

## Enhanced Mineral Uptake by *Zea mays* and *Sorghum bicolor* Roots Inoculated with *Azospirillum brasilense*†

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Inoculation of corn (*Zea mays*) seeds with *Azospirillum brasilense* strain Cd or Sp 7 significantly enhanced (30 to 50% over controls) the uptake of  $\text{NO}_3^-$ ,  $\text{K}^+$ , and  $\text{H}_2\text{PO}_4^-$  into 3- to 4-day- and 2-week-old root segments. No gross changes in root morphology were observed; altered cell arrangement in the outer four or five layers of the cortex was seen in photomicrographs of cross sections of inoculated corn roots. The surface activity involved in ion uptake probably increased, as shown by the darker staining by methylene blue of the affected area. Shoot dry weight increased 20 to 30% in inoculated plants after 3 weeks, presumably by enhancement of mineral uptake. Corn and sorghum plants grown to maturity on limiting nutrients in the greenhouse showed improved growth from inoculation approaching that of plants grown on normal nutrient concentrations. Enhanced ion uptake may be a significant factor in the crop yield enhancement reported for *Azospirillum* inoculation.

Inoculation of crop plants with  $\text{N}_2$ -fixing bacteria of the genus *Azospirillum* was proposed in the mid-1970s as a new approach in providing fixed nitrogen, thereby decreasing fertilization requirements or increasing yield or both (3). Results have been highly variable. Recent experiments in Israel have demonstrated the anticipated benefit in that geographic area: the dry weight (1, 7, 12) and total nitrogen content of maize, sorghum, and *Setaria italica* inoculated with *Azospirillum* spp. increased significantly. Yields of several field-grown grain and forage crops also increased significantly in multiple-year experiments at several different locations (9). Growth-room experiments with  $^{15}\text{N}_2$  (2, 5) or by the  $^{15}\text{N}$  isotope dilution method (6) confirmed incorporation of  $\text{N}_2$  into inoculated plants, although the transfer of newly fixed N from the bacteria to the plant is small, based on studies with a model system (Y. Okon, P. G. Heytler, and R. W. F. Hardy, manuscript in preparation). We report here a significant enhancement of mineral ion uptake by roots of *Zea mays* and *Sorghum bicolor* and associated enhanced growth after inoculation.

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### MATERIALS AND METHODS

*Azospirillum brasilense* (11) strains Cd (ATCC 29729) and Sp 7 (ATCC 29145) and a *Pseudomonas*-like soil isolate were grown in nutrient agar (Difco Laboratories, Detroit, Mich.) at 30°C. Cultures (24 h) were washed from the agar with 0.05 M potassium phosphate buffer at pH 6.8. A 1-ml amount of the washed bacterial suspension containing approximately  $10^8$  CFU was used as the inoculum.

Corn (*Z. mays* L. Pioneer Hybrid 3320) seeds were disinfected with 20% chlorine bleach for 10 min and placed embryos down in Pyrex glass utility trays (20.32 by 30.48 by 5.08 cm) lined with four layers of bleached paper towels and irrigated with 150 ml of 0.2 mM  $\text{CaCl}_2$ ; the treated trays received 1 ml of bacterial inoculum. The trays were covered with plastic food wrap, perforated to allow for air exchange, and placed in a dark growth room, which was maintained at 30°C and 90% relative humidity. On day 2, another 150 ml of 0.2 mM  $\text{CaCl}_2$  was added to each tray. Segments (2 cm long) excised at 0.5 to 2.5 cm from the root tip of 3-day-old etiolated roots were washed at 30°C with 0.2 mM  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$ -0.2 mM  $\text{CaCl}_2$  (pH 6.0) for 4 h and transferred to  $\text{K}^+$  (containing about 12,500 dpm of  $^{86}\text{Rb}^+$  per ml) and  $\text{H}_2\text{PO}_4^-$  (containing about 12,500 dpm of  $\text{H}_2^{32}\text{PO}_4^-$  per ml) nutrient solution to determine the uptake of these ions (4, 10).

