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Enhanced Growth of Wheat and Soybean Plants Inoculated with *Azospirillum brasilense* Is Not Necessarily Due to General Enhancement of Mineral Uptake†

YOAV BASHAN,[1]‡* S. KENT HARRISON,[1] AND ROBERT E. WHITMOYER[2]
Department of Agronomy, Ohio State University, Columbus, Ohio 43210,1 and Laboratory of Electron Microscopy, Ohio Agricultural Research and Development Center, Wooster, Ohio 44691 [2]

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The capacity of *Azospirillum brasilense* to enhance the accumulation of K^+, P, Ca^{2+}, Mg^{2+}, S, Na^+, Mn^{2+}, Fe^{2+}, B, Cu^{2+}, and Zn^{2+} in inoculated wheat and soybean plants was evaluated by using two different analytical methods with five *A. brasilense* strains originating from four distinct geographical regions. A *Pseudomonas* isolate from the rhizosphere of *Zea mays* seedlings was included as a control. All *A. brasilense* strains significantly improved wheat and soybean growth by increasing root and shoot dry weight and root surface area. The degree of plant response to inoculation varied among the different strains of *A. brasilense*. All strains were capable of colonizing roots, but the best root colonizer, *Pseudomonas* sp., had no effect on plant growth. The numbers of organisms of Brazilian strains Sp-245 and Sp-246 colonizing roots were similar regardless of the host plant. Numbers of organisms for the other strains were directly dependent on the host plant. The main feature characterizing mineral accumulation in inoculated plants was that all inoculation treatments changed the mineral balance of the plants, but in an inconsistent manner. Enhancement of mineral uptake by plants also varied among strains to a great extent and was directly dependent on the strain-plant combination; i.e., a strain capable of increasing accumulation of a particular ion in one plant species or cultivar often lacked the ability to do so in another. Minerals in inoculated plants were not evenly distributed in different plant tissues, and the changes varied among groups of plants within each bacterial strain inoculation treatment. We suggest that, although *A. brasilense* strains are capable of changing the mineral balance and content of plants, it is unlikely that this ability is a general mechanism responsible for plant improvement by *A. brasilense*.

Inoculation of plants with *Azospirillum* strains alters root morphology, increases numerous plant shoot growth parameters, and eventually increases the yield of many cereal crops (33), vegetables, and other agricultural plants (10, 14, 34). These changes have been attributed to inoculation-induced enhancement of mineral uptake in plants. Variations in improvement of NO^{3-}, NH^{4+} (12, 23, 26, 27, 29, 32), P (26, 29, 35), K^+ (26), Rb^+ (27), and Fe^{2+} (3) uptake through inoculation in wheat, sorghum, rice, corn, and the hybrid *Sorghum bicolor* x *Sorghum sudanense* plants have been demonstrated. It was proposed that enhancement of mineral uptake by plants should result in an increased accumulation of both dry matter and minerals in the stem and leaves of the plant. During the reproductive period, the accumulated minerals would be transferred to the reproductive parts of the plants and ultimately would result in higher yields (21, 23, 29). However, these studies used few *Azospirillum* strains, and it is unknown whether most *Azospirillum* strains possess this ability.

The objectives of this study were as follows. (i) We wanted to focus on the basic question as to whether a general enhancement in uptake of minerals other than nitrogenous compounds is a common mechanism induced in plants by *Azospirillum brasilense*. In this analysis we employed several common *A. brasilense* strains that originated from different regions of the world to determine their effect on the mineral content of inoculated plants. The evaluation was carried out in wheat, which is a common model plant for *Azospirillum* spp., and in soybean plants since it has been previously shown that dicotyledonous plants also respond positively to *Azospirillum* inoculation (10, 11, 14). (ii) We also wanted to evaluate whether this enhancement is correlated with increased plant growth.

MATERIALS AND METHODS

Organisms and growth conditions. *A. brasilense* Cd (ATCC 29710) originated from *Cynodon dactylon* plants inoculated with the Brazilian strain Sp-7 in California. Strains Sp-7 and Cd have the same DNA homology but differ in physiological and immunological features (15, 25). *A. brasilense* Sp-245 and Sp-246 originated from Rio de Janeiro, Brazil (2), and Somali 67 is a strain that originated from a desert in Somali, East Africa (16). *A. brasilense* OH 88028 and *Pseudomonas* sp. strain OH 88004 (8) were isolated from maize roots grown in a Crosby silt loam soil in Columbus, Ohio. Wheat plants (*Triticum aestivum*) cv. Deganit (spring wheat) and cv. Tikal (winter wheat) and soybean plants (*Glycine max*) cv. Pella were used as test plant species in this study.

