

Enhancement of Wheat Root Colonization and Plant Development by *Azospirillum brasilense* Cd. Following Temporary Depression of Rhizosphere Microflora

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Inoculation of wheat with *Azospirillum brasilense*, combined with the application of four fungal and bacterium-inhibiting substances to which *A. brasilense* is resistant in the soil, decreased the rhizosphere population, while it increased wheat root colonization by *A. brasilense*, even in cases of poor inoculation. The inoculation significantly increased the following wheat plant parameters as well: plant dry weight, number of tillers per plant, spikelet fertility, harvest index, and grain yield. This model may provide a new approach to improve control of root colonization by beneficial bacteria.

During the last decade, interest has been increasing in rhizosphere bacteria, particularly *Azospirillum* strains, because of their possible contribution to the yields of various cereals (2, 7, 9, 10, 13, 14, 16-20).

Inconsistent results were common in field experiments in which the effect of cereal root colonization by bacteria was followed; large yield increase, negligible increase, and reduction of yield were recorded in apparently similar experiments. Yet, because most scientific reports deal with positive effects, one may assume that inoculation is usually successful. The commercialization of plant inoculation, therefore, has received much attention during the last decade (15, 21, 22).

The mechanism involved in successful inoculation is unknown, although some avenues have been suggested in recent experiments (7, 15). Therefore, it is practically impossible to simulate the exact conditions and factors contributing to any success in field experiments. Additionally, root colonization cannot yet be enhanced by external application of beneficial bacteria, because the soil acts as a biological buffer against most nonresident bacteria.

In this study are presented results of an attempt to enhance wheat root colonization of *Azospirillum brasilense* Cd. by temporarily depressing soil microflora. The effects of this inoculation on various plant parameters were evaluated.

MATERIALS AND METHODS

Organisms and growth conditions. *A. brasilense* Cd. (ATCC 29710) and wheat plants (*Triticum aestivum* cv. "Hazera-18" or "Deganit") were used as model organisms throughout this study.

After surface disinfection (3-min soaking in 1% NaOCl and thorough washing with tap water to eliminate traces of hypochlorite), seeds were imbibed for 3 h in tap water and germinated in the darkness on wet filter paper at $25 \pm 3^\circ\text{C}$ for 48 h. Five seeds were then planted (1-cm depth) in each pot containing 5 liters of Rehovot brown-red degrading sand-soil and thinned to three plants per pot after the full emergence of the first leaf. Each pot was fertilized once with 5 g of Osmocot, a slow-release fertilizer (total N, 18% derived from NH_4NO_3 ; total P, 6% derived from P_2O_5 ; total K, 12% derived from K_2SO_4 ; Sierra, Holland), and 5 ml of Hoagland microelement solution.

In the summer, pots were kept at $22 \pm 3^\circ\text{C}$ in an environ-

mentally controlled greenhouse, and in the autumn and winter they were kept outdoors under a white net (to avoid damage by birds). Whenever needed, plants were drip irrigated with a constant amount of water, using droplets of water (flow rate, 4 liters/h) (Netafim, Israel) per pot. Pot experiments were of equal design and of equal length.

Plants were also grown in speedling trays, with a single plant per hole, in sterile vermiculite under a completely controlled environment in a growth chamber (Conviron model EF7H; Controlled Environments, Winnipeg, Canada).

Application of bacteria and bacterial inhibitors. Bacteria, grown in a nutrient broth (Difco Laboratories, Detroit, Mich.) medium for 24 h at $30 \pm 2^\circ\text{C}$, were harvested by centrifugation at $12,000 \times g$ for 10 min and washed twice with sterile tap water. At the first leaf stage the pots were inoculated (twice with a 1-week interval) with 10^6 CFU/g of soil (Y. Bashan, Soil Biol. Biochem., in press). Control plants were irrigated with tap water at the time of inoculation. The soil surface in the pots was covered with a 2- to 3-cm layer of sterile vermiculite, to prevent bacterial dispersion during rainy periods. In addition, each pot was placed on a separate small bench (30-cm high) to avoid cross contamination by bacteria from the dripping of excess irrigation water from the bottom of the pots.

