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NITROGEN FIXATION BY ASSOCIATIVE GRASS-BACTERIA SYSTEMS

BY

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TABLE OF CONTENTS

	Page
SUMMARY OF PROGRESS ON GRASS BACTERIA ASSOCIATIONS	1
INTRODUCTION	7
LIST OF TABLES AND FIGURES	8
I ESTABLISHMENT OF CONSISTENTLY REPRODUCIBLE, AXENIC PLANT-BACTERIA SYSTEMS	9
II COLLECTION AND CHARACTERIZATION OF GRASS-RHIZOSPHERE INHABITING, N_2-FIXING BACTERIA	10
Statewide survey of naturally occurring grass-bacteria associations	10
Microbial physiology	10
Microbial genetics	11
III ECOLOGICAL FACTORS THAT LIMIT OR ENHANCE PLANT-BACTERIA ASSOCIATIONS	13
Laboratory studies	13
Field studies	13
Greenhouse studies	15
Phytohormone production by *Azospirillum* sp.	16
Cell suspension studies	17
IV CARBON DIOXIDE AND NITROGEN FIXATION BY PLANTS AND ASSOCIATED RHIZOSPHERE BACTERIA	18
PUBLICATIONS	19
TABLES & FIGURES	21
CROSS LINKAGES	28
STUDENTS AND TRAINING	30
WORKSHOPS, SEMINARS, ON-SITE VISITS, & CONSULTANTS	31
AID. EQUIPMENT LIST	34
RESEARCH PLANS 1981-82	35
EXPENDITURES FOR PAST YEARS	39

NITROGEN FIXATION BY ASSOCIATIVE

GRASS-BACTERIA SYSTEMS

SUMMARY

AID/ta-C-1376

Field Inoculation and Acetylene Reduction Experiments

Replicated field plots of corn, sorghum, pearl millet, digitgrass, buffelgrass and guineagrass were inoculated over a six-year period. Pearl millet and guineagrass responded to inoculation by producing higher dry matter yields a number of times, but not consistently. A 20% yield increase was generally required before statistically significant differences were detected in some experiments and inoculated plots yielded up to 40% greater dry matter yields than controls. Yield responses were observed only when a light nitrogen fertilizer application was made. No response was observed with zero and heavy nitrogen application. Acetylene reduction (AR) on soil cores from these plots indicated maximum activity was also obtained with a light nitrogen fertilization.

Inoculation increased AR only occassionally, and AR usually did not correlate with yields. A double-marked strain of *Azospirillum brasilense* (streptomycin and rifampicin resistant) could be recovered on plates of selective medium for only a few weeks after inoculation. In other inoculation experiments, populations of other strains of *A. brasilense*, observed using immunofluorescence and strain specific antisera, decreased to a low level within a few weeks. However, this low level could be detected from season to season. These data indicate inoculation has not established higher numbers of *Azospirillum* in our soils.

Factors affecting AR in the field were evaluated by assaying over 1500 soil-root cores. Lowering O_2 tension from ambient to about 2% O_2 increased AR activity five fold. In our porous soils, excessive O_2 is a major deterent to high activity. Cores containing living plant roots had AR activities 10 fold higher than identical cores without roots. Shading plots with 50% and 75% shade reduced plant growth and modified plant composition but also reduced AR by 25 and 56% respectively, below the unshaded control. Acetylene reduction increased as the season progressed, as soil moisture increased and as soil pH increased from 5.8 to 7.2 (the pH range of the experiments).

Crop species varied in their ability to support AR, with corn highest followed by pearl millet and guineagrass. Screening for higher activity in corn, pearl millet, sorghum, bahiagrass, guineagrass, digitgrass, chloris, bermudagrass, and buffelgrass has shown that differences exist and suggests that there is a potential for improving nitrogenase activity (AR) through breeding in most species.

Search for Highly Active Associative N_2-Fixing Systems

The need for better adapted N_2-fixing bacteria and more active associative N_2-fixation prompted a search for highly active plant species in natural soil-plant systems. A statewide survey involving county extension agents brought in cores from 57 of Florida's 67 counties. Five very active sites were identified in this survey giving top AR activities up to 1226 n-moles core^{-1}hr^{-1}. Soil from two of these sites has been used for greenhouse experiments and for isolation of N_2-fixing bacteria. These systems will be dissected and reconstituted to characterize the necessary components of these highly active sites.

A search was also made among native and introduced grasses, and non-legume weeds to identify those capable of fixing a major portion of their required nitrogen.

Acetylene reduction was conducted on about 60 non-legume plant species and in general, the highest activity was found among plants adapted and growing on wet sites (or at least potentially wet sites). Condition of the grass appeared to influence AR a great deal. Mature, senescing grass usually had low AR. High activity appeared to be more site specific than species specific. Bahiagrass has given as high an activity as any other species.

