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NITROGEN FIXATION BY ASSOCIATIVE GRASS-BACTERIA SYSTEMS

BY

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INTRODUCTION

This is the final report of the research conducted on associative N_2 fixation by the interdepartmental University of Florida N_2 Fixation Group on Contract AID/ta 1376. This report consists of highlights, in abstract form, of the research listed according to outlined research objectives. The number following each abstract title is the publication number listed in the publication section. If more information is desired, copies of those publications may be obtained from the authors. Ninety seven publications and abstracts have been published under this contract.

Main charges of this contract were: 1) To evaluate the potential of associative N_2-fixation to supply nitrogen to grass crops and to reduce their requirement for fertilizer nitrogen, and 2) To develop the biology of the associative N_2-fixation phenomenon. Brazilian research indicated that agronomically significant amounts of nitrogen were being fixed by the free-living bacterium, Azospirillum brasilense. This stimulated early work to evaluate the effect of this bacterium on grass crop productivity and to verify the earlier research findings. Highlights of our work are listed below.

Inoculation experiments, mainly with Azospirillum, conducted early in this contract indicated that inoculation could significantly enhance yields of grasses. Continuation of these experiments pointed out that those responses were erratic and could be obtained only part of the time under our soil and climate conditions. Failure of inoculation to enhance N_2-fixation (measured by acetylene reduction) along with yield enhancement in early experiments, created real concern whether N_2-fixation was the main mechanism responsible for the yield responses. Later inoculation experiments showed significant yield responses, due to inoculation, while nitrogenase activity remained very low and non-responsive to inoculation. This is a convincing argument that N_2-fixation was probably not an important mechanism in causing yield enhancement.

Discovery that Azospirillum brasilense produced plant hormone-like substances in culture provided an alternative hypothesis because those substances often stimulate root growth which may be the cause of the yield stimulation. Both in vitro and pot inoculation experiments verified that Azospirillum caused root proliferation.

Early reports indicated Azospirillum infected grass roots and colonized living root cells. Our studies showed that the plant-bacterial interaction was mainly on the root surface. In some cases, bacteria would colonize lysed cells and voids between intact cells of the root cortex.

Numerous studies were made to define the soil and climatic factors affecting associative N_2-fixation and also the factors affecting introduced populations of N_2-fixing bacteria into the soil. Generally, we found that populations of N_2-fixing bacteria introduced into the soil decline rapidly in both pot and field evaluations. Low soil moisture and organic matter seemed to cause the most rapid population decline and only in axenic conditions did populations of N_2-fixers maintain themselves. Soil moisture appeared to be the most important factor affecting nitrogenase activity.

Highly active associative N_2-fixing sites were found at various locations in Florida and, in each case, they were moist sites. We concluded that to maintain high nitrogenase activity in our soils, high soil moisture is required. We also found that most associative N_2-fixation is plant-dependent; nitrogenase activity was 10 fold higher when living plant roots were present than when they were absent.

The physiology and genetics of Azospirillum were studied in detail and information obtained was used to reclassify Spirillum lipoferum to Azospirillum brasilense. Azospirillum were found to carry as many as eight megaplasmids of 40 to 1350 Mdal. Nif genes were located on one or more of those plasmids and auxotrophs could be transformed to prototrophs by plasmid DNA.

Our data show that under ideal, moist conditions for maximum nitrogenase activity, fixed nitrogen contributions of up 20 kg ha^{-1} yr^{-1} could be obtained from associative N_2-fixation. However, under Florida's usual cropping conditions little nitrogen was contributed to the system. ^{15}N studies showed that the nitrogen fixed by associative N_2 fixation was available and most of it was taken up by plants in the first season.

REPORTS

I. ESTABLISHMENT OF CONSISTENTLY REPRODUCIBLE, AXENIC PLANT-BACTERIA SYSTEMS.

a. *Digitaria* Genotypes Evaluated in Brazil for Nitrogenase Activity, Yield and IVOMD. (1)

Twenty-six digitgrass breeding lines were compared with four commercial cultivars of digitgrass and bermudagrass to determine possible dinitrogen fixation, nitrogen content, *In Vitro* organic matter digestibility (IVOMD), and yield. Commercial lines tested were 'Pangola', 'Transvala' and 'Slenderstem' digitgrass, and 'Coastcross-1' bermudagrass. Harvests were made every 28 days during 1974 at Km47, EMBRAPA, near Rio de Janeiro. During the year, accumulative dry matter (oven-dryed) of the lowest yielding line was 16,711 kg/ha/yr and the highest line yielded over 30,000 kg/ha/yr. Commercially available cultivars were intermediate in production, and not significantly different from each other at the 0.05 level. Seven of the breeding lines of digitgrass had yields surpassing Transvala. IVOMD data revealed that Transvala, and Slenderstem were significantly higher in digestibility than Coastcross-1. Pangola, with 61.9% IVOMD was not significantly different from Coastcross-1 (60.6% IVOMD). Four of the genetic lines were statistically superior in IVOMD with over 67% digestibililty. Nitrogenase activity by acetylene reduction, a measure of the dinitrogen fixed on roots was monitered, with rates of over 500 gN_2/ha/day possible under favorable conditions. Nitrogen content of soil and plant material harvested from each plot was also monitered during the year.

b. Nitrogen Fixation in Grasses Inoculated with *Spirillum lipoferum*. (3)

Field-grown pearl millet (*Pennisetum americanum*) and guinea grass (*Panicum maximum*), lightly fertilized and inoculated with *Spirillum lipoferum*, produced significantly higher yields of dry matter than did uninoculated controls. Up to 42 and 39 kilograms of nitrogen per hectare were replaced by inoculation for pearl millet and guinea grass, respectively. The data demonstrate that nitrogen fixation by these grass-*Spirillum* systems is efficient and is achieved at a reasonable energy cost to the plant.

c. Yield Increases of Tropical Grain and Forage Grasses after Inoculation with *Spirillum lipoferum* in Florida. (8)

During the spring and summer of 1975, Spirillum lipoferum was inoculated into maize, sorghum, pearl millet, Panicum maximum (guinea-grass), Digitaria decumbens (digit-grass) and other forage grass in field and glasshouse experiments. Results showed that: (1) corn, sorghum and millet stimulate nitrogenase activity; (2) inoculated pearl millet and guinea-grass yielded significantly more than uninoculated plots; (3) some nitrogen fertilizer is needed to enhance the biological N_2 fixation; (4) strains of bacteria similar to Spirillum are present in some Florida soils; (5) Spirillum can be identified in the root by a fluorescent anti-body labelling technique.

d. Yield Increases of Tropical Grasses After Inoculation with Spirillum lipoferum. (11)

For three years, higher dry matter yields have been obtained from grasses inoculated with tropical strains of the bacterium Spirillum lipoferum than uninoculated counterparts. During the first year, 1974, inoculated Panicum maximum (guinea grass) and Digitaria decumbens (digit grass) produced 80% and 61% more protein than uninoculated controls. In 1975, inoculation produced significantly higher protein and dry matter yields in Pennisetum americanum (pearl millet) and P. maximum. Projected yields using regression analysis of both pearl millet and guinea grass indicated indicated that about 40 kg $ha^{-1}yr^{-1}$ were replaced by inoculation. Although overall production was lower during 1976, yield responses due to inoculation were similar to those of 1975 in guinea grass.

Nitrogen fertilizer was required to enhance response to inoculation and higher rates of nitrogen fertilizer repressed response. Acetylene reduction verified that nitrogen fixation occurred, but did not correlate well with yield increases.

Immunofluorescent techniques were used to specifically identify and monitor Spirillum lipoferum populations in the soil. These data are being used to evaluate inoculation procedures.

Inoculation with Spirillum lipoferum has given yield increases for three consecutive years in Florida. This indicates: (1) response to inoculation can be repeated, (2) economically important amounts of nitrogen can be replaced by inoculation, (3) a high potential exists for developing grass-bacterial systems.

e. Effect of Inoculation with Azospirillum and Azotobacter on Turf-type Bermuda Genotypes. (12)

Eight selected bermudagrass (Cynodon dactylon L. Pers.) genotypes were inoculated with a mixed culture of the N_2-fixing bacteria, Azospirillum and Azotobacter, to determine plant growth response to inoculation under greenhouse conditions. Top, crown, and root growth and N content were measured. At low N fertility, top growth was increased 17% as a result of inoculation. Inoculation also resulted in increased total N accumulation in the top growth. Crown and root growth was not increased by inoculation and total dry weight of all plant parts was not significantly increased. The high fertility treatments gave significantly higher clipping yields, as expected, but no significant differences were observed between the inoculation treatments. No leaf color differences were observed as a result of inoculation. Bermudagrass genotypes were not significantly different in response to inoculation treatments.

f. The Biology of Azospirillum-Sugarcane Association. II. Ultrastructure. (15)

Tissue cultures of sugarcane support abundant growth of Azospirillum brasilense (SP 7). Visible after 1-2 weeks as a white or pink slime, this growth reaches 2×10^8 bacteria/mm^2 on the surface of callus. Growth of the bacterium is strictly extracellular in viable callus, and instances of intracellular growth result from rupture of the cell wall during senescence of callus tissue. A significant proportion of the bacterial population on callus is pleomorphic. Varying the nitrogen source in the nutrient medium caused no obvious effect on callus cell structure. The presence of the bacterium caused structural alterations in callus cells which did not inhibit overall growth of the bacterium. Growth of callus as tight groups of cells lacking intercellular spaces may be important for the establishment of a long-term association with Azospirillum. The interface of bacteria and live callus tissue is at the surface of tight cell groups. Browning of the surface cell layers of these groups in the presence of Azospirillum is not of the rapid nature known for hypersensitivity reactions. Rather, this production of phenolics appears to be due to the accumulation of extracellular bacterial metabolites. The ultrastructure of this and other callus reactions is described. As evidenced by organogenesis, the associated culture have remained viable for at least 18-20 months.

g. Response of Pearl Millet Inbreds and Hybrids to Inoculation with Azospirillum brasilense. (16)

Plant yield and acetylene reduction were measured on six hybrids and 15 inbreds (including the hybrid parents) of pearl millet, Pennisetum americanum (L.) K. Shum., after field inoculation with Azospirillum brasilense, Sp 13t

