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BY

THE UNIVERSITY OF FLORIDA

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I. INTRODUCTION

This document reports the research accomplished in 1979, and in the first four months of 1980 for the Contract AID/ta-C-1376 Mod. #3, entitled "Nitrogen Fixation by Associative Grass Bacteria Systems." The outline of "Article 1 - Statement of Work" of the contract has been used as a guide in preparing this report. The following interdisciplinary team members have cooperated in the research and have contributed the information for this report:

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Research from the following graduate students and postdoctoral fellows is also included in this report:

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* Dr. Albrecht joined the University of Florida faculty in Jan. 1979 and has contributed extensively to the project objectives.

**Dr. Tyler suffered a stroke while attending N_2-Fixation meetings in Sussex, England in September 1979. He is recuperating at his home, and his 20% participation in the project has been largely shifted to Mr. J. R. Milam with assistance from Drs. Smith, Schank and Albrecht.

II. RESEARCH REPORTS

A. Establishment of axenic plant-bacteria systems.

The development of axenic plant-bacteria systems will permit the establishment of associations under chemically, physically and biologically controlled conditions. Glass lysimeters have been fabricated to accomodate mature tropical grass plants. These lysimeters are now being modified to permit increased flexibility, greater capacity for both root and leaf systems and a subirrigation system. Experiments performed during the past year in these systems, using Sorghum vulgare inoculated with either Azotobacter vinelandii, Azospirillum brasilense or Klebsiella pneumonae, and compared to gnotobiotic controls, showed that plant growth rates were invariably increased when any bacterial species were present. The increased growth rate was found in both the roots and shoots.

Azospirillum brasilense strain 13tSR2 (a doubly-marked antibiotic resistant mutant of strain 13t) was studied for persistence in these chambers. A bacterial population can be maintained for as long as 6 months, but the numbers of bacteria remain low, regardless of quantity of plant roots present, addition of carbohydrates or the moisture level, within reasonable limits, maintained in the chambers.

In another series of experiments Cynodon doctylon (bermuda grass), Digitaria decumbens (digit grass), Pennisetum americanum (pearl millet), or Sorghum species were grown in either nutrient solutions or soil. Either A. brasilense, A. vinelandii or K. pneumonae were added as inoculants. The bacteria normally produced phytohormone-like materials and auxin, cytokinin and gibberellin-like material have been isolated and identified. Inoculation usually produced root branching and root hair proliferation and in some, but not all, cases a stimulation of plant growth. Only the gibberellin was directly associated with stimulation of plant growth. The magnitude of the beneficial stimulation varied with plant genotype, plant

age and environmental conditions. Growth of young plants was suppressed by inoculation with bacteria that produced auxin abundantly. Inoculation was most effective, as a stimulus, when plants were grown with moderate nitrogen levels and otherwise adequate nutrient regimes. (See Sections B-3 and C-2 for additional reports on bacterial produced phytohormones.)

A nif$^-$ mutant (a strain unable to produce nitrogenase) has recently become available (see Section B-3 below). The effect of inoculation with this strain will be evaluated with the axenic chambers. Experiments are in progress to determine the ability of this mutant to produce phytohormones.

B. Collection and characterization of grass rhizosphere N_2-fixing bacteria.

It is important to isolate N_2-fixing bacteria capable of associating with plant roots. Isolation efforts have concentrated on Florida's natural grasslands, cultivated and non-cultivated soils. The investigation of the physiology and molecular biology of these N_2-fixing bacteria is also important, because these studies may provide valuable insights into plant bacterial associations or may produce new useful strains of N_2-fixing bacteria.

1. Isolation of N_2-fixing bacteria.

Acetylene reduction (AR) evaluations of field cores indicated that nitrogenase activity in uninoculated plots was as high, on the average, as in inoculated plots. In some of the non-inoculated cores, reasonably high rates (over 500 $nM \cdot core^{-1} \cdot hr^{-1}$) were observed. Nitrogen-fixing bacteria were isolated from roots found in the most active cores.

An isolation technique was selected without an enrichment step in order to avoid the drastic shifting of natural N_2-fixing bacterial populations by artificial media. This method consisted of selecting very active (high AR) cores, grinding 2 g of root tissue and some rhizosphere soil with a mortar and pestle in water containing a trace of detergent, filtering through a 5 micron filter, diluting and plating on various bacterial growth

media (with and without nitrogen). Nitrogen free plates were incubated in a low O_2 atmosphere (2% O_2). Plates containing nitrogen were incubated in air. Random individual colonies were picked and inoculated in nitrogen-free semisolid deeps containing various carbon sources. Acetylene reduction assays were conducted when bacterial growth occurred, and AR positive isolates were retained.

The plating media was either trypticase soy agar (TSA) or nitrogen-free media salts (Döbereiner's) with one of the following carbon sources: sucrose (SR) at 10g/1; succinate (SNF) at 10g/1 or mannitol (MNF) at 10g/1 or a combination of all three ("3C Media") at 5g/1 each. Buffering capacity was increased by using 5 times the normal phosphate concentration. Semisolid agar deeps for AR's were all nitrogen free and contained one or all of the carbon sources. Acetylene reduction was done on cultures grown in deeps of the same medium as origninally isolated except those isolated on TSA were assayed in the 3C medium.

Results suggest that high yields of N_2-fixing bacteria can be isolated from Florida soils without an artificial enrichment step. Presumably the plant root provided some enrichment. Predictably, different plating media gave different results. The highest proportions of N_2-fixers (above 50%) were isolated from either nitrogen-free sucrose or manitol media. When isolates were selected on the basis of large colony size the frequency of N_2-fixing bacteria isolated could be as large as 84%. A higher percentage of N_2-fixing bacteria was always found when colonies were selected for size rather than using a random colony selection, regardless of the plating medium used. Nitrogen free "3C" medium became the plating medium of choice because it appeared to allow development of a broad spectrum of N_2-fixing bacteria with the least selection pressure and presented a more realistic estimate of the total population of N_2-fixing bacteria in the soil cores.

In most cores a low frequency of N_2-fixers were isolated from the nitrogen free succinate plates, indicating that few N_2-fixing bacteria in Florida soils are able to utilize succinate, and possibly most Krebs cycle acids, as an energy source. This low frequency of N_2-fixers that could metabolize succinate was also found in cores from areas previously inoculated with A. brasilense.

The isolated bacteria are being characterized, identified, and evaluated for their ability to associate with plant roots (see Section C below). Some N_2-fixing bacteria were isolated using an enrichment technique similar to Döbereiner's. Also several bacteria were isolated from the rhizosphere of field grown S. vulgare plants located 12 miles west of Gainesville. These included both N_2-fixing and non N_2-fixing bacteria. These soil bacteria are being identified and will be used in competition studies. A statewide search, county by county, is being initiated to obtain additional N_2-fixing bacteria. This search, using county agents, is being standardized and a methodology has been developed for the agents to use in sending samples to the University of Florida.

