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NITROGEN FIXATION BY ASSOCIATIVE  
GRASS-BACTERIA SYSTEMS

BY

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## INTRODUCTION

The main objectives of this contract are to determine the nitrogen fixation potential of nitrogen-fixing bacteria in association with grasses and cereal crop plants and to develop systems with potential applications which reliably provide nitrogen to plants growing in nitrogen-deficient soils in developing countries. The principal organism used in these experiments was Azospirillum brasilense. The crop plants included pearl millet, sorghum, corn and forage grasses. The experiments ranged from axenic single bacterial strain and plant combinations to field experiments.

Besides the continued analysis of the soil cores with high acetylene reduction activities (ARA) (described in last year's report) and field testing of the interaction between different bacterial strains and plant genotypes, considerable effort was expended in the design and execution of experiments to test the flow of N to the plant from the bacterium and/or soil, using  $^{15}\text{N}$  as the tracer. The results of one such experiment are presented in section Id.

A second experiment was designed to evaluate the rate of flow of organic nitrogen from the bacterium to the corn plant. It is generally accepted that living A. brasilense cells do not excrete fixed N as ammonia. The experiment described in section IVa was designed to test the rate at which N previously assimilated into cell material is converted into plant tissue.

The major difficulty in conducting  $^{15}\text{N}_2$  fixation experiments with grass-bacteria associations results from the relatively low level of nitrogenase activity. Long incubation periods in tightly sealed growth chambers are required. One of our major efforts during the year was the design and construction of suitable growth chambers for long term  $^{15}\text{N}_2$  fixation experiments. Several chambers of the type described in section Ie will be in constant use during the coming year, for determination of actual nitrogen fixation rates and nitrogen-transfer rates from the bacterium to the plant.

Another achievement during the last year was the construction of an inexpensive but highly efficient and reproducible soil inoculation system described in detail in section IIIg.

The results of these and other major field and laboratory experiments are presented in the following pages.

I. Establishment of consistently reproducible, axenic plant-bacteria systems.

a) Screening several strains and species of bacteria for plant stimulation.

Plant genotype and bacteria strain relationships were studied using axenic soil systems in two greenhouse experiments. In the first experiment, quart Mason jar assemblies containing autoclaved soil, seeded with sorghum, and inoculated with four isolates of nitrogen-fixing bacteria were compared with each other and with non-inoculated (sterile) and non-axenic (natural soil) controls. Manual surface irrigation, used in the first experiment, presented problems of moisture control and contamination in the axenic systems. An improved irrigation system was designed for the second experiment. The system consisted of two-liter plastic jars with drip irrigation, gravity drainage (into potassium permanganate) and seals of waxed sand and cotton plugs to prevent contamination. This experiment compared six nitrogen-fixing bacterial isolates and root washings from field grown grass used as inoculum, and a sterile control (all utilized autoclaved soil). The number of bacteria added to each jar was  $1.8 \times 10^5$  per gram of soil. In addition, natural unautoclaved soil was also included. The autoclaved soil, natural soil and root washings were from an active site with a previous ARA of 1,094 nmoles ethylene evolved $\cdot h^{-1}$ . Uniform sorghum plants (from surface disinfected seeds) were established in the jars.

Data from both experiments indicated that autoclaving can change the soil properties so drastically that ARA comparisons between the soils are difficult. Bacteria, based on ARA, were slow in establishing in all autoclaved soil treatments. The "natural" soil had higher moisture content, because of poor drainage, as compared to the autoclaved soil. This greatly reduced root and top growth, but increased the nitrogen concentration in the roots. Acetylene reduction values were highly correlated with moisture content.

Among the autoclaved soil treatments, a significant yield increase was observed in the treatment where root washings were used as inoculum. None of the other parameters studied (percent moisture, root growth, plant nitrogen content, or acetylene reduction) proved to be significant.

Bacterial counts (using the most probable number (MPN) technique in a nitrogen-free malate medium) showed significant differences. These values ranged from  $4.9 \times 10^5$  /gram of soil with Azospirillum brasilense strain CD (ATCC 29729) when used as inoculum to only  $2.4 \times 10^3$  in the unautoclaved "natural" soil. The number of bacteria among inoculation treatments varied between  $4.9 \times 10^5$  and  $4.9 \times 10^3$  per gram of soil. MPN counts in a medium containing sucrose, mannitol and malate as carbon sources gave  $1.4 \times 10^5$  bacteria/gram of soil with A. brasilense strain JM125A2 as inoculum to only  $2.1 \times 10^3$  in the autoclaved "sterile" soil. It should be mentioned that these bacterial counts were made at the end of the experiment, 85 days after inoculation.

At the termination of the experiment, the autoclaved "natural" soil was also significantly higher in K, P, Al, Mg, and Fe. Despite problems of growing plants in closed systems such as this, it is essential in order to understand the interaction of bacteria, plants and soils.

b) Bacterial strain-plant genotype specificity studies: Sorghum variety testing.

Bacterial strain-plant genotype studies were accomplished primarily on sorghum (Sorghum vulgare) varieties during 1981. Preliminary field trials in 1976 had shown a great variation in acetylene reduction activity (ARA) in the fifty-one sorghum genotypes tested. Selections were made of "high" and "low" ARA lines and a series of replicated axenic test tube experiments were made. Following the successful completion of the test tube experiments, field trials were conducted using the same sorghum lines.

In the first series of axenic test tube experiments, it was possible to only make paired comparisons using two sorghum varieties. Five treatments (4 N<sub>2</sub> fixing bacteria and one sterile control) were imposed. In all, 14 different sorghum varieties were used. Mean ethylene produced in nanomoles per tube per hour are presented in Table 1.

Data from this table clearly indicated that Azospirillum brasilense strains JM125A2, and S145, both Florida isolates of Azospirillum brasilense, were superior in ARA compared to strains 13t and S85-9. For this reason, the following experiments used these two strains.

Two experiments compared 11 varieties of sorghum, grown in test tubes containing Fahraeus medium (nitrogen and carbon-free) and cultured axenically. The purpose of these experiments was to see how well the test tube results would correlate with the field assay. Soil cores (8 cm diameter) containing decapitated sorghum plants were taken from the field. Acetylene reduction was measured by adding 10% acetylene to the cores and incubating overnight. Comparisons of the field assays with the test tube runs 12 and 14 are given in Table 2.

All experiments showed differences ( $P=.10$ ) in ARA between the sorghum varieties tested. Spearman's rank correlations, comparing field assays with test tube screening methods may be used to approximate field results. The results from this experiment indicate that the Coker 7723 variety was significantly higher in ARA than Penngrain YE, Funks 522 and Savanna-5. Silomaker, FS25a, and Hunt varieties were significantly better than Savanna-5. Root dry weight was not correlated with ARA in these experiments.

These results clearly show that some bacterial strains in association with certain plant genotypes produce significantly higher nitrogenase activity. This information is of great value in designing the N<sub>2</sub>-fixation experiments to test the role of nitrogen fixation in associative systems.

c) Establishment of Associative Nitrogen-fixing Systems.

Axenic root-bacteria associations were established by inoculating sorghum seedlings growing in test tubes with nitrogen-fixing bacteria. The degree of association of four bacterial strains with four sorghum genotypes was evaluated by visually rating the amount and location of bacterial growth and by assaying for ARA. Most of the time (see Table 3), active associations were established and tubes with plants had higher AR than 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 containing autoclaved soil to see whether the already established ARA would be maintained. Acetylene reduction assays were continued three more times (See Table 3). It was found that the ARA was at rates equivalent to two non-autoclaved soils, Putnam and Madison, (both highly active natural systems) which were included as controls because of their high ARA.

As a final check on the establishment of the system, the plants from the Mason jar assemblies were transplanted into a field which usually had low ARA. Two more acetylene reduction assays were done (See Table 3). Highest ARA's were from the natural (unautoclaved) soils, with lower rates from all inoculated treatments.

d) Quantification of nitrogen fixed by plants in  $^{15}\text{N}$  studies:  
Evaluation of  $\text{N}_2$ -Fixation in Bahiagrass *Paspalum notatum*  
Flugge.

The potential for nitrogen fixation in 21 genotypes of bahiagrass, *Paspalum notatum* Flugge, was investigated in a greenhouse study using a artificial growth medium (95% coarse builder's sand and 5% bentonite clay, v/v, 17 ppm total N, pH 8.4). Nitrogen fertilizer was supplied in the form of  $\text{KNO}_3$  (2.23 atom %  $^{15}\text{N}$ ). Nitrogen fixation and the response due to inoculation with a mixture of seven diazotrophs was estimated by several techniques: 1) yield, measured by top dry weight (TDW) and grams nitrogen top (GNT); 2) acetylene reduction (AR); and 3) dilution of  $^{15}\text{N}$ .

Genotypes were significantly different ( $p=.0001$ ) for TDW and GNT. A significant increase in TDW and GNT ( $p=0.03$  and  $0.005$ , respectively), due to inoculation, was observed. TDW adjusted means for inoculated and control plants were 5.73 and 5.40 grams, respectively. The corresponding adjusted means for GNT were 0.024 and 0.022 grams.

