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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.

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-μm 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 1×10^6 CFU ml^{-1}. 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 ± 2 °C under light intensity of 70 μmol photons m^{-2} 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_2O_5, total phosphorus, K_2O, MgO, and Fe_2O_3, 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 Kjeltec 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 (μg 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_2 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_2O, Fe_2O_3, 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_2O_5) 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^{-1} dw seeds), from 760×10^6 to $>1 \times 10^6$, only the combination of cholramphenicol and tetracycline