(reclassified from Spirillum lipoferum Beijerinck). During the first year of testing inoculation responses were investigated among all plant genotypes by measuring plant dry weight, % N, total N, and acetylene reduction activity. A smaller population of genotypes were tested a second year for repeatability of inoculum response. The first year, one hybrid, Tift 23DA x Tift 186 ('Gahi 3'), gave significantly higher plant dry weight and total N in response to inoculation. Inoculated Gahi 3 produced 31.7% more dry weight and 37.4% more total plant N when compared to autoclaved inoculum controls. No inbred was found to respond. Acetylene reduction values were low among all genotypes (range 0.54 nmole/g dry root x hour) and did not support yield effects or confirm inoculum treatments. Inoculation responses were found to be repeatable for all genotypes tested during the second year (positive or negative) but not of a statistically significant magnitude. Of the genotypes tested both years, a combined analysis of the 2-year yields revealed significant dry weight increases after inoculation of 19.2 and 14.0% with Gahi 3 and the inbred, Bil 3B, respectively. Again, acetylene reduction values did not explain any data. A N balance study was conducted in greenhouse containers on inoculated Gahi 3 plants in an attempt to repeat the yield differences observed in the field and to monitor inputs of N into the soil-plant system. No significant yield increase of N into the soil-plant system was found due to inoculation. Sampling error possibly negated an accurate measurement of all N.

h. Responses of Two Pearl Millets Grown in vitro After Inoculation with Azospirillum brasilense. (19)

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

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

i. The Biology of Azospirillum-sugarcane Association. I. Establishment of the Association. (22)

Callus cultures of tobacco, pearl millet, and centipede grass do not survive when inoculated with the free-living, nitrogen-fixing bacterium Azospirillum. The rapidly multiplying bacteria overgrow the callus, and death of the plant tissues ensues in about a week after inoculation. However, so long as the callus cells survive, the bacteria show nitrogenase activity if the callus is being grown on nutrient media totally or largely devoid of combined nitrogen. Under similar conditions, sugarcane callus cultures grow for periods of more than 18 months with regular sub-culture, and considerable amounts of nitrogenase activity can be demonstrated in such associated cultures. Plantlets have been regenerated from sugarcane callus tissues inoculated with Azospirillum.

j. Biology of Azospirillum-Sugarcane Association: Enhancement of Nitrogenase Activity. (24)

Azospirillum brasilense was reisolated from associations with callus tissue cultures of sugarcane and compared with stock cultures of the inoculated bacterium and related strains. Although the reisolate had a growth rate similar to stock cultures, it exhibited a severalfold increase in maximum specific activity of nitrogenase. The reisolate and the parent culture had similar ultrastructure. The general ultrastructure of Azospirillum is described. The bacterium was capsulated when grown on nitrogen free nutrient agar plates and on callus, but was not capsulated when growing in a subsurface zone in N-free semisolid nutrient agar, except rarely in aging cultures. Capsulation may be a protective mechanism against unfavorable pO_2 under dinitrogen-fixing conditions. Pleomorphism occurred in capsulated forms, and the ultrastructure of these forms is described.

k. Plant Yield and Nitrogen Content of Digitgrass in Response to Azospirillum Inoculation. (31)

Two Australian soils, a vertisol (pH 6.8, 0.299% N) and a sandy yellow podzol pH 6.2, 0.042% N), were used with digitgrass, Digitaria sp. X46-2 (PI 421785), in a growth room experiment. Comparisons were made between plants inoculated with live and autoclaved bacterial suspensions of Australian and Brazilian isolates of Azospirillum brasilense. Seedlings were inoculated on days 10 and 35. Acetylene-reducing activity was measured five times during the experiment. Dry matter yields of the digitgrass on the podzol (low N) inoculated with live bacteria were 23% higher than those of the controls. On the vertisol (high N), yield increases from inoculation with live bacteria were 8.5%. The higher-yielding plants had significantly lower percent nitrogen, but when total nitrogen of the tops was calculated, the inoculated plants had a higher

total N than did the controls (P = 0.04). Acetylene - reducing activity was variable in the experiment, ranging from 0.5 to 11.9 mol of C_2H_4 $core^{-1}$ day^{-1}. Live bacterial treatment induced a proliferation of roots, possible earlier maturity, higher percent dry matter, and a higher total N in the tops.

l. Bioenergetics of Asymbiotic Nitrogen Fixation. (33)

Rising energy costs have prompted renewed interest in biological nitrogen (N_2) fixation to reduce agricultural requirements for chemical nitrogen fertilizers. When the large energy requirement for reduction of gaseous nitrogen is supplied by biological conversion of solar energy, fossil fuels are spared for other uses. Nodulated legumes are the best known N_2-fixing plants in agriculture, but most of the nitrogen used for growth and maintenance of forests, grasslands, and aquatic habitats is provided by a variety of non-symbiotic algal and bacterial species. Recently these organisms have been studied intensively to identify those able to make a practical contribution to agriculture. We are studying non-symbiotic N_2-fixing bacteria in close associations with roots of crop plants, and believe they have great potential for agricultural use. As other N_2-fixing organisms they ultimately derive their energy from the sun as products of photosynthesis. The reduced carbon available from roots of the photosynthesizing host plants is a critical element in productivity of these root-associated organisms. Some studies indicate that plants can lose as much as 18-25% of their photosynthetic products from the roots, and that 5% or more of this can be used by associated microorganisms. The efficiency of N_2-fixation by some of these bacteria has been determined to be in the range of 10-50 mg cell nitrogen fixed/g carbon substrate utilized. This would be equivalent to 5-15 kg N/ha/yr^{-1} fixed by these organisms. In natural ecosystems this is a highly significant quantity. In primitive farming systems also this quantity of nitrogen is significant, but it is only a fraction of that required by intensive agriculture. We have generated a model from metabolic data suggesting that these systems could provide as much as 1,000 kg/ha/yr if all the available photosynthetic products were used for this purpose. A more reasonable projection, allowing for losses to competitive organisms and other demands, may be 50-100 kg/ha/-growing season. Factors which limited the production of nitrogen by associative systems in agriculture will be examined, and both positive and negative effects on yield will be projected.

m. Establishment of Associative N_2-Fixing Systems. (41)

Monoxenic root-bacterial associations were initiated in test tubes containing agar medium free of nitrogen and

carbohydrates. Seedlings of five sorghum genotypes were inoculated with four strains of bacteria. The degree of association was evaluated by visually rating the amount and location of bacterial growth and by assaying for acetylene reduction activity (ARA). Active ARA associations were established 83% of the time, in that tubes with plants had higher ARA than the no plant controls. Sterile controls remained sterile and had no ARA. The plants and intact agar-root systems were transferred axenically to mason jar assemblies containing autoclaved soil to see whether the active, already established associations would be maintained. Acetylene reduction assays were repeated three more times on the jar assemblies. The ARA rates in these pre-established systems were equivalent to two non-autoclaved, but highly active natural soils from Putnam and Madison Counties, which were included as standards because of their high ARA. Intact soil-plant cores from the mason jar assemblies were transplanted into a field with low ARA and were re-assayed for ARA two more times. Highest ARA was from the natural soil-plant transplants, with lower rates from all inoculated treatments. This suggests that the complex natural systems are better able to persist in competitive situations, than the inoculated associations established from selected N_2-fixing strains.

n. Status and Evaluation of Associative Grass-Bacteria N_2-fixing Systems in Florida. (45)

In the past 10 years, the associative grass-bacteria N_2-fixing system has been studied extensively in several research locations around the world. Initial claims of very high rates of biological N_2 fixation nearly equal to legumes have not been verified and do not generally have credibility in the scientific community. However, several research groups have shown that associative N_2-fixation does occur and that yields of grass crops may be increased by inoculation with N_2-fixing bacteria, mainly Azospirillum species. Maximum rates of fixation are usually 20-30 kg/ha (10-fold less than reported in 1975). We have found that populations of the inoculated bacteria generally declined rapidly following inoculation, particularly under natural soil conditions. Inoculation experiments conducted in Florida and New Mexico in 1982 verified these conclusions. There is some evidence that increased growth (dry matter yield) of grass crops after inoculation is due to the stimulatory effect of phytohormones produced by the bacteria.

o. Responses of Sorghum and Pennisetum Species to the N_2-Fixing Bacterium Azospirillum brasilense. (50)

Three field inoculation experiments, two in Florida and one in New Mexico, were conducted with Azospirillum

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

p. Adsorption and Infection of Grass Roots by Azospirillum brasilense SP 7. (59)

The association between grass roots and Azospirillum brasilense SP 7 was investigated by the Fahraeus slide technique using a nitrogen-free medium. Young inoculated roots of pearl millet and guinea grass had more mucigel, root hairs, and lateral roots than uninoculated controls. The bacteria were found within mucigel accumulating on the root cap and along the root axis. Azospirillum brasilense also adsorbed firmly to root hairs and undifferentiated epidermal cells. Some of the bacteria were oriented in a polar fashion on root hair cell walls. Supplementing the medium with fixed nitrogen ions suppressed the firm adsorption of the bacteria to the roots. Ultrastructural examination of the pearl millet rhizosphere revealed Azospirillum cells enclosed within a common slime layer that had a high affinity for electron-dense materials normally associated with the plant cell walls. Preliminary studies revealed thar root exudate from pearl millet contains substances which bind to Azospirillum and promote their firm adsorption to the root epidermal cells of this host. These substances are non-dialyzable and are inactivated by protease. Azospirillum brasilense enters

root tissue through void spaces created by profuse epithelial desquamation and lateral root emergence. The bacteria invade the middle lamella and show signs of hydrolysis which follow the contour of the bacterial cell walls. However, adjacent cortical cells retain active cytoplasm, and the bacteria were not found within living host cells. Pectolytic activity is detected in pure cultures of A. brasilense induced with purified pectin. These studies show that A. brasilense is invasive on these grass roots and that there is intimate but limited colonization of the middle lamella.

q. Factors Affecting Introduced Populations of N_2-Fixing Bacteria in the Rhizosphere. (71)

Several factors affecting the introduction and maintenance of large numbers of N_2-fixing soil bacteria into the rhizosphere of selected crop plants were examined. Plants were grown in a greenhouse. Several methods were utilized to introduce N_2-fixing bacteria into the rhizosphere. Nitrogenase activity was estimated by C_2H_2 reduction, organic material released by the roots determined by ^{14}C labelling and bacterial populations measured by dilution plate counts. Rates of nitrogenase activity and numbers of soil bacteria were positively correlated to soil moisture. In monoxenic systems, the numbers of N_2-fixing bacteria were found to be positively correlated with soil organic matter content. Soil respiration and subsequent plant dry matter yields were higher with inoculation. Also, the amount of ^{14}C label in the rhizosphere was greater in the inoculated plants. General trends suggest that low soil moisture and organic matter will limit associative nitrogen fixation and growth of soil N_2-fixing bacteria.

r. Evaluating Plant-bacterial Interactions Which Affect Rhizosphere Nitrogen Fixation. (84)

Several factors affecting the interactions of plants and N_2-fixing rhizosphere bacteria were examined. Plant-bacterial systems were grown in a greenhouse, and different methods were used to inoculate bacteria into the rhizosphere. Nitrogenase activity was determined by C_2H_2 reduction, and bacterial populations estimated by most probable number and dilution plate counts. The populations of inoculated N_2-fixing bacteria and nitrogenase activity were correlated positively with soil moisture and soil organic matter content. In axenic systems, growth of inoculated organisms was influenced by selected plant breeding lines. Introduced populations of N_2-fixing organisms increased in axenic systems, but their numbers were greatly reduced in non-sterile environments, suggesting that biotic factors are responsible for the reduction in number of N_2-fixing bacteria. Nitrogen fertilizer did not alter N_2-fixing

populations in axenic systems, although in sufficient quantity it did suppress nitrogenase activity. Low soil moisture or organic matter levels and microbial competition are much more significant than soil nitrogen levels in limiting populations of introducted N_2-fixing bacteria.