Unless otherwise indicated, each pot was treated with a 10-ml (pipet application) fresh mixture of the following substances (in milligrams per liter): cycloheximide, 180; streptomycin sulfate, 150; sodium deoxycholate, 150; 2,3,5-triphenyltetrazolium chloride, 10. Two such treatments were carried out immediately after inoculation, and three more were carried out at one-third the original concentration at weekly intervals. After every treatment each pot was irrigated with 0.5 liters of water to ensure dispersal of substances throughout the soil.

In speedling trays, 5 ml of the inhibiting mixture, at one-fifth the original concentration, was applied to each hole.

Bacterial enumeration in roots. *A. brasilense* Cd. was counted in roots by two different methods. The first was based on selective enrichment in selective media, and the most-probable-number determination (3) was used for the enumeration of *A. brasilense* Cd. in numbers smaller than 10^4 CFU/g of root. The second method was based on an

enzyme-linked immunosorbent assay developed especially for specific *A. brasilense* Cd. detection (H. Levanony, Y. Bashan, and Z. E. Kahana, FEMS Symposium on Microbial Communities in Soil, Copenhagen, Denmark, p. 77-77a) and was used for counting 10^4 or more *A. brasilense* Cd. per g of root.

Total bacterial counts in the rhizosphere were performed by placing 1 g of whole young roots in a 100-ml Erlenmeyer flask containing 30 ml of sterile saline (0.85% NaCl). After vigorous shaking (200 strokes per min) at 30°C for 30 min, the solution was serially diluted, plated on solid nutrient agar (Difco), incubated at $25 \pm 2^\circ\text{C}$ for 72 h, and counted.

Plant parameter measurements. The following plant parameters were quantitated: yield per pot, plant, main spike, and tiller (in grams); foliage dry weight (in grams); changes in plant height until full heading (in centimeters); rates of germination (in days) and growth (in centimeters); heading date (in days); root surface area (gravimetric method [5]); number of tillers, spikelets on the main spikes, and grains per spikelet on the main spikes; mean weight of grains on the main spike; protein content of grains (with an automatic Technicon Infralyser 400 [percent]); and harvest index (grain weight divided by plant weight).

Experimental design and statistical analysis. Pot experiments (160 pots per experiment) were conducted in a randomized block design (16 pots per block) in 10 replicates (four pots per treatment). Tray experiments, conducted in five replicates (20 trays each), were repeated three times. The results that are given are from one representative experiment. Significance is given by $P < 0.05$ by bifactorial analysis or by standard error.

RESULTS

Effect of bacterium-inhibiting substances on the growth of wheat rhizosphere bacteria. Seventy different rhizosphere bacteria, isolated from roots of wild cereals or from cultivated wheat plants, were tested for their ability to grow on nitrogen-free BL medium and on rich King-B medium supplemented with the four inhibitory substances (3). In general, none of the bacteria grew as well as *A. brasilense* Cd. (3). When homogenates obtained from wheat roots were spread over plates containing these media, very few colonies developed (1.2×10^1 to 2.4×10^2 CFU/g of root on BL medium compared with 10^6 to 10^8 CFU/g of root on King-B medium), showing the marked inhibitory activity of these substances.

Effect of bacterial inhibitors applied to the soil on the total rhizosphere bacterial population and root colonization by *A. brasilense* Cd. A single application of bacterial inhibitors to the soil concomitantly with *A. brasilense* Cd. inoculation reduced the total number of bacteria in wheat rhizospheres, while the rate of decline of *A. brasilense* Cd. was slower in the presence of the inhibitors compared with that of the untreated inoculated control plants (Fig. 1A). However, this improvement in *A. brasilense* Cd. colonization was only temporary, decreasing within 4 weeks to a rate similar to that in the untreated controls. Application of the inhibitors 1 week after inoculation only very slightly and temporarily reduced the total rhizosphere population and left root colonization by *A. brasilense* Cd. unaffected (Fig. 1B).

