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Establishment of Associative N_2-Fixing Systems[1]

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ABSTRACT

Monoxenic root-bacteria associations were initiated in test tubes containing agar medium free of nitrogen and carbohydrates. Seedlings of five sorghum genotypes were inoculated with four strains of bacteria. The degree of association was evaluated by visually rating the amount and location of bacterial growth and by assaying for acetylene reduction activity (ARA). Active ARA associations were established 83% of the time, in that tubes with plants had higher ARA than the no plant controls. Sterile controls remained sterile and had no ARA. The plants and intact agar-root systems were transferred axenically to mason jar assemblies

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containing autoclaved soil to see whether the active, already established associations would be maintained. Acetylene reduction assays were repeated three more times on the jar assemblies. The ARA rates in these pre-established systems were equivalent to two non-autoclaved, but highly active natural soils from Putnam and Madison Counties, which were included as standards because of their high ARA. Intact soil-plant cores from the mason jar assemblies were transplanted into a field with low ARA and were re-assayed for ARA two more times. Highest ARA was from the natural soil-plant transplants, with lower rates from all inoculated treatments. This suggests that the complex natural systems are better able to persist in competitive situations, than the inoculated associations established from selected N_2-fixing strains.

Additional Index Words: Acetylene reduction, Nitrogenase activity, Soil-grass cores, Axenic associations, Inoculation, Diazotrophs, Ethylene production.

INTRODUCTION

Associative grass-bacteria are reported to fix low but agronomically important amounts of nitrogen (1, 2, 6, 7, 12, 20). Yield (dry matter) increases after *Azospirillum* inoculation have been reported from numerous locations, viz, Israel (8, 9), Florida (1, 13, 14), India (16), Bahamas (17), and Australia (11, 22). Plant growth substances are produced by the bacteria (4, 18), and increased metabolism may cause an increase in dry matter yields. Mechanisms suggested include a proliferation of the grass roots, followed by increased ion uptake so that inoculated plants can grow faster and larger, with a possible earlier maturity (11, 19).

Root-bacteria associations under axenic conditions have been well documented in grasses (7, 9, 10, 19, 20). It has been much more difficult to quantify N_2-fixing bacteria under field conditions and to correlate bacterial numbers with acetylene reduction activity (ARA) (5, 7, 14, 16, 20, 21, 22, 23). Natural or native systems of grass-bacteria associations with very high ARA's are known to exist, and have been studied for several years in Florida soils (15). A more complete understanding of these natural systems is needed.

Previous experiments with monoxenic inoculation with *A. brasilense* had clearly indicated that N_2-fixing bacteria associations existed with the grass root in test tube conditions (10, 19). It was the purpose of this series of experiments to carefully follow this association into greenhouse and field conditions, and to determine if already established monoxenic systems could be maintained in a progressively more natural environment. Acetylene reduction activity studies were conducted throughout the experiments, to determine if specific bacteria associated better with some sorghum strains than with others.

METHODOLOGY

Four N_2-fixing bacterial genotypes were used as inoculum: *Azospirillum brasilense* strain 13t (from Brazil), *A. brasilense* strain JM125A2 (from Florida), *Enterobacter agglomerans* strain S85-9 (from Florida), and *A. brasilense* strain S145 (from Florida). The five *Sorghum bicolor* genotypes used were Georgia Experimental Station inbreds B68181 and B68027 (3), cv. 'Savanna-5', cv. 'Coker 7723' and an experimental line FS 25a. Savanna-5 seeds were supplied by Northrup-King, Coker 7723 seed was obtained from the Coker Seed Company, and line FS 25a from DeKalb, Inc.

For the axenic test tube inoculations, seeds of the five sorghum genotypes were surface-sterilized using 5 minute consecutive soaking in ethanol, sodium hypochlorite (2.5%), and hydrogen peroxide (30%). Sterile water rinses were used between chemicals and at the end of seed treatment. Seeds were pre-germinated on 0.5% sucrose agar (to verify they were axenic) and then were transferred aseptically to the test tubes containing a modified nitrogen- and carbon-free Fahraeus medium (19) with 0.3% agar. Tubes were kept sterile (controls) or were inoculated with the different diazotrophs.