II. COLLECTION AND CHARACTERIZATION OF GRASS-RHIZOSPHERE INHABITING NITRON-FIXING BACTERIA.

a. Serological Studies of Spirillum lipoferum. (2)

Serological tests including tube agglutination, direct and indirect immunofluorescence, immunodiffusion, immunoelectrophoresis and capillary precipitin techniques were examined for possible use in the detection and identification of Spirillum lipoferum. High titer goat antiserum was prepared against S. lipoferum strain 13t. This antiserum was more reactive with 7 day old cells than with 3 day old cells (agglutination titers of 640 versus 160) of S. lipoferum 13t. The antiserum reacted with a wide variety of S. lipoferum strains. The antiserum possessed immunological specificity and bound to surface cross reactive antigens of Panicum maximum 285 grass roots which supports a N_2-fixing association with S. lipoferum. S. lipoferum could be distinguished from Azotobacter paspali by analysis of their soluble antigens using immunodiffusion and immunoelectrophoresis. These findings indicate that various serological techniques can aid in the detection and identification of S. lipoferum.

b. Fluorescent Antibody Technique to Identify Azospirillum brasilense Associated with Roots of Grasses. (18)

Bacteria associated with roots of grasses from Florida, Ecuador and Venezuela were isolated and their N_2-fixing ability was demonstrated by C_2H_2 reduction assay. The bacterial isolates have been classified as Azospirillum brasilense (formerly Spirillum lipoferum). These N_2-fixing isolates have been compared with several Brazilian strains. Fluorescent antibody (FA) techniques were used to assist identifying isolates of N_2-fixing bacteria from grass roots. Tests with antisera prepared against four strains of Azospirillum were used to define serological groups. Antigen-antibody specificity was demonstrated using both Azotobacter and Azospirillum antisera against known species of other soil microorganisms and numerous unidentified soil bacteria. Several applications of the FA technique are suggested to identify N_2-fixing bacteria associated with grass roots.

c. Isolation of Azospirillum from Diverse Geographic Regions. (21)

We have isolated Azospirillum (Spirillum lipoferum) from roots of grasses of several genera collected from a number of tropical and subtropical-temperate locations. Pure cultures were obtained from a small percentage of samples; no higher percentage was secured from tropical than from other grasses. Acetylene reduction and distinctive growth in N-free soft agar deeps were inadequate to identify this genus, although helpful in initial screening. Fluorescent antibody tests with antiserum against characterized strains were helpful. There is some evidence that this genus of bacteria may be favored in the rhizoplane.

d. Methods to Demonstrate the Megaplasmids (or Minichromosomes) in Azospirillum. (28)

With the exception of resistance to a limited number of antibotics, we have found Azospirillum very difficult to mutate. Exceptional repair does not seem to be involved, since the UV survival curve of Azospirillum is similar to that of Escherichia coli. One possible explanation for such a refractory response to mutagenesis is genetic redundancy, either as polyploidy or as multiple genome copies. Since a gene transfer system was unavailable during the early stages of this work we endeavored to compare the genetic molecules of Azospirillum to those of other widely studied microbes.

The first evidence of the multiplasmid nature of the genus was obtained from electron microscopy of DNA from Azospirillum. Kleinschmidt spreads of material from the lower band of dye bouyant density gradient preparations of gently lysed cells were examined. Each strain contained many different plasmids, some quite large, and each strain had a unique array of sizes. Since nothing could be learned of the plasmids seen by EM we turned to gel electrophoresis for resolution of the plasmids. Because the act of pipetting lysed cells into gels would shear large plasmids and alter mobility in gels, the Eckhardt technique was adopted. This procedure, which consists of lysing cells in the wells of vertical gels, was modified until large molecules of Azospirillum and several other genera could be resolved. We demonstrated the resolution of 7 or 8 DNA bands from each of 8 strains of Azospirillum. Estimates were made of the molecular weights of the DNA in the bands of one of the strains. Two methods (namely curing and transformation) were used to ask what genetic characters are carried on plasmids.

e. Polygalacturonic Acid Transeliminase Production of Azospirillum Species. (31)

Polygalacturonic acid transeliminase (PATE) was produced by all of six Azospirillum strains studied. Characteristics were similar to those of PATE from other bacteria: activity was maximal at pH 8.0 and was stimulated by $CaCl_2$. Polygalacturonic acid was used more readily than pectin as a substrate. Polygalacturonic acid in the medium stimualted PATE production by several but not all strains. In all cases some of the PATE produced in cultures remained bound to cell walls. In one strain, most remained cell wall bound. When nitrogen was supplied as amino acids rather than ammonium salts, the ratio of free to bound enzyme was increased. The strains studied varied considerably in response to nutrient amendments and in maximum PATE activity.

f. L-Arabinose Metabolism in Azospirillum brasilense. (34)

An oxidative pathway by which L-arabinose is converted to -ketoglutarate in crude extracts of Azospirillum brasilense has been. Specific activities of enzymes involved in the pathway were determined, and several pathway intermediates were identified.

g. Peroxidase-Antiperoxidase Labeling of Azospirillum brasilense in Field-Grown Pearl Millet. (39)

Bacterial cells of Azospirillum brasilense (Tarrand) from their natural habitat were labeled with peroxidase-antiperoxidase (PAP), identified, and observed using the transmission electron microscope.

Pure cultures of A. brasilense, axenically inoculated pearl millet root samples, and field-grown inoculated pearl millet root samples were embedded in Luft's araldite. Thin sections were treated using the immunological PAP method. Identification was possible because of the heavy outlining of the cells with a dense deposit of osmium.

Pleomorphic forms of A. brasilense were observed in axenic pearl millet root cultures. Encapsulated forms were larger than vibroid forms, and both types reacted with antiserum against the bacterial stain.

h. Partial Purification and Properties of an L-arabinose Dehydrogenase from Azospirillum brasilense. (40)

An L-arabino-aldose dehydrogenase responsible for the oxidation of L-arabinose to L-arabino-γ-lactone has been purified 59-fold from L-arabinose grown cells of Azospirillum brasilense. The dehydrogenase was found to be specific for substates with the L-arabino-configuration at carbons 2, 3, and 4. K_m values for L-arabinose of 75 and 140 μM were found with NADP and NAD as coenzymes, respectively. The enzyme had a pH optimum of 9.5 in

glycine buffer and was stable when heated to 55°C for 5 min. No enhancement of activity in the presence of any divalent cation or reducing agent tested was found. L-Arabinose dehydrogenase had a molecular weight of 175,000 as measured by the gel filtration technique.

i. Taxonomy of the Diazotroph of Tropical Grasses Called Spirillum lipoferum. (57)

Thirty strains of Spirillum lipoferum isolated by J. Dobereiner of Brazil and by us have been examined by an array of morphological, physiological and biochemical techniques. All isolates were recovered from roots or rhizospheres of Panicum, Zea, Pennisetum, Musa or other plants in Brazil, Ecuador, Florida or Venezuela, and all reduce acetylene to ethylene in pure culture.

All isolates are straight or slightly curved, Gram negative rods with poly-β-hydroxybutyrate inclusions and a dominant monopolar flagellum, with numerous smaller lateral flagella, which develop only at 30°C on agar medium. A few exhibit cellular shapes slightly different from the majority. There are two physiological groups, based on mode of respiration and several other reactions. Phenotypically, most of the strains are intermediate between described species of Spirillum and Pseudomonas. DNA base ratios substantiate this assessment.

j. New Features of Azospirillum Isolates and of an Antibiotic-Resistant Strain. (60)

Additional Azospirillum strains have been isolated from grass roots and soil collected in South Africa and Florida. These strains, and cultures obtained from other workers in Puerto Rico, Canada, and South Africa, have phenotypic and genotypic features of A. brasilense and A. lipoferum with minor aberrations.

Isolates M82 and Br17, from Brazil, have been shown to be mixtures. When separated by colonizal selection, each mixture yields phenotypes of A. brasilense and A. lipoferum.

The intracellular granules of one strain have been identified as poly-β-hydroxybutyrate by isolation and chemical identity. Pigments of two highly colored strains have been shown to contain both polar and non-polar xanthophyll-like and carotenoid pigments by chromatographic separation followed by spectral absorption identity. Cytochrome C ferrohemochrome has been identified in alkaline pyridine extracts from cells previously extracted for carotenoid pigments.

A mutant of strain 13t, resistant to streptomycin and rifampicin, possesses the features of the wild-type strain including nitrogenase. It grows well in a substrate-buffered, N-containing medium with equimolar concentrations of succinate and fructose and a low level of phosphate. The concurrent metabolism of the two C sources prevents the increase of alkalinity above pH 7.5 which occurs in succinate broth even with high phosphate. Preliminary results with wild-type strain 13t suggest that active acetylene reduction is prolonged in a substrate-buffered, N-free medium.
cells.

k. Transformation of Azospirillum: Biosynthetic Genes in Plasmid DNA. (68)

Azospirillum species have been found to contain up to 8 plasmids ranging from 60 to over 400 M.D. Auxotrophs were transformed to prototrophy at high frequency by plasmid DNA separated by dye bouyant density centrifugation. Resistance to rifampicin, streptomycin and spectinomycin were also transferred with this DNA. Plasmid species were separated by gel electrophoresis and supernatants from gel bands electrodialyzed or crushed were used for transformation. DNA from some of the separated bands was found to transform for biosynthetic markers. These findings suggest that Azospirillum may be a multilinkage group procaryote.

l. Multiple Large Plasmids of Azospirillum. (70)

Six strains of Azospirillum brasilense and two of A. lipoferum were found to possess as many as eight different sized plasmids ranging from 60 to well over 400 megadaltons. Isolation and separation of these very large plasmids was achieved by lysing cells in the slots of agarose gels, subjecting the lysate to electrophoresis at 2 mA for 6 hours, and then continuing electrophoresis at 30 mA for another 12-16 hours. Optimal plasmid recovery required growth in minimal medium and lysis at or below 10°C. Acridine orange (AO) treatment of one of the wild strains permitted isolation of mutants unable to grow on ethanol or butanol as sole carbon source; all such strains showed increased sensitivity to cadmium. All of the mutants were shown to have lost a common plasmid, but one of them appeared to have lost a second plasmid as well as portions of four others. AO treatment of the latter resulted in isolation of a mutant unable to reduce acetylene or grow on nitrogen-free media. The plasmid profile of this nif^- mutant appeared identical to that of its parent strain.