For microscopic examination, 2-mm root segments were fixed in 5% cold glutaraldehyde-phosphate buffer at pH 7.0 (G. Milloning, J. Appl. Physiol. 32:1637, 1961) for 1 h. After one wash with phosphate buffer, root segments were postfixated with 2% cold  $\text{OsO}_4$  in phosphate buffer at pH 7.2 for 1 h. After gradual dehydration with ethanol, root segments were transferred in 100% propylene oxide, infiltrated with Spur

TABLE 1. Effect of *A. brasilense* on  $\text{NO}_3^-$  uptake in corn plants

Inoculum	Total Amt of $\text{NO}_3^-$ removed from culture solution <sup>a</sup> ( $\mu\text{mol}$ )		
	2 h	24 h	48 h
Control	0.90 $\pm$ 0.05	4.51 $\pm$ 0.58	7.20 $\pm$ 0.86
<i>A. brasilense</i> Cd	0.85 $\pm$ 0.07	4.60 $\pm$ 0.74	10.05 $\pm$ 1.04
<i>A. brasilense</i> Sp 7	0.91 $\pm$ 0.06	4.55 $\pm$ 0.49	10.95 $\pm$ 1.55

<sup>a</sup> Nine-day-old plants were used. Numbers are means and standard deviations from 12 plants in six hydroponic culture bottles.

(Polyscience, Inc.), and polymerized overnight at 16°C. Root sections (5  $\mu\text{m}$ ) were then stained with methylene blue and examined with a light microscope.

Nitrate uptake was measured with 9-day-old corn plants germinated in the dark room for 3 days and transferred to a 500-ml hydroponic culture bottle (two plants per bottle) containing 300 ml of  $\text{NO}_3^-$ -free, half-strength Hoagland solution with constant air bubbling. The bottles were maintained overnight in a growth room at 27°C with 63% relative humidity. Bacterial inoculum (1 ml) was added to the bottle after 4 days,

and 4 ml of a 1 M  $\text{KNO}_3$  solution was added 24 h later. The disappearance of  $\text{NO}_3^-$  from the solution was monitored with an Orion  $\text{NO}_3^-$  ion-specific electrode.

Four corn seeds were germinated in 15.24-cm pots with vermiculite, and 1 ml of bacterial inoculum was added to each pot. The pots were kept in a growth room under the conditions mentioned above, and Hoagland solution was used continuously as nutrient solution. Two weeks later, one set of plants was removed from the pot and carefully washed with distilled water, and fresh and dry (48 h in a 80°C air-forced oven) weights of plant root and shoot systems were taken. Another set of plant roots was used for  $\text{K}^+$  and  $\text{H}_2\text{PO}_4^-$  uptake determination.

Sorghum (*S. bicolor* SC-599-6 nonsenescent) and corn plants were grown in 25.4-cm pots (two plants per pot) with Metro mix 350 (Grace Horticultural and Agricultural Product Co., Cambridge, Mass.). They were fertilized twice weekly with 20-20-20 fertilizer (A. Peter Co., Allentown, Pa.) at a rate of 226.8 g/7.57 liters of water per  $9.29 \times 10^4 \text{ cm}^2$ . Control plants and those inoculated with 1 ml of bacterial inoculum per pot were fertilized once with 20-20-20 fertilizer and once with 300 ml of  $\text{NO}_3^-$ -free Hoagland solution per week. The plants were kept in a greenhouse with an automatic watering device at temperatures of 75°F by day and 65°F by night with 55% relative humidity.

All control plants received 1 ml of autoclaved bacterial inoculum for comparison.