Inoculum was cultured as previously reported in a succinate medium (12). To avoid nutrient carry-over, cells were spun in a refrigerated centrifuge (2500 rpm for 10 minutes) and washed three times in sterile water. Inoculation rate was 10^7 cells per test tube in 30 ml of medium in a 25 x 200 mm tube. Observations on root colonization and root development were made on the test-tube-grown plants. Acetylene reduction was measured by adding 10% acetylene to the tubes, incubating for 24 h at 30C, then analyzing for ethylene by gas chromatography.

Time course AR measurements were taken during incubation to determine linearity of ethylene evolution on 2, 6 and 9-day-old seedlings of cv. Savanna-5. Scanning electron microscope observations were also made on these associations.

One quart mason jar assemblies were prepared by placing 3 cm of river gravel in the bottom for drainage, then uniformly packing the jars full of soil from Madison and Putnam County sites (15) which had previously shown high ARA. Entire assemblies were autoclaved except those kept "natural". The intact root-agar mass was transplanted from the test tubes into the mason jars in a laminar flow hood. Sterilized river gravel (3 cm depth) was placed on the surface of the soil surrounding the lower stem of the seedlings, and sterilized cotton covered the river gravel in the upper neck of the jar. After transplanting, the seedlings were moved into a greenhouse. Control treatments in the mason jars included both autoclaved and non-autoclaved Madison and Putnam soils with and without plants. Plants were grown in the greenhouse for 8 weeks and were watered using autoclaved water applied beneath the cotton with a syringe. Acetylene reduction assays were conducted on the mason jar assemblies by placing the entire assembly inside a plexiglass chamber and adding 10% acetylene, incubating for 18 to 20 h, then analyzing for ethylene. Assays for ARA were done at two week intervals, beginning 3 weeks after transplanting.

The field planting consisted of transplanting the intact contents of the mason jars directly to the field. The field site chosen was a well drained Arredondo fine sand (loamy siliceous hyperthermic Grossarenic Paleudult) with a history of low ARA. Mason jars were broken in order to keep the soil-root mass intact for transplanting. Soil-root cores from the field were assayed for ARA as previously reported (15) 2 and 4 weeks after transplanting to the field.

