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SHORT COMMUNICATION

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Increased acidification in the rhizosphere of cactus seedlings induced by *Azospirillum brasilense*

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Abstract Acidification of the rhizosphere of cactus seedlings (giant cardon, *Pachycereus pringlei*) after inoculation with the plant growth-promoting bacterium *Azospirillum brasilense* Cd, in the presence or absence of ammonium and nitrate, was studied to understand how to increase growth of cardon seedlings in poor desert soils. While ammonium enhanced rhizosphere and liquid culture acidification, inoculation with the bacteria enhanced it further. On the other hand, nitrate increased pH of the rhizosphere, but combined with the bacterial inoculation, increase in pH was significantly smaller. Bacterial inoculation with ammonium enhanced plant growth.

Introduction

Nitrogen nutrition and inoculation with the plant growth-promoting bacterium (PGPB) *Azospirillum brasilense* positively and significantly affect survival, development, and growth of seedlings and transplants of the giant columnar cardon cactus [*Pachycereus pringlei* (S. Wats) Britt. and Ross] (Bashan et al. 1999; Carrillo-Garcia et al. 2000a). This tree-shaped dominant cactus of the southern Sonoran desert is possibly a main contributor to soil stabilization on a large scale in the deserts of Baja California, Mexico (Bashan et al. 1999).

Nitrogen nutrition and phosphorus availability are well documented for affecting proton efflux, and consequently, acidification of the rhizosphere in many plant species (Kirk and Du 1997; Marschner 1986). Ammonium fertilizer increases proton extrusion from the roots to the rhizosphere, whereas nitrate fertilizer reduced proton extrusion in plants (Bashan and Levanony 1989). Inoculation of several major crop plants with *A. brasilense* increased proton extrusion and rhizosphere acidification (Bashan 1990; Bashan et al. 1989, 1992) via changes in the membrane potential of the roots (Bashan 1991; Bashan and Levanony 1991). Also, stimulation of the ionic transport system, including enhanced proton extrusion in *Brassica napus*, occurred after inoculation with the PGPB *Achromobacter* sp. (Bertrand et al. 2000).

In the southern Sonoran desert, cactus seedlings are restricted to development under the canopy of mature mesquite trees, where the "resource island" created by the tree provides improved mineral nutrition, shade, abundant mycorrhizal hyphae, and increased water availability (Bashan et al. 2000; Carrillo-Garcia et al. 1999). In barren desert areas, where soil is extremely poor and supports only a handful of plant species, almost no cactus seedlings grow. Cardon seedlings greatly benefit from nitrogen and phosphorus nutrition (Carrillo-Garcia et al. 2000a), normally in short supply under desert soil conditions. Where revegetation is planned in poor, eroded desert soils, this study evaluates how to increase growth of cardon seedlings by enhancing acidification of the rhizosphere when subjecting cardon roots to simultaneous nitrogen nutrition and inoculation with a plant growth-promoting bacterium.

Materials and methods

Organisms

Seeds of the giant cardon cactus (*Pachycereus pringlei*) were obtained from wild plants in August 1998. Before each experiment, seeds were washed with tap water and disinfected (Puente and Bashan 1993), and allowed to germinate in a sterile Petri dish containing 10 ml distilled water. The plate was wrapped in dark brown paper and incubated in a growth chamber at 27±1°C, 70% relative humidity for 16 days. After 5 days, seedlings were exposed to a 12 h photoperiod at 200 µmol m^{-2} s^{-1}.

The plant growth-promoting bacterium *A. brasilense* Cd (DSM 1843, Germany) was cultivated and prepared for inoculation ac-