2. Microbial physiology.

An oxygen and pH controller has been developed which will allow the study of growth characteristics and N_2-fixing capabilities of selected rhizosphere bacteria under closely controlled conditions. Most of the initial work has been done on A. brasilense. The observations of other workers on generation time and maximum AR rates of this bacteria have been confirmed. Now other N_2-fixing isolates will be examined to determine growth rates and nitrogen-fixing capabilities with varying substrates, partial pressures of oxygen, and pH. The goal here is to be able to pick the best isolates for field experiments.

Electron transport components and TCA cycle dehydrogenases have also been investigated. Ammonium grown and N_2-fixing cells have been broken in a French press and membrane and soluble fractions separated by ultracentrifugation. Reduced-oxydized spectra have been run on each fraction. Both cytochromes B and C have been located in the fractions along with a cytochrome oxidase and possibly a flavoprotein. Physiological and artificial electron donors are being used to reduce the system. Electron trasnport inhibitors are also being used to better define the system.

The membrane and soluble fractions are also being assayed for TCA cycle dehydrogenases (isocitrate, 2-ketoglutarate, succinate, malate). The assays have shown an expectedly high activity for these enzymes, with a usual distribution in soluble and nonsoluble fractions.

Investigation of arabinose metabolism has shown that A. brasilense catalyzes an uncommon degradative pathway. The arabinose is first oxidized to arabonate by an NAD(P)$^-$ dependent dehydrogenase, then taken through two dehydratase enzyme steps to 2-ketoglutarate semialdehyde, and finally oxidized to 2-ketoglutarate by an NAD dependent dehydrogenase. The first two enzymes in this pathway are being purified and characterized. This pathway has also been found in some Pseudomonads.

Weak pectolytic activity was deomonstrated by several of the bacterial strains, and in pure cultures some grew well without organic nitrogen and with pectin as their only carbon source. Electron micrographic examination indicated that some bacterial growth occurred in the pectin-rich middle lamellar tissue of roots. However, most proliferation of the inoculant organisms was inside or adjacent to lysed epidermal or root hair cells, where cytoplasmic constituents were available as growth substrates.

3. Microbial genetics.

Early attempts to develop the bacterial gene transfer procedures of transduction and conjugation respectively, were unsuccessful because no viruses were found, or of limited value because of low transfer frequencies. Although geneticists in India reported low levels of transformation frequency, we have found that a new method for transforming Escherichia coli will yield a very high frequency of transformation in A. brasilense. Further, the fraction consisting of highly purified plasmid DNA will transform the biosynthetic genes for several amino acids, and a purine as well as the genes for two ribosomal proteins (as resistance to streptomycin and spectinomycin) and the B subunit of RNA polymerase (as resistance to rifampicin). Separation of the plasmids by gel electrophoresis, as described below, is being carried out so as to determine which plasmids carry these genes.

Previously we reported that electron microscopy of plasmid DNA of A. brasilense revealed that it consists of many sizes of plasmids and that each strain carried a different array of plasmid sizes. In an effort to confirm this observation in other ways and to separate the plasmid sizes for characterization, methods were developed to separate the plasmids by gel electrophoresis. This proved difficult because of the very large size of some of the plasmids but repeatable separation is now being accomplished. The results confirm the existance of several plasmids in each strain and the difference in plasmid make up of each strain. Such differences are of value for strain identification.

Strains have been isolated from cultures grown in curing conditions that have lost the ability to carry out three different functions - a property expected of a strain that had lost a plasmid. Gel electrophoresis of these isolates did indeed show an alteration in the plasmid banding pattern and the loss of a plasmid. One of the latter isolates was grown in curing conditions

again and isolates were examined for altered properties. Among 250 such colonies, one was found unable to grow on nitrogen free agar or to reduce acetylene - it is not an auxotroph. This culture is being evaluated for grass stimulation in field trials.

Genetic studies on A. brasilense were designed to gather information on the possible mechanisms by which these bacteria stimulated the growth of grasses. The approach was to isolate mutants unable to fix nitrogen (nif^-), mutants unable to synthesize the bacterial produced phytohormones (bpp^-) or mutants unable to associate with grass roots.

Early attempts to induce nif^- mutants with common mutagens was unsuccessful. Even nitrosoguanidine, which was used to make nif^- mutants in several other nitrogen fixers, did not induce stable nif^- strains. We have found, as have the group at the Pasteur Institute, that unstable strains unable to reduce acetylene are found as a rare event but soon revert to wild type.

A strong enrichment technique was devised using a chemostat, to control pH and O_2 levels, together with a procedure similar to the penicillin enrichment devised to isolate auxotrophs in E. coli. When the culture exhausted the limited NH_4^+ in the culture, the parental types began fixing nitrogen (as determined by AR) cycloserine was added to kill the nif^+ cells without affecting any nif^- mutants present. Two cycles of this enrichment failed to yield nif^- mutants after nitrosoguaindine treatment. A stable nif^- mutant was isolated however as a result of the molecular biology studies described above (see Section A for pending experiments with the nif^- mutant).

Azospirillum brasilense metabolizes tryptophan into indole acetic acid (IAA). We have developed an biological assay for the production of IAA to use in the screening of survivors of nitrosoguanidine. Pennisetum americanum seedlings (sterile) placed on the surface of agar containing tryptophan and A. brasilense develop a stubby root of ¼ - ½" length. Omission of either or both (tryptophan or bacteria) allows normal root development. Indole acetic acid

mutants of A. brasilense would not affect seedling roots in this manner. We are currently screening mutagenized cells for this property. Some plant mutants are available that require gibberellin or cytokinin for development. These are being collected for evaluation as screening agents for detection of mutants (see Sections A and C-2 for description of other experiments with bpp).

The role of roots in attracting or supplying nutrients to N_2-fixing bacteria has been investigated using multiple auxotrophs of A. brasilense. A strain requiring methionine, leucine, tryptophan and adenine would not grow in or on Döbereiner's salts agar with NH_4^+ as the sole nitrogen source. Growth of this auxotrophic strain was present along roots of P. americanum seedlings grown in the same agar, suggesting that the required three amino acids and purine were supplied in the root exudate.

C. Examination of bacterial strain-plant genotype specificity.

There is evidence for varietal differences in the response of plant genotypes to inoculation with N_2-fixing bacteria. This type of response offers opportunities to increase any N_2-fixation or plant yield increases afforded by the association through plant breeding and selection. The majority of work in this area has been in the field, although experiments in both the laboratory and greenhouse have been carried out in support of the field work.

1. Field work.

Evaluations to assess the contribution of plant roots to associative N_2-fixation (measured by AR) were made on matched field cores with and without plant roots. Polyvinyl chloride (PVC) tubes 3" diameter and 12" long were installed between plants in inoculation nurseries of Zea mays (corn), P. americanum (pearl millet) and Panicum maximum (guinea grass) soon after planting and inoculation. Tubes 6" long were found to be inadequate as they soon became colonized by roots growing under the tube then upward into the core soil. Tubes were maintained weed free and received similar

treatment to the rest of the plot. However, conditions within the PVC tubes were not identical to the root colonized outside soil as soil moisture and fertility were higher inside. These differences were not believed to interfere with the nitrogenase evaluations. Higher moisture would tend to increase AR, while the NO_3^- or NH_3^+ levels were not believed high enough to interfere.