Of special interest were the grasses and weeds that appeared to grow much better than anticipated from soil nitrogen content. *Panicum virgatum* was selected for detailed study because of its relatively high vigor on unfertilized, nitrogen-poor sites. This species had good AR activity but was not greatly superior to other species of much lower vigor on the low nitrogen sites. We found that *P. virgatum* tissue contained a much lower nitrogen content and therefore produced more plant biomass per unit nitrogen.

Mean estimates of N_2-fixation of *P. virgatum* in the field by ^{15}N dilution and AR (cores) were 20.8 and 6.5 kgN^{-1} ha^{-1}, respectively.

Physiology of Associative N_2-Fixation

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

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

Low levels of plant cell wall-hydrolyzing enzymes have been detected in cultures of symbiotic (Rhizobium) and associative (Azospirillum) bacteria. Assay methods have been developed for identifying the various enzymes present, using a highly sensitive agar plate assay method incorporating cell wall polysaccharides or derivatives as substrates for the enzymes. Studies are in progress to quantitate the enzymes present in N_2-fixing bacteria, relate this information to the plant infection characteristics of the organisms, and determine best methods of establishing effective associative combinations under field conditions.

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

Mode of Association or Infection

Early light-microscopy studies suggested that a root infection existed, with bacteria invading living root cells. Electron microscopy later showed that the light microscope lacked the resolution to elucidate the association. Extensive electron microscopy of both axenically- and field-grown roots using Peroxidase anti-peroxidase (PAP) failed to show that Azospirillum invade intact plant cells. Azospirillum were found in the mucigel layer on root surfaces, free spaces within the cortex, within lysed cells, and in breaks in the epidermis. Pure cultures of Azospirillum possessed weak pectolytic activity that may permit dissolution of middle lamella and entry of bacteria between cells.

Axenically grown Azospirillum - pearl millet associations were documented by electron microscopy. Young inoculated roots produced more mucigel, root hairs and lateral roots than did uninoculated controls. The bacteria adsorbed firmly to root hairs and undifferentiated epidermal cells. Addition of fixed nitrogen suppressed adsorption while a root exudate containing a non-dialyzable, protease-sensitive substance which bound to Azospirillum was found to promote adsorption.

Azospirillum Genetics, Characterization and Physiology

Double-marked strains of Azospirillum were produced and used to evaluate factors affecting their survival in laboratory soil and field plots. A mutant unable to fix nitrogen was isolated for evaluation of its ability to stimulate grass yields in inoculated plots.

A multiple auxotroph was isolated that was unable to synthesize several amino acids but was able to grow well in association with plant roots showing that plants supply carbohydrates and amino acids.

All Azospirillum isolates examined contained plasmids differing is size, ranging in size from 100 to >1800 mega-daltons. This was determined by contour measurements of isolated plasmid DNA examined by electron microscopy. A unique method was refined permitting separation of these large plasmids by gel electrophoresis. This opened the way for detailed study of Azospirillum plasmids. By means of curing processes and electrophoretic examination, plasmids of Azospirillum have been characterized and several biosynthetic genes mapped on them. Ability to manipulate plasmids has opened the way to make genetic modifications in this species. Transformation techniques were developed for genetic analysis and manipulation. The multiple auxotrophs mentioned above were transformed to prototrophy using DNA from purified plasmid DNA.

Several hundred Azospirillum and other N_2-fixing bacteria strains were isolated from active N_2-fixing sites in Florida and grass roots imported from Africa and Latin America. Many of these have been characterized using approximately 100 physiological and morphological characteristics. The isolates tested are distinct from Spirillum and fix N_2 in the free-living state. Two species are defined; A. brasilense is strictly aerobic, whereas A. lipoferum is weakly fermentative and requires biotin. Strains differ in specific nitrogenase activity; efficiency of acetylene reduction approaches that of Azotobacter but at lower pO_2. Comparisons of mass culture (strains 13t and JM125A2) in nitrogen-containing media using high phosphate buffer vs. substrate buffering showed best results from low phosphate substrate-buffered media. Growth curves of strain 13t were established using a chemostat.

Tissue Culture Experiments

Sugarcane callus-*Azospirillum* associations have been established and successfully repeated. High nitrogenase activity has been maintained in these cultures for several months with periodic transfers. A successful tobacco-*Azospirillum* association has also been accomplished.

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

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

Plant Genotype-Bacterial Strain Screening

A simple, quick method of screening plant genotypes, evaluating bacteria on plants and studying plant bacteria interactions was developed. This method involves growing sterile seedlings in test tubes (size dependent upon plant species) on nitrogen- and carbon-free Fahraeus medium inoculated with the selected bacteria. Acetylene reduction and bacteria-root associations have been found to be reasonably consistent. Plant genotype ranking of this highly artifical system have been compared to some field data with positive correlation. This method was developed from studies evaluating several growth media and container types. Twenty genotypes of corn, sorghum and millet have been evaluated using this system, and ten bacterial strains have been used.