Plants were grown in pure quartz sand (Millwood Silica Co.) or in coarse vermiculite in 800-ml pots (4 to 10 plants per pot). Plants were irrigated daily with 40 ml of half-strength Hoagland nutrient solution and were maintained at 25 ± 3°C with 16 h of light (400 μE/m^2 per s) in a growth chamber (Environmental Growth Chamber Co.) for the entire growth period.

Bacterial inoculation. Seeds were disinfected in 0.5% aqueous NaOCl for 5 min under continuous shaking (120 rpm). The seeds were then thoroughly rinsed with tap water (average bacterial population, 200 to 500 CFU/ml) until all

* Corresponding author.
†Manuscript no. 226-89 from Ohio Agricultural Research and Development Center, Wooster, Ohio.
‡Present address: Karme Yosef, M. P. Ayalon 72910, Israel.

traces of NaOCl were removed. This procedure removed most of the bacteria naturally occurring on the surface of wheat and soybean seeds (average remaining bacterial population, 10 to 100 CFU/100 seeds) but failed to eliminate internal seed-borne saprophytic *Erwinia* sp. (10 to 1,000 CFU/100 seeds), which could be detected in most seed batches but which produced no apparent effect on seed germination or seedling development.

Bacteria were cultured from a single colony into a stock culture. A 1-ml sample of bacterial suspension was then transferred into 50 ml of nutrient broth (Difco Laboratories) and incubated in a shaker at 200 rpm at 30 ± 2°C for 16 to 18 h until the logarithmic phase of growth. Bacterial cells were harvested by centrifugation (7,000 x g, 10 min) and then washed once in 0.06 M potassium phosphate buffer supplemented with 0.15 M NaCl and once with sterile tap water. The number of bacteria for inoculation was adjusted to 10^6 CFU/ml in tap water (10^6 CFU/ml is the optimal inoculation level for *A. brasilense* [4, 23]). Seeds were inoculated by immersing them into the bacterial suspension and subjecting the mixture to a light vacuum created by a small pump. The vacuum was released abruptly to introduce the bacteria into seed cavities. The seeds were imbibed in the bacterial suspension for an additional 2 to 4 h and carefully placed on the surface of wetted sand in the pots and covered with a 2- to 3-cm layer of sand or vermiculite and transferred to the growth chamber.

Bacterial counts on roots. Bacterial counts on roots of inoculated plants were carried out as follows: roots were excised, placed in 10 ml of phosphate-buffered saline, and homogenized. The slurry was then serially diluted in phosphate-buffered saline and plated on BL semiselective N-free medium for strain Cd (5), OAB N-free medium for the other *Azospirillum* strains (31; medium was modified as described in reference 5), and nutrient agar (Difco) supplemented with 200 mg of streptomycin sulfate per liter for the *Pseudomonas* sp. Although few other unidentified bacterial strains were able to grow on this medium, *Pseudomonas* sp. was identified by its colony morphology and by a unique fluorescence that appeared when the plates were illuminated with UV light. The plates were incubated at 30 ± 2°C for 72 to 96 h, and colonies were counted.

Plant tissue mineral analysis. Mineral content in plant tissues was analyzed by two methods: (i) X-ray microanalysis with an Edax unit (Edax Co.) attached to a scanning electron microscope (ISI 40; International Scientific Instruments Co.) for K^+, Mg^{2+}, P, and S; and (ii) inductively coupled argon plasma atomic emission spectroscopy (ICP spectroscopy) (model 975; Jarrell-Ash Co.) for K^+, Mg^{2+} P, S, Fe^{2+}, Ca^{2+}, Na^+, Mn^{2+}, B, and Cu^{2+}.