m. Genetic Studies With N_2-Fixing Enterobacter Strains From The Rhizospheres of Grasses. (74)

Enterobacteria form a substantial part of the microflora in the rhizosphere of grasses. Making them fix nitrogen seems a promising approach to save on nitrogen fertilizer. As a model system, Enterobacter cloacae from the rhizosphere of Festuca heterophylla is being studied. Onto such bacteria, the gene group of N_2-fixation from Klebsiella was transferred, using the composite self transmissible plasmid pRD1, and E. coli cells as donors. The plasmid with all genes could be stabilized in the recipient bacteria via integration into their chromosome. Stable hybrids were nitrate reductase negative, hence integration is in a gene essential for the production or function of this enzyme. The hybrids fix more N_2 than the donor cells. In pot cultures, they compete successfully with wild type bacteria. However, they do not utilize nitrate, hence do not compete with the host plants for nitrate fertilizer, added as starter. A method for obtaining such hybrids from other Enterobacteriaceae is being developed, which employs chlorate resistance as a means to select for nitrate reductase negative hybrid cells.

n. Nif Gene Location in the Megaplasmic Bacterium Azospirillum. (79)

Each isolate of the two species of Azospirillum has previously been found to carry a different array of 7 or 8 DNA molecules separable by gel electrophoresis. The sizes in one strain range from 40 Mdal up to a minimum estimate of 1350 Mdal and add up to at least 4300 Mdal. A single prototrophic strain unable to reduce actylene or grow in N-free medium had been isolated after serial curing treatments. Since the plasmids in this strain were indistinguishable, by gel electrophoresis, from the parental strain, no information was provided as to which of the 7-8 molecules carried the nif genes. However DNA homologous to the nif KDH DNA from Klebsiella was seen on the same molecule in all strains examined when such a probe was applied to Southern blots of the separated molecules.

III. ECOLOGICAL FACTORS WHICH LIMIT OR ENHANCE PLANT-BACTERIAL RESPONSES.

a. Response of Nonleguminous Plants to Root Inoculation With Free Living Diazotrophic Bacteria. (13)

Pearl millet, Pennisetum americanum (L) Shum cv Gahi 3, plants achieved higher rates of growth after inoculation with a diazotrophic bacterial culture, Azospirillum brasilense (formerly Spirillum lipoferum) strain 13t. However

nitrogenase activity increased only when competing bacteria were excluded. Cytokinins and other plant growth substances were identified as metabolic products in bacterial cultures, and treating plants with kinetin or gibberellic acid induced dry weight increases similar to those obtained from inoculation treatments. These results suggest that their production of growth-modifying phytohormones, rather than, or in addition to their fixation of dinitrogen, explains the plant responses to inoculation with Azospirillum.

b. Process of Infection of Panicum Maximum by Spirillum lipoferum. (14)

The mode of infection of Panicum maximum by Spirillum lipoferum strain 7 in axenic culture was investigated. The bacteria were adsorbed to the surfaces of the roots 24 hours after inoculation and were observed in the middle lamellae of the root cells within a week. The bacteria remained intercellular even in one month old cultures. The following evidence indicates that the entry of the bacteria into the middle lamellae is mediated by pectolytic enzymes: a) zones of hydrolysis around colonies on pectin agar plates; b) growth of bacteria in pectin broth media; and c) ultrastructural evidence of hydrolysis of middle lamellae that were closely associated with the bacteria.

c. Plant Growth Substances Produced by Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.). (20)

Azospirillum brasilense, a nitrogen-fixing bacterium found in the rhizosphere of various grass species, was investigated to establish the effect on plant growth of growth substances produced by the bacteria. Thin-layer chromatography, high-pressure liquid chromatography, and bioassay were used to separate and identify plant growth substances produced by the bacteria in liquid culture. Indole acetic acid and indole lactic acid were produced by A. brasilense from typtophan. Indole acetic acid production increased with increasing tryptophan concentration from 1 to 100 μg/ml. Indole acetic acid concentration also increased with the age of the culture until bacteria reached the stationary phase. Shaking favored the production of indole acetic acid, especially in a medium containing nitrogen. A small but biologically significant amount of gibberellin was detected in the culture medium. Also at least three cytokinin-like substances, equivalent to about 0.001 μg of kinetin per ml, were present. The morphology of pearl millet roots changed when plants in solution culture were inoculated. The number of lateral roots was increased, and all lateral roots were densely covered

with root hairs. Experiments with pure plant hormones showed that gibberellin causes increased production of lateral roots. Cytokinin stimulated root hair formation, but reduced lateral root production and elongation of the main root. Combinations of indole acetic acid, gibberellin, and kinetin produced changes in root morphology of pearl millet similar to those produced by inoculation with A. brasilense.

d. Association of Azospirillum with Grass Roots. (26)

The association between grass roots and Azospirillum brasilense Sp 7 was investigated by the Fahraeus slide technique, using nitrogen-free medium. Young inoculated roots of pearl millet and guinea grass produced more mucilaginous sheath (mucigel), root hairs, and lateral roots than did uninoculated sterile controls. The bacteria were found within the mucigel that accumulated on the root cap and along the root axes. Adherent bacteria were associated with granular material on root hairs and fibrillar material on undifferentiated epidermal cells. Significantly fewer numbers of azospirilla attached to millet root hairs when the roots were grown in culture medium supplemented with 5 mM potassium nitrate. Under these growth conditions, bacterial attachment to undifferentiated epidermal cells was unaffected. Aseptically collected root exudate from pearl millet contained substances which bound to azospirilla and promoted their adsorption to the root hairs. This activity was associated with nondialyzable and protease-sensitive substances in root exudate. Millet root hairs adsorbed azospirilla in significantly higher numbers than cells of Rhizobium, Pseudomonas, Azotobacter, Klebsiella, or Escherichia. Pectolytic activities, including pectin transeliminase and endopolygalacturonase, were detected in pure cultures of A. brasilense when this species was grown in a medium containing pectin. These studies describe colonization of grass root surfaces by A. brasilense and provide a possible explanation for the limited colonization of intercellular spaces of the outer root cortex.

e. Induction of Nitrogenase Activity in Azospirillum brasilense by Conditioned Medium from Cell Suspension Cultures of Pennisetum americanum (Pearl Millet) and Panicum maximum (Guinea Grass). (35)

Nitrogenase activity (= acetylene reduction activity) was observed in Azospirillum brasilense cultures grown on 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.

f. Statewide Search for Highly Active Associative N_2-Fixation Systems. (36)

A search was conducted in 57 of Florida's 67 counties for highly active, associate (grass-bacteria) N_2-fixing sites. Two hundred sixty-four soil-grass cores were analyzed for nitrogenase activity by the acetylene reduction method (AR). Cores that were collected by Extension Service personnel, shipped to Gainesville and analyzed after standardization in the greenhouse gave results similar to cores that were taken and analyzed directly.

Four sites had cores which evolved over 1000 nM ethylene $core^{-1}hr^{-1}$, which is very high for grass-root-soil cores. Nineteen sites had cores with other 500 nM $core^{-1}hr^{-1}$ and 34 cores had over 300 nM $core^{-1}hr^{-1}$. Paspalum notatum Flugge was the most prevalent grass species in the highly active cores, and Cynodon dactylon (L.) Pers. was the next most prevalent.

Soil moisture and zinc content were positively correlated and calcium content was negatively correlated with AR activity. Elevated soil moisture was required for high AR, but not all cores with elevated soil moisture were active.

Acetylene reduction was positively correlated with CO_2 concentration and negatively correlated with O_2 concentration in the cores at the end of incubation. These active sites are being evaluated further to determine the necessary requirements and best organisms for active associative N_2-fixation.

g. Ecological Factors Affecting Survival and Activity of Azospirillum in the Rhizosphere. (38)

Azospirillum inoculates disappear rapidly when inoculated into the rhizosphere. We have attempted to identify conditions that would increase survival. After inoculating by various methods, we estimated N_2 fixation by C_2H_4 reduction, organic material (released by plant roots) by ^{14}C labelling, and respiration by IR CO_2 analysis. Dilution plate counts and MPN techniques were used to determine bacterial numbers. Nitrogenase activity and numbers of soil bacteria (including Azospirillum) were positively correlated with soil moisture. In monoxenic systems, Azospirillum populations were positively correlated with soil organic matter content. Soil respiration and subsequent plant dry matter yields were greater in some but not all cases after Azospirillum inoculation. Also, the

amount of ^{14}C label in the soil solution was increased by inoculation.

We found that plant growth hormones produced by the inoculants often stimulated early growth of the host plants, but in both field and greenhouse investigations population levels of the introduced bacteria were too low to fix amounts of nitrogen that would be significant in an intensively managed crop production system. Our data indicate that microbial competition limits Azospirillum growth and that suboptimal levels of moisture and carbon substrates in the soil limit nitrogenase activity. Further research on methods for increasing survival and growth of associative N_2-fixing bacteria in the soil is badly needed.

h. Water Tolerance of Pennisetum Hybrids. (43)

Waterlogging tolerance of several genotypes from crosses between pearl millet (Pennisetum americanum (L.) K. Schum.) and elephantgrass (Pennisetum purpureum Schum.) were evaluated at the University of Florida, Gainesville, in 1983. The crosses were made in a greenhouse in the late fall of 1982 using three varieties of pearl millet as the female parent, and 10 selections of elephantgrass as the male parent. Seeds were collected, planted in a greenhouse, and seedlings were transplanted to the field in April 1983 in six blocks. In June, five of the blocks began receiving a water treatment to determine if differences existed among crosses and among plants within crosses in their ability to tolerate waterlogged soil conditions. The water treatment lasted 12 weeks, and consisted of sprinkle irrigating the plots for 14 days followed by no irrigation for 7 days. The remaining block was not irrigated, and was used as the control.

Visual scores were given to each row after each irrigation cycle, and to each plant after the final cycle. The plants were then harvested, and evaluated for dry matter yield, in vitro organic matter digestibility (IVOMD), neutral detergent fiber (NDF), and N concentration to determine if there were differences in forage quality.

According to Duncan's multiple range test, differences were observed both among crosses, and among plants within crosses with the visual scoring technique. A block effect was not evident, indicating that the differences were genotypic rather than environmental.