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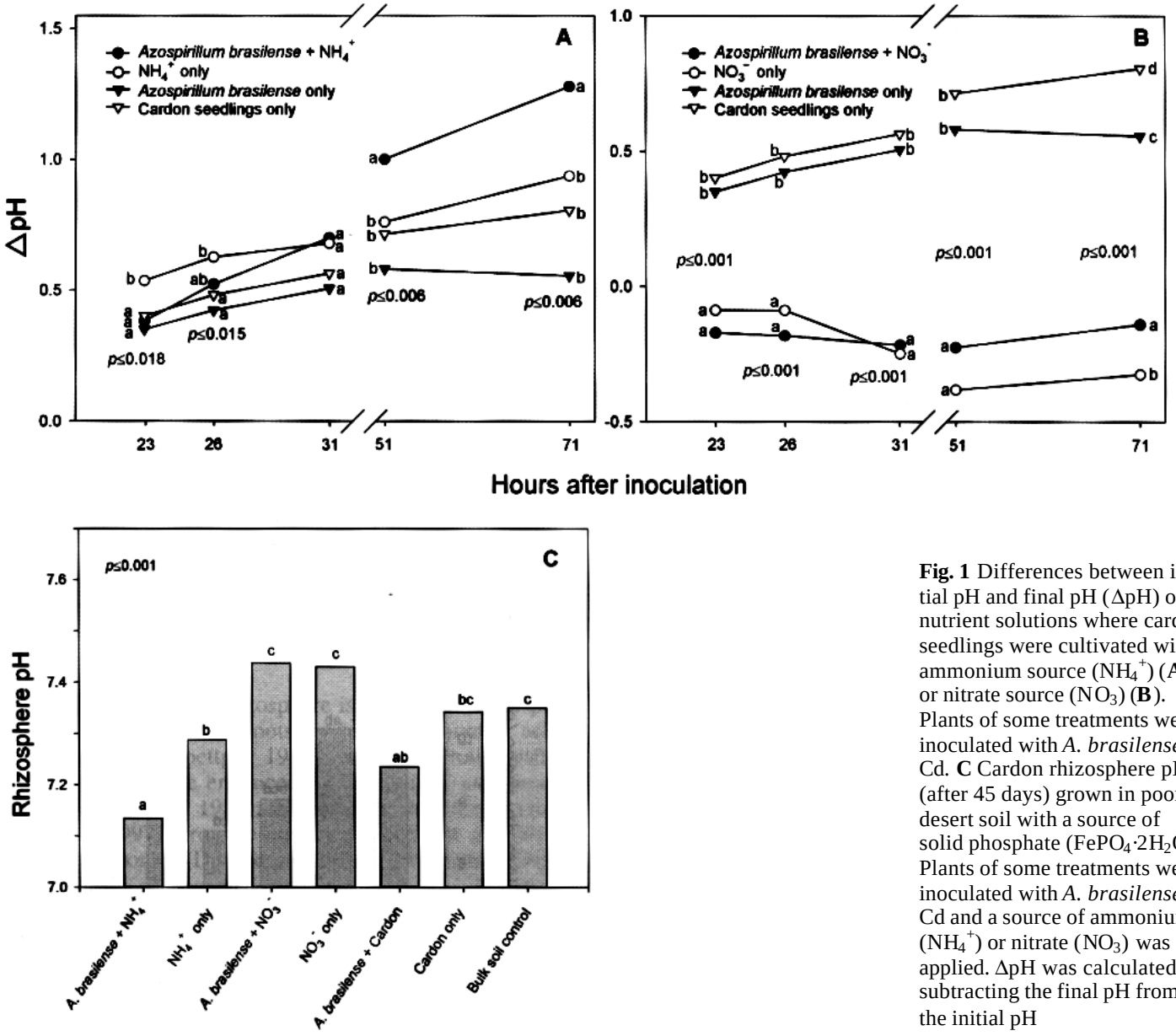

Fig. 1 Differences between initial pH and final pH (ΔpH) of nutrient solutions where cardon seedlings were cultivated with ammonium source (NH_4^+) (**A**) or nitrate source (NO_3) (**B**). Plants of some treatments were inoculated with *A. brasilense* Cd. **C** Cardon rhizosphere pH (after 45 days) grown in poor desert soil with a source of solid phosphate ($FePO_4 \cdot 2H_2O$). Plants of some treatments were inoculated with *A. brasilense* Cd and a source of ammonium (NH_4^+) or nitrate (NO_3) was applied. ΔpH was calculated by subtracting the final pH from the initial pH

cording to the standard methods for this species (Bashan et al. 1993). Tests for the ability of *A. brasilense* Cd to grow on solid $FePO_4 \cdot 2H_2O$ were performed in N-free OAB standard medium (Bashan et al. 1993), where the soluble phosphate was replaced by the solid iron phosphate at the same concentration.

