing potential in degraded arid lands and increasing the population of diazotrophs.

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N. E. Lopez-Lozano · Y. Bashan
Environmental Microbiology Group, The Northwestern Center for Biological Research (CIBNOR), Av. IPN 195, La Paz, B.C.S. 23096, Mexico

N. E. Lopez-Lozano
División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), Camino a la Presa San José 2055, Lomas 4ta. sección, San Luis Potosí, S.L.P. 78216, Mexico

M. G. Carcaño-Montiel
Laboratorio de Microbiología de Suelos, Centro de Investigaciones en Ciencias Microbiológicas, Benemérita Universidad Autónoma de Puebla, Edificio 103-E. Ciudad Universitaria, Puebla, Puebla 72570, Mexico

Y. Bashan
The Bashan Institute of Science, 1730 Post Oak Court, Auburn, AL 36830, USA

Y. Bashan (✉)
Department of Entomology and Plant Pathology, Auburn University, 301 Funchess Hall, Auburn, AL 36849, USA
e-mail: ybb0001@auburn.edu

Y. Bashan
e-mail: ybashan@cibnor.mx

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Introduction

Ecological restoration is the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed. Ruiz-Jaen and Aide (2005) proposed that a restored ecosystem has many attributes. These include similar diversity and community structure as reference sites, presence of indigenous species, presence of functional groups necessary for long-term stability, normal functioning, integration with the landscape, and self-sustainability. It is very difficult to recover all these attributes after severe perturbation, in part because natural revegetation in highly disturbed areas is very slow, especially in arid lands. A shortcut strategy to long vegetative succession process and promote the potential return of most of the original attributes requires establishment of climax plants to accelerate revegetation (Moore and Russell 1990; Bashan et al. 2009a, b).

Restoration or rehabilitation projects had focused on aboveground vegetative communities. This has been done routinely for years without attention to the microorganisms involved, assuming that these will appear once trees and shrubs are established. Microbial populations in arid soils perform essential functions critical for supporting plant growth and revegetation success. Specifically in terrestrial arid ecosystems, next to water (Bacilio et al. 2011), nitrogen (N) is regarded as one of the most limiting factors for productivity (LeBauer and Treseder 2008; Bashan and de-Bashan 2010). In soils with high levels of carbonates, P or even Fe availability restrains productivity. The biological activity of microorganism can help to provide these essential nutrients. N supply in arid lands is mostly incorporated via biological fixation of atmospheric N_2 (Belnap 2002) because atmospheric deposition of nitrogen by lightning and fertilization is typically low (Peterjohn and Schlesinger 1990). With the essential role of N_2 fixation to ecosystem processes, the microorganisms can be used as an ecological indicator of the success of reforestation in arid zones and as a sensitive measure of environmental perturbations (Vovides et al. 2011), independent of the plant species.

Arid lands have heterogeneous distribution of resources, with greater nutrient concentrations and microbial densities occurring in vegetated areas than in bare soils. Nursing of various plant species by a pioneer plant is presumably aided by a large community of soil microorganisms, among them N_2 fixers and P-solubilizers. A well-known nurse plant–seedling association, known as a resource island, occurs between leguminous small trees and shrubs, cacti, and desert succulents (Bashan and de-Bashan 2010). The legume plants create, within the zone of their roots, a favorable environment for N_2-fixers. Unfortunately, information about the N_2 fixers in the rizhosphere of arid lands is limited, despite the fact that these microbial groups are necessary for the establishing of plants during secondary succession. In most arid areas, it is common to have a few dominant groups of N_2 fixers (Nagy et al. 2005; Gundlapally and Garcia-Pichel 2006). The most studied N_2 fixers in arid lands are the diazotrophs of microbial crusts, where free-living cyanobacteria are the principal contributors to the N budget of the crust. Mature crusts, with greater biomass, fixation rates, and stabilizing activity, contribute more to soil fertility than pioneer crusts. Molecular fingerprinting profiles showed that *nif*H sequence types were very similar between young and mature crusts. However, quantitative PCR showed that developed crusts contained approximately 30-fold more *nif*H gene copies than did young crusts. This suggests that the difference in N_2 fixation rates between the two crusts is most likely a consequence of the number of N_2-fixing organisms, rather than the result of a difference in composition of diazotroph species (Yeager et al. 2004). After severe disturbance, it may take up to 50 years for a crust to regain its original thickness and up to 250 years for mosses and lichens to reappear and flourish (Bashan and de-Bashan 2010). Although microbial crusts are important components of many desert ecosystems, there is very little evidence that N fixed in them becomes available to higher plants. Considering this occurrence, N_2 fixers in the rhizosphere of plants become more relevant.

To address the question of impact of restoration on potential nitrogen fixation in arid zone, we analyzed a specific case of field restoration with trees/shrubs and cacti which started 10 years ago (Bashan et al. 2009a, b; 2012). We hypothesized that the N_2-fixing community of rhizosphere soil samples from a long-term successfully restored area would resemble those of undisturbed soil regarding microbial abundance, diversity, and potential N_2-fixing activity and be greater than those microbial communities in disturbed soils. We also hypothesized that the combination of young cardon cactus

under the canopy of the mesquite nurse tree would improve N_2-fixing characteristics of the restored soil.

Consequently, the objectives of this study were to determine abundance, diversity, and potential N_2-fixing activity of the rhizosphere N_2-fixing communities below the dominant plant species, *Pachycereus pringlei* (giant cardon), *Prosopis articulata* (mesquite amargo), and *Jatropha cinerea* (Arizona nettlespurge, also known in Mexico as jatropha), in comparison with adjacent disturbed and undisturbed areas.