An intermediate level of evaluating plant genotype-bacterial strain responses and interaction in soil and in greenhouse was developed. This system utilized soil packed in 1 qt. Pyrex jars, sterilized by autoclaving. Seedling plants were transplanted into the quart jars using aseptic techniques and were kept under beakers until sterile river gravel and sterile cotton could be installed around the plants to prevent algal bloom and bacterial contamination. Irrigation was applied using a syringe, as

needed, from the top. Useful AR data was obtained but the system did not remain axenic and excessive labor was required for watering. Inoculated associations were very slow to establish and had low activity compared to natural soil controls. This system has been modified with drip irrigation and microbiological seals of waxed sand.

Training

Although the major thrust of this contract has been research and the development of methodology for grass-bacteria association studies, a large amount of training has occurred.

Twenty-five students, (MS, Ph.D, Post Doc. and Other) from 8 countries have participated in training programs. Only a few have been supported from contract funds. Of the 25 students, 4 completed the Master's degree and 7 have completed the Ph.D degree. We also have had 7 post-doctoral persons, and three visiting professors here on sabbatic leaves.

Two workshops have been held on the University of Florida campus on grass-bacteria association work. Over seventy distinguished visitors from all over the world have visited us to exchange information, present seminars and consult. A detailed list of these visiting scientists is given at the end of this report.

INTRODUCTION

This document reports the research accomplished from 4/30/80 through 4/3/81 for the Contract AID/ta-C-1376 Mod. #3, entitled "Nitrogen Fixation by Associative Grass-Bacteria Systems." The outline of "Article 1 - Statement of Work" of the contract has been used as a guide in preparing this report. The following interdisciplinary team members have cooperated in the research and have contributed the information for this report:

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Research contributions of the following graduate sutdents and postdoctoral fellows also is included in this report:

Robert Green
Dr. Elza Menezes
Dr. M. E. Mitchell
Dr. Norman Novick
Dr. Tran Minh Tien
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LIST OF TABLES AND FIGURES

Table		Page
1	Comparison of actylene reduction from selected "high activity" and "low activity" grass sites	21
2	The influence of polyethylene glycol-induced water stress on the association of *Azospirillum brasilense* and pearl millet	22
3	Soil type and drainage, plot treatments, and plant species in nursery experiments of 1979 and 1980	23
4	Statistical analysis of main factors affecting acetylene reductions.....	24
5	Ethylene production by *Azospirillum brasilense* strain SR2 cultured on sorghum root powder with cellulase and pectinase	25

Figure		Page
1	Active N_2-fixing sites in Florida, 1980	25a
2	Effect of shading on actylene reduction	26
3	Effect of N fertilizer on acetylene reduction (4 nurseries)	27

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

An *in vitro*, axenic screening technique was develoed to study plant response to bacterial inoculation, bacterial association with roots, and actylene reduction (AR) with N_2-fixing bacterial isolates. The test was used first with pearl millet, *Pennisetum americanum* (L.) Schum. and later was used with corn, *Zea mays* L. and sorghum, *Sorghum bicolor* (L.) Moench. *Azospirillum brasilense* and other N_2-fixing bacterial isolates from Florida were used in the tests. Seeds were surface sterilized and germinated on tryptic soy agar or on 5% sucrose agar in order to verify that the seeds were uncomtaminated. Seeds then were transferred aseptically to test tubes containing Fahraeus nitrogen- and carbon-free medium. Tubes were inoculated with different N_2-fixing bacteria and seedlings were grown for 1 to 2 wks. Observations on bacterial colonization of roots and root development were made. Acetylene reduction was measured by analyzing for ethylene with a gas chromatograph, after adding 10% acetylene to the tubes and incubating for 24 hr.

Acetylene reduction was monitored in test tubes containing noninoculated plants, plants inoculated with *A. brasilense*, and bacteria without plants. The acetylene reduction values usually were higher 7 days after inoculation than either 4 or 12 days after inoculation.

Root-bacteria associations were observed and rated. Strong associations were observed between *A. brasilense* strain 13t and corn seedling roots. S141A and S145 (from Florida) were very effective in colonization of Gahi-3 pearl millet roots, and JM125A2 appeared to be the most effective in colonizing sorghum roots. Some bacterial strain-plant genotype interactions were observed when different sorghum varieties were tested.

After 2 wks. the experiments with millet and corn were terminated and roots were oven dried and weighed. Root development was inhibited partially by some of the bacterial strains when compared with controls. Inoculated sorghum seedlings with both high and low AR values have been transplanted to pots, and will be transplanted to the field to continue the screening procedure.

