# Microbial Populations and Activities in the Rhizoplane of Rock-Weathering Desert Plants. II. Growth Promotion of Cactus Seedlings

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***Abstract:*** **Four bacterial species isolated from the rhizoplane of cacti growing in bare lava rocks were assessed for growth promotion of giant cardon cactus seedlings *(Pachycereus pringlei)*. These bacteria fixed $N_2$, dissolved P, weathered extrusive igneous rock, marble, and limestone, and significantly mobilized useful minerals, such as P, K, Mg, Mn, Fe, Cu, and Zn in rock minerals. Cardon cactus seeds inoculated with these bacteria were able to sprout and grow normally without added nutrients for at least 12 months in pulverized extrusive igneous rock (ancient lava flows) mixed with perlite. Cacti that were not inoculated grew less vigorously and some died. The amount of useful minerals (P, K, Fe, Mg) for plant growth extracted from the pulverized lava, measured after cultivation of inoculated plants, was significant. This study shows that rhizoplane bacteria isolated from rock-growing cacti promote growth of a cactus species, and can help supply essential minerals for a prolonged period of time.**

***Key words:*** ***Azospirillum, Bacillus,*** **cactus, cardon,** ***Citrobacter,*** **desert, $N_2$-fixation,** ***Pachycereus,*** **phosphate solubilization,** ***Pseudomonas,*** **rock degradation, rock weathering, soil formation.**

## Introduction

Plant growth-promoting bacteria (PGPB), belonging to diverse genera, are free-living soil, rhizosphere, rhizoplane, and phylosphere bacteria that, under some conditions, are beneficial for plants, as their name implies (Bashan and Holguin, 1998). Most are known in agriculture. Two groups of PGPB promote plant growth differently. One group affects the metabolism of plants directly by providing substances that are usually in short supply. These bacteria fix atmospheric nitrogen, dissolve phosphorus and iron, and produce plant hormones. Additionally, they improve plant stress tolerance to drought, salinity, metal toxicity, and pesticide load (Bashan and Holguin, 1997). A second group, biocontrol PGPB, promotes plant growth indirectly by preventing the deleterious effects of phytopathogenic microorganisms (Glick and Bashan, 1997).

Very slow, direct microbial rock weathering is common in all climate zones (Sun and Friedmann, 1999), but information on weathering mechanisms is scarce. Acid-producing microorganisms can degrade minerals, benefiting microbes and plants, by making inorganic nutrients available (Hinsinger et al., 1992; Hinsinger and Gilkes, 1993, 1995; Illmer et al., 1995). Examples of these microbes are: phosphate-dissolving bacteria, abundant in agricultural fields (Chabot et al., 1996), in mangrove ecosystems (Vazquez et al., 2000), and on rocks (Chang and Li, 1998), bacteria that dissolve insoluble Fe from rocks (Adams et al., 1992), ectomycorrhizal fungus *Laccaria laccata* and the phosphate-dissolving bacteria *Agrobacterium radiobacter* and *Achromobacter* sp. in the rhizospheres of pine and beech (Leyval et al., 1990).

Recently, we reported on several species of desert plants, mainly cacti, growing on cliffs, rocks, and ancient lava flows in hot desert areas in Baja California, Mexico that noticeably weather these igneous rocks and cliffs (Bashan et al., 2002). We characterized several bacterial strains capable of weathering powdered igneous rocks *in vitro* with potential PGPB traits (Puente et al., 2004).

This study tests the hypothesis that these potential PGPB participate in mineral weathering in nature, and support better plant growth, possibly by supplying nitrogen, soluble phosphorus, and other essential minerals.

## Materials and Methods

### *Organisms*

Seeds of the giant cardon cactus (*Pachycereus pringlei* [S. Wats] Britt. and Ross) were collected in the field (Bashan et al., 2002) for use as test plants. Plantlets were tested with four rhizoplane bacterial species: *Bacillus chitinolyticus, B. subtilis* var. 2, *Citrobacter* sp., and *B. pumillus* var. 2 (Puente et al., 2004), and two PGPB controls: *Pseudomonas putida* R-20 (Osburn et al., 1983; Meyer and Lindrman, 1986) and *Azospirillum brasilense* Cd (ATCC 29710). The rhizoplane bacteria, isolated from the roots of several young cacti species growing in rocks without soil, were tested as PGPB. *In vitro,* they showed several physiological traits potentially useful as PGPB, $N_2$-fixing, mineral-solubilizing (including P), and producers of organic acids (Puente et al., 2004).