Material and methods

Study site

The sampling sites are located at El Comitan, 17 km northwest of La Paz, Baja California Sur (Southern Sonoran Desert), in the "Laura Arriaga ecological and research reserve" (24° 07′ 31.91″ N, 110° 25′ 41.80″ W). The area is an alluvial coastal plain of desert scrubland. Mean annual precipitation is 180 mm, mainly from tropical storms in late summer. Yet, many years have only 50–100 mm and droughts are common. Day temperatures in summer can exceed 40 °C, and extreme solar radiation (up to 2500 μmol photon m^2 s^{-1}) is common. The site was cleared of vegetation in the early 1980s for a road that was not built. Part of the site was restored in 2004 in an area of ~2000 m^2, using three species of trees mesquite amargo (*P. articulata* S. Watson), yellow palo verde (*Parkinsonia microphylla* Torr.), and blue palo verde or palo Junco (*Parkinsonia florida* Benth. (ex A. Gray) S. Watson) and a columnar-shaped cactus, the giant cardon (*P. pringlei* (S. Watson) Britton & Rose) (Bashan et al. 2000b). All trees were inoculated when transplanted to the field with two plant growth-promoting bacteria (PGPB), *Azospirillum brasilense* Cd (DSM 1843, the type strain of *A. brasilense,* Braunschweig, Germany) and *Bacillus pumilus*, strain RIZO1 no. FJ032016, GenBank (Hernandez et al. 2009), and an inoculum of arbuscular mycorrhizal fungi, mainly *Glomus* sp. combined with a consortium of unidentified native mycorrhizae species found in resource islands in the same area (Halvorson et al. 1994) under mesquite in the southern Sonoran Desert (Carrillo-Garcia et al. 1999; Bashan et al. 2000a). The site was maintained until 2009 by eliminating invasive grasses, excluding cattle, and preventing soil erosion; it was abandoned since then. Soil characteristics and the seven restoration trials were described in detail by Bashan et al. (2009a, b; 2012). Because the area is part of a protected natural reserve, there has been no impact by humans or free-ranging cattle for 10 years. The undisturbed area is located to the west of the restored area in the same reserve; it was never disturbed and contains the breadth of vegetation of the area (León de la Luz et al. 1996). The remains of the cleared area that was not restored was used as a negative control for the restoration trials. Currently, the disturbed site is dominated by *J. cinerea*, the most important pioneer plant in the region (Perea et al. 2005), and the invasive buffelgrass *Cenchrus ciliaris* L. that was eliminated from the restored area in 2004. We also analyzed differences in rhizosphere N_2-fixing communities under cardon, mesquite, cardon under the canopy of mesquite, nettlespurge (*Jatropha*), and where soil was without plants (open ground).

Sampling

Undisturbed and disturbed areas of 25 m × 25 m next to the restored site were selected randomly. In November 2013, five cardon (approximately 1 m high), five mesquite (2 m high), five cardon under the canopy of mesquite in the restored and undisturbed sites (1 and 2 m high, respectively), and five nettlespurge (~2 m high) in the disturbed site were chosen randomly; the only attribute that we considered was height, representing age of plants in these species. Rhizosphere soil samples below these plants and soil samples under open spaces were collected from each area after litter or rocks were removed. From the first 15 cm associated with the rhizosphere of these plants, 500 g of soil were collected with a small spade. From these, 10 g of each sample were stored at −80 °C for DNA extraction, and the remaining soil was stored in black plastic bags at 4 °C until processed in the laboratory for physicochemical analysis (less than 1 month). For acetylene reduction assays (ARA), soil samples were taken next to the base of the plant and from the open ground with 50-mL tubes to recover intact cores. The tubes were sealed and stored at 4 °C until the assays was performed (within 1 week). Sampling was done twice; results are presented for one of the sampling events.

Physicochemical analysis

Soil pH was measured in deionized water (soil/solution ratio, 1:2 *w*/*v*), using a pH meter (211, Hanna

Instruments, Woonsocket, RI). Organic matter, soluble P, and soil texture were determined, using standard techniques described in Jackson (1958). Total N was determined by the Dumas method in a nitrogen–protein analyzer (FP528, LECO Corp., St. Joseph, MI), nitrate by the method described by Strickland and Parsons (1972), and ammonium by the phenolhypochlorite method adapted to microplates (Solórzano 1969).

Acetylene reduction assay

N_2 fixation was assessed indirectly using the acetylene reduction assay measuring ethylene formation from acetylene. Three replicates per soil sample of 100 g were placed in 120-mL sterile serum vials and hydrated with 20 mL solution with 1 % D-glucose and 1 % malic acid. This was done because our goal was not to determine the actual N fixation rates, but rather compare potential N fixation between sites. Afterwards, the vials were hermetically sealed with serum stoppers and 20 % of the vial headspace was substituted with acetylene. The vials were incubated for 72 h at 30 °C. Then, 100 μL of the headspace gas was withdrawn and injected in a mass gas chromatograph (Finnigan Focus GC, Thermo Scientific, Waltham, MA) with a flame ionization detector and fitted with a column (TG-Bond Alumina, no. 26001-6080, serial no. 992068, Thermo Scientific), using nitrogen gas as a carrier. The temperature of the injector and the detector was 200 °C, and the total analytical time per sample ranges from 4 to 6 min. The ARA is expressed in nanomoles of ethylene produced per dry soil gram per day.