II. COLLECTION AND CHARACTERIZATION OF GRASS-RHIZOSPHERE INHABITING, N_2-FIXING BACTERIA

A. Statewide Survey of Naturally Occurring Grass-Bacteria Associations

In 1980, a systematic search was conducted for grass-bracteria associations with high N_2-fixing capability from 57 Florida counties (Fig. 1). With the cooperation of the Florida State Extension Service, soil-plant cores (11.0 cm diameter x 15.0 cm length in plastic cylinders) were obtained from areas with little or no nitrogen fertilization where plant growth was better than expected. These cores were standardized for their plant growth and soil moisture and then were assayed for nitrogenase activity by measuring acetylene reduction. Metal cylinders (8.5 and 11.0 cm diameter) also were used for assays and the two systems were compared. Sites with the highest ethylene production were resampled. Ethylene evolution remained relatively high in nearly all sites with high original activity, and low activity sites remained constantly low in ethylene evolution (Table 1). High rates of ethylene production for Paspalum notatum 'Pensacola', P. notatum 'Argentine', Imperata cylindrica (Cogan's grass), and Eremochloa ophiuroides (centipedegrass) were 1227, 638, 629, and 522 n-moles per hr per 95 cm^2 of core surface, respectively. Dinitrogen-fixing bacteria have been isolated from the active sites.

After 2 wks. the experiments with millet and corn were terminated and roots were oven dried and weighed. Root development was inhibited partially by some of the bacterial strains when compared with controls. Inoculated sorghum seedlings with both high and low AR values have been transplanted to pots, and will be transplanted to the field to continue the screening procedure.

B. Microbial Physiology

Metabolic patterns of A. brasilense were characterized by investigating several catabolic pathways. The eventual goal of such research is to facilitate the manipulation of various edaphic factors to match the metabolic requirements of this bacteria.

Under dinitrogen-fixing conditions, A. brasilense showed comparable generation times using L-arabinose or malate as sole carbon and energy sources. This root-associated bacterium was found to metabolize L-arabinose through an oxidative pathway that also has been found in certain species of Pseudomonas and fast-growing Rhizobium. L-arabinose is converted to L-arabono-γ-lactone by an NAD(P) dependent dehydrogenase, hydrolyzed to L-arabonic acid by lactonase, and dehydrated to L-2-keto-3-deoxyarabonate (L-KDA) by dehydratase activity. In crude extracts NAD is reduced rapidly if potassium L-arabonate or DL-KDA is added as substrate. L-2-keto-3-deoxyarabonate has been found to reduce NAD at three times the rate of L-arbonate.

Alpha-ketoglutarate accumulated in crude extracts to which L-arbonate and NAD were added. It is proposed that L-KDA is dehydrated to alpha-ketoglutaric semialdehyde which them is oxidized to alpha-ketoglutaric by an NAD dependent dehydrogenation reaction. L-arabinose dehydrogenase

and L-arabonate dehydratase have been partially purified and characterized. The NAD(P) dependent dehydrogenase, which has been found to be specific for the L-arabono-configuration, has been purified 59-fold. No enhancement of activity in the presence of any divalent cation or reducing agent tested has been found.

The Km values of 75 μM and 140 μM were found with NADP and NAD as cofactors, respectively. The enzyme has a pH optimum of 9.5 in glycine buffer and was stable when heated to 55°C for 5 min. The enzyme product has been identified as L-arabono-γ-lactone. L-arabonate dehydratase has been purified 38-fold. The presence of $MgCl_2$ and $MnCl_2$ has been found to increase significantly enzyme activity. The pH optimum for the dehydratase has been found to be 7.8. The enzyme product was identified as L-2-keto-3-deoxyarabonate. In futher studies TCA cycle dehydrogenases have been assayed and indicate an active TCA cy le. The presence of b and c type cytochromes has been confirmed and their distribution between membrane and soluble fractions determined.

C. Microbial Genetics

We have shown previously by electron microscopy and gel electrophoresis that *A. brasilense* and *A. lipoferum* carry several plasmids. Each strain carries a different array of plasmids of various sizes. The sizes were shown to vary from small plasmids, typical of those seen in other bacteria, to plasmids larger than any previously reported in bacteria. Gel electrophoresis techniques have been improved to resolve the plasmids, determine their sizes, and try to determine some of the genetic functions carried on these plasmids.

Factors affecting the ability to separate these plasmids were found to include: (i) growth medium; (ii) age of the cells; (iii) time and temperature of cell lysis; (iv) initial and secondary current used to move DNA into and through the gel, as well as the length of time at each setting; and (v) agarose gel concentration.

Cells in late logarithmic-growth stages in minimal medium were chilled and lysed in wells of cold vertical gels made of 0.7% agarose. Standarized lysing mixtures and layering procedures were employed. The current was set at 2mA for 6 hr and then increased to 30 mA for 16-40 additional hours. These procedures were used to examine several isolates of *Azospirillum*. Those characterized carried from six to eight plasmids varying in size from 100 to >1800 mega-daltons (mD). The largest plasmid (1850 mD), from strain 125A2, is smaller than the *Escherichia coli* chromosome (2700 mD). No DNA band was seen on gels between the 1850 mD plasmid band and the position of the *E. coli* chromosome. This may indicate that *Azospirillum* carry all of their genetic information on six to eight units, all smaller than the *E. coli* chromosome. The total amount of DNA in the plasmid bands is four times the molecular weight of the *E. coli* chromosome.