Table 1 (following page) shows the results of the experiments described above. Acetylene reduction of 53 cores without roots ranged from 0 to 51 $nM \cdot core^{-1} \cdot hr^{-1}$ with a mean of 14 $nM \cdot core^{-1} \cdot hr^{-1}$ while that of 64 cores containing roots (taken near the core without roots) ranged from 36 to 432 with a mean of 164 $nM \cdot core^{-1} \cdot hr^{-1}$. The cores with roots had over 10 times more activity than rootless cores. The level of activity without roots could be considered as an insignificant level of N_2-fixation.

Panicum virgatum L. (switchgrass), a grass that has been observed to grow much better than expected compared to other grass species on areas of low nitrogen fertility, was studied to evaluate its N_2-fixing potential, nitrogen use efficiency and genetic variation for the above two characteristics.

In the field, over a two-year period at two locations, significant variations were observed for yield, percent nitrogen, nitrogen fertilizer use efficiency and nitrogen fixation estimated by the total nitrogen difference method. Responses were infuenced by maternal line origin, location, plant establishment and applied nitrogen fertilizer level. Broad-sense heritabilities for the field measured parameters were generally low, indicating that breeding progress will be slow. In the greenhouse, four populations of *P. virgatum* showed significant differences in yield, nitrogen fertilizer use efficiency and nitrogen fixation estimated by total nitrogen difference and by ^{15}N dilution. Responses were influenced by maternal line origin, selected population and plant establishment.

TABLE 1. Mean acetylene reduction rates of field plot cores containing and devoid of plant roots.

Nursery	Plant Species	Date	Mean AR $nM \cdot core^{-1} \cdot hr^{-1}$			
			Killed Inoculum		Live Inoculum	
			Roots	w/o Roots	Roots	w/o Roots
Agronomy Farm	Corn	8/21	36	0	118	0
	Millet	8/21	84	0	72	0
BRU	Corn	7/26	79	0	183	16
	Millet	7/26	128	0	143	0
	Millet	8/23	80	15	102	7
	GG*	8/23	182	7	182	22
	Millet	8/28	432	35	327	18
	Corn	8/28	233	18	415	36
	Millet	9/5	51	13	151	13
	Corn	9/5	126	51	164	13
Overall Average			143	14	186	13

Overall mean w/roots 164 (64 cores)
w/o roots 14 (53 cores)

* guinea grass.

Mean estimates of nitrogen fixation in the field by total nitrogen difference and AR cores were 20.8 and 6.5 kgN/ha, respectively, and the two estimates were positively correlated ($P>0.001$). Mean estimates of N_2-fixation in the greenhouse by total nitrogen difference and ^{15}N dilution were 2.4 and 9.5 kgN/ha, respectively, but in two combined harvests, the estimates were negatively correlated ($P=0.02$).

In all cases, %N in P. virgatum was lower than Paspalum notatum ('Pensacola' bahiagrass) which was included as a check species. Dry matter yields were much higher than in P. notatum. The lower percent nitrogen content of the P. virgatum could be a factor in its greater biomass production with limited nitrogen and explain the observations of better than expected growth in nitrogen deficient sites.

Field data suggest differences in the response of several plant genotypes to bacterial inoculation. Experiments have been done in both the greenhouse and laboratory to evaluate the effects on both plant and bacteria to inoculation and work has been done to develop a rapid, simple and reproducible method to screen large numbers of plant genotypes.

2. Greenhouse work.

Earlier it was reported that A. brasilense could produce phytonormones (bpp) in culture filtrates. To determine if this bacterium can produce bpp when living in associations with plants, greenhouse grown S. vulgare, P. americanum and Lycopersicon esculentum (tomato) plants were inoculated with A. brasilense strain 13tSR2. Plants were harvested one month after planting. Number of bacteria recovered from the soil and rhizosphere were determined and bpp were extracted from the soil and measured. Bacteria recovered from the soil ranged from 2.5 to 4.2×10^3 ·g soil^{-1} and 3×10^2 to 1.8×10^3·mg dry root^{-1}. Only gibberellic acid-like compounds could be detected by bioassays (lettuce hypocotyl test) from soil extracts. Although the amount of GA-like compounds in inoculated soil was 3 to 7 times greater than non-inoculated soil, the amount is very

small, about 0.3 to 0.7 $\mu g \cdot kg$ soil^{-1}.

Cytokinins and IAA-like substances could not be detected in the soil by either chromatogrphic methods or bioassays. The recovery of IAA, GA_3 and cytokinins (zeatin, zeatin riboside and kinetin) added to the control soil was very low, and recovery was reduced with increased incubation. This suggests that bpp produced in the soil may be higher than reported (see Sections A and B-3 for additional information on bpp). Reference 9 in the appendix presents published data on plant growth substances produced by A. brasilense.

3. Laboratory experiments.

Growth responses of two P. americanum genotypes were studied in vitro using soil in both test tubes and small plastic bags (Reference 6, Appendix). Inoculation with live bacterial cells caused root hair proliferation, however in this set of experiments shoot development was reduced and both genotypes exhibited lower total nitrogen contents. The limited volume for root development, coupled with low light intensity and lower than optimum temperatures, did not permit adequate plant development to assess plant bacterial associations.

Plant "growth pouches" were also used in replicated inoculation experiments. Pennisetum americanum was inoculated with A. brasilense in dilutions of 10^8 10^7, 10^6 and 10^5 cells/ml. Root elongation was measured and acetylene reduction (AR) assays performed. This highly aerated system gave excellent root growth, but very low AR values. Experiments in "growth pouches" plastic bags and test tubes indicate that improvements in these systems are necessary for screening grass-bacteria associations.

An interesting methodology for studying the bacterization of roots was presented at the 1979 American Society of Agronomy meetings (Gilmore, Thorne,

Beck and Gilmore, ASA Abstracts 1979). This methodology was employed to test different substrates as rooting media. It was found that the reported substrate favored bacterial growth, at the expense of "normal" root development. Carbon and nitrogen free Fahraeus agar offered the optimal rooting medium for screening associations. Reproducible results have been obtained using this media. Data from three trials using P. americanum and an additional three trials using Z. mays (McNair #5084F) are presented in Reference 5 (Appendix). Bacterial colonization of the roots was clearly observed and AR measured.

P. americanum, variety Gahi-3, and Z. mays, variety McNair 5084F, were screened using the Fahraeus test tube procedure as outlined above. Seven different N_2-fixing bacterial isolates growing in association with plants were compared with sterile plants. Mean AR of sterile plants (no bacteria) was 1.5 nanomoles per tube per day. Response of associations are presented in Table 2 (next page). Root growth (dry weight) at harvest was significantly lower (P=.05) with bacterial inoculation treatments.

Pennisetum americanum was screened against 26 different strains of N_2-fixing bacteria in replicated test tube experiments. Under the conditions of the experiment little or no plant stimulation, whether in increased dry matter production or increased root growth, was observed (see Section A for additional information on axenic systems).

Fluorescent antibody and electron microscopy studies.

Fluorescent antibody staining is a useful qualitative tool to study sites of N_2-fixing bacteria on plant roots and investigate the colonization of roots and soil by these microorganisms.