Molecular analysis

Soil DNA was extracted using the FastDNA SPIN Kit for Soil (116560200, MP Biomedicals, Santa Ana, CA). One additional cleaning step was added, supplementing 1 mL guanidine thiocyanate to the binding matrix in the SPIN Filter. The binding matrix was gently resuspended and centrifuged at 14,000 × *g* for 1 min, then the wash step of the protocol was continued. DNA was stored at −80 °C until further processing. To assess quantity and purity, the crude DNA extracts were run on 1.5 % agarose gels at 100 V for 1 h in 0.5 Tris–acetate–EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 8.0), using a fixed amount (2 μL) of a 1-kb DNA Ladder (10787018, Invitrogen, Carlsbad, CA) as the molecular size and quantity marker. After staining (SYBR Gold Nucleic Acid Gel Stain (S-11494, Life technologies, Carlsbad, CA)), DNA quality was determined based on the degree of DNA shearing (average molecular size), as well as the amounts of co-extracted compounds.

Quantification of nitrogen-fixing microbial communities

The abundance of nitrogen fixers was quantified by qPCR targeting the *nifH* gene, using the primers FPGH19 (Simonet et al. 1991) and PolR (Poly et al. 2001). Absolute quantification of each soil sample used the StepOne Real-Time PCR System (4376600, Applied Biosystems, Carlsbad, CA). Three reactions per replicate were performed using SYBR Green PCR Master Mix (Applied Biosystems). The specificity of the amplification products was confirmed by melting curve analysis, and the expected size (~400 bp) of the amplified fragments was checked in a 1.5 % agarose gel stained with SYBR Gold. The standard curves were obtained using serial dilutions of the vector plasmid, containing a cloned fragment of the gene of *A. brasilense* (pGEM-T Vector Systems, Promega, Madison, WI). The primer concentrations were optimized to close to 100 % efficiency. Inhibition in the PCR reactions was tested by mixing serial dilutions of DNA extracted from soil against a known amount of standard DNA before qPCR. The Ct values of the standard DNA did not change in the presence of the diluted soil DNA, indicating the absence of severe inhibition.

Denaturing gel electrophoresis analysis of the *nifH* gene

Denaturing gel electrophoresis analysis (DGGE) of a nested PCR was conducted for *nif*H genes (Diallo et al. 2004; Pereira e Silva et al. 2011). For the first PCR reaction, the primers FPGH19 (5′-TACGGCAARGGTGGNATHG-3′; Simonet et al. 1991) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′; Poly et al. 2001) were used. Two uL of the first PCR product were used as a template in the second reaction, where the primer PolF contains a GC clamp (5′-TGCGAYCCSAARGCBGACTC-3′) and AQER (5′-GACGATGTAGATYTCCTG-3′) (Poly et al. 2001). The integrity and size of the PCR products were determined by a 1.5 % (*w*/*v*) agarose TAE gel, stained with SYBR Gold. All soils were PCR-amplifiable, resulting in a single band of the expected size

(360 bp). DGGE profiles were generated, using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). PCR products (250–300 ng per lane) were loaded onto 6 % (*w*/*v*) polyacrylamide gels, 1 mm thick, in 1× TAE buffer with a 40–65 % denaturant gradient [100 % denaturant corresponded to 7 M urea and 40 % (*v*/*v*) deionized formamide to separate the generated amplicons]. Electrophoresis was performed at a constant voltage of 200 V for 6 h at 60 °C. The gels were stained for 30 min in 0.5 TAE buffer with SYBR Gold (final concentration 0.5 mg L^{-1}). Images of the gels were obtained with the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories) and stored as TIFF files. The images were analyzed using Quantity One 1-D Analysis 4.6.7 software to determine the Shannon diversity index, based on the number and intensity of the bands. Three samples of each treatment were chosen to analyze the similarities between the DGGE profiles, using the Jaccard similarity coefficient. Cluster analysis and Kruskal's non-metric multidimensional scaling (NMDS; Venables and Ripley 2002) were used in the vegan package for R (R Development Core Team 2008). Kruskal's stress coefficient was used to reflect the goodness of fit of the model. Values of Kruskal's stress <0.1 are considered a good fit. The brighter bands of the DGGE were excised from the gels, purified, reamplified, and sequenced. The identity of the sequences was confirmed by comparing them with the NCBI database using BLAST. For each sequence, the best hit was downloaded and incorporated to the alignments as a reference of the identity of the sequence. We used the program ModelTest (Posada and Crandall 1998) to determine the nucleotide substitution model with the best fit to the data. Tree reconstruction was performed with MEGA 6.0 (Tamura et al. 2007), using the neighbor-joining method with 1000 bootstrap replicates.

Statistical analyses

Statistical analyses were conducted using R for all samplings (R Development Core Team 2008). Sampling site and type of plant (including soil without plants) were assessed as dependent variables of soil parameters (pH, NO_3^-, NH_4^+, P, organic matter, texture (sand, silt, clay), activity of N_2 fixation, N_2 fixer abundance, and diversity) using one-way ANOVA. Normality of residuals was checked with the Shapiro–Wilk test. The post hoc Tukey test was used to determine group differences for significance. Moreover, to understand the variation of the data, we analyzed three dependent variables, ARA (in nmoles ethylene g^{-1} soil day^{-1}), abundance of the *nif*H gene (log converted to improve fit of model, in gene copies g^{-1} dw), and diversity, using the Shannon index. For each of these dependent variables, a general linear model was determined. The explanatory variables considered in these models were as follows: sampling site, type of plants, pH, NO_3^-, NH_4^+, P (mg soil kg^{-1}), organic matter, and texture (sand, silt, and clay in %). The starting model for all three dependent variables included all explanatory variables. For each dependent variable, the corresponding model that best fits data was obtained by removal of non-significant variables using backward deletion tests. Normality of residuals was checked with the Shapiro–Wilk test. All analyses set significance at $P<0.05$. Results show mean and standard error of the mean.

