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## Endophytic bacteria in cacti seeds can improve the development of cactus seedlings

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## ABSTRACT

A plant–bacterium association between the giant cardon cactus *Pachycereus pringlei* and endophytic bacteria help seedlings establish and grow on barren rock. This cactus, together with other desert plants, is responsible for weathering ancient lava flows in the Baja California Peninsula of Mexico. When cardon seeds are inoculated with endophytic bacteria, the seedlings grow in pulverized rock for at least a year without fertilization and without showing distress. The bacteria–plant association released significant amounts of necessary nutrients from the substrate. When endophytic bacteria were eliminated from the seeds by antibiotics, development of seedlings stopped. In complementary experiments of sterile seeds inoculated with the same endophytic bacteria, plant growth was restored. This study and the previous one show that, under extreme environmental conditions, a symbiotic relationship is present between endophytic bacteria and their cactus host.

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## 1. Introduction

Several species of desert plants, mainly cacti, grow without soil on rocky cliffs, large rocks, and ancient lava flows in hot desert areas of the Baja California Peninsula of Mexico where weathering is not apparent (Bashan et al., 2002, 2006). Higher plants are known to considerably affect the kinetics of dissolution of basalt in other environments (Hinsinger et al., 2001). Chemical weathering of lava flows beneath mature forests continues unabated for thousands of years after initial colonization (Cochran and Berner, 1996). In Iceland, the rate of weathering of rock by higher plants is two to five times higher in vegetated areas than in barren areas (Moulton and Berner, 1998). Involvement of microorganisms in these processes is inherent because they are widely present in bulk soil (Berthelin et al., 1991). Mycorrhizal fungi in European coniferous forests that grow on shallow granite rock are able to penetrate and dissolve the rocks. Dissolved products are translocated by the host plant roots,

bypassing the soil solution and bypassing competition for nutrient uptake by other organisms (van Breemen et al., 2000). *Frankia* and *Alpova diplophloeus* assisted growth, nitrogen fixation, and mineral acquisition by *Alnus tenuifolia* (Yamanaka et al., 2003).

In deserts, the rhizoplane population of rock-dwelling cacti contains many plant growth promotion traits, such as a capacity to dissolve minerals, fix nitrogen, and promote plant growth (Puente et al., 2004a,b). These plants contain endophytic bacteria with similar growth-promoting traits (Puente et al., 2009).

This study explored the potential of these endophytic bacteria to promote plant growth of cardon cacti. We hypothesized that the endophytes are essential for normal growth of cactus and, if removed, plant growth is impaired.

## 2. Materials and methods

## 2.1. Organisms

Seeds of the cardon cactus (*Pachycereus pringlei* [S. Watson] Britton & Rose) were used in all experiments. The following strains of endophytic bacteria were used and their nucleotide sequences were deposited in GenBank: EF123226 *Klebsiella* sp. SENDO 1, EF123227 *Acinetobacter* sp. SENDO 1, EF123229 *Pseudomonas* sp. SENDO 2, EF123230 *Bacillus* sp. SENDO 6, EF123231 *Klebsiella* sp. SENDO

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2, EF123233 *Staphylococcus* sp. SENDO 2 (Puente et al., 2009). Two positive controls, the plant growth-promoting bacteria (PGPB) *Pseudomonas putida* R-20 (Meyer and Linderman, 1986) and the PGPB *Azospirillum brasilense* Cd, ATCC 29710 were also used. Additionally, several mixtures of bacteria were tested: each of the six endophytes listed above, endophytes with each of the PGPB, and endophytes with both PGPBs.

### 2.2. Inoculation of cardon cacti grown in pulverized rock with endophytic isolates

Ancient extrusive igneous rocks (lava flows) were subjected to bacterial weathering experiments after being oven-sterilized (250 °C, overnight), pulverized in a mill, and sieved to obtain <90- $\mu\text{m}$  particles (rock flour). Rock flour (4 g) was mixed with 23 g pulverized perlite and placed in small black pots. For a negative control, pots were filled with 27 g perlite. Cardon seeds were washed thoroughly with 2% detergent (Tween 20) for 10 min to remove residual dust. Seed surfaces were disinfected with 3% commercial hypochlorite bleach for 5 min and then rinsed continuously for 10 min with sterile distilled water. Seeds were inoculated with a selected species of bacteria by a standard vacuum infiltration technique (Puente and Bashan, 1993) at a concentration of  $1 \times 10^6$  CFU ml<sup>-1</sup>. Briefly, seeds were inoculated by dipping them in bacterial suspensions for 5 min under a vacuum of 600 mm Hg. Then, the vacuum was released abruptly, allowing the bacteria to penetrate seed cavities that were previously filled with air.