Nutrient solutions

The base of all nutrient solutions was 25% Hoagland's nutrient solution (HNS) (Taiz and Zeiger 1998) containing the following ingredients (mg/ml): KNO_3 (101.1), $Ca(NO_3)_2 \cdot 4H_2O$ (236.16), $NH_4H_2PO_4$ (115.08), $MgSO_4 \cdot 7H_2O$ (246.49), KCl (1.864), H_3BO_3 (0.773), $MnSO_4 \cdot H_2O$ (0.169), $ZnSO_4 \cdot 7H_2O$ (0.288), $CUSO_4 \cdot 5H_2O$ (0.062), $NaMoO_4 \cdot 2H_2O$ (0.057), and NaFeEDTA (30). The water potential (Ψw) of -0.2 MPa (the Ψw of 25% HNS) was found to have no harmful effect on cardon seedlings. Therefore, all variations in nutrient solutions were adjusted to Ψw of -0.2 MPa by exactly replacing ions to obtain this Ψw. The Ψw, of each new solution was calculated after measuring the respective electrical conductivity of each solution. After autoclave sterilization, pH values were adjusted with 0.1 N NaOH and 0.1 N HCl to 5.0, 5.5, and 6.2. Nutrient solutions used to estimate acidification of cardon plantlet rhizospheres were: solution a: (25% HNS without $NH_4H_2PO_4$ supplemented with KH_2PO_4); solution b: [25% HNS without $Ca(NO_3)_2$ and KNO_3 supplemented with KCl and $CaCl_2$]; and solution c: (25%o HNS without nitrogen sources supplemented with KCl, KH_2PO_4 and $CaCl_2$). All solutions were adjusted to pH 6.2 (a preliminary experiment showed best growth of cardon cactus at this soil pH, data not shown).

Acidification of the solutions containing cardon seedlings

To measure acidification caused by inoculated cardon seedlings, the following six treatments were performed: inoculated cardons in solution a (nitrate source); inoculated cardon in solution b (ammonium source); inoculated cardon in solution c (without nitrogen sources); controls: non-inoculated cardons in solution a, b, or c. Plants (24 per treatment) exhibiting growing roots were immersed in *A. brasilense* Cd liquid inoculum (10^6 cfu/ml in 0.85% saline solution) for 5 min at ambient temperature. Two plants were later

transferred to Petri dishes containing 10 ml of one of the nutrient solutions (12 plates per treatment), and incubated in the growth chamber for 72 h in the conditions described above. The pH of the solution in each plate was measured directly by a portable micro pH meter (TwinpH, Horiba) at 23, 36, 31, 51, and 71 h after inoculation.

Acidification of soil containing cardon seedlings and phosphate solubilization

Plant-free, poor desert soil (passing through 1 mm sieve), as previously described (Bashan et al. 2000; Carrillo-Garcia et al. 1999) was used for all experiments. After soil sterilization (180°C for 72 h), 17 g soil [in phosphate solubilization experiments, also supplemented with 25 mg of $FePO_4 \cdot 2H_2O$ (Sigma-Aldrich)] was packed in each well serving as an individual pot (tissue culture plates with six wells per plate; Nunclon Multidishes). Treatments were similar to the in vitro, soilless experiment. Wells were irrigated every 4 days alternately with 3 ml distilled water and 3 ml of their respective solution, and maintained in the growth chamber for 45 days, as were the in vitro experiments. Seedlings with the rhizosphere soil attached were then extracted. Each seedling was placed in a separate Eppendorf tube containing 1.5 ml distilled water. Tubes were agitated in a vortex for 2 min. The pH and *A. brasilense* Cd populations were determined, as described below. Solubilization of iron phosphate was measured indirectly by measuring soluble phosphate (orthophosphate) formation in the soil by the ammonium paramolybdate method (Olsen and Sommers 1982).

Dry weight determination and bacterial counts

After 45 days, bacterial colonization of roots was assessed (Puente and Bashan 1993). After drying the plants at 70°C for 72 h in a forced draft oven, plants were weighed immediately.

Experimental design and statistical analysis

All experiments were done in a completely randomized fashion in six replicates. A replicate consisted of four individual plants (24 plants per treatment). Apart from six preliminary experiments (not reported here) designed to establish the growth parameters of the reported experiments, the in vitro experiment was repeated three times and the soil experiment twice. Results of each representative experiment were analyzed by two-way ANOVA, using Statistica software (Statsoft, Tulsa, Okla.).