Results

Physicochemical characteristics of soils

Soil analyses were performed across sampling sites. As expected, the data revealed significant differences between various parameters (Table 1). Differences in nitrate content were significant between the restored site (3.30 ± 0.85 mg soil kg^{-1}) and the disturbed site (7.49 ± 1.37 mg soil kg^{-1}). Organic matter content was significantly higher ($P<0.001$) at the undisturbed site (1.58 ± 0.1) than at the restored (0.94 ± 0.12) and disturbed sites (0.90 ± 0.03). Small, non-significant variations in pH, NH_4, P, and soil texture (and, silt, clay) were found between sites (Table 1). pH was significantly higher in the rhizosphere of cardon (7.17 ± 0.07) than in soil without plants (5.66 ± 0.18). We found significantly higher concentration of nitrate in the rhizosphere of cardon growing with mesquite (7.48 ± 1.19 mg soil kg^{-1}) than in the rhizosphere soil of cardon growing alone (2.63 ± 0.65 mg soil kg^{-1}). P concentration was significantly higher in the rhizosphere of cardon growing with mesquite (23.65 ± 2.24 mg soil kg^{-1}) than in the rhizosphere of cardon and mesquite growing alone, 12.80 ± 1.81 and 14.28 ± 1.42 mg soil kg^{-1}, respectively (Table 1).

Abundance and potential activity of N_2-fixing bacteria

N_2-fixing bacteria quantified by *nif*H gene by qPCR showed significantly lower abundance in the disturbed site (4.61 ± 0.18 log gene copies soil g^{-1}), compared

Table 1 Soil physicochemical parameters (mean and ± SE) in the undisturbed, restored, and disturbed sites in the southern Sonoran Desert

	pH	NO_3 (mg soil kg^{-1})	NH_4 (mg soil kg^{-1})	P (mg soil kg^{-1})	OM (%)	Sand (%)	Silt (%)	Clay (%)
Sampling site								
Undisturbed	6.51 ± 0.20	7.47 ± 1.22^{a}	0.48 ± 0.08)	16.33 ± 1.77	1.58 ± 0.10^{a}	19.85 ± 2.34	76.44 ± 2.07	4.37 ± 0.48
Restored	6.90 ± 0.12	3.30 ± 0.85^{b}	0.44 ± 0.07	17.04 ± 1.89	0.94 ± 0.12^{b}	13.76 ± 3.22	81.39 ± 2.97	5.07 ± 0.33
Disturbed	6.21 ± 0.24	7.49 ± 1.37^{a}	0.68 ± 0.07	14.14 ± 2.60	0.90 ± 0.03^{b}	13.03 ± 0.33	82.17 ± 0.86	4.60 ± 0.89
Plant species								
Cardon	7.17 ± 0.07^{a}	2.63 ± 0.65^{a}	0.36 ± 0.10	12.80 ± 1.81^{b}	0.99 ± 0.18	13.31 ± 1.87	82.01 ± 1.82	4.48 ± 0.45^{ab}
Mesquite	6.47 ± 0.16^{ab}	6.28 ± 1.93^{ab}	0.36 ± 0.07	14.28 ± 1.42^{b}	1.33 ± 0.14	24.10 ± 4.78	71.82 ± 4.25	3.77 ± 0.60^{b}
Nursling cardon	6.56 ± 0.27^{ab}	7.48 ± 1.19^{b}	0.66 ± 0.07	23.65 ± 2.24^{a}	1.45 ± 0.18	10.10 ± 2.28	84.41 ± 1.97	5.86 ± 0.20^{a}
Jatropha	6.77 ± 0.26^{ab}	8.68 ± 2.32^{ab}	0.68 ± 0.07	13.20 ± 0.90^{b}	0.90 ± 0.06	12.60 ± 0.25	81.88 ± 0.85	6.08 ± 0.86^{a}
Soil without plants	5.66 ± 0.18^{b}	6.00 ± 0.98^{ab}	0.67 ± 0.18	14.70 ± 4.30^{ab}	0.90 ± 0.03	13.47 ± 0.70	82.57 ± 2.08	3.12 ± 0.99^{b}

Letters indicate significant differences between sampling site or between sampled plants (ANOVA, $P < 0.05$); no letters indicate no significant differences

OM organic matter

with that in the undisturbed and restored sites, 5.48 ± 0.15 and 5.33 ± 0.15 log gene copies soilg^{-1}, respectively. No significant differences in diazotrophs at undisturbed and restored sites were found (Fig. 1a). Grouping samples according to plant species showed that the rhizosphere of cardon growing with mesquite had significantly higher ($P < 0.001$) *nif*H gene abundance than the rhizosphere of cardon growing independently, 5.00 ± 0.09 and 5.63 ± 0.19 log gene copies soil g^{-1}, respectively. As expected, soil without plants showed the lowest *nif*H gene copy numbers, 4.24 ± 0.14 log gene copies soil g^{-1} (Fig. 1b).

Across all samples, potential N_2 fixation measured by ARA had small variations and was low, as expected for desert soils. Soil samples from the restored site yielded significantly higher ($P < 0.001$) ethylene (2.72 ± 0.03 nmol ethylene soil $g^{-1} \cdot day^{-1}$) than did samples from the undisturbed and disturbed sites, which were identical at 2.42 ± 0.05 and 2.42 ± 0.06 nmol ethylene soil g^{-1} day^{-1}, respectively (Fig. 2a). This suggests higher potential N_2 fixation at the restored site. Analysis of data sorted by plant species only showed significant differences between mesquite and soil without plants, 2.66 ± 0.06 and 2.39 ± 0.09 nmol ethylene soil g^{-1} day^{-1}, respectively (Fig. 2b).