Genotypes were also significantly different ( $p=.0001$ ) for total N balance. Inoculated and uninoculated systems (plant and soil) gained 0.085, and 0.082 g N, respectively. This difference, although small, is significant ( $p=.0001$ ). Nitrogen balance calculations indicated that 85% of the total N is in the soil and 70% of this portion is organic.

Acetylene reduction estimates of nitrogen-fixation, although variable, confirmed that control plants, as well as inoculated plants, were fixing a considerable amount of nitrogen.  $^{15}\text{N}$  data indicated a

10 to 30% N loss from the plant-soil system, likely due to denitrification. Bahiagrass assimilated 22-38% of the labelled fertilizer.

We tentatively conclude that nitrogen-fixation was occurring at a considerable rate in both treatment and control systems. Nitrogen balance calculations support the above statement. Although small, a significant increase of N occurred in inoculated systems. Most of the fixed nitrogen is in the organic portion of the soil, probably in fine root tissues.

Results from this experiment confirm other reports in the literature indicating that control plants fix  $N_2$  in greenhouse and field experiments. This can lead to erroneous interpretations in comparisons among "A" values as often used in associative  $N_2$ -fixation. However, we believe that growing plants in N-deficient growth media as described, circumvents confounding soil and fixed N effects. Without this complication, control plants that fix nitrogen are acceptable and statements concerning N accumulation become possible.

e) Design of a growth chamber for longterm  $^{15}N_2$  studies.

We have designed and constructed growth chambers to allow growth of small plants as well as for testing soil cores in a closed system for long periods of time. These systems permit determinations of  $O_2$ ,  $CO_2$ ,  $C_2H_2$  and  $C_2H_4$  levels in the atmosphere. Facilities have been incorporated into the systems which allow the adjustment of the  $O_2$ ,  $CO_2$ , and  $C_2H_2$  and  $C_2H_4$  concentrations and monitoring of temperature and relative humidity (RH). A newly designed cooling system in the gas circulation line will provide extensive control of both the temperature and RH in the chamber. The overall volume of these chambers has been minimized, thus permitting experiments using  $^{15}N_2$  gas in concentrations unattainable in larger systems because of procurement costs. These chambers allow plant samples to be maintained for long periods of time and permit the measurement of nitrogen fixation using  $^{15}N_2$  in systems exhibiting low rates of nitrogenase activity.

## II. Collection and characterization of grass-rhizosphere inhabiting nitrogen-fixing bacteria.

The loss of pigmentation by A. brasilense strain CD under certain conditions, prompted a partial examination of the pigments and the changes in pigmentation as affected by selected edaphic conditions. The non-polar petroleum ether-soluble pigments were extracted from CD cells that had been grown in batch culture and collected by centrifugation. The pigments were separated by two-dimensional thin-layer chromatography. Preliminary characterization of their chromatographic properties (Rf values) and visible spectra suggest the pigments are C40-isoprenoids, similar to  $\beta$ -carotene and lycopene.

### a) Regulation of nitrogen fixation

Our studies on the regulation of nitrogen fixation utilize Klebsiella pneumoniae strain M5A1. Previous experiments showed that the nif gene product is essential for active nitrogen fixation. We have isolated and characterized suppressor mutants that map in a region of the bacterial chromosome apart from the original nif mutation. Unfortunately these suppressor mutations are very unstable for continued use. Our present and future work involves the biochemical characterization of the nif induced defect in nitrogen fixation.

### b) Genetics of Azospirillum: Role of plasmids.

Earlier proposals included strain construction ("strain breeding") by moving genes for desirable properties (e.g. high nif activity) from one strain to another strain having other desirable properties. For these purposes it would be useful to know which of the 6-8 different DNA molecules in Azospirillum carries the nif genes. Since earlier analysis of a nif mutant by gel electrophoresis did not reveal any information as to the location of the genes controlling nitrogen fixation, recombinant DNA techniques, used successfully in other bacterial systems, were used here.

Specifically, a recombinant plasmid (pWK27) was used because it carries the two highly conserved structural genes (nif KDH) from Klebsiella. Purified pWK27 plasmid DNA was used as a radioactive probe to reanneal with the separated DNA bands. The bands had been previously resolved by gel electrophoresis and transferred to nitrocellulose by Southern blotting (J. Mol. Biol., 1975. 98: 503).

This procedure showed that at least some of the genes for nitrogen fixation are present on the largest DNA molecule in all of the strains of Azospirillum examined. If this largest molecule is the chromosome and if it carries the nif genes, and these are not duplicated on any plasmid, how then can we explain the refractory nature of nif genes to mutagenesis? The nif genes could possibly be duplicated on the largest molecule in tandem or scattered form.

Improved analytical treatment of gel electrophoresis data was developed to more accurately estimate molecular weights of the DNA molecules (in Mdal) in Azospirillum brasilense strain JM125A2 (see below).

pAz7	.....	46
pAz6	.....	130
pAz5	.....	345
pAz4	.....	385
pAz3	.....	600
pAz2	.....	1130
pAz1	.....	1350

High doses of UV light immobilize the DNA molecules of Azospirillum in the electrophoresis gel. This indicates the DNA molecules in the gel are supercoiled molecules rather than open circles or linear molecules, as has been suggested by some workers.

Attempts to develop more drastic and successful procedures for mutagenesis for Azospirillum have not been fruitful. The following modifications of mutagenesis procedures were evaluated:

- 1) Very long segregation times after mutagenesis.
- 2) Variations in exposure time, and concentration and pH of mutagens (ethyl violet and acridine orange).
- 3) Variations in nitrosoguanidine concentrations and exposure times.

Derepressed nif mutants were easily found after nitrosoguanidine mutagenesis by testing survivors for ability to reduce acetylene when grown in the presence of excess  $\text{NH}_4^+$ . None of the mutants found were stable however and thus were unsuitable for field experimentation. An explanation for the instability might be that the mutated gene reverted at a normal and reasonable frequency, and since the derepressed mutant cells expended so much energy fixing nitrogen, they grew much slower than the revertants in the population. The revertants then overgrew the mutants and became the predominant cells in the population. Cultures of derepressed mutants of Azospirillum obtained from other labs were either unable to grow or were contaminated.

### III. Ecological factors which limit or enhance plant-bacterial responses.

#### a) Search for Natural, Highly Active Associative Nitrogen-Fixation Systems--Continued Effort.

In the search for highly active associative nitrogen-fixation sites (preliminary report last year), 264 grass-soil cores were analyzed. Four sites had cores that evolved over 1000 nmoles ethylene  $\text{core}^{-1}\text{h}^{-1}$ , which is very high for grass-soil cores. Nineteen sites had cores over 500  $\text{nmoles core}^{-1}\text{h}^{-1}$ , and 34 cores had over 300  $\text{nmoles core}^{-1}$  (see Table 4 and Fig. 1). Paspalum notatum was the most prevalent grass species in the highly active cores (it was also the most prevalent species in the study), followed by Cynodon dactylon.

A comparison of soil factors from active and inactive sites by stepwise multiple regression analysis indicated that soil moisture was the first factor included in the model ( $r=0.7$ ,  $p=0.0001$ ) and was highly correlated with high ARA activity (see Table 5 and Fig. 2). In addition, both Ca and Zn concentration gave significant effects ( $p=0.009$  and  $0.039$ , respectively). Calcium content was negatively correlated and zinc content positively correlated with high ARA activity.

Acetylene reduction was positively correlated with  $\text{CO}_2$  concentration and negatively correlated with  $\text{O}_2$  concentration in the cores at the end of the AR assay incubation period (see Table 6). This suggests that higher metabolic activity is occurring in the active cores (probably from plant metabolism).

Good ARA has continued, though not always above 500  $\text{nmoles ethylene evolved core}^{-1}\text{h}^{-1}$ . We have verified that ARA depends on good soil moisture. When soil moisture is low, ARA is always low.

These active sites are being used for sources of cores and soil for greenhouse and laboratory experiments. It is of special interest to characterize these systems in order to understand why they are active and to obtain improved biological systems. Additional active sites are being added to our list.

#### b) Inoculation with Azospirillum brasilense strain CD.

An inoculation experiment conducted at the Hague Experimental Farm with the Azospirillum brasilense strain CD, (used successfully in Israel) gave a significant dry matter yield increase due to inoculation. This increase was observed with Funks 522 sorghum, but not with Gahi-3 pearl millet.

Reports of the high success rate of inoculation experiments in Israel, where the Azospirillum brasilense strain CD was used as inoculum, prompted us to try the same strain in Florida inoculation studies. Since one of the most severe limitations to obtaining a response from inoculation is the inability of inoculated bacterial cells to survive and establish in the rhizosphere, we devised a new inoculator that

would handle the inoculum carefully and place it directly on the seed in the seed furrow (see section IIIf for details).

We selected the Florida experimental farm area at Hague because it had never been used in Azospirillum studies. Twelve replications were used in this experiment, with two inoculation treatments, live and killed cells, and two grass species, Gahi-3 pearl millet, and Funk's 522 sorghum. A preliminary soil test showed the pH to be 5.9, and therefore 2 MT/ha of hydrated lime was added. Adequate phosphorus and potassium fertilizer with minor elements was applied prior to seeding. Nitrogen fertilization was applied uniformly at the rate of 30 kg/ha on all plots. Irrigation was not available, and rainfall was marginal during the experiment, but the plants did not wilt until near the harvest date. Three assays for ARA were conducted during the growing season.