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*Bacterial growth conditions*

Bacterial isolates were grown in nutrient broth (Merck) at 30 ± 1 °C for 18 h at 120 rpm (incubator shaker series 25; New Brunswick, Edison, NJ), and harvested by centrifugation at 1000 *g* for 20 min. The pellet was suspended in 0.85% saline solution to a final concentration of $10^6$ colony-forming units (cfu) $ml^{-1}$.

*Inoculation of cardon cacti with rhizoplane isolates growing in pulverized rock*

Ancient lava rocks (for origin and composition, see Bashan et al., 2002) were submerged in 1 N HCl solution overnight at 28 – 33 °C to eliminate organic matter, then rinsed several times with de-ionized water and dried at 160 °C for 2 h. These rocks were pulverized in a mill (Sprecher and Schun Industrial Control, Germany), and sieved to 120-µm particle size. A mixture of 4 g powder and 23 g perlite (1.0 ± 0.25 mm, 0.04% N, 0.01% P, 0.06% K; Miracle-Gro Lawn Products, New York) was placed in small black pots. Negative control pots were filled with 27 g perlite only. Seeds were inoculated with each bacterial species by the standard vacuum infiltration technique of Puente and Bashan (1993) for this plant species. Briefly, disinfected seeds (2% Tween-20 [Sigma] for 10 min, rinsed, 3% commercial NaOCl for 5 min, thoroughly washed in sterile tap water for 10 min) were immersed in a bacterial suspension ($10^6$ cfu $ml^{-1}$ in 0.85% saline solution) and subjected to a vacuum of 600 mm Hg for 5 min. The vacuum was released abruptly to allow penetration of bacteria inside the seed's cavities. Ten seeds were placed on the substrate surface of each pot previously irrigated with 50 ml distilled water, and covered with a 5-mm layer of substrate. The pots were incubated in a growth chamber (Biotronette Mark III, Melrose Park, IL) at 30 ± 2 °C under a photon flux density of 250 ± 3 µmol $m^{-2}$ $s^{-1}$ for 12 months. Pots were irrigated every 15 days. Irrigation regime is given in Table **1**. Although all pots had drainage holes, the quantity of irrigation was adjusted to avoid drainage. Substrates were analyzed for mineral content and pH values before and after the experiment (described later). At the end of the experiment, plants were extracted and photographed, and height, volume (Bashan et al., 1999), root length, and dry weight (drying oven, 50 °C, 120 h) were measured. Total nitrogen content of the plants was measured by automatic micro-Kjeldahl after digestion (Digestion System 12.1009, and Kjeltec Auto 1030 Analyzer, Tecator, Höganäs, Sweden).

*Mineral analyses*

Before and after plants were grown in the crushed rock, the rock was analyzed for $P_2O_5$, $K_2O$, MgO, and $Fe_2O_3$, using EPA microwave digestion method #3015 (nitric acid) (Kingston, 1994) with an atomic absorption spectrometer (GBC Scientific Equipment, Australia). Total concentration of phosphate was determined according to Jackson (1958).

*Experimental design and statistical analysis*

Plants were inoculated in pots (ten replicates), thinned to three seedlings per pot. Pots were distributed randomly in the growth chamber, and rearranged occasionally during the 12-month incubation period. Percentage data were converted to arcsine before analysis. Statistical analysis was done either by one-way analysis of variance (ANOVA) or Student's *t*-test at $p \leq 0.05$, using Statistica software (Statsoft, Tulsa, OK). Numerical data for the mean of treatments are accompanied by standard error.

