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Responses of *Sorghum* and *Pennisetum* Species to the N_2-Fixing Bacterium *Azospirillum brasilense*†

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Three field inoculation experiments, two in Florida and one in New Mexico, were conducted with *Azospirillum brasilense* Cd. Each of the Florida experiments evaluated two crop species. One species in each of the Florida experiments responded to inoculation with a significant dry matter yield increases of 11 to 24% and nitrogen yield increases of 9 to 39%. No inoculation response was noted in the New Mexico experiment. The responding species were *Sorghum bicolor* (L.) Moench (sorghum) and the interspecific hybrid between *Pennisetum americanum* (L.) K. Schum. (pearl millet) and *P. purpureum* Schumach. (napiergrass). Nonresponding species were pearl millet (Florida) and *Sorghum sudanense* (Piper) Staph. (New Mexico). Acetylene reduction activity of inoculated plots in Florida was low, showing no increase over the natural uninoculated background rates and, in one case, was negatively correlated with yield. Acetylene reduction activity was not measured in New Mexico. In Florida, *A. brasilense* populations were found to decline from 5×10^3 to 5×10^2 bacteria g of soil^{-1} in about 3 weeks (quadratic regressions). Continued decline to less than 10^2 by week 5 indicated that the inoculated bacteria did not become established in the soil in high numbers. The *A. brasilense* population declined at about the same rate in the New Mexico experiment. The erractic inoculation responses in these experiments are similar to those observed in earlier work at the University of Florida. The lack of acetylene reduction activity response to inoculation and the rapid population decline of the inoculated bacteria suggest that N_2 fixation is not the major mechanism causing yield responses after inoculation.

Reports (8) of very high N_2 fixation in tropical grass-*Spirillum* sp. (now *Azospirillum* sp.) systems in Brazil in 1974 stimulated renewed interest in associative N_2 fixation. It became an important research priority to determine whether associative N_2 fixation would benefit grass crop productivity and reduce nitrogen fertilizer requirements.

In 1975, increased dry matter yields of 21% for pearl millet and 18% for guineagrass were obtained by inoculating with the N_2-fixing bacterium, *Spirillum lipoferum* (18), now known as *Azospirillum brasilense*. We estimated that an additional 42 and 39 kg of nitrogen fertilizer per hectare (ha) would have been necessary to produce similar yields in uninoculated plots. In various subsequent experiments, yield increases (11 to 32%) have been obtained by inoculation (5, 17, 20). In about half of the field trials, however, yield increases were not obtained. We have been unable to improve the repeatability of the yield response through management, host plant, bacteria genotypes, or improved inoculation techniques.

Other workers in various parts of the world have reported favorable responses to inoculation with *Azospirillum* sp. and other N_2-fixing bacteria. As in Florida, however, not all experiments have been successful. In Oregon and Beltsville, yields were not stimulated by inoculation (3, 16). Wisconsin data were mixed; some maize genotypes responded, whereas others did not (1). Reports from the Bahamas (23), India (22), Egypt (11), and Belgium (15) showed yield responses in various grass crops and forages, but they have not demonstrated that the bacteria-root associations could be relied upon to improve yields. In most areas where commercially significant yield responses have been reported, commercial use has been discouraged by lack of dependable responses.

Reports from Israel of consistent yield responses due to inoculation with *A. brasilense* (7, 12, 13) renewed hope of commercial use. Although Israeli soil and climatic conditions are unique, that success has been attributed, at least in part, to the use of a strain of *A. brasilense*, strain Cd (9).

In this paper we report inoculation experiments conducted in Florida and New Mexico with *A. brasilense* Cd as the inoculum to determine whether it would produce consistent yield responses in Florida's slightly acid or New Mexico's highly calcareous, high pH soils. Acetylene reduction responses to inoculation and *A. brasilense* persistence in the soil were also studied.

MATERIALS AND METHODS

Gainesville experiment 1. In the first experiment conducted near Gainesville, Fla., *Sorghum bicolor* (L.) Moench (sorghum) var. Funk G522 and *Pennisetum americanum* (L.) K. Schum. (pearl millet) var. Gahi-3 were inoculated with *A. brasilense* Cd. The soil in this field was a Sparr fine sand (a loamy, siliceous, hyperthermic Grossarenic Paleudult) with an initial pH of 5.9. Hydrated lime was applied at the rate of 2,000 kg ha^{-1} to raise the pH to 6.0. Fertilizer was applied before planting at the rate of 20 kg of P and 70 kg of K ha^{-1}. Trace elements were applied as glass frits (FTE 501) at the following rates (grams per hectare): B, 140; Cu, 140; Fe, 810; Mn, 340; Mo, 10; and Zn, 315. Nitrogen (as ammonium nitrate) was broadcast 1 week after planting at the rate of 30 kg ha^{-1}. Weeds were controlled by hand and tractor cultivations. Methomyl was used as necessary to control insects.