Differences were also observed among crosses, and among plants within crosses in regard to IVOMD, NDF, and N concentration. A negative correlation was observed between IVOMD and NDF, but no correlation was evident between N concentration was IVOMD or NDF.

i. Sorghum Inoculated with Azospirillum in Autoclaved vs. Non-Autoclaved Soil. (46)

Plant genotype and bacteria strain relationships were studied using axenic soil systems in greenhouse experiments. Both glass and plastic jar assemblies were tested using sorghum plants, N_2-fixing bacterial inoculation, and appropriate controls. Based on acetylene reduction activities, bacteria were slow to become established in all autoclaved soil treatments. Among the autoclaved soil treatments, a significant yield increase was obtained from the treatment which used native soil-root microorganisms. Bacterial survival measured by using the most probable number (MPN) technique in nitrogen-free succinate media showed significant differences among inocula. Highest count of N_2-fixing bacteria was 4.9×10^5 per gram of soil where Azospirillum brasilense strain CD, was used as inoculum.

j. The Influence of Shading on Associative N_2 Fixation. (49)

The effect of reduced solar radiation on associative N_2-fixation and plant parameters was studied in three field experiments (1978-80). 'Gahi-3' pearl millet (Pennisetum americanum (L.) K. Monch.) field plots were shaded with saran shade cloth that reduced solar radiation by 50% and 75%. Acetylene reduction activity (ARA) was reduced by shading in one of the three experiments. The two non-responding experiments were conducted on a well-drained, low-activity site (ARA means ranging 17-68 n moles ethylene $core^{-1}h^{-1}$), the responding experiment was conducted on a poorly drained, high-ARA site.

Shading affected the plants drastically, reducing fresh weight and dry matter yields up to 46% (50% shade) and 57% (75% shade). Shading also reduced dry matter percentage from 19.6 (no shade) to 15.3 (75% shade) and increased nitrogen content from 0.6% (no shade) to 1.53% (75% shading). However, shading did not affect protein yield. Inoculation with Azospirillum brasilense had no measurable effect on yield or acetylene reduction in the first two experiments.

In the third experiment, shading reduced mean ARA of inoculated plots over 100% but had no significant effect on control plots. Inoculation significantly increased ARA in the non-shaded plots but not in shaded plots. Acetylene reduction activity was high, with means ranging between 208 and 465 n moles ethylene evolved $core^{-1}h^{-1}$. Soil moisture and millet growth stage also affected acetylene reduction activity.

k. Why Does Inoculation With Azosporillum brasilense (Spirillum lipoferum) Increase Yield of Grasses? (61)

Higher forage yields have been achieved by inoculating various grass species with Azospirillum brasilense (formerly Spirillum lipoferum). However we find that acetylene reduction assays indicate increased nitrogenase activity only in axenic systems. This has led us to suspect that in the field plant responses are to inoculation effects other than or in addition to nitrogen fixation. It is possible that the organism enhances nutrient accumulation or that growth hormones produced by the bacteria directly influence plant growth.

We found that Azospirillum cells or a diffusate from the cells in the nutrient medium stimulated development of secondary roots and root hairs. This could permit greater rates of nutrient absorption, which in turn could be expected to increase the rate of plant growth. We found also that growth substances produced by Azospirillum brasilense increase the rate of dry matter accumulation in both tops and roots of some plants. Thus, these investigations indicate that Azospirillum brasilense is capable of promoting plant growth without increasing nitrogen fixation in the rhizosphere. Nitrogen fixation may increase under field conditions as a result of inoculation, but using acetylene reduction assays we were able to detect this in axenic systems only.

l. Growth Responses and Acetylene Reduction of Several Grass/Bacteria Associations Grown in vitro. (63)

A screening technique has been developed to evaluate plant responses, root-bacteria association and acetylene reduction of specific grass-bacteria combinations. Axenic systems were established by inoculating corn or millet plants with Azospirillum or other N_2-fixing bacteria. The seedlings were grown in test tubes for 2 weeks on a Fahraeus, nitrogen and carbon free, medium. In addition, bacterial populations of Azospirillum were studied at the end of the growth period using fluorescent antibody labeling. Both pearl millet and corn had high acetylene reduction values in several of the innoculation treatments. Root development was partially inhibited by some of the bacterial strains and enhanced by others. Roots were scored for root-bacteria colonization with the Azospirillum types appearing to associate best. Fluorescent antibody reactions with Azospirillum clearly showed masses of bacteria at the root-agar interface.

m. Factors Affecting Nitrogenase Activity of Grass Field Plots. (64)

Factors affecting the nitrogenase activity of field plots inoculated with the nitrogen fixing bacterium, Azospirillum brasilense, were evaluated using acetylene reduction assays. Over a two period, nine nurseries on three soil types, containing three grass species were studied. Two of the soils were well drained, fine sands, the other was poorly drained, fine sand. The three species were corn, pearl millet and guineagrass. The effects of the following factors were evaluated: inoculation, nitrogen fertilizer, shading, days into the growing season from June 1 (Day), plant genotype and soil factors of moisture, pH, P, K, NO_3^- and NH_4^+. Soil factor evaluations (except moisture) are not completed for 1980.

Acetylene reduction (AR) was conducted on 8.5 and 11 cm dia cores by incubating them about 18 hr in 10% acetylene at 30°C. Oxygen was reduced in the cores to about 6% for the assay. The 1056 cores evaluated were taken over a 100 day period in both 1979 and 1980. Highly variable acetylene reduction data were transformed using a log transformation to stabilize variances for statistical analyses of variance and multiple regressions (SAS GLM Procedure).

n. Influence of Soil Moisture and Organic Substances on $N_2(C_2H_2)$-Fixation by Sorghum and Millet. (65)

Several factors affecting nitrogenase activity and growth of N_2-fixing soil bacteria in association with sorghum and millet roots were examined. Plants were grown in a greenhouse and nitrogenase activity was determined by acetylene reduction. Organic material released by the roots was determined by ^{14}C labelling studies. Rates of nitrogenase activity and numbers of soil bacteria were positively correlated to soil moisture. Nitrogenase activity increased to a maximum at 7 weeks after germination. Soil pH remained constant during the course of the experiment.

In monoxenic systems, the numbers of the N_2-fixing soil bacterium Azospirillum brasilense were found to be positively correlated to soil organic matter content. Plant dry matter yield, root and soil respiration were higher with inoculation. The amount of ^{14}C label in the rhizosphere also was greater in the inoculated plants. General trends suggest that low soil moisture and organic matter will severely limit associative nitrogen fixation and growth of soil N_2-fixing bacteria.

o. Effect of Soil Conditions on Survival and Activity of Diazotrophic Bacteria. (73)

Seedlings of Sorghum bicolor and Setaria italica were grown in nutrient solutions, sand, and sandy loam soil, and inoculated with strains of Azospirillum, Klebsiella and Azotobacter. Carbon substrates, pH, and oxygen levels were varied to determine their effects on survival and N_2 fixation activity of the bacteria. Some treatment combinations supported growth of the inoculant strains, but N_2 fixation activity was low in all cases. Further tests, comparing growth of the inoculant strains in cultivated and uncultivated soils, indicated that Azospirillum is more likely to become established in a rhizosphere which has not been disturbed by cultivation.

p. Acetylene Reduction Activity of several Sorghum and N_2-Fixing Bacterial Associations. (76)

Fifty-one Sorghum vulgare (L.) Moench lines were tested in replicated field plots for acetylene reduction (AR). Range of AR activity was from 0 to 1934 nanomoles per gram of dry root per hour. In order to further study these highly variable field responses, an in vitro screening technique has been developed to evaluate plant responses, root bacteria associations, and acetylene reduction of specific grass bacteria combinations. Axenic systems using diverse sorghum germplasm were established by inoculating sorghum plants with Azospirillum or other N_2-fixing bacteria. The seedlings were grown in test tubes for 10 days on a Fahraeus, nitrogen and carbon free medium. In addition, bacterial populations of Azospirillum were studied at the end of the growth period using fluorescent antibody labeling. Roots were scored for root-bacteria associations. Photos of the root-bacteria associations will be displayed.

q. Survival of Root-Associated Bacteria in the Rhizosphere. (85)

Results of root and soil inoculation studies are frequently analyzed only in terms of favorable effects on the host plants. However when these effects are not found it is impossible to identify reasons for failure of the experiments. It would be possible to do so at least in some instances however, if data were collected to indicate survival and activity of the inoculant organism.

Our data from experiments with Azospirillum, Azotobacter, and Klebsiella suggest that failures in most cases indicate poor survival capability of the organism rather than its incapability to interact with the host plant genotype. Favorable effects on plant growth occur consistently when the inoculant strains proliferate in the

rhizosphere. We continue to find however that these favorable effects occur even when N_2 fixation in the rhizosphere is not increased.

Further progress in developing useful bacterial strains to enhance yields of nonlegumes may not be achieved until methods are identified to assure their growth in the rhizosphere. There is an urgent need to identify environmental conditions and host plant characteristics, which significantly enhance survival and activity of beneficial rhizosphere bacteria.

r. Effect of Moisture and Nitrogen on Nitrogenase Activity. (90)

Many factors can effect the fixation of nitrogen by free-living bacteria associated with the roots of higher plants. Water is required for movement and metabolism. Nitrogen can repress the formation of the nitrogenase enzyme complex. This report presents the effect of both moisture and nitrogen on nitrogenase and populations of nitrogen-fixing bacteria. Plant bacterial associations were grown in the greenhouse, nitrogenase was estimated by acetylene reduction and bacterial numbers by the dilution-pour plate technique.

Soil moisture was found to greatly effect both nitrogenase activity and the numbers of soil bacteria. There was a high positive correlation between nitrogenase activity and soil moisture, with activity declining to negligable amounts when soil moisture reached 50% of field capacity. Soil moisture stress, as applied by polyethylene glycol, greatly inhibited nitrogenase activity. In addition, soil moisture reduced the number of soil microorganisms and greatly reduced the number of nitrogen-fixing bacteria, both native and introduced. Introduced populations of nitrogen-fixing bacteria were greatly reduced at any level of soil moisture, indicating that these organisms may not be as competitive as originally thought.

Nitrogen was provided as either nitrate or urea. The type of nitrogen had little effect on the population dynamics of the organisms in the rhizosphere. The amount of nitrogen did influence the production of the nitrogenase enzyme complex. Although lack of nitrogen did not inhibit the formation or activity of nitrogenase, it was found that small amounts of combined nitrogen, either as urea or nitrate, increased the nitrogenase activity of the associations. Increasing quantities of combined nitrogen in the rooting medium did depress nitrogenase activity and eventually inhibited the activity entirely.

IV. CARBON DIOXIDE AND NITROGEN FIXATION BY PLANTS AND ASSOCIATED RHIZOSPHERE BACTERIA.

a. Partitioning of Nitrogen from ^{15}N-Labelled Bacteria into Soil and Plants. (72)

The fate of reduced nitrogen from N_2-fixing rhizosphere bacteria is not adequately understood. This study was undertaken to examine the translocation of nitrogen, introduced by N_2-fixing bacteria, in the soil. Living cells of *Azospirillum brasilense* strain JM125A2 and *Klebsiella pneumoniae*, uniformly labelled with ^{15}N were introduced, at several inoculation rates, into the rhizosphere of greenhouse-grown *Zea mays*. Total nitrogen, ^{15}N to ^{14}N ratios, plant yield and soil bacteria populations were determined at plant maturity. Plant dry weight yields were directly proportional to the rate of bacterial addition. Large populations of N_2-fixing bacteria were not maintained in the inoculated soils. However, there was an increase in denitrifying bacteria during the experiment. Nitrogen content of the plant tissue increased with increased rates of added bacteria. The fact that ^{15}N was incorporated into the plant tissue demonstrates that plants were able to take up nitrogen supplied by the root-associated microorganisms. The study indicates that 60-70% of the nitrogen incorporated in the bacterial biomass can be removed from the soil by plant growth.