Results showed a dry matter yield increase (significant at  $P=0.015$ ) from the live bacterial cell application on 'Funks 522' variety of sorghum. The dry matter yield increase of 11% is the smallest statistically significant yield increase that we have been able to measure. The C.V. for the experiment was 8.5, which is very good for field plots in Florida. Samples were taken from the plots for protein determination but analysis has not been completed. The Gahi-3 variety of pearl millet did not show a corresponding yield increase.

ARA did not correlate with the inoculation in either plant-bacteria system. However, AR data showed a high positive correlation with the moisture content of the soil. This is an ecological factor which is extremely important if high ARA is to be observed. One other ecological consideration was the observation that inoculated plots of Gahi-3 had a significantly higher ( $P=0.03$ ) soil moisture content, as well as higher ARA.

c) Inoculation with *A. brasilense* strains CD and CDSR in a peat slurry--DD82.

Successful inoculation of tropical grasses with nitrogen-fixing bacteria for yield increases and/or total protein increase continues to be a major commitment of the Florida group. Mass cultures of selected organisms were produced and then applied in various ways to different grass species. In 1982, the major field experiment at Florida was designated DD82 and although the available data is still incomplete, some interesting observations have been made. A similar large field experiment was sub-contracted to New Mexico State University which will be reported in the next annual report.

The Florida experiment is testing two species of grass ('Tifleaf' pearl millet and an intraspecific hybrid between 23DA pearl millet and N75 Dwarf elephant grass), three inoculation treatments (killed bacteria control, live strain CD, and a live spontaneous streptomycin and rifampicin resistant mutant strain of CD), four fertilizer treatments (0, 30, 60, and 120 kg N/ha) in 10 replications in a split-plot design. Inoculation methodology used a peat slurry as described in section IIIf. Samples of soil were removed from the rows prior to inoculation

and at weekly intervals from the three treatments at the 0 and 60 fertilization levels for bacterial counts (MPN method). In the first harvest, both strains showed a significant nitrogen fertilizer effect on dry matter yield, (see Fig. 3) but no significant effect due to inoculation. Bacterial counts are plotted for each week after inoculation (see Fig. 4). The grasses were harvested on the 78th day after planting. Plots were re-fertilized, and a second harvest will be made in September 1982. Bacterial counts will continue, but at triweekly intervals.

We have found that the bright pink color of strain CD permits visual identification of strains CD and CDSR, which facilitates counting. Growth of strain CDSR in tubes containing a selective medium (with streptomycin and rifampicin) have also verified that counts of strain CD using the pink color marker is reliable. It is important to note that the bacterial counts from the control plots have always been zero for strains CD and CDSR. This indicates, that in the 12 week period, there was no horizontal movement of strains CD or CDSR to adjacent control plots. Ancillary experiments verified that the inoculated bacteria did not migrate large distances. Samples taken 12 inches from point of inoculation were always negative for strains CD or CDSR, but samples taken 6 inches away sometimes had reduced counts of strains CD and CDSR (when compared to counts directly in the inoculated row). These movement studies were done 13 days and 59 days after inoculation. More results from the DD82 experiment will be presented in next year's report.

#### d) Survival of *Azospirillum* in peat

Recent experiments have indicated the rapid loss of introduced nitrogen-fixing bacteria in the rhizosphere. To provide sufficient viable bacteria in the rhizosphere of inoculated plants, we have been investigating inoculation methodology. Many of the early inoculation trials have used liquid inoculation methods. However, the large volumes of liquid required present storage and transportation problems. A survey of selected inoculation techniques, including liquid, vermiculite base, peat base, pelletized, and seed coated, suggested a peat base inoculum would provide greater survival rates for the introduced microorganism.

These results, in addition to the fact that peat is a convenient material, prompted the examination of the survival of *A. brasilense* in peat. The method chosen for investigating the survival in peat was to follow the bacterial populations, employing an antibiotic resistance marker.

The population size inoculated into peat appeared to affect the survival of the organism. As the size of the initial population decreased from  $7.5 \times 10^8$  to  $6.2 \times 10^2$ , the rate of loss of viability decreased from  $4.4 \times 10^6$  to 5.1 cells per day, as determined by a total loss of organism viability over a 152 day incubation period. Although there is a decrease in the total numbers of bacteria, which is evident in all treatments and positively correlated with inoculum size, the results suggest that *A. brasilense* can be maintained in relatively

large numbers on a peat carrier for as long as 150 days.

The pH of the peat medium exerted slight effect for the first 8-16 days of the culture. Survival was best on peat adjusted to 7.0. However, the difference in survival between pH 6.5 and 7.0 was slight after 2 months. This corresponds to the optimum pH for many of the physiological processes of this organism in liquid culture. Moisture content of the peat carrier seems to have little effect on survival for the first 20 days, except at very low levels. The optimum range for survival was 40-50% moisture. Sixty percent moisture was detrimental to bacterial survival.

Sterilization of the peat improved the survival of A. brasilense. The greater survival of A. brasilense on sterile peat suggests that biological factors affect the survival of the nitrogen-fixing bacteria. Dry matter size, aeration status, and general nutrition, that can influence bacterial growth on peat will be incorporated into this experimental framework as a more comprehensive interpretation of microbial growth on selected carriers will be investigated in the future.

e) The Effect of Cellulose on Acetylene Reduction in Formerly Identified Active Soils.

Our previous data indicated that the presence of plant roots in cores increased the AR in those cores 10 fold over no-root controls. Since plants lose a considerable amount of fixed carbon (up to 25%) to the rhizosphere, we hypothesize that the major portion of increased AR activity may be due to the energy contributed by the plant material. Plant material lost to the soil rhizosphere is in both soluble and insoluble forms. Soluble forms, i.e., sugars, amino acids, etc., have been shown to be good substrates for nitrogen-fixing bacteria. On the other hand, most nitrogen-fixing bacteria cannot hydrolyze many insoluble plant materials such as cellulose, hemi-cellulose and lignin.

An experiment was conducted to determine whether soils of previously identified high ARA sites would respond to the added cellulose. Duplicate soil cores were obtained from two sites each at Union County, Madison County and Jefferson County, that had previously yielded active cores (over 500  $\mu$ moles acetylene reduced per core per hr). Fifteen gram (dry wt.) samples of soil were standardized to 20% moisture and incubated with 75 mg cellulose, in a 2% O<sub>2</sub>-98% N<sub>2</sub> atmosphere at 30°C. Duplicate cores were each replicated two times giving four reps per soil. Acetylene reduction assays were conducted after incubation for six and 12 days.

Three of the six soils responded with increased ARA. Both (all but one replicate) of the Madison County soils responded and only one replicate of one Jefferson County soil responded. Union County soils did not respond. Experimentation is being continued to evaluate more soils for response to cellulose, ground bahiagrass root, glucose, manitol, succinate, and a non-additive control. We also plan to evaluate the substrate effect on the relative populations of nitrogen-fixing bacteria compared to the total bacteria and to isolate the organisms

responsible for this response to insoluble substrates. These organisms could be important in the maintenance of high ARA in these sites.

#### f) A Plot Inoculator for Liquid Inoculum

An inoculator was constructed capable of precisely placing metered amounts of liquid inoculum ranging in consistency from water to viscosity of peat slurry. This simple inoculator can be mounted easily on a wide variety of planting or cultivating equipment used both experimentally and commercially, and it is easy to operate. It consists of two peristaltic pump units (each driven by a 12-volt Ford windshield wiper motor unit), two 20-liter carboys (for inoculum reservoirs) fitted with propeller-type stirrers driven by 12-volt automotive heater motors, and distribution tubing (1 cm i.d. tygon). The 12-volt power to operate all motors is supplied by the tractor's battery.

Separate inoculator units and distribution tubing apply live and control inocula and permit application to be changed from live to control or vice versa with a flip of a switch. This permits a randomized plot arrangement with only one inoculator-planter pass over the field. Inoculum can be distributed directly over the seed in the furrow by placing the distribution tube directly behind the seed furrow opener. One or two row plots can be inoculated by attaching one or two pump heads to each pump unit along with the appropriate distribution tubing. Motor switches are mounted on a switch umbilicum operated by the tractor driver or someone following behind.

This inoculator permits flexibility in inoculum formulation since it can apply liquid cultures, water bacterial suspensions, and slurries containing peat or other solid carrier materials of at least 10% (wt/vol) of the total volume. The use of peat requires that tubing have minimal restrictions. We found that peat would accumulate at any orifice of 4mm or smaller and plug up the tubing. Rate of application is 600 ml/min for each pump head (Cole-Parmer model 7018) on low speed and 860 ml/min on high speed. Maximum rate of application is about 0.2 ml/cm of row at slow tractor speed and less at higher speeds. To obtain an inoculation rate of  $10^8$  bacteria per cm of row, the inoculum would have to contain  $5 \times 10^8$  bacteria per ml. Increasing tractor speed or diluting the inoculum will reduce the inoculation rate.