Ten seeds were placed on the surface of the substrate in each pot, which had been irrigated with 50 ml distilled water, and then covered with a 5-mm layer of dry substrate. The pots were incubated in a growth chamber (Biotronette Mark III, Barnstead International, Dubuque, IA) at  $30 \pm 2$  °C under light intensity of  $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 12 h for 12 months. Pots were irrigated every 15 days with 25 ml basal Hoagland's nutrient solution without phosphorus and nitrogen (for the two strains of *Klebsiella* sp., mixture of endophytic bacteria, and controls) or with nitrogen, but without phosphorus (for *Staphylococcus* sp., *Acinetobacter* sp., two strains for *Pseudomonas* sp.). The perlite negative controls were also irrigated with phosphorus. The positive control plants were inoculated with a phosphate solubilizing bacterium, *P. putida*, and grown in perlite only, irrigated with Hoagland's nutrient solution containing nitrogen, while those inoculated with the diazotroph *A. brasilense* and grown in perlite were irrigated with Hoagland's nutrient solution with phosphorus but without nitrogen. Uninoculated plants served as controls. One additional positive control was irrigated with Hoagland's nutrient solution without phosphorus or nitrogen and all bacteria and the other positive control was irrigated with complete Hoagland's nutrient solution. Before and after plants were grown in the rock flour with perlite, the rock was analyzed for P<sub>2</sub>O<sub>5</sub>, total phosphorus, K<sub>2</sub>O, MgO, and Fe<sub>2</sub>O<sub>3</sub>, as described in the previous paper (Puente et al., 2009). At the end of the experiment, plants were extracted and photographed. Height and volume (Bashan et al., 1999) and root length and dry weight were measured. The drying oven was set at 50 °C for 120 h. Total nitrogen content of the plants was measured by an automatic, micro-Kjeldahl method after digestion of the samples (Digestion System 12.1009 and Kjeltac Auto 1030 Analyzer, Tecator, Höganäs, Sweden).

### 2.3. Elimination of endophytic bacteria in seeds treated with antibiotics

Since all batches of cardon seeds collected from wild plants contained bacterial endophytes, an attempt was made to eliminate them and produce endophyte-free seedlings by soaking seeds in the following antibiotic solutions ( $\mu\text{g ml}^{-1}$ ) for 20 min: chloramphenicol (500); streptomycin sulfate (200), tetracycline (12), penicillin G

(500), rifampicin (150), and mixtures of all antibiotics at the above concentrations. To assay for sterile seeds, 1 g of surface-disinfected seeds was pulverized with a pestle and mortar in 10 ml PBS. The homogenized slurry was transferred to tryptic soy broth (50 ml) and incubated with agitation (120 rpm) for 24 h at 30 °C. Then, 1 ml aliquots were taken from each suspension of bacteria and assayed for cultivable bacteria by the plate count method, total bacteria by the FITC method, and viable bacteria by the FDA method (Puente et al., 2009). For comparison, 1 g of seeds was treated with 1 ml of each of the antibiotics in test tubes and incubated for 3 h under the same conditions. The same assays for bacteria were made with the three methods listed here to decide which antibiotic (or combination) worked best. Seeds of lodgepole pine *Pinus contorta* Dougl. ex Loud., which are susceptible to antibiotic applications, served as a control for the germination tests.

### 2.4. Field emission scanning electron microscopy

For this test, root samples (0.5–1.5 cm long) were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) immediately after field sampling and sliced in half with a sterile razor. The following day, roots were rinsed in cacodylate buffer, dehydrated in a series of increasing ethanol concentrations from 30 to 100% for 20 min each, and dehydrated with isoamyl acetate. After dehydration, samples were dried with CO<sub>2</sub> in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD). The dried samples were fixed to stubs with double-sided adhesive tape and coated with 30 nm 60:40% gold:palladium alloy foil in a sputter coater (Edwards S150B) and then examined at 7 kV with a field emission scanning electron microscope (AmRay 3300FE, Advanced Metals Research, Bedford, MA, USA).