Plots consisted of single 0.9-m-wide rows, 9 m long. A randomized complete block design was used with 12 replications. The two plant species were arranged systematically in pairs within the blocks and analyzed separately. To prevent

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shading, the taller species, pearl millet, was always planted on the north with sorghum on the south, and these pairs received either the live or killed inoculum. Inoculum treatments were separated by sorghum border rows. Seed was planted by drilling seeds 3 cm deep at a rate to give 50 to 60 plants per m of row.

The inoculum, *A. brasilense* Cd (ATCC 29729), was cultured in a New Brunswick Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) in 10-liter batches. The inoculum was grown in an aerated mineral salts succinate medium containing 1.0 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) per liter and 0.5 g of $(NH_4)_2SO_4$ per liter. The killed inoculum used as control inoculum was autoclaved for 60 min at 121°C.

Inoculation was accomplished by metering live or autoclaved culture directly behind the planter shoe onto the seed in the furrow with a peristaltic pump mounted on the planter frame. The seed furrow was closed immediately to prevent inoculum dessication. The inoculation rate was approximately 1.5×10^7 cells per cm of row. Two weeks after planting and initial inoculation, the nursery was reinoculated by metering similar inoculum into the soil 5 cm deep about 15 cm on both sides of the seedling rows.

Acetylene reduction activity (ARA) was measured three times on soil-root cores (8.5 cm in diameter by 17 cm long) taken in 37-cm-long steel coring tubes. Tubes were made gas tight with rubber caps over the open ends and septum stoppers in the welded lid of the other end. The cores were flushed with either argon or helium to an oxygen concentration of about 6%, and then 10% of the core atmosphere was replaced by acetylene. Incubation was at 30°C for 16 to 20 h, and then the evolved ethylene was measured by gas chromatography.

Sixty-four days after planting, plants were harvested from the center 6 m of the inoculated and control rows by using a Carter flail harvester. Fresh-weight and oven-dry yields, percent dry matter, and nitrogen content measurements were made. Plant nitrogen was determined by Kjeldahl digestion followed by Technicon Autoanalyzer analysis (10). Statistical analyses of variance and correlation were run.

Gainesville experiment 2. Two crop species were also inoculated in the second Gainesville experiment. Those species were *P. americanum* var. Tifleaf and an interspecific hybrid of *P. americanum* (inbred 23DA) and *P. purpureum* Schumach (strain N75 napiergrass). This experiment was conducted on a well-drained, Arredondo fine sand (a siliceous, hyperthermic, Grossarenic Paleudult) with an initial soil pH of 6.5 making liming unnecessary. Fertilizer was applied as in the first Gainesville experiment, except nitrogen was applied at four different rates of 0, 30, 60, and 120 kg of N ha^{-1} (applied as ammonium nitrate). After the first harvest, 16 June 1982, N fertilizer was reapplied. Weed and insect control were simlar to those in the first experiment, and the plots were sprinkle-irrigated weekly. A second harvest was made on 1 September 1982.

The plots consisted of single 0.9-m-wide rows, 4.6 m long. Plots were laid out in a split plot design replicated 10 times. Plant species were systematically arranged in pairs, consisting of one row each of pearl millet and the hybrid, and were analyzed separately. Three pairs were located in each N fertilizer treatment and received the Cd, CdSR, or killed inoculum. Strain CdSR is a double-marked natural mutant of strain Cd with resistance to streptomycin and rifampin that facilitates its recovery from soil. Nitrogen fertilizer rates were the main plots, and inoculum treatments were the subplots. Fertilizer N treatments were separated by border rows that received no inoculum treatment. (These border rows were also harvested as a nonamended control.)

The pearl millet was planted 30 March 1982 and inoculated as in the first experiment, except that just before application the inoculum cultures were mixed with 10% (vol/wt) peat carrier (Nitragin Co., Milwaukee, Wis.) which had been neutralized by adding 300 g of $CaCO_3$ to each kg of peat. Approximately 1.3×10^7 cells, grown as described above, were applied per cm of row. The control inoculum was a mixture of autoclaved strains Cd and CdSR.