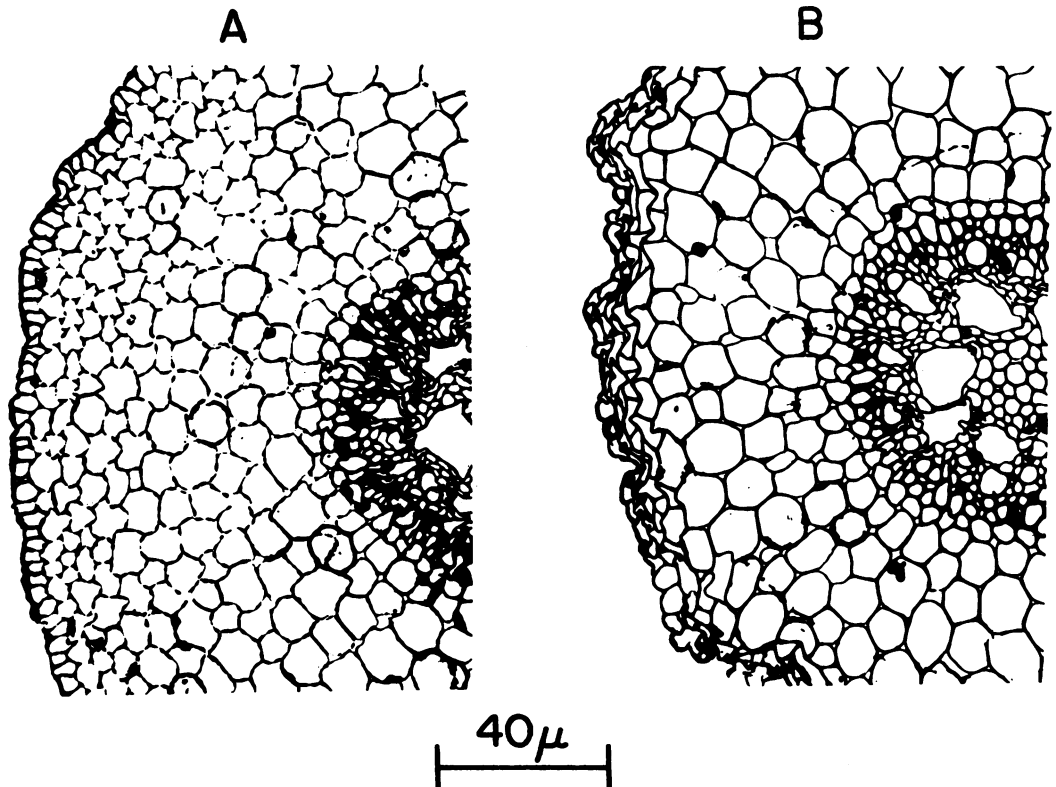


FIG. 1. Light micrographs of the cross section of corn roots. (A) Control tissue. (B) Sp 7-inoculated tissue. Bar, 40  $\mu\text{m}$ .

TABLE 2. Effect of *A. brasilense* on  $K^+$  and  $H_2PO_4^-$  uptake into corn root segments

Seedlings and inoculum	Uptake ( $\mu\text{mol/g}$ [fresh wt] per h) (%)	
	$K^+$ ( $^{86}\text{Rb}^+$ )	$H_2^{32}\text{PO}_4^-$
3-day-old etiolated seedlings <sup>a</sup>		
Control	2.30 $\pm$ 0.54 (100)	0.89 $\pm$ 0.08 (100)
<i>A. brasilense</i> Cd	3.63 $\pm$ 0.38 (158)	1.06 $\pm$ 0.06 (132)
<i>A. brasilense</i> Sp 7	3.83 $\pm$ 0.44 (166)	1.07 $\pm$ 0.09 (134)
2-week-old seedlings <sup>b</sup>		
Control	0.128 $\pm$ 0.015 (100)	0.098 $\pm$ 0.004 (100)
<i>A. brasilense</i> Cd	0.152 $\pm$ 0.009 (119)	0.145 $\pm$ 0.003 (150)
<i>A. brasilense</i> Sp 7	0.164 $\pm$ 0.011 (128)	0.131 $\pm$ 0.007 (134)
<i>Pseudomonas</i> -like soil isolate	0.101 $\pm$ 0.010 (79)	0.083 $\pm$ 0.008 (84)

<sup>a</sup> Root segments were washed (aged) for 4 h in  $K\text{-}PO_4$  solution (see text). Numbers are means and standard deviations of three experiments with triplicate samples.