The nif-DNA in the gel bands has been extracted by two methods and used in transformation studies to determine the genes carried on each plasmid. Biosynthetic genes have been found on each of the plasmids examined. Some genes are found in more than one plasmid and these multiple gene copies may explain the difficulty experienced in mutagenizing these bacteria.

III. ECOLOGICAL FACTORS THAT LIMIT OR ENHANCE PLANT-BACTERIUM ASSOCIATIONS

A. Laboratory Experiments

Duplication of the work of J. Bouton, where a heterogenous population of pearl millet was screened for consistent quantitative differences in acetylene reduction when plants were inoculated with A. brasilense, was attempted. Using Bouton's seed lines that were associated with high or low AR and an analagous system with A. brasilense strain 13t, it was found that the "high AR" millet lines had greater acetylene reducing capacity than the "low AR" lines. However, the amount of AR was much lower and variability was greater than he reported. Investigations of the chemical composition of the roots of both "high and low AR" lines are in progress.

Attempts were made to define this experimental system. It was found that a range of small-seeded plants, including both monocots and dicots with C-4 and C-3 photosynthesis, had AR capability when associated with A. brasilense. Agar concentration in the system also affected AR, presumably by altering the O_2 concentration of the root medium. Activity was found to be greatest at 0.2% agar. The mineral salts composition in the medium also affected AR rates. Activity was greater in Hoagland's solution than either Jensen's or Thornton's solutions. The latter two solutions have greater Ca^{++} concentrations and insoluble fractions after autoclaving, either or both of which may inhibit the plant-bacteria association or the nitrogenase activity of A. brasilense.

Polyethylene glycol (PEG) was used in the rooting medium to determine the affect of water stress on the plant-bacteria association in this system (Table 2). Nitrogenase activity was present only where no PEG was present. Plant growth was decreased with increasing water stress (PEG induced), however, numbers of viable bacteria appear to vary slightly with increased stress.

It also was found that pearl millet responded differently, in terms of nitrogenase activity, to different strains of inoculum. This data substantiates other reports that there is bacterial strain-plant cultivar specificity.

B. Field Studies

Factors affecting the nitrogenase activity of field plots inoculated with A. brasilense, were evaluated using acetylene reduction assays. Over a 2-yr. period, nine nurseries on three soil types containing three grass species were studied (Table 3). Two of the soils were well-drained, fine sands, the other was a poorly-drained, fine sand. The grass species were corn, pearl millet, and guineagrass. The effects of the following factors on nitrogenase activity were evaluated: inoculation with A. brasilense; nitrogen fertilization; plant shading; plant growth stage; plant genotype; soil moisture and pH; and soil P, K, No_3^-, and NH_4^+ concentrations.

Acetylene reduction (AR) was conducted on 8.5- and 11-cm-dia soil and plant cores after incubating them for about 18 hr in 10% acetylene at 30°C. Oxygen was reduced in the cores to about 6% during the assay. The 1056 cores evaluated were taken between July 1979 and October 1980. Highly variable acetylene reduction data were transformed using a log transformation to stabilize variances for statistical analyses of variance and multiple regressions.

Analyses of variance showed only the one nursery had a significant inoculation response (Table 4). Fifty percent shade reduced mean dry matter yields from 7798 to 5946 kg ha^{-1} and 75% shade reduced yields from 7798 to 3403 kg ha^{-1}. In 1979, shading did not affect AR activity, but in 1980 there was an inoculation and shade interaction with shade reducing AR in only the inoculated plots [AR = 18.1 - 129.0 (shade) + 24.9 (soil moisture) + 46.2 (day)] (Fig. 2). The 1979 shading test was conducted on well-drained, fine sand and AR activity was very low (mean = 51 n-moles $core^{-1}$ hr^{-1}); the 1980 test was conducted on poorly-drained, fine sand and AR activity was higher (mean = 323 n-moles $core^{-1}$ hr^{-1}).

Light nitrogen fertilization produced higher AR activities than either no nitrogen or heavier applications in each of the four fertilizer nurseries (Fig. 3). The analysis of individual nurseries did not give significant nitrogen effects, but combined analysis of the four nurseries gave a significant quadratic effect [AR = 80.9 + 0.52 (FERT) - 0.006 $(FERT)^2$]. These AR responses resemble the yield responses to inoculation we reported earlier.

Soil moisture had a significant effect on AR in four of the nine nurseries in 1979 and in three of four nurseries in 1980 (Table 4). The rainfall in 1979 was quite uniform, but in 1980 several wet-dry cycles occurred. Effects of differences in soil pH were significantly different in four of the five nurseries in 1979. However, pH soils generally had higher AR activities in three of the four nurseries. The range of pH was 5.7 to 7.2. In a follow-up experiment, applications of lime at the rate of 0, 2, and 6 metric tons ha^{-1} did not affect AR activity. The pH effect may be direct or due to some other associated factor. Soil P and K were positively correlated with AR in one nursery each.