A single unit version of this inoculator was used to inoculate the 1981 Hague experiment with live and killed cultures of Azospirillum brasilense strain CD. With this low viscosity culture, anti-drip mechanisms from spray nozzles were used to prevent dripping between plots. This single unit was used also to inoculate the 1982 Agronomy Farm pearl millet-Pennisetum hybrid nursery (experiment DD82) with culture containing a 10% peat (Nitragin Co.) suspension (vol/wt). The double unit inoculator described above was used to inoculate two corn experiments on the Agronomy Farm and then to inoculate the New Mexico experiment, all with 10% peat added to the appropriate culture.

#### IV. Carbon dioxide and nitrogen fixation by plants and associated rhizosphere bacteria.

##### a) Studies to quantify the CO<sub>2</sub> and N<sub>2</sub> fixed by plants and rhizosphere associated organisms respectively.

The translocation of fixed nitrogen from the nitrogen-fixing bacteria to a crop plant is not adequately understood. It has been reported that bacterial cell nitrogen labelled with <sup>15</sup>N, is recovered principally in acid hydrolyzable and acid insoluble humic fractions (Knowles, R. and D. T. H. Chu. 1969. Can. J. Microbiol. 15: 223-228). We have initiated a study to test the hypothesis that during a growing season nitrogen from nitrogen-fixing bacteria in the soil biomass can become incorporated into the plant tissue. If there is incorporation of <sup>15</sup>N in the plant tissue, the experiment will be continued into successive growing seasons to determine the rate of nitrogen incorporation. Cells of Azospirillum brasilense strain JM125A2 and Klebsiella planticola uniformly labelled with <sup>15</sup>N were added to non-sterilized soil during the growth of Zea mays. At maturity the plants, including roots, were harvested and dry matter yields were determined. Nitrogen in the soil and plant samples are being determined by standard techniques and isotopic nitrogen by mass-spectrometry analysis. Total soil bacteria, denitrifying bacteria and nitrogen-fixing bacteria were estimated using MPN techniques on the appropriate media. Yields show that inoculation, with either strain of bacteria, produced a positive plant response. Early analysis of plant nitrogen suggests that bacterial nitrogen was translocated from the nitrogen-fixing bacteria and made available to the corn plant during the growing season. Effects of nutrients, organic nitrogen, aeration and other variables on incorporation of bacterially fixed nitrogen will be studied in future experiments.

Nitrogenase activity (=acetylene reduction activity) was observed in Azospirillum brasilense cultures grown in conditioned medium obtained from embryogenic pearl millet (Pennisetum americanum) and Guinea grass (Panicum maximum) cell suspension cultures, but no bacterial growth or activity was observed on unconditioned medium. A peak of maximum activity was obtained with medium harvested mid-way through a 13 d culture period. Non-embryogenic suspension cultures of pearl millet and Guinea grass supported little or no bacterial growth or nitrogenase activity.

Table 1. Study of bacterial strain-plant genotype specificity using axenic test tube method. Five treatments (four nitrogen-fixing bacteria and one sterile control) were compared using 14 sorghum varieties (tested in pairs). Mean ethylene produced is given in nanomoles per tube per hour.

CULTIVAR	13t	JM125A2	S85-9	S125	STERILE
SX 16	18.5	227.6	1.6	32.4	0
FS25a	18.8	197.6	0	13.9	0
Pennsilage	0	63.5	0	12.2	0
Penngrain YE	0	70.2	0	9.9	0
B83E (Inbred)	0	18.7	0	1.0	0
B97E (Inbred)	0	28.2	0	5.7	0
B68181 (Inbred)	50.7	40.4	0	48.7	0
B68027 (Inbred)	53.7	34.7	0	55.0	0
Savanna-5	21.6	27.2	0	28.8	0
Coker 7723	27.4	40.0	0.4	33.4	0
Silomaker	0	19.7	0.3	25.9	0
Funks G83F	0	35.4	0.3	29.4	0
Funks 522	3.4	18.3	0.3	51.4	0
Hunt 128GDR	5.0	28.1	0	44.3	0

Table 2. Comparison of axenic test tube method and field assay for testing varieties of sorghum for plant-bacteria associative specificity. All results are expressed as nanomoles ethylene evolved per hour.

SORGHUM CULTIVAR	TEST TUBES		FIELD
	RUN 12	RUN 14	ASSAY
SX-16	42.4 <sup>1</sup>	10.0 <sup>1</sup>	283 <sup>2</sup>
G83-F	35.2	10.4	236
FS25A4	33.2	5.4	404
HUNT 128GDR	21.6	6.8	346
FUNK 522	19.0	9.2	165
PENNGRAIN YE	16.2	3.4	209
GATORGRAIN	16.0	1.2	210
COKER 7723	14.8	12.2	330
PENNSILAGE	10.2	3.2	214
SAVANNA-5	7.2	4.6	162
SILOMAKER	5.6	7.8	309

<sup>1</sup> Each figure in this column is the mean of 5 replications.

<sup>2</sup> Each figure in this column is the mean of 10 field cores.

Table 3. Summary Table on ARA in Test Tubes, Mason Jars and Field.<sup>1</sup>

Experimental Method Used	No. Assayed	BACTERIAL SYSTEM					
		Natural	13t	JM125	S85-9	S-145	None
Test tubes	99	—	18.6	26.2	0	28.3	0
Mason jars	154	156.5	65.9	174.9	85.6	160.4	114.0
Field cores	144	166.1	111.8	71.5	71.3	91.9	53.8

<sup>1</sup> Results expressed as nanomoles ethylene evolved per hour.

Table 4. Summary of grass species and number of cores with AR activities over 300 nM ethylene evolved core<sup>-1</sup>hr<sup>-1</sup>

AR Activities					
Over 1000 nM		Over 500 nM		Over 300 nM	
<u>No. Cores</u>	<u>Species</u>	<u>No. Cores</u>	<u>Species</u>	<u>No. Cores</u>	<u>Species</u>
3	<u>Paspalum notatum</u>	9	<u>P. notatum</u>	14	<u>P. notatum</u>
1	<u>Cynodon dactylon</u>	3	<u>C. dactylon</u>	4	<u>C. dactylon</u>
4	TOTAL	2	<u>Heteropogon sp.</u>	2	<u>Heteropogon sp.</u>
		1	<u>Ermochloa ophiuroides</u>	2	<u>Paspalum urvillei</u>
		1	<u>Imperata cylindrica</u>	2	<u>Panicum sp.</u>
		1	<u>Panicum repens</u>	2	<u>Stenotaphrum secundatum</u>
		1	<u>Panicum sp.</u>	1	<u>E. ophiuroides</u>
		1	Unknown	1	<u>I. cylindrica</u>
		19	TOTAL	1	<u>Sporobolus poiretti</u>
				1	<u>Panicum hemitomon</u>
				1	<u>Panicum repens</u>
				1	<u>Hemarthria altissima</u>
				1	<u>Pennisetum americanum</u>
				1	Unknown
				34	TOTAL

Table 5. Mean soil factor values for active and inactive sites and their relation to AR analyzed by multiple regression.

Factor (Units)	Acetylene Reduction Activity Group	
	High	Low
AR (nM Core <sup>-1</sup> hr <sup>-1</sup> )	597	35
pH (pH units)	6.1	6.5
Ca (ppm)*	882	1518
Mg (ppm)	37	72
K (ppm)	21	23
P (ppm)	58	35
Fe (ppm)	45	11
Mn (ppm)	6.2	3.7
Zn (ppm)*	2.4	0.9
Moisture (percent)**	14.0	7.5

\* Significant 0.05 and \*\* at the 0.01 P level calculated by SAS Stepwise Multiple Regression Procedure.

Table 6. Mean CO<sub>2</sub> and O<sub>2</sub> values of cores from active and inactive sites as estimated of core respiration during incubation.

Variable	Low Activity sites	High Activity sites
Acetylene Reduction*	35	59.7
% CO <sub>2</sub>	10.0	14.3
% O <sub>2</sub>	5.1	2.5

\* Acetylene reduction in nM ethylene evolved core<sup>-1</sup>hr<sup>-1</sup>.