A limited quantity of hybrid seed prevented direct seeding, so seeds were germinated and established in plastic cells in the greenhouse. Seedlings were inoculated by applying approximately 4.5×10^8 cells of the appropriate inoculum (containing 10% peat as above) into the soil surrounding each seedling. Inoculated seedlings were transplanted into the field 31 March 1982, 0.5 m apart in the row, and sprinkle irrigation was used as necessary.

Counts of *A. brasilense* were made by the most probable number method. Succinate N-free media with and without those antibiotics were used in counting. Soil for counting was taken by coring 2-cm-diameter, 9-cm-long soil cores from the center of the rows (where the inoculum was placed) and through plant root systems. Two replicate soil samples of each inoculum treatment from the plots with 0 and 60 kg of N ha^{-1} were counted. Each soil sample was made up of pooled soil from five plots, so all 10 replicate plots were represented. Counts were made weekly for the first 12 weeks and then at longer intervals during fall, winter, and spring.

The center 3.7 m of the rows was harvested 76 days after planting (first harvest) and 76 days later (second harvest) with the same equipment as in the first experiment. Plant parameter measurements and analyses were also made as stated above. In addition to the statistical analyses mentioned above, the statistical analysis system (SAS Institute, SAS Circle, Cary, N.C.) general linear model procedures were used.

New Mexico experiment. The third experiment was conducted on the New Mexico State Agricultural Experiment Station farm near Las Cruces. That site was selected because its calcareous, fine-textured soil differed from the sandy, slightly acidic soils of Florida. The New Mexico soil is a Glendale clay loam (classified as mixed calcareous thermic family of typic Torrifluent) with a pH of 7.95. A uniform application of 73 kg of P ha^{-1} was incorporated before planting. Potassium was not applied because adequate amounts existed in the soil. Four nitrogen fertilizer (ammonium nitrate) rates of 0, 30, 60, and 120 kg ha^{-1} were applied before planting and again after the first harvest. Flood irrigation was used the same day that fertilizer was applied and at weekly intervals afterward, as is conventionally practiced in the area, to attain maximum vegetative growth.

A split plot experimental design was used with nitrogen fertilizer rates comprising the main plots and inoculation treatments as subplots. Plots consisted of four rows, spaced at 2.6-m intervals, and were 6.1 m in length. Nine replicates were used. Seed of *Sorghum sudanense* (Piper) Staph. (sudangrass) var. ACCO Sweet Sioux IV was planted by drilling at the rate of 28 kg ha^{-1}.

Inoculation was done at planting as was described above for the second Gainesville experiment, except only live and killed strain Cd were used. The inoculum was prepared by washing the cells of a 10-liter culture, suspending them in 400 ml of fresh culture medium, and then mixing the concentrated cell suspension with 1 kg of peat neutralized with 300 g of

TABLE 1. Mean yields and other plant parameters for the first Gainesville experiment

Species	Inoculation treatment	Dry matter yield (kg ha^{-1})	Fresh wt (kg ha^{-1})	Dry matter (%)	Nitrogen content (%)	ARA[a] (nmol)	Soil moisture[b] (%)
Sorghum (Funk G552)	Live	5,951[c]	30.2[c]	24.1[c]	1.33[c]	55.3[c]	5.6[c]
	Killed	5,385[d]	28.3[d]	23.0[c]	1.35[c]	64.7[c]	5.6
	Mean	5,668	29.3	23.6	1.34	60.0	5.6
Pearl millet (Gahi-3)	Live	10,733[c]	61.7[c]	21.3[c]	1.18[c]	40.0[c]	5.0[c]
	Killed	10,848[c]	63.1[c]	20.9[c]	1.26[c]	36.0[c]	4.8[c]
	Mean	10,791	62.4	21.1	1.22	38.0	4.9

[a] ARA in nanomoles per core per hour. Each ARA value is the mean of three sampling dates, August 4, 18, and 24.
[b] Soil moisture as a percent of dry soil. Each moisture value is the mean of three sampling dates, August 4, 18, and 24.
[c,d] Means followed by the same letter (within a species) are not significantly different at the $P = 0.05$ level.

$CaCO_3$. This peat inoculum had a moist consistency, but was not sticky and was easily handled and transported. Just before inoculation, a peat-bacteria suspension, similar to that used in the second Gainesville experiment, was made by adding 10 liters of distilled water to the peat inoculum.