Pearl millet genotypes had a significant effect on nitrogenase activity as measured by acetylene reduction in core assays (Table 4). Pearl millet genotypes differed in AR activity, but the genotypes with highest activities were not those that responded with yield increases due to inoculation in previous studies. In additional field studies, corn had higher AR activity than pearl millet or guineagrass.

The log transformation coupled with computer analyses using Statistical Analysis Systems (SAS) General Linear Model (GLM) procedures appears to be an effective method of analyzing variable AR data. In our studies, day of sample, soil pH (or an associated factor),

soil moisture, and nitrogen fertilization affected AR activity the most (listed in probable order of importance). Shading sometimes reduced activity, while inoculation, addition of lime, and soil P, K, NO_3^-, and NH_4^+ had little affect on AR.

C. Greenhouse Studies

Greenhouse studies are being conducted to determine the effects of seven different inoculation techniques (including pelleted, liquid, and granular forms of inoculum) on the establishment of *Azospirillum* spp. in the rhizospheres of millet and sorghum. Inoculation techniques will be chosen for field tests based on the results of the greenhouse tests.

Some experiments have been done to evaluate methods for introducing bacteria into soil and for monitoring their longevity. Peat was chosen as the vehicle for the bacteria and *A. brasilense* strains 125 and 13tSR2 were used in these studies.

Strain 13tSR2 was put in non-sterile peat, at four concentrations to evaluate the effects of inoculum concentration and competition with other microorganisms on bacterial numbers over time. Numbers of colony forming units of 13tSR2 decreased about one order of magnitude after 10 days (e.g., 1.28×10^5 to 1.12×10^4 colony forming units/g peat when applied at a rate of 4×10^5 cells/g peat) and then remained relatively stable. This held true for all inoculum levels. Total numbers of bacteria recovered from the peat remained constant at about 2×10^8 cells per gram. This suggests that a large number of cells of *A. brasilense* can be mixed into non-sterile peat and relatively high populations will be maintained.

To determine the effect of pH on the longevity of *A. brasilense*, peat samples were adjusted to five pH levels (5.5, 6.0, 6.5, 7.0, and 7.5) and the peat was sterilized by autoclaving for 2 hr on three alternative days. Again, there appeared to be an initial drop in bacterial numbers, however, the twofold to threefold reductions were not as large as those in a non-sterile system. There seemed to be little effect of pH on the numbers of cells that survived in the peat, as determined by serial dilutions and plate counts, although growth seemed to be better at pH 6.5-7.0 than at pH 5.5 and 7.5.

The initial decrease in bacterial numbers observed may be due to the method used to transfer the bacteria. They were grown in nutrient broth, spun down in a centrifuge, washed with sterile 0.085% NaCl solution, and resuspended in a fresh aliquot of the same solution. The change in nutrient status, from nutrient broth to peat, may be responsible for the tenfold reduction in viable cells. The effect of nutrient status on bacterial survival currently is being studied.

Short-term experiments were carried out to determine the total quantity of soluble carbon compounds lost by sorghum roots in their first 20 days of growth. Extrapolations based on these other

observations then were used to estimate the rate of nitrogen fixation that could be supported by the energy substrates provided by the roots. This experiment suggests that the roots of a healthy sorghum plant approaching maturity might lose carbon compounds at a rate of about 3 g per week. Although this represents a significant quantity of energy substrate, other evidence indicates that only a small portion of such substrates is available to free-living heterotrophs to support nitrogen fixation. The access to relatively small amount of carbon substrates and the low efficiency of conversion where these organisms are involved, suggest that the rate of nitrogen fixation would fall in the range of a few grams per hectare per week.

To determine the extent to which nitrogenase activity is coupled to rates of enzymatic degradation of plant tissue, an A. brasilense strain was incubated with cellulase or pectinase in the presence of a sorghum root powder. Both the active and the denatured enzymes greatly increased AR in 4-day-old cultures (Table 5). However, activity declined to zero within 10 days, whereas in a control with malate as the energy source, activity was sustained. The reason for this difference remains to be explained.

D. Phytohormone Production by Azospirillum sp.

The effects on plant growth of growth substrates produced by A. brasilense were studied. A technique was developed for extracting and concentrating plant-growth substances from both culture media and soil using a small column or cartridge packed with reversed-phase, stationary-phase material. Basic plant-growth substances (cytokinins) and acidic plant-growth substances [Indole Acetic Acid (IAA) and gibberellins] were separated and extracted from culture media and soil samples after adjusting the pH of sample solutions to 7.0-7.5 and 2.5-3.0, respectively.