Chronological list of N_2 publications written by UF researchers under USAID Contract

1. Schank, S. C. 1975. Digitaria genotypes evaluated in Brazil for nitrogenase activity, yield and IVOMD. Proc. 32nd Southern Pasture Forage Crop Improvement Conference. 79-86. May 20-22, 1975. Longview, Texas.

2. Dazzo, Frank B. and James R. Milam. 1976. Serological Studies of Spirillum lipoferum. Proc. Soil Crop Sci. Soc. Fla. 35:121-126.

3. Smith, Rex. L., J. H. Bouton, S. C. Schank, K. H. Quesenberry, M. E. Tyler, J. R. Milam, M. H. Gaskins and R. C. Littell. 1976. Nitrogen fixation in grasses inoculated with Spirillum lipoferum. Science 193:1003-1005.

4. Schank, S. C., Rex L. Smith, K. H. Quesenberry and J. H. Bouton. 1976. Nitrogen fixation by grasses in the field. 33rd Pasture and Forage Crop Improvement Conference. Proc. pp 57-62.

5. Smith, Rex. L., J. H. Bouton, S. C. Schank, K. H. Quesenberry, M. E. Tyler, J. R. Milam, M. H. Gaskins and R. C. Littell. 1977. Technical Comments. Science 195:1362.

6. Quesenberry, K. H., Rex L. Smith, S. C. Schank and J. H. Bouton. 1977. Nitrogen fixation by tropical grasses. Proceedings of a Forage Legume Conference. p. 20-22. Noble Foundation, Ardmore, Oklahoma.

7. Schank, S. C., J. M. Day and Enesio Delgado de Lucas. 1977. Nitrogenase activity, nitrogen content, in vitro digestibility and yield of 30 tropical forage grasses in Brazil. Trop. Agric. 54:119-125.

8. Smith, R. L., J. H. Bouton, S. C. Schank and K. H. Quesenberry. 1977. Ch. 25. Yield increases of tropical grain and forage grasses after inoculation with Spirillum lipoferum in Florida. In: Biological Nitrogen Fixation in Farming Systems of the Tropics. Ayanaba, A. and P. Dart (Eds). John Wiley & Sons. 377 pp.

9. Smith, Rex L., S. C. Schank and K. H. Quesenberry. 1977. Biological nitrogen fixation: Opportunities for greater utilization of solar energy in food production. Am. Chem. Soc. Agric. and Food Chem. Symp. on Renewable Resources. New Orleans.

10. Quesenberry, K. H., R. L. Smith and S. C. Schank. 1978. N_2-fixation research with tropical grasses. Proc. 35th Southern Pasture and Forage Crop Improvement Conference. SEA-USDA. pp. 33-34.

11. Smith, Rex L., S. C. Schank, J. H. Bouton and K. H. Quesenberry. 1978. Yield increases of tropical grasses after inoculation with Spirillum lipoferum. In: Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria. Granhall, U. (Ed). Ecol. Bull. (Stockholm) 26:380-385.

12. Baltensperger, A. A., S. C. Schank, Rex L. Smith, R. C. Littell, J. H. Bouton and A. E. Dudeck. 1978. Effect of inoculation with Azospirillum and Azotobacter on turf-type bermudagrass genotypes. Crop Sci. 18:1043-1045.

13. Gaskins, M. H. and D. H. Hubbell. 1979. Response of nonleguminous plants to root inoculation with free living diazotrophic bacteria. In The Soil-Root Interface. J. L. Harley and R. S. Russell, (Eds). Academic Press.

14. Umali-Garcia, M., D. H. Hubbell, M. H. Gaskins. 1978. Process of infection on Panicum maximum by Spirillum lipoferum. Ecol. Bull. 26:373-379.

15. Berg, R. H., V. Vasil and I. K. Vasil. 1979. The biology of Azospirillum -sugarcane association. II. Ultrastructure. Protoplasma 101:143-146.

16. 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.

17. Hubbell, D. H., T. M. Tien and M. H. Gaskins. 1979. Physiological interaction in the Azospirillum grass root interaction. Proc. Intl. Wkshp Assoc. N_2 Fix.

18. Schank, S. C., Rex L. Smith, G. C. Weiser, M. E. Tyler, J. H. Bouton, K. H. Quesenberry, David Zuberer, J. R. Milam and R. C. Littell. 1979. Fluorescent antibody techniques to identify Azospirillum brasilense associated with roots of grasses. Soil Biol. Biochem. 11:287-295.

19. Schank, S. C., Rex L. Smith and G. C. Weiser. 1979. Responses of two pearl millets grown in vitro after inoculaton with Azospirillum brasilense. Proc. Soil Crop Sci. Soc. Fla. 39:112-115.

20. Tien, T. M., M. H. Gaskins and D. H. Hubbell. 1979. Plant growth substances produced by Azospirillum brasilense and their effect on the growth of pearl millet (Pennisetum americanum L.). Appl. Environ. Microbiol. 37:1016-1024.

21. Tyler, M. E., J. R. Milam, R. L. Smith, S. C. Schank and D. A. Zuberer. 1979. Isolation of Azospirillum from diverse geographic regions. Canadian J. Micro. 25:693-697.

22. Vasil, V., 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.

23. Albrecht, S. L. and Y. Okon. 1980. Cultures of Azospirillum. pp. 740-750. In: Methods in Enzymology. Colowick, S. P. and N.O. Kaplan (Eds).

24. Berg, R. H., M. E. Tyler, N. J. Novick, V. Vasil, and I. K. Vasil. 1980. Biology of Azospirillum-sugarcane association: Enhancement of nitrogenase activity. Appl. Env. Micro. 39:642-649.

25. Hubbell, D. H. and M. H. Gaskins. 1980. "Cryptic" plant root-microorganism interactions. What's New in Plant Physiology 11:17-19.

26. Umali-Garcia, Mercedes, D. H. Hubbell, M. H. Gaskins, and F. B. Dazzo. 1980. Association of Azospirillum with grass roots. Appl. Environ. Microbiol. 39:219-226.

27. Albrecht, S.L., Y. Okon, J. Lonnquist, and R. H. Burris. 1981. Nitrogen fixation by corn-Azospirillum associations in a temperate climate. Crop Sci. 21:301-306.

28. Wood, A. G., E. M. Menezes, C. Dykstra, and D. E. Duggan. 1981. Methods to demostrate the megaplasmids (or minichromosomes) in Azospirillum. Azospirillum Genetics, Physiology and Ecology Workshop. Univ. Bayreuth, Germany.

29. 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. Vose, P. and A. Ruschel (Eds). CRS Press, Inc., Boca Raton, Florida.

30. 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. Micro. 41:342-345.

31. Tien, T. M., H. G. Diem, M. H. Gaskins and D. H. Hubbell. 1981. Polygalacturonic acid transeliminase production by Azospirillum species. Can. J. Microbiol. 27:426-431.

32. 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 N_2 Fixation, Vol. I. Vose, P. and A. Ruschel (Eds). CRS Press, Inc., Boca Raton, Florida.

33. Albrecht, S. L. and M. H. Gaskins. 1982. The bioenergetics of asymbiotic nitrogen fixation. In: The Use and Conservation of Renewable Energies in the Bio-Industries. F. Vogt (ed). Pergamon Press, Oxford.

34. Novick, Norman J. and Max E. Tyler. 1982. L-arabinose metabolism in Azospirillum brasilense. J. Bact. 149:364-367.

35. 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. 106:139-147.

36. Smith, R. L., S. C. Schank, J. R. Milam and R. C. Littell. 1982. Statewide search for highly active associative N_2-fixing systems. Soil Sci. Soc. Fla. Proc. 41:122-126.

37. Albrecht, S. L., M. H. Gaskins, J. R. Milam, S. C. Schank and R. L. Smith. 1983. Ecological factors affecting survival and activity of Azospirillum in the rhizosphere. In: Experientia Supplementum, Azospirillum II. Klingmuller, W. (Ed). 48:138-148. Birkhauser Verlag, Basel.

38. Klingmuller, W., K. T. Shanmugam and M. Singh. 1983. Nif- hybrids of Enterobacter: selection for nif gene integration with chlorate. Mol. Gen. Genet. 191:221-224.

39. Matthews, Sharon W., S. C. Schank, H. C. Aldrich and Rex L. Smith. 1983. Peroxidase- antiperoxidase labeling of Azospirillum brasilense in field-grown pearl millet. Soil Biol. Biochem. 15:699-703.

40. Novick, Norman J. and Max E. Tyler. 1983. Partial purification and properties of an L-arabinose dehydrogenase from Azospirillum brasilense. Can. J. of Microbiol. 29:242-246.

41. Schank, S. C., R. L. Smith and R. C. Littell. 1983. Establishment of associative N_2-fixing systems. Proc. Soil Crop Sci. Soc. Fla. 42: 113-117.

42. Albrecht, S. L. and M. H. Gaskins.. 1984. Effect of moisture levels in the soil on growth and nitrogenase activity of root-associated N_2-fixing bacteria. Plant and Soil. (Submitted)

43. Alphs, D. A. and S. C. Schank. 1984. Water tolerance of Pennisetum hybrids. Proc. Soil Crop Sci. Soc. Fla. 43:193-196.

44. Hubbell, D. H. and M. H. Gaskins. 1984. Associative N_2-fixation with Azospirillum. pp 201-224. In: Biological Nitrogen Fixation. M. Alexander (Ed). Plenum Publ.

45. Schank, S. C. and R. L. Smith. 1984. Status and evaluation of associative grass-bacteria N_2-fixing systems in Florida. Proc. Soil Crop Sci. Soc. Fla. 43:120-123.

46. Schank, S. C., Rex L. Smith, J. R. Milam and R. C. Littell. 1984. Sorghum Inoculated with Azospirillum in autoclaved vs. non-autoclaved soil. Proc. Soil Crop Sci. Soc. Fla. 44: (Submitted).

47. Sims, J. R., R. S. Smith, S. H. West, W. C. Lindemann, and L. R. Frederick. 1984. Biological nitrogen fixation research and technology assessment for Egypt. I. Scientists, publications and extension. J. Agron. Educ. 13:19-23.