<sup>b</sup> Freshly cut root segments were used. Numbers are means and standard deviations of two experiments with four replicate samples.

TABLE 3. Effect of *A. brasilense* on the growth of corn plants<sup>a</sup>

Inoculum	Fresh wt (%)	Dry wt (%)	Fresh wt (%)	Dry wt (%)
Control	2.19 $\pm$ 0.78 (100)	0.144 $\pm$ 0.011 (100)	2.78 $\pm$ 0.51 (100)	0.262 $\pm$ 0.018 (100)
<i>A. brasilense</i> Cd	2.18 $\pm$ 0.64 (99)	0.156 $\pm$ 0.020 (108)	4.38 $\pm$ 0.76 (157)	0.420 $\pm$ 0.020 (160)
<i>A. brasilense</i> Sp 7	1.81 $\pm$ 0.77 (93)	0.136 $\pm$ 0.015 (94)	3.46 $\pm$ 0.98 (125)	0.319 $\pm$ 0.015 (122)
Complete	2.38 $\pm$ 0.55 (107)	0.152 $\pm$ 0.018 (106)	3.33 $\pm$ 0.89 (120)	0.291 $\pm$ 0.032 (111)

<sup>a</sup> Numbers are means and standard deviations from six 15-day-old corn plants. Weight is in grams.

## RESULTS

A 40 to 50% increase in  $\text{NO}_3^-$  removal by 1-week-old hydroponically grown corn seedlings inoculated with *A. brasilense* Sp 7 or Cd was found 48 h after inoculation, with no effect at 24 h (Table 1). In the control experiment with *A. brasilense* in the hydroponic culture medium without corn plants, no detectable loss of  $\text{NO}_3^-$  was observed after 48 h. We concluded that the increased uptake of  $\text{NO}_3^-$  is not attributable to an effect of bacteria alone, such as dinitrification. The enhanced ion uptake phenomenon extended to  $K^+$  and  $H_2PO_4^-$ , based on measurements with root segments of young corn seedlings. Inoculation increased the uptake capability 32 to 66% for  $K^+$  and  $H_2PO_4^-$  by 3-day-old etiolated root segments; the increase was 19 to 50% for 2-week-old seedlings (Table 2). The increased ion uptake produced by *A. brasilense* inoculation is not a general response to bacterial colonization, since a *Pseudomonas*-like soil isolate gave no beneficial effect. The reason for the 15 to 20% inhibition of  $K^+$  and  $H_2PO_4^-$  uptake by corn roots inoculated with the *Pseudomonas*-like soil isolate is unknown.

Growth parameters, including fresh and dry weight, were determined for corn and sorghum plants grown to maturity on limiting nutrients with and without *A. brasilense* inoculation (Ta-

bles 3 to 6). The root weight of the 15-day-old corn plant was unaffected, whereas the dry weight of the shoot increased 22 to 60%. The mature dry weight was increased 27 to 34% by inoculation, about half of that produced by non-limiting nutrients. Similar responses were produced in 55-day-old sorghum, with a 16 to 21% increase in shoot dry weight, equivalent to that for nonlimiting nutrients. Nonlimiting nutrient-grown sorghum plants flowered at an average of 64 days, whereas 64, 63, and 66 days passed before flowering took place in *A. brasilense* Cd- and Sp 7-inoculated and uninoculated limited nutrient-grown plants. Leaf size, rate of appearance (Table 5), and date of flowering in sorghum suggested faster development by either inoculat-

TABLE 4. Effect of *A. brasilense* on the shoot weight of matured corn plants<sup>a</sup>

Inoculum	Fresh wt (%)	Dry wt (%)
Control	351 $\pm$ 40	75.7 $\pm$ 6.8 (100)
<i>A. brasilense</i> Cd	349 $\pm$ 52	95.9 $\pm$ 7.2 (127)
<i>A. brasilense</i> Sp 7	354 $\pm$ 47	101.7 $\pm$ 5.6 (134)
Complete	462 $\pm$ 68	126.7 $\pm$ 10.8 (167)

<sup>a</sup> Numbers are means and standard deviations from 12 plants. Weight is in grams.