Plant-growth substances in extracts from culture media and soil were separated and identified by thin-layer chromatography, high performance liquid chromatography, and bioassays. Indole-3-lactic acid, IAA, and tryptamine were produced from tryptophan by A. brasilense. Seven strains of A. brasilense, among nine strains studied, produced very high quantities of IAA (21.6-26.0 μg/ml) from tryptophan after 2 wk in agitated cultures. The other two strains produced 0.3 and 0.6 μg/ml IAA. Production of IAA increased as the tryptophan concentration in the growth media was increased from 1 to 100 μg/ml. Concentration of IAA also increased with the age of the culture unitl the bacterial culture reached the stationary growth phase. Shaking favored the production of IAA, especially in a medium containing nitrogen. A small but biologically significant amount of gibberellin was detected in culture supernatants that increased with incubation time, but total gibberellin in cells decreased as the bacterial population decreased after 1 to 3 wk in agitated culture. Substances that inhibited the growth of lettuce seedlings were present in the culture media. A minute amount of cytokinin-like substances also was detected (equivalent to about 10^{-4} to nearly 10^{-3} μg/ml zeatin). Adding adenine or ammonium chloride and shaking the culture did not affect the cytokinin production.

Of the plant substances studied, only gibberellin-like compounds (0.3-0.7 μg/kg dry soil) were detected in soil extracts. Inoculating soil with an A. brasilense culture caused gibberellin activity in the soil to increase.

The survival of Azospirillum sp. in a peat-based inoculant was affected by the pH of the peat and by incubation temperature. The bacterium could be maintained at 10^8 cells per gram of peat for at least 3 months by adjusting the pH to 6.4-6.8 and incubating at 25 or 35C. In the sorghum rhizosphere, the introduced Azospirillum sp. declined rapidly during the first week after inoculation and declined slowly thereafter. Two months after inoculation, the Azospirillum sp. population in the soil was 10^3 to 10^4 cells per gram of wet soil, depending on the amount of inoculant added initially. The bacterial population on roots was dependent on the number of bacteria in the surrounding soil. Bacteria in the sorghum rhizosphere were easily removed by washing the roots and numbered 10^2 to 10^3 cells per gram of wet roots 2 months after inoculation.

The morphology of pearl millet roots changed when plants in solution culture were inoculated. After inoculation the number of lateral roots was increased, and all of the laterial roots were covered densely with root hairs. Experiments with pure plant-growth substances showed that gibberellin caused increased lateral root production. Cytokinin stimulated root hair formation but reduced lateral root production and elongation of the main root. Combinations of IAA, gibberellin, and kinetin produced changes in root morphology of pearl millet very similar to those produced by inoculation with A. brasilense.

E. Cell Suspension Studies

Nitrogenase activity AR was observed in Azospirilum brasilense cultures grown on conditioned medium from embryogenic pearl millet and guineagrass cell suspension cultures, but no bacterial growth or AR was observed on unconditioned medium. A peak of maximum activity was obtained with medium harvested midway through a 13-day culture period. The time of the peak activity could be shifted slightly by changing the concentration of 2,4-D in the medium, apparently because such changes altered the rate of growth of the suspension cultures. The decline in activity later in the culture period followed a decline in the concentration of glucose and fructose in the medium as well as a slight increase in total nitrogen. Non-embyrogenic suspension cultures of pearl millet and guineagrass supported little or no bacterial growth or AR.

A trans-filter apparatus, in which plant cell suspension cultures are spearated from A. brasilense by 0.45μ membrane filter, has been utilized to study the effect of plant cells on ARA. Cell suspension cultures from pearl millet and several other species induced AR in A. brasilense, but no detectable transfer of nitrogen took place from the bacterial to the plant cells. These experiments were carried out using ^{15}N gas.

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

Pearl millet plants were grown in large glass tubes (3 x 30 cm) in sand, watered with Hoagland's solution (without N), and treated with either live or autoclaved cells of A. brasilense strain 13t. After 4 wk, plants were exposed to $^{14}CO_2$ for 5 hr, followed by "cold" CO_2 for 48 hr, after which the plants were harvested. Rates of photosynthesis, measured by CO_2 incorporation, did not differ significantly between treatments, nor did dark respiration as measured by CO_2 release. Soil respiration did not show significant differences between treatments. More ^{14}C labelled material was found in the leachate from plants inoculated with live bacteria than in that from plants inoculated with autoclaved bacteria. The importance and mechanism of this result are under investigation, as are attempts to identify the compounds released into the rhizosphere.

An experiment was carried out to determine whether respiration rates of root systems might be used to identify the rate of microbial activity in the rhizosphere and to determine whether such measurements would indicate reliably the extent of root-microbial interactions following inoculation with nitrogen-fixing bacteria. The data collected to this point have not been encouraging. Inoculated plants do not have consistently higher root respiration rates under the experimental conditions used. Further tests are in progress.

PUBLICATIONS
1980-1981

1. 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.