In the untreated inoculated control plants, the rhizosphere bacterial population was constant, increasing slowly during the course of the experiment. The *A. brasilense* Cd. population, on the other hand, decreased sharply to a relatively low level only 3 weeks after inoculation (Fig. 2A).

Four successive inhibitor applications at weekly intervals

significantly reduced the total rhizosphere bacterial population. This inhibitory effect lasted for as long as 2 weeks, after which the bacterial population returned to its initial level. *A. brasilense* Cd. counts in the wheat rhizosphere increased during the period of inhibitor application, but later it decreased slowly (Fig. 2B).

Increased root colonization by *A. brasilense* Cd. following the application of bacterial inhibitors. Monitoring of the success of plant root colonization by measuring the population of *A. brasilense* Cd. in the roots of each plant (100 plants) after one bacterial and three inhibitor applications showed increased percentages of colonized plants in the inhibitor-treated plants compared with those in plants inoculated with bacteria only. This increase in successful colonization was detected in plants grown in vermiculite or soil (Fig. 3).

Effect of bacterial inhibitors combined with bacterial inoculation on various wheat plant parameters. The effect of inoculating wheat plants after the application of bacterial inhibitors was measured in three experiments conducted in the summer (day temperatures, 24°C [minimum] to 30°C [maximum]), autumn (22°C [minimum] to 28°C [maximum]), and winter (2°C to 20°C [maximum]).

In general, most plant parameters showed some reduction following inhibitor application. The trends of plant parameters were similar in all treatments, regardless of the season. All but two parameters presented here are from the winter experiment, corresponding to normal wheat growth conditions in Israel. The other two parameters (days till germination and root surface area) are from the summer experiment.

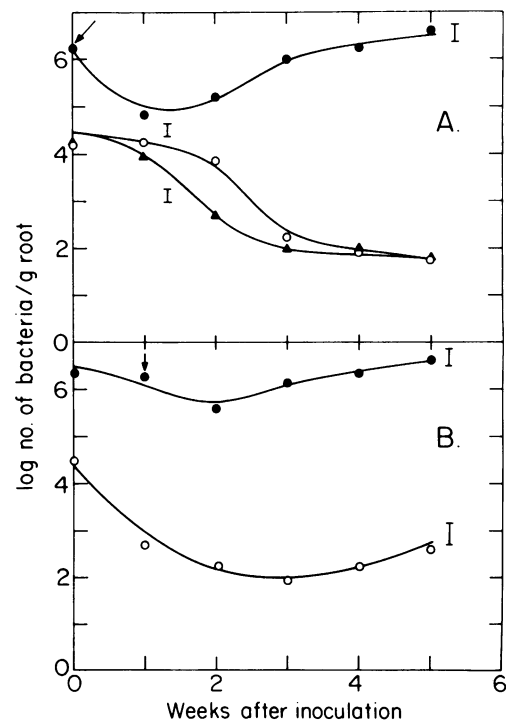


FIG. 1. Effect of a single application of bacterial inhibitors on the total number of rhizosphere bacteria (●) and root colonization by *A. brasilense* Cd. with (○) and without (▲) inhibitory substances. (A) Application at the time of inoculation; (B) application 1 week after inoculation. Arrows represent application of inhibitors. Bars represent standard error of the line.

The effect of bacterial inoculation on plant parameters can be separated into three categories: (i) positive, significant increase, such as yield per plant, pot, main spike, or tiller; number of fertile tillers; main spike fertility; foliage dry weight; harvest index; and root surface area (Fig. 4 a-1 to a-4, 4 b-1 and b-2, 4 c-1 and c-2, and 4 d-4); (ii) no effect, as on plant height, growth rate, grain weight in the main spike, average number of spikelets on the main spike, and protein content of the main spike (Fig. 4 b-3 and b-4, 4 c-3 and c-4, and 4 d-2); and (iii) negative, significant decrease, such as days till germination and heading dates (Fig. 4 d-1 and d-3).