Samples for X-ray microanalysis were cut with a razor blade from the stem and leaves for soybean and from the first leaf of wheat (2 to 3 mm wide). The segments were mounted on a carbon planchet by using silver paint and analyzed immediately without further specimen preparation. The X-ray beam scanned from one end of the segment to the other at 30 kV (Fig. 1), and the results obtained were the average mineral content in the scanned zone. This accurate but qualitative method gives the amounts of each element (in percentage) relative to the other elements in a particular analysis; i.e., the combined percentages of all the elements evaluated equal 100. Results were presented in proportion to K^+ (ion/K^+ ratio), which was the most abundant element detected. Samples for ICP spectroscopy were prepared by cutting the entire plant with scissors at the soil level and drying it in a forced-air oven at 50°C for 48 h. Analysis of all ions (except S) was done by acid digestion of dry-ash samples prepared as follows. A 0.25-g sample of dried ground plant was placed in Vycor (Fisher Scientific Co.) crucibles and heated for 5 h at 550°C. After cooling, 4 ml of 1:3 HCl-water digestion solution was added to the crucible, and it was heated on a hotplate (100 to 120°C) almost to dryness. Then 10 ml 10% HNO_3 was added and diluted to a 50ml volume with deionized water (modification of methods presented previously [l, 20]). Samples for S analysis were digested by the nitric-perchloric acid method as follows. Samples of 0.25 g of dried ground plant tissue were placed in digestion tubes (20 ml); 3 ml of concentrated HNO_3, 2 ml of 72% $HClO_4$, and 5 ml of deionized water were added; and the tissues were predigested overnight at 25°C. Samples were then digested at 160°C for 2.5 h and at 225°C for 4 h. After the digestion period the mixtures were cooled, and 15 ml of deionized water was added to each tube. Samples were reheated for 15 min to dissolve crystals. Mixtures were transferred to 50-ml volumetric flasks, brought to volume with deionized water, and mixed without filtration. The digested samples were transferred to 50-ml Erlenmeyer flasks, and the precipitates were allowed to settle out before a sample was taken for elemental analysis (modification of methods described previously [l, 22]). Elemental analysis was done by ICP spectroscopy. Calibration of the instrument was done through use of known calibration standards.

Measurements of plant growth parameters. Root surface area was determined by the gravimetric method by using $Ca(NO_3)_2$ (13) after all vermiculite particles were washed gently from the roots and the roots were blotted dry with a soft paper towel. Dry weight was determined immediately after the roots and the shoots were dried in a forced-air oven at 50°C for 48 h.

Experimental design and statistical analysis. All experiments were performed two or three times each in a completely randomized design with three or five replicates. A replicate consisted of a pot containing 4 (soybean) or 10 (wheat) seedlings. Results from identical experiments were combined for analysis. Significant differences among treatment means were determined at $P \leq 0.05$ by using either the Fisher least significant difference (comparison between different bacterial isolates by X-ray microanalysis) or the Student t test (comparison between different plant tissues and sampling dates by X-ray microanalysis). All data from ICP spectroscopy are presented directly without analysis to demonstrate the variability in mineral content of similar samples.

RESULTS

Response of wheat and soybean plants to inoculation with *A. brasilense* and *Pseudomonas* sp. Plant response to inoculation was evaluated by measuring root and shoot dry weight and root surface area. Generally, wheat and soybean plants responded positively to inoculation with each of the five strains of *A. brasilense*, as indicated by a significant increase in plant growth parameters compared with that of noninoculated plants (Fig. 2). Plants did not respond to inoculation with the *Pseudomonas* sp. Significant variations were detected in plant responses to different strains. Strain Sp-245 induced a pronounced effect in all plant cultivars and plant parameters. Strains Cd and Sp-246 induced effects that were smaller than that of Sp-245 but greater than those of strains Somali-67 and OH 88028. All growth parameters measured were significantly higher for *A. brasilense*-inoculated plants than for noninoculated plants, regardless of the bacterial strain (Fig. 2).