Over one thousand fluorescent antibody (FA) microscope slides were made and scored in 1979. These trials were primarily to monitor bacterial populations of A. brasilense in field nurseries at intervals during the year, and to observe test tube experiments.

TABLE 2 Mean acetylene reduction by various isolates, alone (in agar) and in association with plants.

Bacteri Strain	Associatio	n moles C_2H_2 reduced/tube/day
13t	+	325.5
	-	206.0
S28-10	+	18.0
	-	0.5
S141A	+	1377.0
	-	64.7
S145	+	1224.2
	-	48.5
S142	+	410.8
	-	0.7
S143	+	849.1
	-	0.0
85-9	+	567.6
	-	15.1

* + is with plants, - in agar only.

Beef Research Unit (BRU) trials included contact slides placed next to Z. mays, P. americanum and P. maximum plants six times during the year. Slides were placed in both plus (inoculated) and minus (autoclaved inoculum) plots at 3 fertility levels 0, 40 and 80 $kg \cdot ha^{-1} \cdot yr^{-1}$. Duplicate contact slides were also placed in a controlled "root-free" area near the root contact slides. Data indicated that minus plots do not remain free of A. brasilense, but gradually, during the year, more and more of the minus plots become infected.

The 1979 Z. mays nursery slides on the agronomy farm were placed and collected at five different times during the year. Plus and minus plots were monitored at 4 different N fertilizer levels viz. 0, 30, 60 and 120 $kg \cdot ha^{-1} \cdot yr^{-1}$. Again the trends were for minus (uninoculated) plots to gradually become infected with A. brasilense. The 120 fertilizer rate had fewer A. brasilense infested plots, overall, than the lower fertilizer rates.

Fertilizer Rate	0	30	60	120
No. of Infected Plots	6	6	4	3
Total plots studied	12	12	12	12

In the old 199 P. maximum nursery, where A. brasilense strain 13t has been inoculated every year for the past 5 years, monitoring commenced in July 1979 and ended in April 1980. Contact slides were placed in the minus and plus plots at 4 fertility levels, 0, 20, 40 and 80 $kg \cdot ha^{-1} \cdot yr^{-1}$. Contact slides were collected at six intervals.

Fertilizer Rate	0	20	40	80
No. of Infected Plots	34	34	39	25
Total plots studied	42	42	42	42

The trend of fewer infected plots at higher fertility levels was again observed. Also, minus plots tended to become infected as the year progressed.

Only 2 out of 16 were infected with A. brasilense on July 10th, but on August 7, 11 of 16 were infected and on December 5, 14 of 16 were infected. Roots from P. maximum plants collected at two week intervals and stained with FA, showed similar results to the FA contact slides.

The P. americanum variety trial of 1979 included 5 varieties: Tift 186; 23DA; Gahi-3; Bil 3-B; and 23DA x Bil 3-B. Only one nitrogen fertilizer level was used, 35 $kg \cdot N \cdot ha^{-1} \cdot yr^{-1}$. Contact slides were placed in the root zone of each variety in inoculated and uninoculated rows. The three sampling dates were separated by two week intervals. Tift 186, which had been found to be a non-responder to inoculation, (Reference 3, Appendix) did not support A. brasilense colonization as measured in these FA tests. Only 4 of 10 plots were infected, while in the other varieties, 7 of the 10 plots were infected. The trend of minus plots becoming infected as the year progressed was again observed, except in Tift 186.

Laboratory experiments to study grass-bacteria associations with soil in test tubes and in plastic bags have been reported (Reference 6, Appendix). The FA results showed that live bacteria persisted in the soil of all live inoculated tubes and bags. Azospirillum brasilense was more prevalent on root surfaces of P. americanum genotype Gahi-3 than Tift 186. At the low inoculation concentration (4×10^5 $cells \cdot tube^{-1}$) the FA root tests showed colonization by A. brasilense in only 1 out of 5 test tubes with Tift 186, but heavy root colonization occurred in all five replications at the higher (4×10^8 $cells \cdot tube^{-1}$) inoculum concentration. The autoclaved cells gradually diminished in fluorescence, and by the 30th day after inoculation were non-reactive. One other observation from the laboratory studies was that cells of A. brasilense line up along mycelial growth in the soil. The possibility that soil fungi may play an important role in keeping bacterial cells alive,

by various mechanisms, in the interim between inoculation and adequate root growth should be investigated.

Electron microscope studies were completed in 1979 (Reference 2, Appendix). Grass-bacteria associations were studied using specific antigen-antibody reactions and the peroxidase-antiperoxidase (PAP) method. Positive identification of PAP labelled A. brasilense was possible because of the heavy outlining of the cells with a dense deposit of osmium. Pleomorphic forms of A. brasilense were observed in the axenic root culture. Encapsulated forms were much larger than the vibrioid forms, and both types reacted with antiserum against either strain 13t or strain JM125A2. Small roots from the field usually had intact cortical regions with few microorganisms present, while large roots contained many microorganisms in the cortical region, and cells were often broken or sloughed. Azospirillum strains 13t and JM125A2 were immunologically identified, both intercellularly and intracellularly, in roots of field grown guinea grass.

The continued observation of A. brasilense strains in uninoculated root sections indicates that the organism can overwinter in Florida soils, and can migrate, by some mechanism, from inoculated to uninoculated plots.

Research using the scanning electron microscope (SEM) to observe bacterial cells specifically labelled with latex spheres has been done. However, all attempts at labelling A. brasilense with latex spheres have been unsuccessful and the study has been suspended.

D. Ecological factors that limit or enhance plant-bacteria interactions.

Several ecological factors probably determine the microbial composition of the rhizosphere. These factors are being investigated in the field and under more controlled conditions in both greenhouse and laboratory.

1. Field work.

Acetylene reduction analyses were conducted on 550 soil-plant cores taken over the growing season from five inoculated field nurseries. Correlations were made with these data, plant parameters (fresh and dry matter yields, percent dry matter, and nitrogen content), soil characteristics (soil moisture, pH, P, K, NO_3^-, and NH_4^+ content) inoculation and nitrogen fertilizer treatments.

No significant correlations were noted between acetylene reduction and the inoculation treatments. However, in a P. maximum experiment (ON 199) positive correlations were found between inoculation and total dry weight of the grass (r^2 = .017, P = 0.064) and percent dry matter (r^2 = 0.26, P = 0.004). Inoculated dry matter mean yield was 9763 kg/ha compared with 8895 kg/ha for the control. Dry matter percentage of inoculated forage was 30.5 whereas that of the control was 29.2. Soil phosphorous was found to be larger in the inoculated plots when compared to the controls, means of 0.187 ppm to 0.169 ppm, respectively. This unexpected finding is being investigated. A negative correlation was noted between inoculation treatment and forage nitrogen content (r^2 = -0.26, P = 0.017) in the P. americanum (millet) variety trial (MILVAR). Inoculated P. americanum had a mean nitrogen content of 0.42% and the control 0.46%. This dilution effect has been reported by other workers.