48. Sims, J. R., R. S. Smith, S. H. West, W. C. Lindemann, and L. R. Frederick. 1984. Biological nitrogen fixation research and technology assessment for Egypt. II. Training, reseaarch and inoculant production. J. Agron. Educ. 13:24-28.

49. Smith, Rex L. and S. C. Schank. 1984. Influence of shading on associative N_2-fixation. Plant and Soil. 80:43-52.

50. Smith, Rex L., S. C. Schank, J. R. Milam and A. A. Baltensperger. 1984. Responses of Sorghum and Pennisetum species to the N_2-fixing bacterium Azospirillum brasilense. Appl. Env. Micro. 47:1331-1336.

51. Smith, Rex L. and S. C. Schank. 1984. Associative N_2 fixation-- an update. Proc. South. Forage Crop Imp. Conf. (In Press)

Chronological list of N_2 abstracts written by UF researchers under USAID Contract

52. Schank, S. C., J. Day, G. T. V. Abrantes and J. Dobereiner. 1975. Growth characteristics, nitrogenase activity and yield of two cultivars of Digitaria decumbens ('Transvala' and 'Pangola') digitgrass in Brazil. Abstracts Southern Agric. Workers Conf. New Orleans. 2:12.

53. Smith, Rex L., M. H. Gaskins, S. C. Schank, and D. H. Hubbell. 1975. Nitrogen fixation by a tropical grass- bacteria association in Florida. Abstracts Southern Agric. Workers Conf. New Orleans. 2:12.

54. Schank, S. C., J. Day, E. Delgado de Lucas, J. Dobereiner. 1975. Nitrogenase activity, Nitrogen content, IVOD and yield of thirty forage grasses in Brazil. Agronomy Abstracts. 1975 Annual Meeting, Society of Agronomy 67:132-133.

55. Smith, Rex L., S. C. Schank, M. H. Gaskins and D. H. Hubbell. 1975. Nitrogen fixation by grass-bacteria associations-a progress report. Agron. Abst. 67:133.

56. Smith, Rex L., S. C. Schank, J. H. Bouton and K. H. Quesenberry. 1976. Yield increases of tropical grasses after inoculation with Spirillum lipoferum. Intern. Symp. Environ. Role of N_2-Fixing Blue-green Algae and Asymbiotic Bacteria. Abstracts p. 65. Uppsala, Sweden. Sept. 20-24, 1976.

57. Tyler, M. E., J. R. Milam, and D. A. Zuberer. 1977. Taxonomy of the diazotroph of tropical grasses called Spirillum lipoferum. p.77 In International Symposium on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Brasilia, Brazil. 116 pp.

58. Schank, S. C., Rex L. Smith, G. C. Weiser, M. E. Tyler, J. H. Bouton, K. H. Quesenberry, D. Zuberer and J. R. Milam. 1977. Fluorescent antibody techniques to identify N_2-fixing bacteria from Florida, Ecuador, Venezuela and Brazil in grass roots. Agron. Abst. Nov. 13-18, 1977. p. 45.

59. Umali-Garcia, M., D. H. Hubbell, M. H. Gaskings, and F. B. Dazzo. 1978. Adsorption and infection of grass roots by Azospirillum brasilense Sp 7. p.24. Steenbock-Kettering International Symposium on Nitrogen Fixation. Madison, WI. June 12-16. 87pp.

60. Tyler, M. E., D. A. Zuberer and J. R. Milam. 1978. New features of Azospirillum isolates and of an antibiotic-resistant strain. Steenbock-Kettering Intern. Symp. on Nitrogen Fixation. Madison, WI, June 12-16, 1978.

61. Tien, T. M., M. H. Gaskins, D. H. Hubbell, and M. Umali-Garcia. 1978. Why does inoculation with Azospirillum brasilense (Spirillum lipoferum) increase yield of grasses? p.23. Steenbock-Kettering International Symposium on itrogen Fixation. Madison, WI. June 12-16. 87pp.

62. Schank, S. C., K. L. Weier and I. C. MacRae. 1979. Yield and nitrogen studies of a digitgrass after inoculation with Azospirillum on two Australian soils. Agronomy Abstracts. 1979. American Society of Agronomy 71:163.

63. Schank, S. C. and Rex L. Smith. 1980. Growth responses and acetylene reduction of several grass/bacterial associations grown in vitro. Can. J. Genet. Cytol. 22:677.

64. Smith, Rex L. and S. C. Schank. 1981. Factors affecting nitrogenase activity of grass field plots. p. 495 In Current Perspectives in Nitrogen Fixation. Australian Academy of Science. Canberra. 534 pp.

65. Albrecht, S. L., M. E. Mitchell, and M. H. Gaskins. 1981. Influence of soil moisture and organic substances on N_2 (C_2H_2)-fixation by sorghum and millet. (Abstr.) Plant Phys. Supp. 67:77

66. Gaskins, M. H., D.H. Hubbell, and S.L. Albrecht. 1981. Interactions between grasses and rhizosphere nitrogen-fixing bacteria. p. 143. (Abstr.) XIV International Grassland Congress, Lexington, KY. 483 pp.

67. Gaskins, M.H. 1981. Metabolite exchange between host and associative organisms. p. 323. In: Current Perspectives in Nitrogen Fixation. Australian Academy of Science, Canberra. 534 pp.

68. Menzes, E.M. and D.E. Duggan. 1981. Transformation of Azospirillum biosynthetic genes in plasmid DNA. p. 131. Abstracts of 81st Annual Meeting of the American Society for Microbiology, Dallas, TX. 365 pp.

69. Schank, S. C. and Rex L. Smith. 1981. Statewide survey for grass- bacteria associations. p. 7. Abstract Southern Assoc. Agric Sci. Meeting, Atlanta, GA. 18 pp.

70. Wood, A.A. and D.E. Duggan. 1981. Multiple large plasmids of Azospirillum. p. 131. Abstracts of 81st Annual Meeting Amer. Soc. Microbiol., Dallas, TX. 365 pp.

71. Albrecht, S. L., M. H. Gaskins, Jiang You-yi and J. R. Milam. 1982. Factors affecting introduced populations of N_2 fixing bacteria in the rhizosphere. p. 24. Abstracts Second Int. Symp. N_2-Fixation Non-Legumes, Banff, Canada.

72. Albrecht, S. L., J. R. Milam and M. H. Gaskins. 1982. Partitioning of nitrogen from ^{15}N-labelled bacteria into soil and plants. Abstracts of 82nd Annual Meeting Amer. Soc. Microbiol., New Orleans.

73. Gaskins, M. H., S. L. Albrecht and J. R. Milam. 1982. Effect of soil conditions on survival and activity of diazotrophic bacteria. Abstracts Second Int. Symp. N_2-Fixation Non-Legumes, Banff, Canada.

74. Klingmuller, W., A. Kleeberger and K. Shanmugam. 1982. Genetic studies with N_2-fixing Enterobacter strains from the rhizospheres of grasses. Abstracts Second Int. Symp. N_2-Fixation Non-Legumes, Banff, Canada.

75. Milam, J. R. and S. L. Albrecht. 1982. Translocation of ^{15}N from inoculated N_2-fixing bacteria into Zea mays. Abstracts Second Int. Symp. N_2-Fixation Non-Legumes, Banff, Canada.

76. Schank, S. C., Rex L. Smith, and J. R. Milam. 1982. Acetylene reduction activity of several sorghum and N_2-fixing bacterial associations. p. 752 In Sorghum in the Eighties, Vol II, ICRISAT, India. 783 pp.

77. Green, R. L., R. L. Smith, R. C. Littell and S. C. Schank. 1982. Evaluation of N_2-fixation in bahiagrass by ^{15}N isotope dilution and other techniques. Agron. Abstracts 74:142.

78. Schank, S. C., R. L. Smith, J. R. Milam and R. C. Littell. 1982. Inoculation of grasses with N_2-fixing bacteria in autoclaved versus unautoclaved soil. Agron. Abstracts 74:197.

79. Wood, A. G. and D. E. Duggan. 1982. Nif gene location in the megaplasmid bacterium Azospirillum. p. 17. Abstracts Second Int. Symp. N_2-Fixation Non-Legumes, Banff, Canada.

80. Green, R. L., R. L. Smith and S. C. Schank. 1983. Evaluation of three methods for measuring the potential of associative N_2-fixation of corn. Agron. Abstracts 75:155

81. Milam, J. R., S. C. Schank, R. L. Smith and A. A. Baltensperger. 1983. Survival of Azospirillum brasilense strain Cd after field inoculations in Florida and New Mexico. p. 33. Abstracts Third Int. Symp. Microb. Ecol. Michigan State Univ. 91 pp.

82. Schank, S. C. and Rex L. Smith. 1983. Summary of grass-bacteria N_2-fixation systems at Florida. Agron. Abstracts 75:161.

83. Smith, Rex L. and S. C. Schank. 1983. Associative grass-bacteria N_2-fixation systems. p. 76. Abstracts. Advances in Gene Technology: Molecular Genetics of Plants and Animals. The Fifteenth Miami Winter Symposium. F. Ahmad, K. Downey, J. Schultz and R. W. Voellmy (eds.) U. Miami School of Medicine. 97 pp.

84. Albrecht, S. L., M. H. Gaskins and J. R. Milam. 1984. Evaluating plant-bacterial interactions which affect rhizosphere nitrogen fixation. p. 47. In: Advances in Nitrogen Fixation Research. C. Veeger and W. E. Newton (Eds). Martinus Nijhoff Publishers, The Hague, 760 pp.

85. Gaskins, M. H., S. L. Albrecht and J. R. Milam. 1984. Survival of root-associated bacteria in the rhizosphere. p. 52. In: Advances in Nitrogen Fixation Research. C. Veeger and W. E. Newton (Eds). Martinus Nijhoff Publishers, The Hague, 760 pp.

86. Milam, J. R., S. L. Albrecht and M. H. Gaskins. 1984. Distribution of labelled nitrogen from N_2-fixing bacteria into Zea mays and soil. p. 351. In: Advances in Nitrogen Fixation Research. C. Veeger and W. E. Newton (Eds). Martinus Nijhoff Publishers, The Hague, 760 pp.

87. Schank, S. C., R. L. Smith, K. H. Quesenberry and R. C. Littell. 1984. Acetylene reduction activity and non-structural carbohydrate content of Hemarthria altissima cv. Bigalta after defoliation. p 520. In: Advances in Nitrogen Fixation Research. C. Veeger and W. E. Newton (Eds). Martinus Nijhoff Publishers, The Hague, 760pp.

88. Smith, Rex L., J. R. Milam and S. C. Schank. 1984. Effect of plant-derived and other carbon substrates on asymbiotic N_2-fixation. p 361. In: Advances in Nitrogen Fixation Research. C. Veeger and W. E. Newton (eds). Martinus Nijhoff Publishers, The Hague, 760pp.