**Table 1** Irrigation regime of the experiment (25 ml solution every 15 days)

| Treatment | Irrigation type |
|---|---|
| Inoculated with native bacteria and grown in rock–perlite mixture | Hoagland's solution without P or N |
| Inoculated with native bacteria and grown in perlite (control) | Hoagland's solution with P but no N |
| Inoculated with *P. putida* in perlite (control) | Hoagland's solution with N but no P |
| Inoculated with *A. brasilense* in perlite (control) | Hoagland's solution with P but no N |
| Control without inoculation | water only |
| Control without inoculation | Hoagland's solution without P or N |
| Control without inoculation | complete Hoagland's solution |

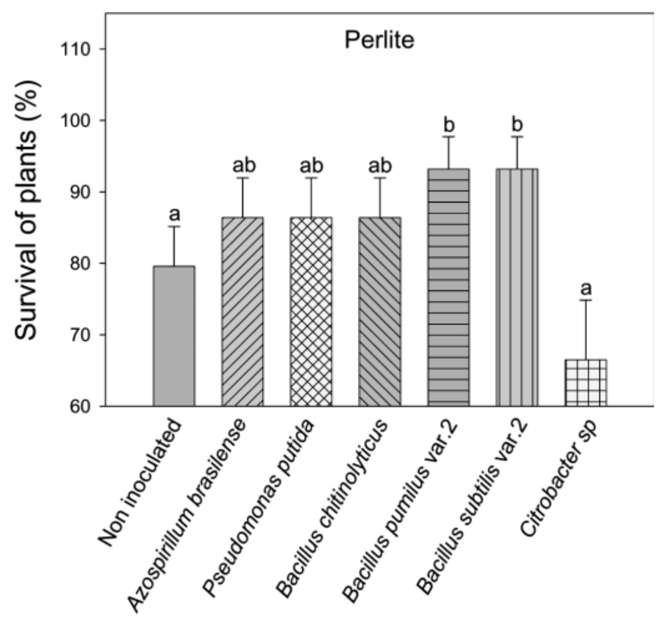


**Fig. 1** Survival of cardon seedlings after growing for one year in perlite and inoculated with rhizoplane bacteria and agriculturally-originated PGPB. Columns denoted by a different letter differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE).

## Results

*Survival of cardon seedlings after inoculation with rock weathering bacteria*

About 80% of cardon cactus plantlets survived for one year, even when grown only in perlite substrate. Inoculation of the plantlets with rhizoplane bacteria or with either of the two control PGPB did not enhance survival in crushed rock supple-

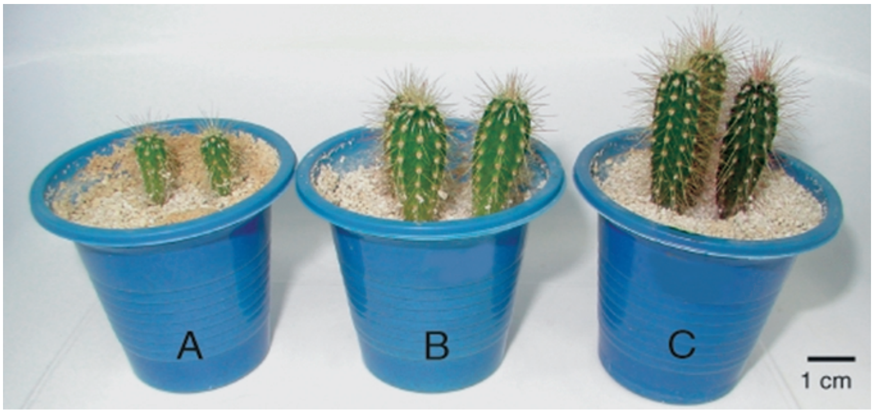


**Fig. 2** Growth promotion by the rhizoplane bacterium *B. subtilis* var. 2 on seedlings of giant cardon cactus growing in crushed extrusive igneous rock supplemented with perlite (**B**) in comparison to plants without bacterial inoculation growing in the same substrate (**A**), and positive control plants without bacterial inoculation, but irrigated with full Hoagland's solution (**C**) 12 months after inoculation of seeds.