Acetylene reduction was correlated with pH in three of the five experiments "MILVAR" (r^2 = 0.31, P - 0.02), "ON 199" (r^2 = 0.45, P = 0.001), and a shading experiment "MILSHADE" (r^2 = 0.68, P = 0.001. The pH ranges in these nurseries were 5.7 to 6.8 in "MILVAR," 6.4 to 7.3 in "ON 199," and 6.1 to 7.2 for "MILSHADE." This suggests that pH may be an important factor affecting nitrogen fixation even when pH is within an acceptable range for crop and pasture production.

Moisture was correlated with AR activity in two experiments, "CORN" ($r^2 = 0.20$, $P = 0.038$) and "MILVAR" ($r^2 = 0.18$, $P = 0.08$). The moisture ranges in these two experiments were 2.8% to 8.5% (by weight) for "CORN" and 2.2% to 10.6% for "MILVAR."

In the BRU nursery, AR activity correlated negatively with soil phosphorous ($r^2 = -0.43$, $P = 0.009$; Range 3.4 to 34 ppm), NH_4^+ ($r^2 = -0.37$, $P = 0.028$; Range 0.8 to 5 ppm) and NO_3^- ($r^2 = -0.37$, $P = 0.027$; Range 0.01 to 18 ppm). Here, the NH_4^+ and NO_3^- were high enough in some cases to lower AR rates and presumably nitrogen fixation. The reason for the negative phosphorous correlation is unknown.

In three of the experiments, variable fertilizer nitrogen treatments were applied. These treatments significantly affected the mean AR rates (Table 3, following page). In every case some fertilizer nitrogen was needed for the highest AR activity but high rates of nitrogen fertilizer reduced AR activity. These data agree with previous experiments indicating some nitrogen fertilizer is necessary for maximum AR, and show that the best yield responses to inoculation occurred when small amounts of nitrogen fertilizer were applied.

In the "MILVAR" trials the varieties gave different AR rates, indicating plant genotype is an important criteria in associative N_2-fixation. *Zea mays* and *P. americanum* (Gahi-3) had similar AR rates while *P. maximum* gave lower rates.

Shading with 50 to 75 percent shade drastically reduced dry matter yields and percent dry matter; increased forage nitrogen content and soil moisture but did not change AR rates. However, the AR rates in this nursery were low and highly variable. Overall AR mean was 51 $nM \cdot core^{-1} \cdot hr^{-1}$.

TABLE 3. Mean acetylene reduction rates ($nM \cdot core^{-1} \cdot hr^{-1}$) at increasing nitrogen fertilization rates.

Nursery	Plant Species	Fertilizer N Treatment* 1	2	3	4
CORN	*Zea mays*	105 (0)	145 (20)	102 (60)	71 (120)
BRU	*Z. mays*, *Pennisetum americanum*, *Panicum maximum*	90 (0)	114 (40)	101 (80)	
ON 199	*Panicum maximum*	53 (0)	96 (20)	53 (40)	49 (80)
$\bar{x}$		83	118	85	60**

* N fertilization rates given in parenthesis in kg N/Ha.

**This mean is of only two values and is biased downward.

Azospirillum spp. have been reported to cause denitrification of nitrate. No signification reduction in soil nitrate due to inoculation was found in these studies. However, the overall soil nitrate means were higher in the control (0.70 ppm) than the inoculated cores (0.63 ppm).

2. Greenhouse studies.

Sorghum vulgare was grown in pots with non-sterile soil as the rooting media and inoculated with *A. brasilense* strain 13tSR2. The association was maintained at 3 soil moisture levels (50, 70 and 90% saturation) in the greenhouse. Low nitrogenase activity (C_2H_2 reduction) was found for all treatments using a soil core technique. The highest activity for inoculated plants was 22 n moles C_2H_4 formed $\cdot hr^{-1} \cdot g$ dry wt. root tissue^{-1}. This activity occurred in the 90% saturation treatment and peaked at 7 weeks after inoculation. Nitrogenase attivity in the 70% saturation treatment was about 1/10 - 1/20 the higher level and was not detectable at the lower level. Re-isolation of the bacteria from the rhizosphere using selective media showed poor survival in all treatments. Bacterial numbers, both 13tSR2 and the normal flora, were highest in the rhizosphere with the highest soil moisture, lowest in the low soil moisture treatment. Numbers of 13tSR2 quickly fell to nearly half the inoculum level, after correction for dilution in the soil. The population stabilized at a low level. Predictably plant yields were increased with increasing soil moisture but not increased by inoculation. Nitrogenase activity increased in the controls during the course of the experiment, reaching levels nearly 4 times the inoculated maximum. This suggests that local N_2-fixing microorganisms may have as great a potential for increasing nitrogen fixation as introduced inoculants.

Preliminary results corroborating earlier data, from model system studies,

indicate an increase of A. brasilense strain 13tSR2 numbers in a monoaxenic system, where soil is the support for S. vulgare roots, and a decrease in bacterial population when sand is used as the support. This data suggests that soil organic matter may provide a substantial portion of the bacterial energy source under some conditions. While the plants grown on sand appear to benefit from the association, as measured by increased dry weight yield, the association seemed detrimental to those plants grown in soil, where there was a decreased dry weight yield.

The rate of leakage of organic metabolites from root into the rhizosphere was affected much less by the inoculation treatments than by soil nutrient levels and other environmental conditions which controlled growth rate of the plants. Indigenous bacterial strains already in the soil metabolized much more of these compounds than did bacteria added to the rhizosphere as inoculants. Under all conditions tested, only small portions of the energy substrates found in the rhizosphere were utilized to support nitrogen fixation. Rates of fixation could be increased moderately by adding carbohydrates or other carbon sources to the soil or by maintaining the soil water content near saturation, but no conditions were discovered in which bacterial fixation served adequately as a source of nitrogen to support plant growth. Nitrogen fixed remained primarily in the bacterial biomass in the soil, and was not released for assimilation by plants in significant quantities.

3. Laboratory experiments.

The survival of A. brasilense strain 13tSR2 in the rhizosphere of S. vulgare was examined. Sorghum vulgare was grown in large glass tubes (25 x 3 cm) containing about 300 g of soil neutralized with 1.5% limestone. A previous study indicated that strain 13tSR2 could maintain very high populations in sterile neutralized peat (about 10^8 cells/g) for nearly two months provided

the temperature was kept between 25 to 35°C. The inoculum of strain 13tSR2, either in peat or water, was mixed into the top 1-2 cm of soil. No significant difference in the number of bacteria recovered from either the soil or the rhizosphere and root fraction was found when the two different methods of inoculation were compared. The number of bacteria in the soil (10 cm from the surface) or in the rhizosphere and root fraction (also 10 cm from the surface) declined rapidly during the first week. The decline in bacterial population was not as rapid during the remainder of the experiment. The number of surviving bacteria was dependant on inoculum size. After 25 days about 0.1% of the original inoculum remained in the soil. No strain 13tSR2 could be detected below 10 cm in either the soil, rhizosphere or root fractions after 25 days (see Section C-4 for other reports on A. brasilense persistance in soil).