Community diversity and structure based on the DGGE of *nif*H gene

Diversity of N_2-fixing communities was determined by DGGE profiles of the *nif*H gene. The number of bands and intensity was used to calculate the Shannon index of each sample. Comparisons of samples from disturbed, undisturbed, and restored sites did not show significant differences in diversity (Fig. 3a). Regarding plant species, only the N_2-

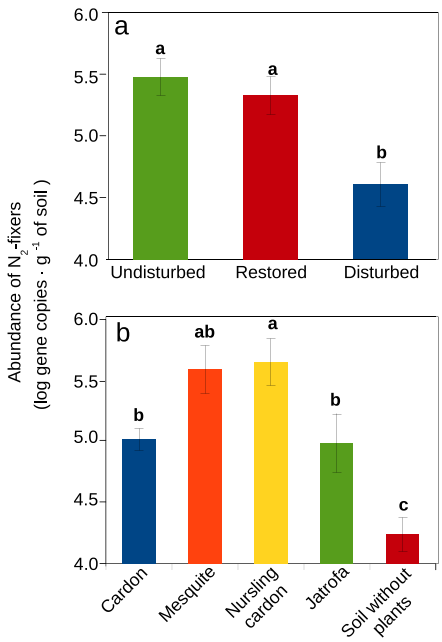

Fig. 1 **a** Real-time PCR quantification of the *nif*H gene in the undisturbed, restored, and disturbed sites in the arid soil of the study area. **b** Quantification of the *nifH* gene in the rhizosphere of different plant species and in soil without plants. Columns denoted by a different *letter*, in each subfigure separately, differ significantly by one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$

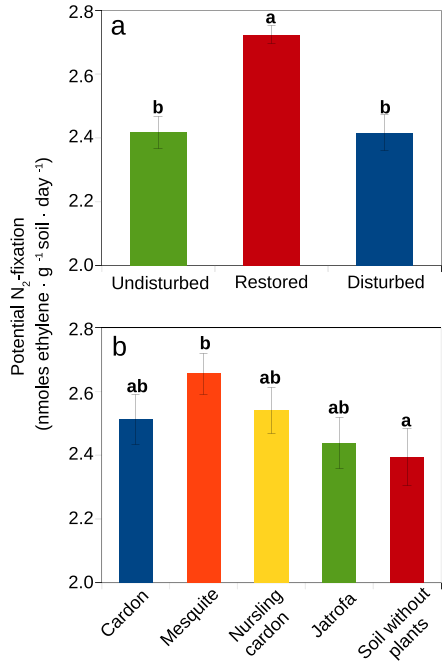

Fig. 2 **a** Potential activity of N_2-fixing bacteria measured by acetylene reduction assay (ARA) in the undisturbed, restored, and disturbed sites in the study area. **b** Quantification of ARA in the rhizosphere of different plant species and soil without plants. Columns denoted by a different *letter*, in each subfigure separately, differ significantly by one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$

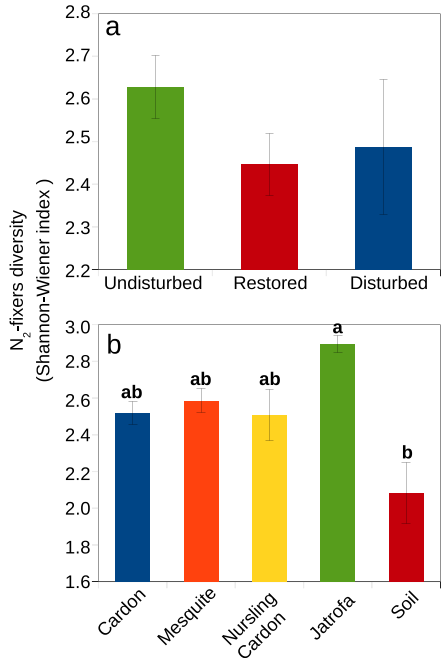

Fig. 3 **a** N_2 fixer diversity showed as Shannon–Wiener index calculated using the number and intensity of the DGGE bands obtained using the *nif*H gene amplifications in the undisturbed, restored, and disturbed sites in the southern Sonoran Desert. **b** Quantification of ARA in the rhizosphere of different plant species and in soil without plants. Columns denoted by a different *letter*, in each subfigure separately, differ significantly by one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$. No *letters* indicate no significant differences

fixing community below *Jatropha* was significantly more diverse than the community in soil without plants, with Shannon indices of 2.89 ± 0.05 and 2.08 ± 0.17, respectively (Fig. 3b). To establish a relationship between diversity, potential activity, and abundance of N_2-fixing communities, correlation tests were performed. No combination showed significant correlation (data not shown).

Overall results of the similarity analysis show distinctive clusters of PCR-DGGE profiles, related to the type of plant present where the sample was taken (Fig. 4a). These clusters were similar to results obtained from Kruskal's NMDS analysis (stress factor $S = 0.0018$; Fig. 4b), which suggested a particular community structure for each kind of plant. Sixteen groups of *nif*H sequences were recovered from reamplifications from DGGE bands. The majority of sequences could not be associated to known sequences in public databases. We found one sequence related to *Azospirillum*, five related to *Rhizobia* and one to *Pseudomonas*. The bands corresponding to these groups were present in all samples (Fig. 5).

