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Responses of Two Pearl Millets Grown in vitro After Inoculation with Azospirillum brasilense[1]

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ABSTRACT

Growth responses of pearl millet [*Pennisetum americanum* (L.) K. Schum.] seedlings inoculated with *Azospirillum brasilense* were studied in test tubes and small plastic bags. Comparisons were made between a pearl millet genotype, 'Gahi-3', which previously had given increased yields after inoculation in field tests, and another genotype, 'Tift 186', which did not respond.

Inoculation with live bacterial cells reduced top growth and gave a lower total nitrogen content in both genotypes. Acetylene reduction was positive but low in live inoculation treatments and zero in the controls. Immunofluorescent assays indicated a high bacteria population of *Azospirillum* in inoculated treatments, whereas the controls were negative. Neither the small test tubes nor the plastic bags provided a suitable environment for screening bacteria-plant associations and predicting their responses in the field.

Additional Index Words: N_2-fixation, Acetylene reduction, N content, Plant yield, Root growth, *Spirillum lipoferum*.

Ample evidence exists that N_2-fixing bacteria-grass root associations do occur (7, 8, 9, 12, 14, 19, 23). Three years of data from Florida (20) have indicated that yield increases are possible after inoculating new stands of forage grasses with *Azospirillum brasilense*.

Similar yield increases after inoculation have been observed in the Bahamas (21) and in India (15). A lack of significant response after inoculation has been reported in Oregon (3) and Wisconsin (1). In Australia (18), a 23% yield increase in tops and a 20% yield increase in root mass were obtained after inoculating digitgrass with *A. brasilense* in a growth room experiment. Baltensperger et al. (2) and Benzion and Quesenberry (4) have also noted positive growth responses after bacterial inoculation. The production of plant growth substances by microorganisms (6, 10, 11, 22) as well as N_2-fixation may cause the yield responses. Increased yield and nitrogen fixation responses have been reported when different grass genotypes are exposed to different bacterial strains (2, 4, 5, 13, 16).

In order to further study the system, we have been searching for a simple, consistent, and repeatable method that would allow the rapid evaluation of grass/bacterial associations. The objectives of this study were: (1) to develop an *in vitro* screening technique which would predict field results; (2) to study changes in root growth caused by inoculation; and (3) to monitor *Azospirillum* populations during the experiment by fluorescent antibody technique (FA).

MATERIALS AND METHODS

Two genotypes of pearl millet, 'Gahi-3' and an inbred parent, 'Tift 186', were tested in two inoculation experiments. Previously, Gahi-3 had responded to inoculation and 186 did not (5). In one experiment, the seedlings were grown in 15 x 200 mm test tubes with fifty grams of autoclaved soil placed in each test tube prior to planting. In the other, 6 oz. (170 gm) "Whirl-Pac" plastic bags were used as containers. The soil was Arredondo fine sand (Grosarenic Palendult; loamy, siliceous, hyperthermic), pH 6.4, low in NH_3 and NO_3, and with an organic matter content of 1.5%.

Seeds were surface sterilized by consecutive 7-minute dips in 70% ethanol, 50% Chlorox, and 3% hydrogen peroxide. After thorough washing in sterile water, they were then germinated on tryptic soy agar (Difco) to test for bacterial contamination. Seeds free of contamination were transplanted into the test tubes or 'Whirl-Pac' plastic bags.

Three inoculation treatments were used: (1) a live inoculum culture of *Azospirillum brasilense* strain JM125A2 (a Florida isolate) at the high rate of 4×10^8 cells per tube; (2) a low rate of the same live bacterial culture, 4×10^5 per tube; and (3) a control, autoclaved JM125A2 culture at the 4×10^8 rate. The experimental design was a split-plot with varieties as main plots and the three inoculation treatments as sub-plots. There were either four or five replications of each millet variety at each bacterial inoculation treatment.

Plants in both experiments were grown adjacent to a window in the laboratory, where light at mid-day was 30 μEm^{-2} sec^{-1}, and average ambient temperature was 25 C. Microscope slides placed in each test tube and Whirl-Pac bag were removed later for fluorescent antibody staining (17). Plants in the test tubes were allowed to grow for 40 days before harvest. Those in plastic bags were set up in sufficient numbers so that four harvests could be made at 10 day intervals, still maintaining four replications in each treatment. Tops and roots were harvested, weighed, and subsampled for microscope studies (17). Visual and microscopic examinations were made and photomicrographs were taken to document root morphological changes due to inoculation. Enumeration of bacteria, acetylene reduction, and nitrogen assays were performed as described previously (5, 10, 17).

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RESULTS AND DISCUSSION

Both top and root development in both experiments were repressed by inoculation (P = .004 and .001). Live inoculum reduced dry weight by about one-half (Fig. 1). This finding was in direct conflict with previous field, greenhouse, and growth room data which had shown an enhancement of dry weight after inoculation. However, plants in these experiments were grown under laboratory conditions in enclosed systems where both temperature and light (25 C and 30 μEm^2 sec^{-1}) were far from field conditions.

Acetylene reduction (AR) measured on the plants in the test tubes after 30 days of growth and again before harvest was low. At 40 days the mean AR value was 5 n moles of ethylene produced per tube per hour in the inoculated tubes (range 0-29), and zero in the control tubes. The plants which showed the higher AR values did not have higher % N or total N in tops or roots. Ethylene production was linear between 0 and 24 hours of incubation in the inoculated tubes.