The two center rows of each plot were harvested at 51 days from planting and then again 46 days later. At the first harvest, 6 m of row was harvested, but only 4.3 m was harvested the second time. Vigor notes were taken 30 days from planting. Fresh weight, dry matter content, and dry matter yield were measured at harvest time. Bacterial counts were made five times at 3-week intervals with the most probable number method. Soil samples consisted of three pooled cores, 2-cm diameter by 9 cm long. Samples were taken 2 cm from the center of the row (where the inoculum had been placed) through the plant root systems. Duplicate samples from two replications in both live and killed inoculum plots were counted. Counting was done as described above for strain Cd. Statistical analyses were conducted as described above.

RESULTS

Gainesville experiment 1. Inoculation significantly increased fresh weight and dry matter yields of the Funk G522 sorghum, but not of the Gahi-3 pearl millet. Yields of the inoculated and control plots on a hectare basis were 5,951 and 5,385 kg, respectively, for an increase of 566 kg ha^{-1} or 11% due to inoculation (Table 1). Inoculation did not affect the plant moisture or nitrogen percentages in either species. Nine percent more nitrogen was incorporated into the shoot tissue of inoculated sorghum plots than control plots, but those amounts were statisically significant only at the $P = 0.07$ level. ARA was not significantly affected by either inoculation or species, but varied considerably from sampling date to sampling date and was correlated with soil moisture. Correlation between ARA and soil moisture is in agreement with our other results (19–21) where correlation coefficients (r) were obtained between 0.22 and 0.70. On the second sampling date, soil moisture was very low (1.9 to 2.4%), near the plant wilting point, and ARA was also very low. The third ARA sampling date also had low soil moisture and low ARA. Irrigation was not available at this site, and rainfall was barely adequate. Other significant correlations with ARA are listed in Table 2. Those were all associated with sorghum, not with pearl millet.

The two species differed in most measured attributes. The sorghum yielded only 53% as much dry matter and 58% as much protein as did the pearl millet. The sorghum hybrid had restricted growth because of genetic dwarfing. Seventy-six kg of N ha^{-1} was incorporated into the above-ground

TABLE 2. Significant correlation coefficients among inoculation treatments, ARA, and plant parameters in the first Gainesville experiment[a]

Parameter	Species	Correlation coefficient					
		ARA	Dry matter yield	Fresh wt yield	Nitrogen content (%)	Dry matter (%)	Soil moisture (%)
Inoculation	Sorghum		0.31[b]			0.34[c]	
	Millet						
ARA	Sorghum		0.30[b]	0.37[c]	0.29[b]		0.60[c]
	Millet						0.48[c]
Dry matter yield	Sorghum			0.94[c]		0.29[b]	
	Millet			0.89[c]		0.26[b]	
Fresh wt yield	Sorghum				0.32[c]		
	Millet						
Nitrogen content (%)	Sorghum					0.26[b]	
	Millet						
Dry matter (%)	Sorghum						
	Millet						

[a] Nonsignificant correlations are not listed.
[b] Significant at the $P = 0.05$ level.
[c] Significant at the $P = 0.01$ level.

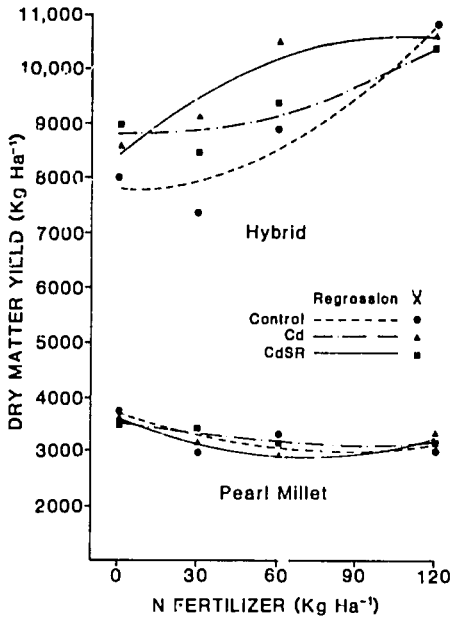

FIG. 1. Second harvest mean dry matter yields at four N fertilizer levels for Tifleaf pearl millet and the *Pennisetum* sp. hybrid are shown (Gainesville experiment 2). Two inoculation treatments, live *A. brasilense* strains Cd and CdSR, were compared to the autoclaved control. With the *Pennisetum* sp. hybrid, statistically significant inoculation responses were noted with strain Cd at the plots receiving 30 and 60 kg of N ha^{-1} (P = 0.006 and P = 0.02, respectively). Inoculation responses to strain CdSR were only statistically significant at P = 0.11 in the plots receiving 30 kg of N ha^{-1}. The Tifleaf pearl millet did not respond to inoculation. High nematode populations were present in the soil of the ratoon regrowth. Statistical analysis was by regression analysis (statistical analysis system general linear model procedures). First harvest dry matter yields are not plotted because no statistically significant responses were noted in either crop species.