Influence of physicochemical and biological soil characteristics on the N_2-fixing community

The effect of physicochemical variables, sampling site, plant species, and site–plant interaction on abundance, activity, and diversity of N_2-fixing bacteria was tested by general linear models. In the final model for potential N_2 fixation activity measured by ARA (ARA = Site + Species), half the variation was explained by the sampling site (36.03 %) and plant species (15.63 %; Table 2). None of the physicochemical variables had a significant influence on the N_2 fixation activity (Table 2). The general linear model for N_2-fixing abundance (abundance = Site + Species + NO_3^- + OM + Sand) showed that about 40 % was explained by the sampling site (22.87 %) and the plant species (28.05 %). Also, pH, organic matter, and amount of sand showed small but significant influence over N_2-fixing abundance, 11.78, 7.06, and 8.54 %, respectively (Table 2). In addition, N_2-fixing diversity (diversity = Site + Species + Sand + Site <> Species, where <> means interaction between

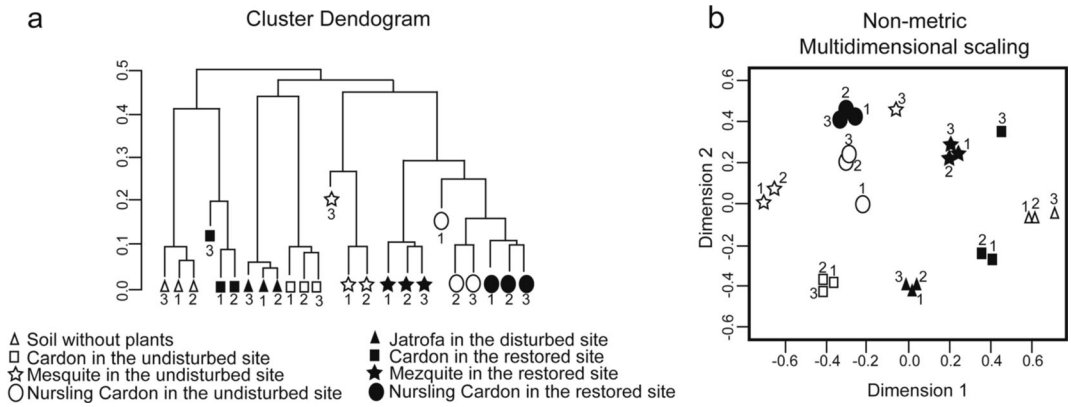

Fig. 4 Comparison of the rhizosphere community structure of N_2 fixers in sampled plants and sites. **a** Dendrogram from cluster analyses obtained using Jaccard's similarity coefficient. **b** Configuration derived from Kruskal's non-metric multi-dimensional scaling analysis of bands. Data with the same *symbols* represent replicates within each treatment. *Numbers* indicate the replicates

the variables) was explained mostly by the kind of plant (34.76 %) followed by sampling site (10.93 %), and the interaction between them (10.20 %). Amount of sand had a small but significant influence on a N_2-fixing diversity of 6.41 % (Table 2).

Discussion

Drastic disturbances that remove vegetation and topsoil deteriorate biological productivity and functional diversity of soil microbial communities (Buckley and Schmidt 2001; Ponder and Tadros 2002; DeGrood et al. 2005). The effect of disturbances can be long-lasting because the natural recovery of the original vegetation is slow and very slow in arid regions (Drezner 2006). Restoration strategies that include native plants, including leguminous N_2-fixing trees, to rehabilitate degraded lands are efficient in reestablishing nutrient cycling processes in tropical forest (Macedo et al. 2008). Few restoration projects using this strategy have been performed in arid lands (Bainbridge 2007), and even fewer using PGPB as aids to restoration. Most studies were done in shadehouses and greenhouses (Carrillo-Garcia

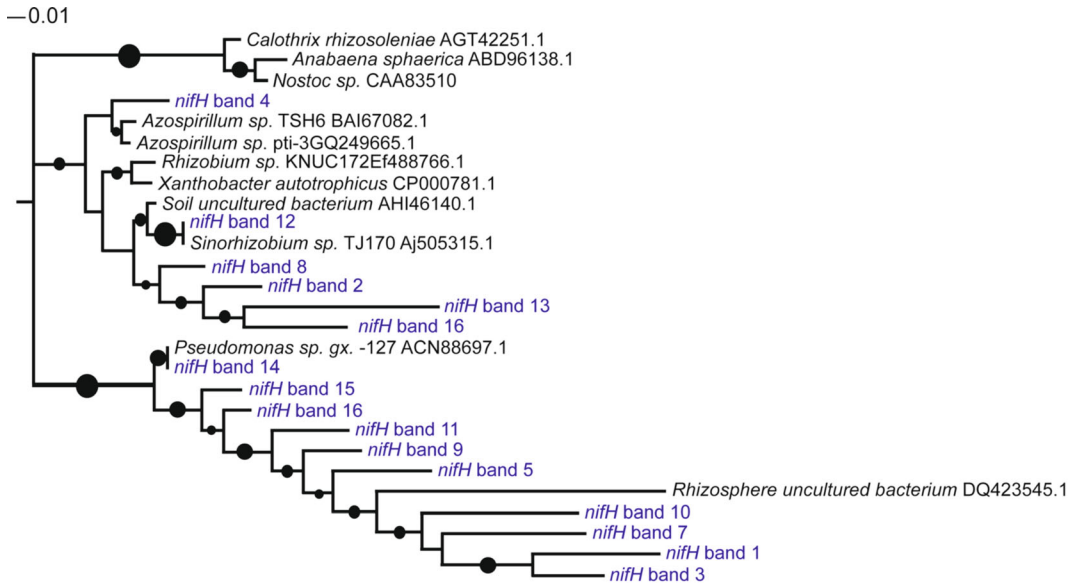

Fig. 5 Neighbor-joining tree for the *nif*H gene sequences reamplified from the DGGE bands. *Grey circles* indicate nodes with bootstrap support >0.5. The sequences from DGGE bands are shown in *blue*