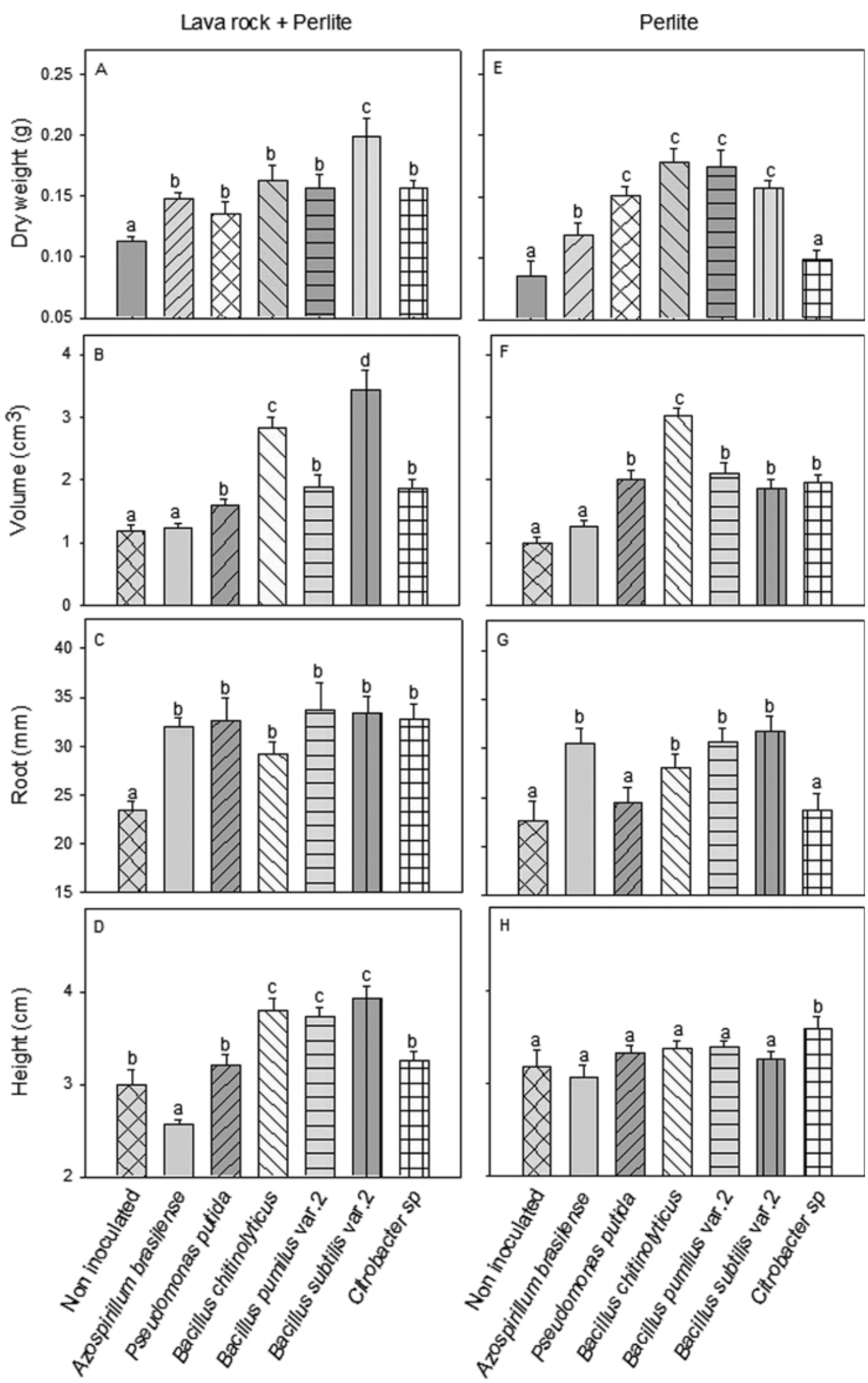


**Fig. 3** Growth promotion effects (dry weight, volume, height, and root length) of rhizoplane bacteria on seedlings of the giant cardon cactus growing in crushed rock supplemented with perlite (**A – D**), and in perlite only (**E – H**), 12 months after seed inoculation. Columns denoted by a different letter in each subfigure differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE).

mented with perlite, since most seedlings survived in the substrate (data not shown). However, when grown in perlite only, plantlets inoculated with *B. pumilus* var. 2 or *B. subtilis* var. 2 survived in significantly larger numbers (Fig. **1**).