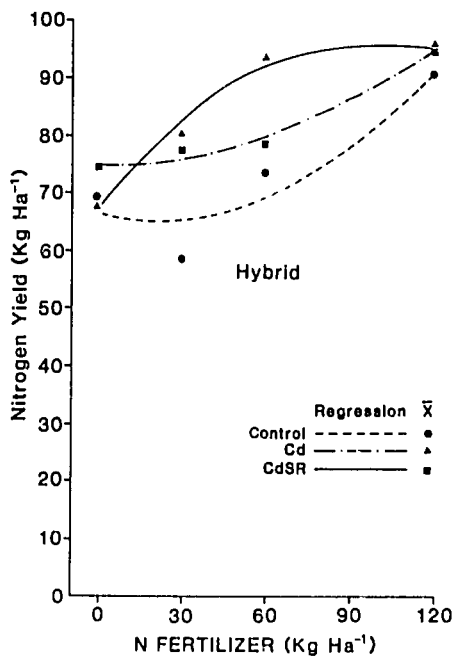

FIG. 2. Mean nitrogen yields in kilograms per hectare of the second harvest of the *Pennisetum* sp. hybrid are shown. (Gainesville experiment 2). The nitrogen yield values were calculated by multiplying the dry matter yield times the percent N. Statistically significant responses to inoculation with strain Cd were observed in the plots receiving 30 and 60 kg of N ha^{-1} (P = 0.003 and P = 0.002, respectively). Inoculation with strain CdSR produced significantly increased nitrogen yields in the plots receiving 30 kg of ha^{-1} (P = 0.06). Statistical analysis was by regression.

sorghum tissue, whereas 132 kg of N ha^{-1} was incorporated into the pearl millet shoots. Only 30 kg of N ha^{-1} had been applied as fertilizer, so considerably more N was taken up than was applied.

The percent dry matter content was higher in the sorghum, but nitrogen content did not differ significantly between the two species. Soil moisture was reduced to a lower level in the pearl millet at the 18 and 24 August sampling dates. Apparently, the greater plant growth of the pearl millet required more water, and the root systems were able to absorb the additional moisture from the relatively dry soil.

Gainesville experiment 2. No significant inoculation responses were noted in the first harvest. The pearl millet yielded more than the hybrid, even though the hybrid seedlings were preestablished and transplanted when the pearl millet was seeded. Both species responded well to the fertilizer nitrogen. However, since pearl millet is an annual, yields were expectedly lower in the second harvest (Fig. 1), whereas yields of the perennial hybrid were higher.

In the second harvest, the hybrid inoculated with both strains Cd and CdSR responded to inoculation with significantly increased dry matter yields (Fig. 1). In plots receiving 30 kg of supplemental N ha^{-1} the strain Cd inoculation yielded 24% more dry forage than the control, and at the 60 kg of N ha^{-1} rate, the increase was 18% (increases significant at P = 0.006 and P = 0.02, respectively). The strain CdSR-inoculated plots fertilized with 30 kg of N ha^{-1} yielded 16% more dry matter than did the controls, but was significant only at P = 0.11. The hybrid also produced significant yield responses for seasonal dry matter yield (both harvests combined) for the plots receiving 30 and 60 kg of N ha^{-1} inoculated with strain Cd (significant at P = 0.02 and P = 0.05, respectively). Plant fresh weight yields followed these same trends. Neither the nitrogen nor moisture content of the plant tissue was changed significantly by inoculation. The nonamended border rows of pearl millet were slightly lower in yield, but this was due to reduced plant populations and was not an inoculation effect.