E. Nitrogen and CO_2 fixation by plants and associated rhizosphere organisms.

Analyses of ^{15}N were completed on a field dilution study and data were calculated using "A" values according to Fried and Broeshart (Plant and Soil 43:707-711, 1975). The P. americanum plots inoculated with A. brasilense fixed 36 kg N/ha when compared with killed inoculum controls. Ammonium sulfate fertilizer labelled with ^{15}N (5% atom excess) was applied to the 0.28 M^2 plots at the rate of 160 kg $N \cdot ha^{-1}$ and then was immobilized by successive weekly sucrose additions. This incorporated the applied nitrogen in a microbial organic form which was resistant to plant uptake. Plant nitrogen was made up of 3.6% fertilizer nitrogen and 96.4% soil nitrogen. Only 4.6% of the fertilizer nitrogen was taken up. Even with this evidence of N_2-fixation due to inoculation these plots did not give a dry matter yield increase due to inoculation.

Plastic chambers have been developed that will enable plants and bacteria to be placed under a $^{14}CO_2$ or ^{15}N enriched environment for short (1-48 hr)

times. Plant-bacterial systems will be exposed to these isotopes and the fate of these isotopes will be determined.

F. Association of N_2-fixing bacteria with plant tissue culture material.

Tissue culture techniques offer a refined method, with controlled conditions, to study plant-bacterial associations. Saccharum officinarum (sugarcane) callus-A. brasilense association experiments have been successfully repeated. High AR activity has been maintained in these cultures for several months with periodic transfers (against controls without callus and controls without A. brasilense). The callus-A. brasilense association has been grown on media with a nitrogen gradient. High AR activity was found on nitrogen free media, and AR activity was observed on media with an initial nitrogen concentration as high as 30 mM nitrogen (see References 1, 2 & 12, Appendix).

Suspension cultures of P. americanum and P. maximum have been established. Good fixation by A. brasilense, under certain conditions, in suspension cultures of P. maximum and embryonic cultures of P. americanum has been achieved. A successful Nicotiana tabacum (tobacco)-A. brasilense association has also been accomplished.

Identification of components necessary for the association (sugars, organic acids, nitrogen containing constituents) are being investigated. ^{15}N experiments are planned for the near future.

APPENDIX

Manuscripts

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3. Bouton, J. H., Rex 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.
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6. Schank, S. C., Rex L. Smith and G. C. Weiser. 1980. Responses of two pearl millets grown in vitro after inoculation with Azospirillum brasilense. Submitted to Soil Crop Sci. Soc. Fla. Proc.
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11. Umali-Garcia, Mercedes, D. H. Hubbel, M. H. Gaskins and F. B. Dazzo. 1980. Association of Azospirillum with grass roots. Appl. Environ. Microbiol. 39:219-226.

12. Vasil, Vilma, I. K. Vasil, D. A. Zuberer and D. H. Hubbell. 1979. The biology of Azospirillum-sugarcane association I. Establishment of the association. Z. Pflanzenphysiol. 95:141-147.

13. Weiser, G. C. 1980. Evaluation of Panicum virgatum L. for nitrogen related agronomic characters. PhD dissertation, University of Florida.

14. Wood, Alvin and D. E. Duggan. 1980. Antibiotic resistant mutants to evaluate survival of Azospirillum in soil. Manuscript completed to be submitted to Appl. and Environ. Microbiol.

WORK PLANS AND CURRENT RESEARCH NEEDS

AID/ta-C-1376

1980-81

The University of Florida Group is continuing the statement of work described on project No. 931-1004 with the same major goal, overall purpose, and research objectives as previously outlined.

New work planned under the six research objectives (in addition to that previously outlined) is outlined below.

A. Establishment of axenic plant-bacteria systems.

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

2. Test and isolate nif$^-$ bacterial phytohormone mutants and compare them with non-mutated lines under axenic systems.

B. Collection and characterization of grass rhizosphere N_2-fixing bacteria.

1. Determine whether liquid chromatography separation/detection techniques can be used as a reliable, routine non-destructive method for quantitatively measuring 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 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. Give a brief look at the electron transport to N_2-ase in Azospirillum sp.

5. Evaluate polyacrylamide gel electrophoresis of total cell protein for identification of strains.

6. Fluorescent antibody screening of colonies on membrane filters used to collect Azospirillum from dilutions of inoculated soils.

7. Identify field isolates from core samples collected by agronomy.

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

   (a) The isolation of mutants that are blocked in the metabolism of NO_2^- to NO/N_2O; NO to N_2O and NO_2^- to NH_4^+.

   (b) Genetic mapping of the various genes and the regulation of these genes.

   (c) Characterize these mutations biochemically.

   (d) Examine the effect of these mutations in the NO_2^- metabolic pathway on nitrogenase biosynthesis.

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

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

C. Examination of bacterial strain and plant genotype specify.

1. Study nif⁻ mutants of Azospirillum for second year in the field.

2. Screen digitgrass and guineagrass genotypes using agar axenic system.

3. Analyze a number of pearl millet genotypes for carbohydrate loss, bacterial infection, 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 acetylene reduction on different grass genotypes at BRU field plots.

6. Observe plant genotype - bacteria assoications in soils cores collected from various counties in Florida.

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

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 nitrifyers, denitrifyers, and N_2-fixers in a partially defined model soil-root system. Variables: plant genotype, levels of N in various forms, temperature, soil aeration, levels of various energy substrates.

2. Examine and characterize microbial ecology populations of the phyllosphere of monocotyledons by several methods - isolation, scanning electron microscopy, acetylene reduction. Evaluation of spray inoculation with leaf isolates.

3. Put in a field experiment at the Chitedze Agricultural Research Station, Malawi, Using technology developed & work in conjunction with Dr. D. McCloud).

4. Study factors affecting nitrogenase activity in the field.

5. Analyze relationships between pH and nitrogenase activity.

6. Discuss cooperative involvement of CIAT in grass-bacterial investigations.

7. Establish inoculation procedures that may enhance grass-bacteria associations.

8. 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 micro-

organisms.

(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.

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

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

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

3. Form a mathematical model to describe the growth and carbon and nitrogen metabolism of N_2-fixing bacteria in the rhizosphere.

4. 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.

(a) Determine whether any of the fixed nitrogen is transferred to the plant cells, by using both $^{15}N_2$ incorporation experiments and $^{15}NH_4Cl$ dilution experiments.

(b) Analyze 'conditioned medium' for sugars, organic acids, and total nitrogen content to determine if there is any correlation between high nitrogenase activity and these factors.

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.