### *Effects of inoculation with rock-weathering bacteria on the growth of cardon seedlings*

Separate inoculations of cardon seedlings with four rhizoplane bacterial species and two known PGPB as controls significantly changed several plant growth parameters over a long period (Fig. **2**). After growing for one year, the dry weight, volume, root length, and height were all significantly greater (Fig. **3**). In general, inclusion of pulverized rock in perlite enhanced the growth of inoculated cardon plantlets over that in perlite alone (compare Figs. **3A–D** to Figs. **3E–H**). The four growth parameters of all plants growing in pulverized rocks and inoculated with rhizoplane bacteria were significantly greater than plants serving as controls. The results of inoculation with the control PGPB *P. putida* were similar to those of the rhizoplane bacteria (Figs. **3A–D**), but inoculation with the control PGPB *A. brasilense* enhanced only dry weight and root length over controls that were not inoculated (Figs. **3A,C**). We could not determine whether the strains persisted after the one-year incubation in roots because molecular markers were not available to identify them.

Compared to plants that were not inoculated, inoculation with the four $N_2$-fixing, rhizoplane bacterial species significantly increased total nitrogen content of the plants one year after inoculation when grown in powdered rock plus perlite (Fig. **4A**). When plants were grown in perlite only, all bacteria increased the nitrogen content of the plants (Fig. **4B**).

### *Weathering of rock minerals in substrate after one year's growth with cardon seedlings*

After one year growing in pulverized rock substrate, cactus plants had significantly reduced the quantities of four minerals essential to plant growth and lowered the pH of the growth substrate. Plants inoculated with any of the treatment species removed more $P_2O_5$ from the substrate than plants that had not been inoculated. Plants inoculated with *B. pumilus* var. 2 removed the most minerals (64.8%) (Fig. **5A**). Plants inoculated with this species also removed the most $Fe_2O_3$ (17.5%). Plants inoculated with *B. subtilis* var 2 removed slightly less $Fe_2O_3$. Plants inoculated with other bacteria removed no more iron than untreated plants (Fig. **5B**). Plants inoculated with each bacterium removed significant amounts of $K_2O$ and MgO, but those with *Citrobacter* sp. removed the most (29.5% $K_2O$, 37.7% MgO) (Figs. **5C,D**). Plants inoculated with control PGPB were less efficient in removing $P_2O_5$ and $Fe_2O_3$ than plants inoculated with rhizoplane bacteria, but were equally efficient as the rhizoplane bacteria at removing $K_2O$ and MgO (Fig. **5**). The pH of all substrates where inoculated plants grew decreased from the initial pH 7.8 to pH 6.47 for *Citrobacter* sp., 6.43 for *B. chitinolyticus,* 6.2 for *B. pumilus* var. 2, and 6.0 for *B. subtilis* var. 2.

## Discussion

Although cacti on the arid Baja California Peninsula, Mexico are well adapted to water scarcity and harsh climatic conditions, transplants to urban areas for decoration or to agricul-