### 2.5. Mineral analyses

After cultivation trials lasting 1 year, plants were removed and the remaining substrate was analyzed for minerals (K<sub>2</sub>O, Fe<sub>2</sub>O<sub>3</sub>, and MgO) by EPA Method 3015-microwave digestion (nitric acid) (Kingston, 1994) with an atomic absorption spectrometer (GBC Scientific Equipment, Dandenong, Victoria, Australia). Concentrations of phosphate (P<sub>2</sub>O<sub>5</sub>) were determined according to Jackson (1958). Substrate without plants but under the same conditions served as control.

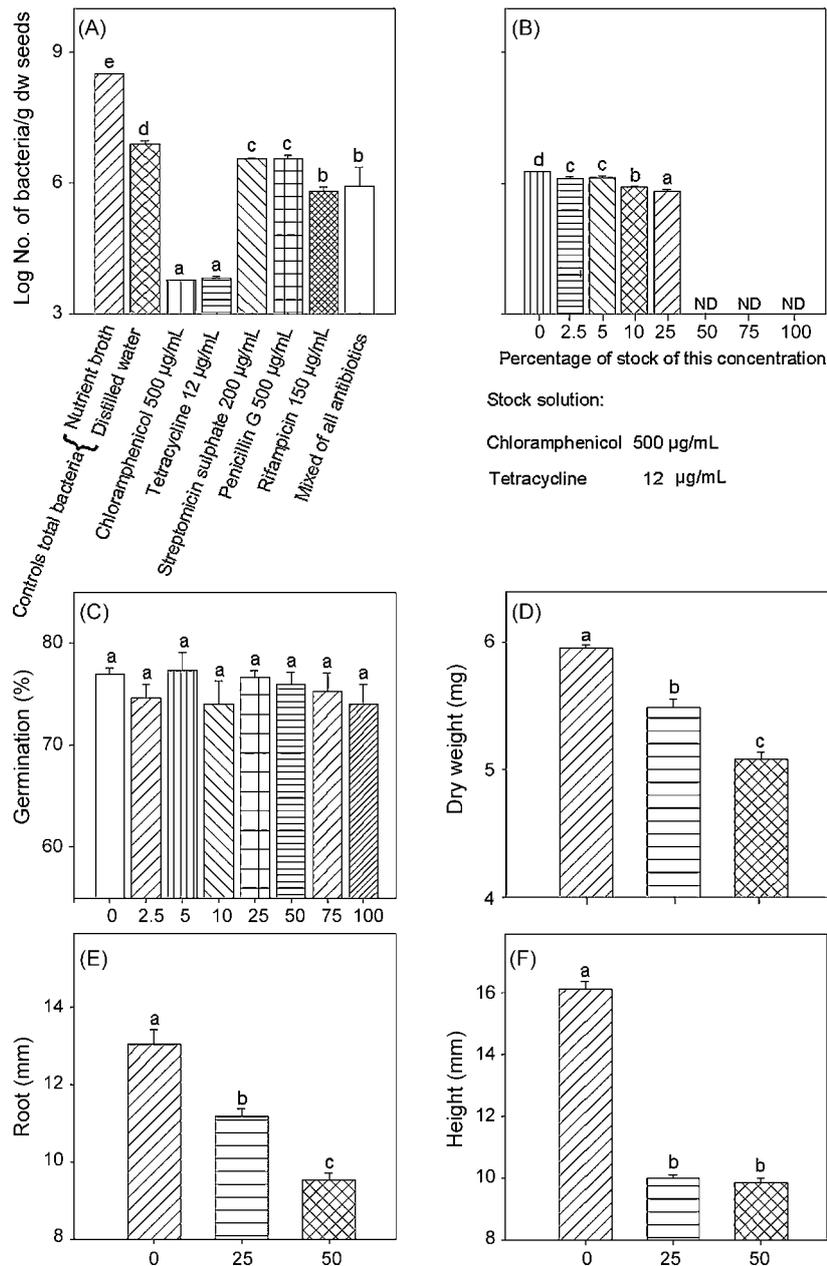
### 2.6. Experimental design and statistical analysis

Plants were inoculated in 10 pot replicates thinned to 3 seedlings per pot. Pots were distributed randomly in the growth chamber, and occasionally rearranged during 12 months of cultivation. Germination tests were made with 5 replicates, where 1 Petri dish containing 25 seeds served as a replicate. Triplicate samples were analytically assayed. One-way ANOVA, followed by Tukey's ad-hoc analysis or Student's *t*-test at  $P < 0.05$  was made with statistical software (Statistica vers. 6, StatSoft, Tulsa, OK). Numerical data are accompanied by standard errors.