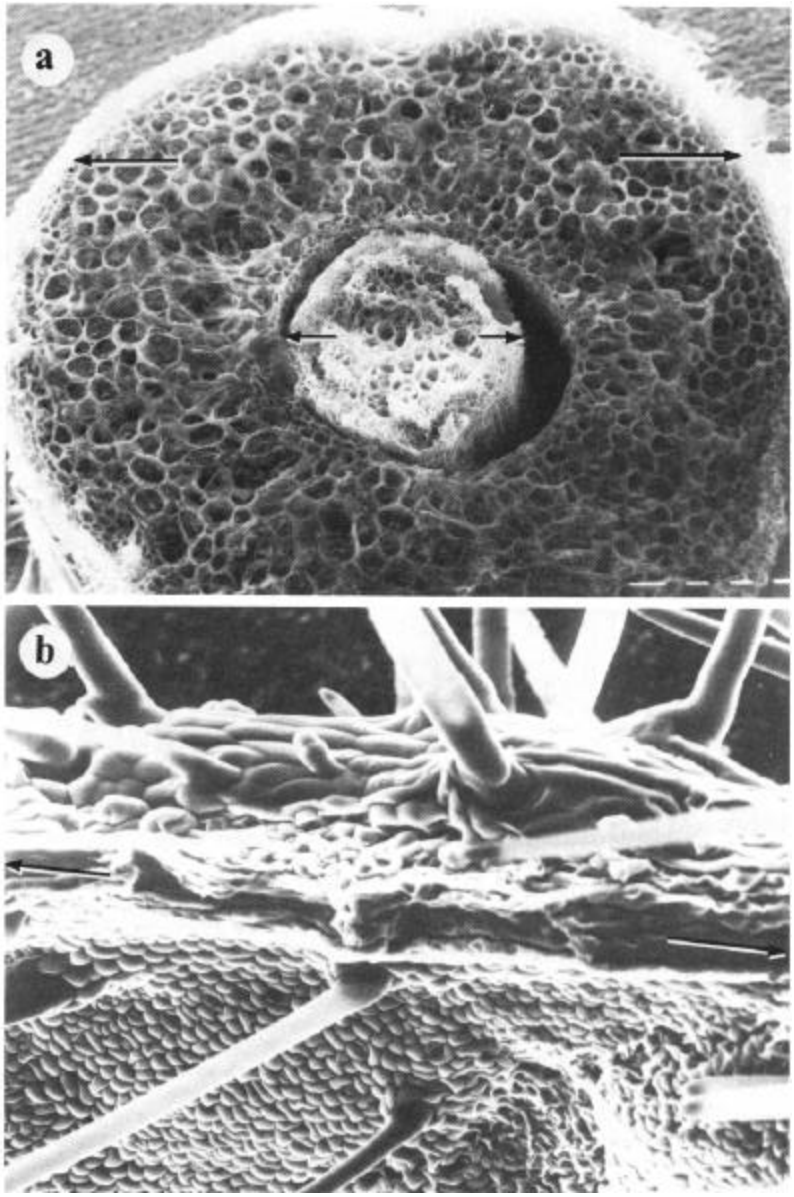

FIG. 1. Site of X-ray microanalysis of noninoculated and inoculated wheat and soybean plants by *A. brasilense* strain,: analysis Sites (between the arrows) in the stele and the cortex (a) and in the leaf (b) of soybean plants.

Root colonization varied greatly among the strains tested. The *Pseudomonas* sp. was the most effective root colonizer, and its cell number in each type of plant reached a population size far above that of any of the *A. brasilense* strains. *A. brasilense* root colonization varied; strain OH 88028 was the least effective root colonizer, and the population sizes of strains Sp-245 and Sp-246 were similar regardless of the plant host. Numbers of strain Cd and Somali-67 organisms colonizing the roots varied and were dependent on the plant cultivar (Fig. 2).

Changes in P, K, Mg, and S ion balance induced in leaves of wheat and soybean plants by different *A. brasilense* strains as analyzed by X-ray microanalysis. Most inoculation treatments, regardless of plant species or bacterial strain, significantly changed the ion balance in plant tissues. However, no common pattern in these changes among different strains was observed. Analysis of the Mg/K balance revealed that only strain Sp-246 induced a significant increase in the ratio of these ions in wheat tissue compared with that in uninoculated plants, whereas in soybean plants its effect was not apparent. Strains Cd and OH 88028 were the only strains that induced an increase in Mg in soybean plants (Fig. 3A).