Immunofluorescence studies from 91 contact slides and from reisolations at the end of both experiments verified that live bacteria persisted in the soil of all inoculated tubes and bags (Table 1). Fluorescent antibody labelled root (FA) preparations showed *A. brasilense* more prevalent on root surfaces of Gahi-3 pearl millet than Tift 186. At the low inoculation concentration, the FA root tests showed root colonization by *Azospirillum* in only 1 out of 5 test tubes with Tift 186, but heavy root colonization occurred in all five replications at the higher inoculum concentration (Table 1). All fungal spores in the soil were not killed by autoclaving, because contact slides showed close associations between *A. brasilense* and fungal hyphae as well.

The total nitrogen (N) content of uninoculated plants averaged 5.9% N in the tops and 1.5 mg total N, while the inoculated treatments averaged 5.4% N in the tops and 0.82 mg total N. Uninoculated plant roots were likewise higher in % N (2.18 vs. 1.78) and in total N (0.35 mg vs. 0.32 mg).

Harvest results from the Whirl-Pac bags are shown graphically in Fig. 2. The linear growth rate is probably due to the low light intensity, lower temperature, and the confined system. Results from Experiment 2 at 40 days (Fig. 2) closely paralleled the 40-day harvest from Experiment 1 (Fig. 1).

Fig. 3 shows root and shoot weight relationships. The ratios differ from those reported by Gaskins and

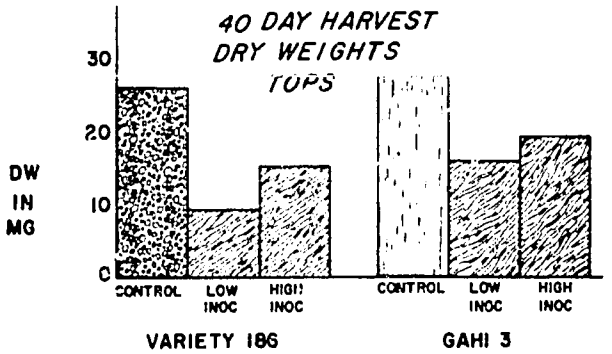

Fig. 1.—Dry harvest weights of two pearl millets grown *in vitro* for 40 days in the laboratory. Inoculated with a Florida isolate of *A. brasilense*.

TABLE 1.—MEAN *Azospirillum* POPULATION SCORES MADE BY FA TECHNIQUE AND MEAN ACETYLENE REDUCTION VALUES IN N MOLES PER TUBE PER HOUR.

Treatment	FA VALUES* Contact Slides	Roots	Soil Re-Isolates	AR	AR Range
186 Dead Inoc	1.0	0.0	0.5	0.0	0
Gahi-3 Dead Inoc	0.3	0.0	0.0	0.0	0
186 Low Live	2.6	0.6	3.0	12.1	0-29.3
Gahi-3 Low Live	3.0	3.0	3.0	5.2	0-26.1
186 High Live	2.8	3.0	3.0	0.0	0
Gahi-3 High Live	3.0	3.0	3.0	8.2	0-16.3

*Each mean in the table is based on 5 replications. A score between 2.5 and 3.0 is considered FA positive.

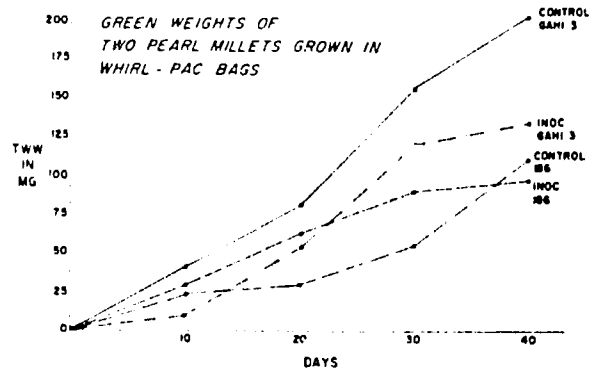

Fig. 2.—Green harvest weights of two pearl millets grown in plastic bags in the laboratory after inoculation with *A. brasilense*.

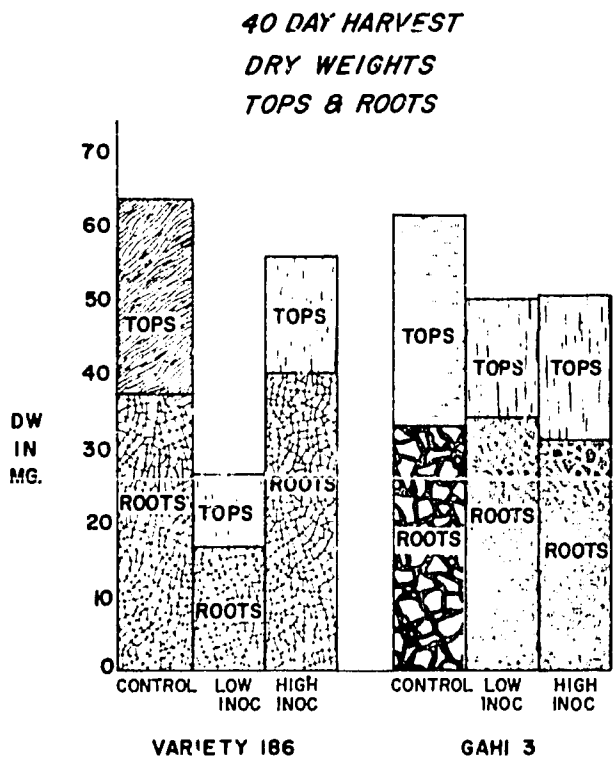

Fig. 3.—Dry harvest weights of tops and roots of two pearl millets grown *in vitro* for 40 days in the laboratory after inoculation with *A. brasilense*.

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