Inoculation with both strains Cd and CdSR also increased the total amount of nitrogen harvested (Fig. 2). The plots receiving 30 kg of supplemental N ha^{-1}, inoculated with strain Cd, yielded 39% more N (also crude protein) in the plant tissue than did the control, and in the plots receiving 60 kg of N ha^{-1} the increase was 27% over the control (increases significant at P = 0.003 and P = 0.002, respectively). Inoculation with strain CdSR increased the harvested N by 32% in the plots fertilized with 30 kg of N ha^{-1}, and this increase was significant at the P = 0.06 level.

There appears to be a net loss of N input at the fertilizer rate of 120 kg N ha^{-1}. Since there was not an appreciable amount of N left in the soil at the end of the experiment, we believe the extra N had leached through the sandy soil. The amount of N in the soil did not inhibit ARA. The low yields and the lack of response of pearl millet to fertilizer in the second harvest was primarily due to the reduced vigor of the ratoon regrowth. In addition, excessive nematode populations were found in the plots of pearl millet that had received the higher rates of N fertilizer.

Acetylene reduction assays conducted on different, but similar, plots in the same area showed no response to

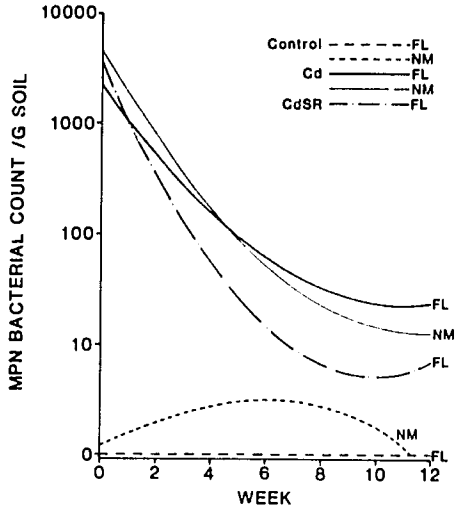

FIG. 3. Quadratic regressions of MPN counts of strain Cd, strain CdSR, and the controls from Gainesville experiment 2 and from the New Mexico experiment. In Gainesville, weekly sampling was done for the first 12 weeks after inoculation. The New Mexico samples were collected on a 3-week basis and were mailed to Florida for most-probable-number counting.

inoculation in either species. Mean ARA values were very low, ranging between 25 and 63 nM core $^{-1}$ h $^{-1}$.

Streptomycin- and rifampin-containing medium was used to facilitate recovery and detection of the double-marked mutant strain CdSR. In addition, we found that strain Cd could be identified by its distinctive pigmentation, which could be visually identified in the culture tubes. Strain CdSR would develop similar pigmentation when plated on rich, nonantibiotic-containing medium, and that characteristic was used to check whether the CdSR strain had undergone significant back mutation toward susceptibility to streptomycin and rifampin. Comparison of the different counting methods showed no real differences between strains Cd and CdSR or evidence for back mutation. Figure 3 shows the decline of those two strains over the course of the experiment. Samples from the fertilizer rates of 0 and 60 kg of N ha^{-1} were counted, but since no differences in *A. brasilense* populations were noted between these rates, the count data were combined. Quadratic regressions are shown in Fig. 3 of strains Cd, CdSR, and the control.

Populations of both strains of *A. brasilense* had fallen to less than 10^2 bacteria per g of soil by the week 6 after inoculation. Indigeneous N_2-fixing bacteria (not *A. brasilense*) were present in about 10-fold greater concentration. Neither strain Cd nor CdSR bacteria were found in the control plots in Florida. Sampling to determine inoculum movement through the soil 6 weeks after inoculation revealed that little movement had occurred. In only a few instances lateral movement of 15 cm was observed, with none observed as far as 30 cm. By the week 17 after inoculation strains Cd and CdSR were barely detectable, and by spring 1983 they could not be recovered in the most probable number tubes.

New Mexico experiment. No significant plant vigor or yield responses to inoculation were noted in the New Mexico experiment. However, there was a trend toward higher yields in the inoculated plots receiving low amounts of supplemental N in the first harvest. *A. brasilense* counts declined in New Mexico (Fig. 3) at about the same rate as in Florida and are plotted on the same graph. The control became contaminated with *A. brasilense* by the week 3, indicating much greater movement of bacteria there than in Florida. This may have been a result of the flood irrigation method that is conventionally used in the west, which results in a temporary but complete saturation of the soil surface.