Table 2 Summary of general linear models to determine which soil property predicts the potential N_2 fixation and N_2 fixer abundance and diversity

	N_2 fixer abundance[a]			Potential N_2 fixation ARA[b]			N_2 fixer diversity[c]		
	DF	*P* value	% SS	DF	*P* value	% SS	DF	*P* value	% SS
pH	1.11	0.087		1.23	0.320		1.2	0.942	
Nitrate	1.16	**0.009**	**11.78**	1.27	0.116		1.6	0.785	
Ammonium	1.9	0.545		1.13	0.806		1.13	0.418	
Organic material	1.16	**0.036**	**7.06**	1.2	0.846		1.11	0.491	
P	1.2	0.762		1.7	0.796		1.15	0.280	
Sand	1.16	**0.023**	**8.54**	1.10	0.936		**1.21**	**0.012**	**6.41**
Silt	1.5	0.710		1.17	0.687		1.5	0.886	
Clay	1.8	0.742		1.6	0.865		1.8	0.938	
Plant species	3.16	**0.003**	**28.05**	4.30	**0.040**	**15.63**	**4.21**	**<0.001**	**34.76**
Sampling site	2.16	**0.003**	**22.87**	1.30	**<0.001**	**36.03**	**1.21**	**0.001**	**10.93**
Plant x site	1.6	0.994		1.15	0.759		**2.21**	**<0.001**	**10.20**

Significant values are in bold

DF degrees of freedom, *% SS* percentage of the total sum of squares explained by each variable in the final model, *X* interaction

[a] Adjusted R^2 0.675; $P = 0.0004$

[b] Adjusted R^2 0.537; $P = 0.0002$

[c] Adjusted R^2 0.754; $P < 0.001$

et al. 2000; Requena et al. 2001; Carrillo et al. 2002; Puente et al. 2004; 2009b; Bacilio et al. 2006; Grandlic et al. 2008; Bashan et al. 2009b; Solís-Domínguez et al. 2011; Lopez et al. 2012) and a handful in the field (Bashan et al. 1999; 2009a, b; 2012; Toledo et al. 2001). Our objective was to use one successful former long-term restoration project to compare the abundance, potential activity, and diversity of diazotrophs in the rhizosphere of the most representative tree species used in this restoration, a project completed 10 years earlier. We compared the restored site to an adjacent undisturbed scrubland area and to a disturbed area nearby. Given the length of time that passed since the trials with PGPB, we did not search for the original inoculated diazotrophic PGPB, which are not known to survive in soil for very long periods (Bashan 1999). We focused on one of the outcomes of the restoration project—restoration of one fundamental function of the restored area under mesquite and cardon, trees that survived better than other trees tested in the restoration project (Bashan et al. 2009a, b; 2012). Our results suggest that, in general, the restored area recovered its N_2-fixing function over time and that different N functional traits tested in this study among the sampling sites were affected principally by the plant species.

Cardon, mesquite amargo, and Arizona nettlespurge (*Jatropha*) are dominant plants in the Southern Sonoran Desert. Cardon is a slow-growing, tree-shaped cactus that can stabilize a large area of topsoil around the plant (Bashan et al. 2009a, b). Their participation in accelerated soil formation from rocks is mediated by associations with rhizoplane and endophitic bacteria (Puente et al. 2004; 2009a, b). Mesquite is a N_2-fixing leguminous tree, well known as a nurse tree that establishes resource islands that facilitate growth of many desert plants (Felker and Clark 1980; Carrillo-Garcia et al. 1999; Bashan and de-Bashan 2010). Both have significant ecological importance and are late succession species. In contrast, *Jatropha*, which we found only in the disturbed area, is the most important pioneer plant in this area (Perea et al. 2005) that commonly disappears in later succession. Its potential as a N_2-fixing plant is unknown, but this possibility, detected in our study, could partly explain its success as a pioneer. The disturbed site is also dominated by buffelgrass (*C. ciliaris*), an invader that threatens arid and semiarid ecosystems because it eventually crowds out native flora and alters ecosystem processes (Marshall et al. 2012). The restored site has been free of buffelgrass since it was mechanically removed in 2004 during restoration and remained buffelgrass-free

ever since. These facts suggest that restoration using cardon and mesquite can enhance, in a yet-to-discover way, resistance to invasion by exotic plants.

Higher abundance of N_2 fixers in the rhizosphere of mesquite was expected, in comparison with the non-leguminous cardon or *Jatropha*. Yet, the highest abundance of N_2 fixers occurred in the rhizosphere of cardon nursed by mesquite, where their roots mingle (Carrillo-Garcia et al. 1999). This occurs in arid and semi-arid areas because legumes, such as mesquite, nodulate, which is a significant attribute to enhance N_2 fixation rates (Sprent and Gehlot 2010). There is experimental evidence to show that some plants benefit from closely associated neighbors, a phenomenon known as facilitation (Padilla and Pugnaire 2006). Key species that minimize the negative environmental effects could be used to improve performance of nearby target species, particularly where restoration fails because of harsh conditions or intense herbivory. This work measured how different plant species (the most abundant and the restoration species) restore potential nitrogen fixation independent of their inherent capacity to nodulate. Although the best known N_2-fixing bacteria in leguminous plants are endophytes, where their importance for the ecosystems process is undeniable (Sprent and Gehlot 2010), there are many free-living N_2 fixers in the rhizosphere of many species of plants that are potential N contributors. Many ecosystems lack large numbers of plants with symbiotic N_2 fixers, and N_2 free-living fixers likely represent the dominant biological source of new N to those ecosystems (Reed et al. 2011). The presence of potentially symbiotic N_2-fixing plants by themselves may not be a reliable indicator of actual N inputs in the field. Symbiotic N_2 fixers with the potential to nodulate do not always fix N_2 in natural environments. Beside the abundance of N_2 fixers, we showed higher potential of nitrogenase activity where mesquite served as a nurse plant in restoration management procedures to improve success of these projects. During the wet growth season in this desert, this may provide some nitrogen to the restored area, possibly explaining the healthy condition of the plantation after one decade.