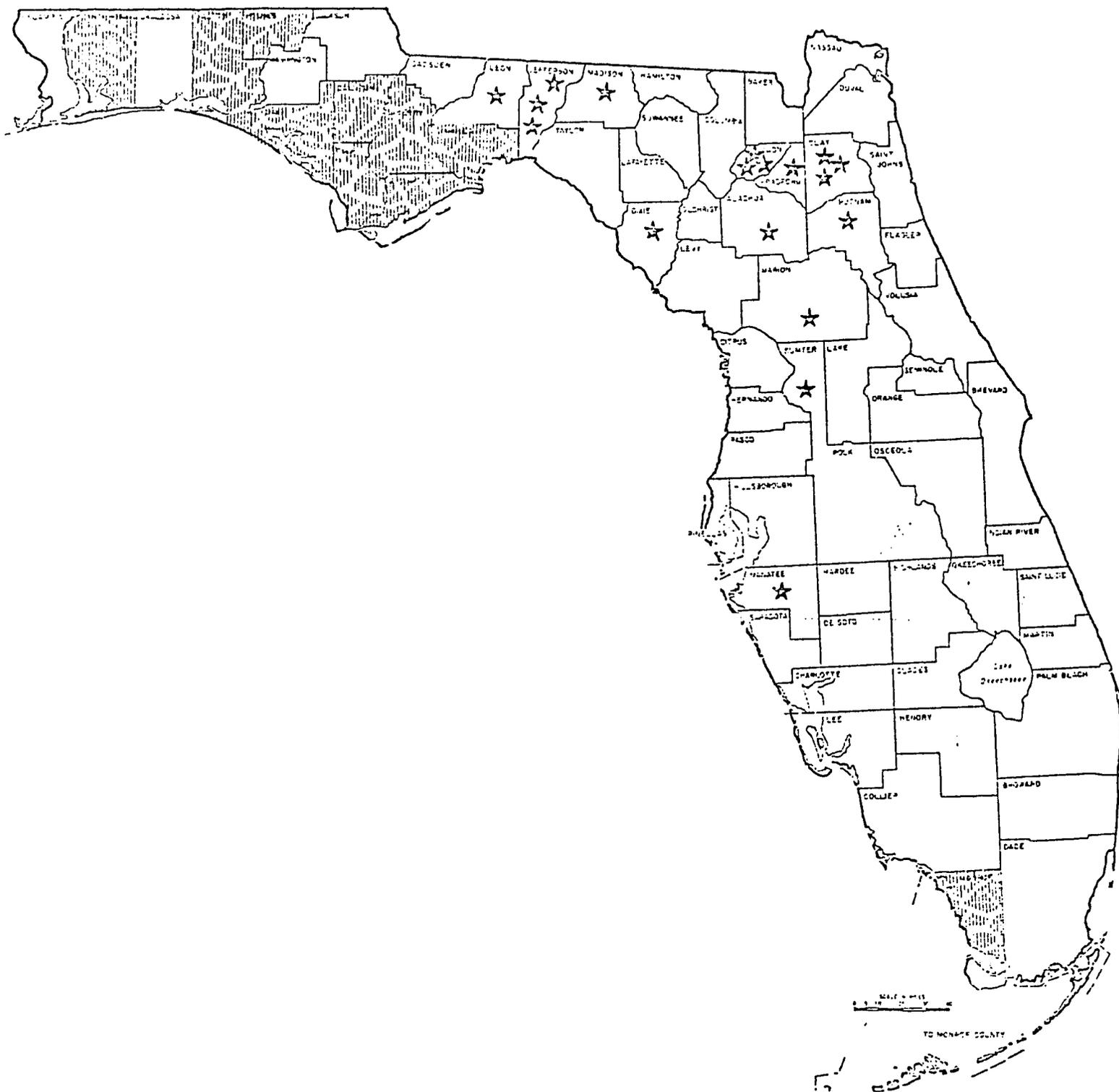
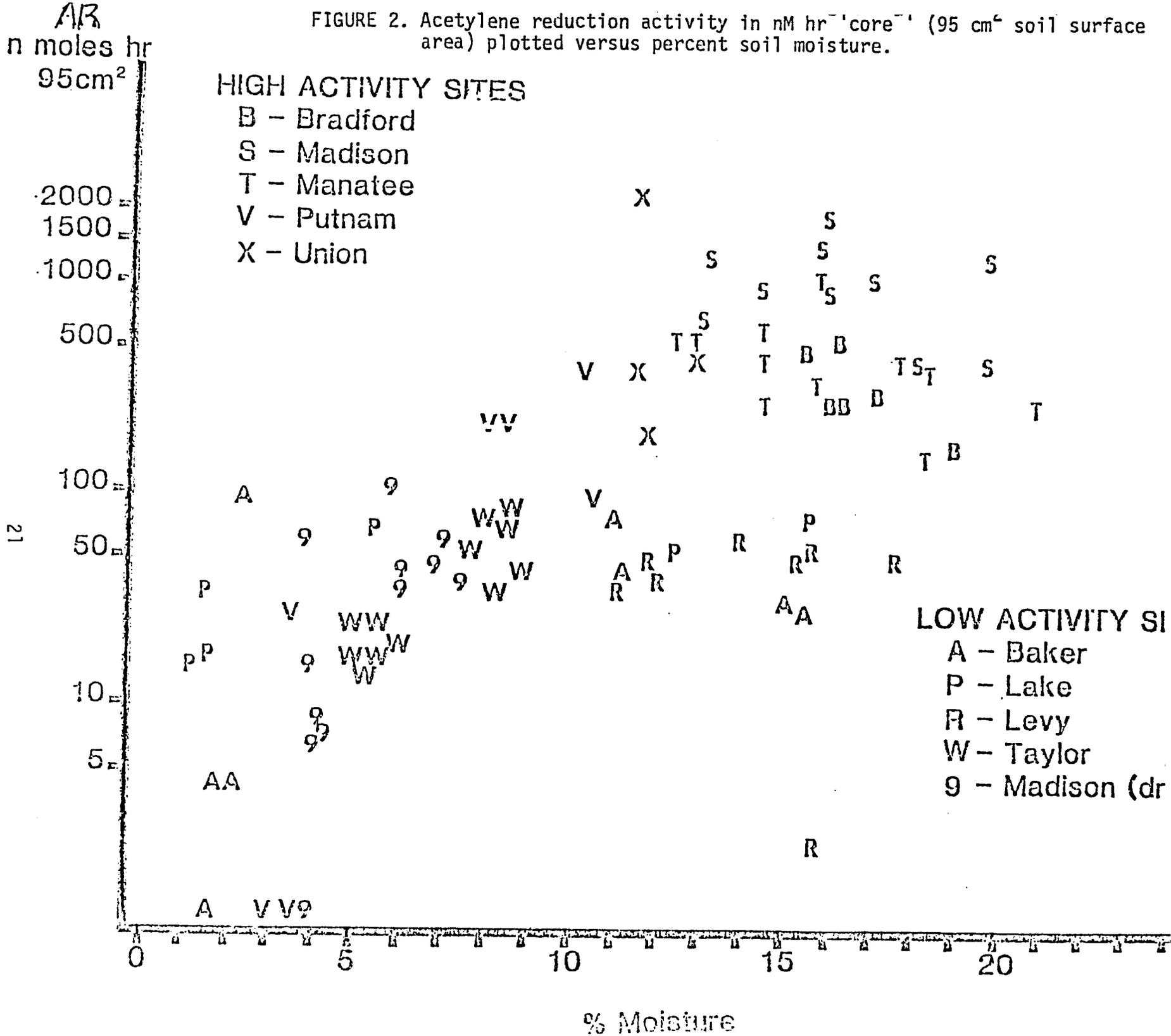


FIGURE 1. Map showing counties sampled and county locations of the 19 highly active (AR) sites. Shaded counties were not sampled. Stars indicate counties producing highly active cores (above 500 nM ethylene core<sup>-1</sup>hr<sup>-1</sup>).

FIGURE 2. Acetylene reduction activity in nM hr<sup>-1</sup> core<sup>-1</sup> (95 cm<sup>2</sup> soil surface area) plotted versus percent soil moisture.