TABLE 5. Effect of *A. brasilense* on the growth of sorghum plants<sup>a</sup>

Inoculum	Leaf 6 (%)		Leaf 7 (%)		Leaf 8
	Length	Width	Length	Width	Appearance (%)
Control	37.4 ± 3.6 (100)	2.19 ± 0.14 (100)	32.2 ± 2.7 (100)	2.73 ± 0.18 (100)	50
<i>A. brasilense</i> Cd	39.3 ± 4.2 (105)	2.34 ± 0.21 (107)	39.1 ± 2.5 (121)	3.10 ± 0.14 (114)	58
<i>A. brasilense</i> Sp 7	40.5 ± 3.4 (108)	2.48 ± 0.18 (113)	42.0 ± 3.4 (130)	3.07 ± 0.13 (112)	92
Complete	35.7 ± 5.3 (96)	2.24 ± 0.23 (102)	37.7 ± 2.8 (117)	3.03 ± 0.19 (111)	100

<sup>a</sup> Numbers are means and standard deviations of leaf size from 12 35-day-old plants. Length and width are in centimeters.

ed or nonlimiting nutrient plants. Inoculated or nonlimiting nutrient plants flower 2 to 3 days earlier than limiting nutrient but uninoculated plants.

There were no gross changes in root morphology or root weight. Alteration of the cell arrangement in the outer four to five layers of cortex cell of corn roots from inoculated 3-day-old corn seedlings was found (Fig. 1). The altered area stained darker with methylene blue, suggesting an increase in the surface activity involved in ion uptake.

#### DISCUSSION

The data presented above demonstrate for the first time that inoculation with either of two strains of *A. brasilense* produces major increases in the uptake of several mineral ions: NO<sub>3</sub><sup>-</sup>, K<sup>+</sup>, and H<sub>2</sub>PO<sub>4</sub><sup>-</sup>. Furthermore, these short-term plant-part experiments also increased the shoot dry matter accumulation by corn and sorghum grown on limiting nutrients. These observations suggest that *A. brasilense* inoculation may improve the efficiency of applied mineral nutrients by helping the plant scavenge limiting nutrients, a role similar to that of mycorrhiza in phosphate recovery. Additional experiments under field conditions will be needed to define the significance of this laboratory phenomenon on crop production (1, 7-9, 12). However, one may suggest that the short-term impact of *A. brasilense* inoculation may be much more important in increasing nutrient recovery than in fixing N<sub>2</sub> and transferring this newly fixed nitrogen to the plant.

TABLE 6. Effect of *A. brasilense* on the shoot weight of 55-day-old sorghum plants<sup>a</sup>

Inoculum	Fresh wt (%)	Dry wt (%)
Control	55.5 ± 4.3 (100)	6.28 ± 0.54 (100)
<i>A. brasilense</i> Cd	64.6 ± 3.8 (116)	7.28 ± 0.50 (116)
<i>A. brasilense</i> Sp 7	63.0 ± 3.3 (114)	7.57 ± 0.60 (121)
Complete	67.4 ± 3.9 (121)	7.48 ± 0.59 (119)

<sup>a</sup> Numbers are means and standard deviations from six plants. Weight is in grams.