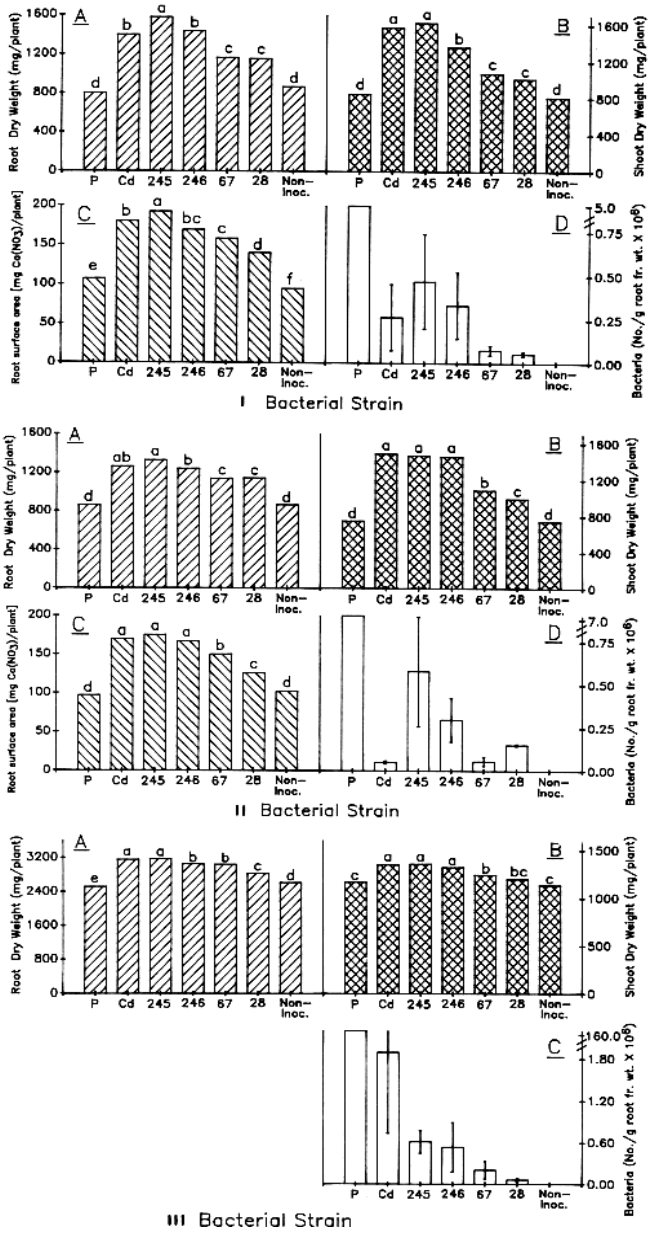

FIG. 2. Response of wheat (cvs. Deganit [I] and Mal [II]) and soybean (cv. Pella [III]) plants to inoculation with various *A. brasilense* strains and a *Pseudomonas* sp.: changes in dry weight of roots (A) and shoots (B), changes in root surface area (C), and the bacterial population size developed in the rhizosphere (D). Columns for each subfigure (separately) followed by a different letter differ significantly at $P \leq 0.05$ according to Fisher's least significant difference. Bars represent standard errors. Each experiment was repeated twice in triplicates. Results are the mean of both experiments. Results presented in panels A and B were obtained from the same experiments. Results presented in panels C and D are from separate experiments.

The P/K ratio in wheat was not affected by any of the *A. brasilense* strains, whereas in soybean plants strains Cd, Sp-245, and OH 88028 increased the ratio but strain Sp-246 had no effect (Fig. 3B). The S/K ratio in wheat was signify-cantly affected only by strain Sp-245, whereas in soybeans strains Cd, Sp-245, and OH 88028 significantly increased this