DD82 EXPT.  
 Dry Matter Yield - Ist Harvest

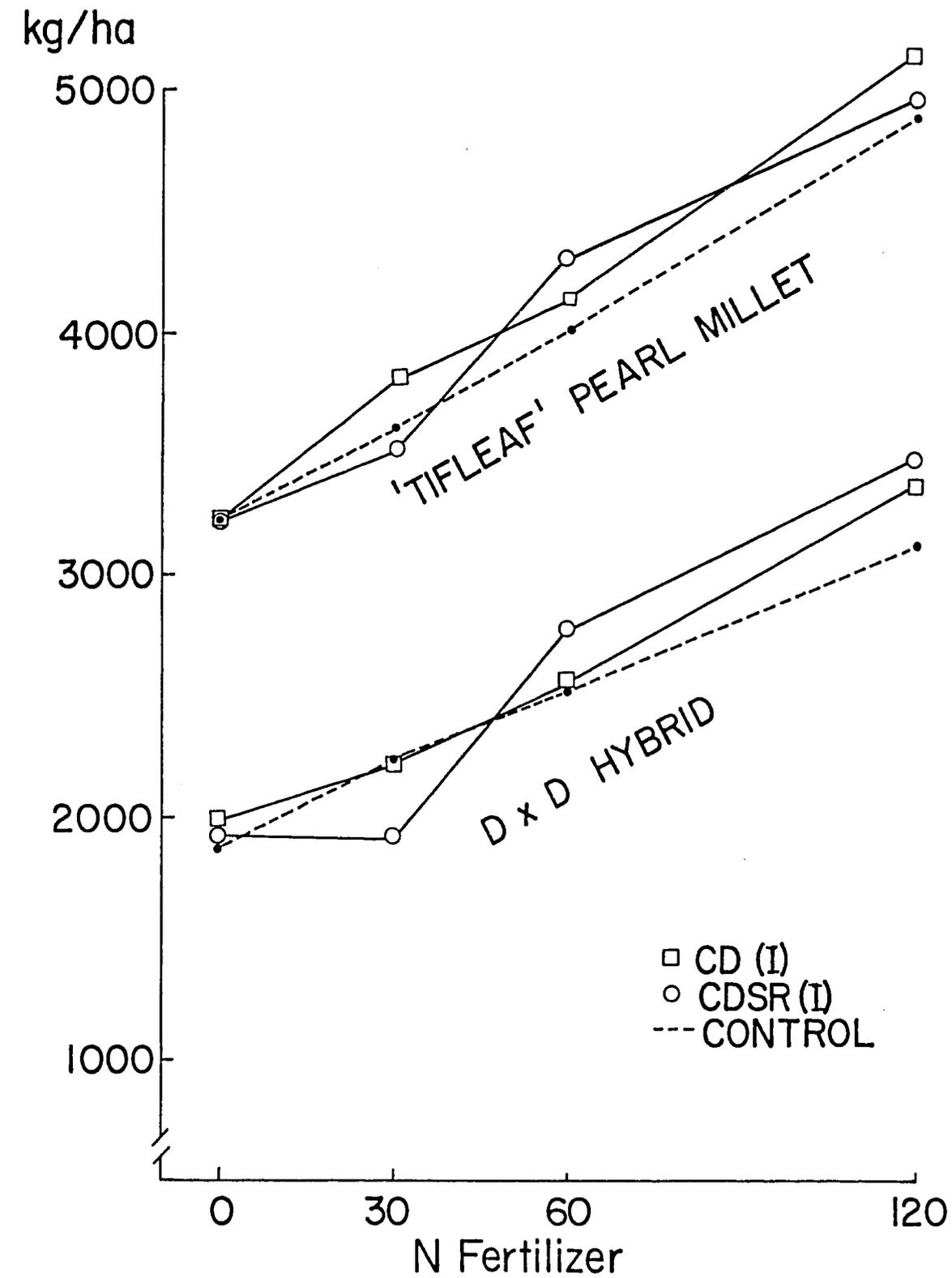
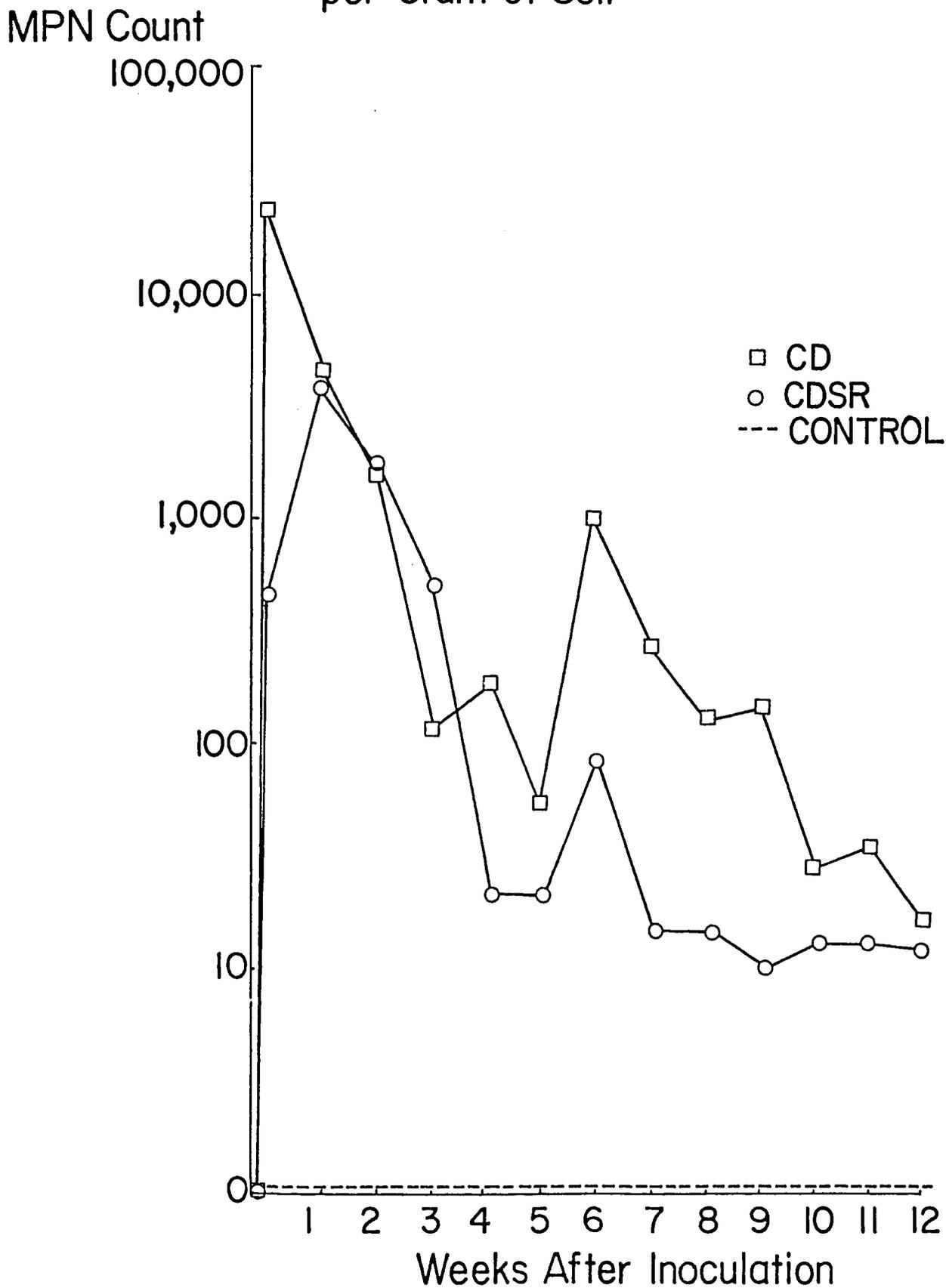


FIGURE 4.

### DD82 EXPT. Number of CD or CDSR Bacteria per Gram of Soil



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2. Albrecht, S. L. and M. H. Gaskins. 1982b. Effect of Moisture Levels in the Soil on Growth and Nitrogenase Activity of Root-Associated N<sub>2</sub>-Fixing Bacteria. (In manuscript form - to be submitted to Plant and Soil).
3. Albrecht, S. L., M. H. Gaskins, Jiang You-yi and J. R. Milam. 1982. Factors affecting introduced populations of N<sub>2</sub>-fixing bacteria in the rhizosphere. Can. J. Microbiol. (In Press).
4. Albrecht, S. L., J. R. Milam and M. H. Gaskins. 1982. Longevity and Growth of Azospirillum brasilense in Peat Culture. (In manuscript form - to be submitted to Applied and Environmental Microbiology).
5. Albrecht, S. L., M. E. Mitchell and M. H. Gaskins. 1981. Influence of soil moisture and organic substances on N<sub>2</sub>(C<sub>2</sub>H<sub>2</sub>) - fixation by sorghum and millet. (Abstract) Plant Physiology 67:77.
6. Dudeck, A. E., R. L. Green, R. L. Smith and A. A. Baltensperger. 1982. Associative N<sub>2</sub>-fixation studies with warm season turfgrasses. To be presented at the Soil and Crop Science Society of Florida Annual Meeting, Oct. 26-28, Tallahassee, Florida.
7. Gaskins, M. H., S. L. Albrecht and J. R. Milam. 1982. Effect of soil conditions on survival and activity of diazotrophic bacteria. Can. J. Microbiol. (In Press).
8. Gaskins, M. H., D. H. Hubbell and S. L. Albrecht. 1981. Interactions between grasses and rhizosphere nitrogen-fixing bacteria. (Abstract) XIV Internat. Grasslands Congress. Lexington, KY p. 143.
9. Green, R. L., R. L. Smith and S. C. Schank. 1982. Screening bahiagrass for associative N<sub>2</sub>-fixation using <sup>15</sup>N and other techniques. To be presented at the Am. Soc. of Agron. Annual Meetings, Nov. 28 - Dec. 3, Los Angeles, CA.
10. Hubbell, D. H. and M. H. Gaskins. 1982. Associative N<sub>2</sub>-fixation with Azospirillum In "Biological Nitrogen Fixation and its Ecological Basis" Conference Proceedings, IIEA, Cen. Ed., Caracas, Venezuela, Jan. 18-29 (In Press).
11. Hubbell, D. H., T. M. Tien, M. H. Gaskins and J. Lee. 1981. Physiological interaction in the Azospirillum-grass root association. In: Associative N Fixation, Vol. I. (P. Vose and A. Ruschel, eds.) CRC Press, Inc., Boca Raton, Florida.