Expenditures (by Outputs) 5/01/79 to 4/30/80	MICRO A89	AGRON A90	SOIL A91	TOTAL
Overhead	$13,833	$ 26,000	0	39,833
A. Establishment of axenic plant-bacteria systems.	$16,064	8,576	0	24,640
B. Collection and characterization of grass rhizosphere N_2-fixing bacteria.	$20,653	$ 12,864	0	33,517
C. Examination of bacterial-strain genotype specificity.	0	$ 17,153	0	17,153
D. Ecological factors that limit or enhance plant-bacteria interactions.	$ 4,589	$ 37,731	0	42,320
E. Nitrogen and CO_2 fixation by plants and associated rhizosphere organisms.	0	$ 9,438	0	9,438
F. Association of N_2-fixing bacteria with plant tissue culture material.	$ 4,589	0	$1,849	6,438
Actually Spent	$59,728	$111,762	$1,849	$173,339

Annual report of AID owned Non-expendable Property in contractor's custody
(University of Florida)
as of 4-30-1980

AID/ta-C-1376-EQUIPMENT

Item	Date of Order	Date of Receipt	Price Paid	Location
Gas Chromatograph #1	3/25/77	5/09/77	$3,997.00 ✓	516 Bartram E
Oxygen Probe	3/27/78	4/17/78	$ 448.96	PSL
Top Load Balance	9/08/77	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 Condenser	11/13/77	12/08/77	$1,530.00 ✓	McC 3197
Double Unit Microferm. Fermentor	3/09/77	4/29/77	$8,688.00 ✓	516 Bartram E
Gas Chromatograph #2	1/08/80	2/06/80	$6,960.00 ✓	PSL
Incubators (2) Mangelsdorf	1/07/80 12/21/79	2/08/80 2/11/80	$ 641.35 $ 436.60	PSL McC 3197
Water Still	1/07/80	2/05/80	$ 825.00	McC 3197
Water Bath Shaker	3/12/80	6/10/80	$1,221.50 ✓	PSL
Laminar Flow Hood	11/14/79	12/10/79	$2,154.00 ✓	PSL
Refrigerator	10/22/79	10/24/79	$ 422.00	McC 1184
Hygrothermograph	3/12/80	4/02/80	$ 550.40	PSL
Convection Oven	1/07/80	2/12/80	$ 769.00	McC 3197
Balance	3/12/80	4/01/80	$1,819.50 ✓	McC 2164
Dissolved oxygen controller & recorder	3/12/80	4/01/80	$ 824.65	516 Bartram E
Single Pen recorder	3/12/80	4/01/80	$ 405.15	PSL
Shaking Water Bath	3/12/80	4/02/80	$1,221.50 ✓	120 ME

Note: According to revised general provision 17, Title to equipment purchased costing more than $1000 is vested in the U.S. Government. Title to equipment costing less than $1000 is vested in the contractor.

NITROGEN FIXATION BY ASSOCIATIVE GRASS-BACTERIA SYSTEMS

SUMMARY
MAJOR RESEARCH ACCOMPLISHMENTS
AID/ ta-C- 1376
University of Florida Group
July 1977- July 1980

Field Inoculation Experiments (R. Smith, S. Schank and K. Quesenberry)

In field research conducted over the past several years, pearl millet, guineagrass, buffelgrass, transvala digitgrass, sorghum, and corn were inoculated with *Azospirillum brasilense*. Pearl millet and guineagrass usually gave significantly higher dry matter yields than uninoculated plots, when some nitrogen fertilizer was also applied. N_2-fixation was studied using the acetylene reduction (AR) assay on both soil root cores and washed roots without pre-incubation. The effect of O_2 tension was studied in soil root cores, with estimated rates of 120, 74 and 25 g N $ha^{-1}day^{-1}$ obtained at O_2 levels of 2%, 6% and 20%, respectively. These data indicated that lower levels of O_2 availability are necessary for higher N_2-fixation rates.

Field trials have been conducted to evaluate effects of plant genotype, shading, mulching with crop residues, soil moisture, time of planting and nitrogen fertilizer on the *Azospirillum*-grass associations. Inoculated Gahi-3 produced 5% more dry matter than the uninoculated control, whereas the inbred parents gave no response to inoculation. Shading with 75 and 50% screens reduced dry matter yields by 54 and 40% respectively. Mulching increase yields of of Gahi-3 significantly over non-mulched. Inoculated non-mulched Gahi-3 produced 6% more dry matter than the non-mulched control. Acetylene reduction rates were generally low and variable. Additional acetylene reductions were conducted on 550 soil-plant cores. Correlation of yield, AR, % dry matter, N content, soil moisture, soil pH, P, K, NO_3 and NH_4 content, inoculation and nitrogen fertilizer treatment were done. Phosphorus was found to be higher in inoculated plots (0.187 ppm) than control plots (0.169 ppm). Acetylene reduction was correlated with soil pH in three of the five experiments, necessitating further research. Soil moisture was also correlated.

^{15}N dilution studies were conducted in field plots for two years, with dry matter yield and nitrogen content measured. Although no inoculation response was observed on yield or nitrogen content, the N_2-fixed on inoculated plots averaged 39.8 kg N/ha higher than the control plots.

Panicum virgatum L. (switchgrass), was observed for yield, percent nitrogen, nitrogen fertilizer use efficiency and nitrogen fixation estimated by the total nitrogen difference method, by acetylene reduction by 15N dilution. Mean estimates of N_2-fixation in the field by total nitrogen difference and by AR cores were 20.8 and 6.5 kg N/ha, respectively.

Growth Room Experiment (S. C. Schank)

In 1978 a cooperative CSIRO - University of Florida experiment was conducted at the Cunningham Laboratory, Brisbane, Australia. This growth room experiment involved inoculation of digitgrass in two Australian soils, using live and autoclaved *Azospirillum* isolates from Australia and Brazil. Dry matter yields of inoculated digitgrass on sandy soil was 23% higher than con-

trol plant yields. On clay soils the yield increase was 8.5% above control plant yields. Nitrogenase activity was variable, ranging from 4-8 g N ha^{-1} day^{-1}. Inoculation resulted in proliferation of roots, earlier maturity, higher percent dry matter and higher total N in the tops.

Plant Physiology Experiments (M. H. Gaskins)

When plants grown in a sterile environment were inoculated with Azospirillum spp., the bacterial growth rate was invariable proportional to the level of fixed nitrogen in the root solution. Various environmental and nutritional conditions were tested unsuccessfully in efforts to establish conditions under which the bacteria utilized atmospheric nitrogen. A tentative conclusion from these studies, to be further tested, is that when the bacteria must rely upon root "exudate" or "lysate" as their source of reduced carbon compounds, the supply of fixed nitrogen is not growth-limiting. This hypothesis implies of course that continuous N_2 fixation under these conditions is not to be expected, since much of the reduced carbon flow to the bacteria would be accompanied by a supply of fixed nitrogen sufficient to suppress N_2ase activity.

The movement of carbon as (^{14}C) from the atmosphere to the soil solution is a quite rapid process. Pulse labelling experiments, with sorghum plants growing in nutrient solutions, indicate from 5 to 10 percent of the carbon fixed by healthy plants can be recovered within 1-2 days in the combined nutrient solution and bacterial biomass of the rhizosphere. However, in experiments involving inoculation with both Azospirillum and Azotobacter, these bacteria capture relatively little of the carbon substrates which escape from the roots. This limited assimilation confirms chemical assay results which have indicated that simple sugars and organic acids constitute a small part of the total "root exudate."

Low levels of plant cell wall-hydrolyzing enzymes have been detected in cultures of symbiotic (Rhizobium) and associative (Azospirillum) bacteria. Assay methods have been developed for identifying the various enzymes present, using a highly sensitive agar plate assay method incorporating cell wall polysaccharides or derivatives as substrates for the enzymes. Studies are in progress to quantitate the enzymes present in diazotrophic bacteria, relate this information to the plant infection characteristics of the organisms, and determine best methods of establishing effective associative combinations under field conditions.