## 3. Results

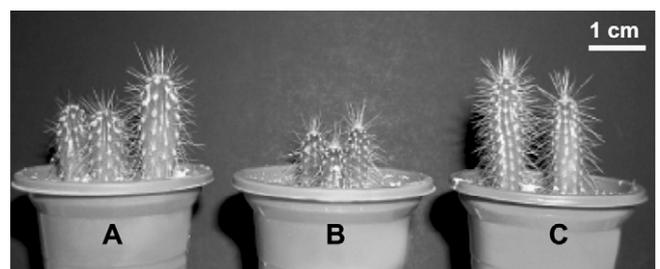
### 3.1. Production of endophyte-free seedlings and the effect of endophytes on seedling development

To evaluate the importance of endophytic bacteria in seeds developing into seedlings, seeds were treated alone and with combinations of antibiotics to eliminate the endophytic populations. While each antibiotic treatment reduced the initial endophytic population (all values in bacteria g<sup>-1</sup> dw seeds), from  $760 \times 10^6$  to  $>1 \times 10^6$ , only the combination of chloramphenicol and tetracycline

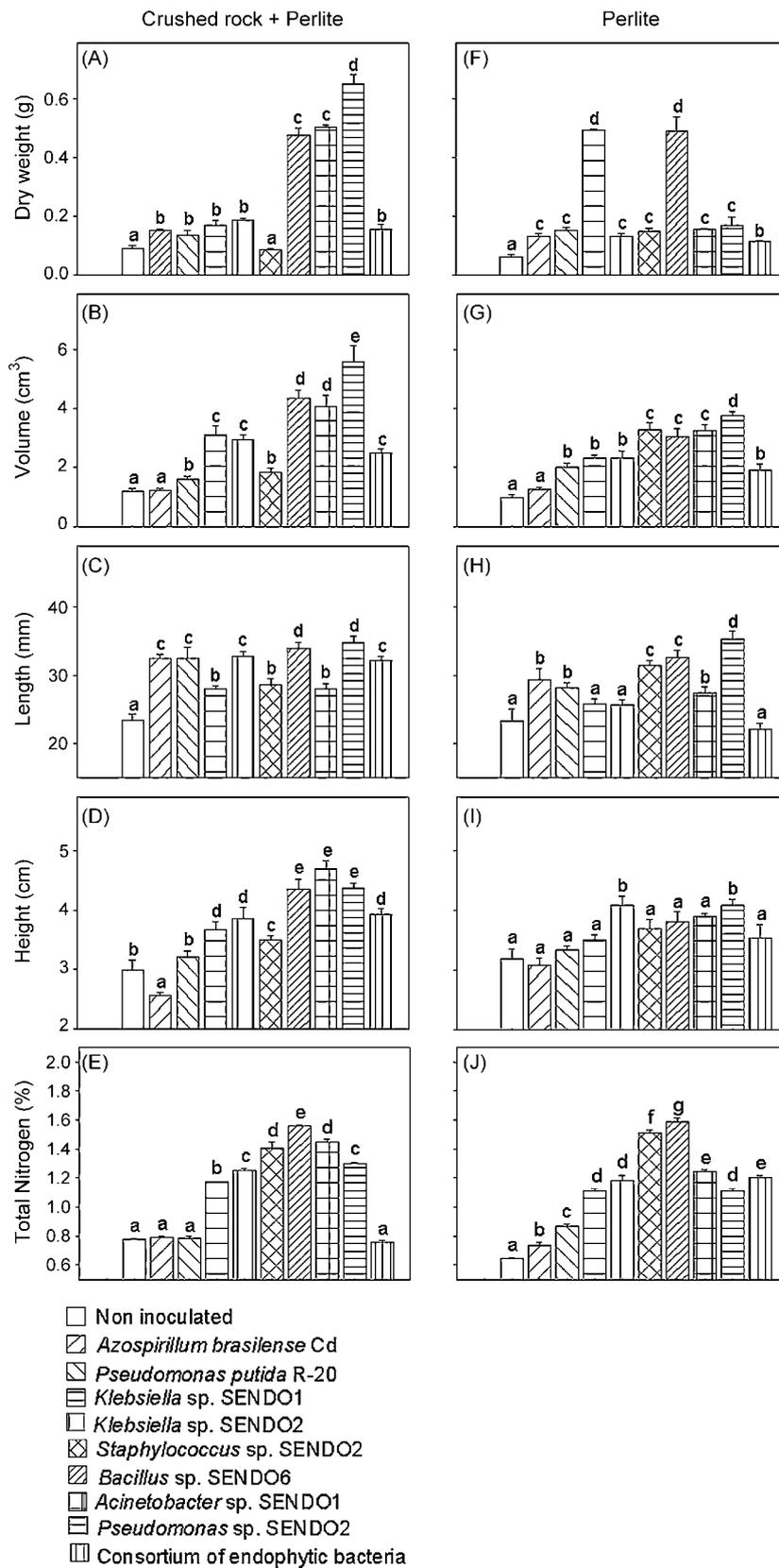


**Fig. 1.** (A) Viable endophytic bacteria count (by FDA) after treatment with different antibiotics. (B) The same counts under different concentrations of an antibiotic mixture (mixture contained: chloramphenicol 500 µg ml<sup>-1</sup> and tetracycline 12 µg ml<sup>-1</sup>). (C) Effect of different concentrations of an antibiotic mixture on germination of cardon seeds. Effect of two concentrations of an antibiotic mixture on dry weight (D), root length (E), and height (F) of cardon seedlings growing for 10 days in pulverized igneous rock substrate after treatment. Columns denoted by a different letter, in each subfigure, differ significantly at  $P < 0.05$  by one-way ANOVA. Bars represent SE. The absence of SE indicates a negligible value. The experiment was repeated five times.

initially reduced bacteria to  $\sim 1 \times 10^3$  (Fig. 1A). Therefore, a mixture of the two antibiotics was further evaluated for later seed treatments. Dilutions of the antibiotic mixture below 50% of the original concentration failed to eliminate endophytes. Concentrations >50% completely eliminated endophytes (Fig. 1B). When germination was evaluated after 11 days, antibiotic mixtures at any dilution (2.5–100%) had no deleterious effect on cactus seed germination (Fig. 1C) and did not affect germination of the pine seeds (*P. contorta*) used as the antibiotic-sensitive control. Without antibiotics treatment (control),  $77 \pm 1\%$  of the seeds germinated; at 100% antibiotic application,  $74 \pm 3.46\%$  germinated. Germination rates were similar at six other dilutions, but none of the differences were statistically significant. However, very young seedlings grown without endophytes were significantly smaller in dry weight, root length, and



**Fig. 2.** Growth promotion of cardon plants growing in volcanic rock for 12 months and inoculated with the endophytic bacteria *Pseudomonas* sp. SENDO 2 (A) and *Bacillus* sp. SENDO 6 (C) compared to plants growing in the same substrate without inoculation with endophytic bacteria. The experiment was repeated twice.



**Fig. 3.** Effects of six endophytic bacteria, two control PGPBs, and one mixture of endophytic bacteria on promotion of growth of giant cardon cactus seedlings (dry weight, volume, height, root length, and nitrogen content) growing in pulverized igneous rock supplemented with perlite (A–E) or exclusively on perlite (F–J) for 12 months after inoculation of seeds. Columns denoted by a different letter in each subfigure differ significantly at  $P < 0.05$  by one-way ANOVA. Bars represent SE. The experiment was repeated twice.

height (Fig. 1D–F). Germinating seeds containing endophytes and the seedlings were irrigated with the successful antibiotic treatment over the following 8 months and showed no negative effects on plant growth (data not shown).