12. Milam, J. R. and S. L. Albrecht. 1982. Translocation of  $^{15}\text{N}$  from inoculated  $\text{N}_2$ -fixing bacteria into Zea mays. Can. J. Microbiol. (In Press).
13. Pence, V. C., N. J. Novick, P. Ozias-Akins and I. K. Vasil. 1982. Induction of Nitrogenase Activity in Azospirillum brasilense by Conditioned Medium from Cell Suspension Cultures of Pennisetum americanum (Pearl Millet) and Panicum maximum (Guinea Grass). Z. Pflanzenphysiol. Bd. 106. S. 139-147.
14. Schank, S. C. and R. L. Smith. 1981. Statewide survey for grass-bacteria associations. (Abstract) Southern Branch Meeting, Am. Soc. Agronomy. 8:7.
15. Schank, S. C., R. L. Smith and R. C. Littell. 1982. Establishment of associative  $\text{N}_2$ -fixing systems. To be presented at the Soil and Crop Science Society of Florida Annual Meeting. Oct. 26-28. Tallahassee. FL.
16. Schank, S. C., R. L. Smith, J. R. Milam, R. H. Berg and R. C. Littell. 1981. Acetylene reduction of sorghum and  $\text{N}_2$ -fixing associations. (Abstract) Sorghum in the 80's - International Symposium - ICRISAT, Hyderabad, India. Nov. 1981.
17. Schank, S. C., R. L. Smith, J. R. Milam and R. C. Littell. 1982.  $\text{N}_2$ -fixing bacteria inoculation of grasses in autoclaved versus unautoclaved soil. To be presented at the Am. Soc. of Agron. Annual Meetings, Nov. 28 - Dec. 3, Los Angeles, CA.
18. Schank, S. C., K. L. Weier, and I. C. MacRae. 1981. Plant yield and nitrogen content of a digitgrass in response to Azospirillum inoculation. Appl. Environ. Microbiol. 41: 342-345.
19. Smith, R. L., S. C. Schank, J. R. Milam and R. C. Littell. 1982. Statewide search for highly active associative  $\text{N}_2$ -fixing systems. Soil Sci. Soc. Fla. Proc. (In Press).
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21. Umali-Garcia, M., D. H. Hubbell, M. H. Gaskins and F. B. Dazzo. 1981. Absorption and mode of entry of Azospirillum brasilense to grass roots. In: Associative  $\text{N}_2$  Fixation, Vol. I (P. Vose and A. Ruschel, eds.) CRS Press, Inc., Boca Raton, Florida.

## CROSS-LINKAGES

### A. Domestic

In the U.S., important cross-linkages with other scientists are maintained by active participation in Regional project S-130. This regional project concerns itself with plant-bacterial root association research, and includes scientists from USDA, Beltsville, Univ. of Georgia, Rutgers Univ., Louisiana State Univ., Texas A & M Univ., Univ. of Nebraska, Tuskegee Institute as well as the Univ. of Florida. Although this project is due to expire Sept. 1982, a request for a 1 year extension of the current project has been made. Objectives are currently being analyzed, and a new proposal for the region is being written. Other important linkages with scientists are maintained at various National meetings, such as ASM (American Society of Microbiologists) and ASA (American Society of Agronomist), where members of our group regularly participate in symposia, or present papers.

Cooperative work with New Mexico State University (Dr. A. A. Baltensperger) was outlined early in 1982, and a full-scale inoculation experiment under 4 levels of N fertilization, was planted at that location. Soil samples from the New Mexico Experiment are routinely sent to Florida, where bacterial counts of the CD strain of A. brasilense are accomplished. Details of this cross-linkage will be reported in next year's experimental results.

### B. Overseas

1. Australian scientists have continued to show interest and cooperation regarding grass-bacterial associations. Dr. A. D. Rovira of CSIRO Division of Soils in Adelaide participated as a consultant and visiting scientist to our group in the past year. Also, a visit from Mr. Keith Weier of CSIRO Division of Tropical Plants and Pastures for 4 days in 1982 stressed research technologies.
2. Cross-linkages with ICRISAT in India continued in the past year, with Dr. S. C. Schank visiting and presenting a paper at the "Sorghum in the 80's" conference at ICRISAT in November 1981. While at Hyderabad, India, Schank discussed research objectives with Peter Dart and S. Wani. Exchanges of preliminary research data, and other research findings is open with ICRISAT. Dr. S. Wani is scheduled to visit the Florida group in August 1982 to further these cross-linkages.
3. Dr. S. H. West has maintained cross-linkages with Egypt and C.I.D. (Consortium for International Development). During the past year, Dr. El-Din from Egypt, Stewart Smith from the Nitragin Company, and Dr. James Sims from the University of Montana met with our group to maintain biological nitrogen fixation contacts. In addition, Dr. West has invited Dr. Victor S. Bedrous, from Cairo, Egypt to work with him in 1983. Another visiting professor, Dr. Mohammed El-Hewl concluded his year's sabbatic leave with us in 1982.

4. Dr. D. H. Hubbell was asked by the Mexican Government to provide a 4-week long training program for two visiting Mexican Scientists in 1982. Scientists scheduled to come are Dr. Manuel Quintero, and Dr. Aquiles Solis, from Durango, Mexico.
5. Dr. Hugh Popenoe, Director of University of Florida's International Programs has been invaluable in maintaining cross-linkages with BIFAD and other governmental agencies in Washington, D.C.
6. Dr. M. H. Gaskins from our group serves in a formal liaison position with TAD (Tropical Agricultural Development), which agency administers grants for the Caribbean, Florida and Hawaii. Excellent cooperation has been continuing with Puerto Rico, (Dr. A. Sotomayer Rios).
7. Israeli contacts have primarily been through a commercial company known as BioTech General. This is the same group that Dr. Yaacov Okon has worked with in past years, and we welcome this added cross-linkage. A BARD proposal with Dr. Okon is still in the planning stages.

STUDENT, POST DOCTORAL, AND VISITING PROFESSOR  
TRAINING PROGRAMS

AID/ta-C-1376  
1977-1982

NAME	Country of Origin	Duration of Training	MS	PhD	Post Doc	Other
Mercedes Umali-Garcia	Philippines	4 years		X		
Sharon Matthews	U.S.	3 years		X		
Alvin G. Wood	U.S.	5 years		X		
Gary Benzion	U.S.	2 years	X			
Christine Carr Dykstra	U.S.	2 years	X			
Howard Berg	U.S.	2 years			X	
Robert Green	U.S.	3 years		X		
Wai-Jane Ho	Taiwan	2 years		X		
Elza Menezes	Brazil	4 years		X		
Norman Novick	U.S.	4 years		X		
Valerie Pence	U.S.	1 year			X	
Tran Minh Tien	Viet Nam	4 years		X	X	
Glen Weiser	U.S.	4 years	X	X		
Arden A. Baltensperger	U.S.	4 months				X
M. E. Mitchell	U.S.	1 year			X	
Mario Sotor	Brazil	1 year		X		
Chiu-Chung Young	Tawian	3 weeks				X
H. G. Diem	Senegal	3 months				X
J. H. Bouton	U.S.	4 years		X		
Joe Salvo	U.S.	2 years	X			
Prem Patel	India	Current		X		
Pushpam Shankar	India	Current		X		
Peggy Akins	U.S.	1 year			X	
Vilma Vasil	India	2 years			X	
Hart Spiller	Germany	1 year			X	
Bruce Bleakley	U.S.	Current		X		
Cathy Meitin	U.S.	Current		X		
Brad Swedlund	U.S.	Current		X		
Mohammed El-Helw	Egypt	Current				X

VISITS, SEMINARS AND SCIENTIFIC  
ACTIVITY AT UNIVERSITY OF FLORIDA (4/30/81-5/1/82)

Dr. Mohammed El-Helw	Egypt	Visiting Scholar
Dr. Mohamed Hassan	Egypt	"
Dr. Jiang You-Yi	China	"
Dr. W. Klingmuller	Germany	"

SHORT-TERM VISITORS (4/30/81-5/1/82)

Dr. Yaacov Okon	Hebrew University, Jerusalem, Israel
Dr. Dale Moss	Oregon State University, Corvallis (Review Team)
Dr. Charles Sloger	USDA, Beltsville (Review Team)
Dr. William Judy	USAID, Washington, D.C. (Review Team)
Dr. Lloyd Frederick	USAID, Washington, D.C. (Tech. Advisor)
Dr. Albert Rovira	CSIRO, Adelaide, Australia
Dr. Edwin George	Forage Research, England
Dr. Yves Savidan	ORSTOM, Trop. Forages, Ivory Coast, Africa
Dr. Ana Popescue	ICCUPT Fundulea, Romania
Galen Mooso	Deseret Ranches, Melbourne, FL
Bob Lamereaux	Deseret Ranches, Melbourne, FL
Paul Genho	Deseret Ranches, Melbourne, FL
Bruce Williams	Auburn University, Alabama
Dr. David Weaver	Auburn University, Alabama
Dr. Jeff Peterson	Auburn University, Alabama
Kitti Vitoonvitalak	Auburn University, Alabama
Dr. James Sims	Montana State University, Bozeman, MN
Dr. Stewart Smith	Nitragin Co., Milwaukee, WI
Dr. Nabil Alla El-Din	Egypt
House Comm. on Science	Washington, D.C.
Dr. G. Oblisami	Coimbatore, India
Dr. Walter Hill	Tuskegee Institute, Alabama
Pansy Rodney	Tuskegee Institute, Alabama
Stafford Crossman	Tuskegee Institute, Alabama
Dr. Fergal O'Gara	Cork, Ireland
Dr. Pat Oriel	Dow Chemical Company
Prof. C. Mi	China
Richard P. Lyman	BioTechnology General, New York
Dr. Haim Aviv	BioTechnology General, Rehovot, Israel
Dr. Zwi Vromen	BioTechnology General, Rehovot, Israel