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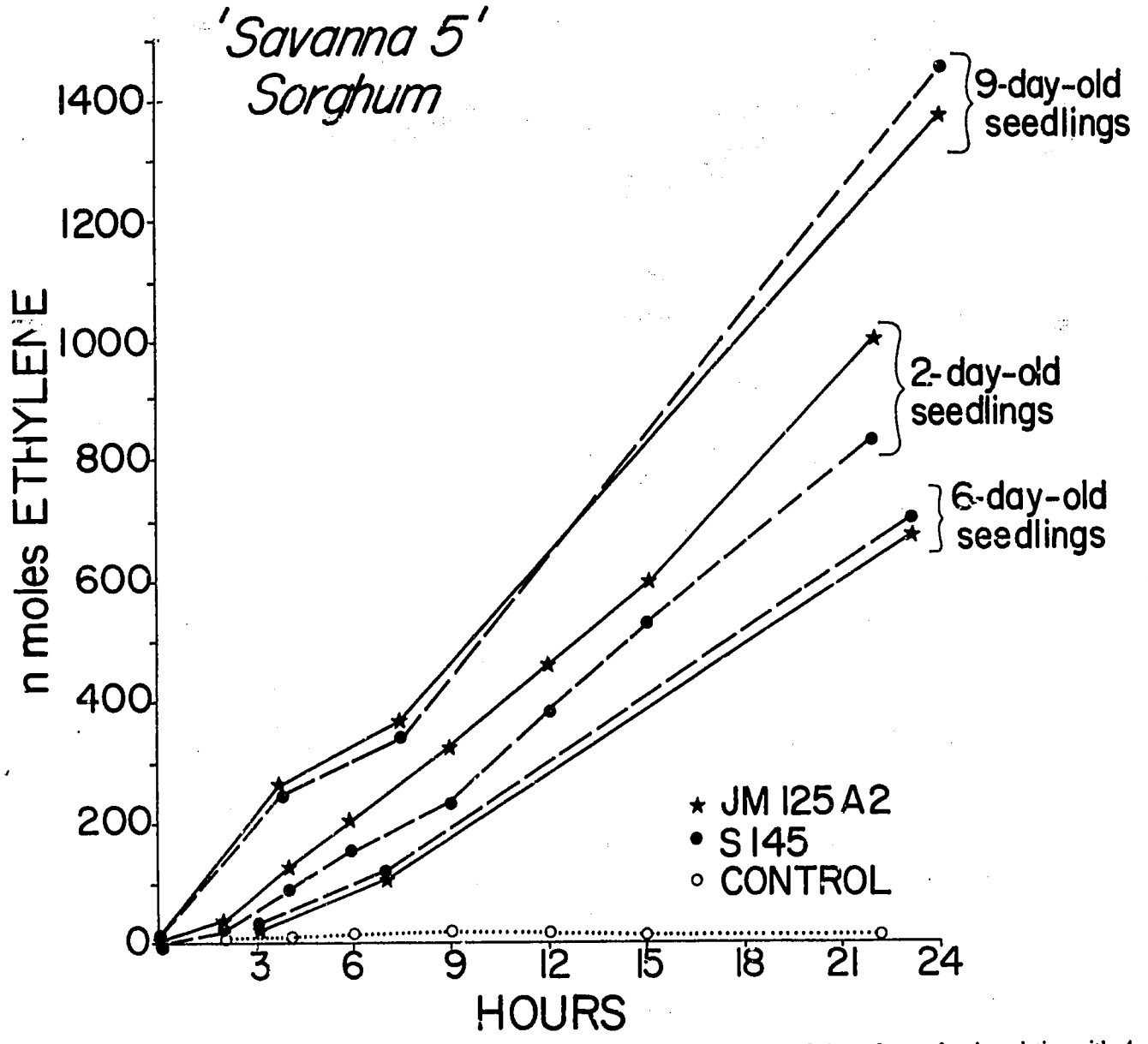

Fig. 1.—Comparative ethylene evolution from cv. 'Savanna-5' sorghum seedlings of 2, 6, and 9 days of age, after inoculation with *A. brasilense*. Note the control plants have no evolution of ethylene.

Analysis of variance (ANOV) and Duncan's multiple range tests were used to analyze the data. Transformed ARA values were used (log transformations) to stabilize the variances in the statistical analyses.

RESULTS AND DISCUSSION

Approximately 400 ARA assays were made on test tube, mason jar, and field cores of sorghum plantings. Bacterial growth in the test tubes was visually observed to be in the rhizosphere of the roots. Once the monoxenic system was established, ARA was monitored, and ranged from 0 in the sterile and *E. agglomerans* inoculated treatments to a high of 28 nmoles tube^{-1} h^{-1} with Florida strain S145 of *A. brasilense* (Table 1). As the plants grew older and the root volume increased in the mason jar assemblies, ARA increased to 160 nmoles jar^{-1} h^{-1}. The non-inoculated controls also became active, indicating sterility was not maintained in the mason jar assemblies. Acetylene reduction activity in the field cores was highest in the natural (non-autoclaved soil) and lowest in the non-inoculated controls (Table 1).

Inoculated test tubes that contained sorghum seedlings were higher in ARA than the no-plant controls. This clearly indicates that plant-dependent acetylene reduction occurred in the monoxenic test tube systems. After transfer into mason jars, these active systems were not significantly different, using ANOV, from natural (non-autoclaved) soil from Madison and Putnam Counties. When moved into the field, the inoculated treatments again were not significantly different in ARA from natural soil. The ANOV test was used again.