The mechanism of increased ion uptake by *A. brasilense*-inoculated plant roots is unknown. The increase may relate to the production of hormones by *Azospirillum* spp. (13-15). Pearl millet seedlings produced more root hairs, lateral roots, and mucilaginous sheath (mucigel) in response to inoculation. These effects were attributed to the auxin, gibberellin, and cytokinins produced. Moreover, *Azospirillum* spp. produced in culture polygalacturonic acid transeliminase, and it was suggested that pectinolytic enzymes play an important role in root invasion by *Azospirillum* spp. Thus, the yield response to *Azospirillum* spp. may result from several factors. Further morphological, physiological, and biochemical studies on the interaction between the host plant roots and *Azospirillum* spp. should provide a better understanding of the enhanced ion uptake mechanism.

#### LITERATURE CITED

- Cohen, E., Y. Okon, J. Kigel, I. Nur, and Y. Henis. 1980. Increases in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen fixing *Azospirillum* spp. *Plant Physiol.* 66:246-249.
- De Polli, H., F. Matsui, J. Dobreiner, and R. Salati. 1977. Conformation of nitrogen fixation by two tropical grasses by <sup>15</sup>N<sub>2</sub> incorporation. *Soil Biol. Biochem.* 9:119-123.
- Dobreiner, J., and J. M. Day. 1976. Associative symbioses and free-living systems, p. 518-538. In W. E. Newton, and C. J. Nyman (ed.), *Proceedings of the 1st International Symposium on Nitrogen Fixation*. Washington State University Press, Pullman.
- Epstein, E. 1972. *Mineral nutrition of plants: principles and perspectives*. John Wiley & Sons, New York.
- Eskew, D. L., A. R. J. Eaglesham, and A. A. App. 1981. Heterotrophic <sup>15</sup>N<sub>2</sub> fixation and distribution of newly fixed nitrogen in a rice-flooded soil system. *Plant Physiol.* 68:48-52.
- Franche, C., and C. Elmerich. 1981. Physiological properties and plasmid content of several strains of *Azospirillum brasilense* and *A. lipoferum*. *Ann. Microbiol. (Paris)* 132:3-18.
- Kapulnik, Y., J. Kigel, Y. Okon, I. Nur, and Y. Henis. 1981. Effect of *Azospirillum* inoculation on some growth parameters and N-content of wheat, sorghum, and Panicum. *Plant Soil* 61:65-70.
- Kapulnik, Y., Y. Okon, J. Kigel, I. Nur, and Y. Henis. 1981. Effects of temperature, nitrogen fertilization and plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (strain Cd). *Plant Physiol.* 68:340-343.
- Kapulnik, Y., S. Sarig, I. Nur, Y. Okon, J. Kigel, and Y.

- Henis.** 1981. Yield increases in summer cereal crops in Israel fields inoculated with *Azospirillum*. *Exp. Agric.* **17**:179–187.
10. **Lin, W.** 1979. Potassium and phosphate uptake in corn roots. Further evidence for an electrogenic  $H^+/K^+$  exchange and an  $OH^-/Pi$  antiporter. *Plant Physiol.* **63**:952–955.
  11. **Neyra, C. A., and J. Dobereiner.** 1977. Nitrogen fixation in grasses. *Adv. Agron.* **29**:1–38.
  12. **Nur, I., Y. Okon, and Y. Henis.** 1980. An increase in a nitrogen content of *Setaria italica* and *Zea mays* inoculated with *Azospirillum*. *Can. J. Microbiol.* **26**:482–485.
  13. **Tien, T. M., H. G. Diem, M. H. Gaskins, and D. H. Hubbell.** 1981. Polygalacturonic acid transeliminase production by *Azospirillum* species. *Can. J. Microbiol.* **27**:426–431.
  14. **Tien, T. M., M. H. Gaskins, and D. H. Hubbell.** 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum L.*). *Appl. Environ. Microbiol.* **37**:1016–1024.
  15. **Umali-Garcia, M., D. H. Hubbell, M. H. Gaskins, and F. B. Dazzo.** 1980. Association of *Azospirillum* with grass roots. *Appl. Environ. Microbiol.* **39**:219–226.