Results

Acidification by cardon roots growing in liquid solutions with different nitrogen sources and inoculated with *A. brasilense*

No change in the pH of the nutrient solution was observed in samples with less than 23 h of exposure. Only from this point on could ΔpH be measured. When inoculation of cardon with *A. brasilense* Cd was accompanied by ammonium nutrition, a high ΔpH was observed (two-way ANOVA; $F_{3,20}$=9.376; P=0.006). The difference reached its peak at 71 h after inoculation (two-way ANOVA; $F_{3,20}$=9.233; P=0.006) (Fig. 1 A). Inoculation with nitrate nutrient reduced the increase in pH (two-way ANOVA; $F_{3,20}$=128.43; P<0.001) caused solely by nitrate (Fig. 1B). *A. brasilense* colonized the roots of all plants similarly;

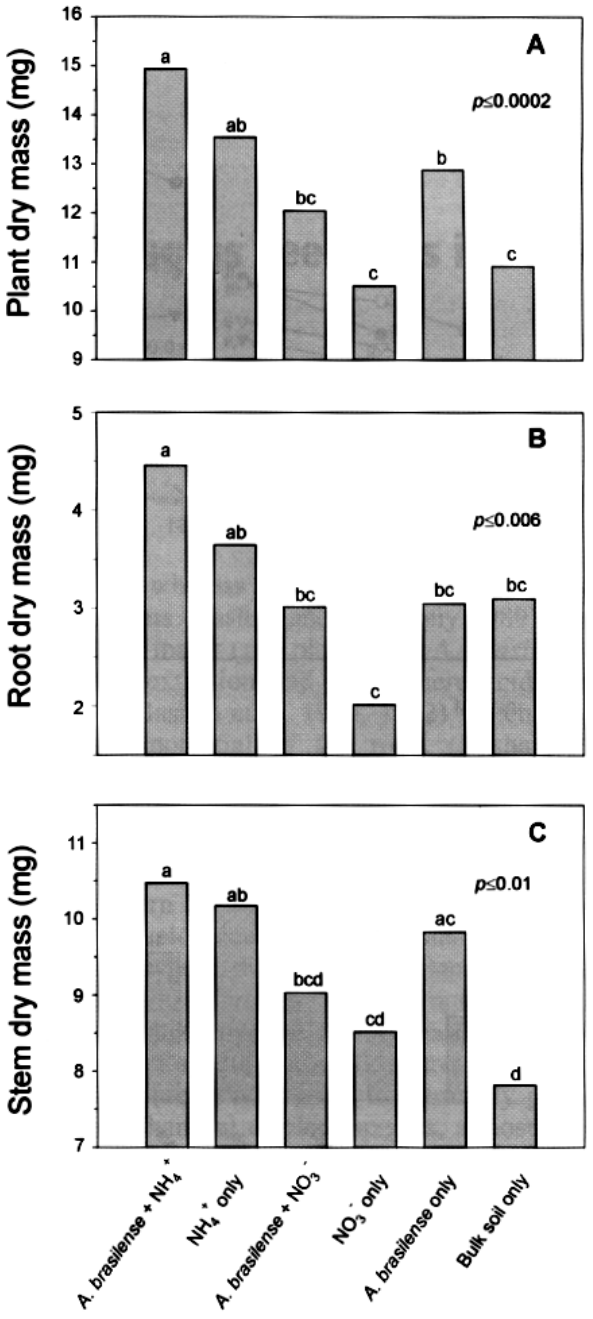

Fig. 2 Total plant (**A**), root (**B**) and stem (**C**) dry mass production (mg) of cardon plants grown in poor soil with a source of solid phosphate ($FePO_4 \cdot 2H_2O$), inoculated with *A. brasilense* Cd and irrigated with a source of ammonium (NH_4^+) or nitrate (NO_3^-)

4.8×10^5 cfu/mg dw root (*Azospirillum* spp. alone) 5.3×10^5 cfu/mg dw root (*Azospirillum* spp. + NH_4^+), and 1.5×10^5 cfu/mg dw root (*Azospirillum* spp. + NO_3^-).

Acidification and phosphate solubilization by cardon roots growing in poor soil with different nitrogen sources and inoculated with *A. brasilense* Cd

To avoid disruption of the soil, pH values were measured only at the end of the experiment. Initial natural soil pH in the well was 7.35. Inoculation of cardon seedlings with

A. brasilense combined with ammonium significantly reduced the pH of the rhizosphere (two-way ANOVA; $F_{3,20}$=8.462; P=0.008), whereas nitrate nutrient increased rhizosphere pH (two-way ANOVA; $F_{3,20}$=19.797; P<0.001) (Fig. 1 C).