Time course studies on cv. 'Savanna-5' for AR activity were conducted on 2, 6, and 9-day-old seedlings (Fig. 1). These studies supported the assay methods used and showed a linear evolution of ethylene up to 24 h. A slightly higher AR for the 9-day-old seedlings was observed. Soil cores from the field (Put-

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TABLE 1.—SUMMARY TABLE ON CONSECUTIVE ARA† IN TEST TUBES, GREENHOUSE AND FIELD PLANTINGS OF SORGHUM.

Experimental Method used	No. Assays‡	Nat§	Bacterial system 13t	JM125	S85-9	S145	None
Test tubes	99	—	19	26	0	28	0
Mason jars	154	156	66	175	86	160	114
Field cores	144	166	112	72	71	92	54

†ARA (Acetylene Reduction Activity) is expressed in nmoles vessel^{-1} h^{-1}.
‡Each test tube, mason jar or field core treatment was replicated 5-7 times.
§Nat = natural or native soil (not autoclaved).

nam soil) also demonstrated linearity over the 27-hour sampling time period.

Electron microscopic observations of cv. 'Savanna-5' showed the rhizosphere bacterial association between the bacteria and the sorghum seedling roots (Fig. 2). Previous EM observations had shown some bacteria occur intercellularly, but not within living root cells (19). Torn or disrupted cortical cells of the root contained *A. brasilense*.

Statistical analyses indicated that two of the five sorghum genotypes were significantly different in ARA from the two sorghum inbreds, but only at the 10% level (Table 2), according to Duncan's multiple range test.

Harvest yields, analyzed by ANOV, at the conclusion of the experiment indicated that 'Savanna-5' was significantly higher in top and root dry weights than 'Coker 7723'. Moisture percentages of the two

Fig. 2.—Scanning electron micrograph (SEM) of cv. 'Savanna-5' sorghum root covered with *A. brasilense* bacterial cells. Three very large epidermal cells of the plant are being sloughed off into the rhizosphere. Magnification is shown on the bar on the photograph.

TABLE 2.—SUMMARY TABLE ON ACETYLENE REDUCTION ACTIVITY OF FIVE SORGHUM LINES GROWN CONSECUTIVELY IN GROWTH ROOM, GREENHOUSE AND FIELD. (SIX ASSAYS).

Sorghum line	Source of seed	ARA†
Savanna-5	Northrup-King	132.8 a*
FS 25a	DeKalb	130.1 a
Coker 7723	Coker Seed Co.	104.7 ab
B68027	Georgia Expt. Sta.	80.3 b
B68181	Georgia Expt. Sta.	61.8 b

†Acetylene reduction activity is measured in nmoles ethylene evolved vessel^{-1} h^{-1}.
*Inbreds B68027 and B68181 were lower ($p=.10$) in ARA than FS 25a and Savanna-5 according to Duncan's Multiple Range Test.

varieties were not significantly different, nor were the ARA values. The two named sorghum varieties were included in the test, because previous results in Florida had indicated both were high yielding varieties and adapted to many regions of the state.

CONCLUSIONS

We conclude from this series of experiments that active acetylene reducing bacterial systems can be established in test tubes with sorghum seedlings. The activity was linear with time, over a 24-h period. When these monoxenic systems were carefully transferred to soil, in order to follow this association into greenhouse and field conditions, we found that the inoculated treatments were not significantly better than the "natural" (non-autoclaved soils). In this series of experiments, the natural soil was from highly active associative N_2-fixing systems previously identified in Madison and Putnam counties (23). The data we have presented in this paper indicate that bacterial inoculation is not yet as effective as some highly active "natural" systems that have evolved in Florida. We plan to continue studying the highly active systems, dissect these systems and look for additional sites that show a natural high AR activity.

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