### 3.2. Effects of inoculation with endophytic bacteria on the growth of cardon seedlings

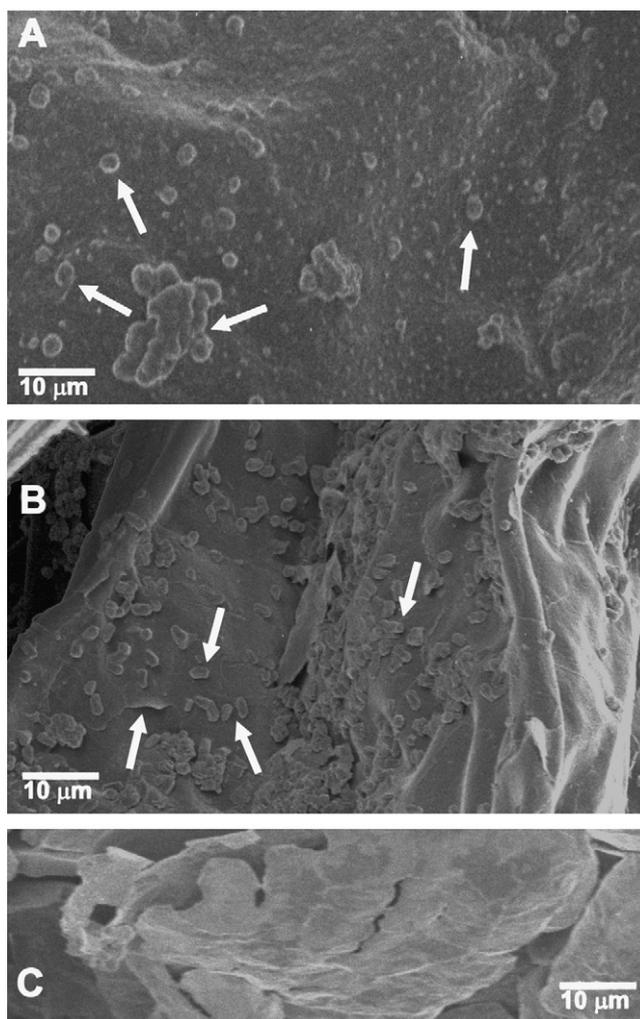
Disinfected cardon seeds were tested with six species of endophytic bacteria and a mixture of all strains. Two known PGPB strains were used as positive controls. These inoculation treatments significantly changed several growth parameters during the experiment lasting 1 year (Fig. 2). Dry weight, volume, root length, and height of inoculated plants were significantly greater in most cases (Fig. 3A–J). The four growth parameters for plants growing in pulverized rock substrate and inoculated with endophytic bacteria were significantly greater than plants serving as controls. The results of inoculation with the positive control PGPB *P. putida* have the same trends, but smaller, to those of the endophytic bacteria (Fig. 3A–D), but inoculation with the other positive control PGPB *A. brasilense* promoted only dry weight and root length over controls that were not inoculated (Fig. 3A and C). We could not determine if the strains persisted on the roots after the 1-year trial because molecular markers were not available to identify them at the time of the experiments. Three endophytic bacteria, *Bacillus* sp. SENDO 6, *Acinetobacter* sp. SENDO 1, and *Pseudomonas* sp. SENDO 2 performed better in the rock-perlite substrate than the other three bacteria, whereas the mixture of all endophytes, while performing better than the controls that were not inoculated, was inferior to single-endophytic inoculations for most tested parameters.

One year after inoculation, plants inoculated with any of the six endophytes or the control PGPB endophytes significantly increased total nitrogen content of the plants grown in pulverized rock-perlite, while the mixture of the six endophytes and the PGPB did not (Fig. 3I). When plants were grown only in perlite, all bacteria increased the nitrogen content of the plants (Fig. 3J).

Histological (not shown) and SEM studies of young cardon cacti grown from endophyte-free seeds, but inoculated with the *Klebsiella* sp. SENDO 2 and *Bacillus* sp. SENDO 6 resulted in root and stem colonization by endophytes during the year after inoculation and first detected after 30 days (Fig. 4). Seedlings that had not been inoculated remained axenic for the duration of the 30-day experiment.

### 3.3. Weathering of rock minerals after growth of cardon seedlings in substrate

After growing for 1 year in pulverized rock-perlite substrate, inoculated cardon cacti had mineralized significant quantities of several minerals essential to plant growth in the substrate. Plants inoculated with any of the bacteria species removed more  $P_2O_5$  from the substrate than uninoculated plants; those inoculated with *Klebsiella* sp. SENDO 2 (30%), *Bacillus* sp. SENDO 6 (29%), *Pseudomonas* sp. SENDO 2 removed the most (26%) phosphate (Fig. 5). Plants inoculated with any of the endophytic bacterial species removed  $Fe_2O_3$  at higher levels than uninoculated plants, where *Klebsiella* sp. SENDO 2, *Staphylococcus* sp. SENDO 2, and *Pseudomonas* sp. SENDO 2 removed the most (34%), while the two control PGPB did not increase the plants' ability to remove more  $Fe_2O_3$  than uninoculated plants (Fig. 5B). Plants inoculated with several of the bacteria removed significant amounts of  $K_2O$ ; *Pseudomonas* sp. SENDO 2, and the mixture of all endophytes removed the largest amount of potassium (32%) (Fig. 5C). All plants inoculated with endophytes removed significantly more MgO than