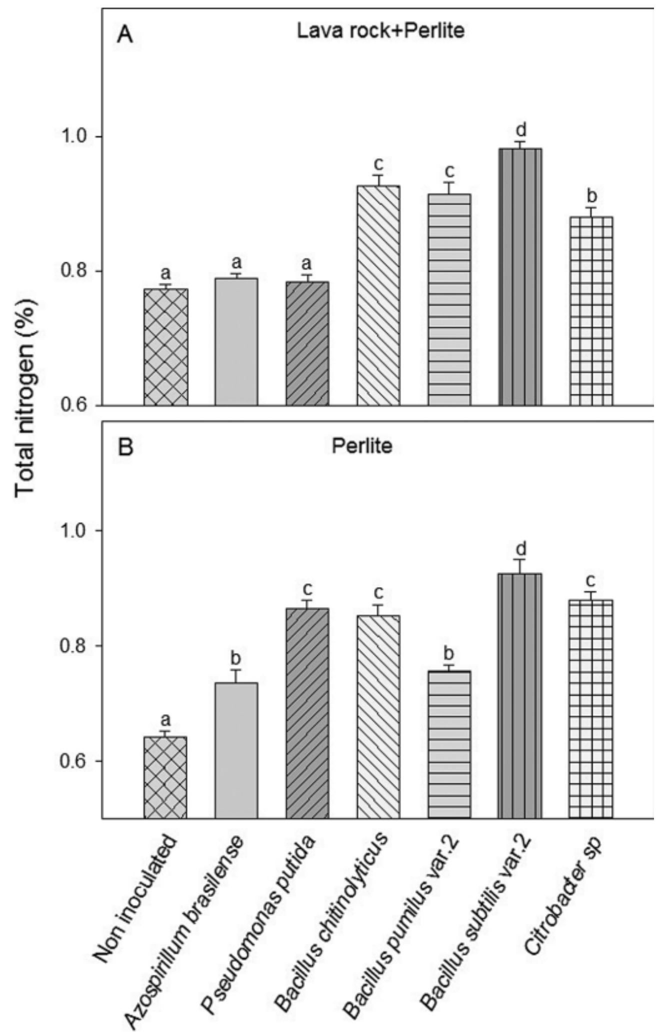


**Fig. 4** Effect of inoculation with rhizoplane bacteria on nitrogen accumulation in seedlings of the giant cardon cactus growing in crushed rock and perlite (**A**) and in perlite only (**B**), 12 months after seed inoculation. Columns denoted by a different letter in each sub-figure differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE).

tural or urban areas for prevention of soil erosion and dust pollution (Bashan et al., 1999) seldom succeed. The leading theory for this failure is that, apart from some agrotechnical difficulties like transportation to the site and initial irrigation regime on site, the nursery-reared and transplanted cacti lacked indigenous microflora, such as mycorrhizal fungi (Carrillo-Garcia et al., 1999; Bashan et al., 2000) and several species of bacteria that assist plant growth.

In agriculture, PGPB are well known for their profound impact on vascular plants with which they are associated, and for many crops they are an integral part of management programmes (Bashan, 1998; Bashan and Holguin, 1997). Yet, desert re-forestation aided by native microflora is a young field of research, and PGPB are seldom used (Bashan, 2003). The diazotrophic, endophytic *Pseudomonas stutzeri* is a potential PGPB found in the desert epiphyte *Tillandsia recurvata* (Puente and Bashan, 1994), but was never used to inoculate desert plants. However, inoculation of cardon cacti with the PGPB

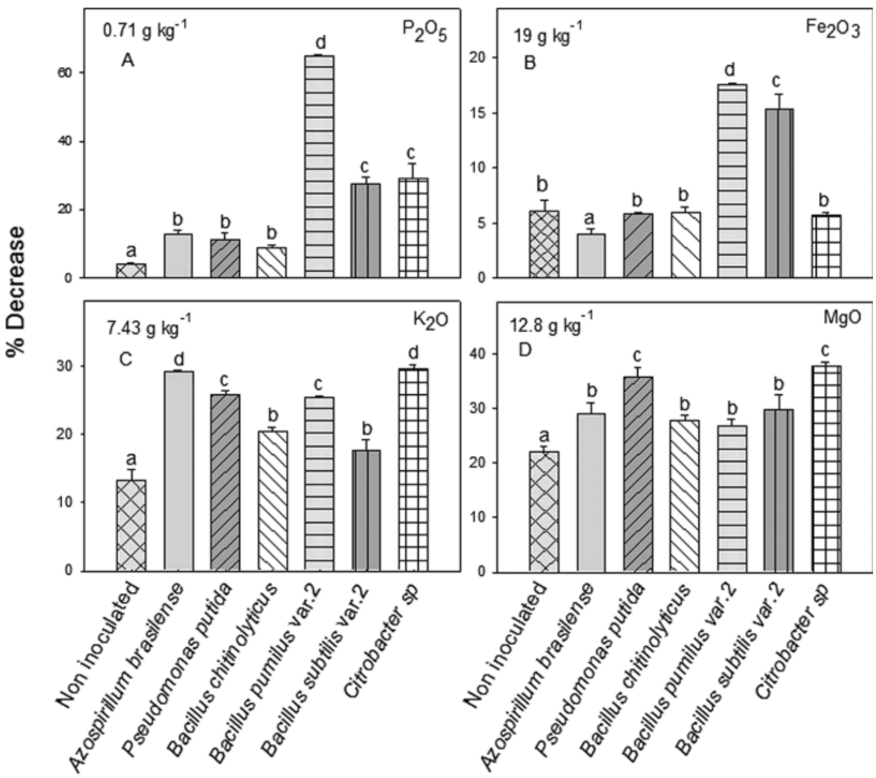


**Fig. 5** Removal of $P_2O_5$, $K_2O$, $Fe_2O_3$, and MgO from the rock substrate in which cardon cacti inoculated with rhizoplane bacteria were grown for 12 months. Numbers in each sub-figure represent the initial concentration of each element in crushed rock. Results are presented as percentage decrease of the mineral. Columns denoted by a different letter in each sub-figure differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE).