## AID/ta-C-1376-EQUIPMENT

Item	Date of Order	Date of Receipt	Price Paid	Location
Gas Chromatograph #1	03/25/77	05/09/77	\$3,997.00	516 Bartram E.
Top Load Balance	09/08/77	10/15/77	\$1,140.45	PSL
Transmitted Halogen Light Source for microscope	11/13/77	12/08/77	\$2,269.00	McC 3197
Phase contrast and Dark Field Condensor	11/13/77	12/08/77	\$1,530.00	McC 3197
Double Unit Microferm Fermentor	03/09/77	04/29/77	\$8,688.00	516 Bartram E.
Gas Chromatograph #2	01/08/80	02/06/80	\$6,960.00	PSL
Incubators (2)	01/07/80	02/08/80	\$ 641.35	PSL
Water Purifier	01/07/80	02/05/80	\$ 825.00	McC 3197
Water Bath Shaker	03/12/80	06/10/80	\$1,221.50	PSL
Laminar Flow Hood	11/14/80	12/10/79	\$2,154.00	PSL
Hygrothermograph	03/12/80	04/02/80	\$ 550.40	PSL
Convection Oven	01/07/80	02/12/80	\$ 769.00	McC 3197
Balance	03/12/80	04/01/80	\$1,819.50	McC 2164
Dissolved oxygen controller & recorder	03/12/80	04/01/80	\$ 824.65	516 Bartram E.
Shaking Water Bath	03/12/80	04/02/80	\$1,221.50	120 ME
Computer Terminal	12/03/81	03/16/80	\$1,105.00	Bldg. 495
Commodore Floppy Disk Drive	12/03/81	01/26/82	\$1,406.78	Bldg. 935

Expenditures (by Outputs) 04/01/81 to 04/01/82	MICRO 89	AGRON 90	SOILS 91	TOTAL
A. Establishment of axenic plant-bacteria systems.	18,820	12,362	-0-	31,182
B. Collection, characterization and modification of grass rhizosphere N <sub>2</sub> -Fixing bacteria.	32,622	14,834	-0-	47,456
C. Examination of bacterial-strain genotype specificity.	3,137	18,130	-0-	21,267
D. Ecological factors that limit or enhance plant-bacteria interactions.	4,392	24,723	-0-	29,115
E. Nitrogen and CO <sub>2</sub> fixation by plants and associated rhizosphere organisms.	-0-	12,362	-0-	12,362
F. Association of N <sub>2</sub> -fixing bacteria with plant tissue culture material.	3,764	-0-	7,250	11,014
Actually Spent	62,735	82,411	7,250	152,396
Overhead				71,647
Total				\$224,043

WORK PLANS AND CURRENT RESEARCH NEEDS  
AID/ta-C-1376 1982-83

Based on the project review held May 28-29, 1981 at the University of Florida, additional emphasis will be placed on using  $^{15}\text{N}$  in experiments. The U. of F. now has a new mass spectrophotomer facility. Other than this minor change in direction, our multidisciplinary group will continue with the same major goal, overall purpose and the same six research objectives. An extension without additional funding was approved, which moved the termination date to November 30, 1982. A one-year extension to the contract was solicited, and we are hopeful for this extension which will extend funding to November 30, 1983. Some of the continuing work planned is outlined below:

A. Establishment of axenic plant-bacteria systems.

1. Test a seedling assay for bacterial stimulation of grasses using inert plant support medium and nutrient solutions.

2. Search for nif<sup>-</sup>, bacterial phytohormone (bph<sup>-</sup>) mutants and compare them with non-mutated lines under axenic systems.

B. Collection, modification and characterization of grass rhizosphere  $\text{N}_2$ -fixing bacteria.

1. Determine whether liquid chromatography separation/detection techniques can be used as a reliable, non-destructive method for quantitating  $\text{N}_2$ -fixation by root associated organisms.

2. Evaluate a system based on photometric determination of N isotopes as a method for quantitating low levels of  $\text{N}_2$ -fixation by root associated organisms.

3. Further test effects of bacterially produced phytohormones for long-term effects on plant growth and yield.

4. Identify field isolates from rhizospheres with high nitrogenase activities.

5. Study processes by which nitrogenase is regulated in Klebsiella pneumoniae utilizing modern biochemical and genetic techniques.

(a) Isolate mutants that are blocked in the metabolism of  $\text{NO}_2^-$  to  $\text{NO}/\text{N}_2\text{O}$ ;  $\text{NO}$  to  $\text{N}_2\text{O}$  and  $\text{NO}_2^-$  to  $\text{NH}_4^+$ .

(b) Map the various genes involved and study the regulation of these genes.

(c) Characterize these mutations biochemically.

(d) Examine the effect of these mutations in the  $\text{NO}_2^-$  metabolic pathway on nitrogenase biosynthesis.

6. Study the role of bacterial produced phytohormones by measuring root responses in the presence of bacterial mutants in various media.

7. Separate plasmids of Azospirillum sp. by gel electrophoresis to characterize the bacterial strains.

8. Microbiological analysis of plant experiments to determine total number of bacteria, including total numbers of  $\text{N}_2$ -fixing and denitrifying bacteria.

9. Act as curator for the collection, purification, maintenance (liquid nitrogen) and distribution of associative  $\text{N}_2$ -fixing bacteria in an effort to establish standardized strains for other investigators (national and foreign).

C. Examination of bacterial strain and plant genotype specificity.

1. Quantitate  $\text{N}_2$  fixation and N uptake efficiency of sorghum and/or wheat genotypes by using  $^{15}\text{N}$  (on low N medium).

2. Mass production of inoculum strains for greenhouse and/or field experiments.

3. Analyze a number of pearl millet genotypes for carbohydrate loss, bacterial infection, and nitrogenase activity.

4. Continue development and evaluation of simple systems for large-scale testing of interactions between plant and root-associated bacterial genotypes.

5. Characterize the effects of the host plant and host modifying factors on acetylene reduction of different grass genotypes.

6. Follow-up study of plant genotype-bacteria associations in most active soil cores collected from various counties in Florida. (Moisture, OM, micronutrients, and isolations).

7. Discuss bacterial strain and plant genotype specificity work in India (ICRISAT), Mali, and Australia, and set up cooperative experiments where warranted.

8. Study the role of non-soluble plant substrates in associative  $N_2$ -fixation, especially cellulose. Attempt to isolate and characterize cellulose using  $N_2$ -fixing systems.

9. Look for bacteria that will interact with roots to give special plant responses such as root hair curling, root lysis, root stimulation or modifications.

D. Define ecological factors that limit or enhance plant-bacteria interactions.

1. Determine (to the extent possible) effects of the variables itemized below on population changes among nitrifiers, denitrifiers, and  $N_2$ -fixers in a partially defined model soil-root system. Variables: plant genotypes, levels of N in various forms, temperature, soil aeration, levels of various energy substrates.

2. Study microbial population dynamics in an Azospirillum-inoculated sorghum rhizosphere.

3. Define the levels of micronutrients (in soil using pot experiments) which may limit AR. (Zn, Mn, Mo, Fe, Mg, etc.).

4. Establish inoculation procedures that may enhance grass-bacteria associations and monitor persistence of inoculated bacteria.

5. Study hydrolytic enzymes produced by bacteria.

E. Quantification of  $CO_2$  and  $N_2$  fixation.

1. Investigate root exudates in relation to associated,  $N_2$ -fixing, soil microorganisms.

(a) Determine the amount of "usable" carbon made available to root microorganisms by selected agronomic crops.

(b) Analyze the types of reduced carbon compounds found in the exudate.

(c) Measure root and microorganism metabolism.

(d) Study the biological factors that affect  $N_2$ -fixation in the rhizosphere.

2. Study the relationships of nitrogen metabolism and possible cycling between crop plants and soil microorganisms.

(a) Determine effects of soil nitrogen on nitrogen metabolism relationships and nitrogen fixation.

(b) Measure the amount of nitrogen lost from the plant into the rhizosphere.

(c) Determine the rate of nitrogen transferred from  $N_2$ -fixing bacteria to crop plants using  $^{15}N$ , and determine whether any of the fixed nitrogen is transferred to the plant cells, by  $^{15}NH_4Cl$  dilution experiments.

3. Use  $^{14}CO_2$  and  $^{15}N_2$  combined labelling to determine, in a model associative system, their transfer between plants and bacteria.

F. Documentation of  $N_2$ -fixing bacteria association using plant tissue techniques.

1. Use defined plant callus-bacteria cultures to examine details of colonization and infection.

2. Establish suspension culture systems with pearl millet.

(a) Determine age of culture and responsiveness to nitrogenase activity.

(b) Determine substances that go from the plant to the bacteria in an associative system.