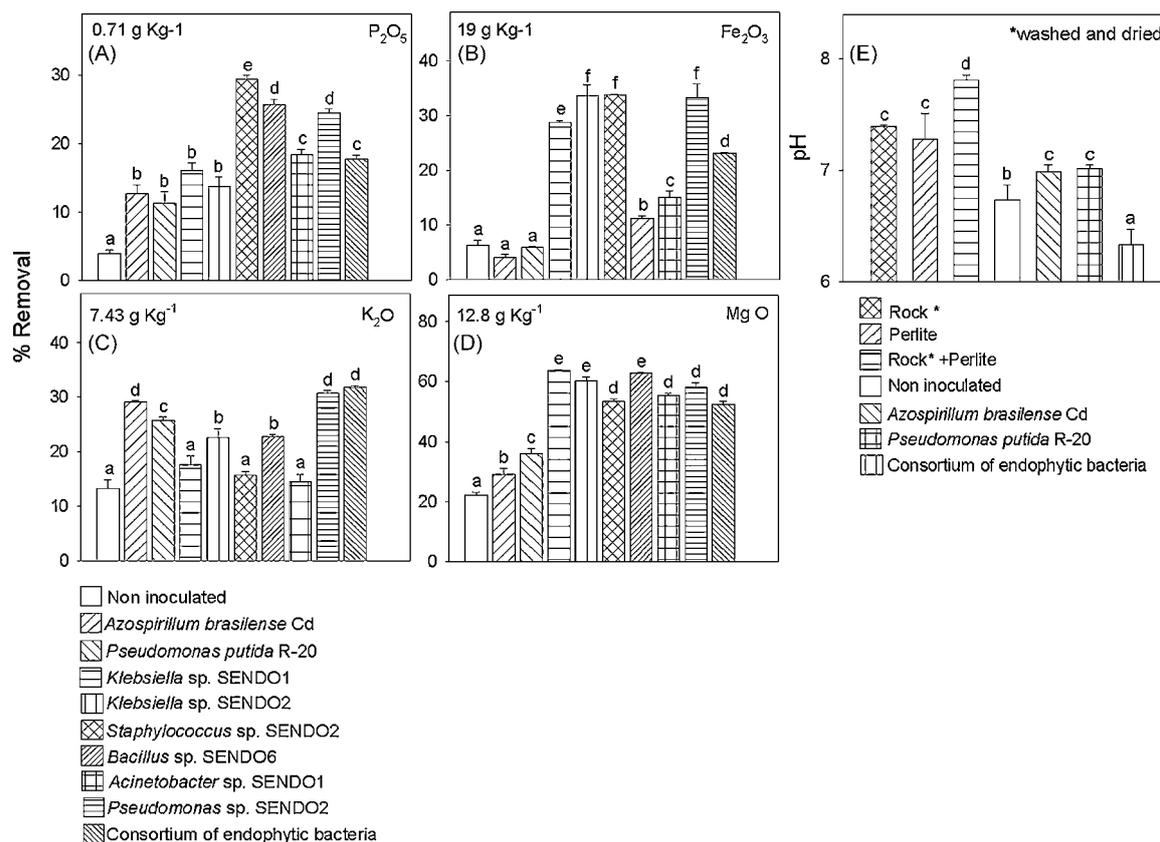


**Fig. 4.** Colonization by endophytic bacteria of the interior connecting tissue between the roots and the stem of cardon plantlets growing from seeds free of endophytes and artificially inoculated with the endophytes *Klebsiella* sp. SENDO 2 (A) and *Bacillus* sp. SENDO 6 (B). (C) Control, uninoculated plants. Arrows indicate bacteria and possible aggregates of bacteria.

uninoculated plants; those with *Klebsiella* sp. SENDO 1 and SENDO 2 and *Bacillus* sp. SENDO 6 removed the most (63%) (Fig. 5D). Plants inoculated with the two control PGPB were less efficient at removing  $P_2O_5$ ,  $Fe_2O_3$ , and MgO than plants inoculated with endophytic bacteria, but were equally efficient as the endophytic bacteria at removing  $K_2O$  (Fig. 5C). The pH of the substrate was lowered by the presence of cacti, but was significantly reduced if the plants were inoculated with the mixture of endophytes (Fig. 5E).