Polygalacturonic acid trans-eliminase (PATE) was produced by six Azospirillum strains studied. Characteristics are similar to those of PATE from other bacteria: activity is maximal at pH 8.0 and is stimulated by $CaCl_2$. Polygalacturonic acid in the medium stimulated PATE production by several but not all strains. PATE was excreted into the culture solution by the bacteria, but in all cases some remained bound to cell walls. In one strain, most remained cell wall-bound. PATE activity varied considerably among the strains of Azospirillum studied. It is possible that Azospirillum uses pectin in the rhizosphere as an energy source for nitrogen fixation, but this has not been determined.

Gibberellins (GA), cytokinins and indoleacetic acid were identified in culture extracts of various diazotrophic soil bacteria (genera Azospirillum, Azotobacter and Klebsiella). GA-cytokinin mixtures, added in low concentration to nutrient solutions, enhanced dry matter accumulation rates of small plants in several experiments. Hormonal stimulation definitely is a contributing element in the enhancement of plant growth following inoculation with free living diazotrophic bacteria.

Electron microscope studies (D. Hubbell, M. Umali-Garcia and S. Mathews)

The events of establishment of axenically grown Azospirillum - pearl millet associations were documented by electron microscopy. Young inoculated roots produced more mucigel, root hairs and lateral roots than did uninoculated controls. The bacteria adsorbed firmly to root hairs and un-differentiated epidermal cells. Addition of fixed nitrogen suppressed adsorption. Root exudate contained a non-dialyzable, protease-sensitive substance which bound to Azospirillum and promoted adsorption. Weak pectolytic activity was detected in pure cultures of the bacteria. Azospirillum was regularly observed in low numbers in intercellular spaces of the outer root cortex but not within living intact cells.

In a second study by electron microscopy, guineagrass roots were analyzed in natural field conditions using an immunological technique peroxidase-anti-peroxidase (PAP). This study supported the axenic work reported above, and showed that A. brasilense abundantly colonizes the mucigel and free spaces within the cortex. Breaks in root epidermis and root cortex layers were particularly favored sites for bacterial colonization. Small roots usually had intact cortical regions with few microorganisms while large roots contained many microorganisms.

In summary the electron microscopic studies have verified and documented the plant-bacteria association and shown exactly where most of the bacteria are present -- in the mucigel, on root surfaces or in the cortex (if cortical cells are broken).

Fluorescent Antibody Studies with Azospirillum (S. Schank, G. Weiser, R. Smith)

The fluorescent antibody (FA) technique allows us to analyze Azospirillum on cut surfaces, in root mucigel and cortex, or on "contact" glass slides placed in the soil. We conclude that FA is an excellent way of identifying and distinguishing A. brasilense strains, JM125A2 and 13t.

Microbiological Experiments (M. Tyler, J. Milam)

Azospirillum nov. gen. (60-70 isolates) has been characterized using approximately 100 physiological and morphological characteristics. The isolates tested are distinct from Spirillum and fix N_2 in the free-living state. Two species are defined; A. brasilense is strictly aerobic, whereas A. lipoferum is weakly fermentative and requires biotin. Strains analyzed were isolated from grass root samples from Africa, Latin America and the United States Strains differ in specific nitrogenase activity; efficiency of acetylene reduction approaches that of Azotobacter but at lower pO_2. Comparisons of mass culture (strains 13t and JM125A2) in Nitrogen containing media using buffer vs. substrate buffered counts showed best results from low phosphate media. Two azospirillum isolates were characterized from sugar cane. Growth curves of strain 13t were established using a chemostat.

(Microbiology and Cell Science) (D. Duggan)

Double marked strains have been used to evaluate factors affecting survival of Azospirillum in laboratory soil and in field plots. A mutant unable to fix nitrogen was isolated and evaluated for its ability to stimulate grass yields in inoculated plots. It was demonstrated that Azospirillum derives nutrients from plant root exudates. A multiple auxotroph unable to synthesize several amino acids and a purine was grown along seedling roots in Hoagland's salt mixture unsupplemented with carbohydrates, amino acids purines or pyrimidines. It was also demonstrated that all Azospirillum isolates examined

contain many plasmids differing in size. This was determined by contour measurements of isolated plasmid DNA examined by electron microscopy and by separation of plasmids by gel electrophoresis. Multiple auxotrophic mutants were induced as well as mutants resistant to several microbial inhibitors. A gene transfer method (transformation) was developed for genetic analysis. It was demonstrated that at least some of the biosynthetic genes are carried on plasmid DNA rather than on the chromosome as is true for all other bacteria examined. This was demonstrated by transforming to prototrophy the multiple auxotrophs mentioned above using DNA from purified plasmid DNA. Thus the genes coding for biosynthesis of several amino acids and for adenine were transformed by plasmid DNA as were the genes for two ribosomal proteins (as streptomycin and spectenomycin resistants) and the RNA polymerase B. subunit (as rifampicin resistance). A bioassay was developed for the biosynthesis from tryptophan and excretion of indole acetic acid (IAA) from Azospirillum and Klebsiella pneumonia. As little as 3 μg per ml. of tryptophan in a culture of either bacterium inhibited root growth of millet seedlings. No inhibition was observed in the absense of tryptophan or bacterium. This assay will be used to screen for IAA mutants which will subsequently be used to evaluate the role of this phytohormone in stimulation of grass yield by Azospirillum and Klebsialla.

Tissue Culture Experiments (I. K. Vasil Botany)

Sugarcane (Saccharum officinarum) callus-Azospirillum association experiments have been successfully repeated. High nitrogenase activity has been maintained in these cultures for several months with periodic transfers (against controls without callus and controls with Azospirillum brasilense). A successful tobacco (Nicotiana tabacum)-Azospirillum association has also been accomplished.

Suspension cultures of pearl millet (Pennisetum americanum), sugarcane, and guinea grass (Panicum maximum) have been established. In one set of experiments suspension cultures of embryogenic cells of pearl millet and guinea grass were transferred to N-free medium and incubated up to 14 days. Plant cells were removed at two day intervals from samples of the suspension cultures and the remaining 'conditioned medium' inoculated with Azospirillum brasilense (SP7). It was found that the conditioned medium supported high nitrogenase activity only for a short interval in this 14 day period. Conditioned medium obtained earlier or later than this period will support little or no activity.

In another experiment embryogenic cells of pearl millet were separated from bacterial cells (SP7) by a 0.45μm membrane filter. The plant cells were kept in suspension by a magnetic stirring rod while the SP7 was left stagnant to grow in a band just below the N-free medium surface. In the absence of plant cells no acetylene reduction was shown by SP7. In the presence of embryogenic pearl millet cells, high nitrogenase activity was maintained for several days.

USDA/SEA (M. Gaskins and S. Albrecht)

The fate of photosynthetically fixed ^{14}C has been followed in Sorghum bicolor seedlings grown in nutrient solution and in pots with field soil. Quantities of ^{14}C lost by roots were, within a broad range, proportional to levels of mineral nitrogen available to upport plant growth, and were increased by bacterial inoculation (various species and combinations). The total amount of ^{14}C lost by roots was greater than anticipated (up to 20% of the amount fixed), but these experiments indicate that most of the carbon lost to the soil solution cannot be used efficiently by the bacteria.