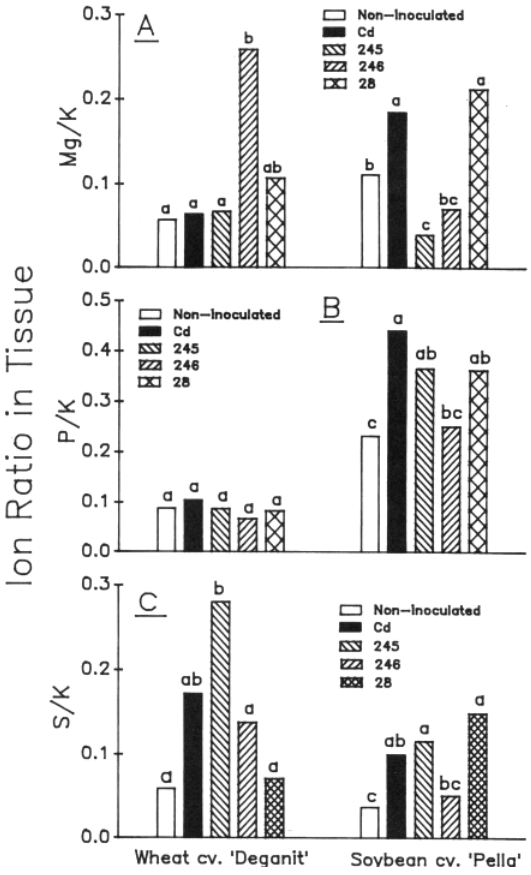

FIG. 3. Changes in Mg/K (A), P/K (B), and S/K (C) ratios induced in leaves of wheat and soybean plants by different *A. brasilense* strains and analyzed by X-ray microanalysis. Columns followed by different letters in each panel and in each plant species (separately) differ significantly at $P \leq 0.05$ according to Fisher's least significant difference. The experiment was repeated three times, and the results were combined and analyzed together.

ratio (Fig. 3C). The differences among *A. brasilense* strains in altering each of the ion ratios in soybeans were not statistically significant.

Changes in ion balance in soybean tissues induced by *A. brasilense* Cd at 4 and 10 days after inoculation as measured by X-ray microanalysis. Analysis of soybean ion balance after inoculation with *A. brasilense* Cd revealed that changes did not occur evenly or simultaneously in all plant parts. However, the ion/K^+ ratio was always higher in inoculated plants. Significant changes in the Mg/K ratio occurred only in the leaf tissue, and these changes were not apparent in either the stele or the cortex (Fig. 4A). On the other hand, significant increases in the P/K ratio occurred in all plant parts regardless of the time after inoculation (Fig. 4B), whereas significant increases in the S/K ratio appeared in the cortex only 4 days after inoculation (Fig. 4C). Despite these variable changes, analysis of ion ratios with respect to time after inoculation revealed significant increases in all ion ratios with time regardless of inoculation. These increases in the ion/K ratio of inoculated plants between 4 and 10 days after inoculation ranged from 49 to 71% depending on the ion, whereas the increases in ion/K ratios of noninoculated