*Azospirillum brasilense* of agricultural origin improved establishment, growth, and survival under controlled, greenhouse, and field conditions (Puente and Bashan, 1993; Bashan et al., 1999; Carrillo-Garcia et al., 2000). Our working hypothesis was that native root-colonizing bacteria from cacti, capable of surviving in the harsh environment, participates in rock and mineral weathering, and supplies plants with released inorganic nutrients and nitrogen from $N_2$ fixation, have more potential as PGPB for arid zone plants than agriculturally-originated PGPB.

When slow-growing cardon seedlings were inoculated with four potential PGPB, and allowed to develop for 12 months, significant growth was detected; plant volume, a critical parameter for cactus plant survival during the first year in the desert (Puente and Bashan, 1993; Bashan et al., 1999), was enhanced. Survival of plants inoculated with *B. pumilus* var. 2 and *B. subtilis* var. 2 was better in perlite alone. All inoculated plants, regardless of the bacterial species, grew much better and were more able to extract essential inorganic minerals from the pulverized rock in which they grew than plants that had not been inoculated. The best PGPBs found in this study were *B. pumilus* var. 2 and *B. subtilis* var. 2. These isolates may serve as potential PGPB for arid zone re-vegetation programmes. The two agriculturally-originated PGPB, while enhancing root growth similar to native bacteria, were nevertheless less efficient in enhancing the parameters of growth of cacti than the arid zone bacterial species. All bacteria used in inoculation treatments were of native rock origin and are nitrogen fixers in the *in vitro* assays, significantly increasing nitrogen content of all inoculated plants. The breakdown of essential plant minerals by inoculated cacti can be attributed to the solubilizing activity of the bacteria colonizing the cactus roots (Gyaneshwar et al., 1998) and to excretion of root exudates containing organic acids (Lynch and Whipps, 1990). Cardon cactus seedlings inoculated with *A. brasilense* excreted more protons and more organic acids, which lowered rhizosphere pH and made more phosphorus available to the plants (Carrillo et al., 2002). This supports the lower pH values found in the rock–perlite substrate after cultivation of the inoculated plants.

It is known that rhizosphere microflora of maize, rice, and pine trees promote dissolution of minerals (Berthelin et al., 1991), and Antarctic lichens altered minerals in the rock on which they grow (Ascaso et al., 1990). This supports our previous studies (Bashan et al., 2002; Puente et al., 2004) that show breakdown of parent material and release of nutrients available to plants in rock cavities where desert plants, heavily colonized with these bacteria, grow.

One can only speculate on the sequence of events leading to establishment of cardon seedlings in lava rock lacking immediately available resources. Seeds of cardon, like many columnar cacti, are disseminated by birds, bats, and small mammals after passing through the digestive system of the animal (Rojas-Arechiga and Vazquez-Yanez, 2000; Valiente-Banuet et al., 1996). Once deposited in a natural cavity or crack in the rock and wetted by an infrequent rain, seeds germinate within a few days (Puente and Bashan, 1993). In the first few days, seeds may be nourished and produce roots on leftover nutrients obtained from the animals' digestive system and on stored material in their cotyledons. Rhizosphere bacterial colonies will build up once initial roots have proliferated, and facilitate nutrient release from rocky substrates and fix nitrogen. These events may make the seedling nutrient-independent for prolonged periods in arid zones, as cacti are very slow growers. This possible scenario has yet to be established.

In summary, this study shows that native arid zone rhizoplane bacteria that weather igneous rock are potential plant growth-promoting bacteria for cultivating cacti in the same environment as areas undergoing programmes of re-vegetation of eroded areas.


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