## 4. Discussion

Plant growth-promoting bacteria (PGPB; Bashan and Holguin, 1998) are commonplace in agriculture (Bashan and de-Bashan, 2005), but are rarely reported for wild plants of environmental importance (Bacilio et al., 2006). Recently, involvement of bacteria in phyto-remediation, in general (Lodewyckx et al., 2001, 2002a; Glick, 2003, 2004; Chaudhry et al., 2005; Reed et al., 2005; Bashan et al., 2008), and more specifically, aiding plants growing on mine tailing was proposed (Grandlic et al., 2008; Mendez et al., 2007; Rosario et al., 2007). Further, endophytic bacteria are well known in crop plants (Hallmann et al., 1997; Lodewyckx et al., 2002b), but largely has not been investigated in wild plants.



**Fig. 5.** (A–D) Removal of P<sub>2</sub>O<sub>5</sub>, K<sub>2</sub>O, Fe<sub>2</sub>O<sub>3</sub>, and MgO from pulverized rock substrate in which cardon cacti, inoculated with six endophytic bacteria, two control PGPBs, and one mixture of endophytic bacteria, were grown. Number at each subfigure represents the quantity of each of the four minerals in the pulverized rock. Results are presented as percentage of depletion of the element. (E) pH changes of substrate of pulverized volcanic rock plus perlite by cactus seedlings inoculated with either a mixture of endophytic bacteria or a control of a PGPB after 1 year of cultivation. Columns denoted by different letters in each subfigure differ significantly at  $P < 0.05$  by one-way ANOVA. Bars represent SE. The absence of SE indicates a negligible value. The experiment was repeated twice.

In desert plants, agricultural PGPB of the genus *Azospirillum* promote the growth of seedlings of the giant cardon cactus and several other cactus species, a process that provides considerable soil stabilization in deserts (Bacilio et al., 2006; Puente and Bashan, 1993; Bashan et al., 1999, 2006; Carrillo-Garcia et al., 2000). Several rhizoplane bacteria isolated from roots of cacti growing in rocky substrates also promote establishment of cardons (Puente et al., 2004b). Our working hypothesis was that endophytic bacteria colonizing the interior of almost every cardon plant have PGPB traits (Puente et al., 2009) and therefore promotes the growth of these plants, a process reported for agricultural crops (Lodewyckx et al., 2002b). Furthermore, certain bacteria are essential for the growth of these cacti under harsh substrate conditions, such as rocks and rocky surfaces.

Direct evidence that endophytic bacteria promote plant growth came from crop studies, including potato and clover (for review Bashan and de-Bashan, 2005). Studies showed that 10–21% of the bacteria recovered from potato tuber fibers, for example, are PGPB, and upon inoculation, promoted plant growth (Sturz, 1995; Sturz et al., 1998). When slow-growing cardon seedlings were inoculated with any of six potential endophytic PGPB and grown for 12 months, significantly increased growth occurred relative to the controls. For example, plant volume, which is a critical parameter for cactus survival during the first year, increased (Puente and Bashan, 1993; Bashan et al., 1999). All inoculated plants, regardless of the bacteria used, grew much better and were more capable of extracting essential inorganic minerals from the pulverized rock substrate than uninoculated controls. The breakdown of essential plant elements from rock minerals by inoculated plants can be attributed to solubilizing activity of the bacteria that colonize the cactus roots

and the root exudates containing organic acids (Lynch and Whipps, 1990; Carrillo et al., 2002). Additionally, nonsymbiotic microflora are known to increase the rate of absorption of Ca and Mg by plants (Berthelin and Leyval, 1982; Berthelin et al., 1991). The exact source of nitrogen used by the plants was not determined. However, several of the endophytic bacteria used were diazotrophs (Puente et al., 2009).

Minerals from igneous and metamorphic rocks contain most of the nutrients required by higher plants for normal growth and development. Geochemical studies of the breakdown of rock minerals and formation of clay minerals have explained the general reaction pathways by which nutrients are released, which is enhanced by disequilibrium between the soil solution and mineral surfaces through the removal of ions by processes such as leaching and nutrient uptake. Rhizosphere processes and other biological activity further enhances breakdown of minerals through the release of hydrogen ions and complex organic compounds that react with mineral surfaces (Harley and Gilkes, 2000). The bacteria used in this study have these abilities (Puente et al., 2009).

Taken together, these two papers raise questions for future research: (1) what is the evolutionary significance of such associations? (2) How does the extra genetic diversity of endophytes contribute to better plant growth in ways that the plant genotype could not satisfy by itself? (3) Are endophytes inoculated into plants also colonize the rhizosphere, which is essential for accelerating biological weathering of rocks?

In summary, this study indicates that endophytic bacteria are essential for the development of cardon seedlings in rocky substrates and promote growth of cacti in soilless environments.

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