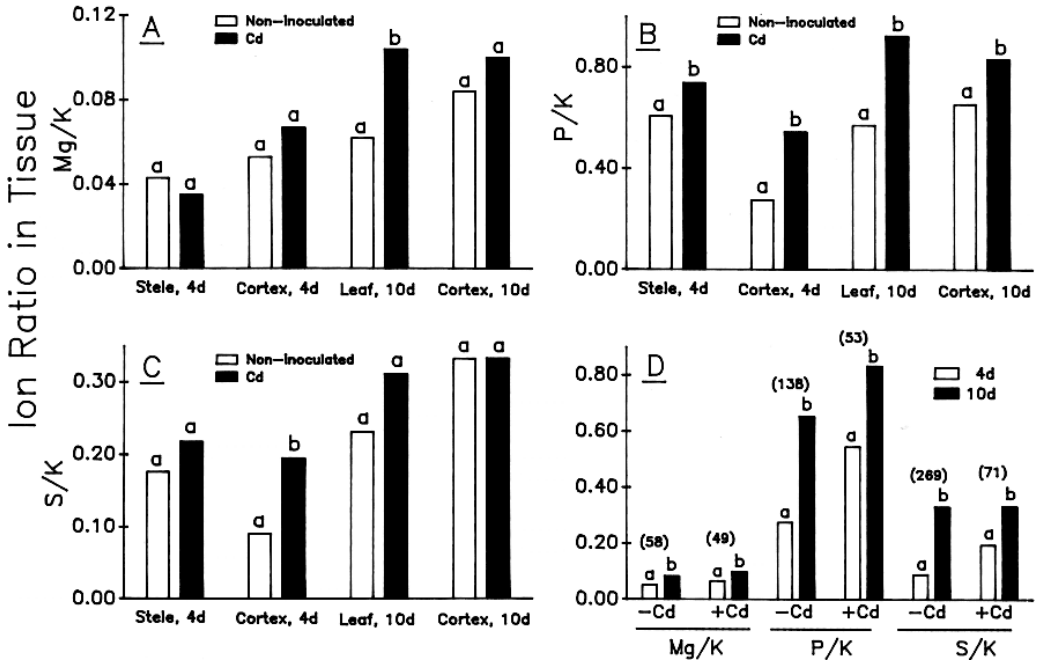

FIG. 4. Changes in Mg/K (A), P/K (B), and S/K (C) ratios in soybean stele, cortex, and leaf as demonstrated by X-ray microanalysis 4 and 10 days after inoculation with *A. brasilense* Cd. Mean pairs for inoculated versus noninoculated plants within a tissue type and ion ratio that are followed by different letter differ significantly according to the Student *t* test at $P \leq 0.05$. (D) Effect of *A. brasilense* Cd inoculation on ion ratios in soybean root cortex 4 and 10 days after inoculation. Mean pairs for 4 days versus 10 days within each inoculation group and ion ratios followed by different letters differ significantly according to the Student t test at $P \leq 0.05$. Differences between ratios obtained 4 and 10 days after inoculation for each inoculation group and ion ratio are given within parentheses in percentages.

plants were more drastic and ranged from 58 to 269% (Fig. 4D).

Changes in ion content of inoculated wheat and soybean plants induced by various *A. brasilense* strains and analyzed by ICP spectroscopy. Quantitative determination of ions in wheat and soybean leaves by ICP spectroscopy revealed that *A. brasilense* strains are capable of changing the relative quantities of several ions in the plant tissues. These changes were characterized by nonuniformity of the response within a plant species to any specific *A. brasilense* strain; i.e., some plants responded by changing the amount of a certain ion in their tissue, whereas a replicate group of plants failed to respond to the inoculation (Fig. 5). This inconsistency in plant response in relation to mineral changes was detected for K^+, Mg^{2+}, P, Ca^{2+}, Fe^{2+}, S, and B. Inoculation had no effect on the content of Na^+, Mn^{2+}, Cu^{2+}, or Zn^{2+} ions.

DISCUSSION

Enhanced mineral uptake in inoculated cereal plants was proposed as a possible mechanism of plant growth enhancement by *Azospirillum* sp. The major element involved was suggested to be N in the form of nitrate in wheat, sorghum, and corn plants (12, 17, 23, 24, 26, 32) or ammonium in rice plants (29). However, other elements such as P and K^+ were also suggested to play a key role in this plant-bacterium interaction. This study aimed to evaluate the relation between enhanced mineral uptake and growth of inoculated plants (21, 26, 27, 29, 35, 37).

Evaluation of the mineral balance and actual content in inoculated plants revealed an effect of *A. brasilense* strains on both parameter. However, no consistent pattern of increased ion content of plant tissues could be found in any bacterial strain-plant combination evaluated. Certain *A. brasilense* strains increased one or more ions in one plant cultivar, whereas other strains only changed the balance and relative content of different minerals. Furthermore, changes, in mineral content of plant tissues were not consistent even within a single plant species and bacterial inoculation treatment; i.e., one group of plants of a given species responded to bacterial inoculation by exhibiting modified mineral content, whereas identically inoculated plants exhibited no effect. The inconsistency in mineral changes found in this study lends support to previous findings that show considerable variability in nitrate accumulation by wheat (24) and phosphorous accumulation in rice after inoculation with *Azospirillum* sp. (29). This inconsistency in plant response at the tissue level resembled the well-known inconsistency in crop yield response to *Azospirillum* inoculation. It is this inconsistency which has to date restricted the progress of *Azospirillum* inoculation technology (36; Y. Bashan and H. Levanony, Can. J. Microbiol., in press).

Other complex factors may contribute to the inconsistency factor: (i) changes in mineral content that are not evenly distributed within plant tissues and are dependent on the specific ion and on the bacterial inoculation strain and (ii) the N_2-fixing ability of *Azospirillum* species. In this study. N_2 fixation was not considered a major factor, since the N content of the Hoagland nutrient solution used prevents N_2 fixation by *Azospirillum* species (12). Additionally, it was recently demonstrated that positive contribution of *A.*