DISCUSSION

Yield increases of 11 to 24% dry matter and 27 to 39% total nitrogen, due to inoculation with *A. brasilense*, are considered great enough to be important to commercial agriculture if they could be obtained consistently. However, nitrogen content was not significantly changed by inoculation. The reduction in the sorghum N and increase in the *Pennisetum* sp. hybrid N content, even though not significant, affected the significance of the total N yield accordingly. Repeatability of response was low in these experiments, even though we used the Cd strain. This emphasizes that the Cd strain is not universally successful. Yield response to inoculation with *A. brasilense* strain Cd was no more consistent than with strains 13t and JM125a2 used previously. Gahi-3 pearl millet was selected as a responding millet cultivar (5) because it has produced significant yield responses to inoculation in several experiments; however, it did not respond in these tests even when Funk G522, grown in adjoining rows, did respond. Tifleaf pearl millet and the sudangrass continued to be nonresponders.

The experimental site in New Mexico was included because its calcareous, fine-textured soil and a warm, dry climate similar to that of Israel, where more dependable inoculation responses were obtained (7, 12, 13). However, those factors apparently do not assure inoculation responses.

Inoculation response appeared to be affected by the rate of nitrogen fertilizer applied. Responses were noted only in the intermediate rates of nitrogen fertilizer. In an earlier report (18), we showed that response to bacterial inoculation may occur only if supplemented nitrogen fertilizer is supplied. This observation has been confirmed by others (13, 15) and in our subsequent experiments (R. L. Smith and S. C. Schank, Plant Soil, in press).

The rapid decline in *A. brasilense* populations in both the Florida and New Mexico experiments demonstrates that those organisms are not competitive in either environment. In the second Gainesville experiment, indigeneous N_2-fixing bacteria (measured by most probable number to an ARA extinction) remained at populations over 10^3. From these data and that of ARA, we concluded that *A. brasilense* is not very important in the total N_2 fixation in these experiments.

The ARA in these experiments was low. Previous ARA measurements in the Gainesville second experiment site have always been low. We have located high ARA sites in Florida where we measured rates of over 1,000 nM ethylene evolved core $^{-1}$ h $^{-1}$ (21). In this study we found ARA to be site dependent and correlated with soil moisture. The soil moisture in the first Gainesville experiment was low, which precluded high ARA rates (21).

Data reported here suggesting that N_2 fixation is not an important mechanism in producing growth responses include the following: (i) ARA measured in both Florida experiments was low and variable and was not affected by inoculation, yet yield responses were obtained; (ii) significant negative correlations of ARA with yield and N content were obtained in the first Gainesville experiment (Table 2); (iii) an extrapolation of amounts of nitrogen fixed from ARA data indicates that less than 3 kg of N was fixed per ha during the season, which was not believed to be significant to crop growth; (iv)

the decline of the inoculated *A. brasilense* population rapidly reduced its importance relative to indigeneous N_2-fixing bacteria in N_2 fixation. Those data indicate that N_2 fixation is neither a necessary nor an important mechanism for stimulating yield responses to inoculation.

Other mechanisms have been proposed. Barea and Brown (4) reported that *Azotobacter paspali* produced plant growth hormones, gibberellic acid, indole 3-acetic acid, and cytokinin, which promoted plant growth. Tien and co-workers (24) demonstrated that *A. brasilense* also produced growth factors that can stimulate plant growth, and they proposed that those hormones are responsible for inoculation responses.

In a previous experiment with eight bermudagrass genotypes (2), inoculation with *A. brasilense* produced higher top growth and total nitrogen accumulation. No leaf color differences were observed among inoculated and control plants. However, leaf greenness (an important turf characteristic) was greatly increased by nitrogen fertilization. This observation also supports indications of growth hormone involvement.

A recent report by Lin et al. (14) indicates that plant nutrient uptake can be stimulated by inoculation with *A. brasilense*. They propose that this may be responsible for inoculation responses. Disease suppression has also been proposed as a mechanism for growth enhancement due to bacterial inoculation (6).

Our results indicate that the lack of consistent inoculation response is the major obstacle to practical use of bacterial inoculation of grass crops. These data further suggest that N_2 fixation is probably not the mechanism producing the increased yields, and that the use of N_2 fixation research methodology per se will not give the most appropriate research approach to this problem. Greater efforts should be made to determine the mechanisms responsible for the growth responses after inoculation.