Factors that determine community structure and abundance of N_2 fixers and their activity remain less understood in natural systems. Because N_2 fixation is one of the most metabolically costly processes (Simpson and Burris 1984), field and laboratory evidence suggests that when fixed N is available in the environment, N_2 fixers preferentially use it rather than fix N_2 (Barron et al. 2008; Cusack et al. 2009; Reed et al. 2011). Our general linear model for abundance of N_2 fixers at all sites showed that NO_3^- and organic matter explained a significant amount of the variation. Lower NO_3^- concentrations at the restored site suggest lower N availability, probably because nitrogen is consumed by the growing plants. This could stimulate the N_2-fixing community and reflect a higher potential of N_2 fixation at this site. At the undisturbed site, the N_2 fixer population, measured by qPCR, did not differ from the restored site, but they are potentially fixing less N_2 because N availability in this site is higher. At the disturbed site, N_2 fixers were less abundant, hence less potential N_2 fixation, with less organic matter as a factor that limits the reducing power needed to fix N_2. Given that N_2 fixation is a high energy-demanding process, microorganisms with this capacity have relatively high demands for ATP. As a consequence, there are high demands for P (Vitousek et al. 2002). An increase of soluble P in the surrounding media could compensate for a higher demand for P. An interesting finding was that the highest soluble P concentration in the soil came from below the resource island of cardon and mesquite. There are at least three plausible explanations for this phenomenon. (1) The rhizoplane and endophyte bacteria of cacti have the capability to solubilize phosphate by weathering the minerals (Puente et al. 2004; 2009a, b; Lopez et al. 2011). (2) A less likely explanation, and still an open question, is that the original inoculant of *B. pumilus*, a P-solubilizing bacteria, survived for a decade in the rhizosphere. Our study did not attempt to recover a genetic sequence of this bacterium from the site. (3) The mycorrhizal potential of this association, commonly associated with P-solubilization and transport in the resource island habitat, is higher than under barren, disturbed land (Bashan et al. 2000a). This study suggests that the cardon–mesquite association is a successful combination with high potential for restoration projects because they maintain an associative microbiota that can potentially satisfy the demands for P needed to fix N_2. However, microbial activity measured in vitro cannot be directly compared to in situ fluxes and turnover rates (Röling 2007) because it is necessary to provide all the elements to activate the microbial populations in samples, specifically, to provide the appropriate humidity and carbon source.

We found no significant difference in diversity of diazotrophs among the sites (Shannon index) based on the number and intensity of bands determined by DGGE

patterns. Analysis of the N_2 fixers' community structure suggests a specific composition for each plant species, but there were no clear differences between sites. A variation in the number of bands was observed between replicates, where only the samples of the soil without plants showed a tendency of having less diversity. Interestingly, the DGGEs bands that show higher variation between samples correspond to unidentified diazotrophs and could not be matched to sequences of cultivable microorganisms in public databases. Sequences related to *Azospirillum*, *Rhizobia*, and *Pseudomonas* were present in all samples, indicating that these groups are widely distributed, as was expected. It should be noted that, although *A. brasilense* strain Cd was inoculated 10 years earlier, this strain was not expected to survive at the time we conducted the analyses. The longest survival time in the rhizophere of three cacti species, including cardon, of *A. brasilense* was 2 years (Bashan et al. 1999). Because the sequence of *A. brasilense* was not specifically targeted in this study, it is still a question whether these arid soils contain indigenous *Azospirillum* or the sequences detected are leftovers from inoculation done a decade earlier. Our results showed that the diversity of N_2 fixers in the arid zone soil does not vary significantly, contrary to their abundance and activity. This suggests that the difference in N_2 fixation rates could be a consequence of the number of N_2-fixing organisms, rather than the differences in diazotroph species composition, as has been suggested previously for microbial crusts (Yeager et al. 2004).

It is important to mention that the primers used in this study are degenerated and thus represent a mix of primers with different binding affinities for different templates. This degeneracy is necessary because it allows for greater coverage of genes that are highly polymorphic, such as nitrogenase. Nevertheless, the number and intensity of bands in a gel do not necessarily provide an accurate status of the microbial community since one microorganism may produce more than one band. Another major deficiency of the DGGE technique is that a single gel can analyze only a limited number of samples and a straightforward and robust protocol for making meaningful DGGE gel-to-gel comparisons remains elusive (Valentín-Vargas et al. 2013). From these drawbacks, the parameters calculated from DGGE fingerprints should be interpreted as indications and not absolute measurements of microbial diversity. For this reason, we decided to limit the analysis to diversity and not compare more deeply the structure of the communities.

In conclusion, our results suggest that successful long-term restoration yields a potential capacity for N_2 fixation to reach levels equal to those in an undisturbed land. Beneficial interactions between cardon and mesquite established by long-term restoration are a promising venue for desert reforestation, not only by long-term survival of the trees but also by their potential contribution to improve the N_2 fixation in degraded arid soils by increasing the population of diazotrophs. Novel unknown diazotrophs, intrinsic to each plant species, are waiting to be discovered and identified.

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