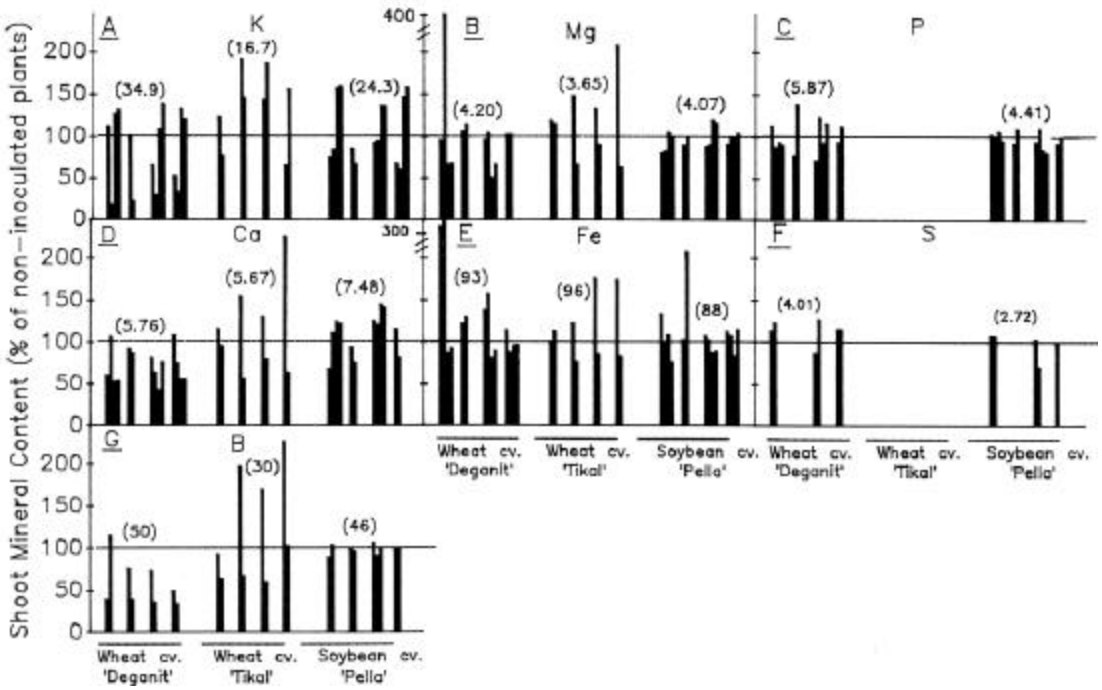

FIG. 5. Ion content of K^+ (A), Mg^{2+}, (B), P (C), Ca^{2+} (D), Fe^{2+} (E), S (F), and B (G) in leaves of wheat cvs. Deganit and Tikal and in soybean cv. Pella after inoculation with four strains of *A. brasilense:* Cd, Somali 67, Sp-245, and Sp-246 (left to right for each plant cultivar; groups of two to four columns per strain). Each column represents one determination, which contains the ion content as a percentage of 10 wheat or 4 soybean plants. The ion content of noninoculated plants is represented in horizontal lines (at 100%), and the quantity of each ion in these plants (in milligrams [A through D, F] or micrograms [E and G] per gram of tissue dry weight) is given for each plant cultivar within parentheses. Missing determinations (<4 per strain in each plant cultivar) were left empty.

brasilense Cd to the growth of tomato seedlings is not by N_2 fixation (11).

Root colonization is always considered a major factor in successful inoculation of plants by beneficial bacteria (38). *Azospirillum* strains are known as good colonizers of wheat (7, 9; Y. Bashan and H. Levanony, Can. J. Microbiol, in press), rice (28), vegetables (10, 19), sorghum (2), and corn (18). However, root colonization ability varied greatly among *Azospirillum* strains. This study presents evidence that high levels of root colonization per se are not solely responsible for the effects induced in plants by rhizosphere bacteria. A rhizosphere pseudomonad with an exceptional ability to colonize wheat and soybean roots failed to induce mineral changes in plant tissues. However, *A. brasilense* strains were much less efficient colonizers yet induced these changes at significantly lower population levels.

Proton efflux from roots is directly related to the mineral uptake of plants (6, 30). Recently, it was demonstrated that inoculation of wheat with *A. brasilense* Cd changes proton efflux from the roots (8). The metabolic pathways responsible for increased proton efflux as well as increased ion uptake are unknown. Since both phenomena are interrelated, elucidation of specific mechanisms at the membrane-enzyme level may help explain the inconsistency in plant response.

In conclusion, this study suggests that, although *Azospirillum* strains are capable of changing the balance and quantity of several minerals other than nitrogen in inoculated plants, it is unlikely that these changes alone can explain the significant increases in plant growth and yield obtained for numerous plant species.

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