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LITERATURE CITED

1. **Albrecht, S. L., Y. Okon, J. Lonnquist, and R. H. Burris.** 1981. Nitrogen fixation in corn-Azospirillum associations in a temperate climate. Crop Sci. **21:**301–306.
2. **Baltensperger, A. A., S. C. Schank, R. L. Smith, R. C. Littell, J. H. Bouton, and A. E. Dudeck.** 1978. Effect of inoculation with *Azospirillum* and *Azotobacter* on turf type bermudagrass genotypes. Crop Sci. **18:**1043–1045.
3. **Barber, L. E., S. A. Russell, and H. J. Evans.** 1979. Inoculation of millet with *Azospirillum*. Plant Soil **52:**49–57.
4. **Barea, J. M., and M. E. Brown.** 1976. Effects of plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. **37:**583–593.
5. **Bouton, J. H., R. L. Smith, S. C. Schank, G. W. Burton, M. E. Tyler, R. C. Littell, R. N. Gallaher, and K. H. Quesenberry.** 1979. Response of pearl millet inbreds and hybrids to inoculation with *Azospirillum brasilense*. Crop Sci. **19:**12–16.
6. **Brown, M. E.** 1974. Seed and root bacterization. Annu. Rev. Phytopathol. **12:**181–197.
7. **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.
8. **Dobereiner, J., and J. M. Day.** 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. p. 518–538. *In* W. E. Newton and C. J. Nyman (ed.). Proceedings of The First International Symposium on Nitrogen Fixation. vol. 2. Washington State University Press, Pullman.
9. **Eskew, D. L., D. D. Focht, and I. P. Ting.** 1977. Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmented *Spirillum lipoferum*. Appl. Environ. Microbiol. **34:**582–585.
10. **Gallaher, R. N., C. O. Weldon, and F. C. Boswell.** 1976. A semiautomated procedure for total nitrogen in plant and soil samples. Soil Sci. Soc. Am. J. **40:**887–889.
11. **Hegazi, N. A., H. Khawas, and M. Monib.** 1981. Inoculation of wheat with *Azospirillum* under Egyptian conditions. p. 493. *In* Current perspectives in nitrogen fixation. Australian Academy Science, Canberra.
12. **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.
13. **Kapulnik, Y., S. Sarig, I. Nur, Y. Okon, J. Kigel, and Y. Henis.** 1981. Yield increases in summer cereal crops in Israeli fields inoculated with *Azospirillum*. Exp. Agric. **17:**179–187.
14. **Lin, W., Y. Okon, and R. W. F. Hardy.** 1983. Enhanced mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Appl. Environ. Microbiol. **45:**1775–1779.
15. **Reynders, L., and K. Vlassak.** 1982. Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. Plant Soil **66:**217–223.
16. **Sloger, C., and L. D. Owens.** 1978. Field inoculation of cereal crops with N_2-fixing *Spirillum lipoferum*. Plant Physiol. **61**(Suppl.)**64:**2.
17. **Smith, R. L., J. H. Bouton, S. C. Schank, and K. H. Quesenberry.** 1977. Yield increases of tropical grain and forage grasses after inoculation with *Spirillum lipoferum* in Florida. p. 307–311. *In* A. Ayanaba and P. J. Dart (ed.). Biological N_2-fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.
18. **Smith, R. L., J. H. Bouton, S. C. Schank, K. H. Quesenberry, M. E. Tyler, J. R. Milam, M. H. Gaskins, and R. C. Littell.** 1976. Nitrogen fixation in grasses inoculated with *Spirillum lipoferum*. Science **193:**1003–1005.
19. **Smith, R. L., and S. C. Schank.** 1980. Factors affecting nitrogenase activity of field grass plots. p. 495. *In* Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra.
20. **Smith, R. L., S. C. Schank, J. H. Bouton, and K. H. Quesenberry.** 1978. Yield increases of tropical grasses after inoculation with *Spirillum lipoferum*. Ecol. Bull. (Stockholm) **26:**380–385.
21. **Smith, R. L., S. C. Schank, J. R. Milam, and R. C. Littell.** 1982. Statewide search for highly active associative N_2-fixation systems. Soil Crop Sci. Soc. Fla. Proc. **41:**122–126.
22. **Subba Rao, N. S., K. V. B. R. Tilak, C. S. Singh, and M. Lakshmi-Kumari.** 1979. Response of a few species of graminaceous plants to inoculation with *Azospirillum brasilense*. Curr. Sci. **48:**133–134.
23. **Tayler, R. W.** 1979. Response of two grasses to inoculation with *Azospirillum* spp. in Bahamian soil. Trop. Agric. **56:**361–366.
24. **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.