DISCUSSION

Soil usually reacts as a biological buffer. Hence, any change in its microbial population is only temporary (1). Soil microflora is believed to be constant most of the time, not growing unless it is nourished. In this respect the soil is energy deficient. The application of beneficial bacteria, particularly under wet conditions, increases the populations of nearby microorganisms, which use these bacteria as nutrients. Beneficial bacteria inoculated into plants compete with many saprophytes for nutrients that leak from the root to the rhizosphere (4, 23, 24). When the applied bacteria fail in this competition, plant roots are not colonized, even when they are inoculated, and there is no visible or measurable effect on plant growth (24).

A. brasilense Cd. is a highly successful yield-increasing bacterial inoculum in Israel, as well as other countries, but it is less successful in the United States (6, 11, 12, 14, 15, 20, 22). In a recent review, Schank and Smith (21) claimed that the success of inoculation with beneficial bacteria, especially *Azospirillum* sp., was short-lived because of inconsistent results.

It seems that enhancing the colonization of beneficial

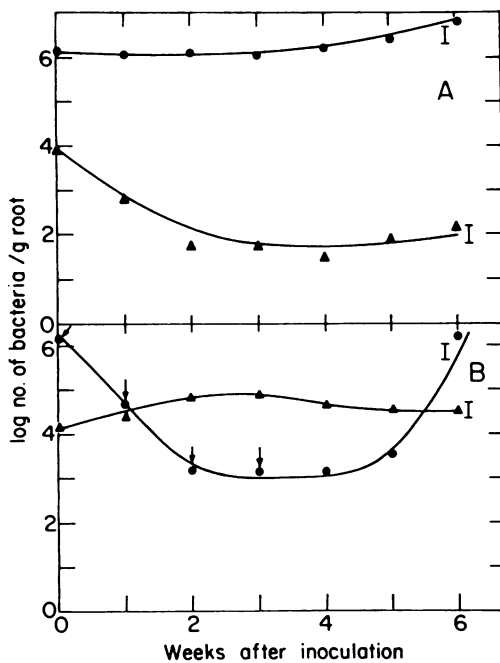


FIG. 2. Total number of wheat rhizosphere bacteria (●) and *A. brasilense* Cd. (▲) after a single inoculation. (A) Without application of inhibitors; (B) during and after four successive applications of inhibitors. Arrows indicate application of inhibitors. Bars represent standard error of the line.

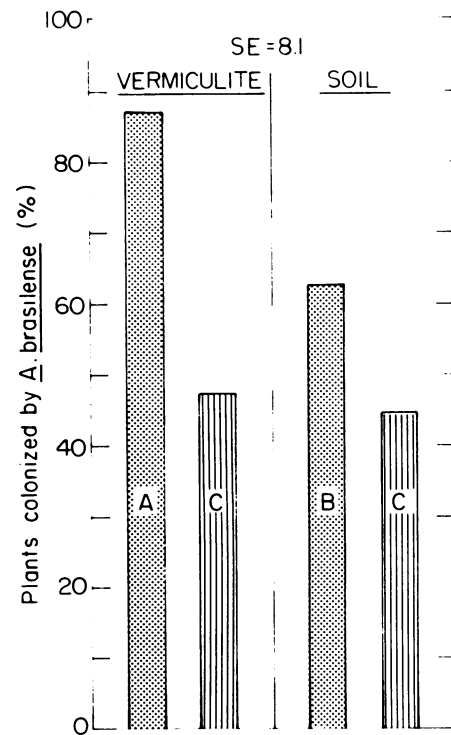


FIG. 3. Successful colonization by *A. brasilense* Cd. of wheat roots after inoculation only (III) and after inoculation plus application of bacterial inhibitors (⊠) in plants grown in vermiculite or in soil. Columns followed by a different letter in each panel differ significantly at $P \leq 0.05$. SE, Standard error.

bacteria in the rhizosphere may render the system more consistent, revealing the potential of the contribution of beneficial bacteria to plants. The basic idea was to reduce the number of bacterial and fungal competitors in the rhizosphere by applying inhibiting substances to which my model organism, *A. brasilense* Cd., was resistant, thus increasing its chances for colonizing root systems.