89. Schank, S. C. and Rex L. Smith. 1984. Ten years of field experiments to evaluate plant responses to grass-bacteria associations. p 81. Abstracts, Fourteenth Steenbock Symposium Nitrogen Fixation and CO_2 Metabolism. University of Wisconsin, Madison, 142pp.

90. Milam, J. R., S. L. Albrecht, and M. H. Gaskins. 1984. The affect of moisture and nitrogen on nitrogenase activity. p.127. Abstracts. The Third International Symposium on Nitrogen Fixation with Non-Legumes. Helsinki, Finland, 130pp.

Chronological list - N_2 theses & dissertations written by students on USAID Contract

91. Bouton, Joseph H. 1977. Response of pearl millet inbreds and hybrids to inoculation with Spirillum lipoferum. Ph.D. Diss., University of Florida, Gainesville. 45 pp.

92. Benzion, Gary. 1978. Interaction of Paspalum notatum genotypes with associative N_2-fixing bacteria. MS thesis, University of Florida.

93. Carr, Christine. 1978. Cryptic plasmids in Azospirillum. MS thesis, University of Florida.

94. Matthews, Sharon W. 1979. A cytological investigation of the association between Azospirillum brasilense and some C-4 grasses. Ph.D. Diss., University of Florida, Gainesville. 102 pp.

95. Umali-Garcia, Mercedes. 1978. Early events in the establishment of an associative symbiosis of Azospirillum brasilense with grass roots. Ph.D. Diss., University of Florida, Gainesville. 105 pp.

96. Wood, Alvin. 1978. Antibiotic resistant mutants to evaluate Azospirillum in soil. MS thesis, University of Florida.

97. Novick, N.J. 1980. Metabolism of L-arabinose in Azospirillum brasilense. Ph.D., Diss., Univ. of Florida, Gainesville. 69 pp.

98. Tien, T. M. 1980. Plant growth substances produced by Azospirillum brasilense and their effects on plant growth. Ph.D. Diss., Univ. of Florida, Gainesville. 82pp.

99. Weiser, G.C. 1980. Evaluation of Panicum virgatum L. for nitrogen related agronomic characters. Ph.D. Diss., Univ. of Florida, Gainesville. 55 pp.

100. Menzes, E.M. 1981. Transformation of Azospirillum with plasmid DNA. Ph.D., Diss., Univ. of Florida, Gainesville. 54 pp.

101. Green, R.L. 1982. Evaluation of associative N_2-fixation in bahiagrass and corn. Ph.D. Diss. University of Florida, Gainesville. 75 pp.

102. Alphs, D.A. 1984. Waterlogging tolerance and forage quality of the interspecific cross between Pennisetum americanum[(L.) K. Shum.] and Pennisetum purpureum Shum.). MS Thesis, University of Florida. 66 pp.

CROSS-LINKAGES DURING THE CONTRACT

1977-1984

A. Domestic

Important cross-linkages were maintained in the U. S. by means of regional research projects on N_2 fixation. During the time-period from 9/30/77 - 9/30/83, the regional project was S-130 entitled "Associative N_2-Fixation in Grasses". The project was revised on 10/1/83 as S-187, entitled "Associative Nitrogen Fixation in Nonleguminous Plants". The Florida group was active in the forming of this regional project in 1977, when Dr. S. H. West was the administrative advisor, and Dr. D. H. Hubbell was the project chairman. 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 were active participants in the regional research projects. Other important linkages with other scientists were maintained at various National meetings, such as ASM (American Society of Microbiology) and ASA (American Society of Agronomy), where members of our group regularly participated in symposia, and/or presented posters or papers.

Cooperative work with New Mexico State University on inoculation (Dr. A. A. Baltensperger) was conducted in New Mexico in 1982, and this research verified the rapid decline in microbial populations after inoculation. This cross-linkage was very important in elucidating ecological factors in more than one environment.

B. Overseas

Many scientists became very interested in the proposed phenomenon of grass- bacteria interactions for biological nitrogen fixation as first observed in Brazil in the early 1970's. Dr. Schank from our group was in Brazil from 1973-74, and with the aid of Dr. Rex L. Smith, who obtained the first cultures of "*Spirillum lipoferum* from Brazil in 1974 we were able to maintain a close liaison with Brazilian scientists.

Australian scientists were especially interested and cooperative in learning more about the grass- bacteria associations. Cross linkages were established and maintained since 1976, when Dr. Alan Gibson of CSIRO in Canberra visited our group. In 1978, S. C. Schank spent 4 months with the CSIRO N-Economy Group in Brisbane. Follow-up visits by Dr. Murray W. Gaskins and Rex L. Smith took place in Australia in 1980. In 1981, Dr. A. D. Rovira of CSIRO Division of Soils in Adelaide, visted as a consultant to our group. The final cross-linkage with Australia was the visit of Keith Weier from CSIRO Division of Tropical Plants (Brisbane) in July 1982.

Cross-linkages with ICRISAT in India were established with visits of Drs. Nambia and Subba-Rao in May 1978 and July 1979. Dr. Peter Dart of ICRISAT talked with Drs. S. H. West and Rex L. Smith of our group in 1980 to formally outline research plans. Then, Dr. Dart visited Florida in March 1981 to finalize a cooperative agreement between Mali, ICRISAT, USAID and Florida. Dr. S. C. Schank reciprocated with a visit to India for three weeks in November 1981, attended the "Sorghum in the 80's" conference and presented seminars in New Delhi, Cuttack, and Hyderabad. Dr. S. Wani, microbiologist from ICRISAT visited the Florida group in August 1982.

Dr. S. H. West has maintained cross-linkages with Egypt and C. I. D. (Consortium for International Development). During 1982, 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 invited Dr. Victor Bedrous, from Cairo, Egypt to work with him in 1983. Dr. Mohammed Hassan from Egypt spent three months in on-the-job training in 1982-83, and Dr. Mohamed Eid, from Egypt was here 2 weeks. Another visiting professor from Egypt, Dr. Mohamed El-Helw, concluded his year's sabbatic leave in Florida in 1982.

Israeli contacts have been an important liaison on the grass-bacteria associations. Dr. Yaacov Okon visited our group twice, and has maintained an active correspondence with our group. We also became involved with a commercial Israeli company known as BioTech General, who discussed inoculation studies with us. Because of the commercial implications, our group did not become actively involved with BioTech General.

The Mexican government asked Dr. D. H. Hubbell and our group to provide a 4-week long training program for two vsiting Mexican Scientists in 1982. Dr. Manuel Quintero, and Dr. Aquiles Solis, from Durango, Mexico participated in this on-the-job training.

Numerous other overseas visitors visited our research project from 1977-84, and we appreciated each of their visits.

STUDENT, POST DOCTORAL, AND VISITING PROFESSOR
TRAINING PROGRAMS
in Chronological Order
AID/ta-C-1376
1977-1984

NAME	Country of Origin	Duration of Training	MS	PhD	Post Doc	Other
Mercedes Umali-Garci	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		
Chui-Chung Young	Taiwan	3 weeks				X
H. G. Diem	Senegal	3 months				X
J. H. Bouton	U.S.	4 years		X		

continued on next page

STUDENT, POST DOCTORAL, AND VISITING PROFESSOR
TRAINING PROGRAMS (concl.)

NAME	Country of Origin	Duration of Training	MS	PhD	Post Doc	Other
Joe Salvo	U.S.	2 years	X			
Prem Patel	India	2 years		X		
Pushpam Shankar	India	2 years		X		
Peggy Akins	U.S.	1 year			X	
Vilma Vasil	India	2 years			X	
Hart Spiller	Germany	1 year			X	X
Bruce Bleakley	U.S.	3 years		X		
Cathy Meitin	U.S.	1 year	X			
Brad Swedlund	U.S.	2 years	X			
Mohammed El-Helw	Egypt	1 year				X
Duane Alps	U.S.	2 years	X			
Manuel Quintero	Mexico	4 weeks				X
Aquiles Solis	Mexico	4 weeks				X
D. Purushothaman	India	6 months			X	
Mohamed Eid	Egypt	2 weeks			X	
Jose diStefano	Costa Rica	2 years		X		
Mohamed Hassan	Egypt	3 months			X	
Jiang You-Yi	China	1 year			X	
W. Klingmuller	Germany	3 months				X

AID/ta-C1376-EQUIPMENT
(items costing over $500)

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	$2,410.00	McC 3197
Phase contrast and Dark Field Condensor	11/13/77	12/08/77	$1,857.00	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	$6,845.00	PSL 935
Incubators (2)	01/07/80	02/08/80	$ 641.35	PSL 935
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
Hygrometer	03/12/80	04/02/80	$ 550.40	PSL
Convection Oven	01/07/80	01/12/80	$ 769.00	McC 3197
Balance	03/13/80	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	03/12/83	04/02/80	$1,221.50	120 MAE
Computer Terminal	12/03/81	03/16/80	$ 800.00	Bldg. 495
Commodore Floppy Disk Drive	12/03/81	01/26/82	$1,407.78	Bldg. 935
Smartface Interface			$1,054.00	Bldg. 495
Printer for CBM computer	04/01/83	04/25/83	$1,621.00	Bldg. 495
Rotohoe tiller & planter	03/01/83	03/30/83	$ 749.00	PSL 935
NEC Spinwriter (for apple)	06/01/83	07/28/83	$2,020.00	Bldg. 661
Refri/Freezer 21'	03/01/83	03/15/83	$ 557.00	Bldg 495
GPI Growth Room			$4,850.00	Bldg.
Percival Biol. incubator			$2,675.00	113 MAE
Upright Freezer	11/01/83	11/25/83	$ 508.00	PSL 935
Udy Grinder	02/01/83	02/20/83	$1,096.00	PSL 935
CBM Disk Drive	04/01/83	04/25/83	$1,560.00	Bldg. 495
Modem, Disk Drive & Printer	03/01/83	03/15/83	$1,597.00	Bldg. 495
Icemaker & bin	12/01/83	12/20/83	$ 913.00	PSL 935

EXPENDITURES BY OUTPUT
(1/1/77 — 11/30/84)

OUTPUT	Department			
	MICROBIOLOGY & CELL SCIENCE	AGRONOMY	SOILS	TOTAL
A. Establishment of axenic plant-bacteria systems.	$49,655	$78,369	---	$128,024
B. Collection, characterization and modification of grass rhizosphere N_2-Fixing bacteria.	$231,724	$26,123	---	$257,847
C. Examination of bacterial-strain genotype specificity.	$16,552	$52,245	---	$68,797
D. Ecological factors that limit or enhance plant-bacteria interactions	$16,552	$182,860	$58,973	$258,385
E. Nitrogen and CO_2 fixation by plants and associated rhizosphere organisms.	---	$182,860	---	$182,860
F. Association of N_2-fixing bacteria with plant tissue culture material.	$16,552	---	---	$16,552
TOTAL (without overhead)	$331,035	$522,457	$58,973	$912,465