Solid iron phosphate ($FePO_4 \cdot 2H_2O$) was converted into soluble phosphate in the proportion of 0.8±0.03 mg *ortho*P/g soil (nitrate nutrition) to 0.91±0.05 mg *ortho*P/g soil (ammonium *Azospirillum* nutrition), but the differences were not significant using two-way ANOVA ($F_{3,20}$=2.922; P=0.076). Treatments that reduced pH the most also produced more dry mass ($F_{3,20}$=11.458; P=0.0002) (Fig. 2A) in roots ($F_{3,20}$=6.075; P=0.006) (Fig. 213) and shoots ($F_{3,20}$=5.383; P=0.01) (Fig. 2C). Colonization level of roots by *A. brasilense* at the end of the experiment was detected in all plants. The population was $3x10^6$ cfu/mg dw roots (ammonium *Azospirillum* nutrition), $2.9x10^6$ cfu/mg dw roots (nitrate *Azospirillum* nutrition), and $1.9x10^7$ cfu/mg dw roots (*Azospirillum* alone). *A. brasilense* Cd was unable to grow (no colonies were developed after 7 days of incubation) in culture medium, where the sole phosphorus source is $FePO_4 \cdot 2H_2O$ (data not shown).

Discussion

Acidification of the rhizosphere is caused either by proton extrusion from the roots, by release of organic acids (Petersen and Boettger 1991), or both. Proton efflux from roots can be enhanced by addition of ammonium ions (Marschner 1986), phosphorus deficiency (Kirk and Du 1997), iron deficiency (Rabotti et al. 1995), alkaline toxicity (Durand and Bellon 1994), inoculation with PGPB of *Azospirillum* (Bashan et al. 1989), or of *Achromobacter* (Bertrand et al. 2000). Proton efflux from roots provides the driving force, which allows the plant to absorb essential minerals from soil (Taiz and Zeiger 1998). Thus, our working hypothesis was that an increase in proton efflux by an external inducer(s) might be beneficial for young seedlings growing under extremely poor soil conditions, as exemplified by giant cardon cacti.

Earlier, we had observed that cardon seedling growth was significantly enhanced by nitrogen and phosphorus nutrition (Carrillo-Garcia et al. 2000a) or by inoculation with *Azospirillum* (Carrillo-Garcia et al. 2000b). *Azospirillum* species are known to increase the growth of numerous crop plants (Bashan and Holguin 1997). One of the mechanisms by which this bacterium affects plant growth is improvement of mineral uptake by the plant (Bashan et al. 1990), especially when these minerals are scarce. In our case, this is the lack of nitrogen and soluble phosphate ions in desert soils. *Azospirillum* is a diazotrophic bacterium, yet it can not supply the full nitrogen requirement of a plant (Bashan and Holguin 1997). Although PGPBs of several genera (*Pseudomonas*, *Bacillus*, and *Rhizobium*) are known to be powerful phosphate solubilizers (Rodriguez and Fraga 1999), the genus *Azospirillum* lacks this capacity and needs soluble phosphorus for growth (Hartmann and Zimmer 1994). Thus, we assumed that the effect of inoculation with this PGPB on plant growth, combined with nitrogen nutrition, might be affecting one or more of the metabolic pathways of the plant. This increases proton efflux from roots and liberation of organic acid, leading to rhizosphere acidification.

Similar to the effect on wheat plants (Bashan 1990; Bashan et al. 1989), ammonium combined with inoculation with *A. brasilense* Cd significantly acidified the rhizosphere of cardon seedlings.

The physiological mechanism by which the acidification of the cardon rhizosphere occurs can only be suggestive with the data we present. *Azospirillum* are auxin-producing bacteria (Bashan and Holguin 1997). Auxins may stimulate the plasma membrane H^+-ATPase to transport protons across the cell wall (Taiz and Zeiger 1998). Addition of ammonium induces even higher root excretion of protons (Marschner 1986), and organic acids (Petersen and Boettger 1991) from roots. The enhanced acidification that was detected might cause two effects: solubilization of solid phosphate, which may supply part of the plants' needs, and enhancement of root growth, since cells grow faster in acid pH than in neutral pH (Taiz and Zeiger 1998). Both features were detected. Solid iron phosphate in the soil decreased in the presence of the plants and the plants grew larger.

In summary, inoculation of cardon seedlings with the PGPB, *A. brasilense* Cd, in the presence of ammonium, enhanced acidification of the rhizosphere, and enhanced plant growth.

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