Results of this study demonstrate the possibility and practicality of temporarily depressing natural plant rhizosphere bacteria. The inhibitory effect lasted longer than the period of substance application. The reduced number of competitors may explain why in treated inoculated plants *A. brasilense* Cd. competed better on rhizosphere sites and colonized root systems in higher numbers than in untreated inoculated plants.

Gaskins et al. (8) showed that the number of bacteria declined rapidly following inoculation into the rhizosphere. Schank and Smith (21) concluded that poor inoculum survival is the most serious problem confronting inoculation technology. Hence, achieving better colonization, as in this study, will ensure the presence of bacteria in plant root systems long after soil microflora returns to normal, at the same time increasing the chances of the bacteria to influence the plant. Furthermore, even if for unknown reasons the bacterial inoculation fails, the depression of competitors will improve the chances of the bacteria that do survive to reach the roots and develop a relatively large population.

The observed effect on plant parameters does not result from the direct application of the inhibitors, because they slightly inhibited plant growth and were chosen only because *A. brasilense* Cd. is resistant to them (3). Hence, the results

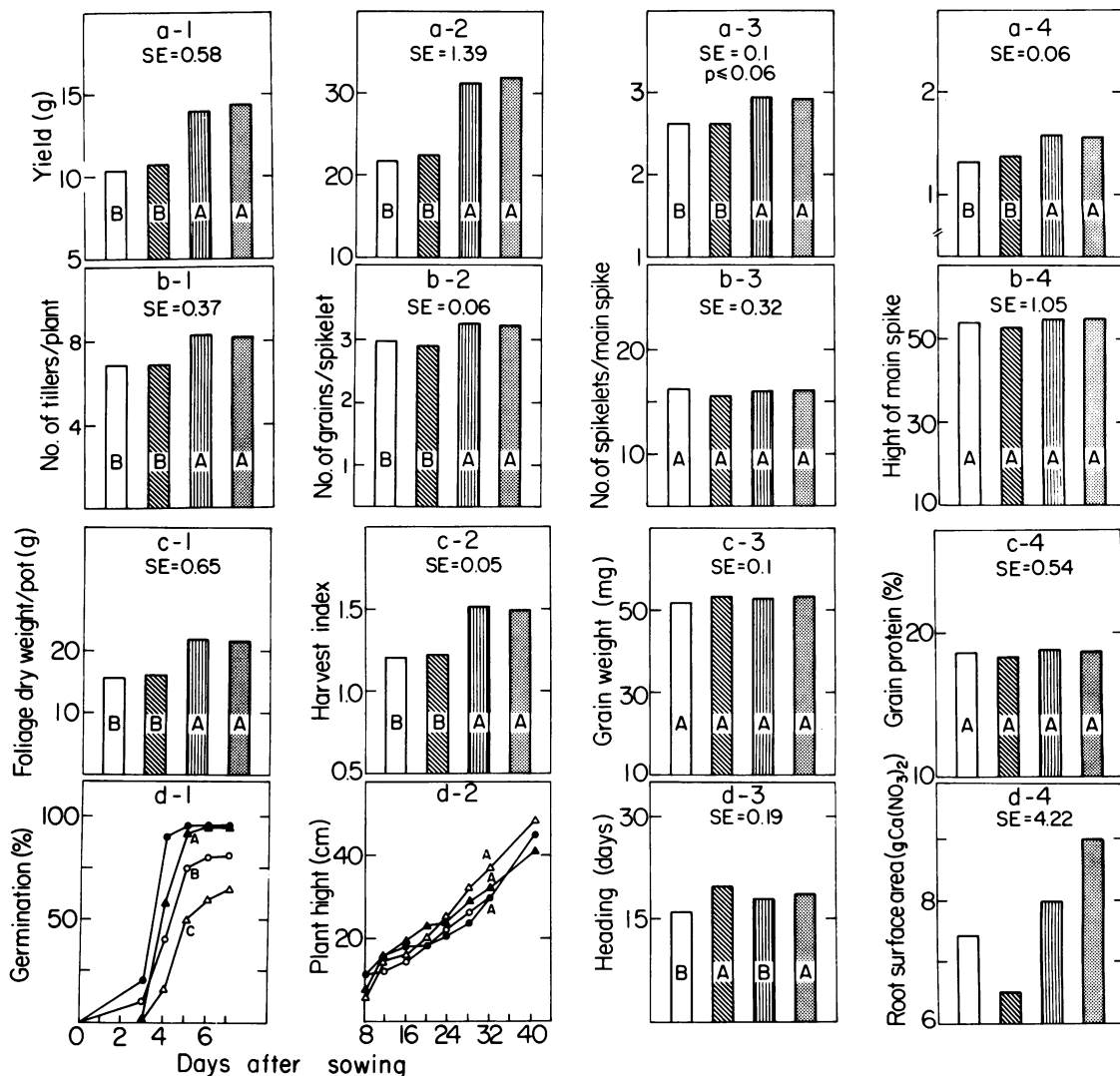


FIG. 4. Effect of application of bacterial inhibitors on various wheat plant parameters in the winter experiment. Symbols: □ and ●, uninoculated, untreated plants; ▨ and ▲, inoculated plants with *A. brasilense* Cd.; ▩ and ○, treated plants with inhibitors only; ▧ and △, inoculated, treated plants. (a) Yield per plant (1), pot (2), main spike (3), and tiller (4). (b) Number of fertile tillers (1), number of grains in spikelets on the main spike (2), number of spikelets on the main spike (3), and height of main spike (4). (c) Foliage dry weight (1), harvest index (2), weight of grain (3), and protein content of grain on the main spike (4). (d) Rate of germination (1) (summer experiment), growth (2), heading (3), and root surface area (4) (summer experiment). Columns or lines followed by a different letter in each panel differ significantly at $P \leq 0.05$. SE, Standard error.

reflect the potential beneficial effects of *A. brasilense* Cd. on wheat plants rather than, as measured in other studies, average bacterial effects influenced by bacterial competition in the rhizosphere.

The inhibitory substances used here are not practical for agricultural usage, as they are expensive and unlicensed for agriculture and have a short life-span in soil and a negative influence on plants. Nevertheless, they demonstrate the potential of this inhibitory model in the rhizosphere. By using other inhibitory substances, such as soil disinfectants, colonization of most field plants may be achieved, thus overcoming the problem of the inconsistency of the inoculation systems that are currently available.

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LITERATURE CITED

- Alexander, M. 1977. Introduction to soil microbiology. John Wiley & Sons, Inc., New York.
- Baldani, V. L. D., J. I. Baldani, and J. Döbereiner. 1983. Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. *Can. J. Microbiol.* **29**:924-929.
- Bashan, Y., and H. Levanony. 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. *Can. J. Microbiol.* **31**:947-952.
- Brown, M. E. 1974. Seed and root bacterization. *Annu. Rev. Phytopathol.* **12**:181-197.
- Carley, H. E., and R. D. Watson. 1966. A new gravimetric

- method for estimating root-surface areas. *Soil. Sci.* **102**: 289–291.
6. Cohen, E., Y. Okon, J. Kigel, I. Nur, and Y. Henis. 1980. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp. *Plant Physiol.* **66**:746–749.
 7. Gaskins, M. H., S. L. Albrecht, and D. H. Hubbell. 1985. Rhizosphere bacteria and their use to increase plant productivity: a review. *Agric. Ecosystems Environ.* **12**:99–116.
 8. Gaskins, M. H., S. L. Albrecht, and J. R. Milam. 1984. Survival of root-associated bacteria in the rhizosphere, p. 52–63. *In* C. Veeger and W. E. Newton (ed.), *Advances in nitrogen fixation research*. Martinus Nijhoff, The Hague.
 9. Kapulnik, Y., R. Gafny, and Y. Okon. 1985. Effect of *Azospirillum* spp. inoculation on root development and NO₃ uptake in wheat (*Triticum aestivum* cv. Miriam) in hydroponic systems. *Can. J. Bot.* **63**:627–631.
 10. Kapulnik, Y., J. Kigel, Y. Okon, I. Nur, and Y. Henis. 1981. Effect of *Azospirillum* inoculation and some growth parameters and N-content of wheat, sorghum and panicum. *Plant Soil* **61**:65–70.
 11. Kapulnik, Y., and Y. Okon. 1983. Benefits of *Azospirillum* inoculation on wheat: effects on root development, mineral uptake, nitrogen fixation and crop yield. *Exper. Suppl.* **48**:163–170.
 12. Kapulnik, Y., S. Sarig, I. Nur, and Y. Okon. 1983. Effect of *Azospirillum* inoculation on yield of field-grown wheat. *Can. J. Microbiol.* **29**:895–899.
 13. Lethbridge, G., and M. S. Davidson. 1983. Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ¹⁵N isotope dilution. *Soil Biol. Biochem.* **15**:365–374.
 14. Millet, E., and M. Feldman. 1984. Yield response of a common spring wheat cultivar to inoculation with *Azospirillum brasilense* at various levels of nitrogen fertilization. *Plant Soil* **80**:255–259.
 15. Okon, Y. 1985. *Azospirillum* as a potential inoculant for agriculture. *Trends Biotechnol.* **3**:223–228.
 16. Patriquin, D. G., J. Döbereiner, and D. K. Jain. 1983. Sites and processes of association between diazotrophs and grasses. *Can. J. Microbiol.* **29**:900–915.
 17. Pedersen, W. L., K. Chakrabarty, R. V. Klucas, and A. K. Vidaver. 1978. Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. *Appl. Environ. Microbiol.* **35**:129–135.
 18. Rennie, R. J., J. R. de Freitas, A. P. Ruschel, and P. V. Vose. 1983. ¹⁵N-isotope dilution to quantify dinitrogen (N₂) fixation associated with Canadian and Brazilian wheat. *Can. J. Bot.* **61**:1667–1671.
 19. Rennie, R. J., and R. I. Larson. 1979. Dinitrogen fixation associated with disomic chromosome substitution lines of spring wheat. *Can. J. Bot.* **57**:2771–2775.
 20. Reynders, J., and K. Vlassak. 1982. Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. *Plant Soil* **66**:217–223.
 21. Schank, S. C., and R. L. Smith. 1984. Status and evaluation of associative grass-bacteria N₂-fixing systems in Florida. *Proc. Soil Crop Sci. Soc. Fla.* **43**:120–123.
 22. Smith, R. L., S. C. Schank, J. R. Milam, and A. A. Baltensperger. 1984. Responses of *Sorghum* and *Pennisetum* species to the N₂-fixing bacterium *Azospirillum brasilense*. *Appl. Environ. Microbiol.* **47**:1331–1336.
 23. Stacey, G., and W. J. Brill. 1982. Nitrogen-fixing bacteria: colonization of the rhizosphere and roots, p. 225–247. *In* M. S. Mount and G. H. Lacy (ed.), *Phytopathogenic prokaryotes*, vol. 1. Academic Press, Inc., New York.
 24. Suslow, T. V. 1982. Role of root-colonizing bacteria in plant growth, p. 187–223. *In* M. S. Mount and G. H. Lacy (ed.), *Phytopathogenic prokaryotes*, vol. 1. Academic Press, Inc., New York.