2. 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. 1

3. Albrecht, S. L. and Y. Okon. 1980. Cultures of Azospirillum Methods in Enzymology. [Eds.] S. P. Colowick and N. O. Kaplan. 69c:740-750. 1

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

5. 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. 2

6. 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. 3

7. Gaskins, M. H. and K. J. Lee. 1981. Effects of bacterial inoculants on carbon metabolism and nitrogen requirements of non-legumes. p. 488. [In] Current Persepctives in Nitrogen Fixation. Austrialian Academy of Science, Canberra. 534 pp. 4

8. Hubbell, D. H. and M. H. Gaskins. 1980. "Cryptic" plant root-microorganism interactions. What's New in Plant Physiology 11:17-19. 2

9. Menzes, E. M. and D. E. Duggan. 1981. Transformation of Azospirillum biosynthetic genes in plasmid DNA. p. 131. [In] Abstracts of 81st Annual Meeting of the American Society for Microbiology. Am. Soc. Microbiol., Washington, DC. 365 pp. 5

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

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

12. Schank, S. C., Rex L. Smith, and G. C. Weiser. 1980. Responses of two pearl millets grown in vitro after inoculation with Azospirillum brasilense. Soil Crop Sci. Soc. Fla. Proc. 39:112-117. 3

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

14. 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. Env. Micro. 41:342-345.

15. Schank, S. C., Rex L. Smith, and J. R. Milam. 1981. Acetylene reduction activity of several sorghum and N_2-fixing bacterial associations. Submitted as an abstract to "Sorghum in the 80's" ICRISAT, Hyderbad, India.

16. Schank, S. C. and Rex L. Smith. 1981. Statewide survey for grass-bacteria ssociations. p. 7. (Abstr.) Southern Assoc. Agric. Sci. Meeting Atlanta, GA. 18 pp.

17. 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.

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

19. Umali-Garcia, Mercedes, D. H. Hubbell, M. H. Gaskins, and F. B. Dazzo. 1980. Association of Azospirillum with grass roots. Appl. Env. Micro. 39:319-326.

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

21. Wood, A. A. and D. E. Duggan. 1981. Mulitple large plasmids of Azospirillum. p. 131. [In] Abstracts of 81st Annual Meeting of the American Society for Microbiology. Am. Soc. Microbiol., Washington, D.C. 365 pp.

22. Wood, Alvin G. and D. E. Duggan. 1981. Antibiotic resistant mutants to evaluate survival of Azospirillum in soil. Manuscript completed to be submitted to Appl. Env. Micro.

23. Wood, Alvin G. and D. E. Duggan. 1981. Multiple large plasmids of Azospirillum. Abstract submitted to ASM meeting, Dallas, TX.

TABLE 1. Comparison of acetylene reduction from selected "high activity" and "low activity" grass sites.

	Location	Grass species	Ethylene (n-moles/hr/cm^2 core surface) 1st assay	2nd assay
"High-Activity"	Madison County	Pensacola Bahia	1226	1057 (12)
	Putnam County	Pensacola Bahia	1094	210 (4)
	Union County	Broad-leaf Bahia	639	739 (6)
		Coastal Bermuda	1088	74**(12)
	Manatee County	Cogan's grass	629	465 (12)
	Bradford County	Centipede	522	311 (6)
"Low-Activity"	Madison County	Pensacola Bahia	33	33 (12)
	Taylor County	Centipede	1	36 (12)
	Lake County	Pampas grass	7	39 (6)
	Levy County	St. Augustine	308	39 (8)
	Baker County	Bermudagrass	25	32 (8)

* The number of cores sampled is shown in the parentheses.

** The coastal bermuda had been cut and was being grazed on the second sampling date.

TABLE 2. The influence of polyethylene glycol-induced water stress on the association of *Azospirillum brasilense* and pearl millet.

Water potential of medium[a] (bars)	Ethylene evolution[b] (nmoles/hr/g dry root)	Bacterial numbers[c] (x 10^9)	Plant dry weight (grams)
0	2.35	8.2	0.2414
-2.5	0	5.0	0.1980
-5.0	0	1.5	0.1956
-7.5	0	2.4	0.1749
-10.0	0	1.1	0.1014

[a]Gahi 3 pearl millet seedlings were grown in 60 ml of Hoaglands' agar in large test tubes in a greenhouse for 10 days.
[b]Nitrogenase activity was measured by quantifying acetylene reduction following the introduction of 10% acetylene into the tubes.
[c]Numbers of colony forming units on nutrient agar plates following dilution plating of the plant-growth medium and roots (10^8 bacteria were placed in each tube initially).

TABLE 3. Soil type and drainage, plot treatments, and plant species in nursery experiments of 1979 and 1980.

Nursery Information		
Nursery	Special Treatment	Plant Species
CORNMIL[1/]	N fertilization	corn, millet
MILVAR[1/]	plant genotype	millet
MILSHA[1/]	shading	millet
OLDGG[1/]	N fertilization	guineagrass
PHMIL[1/]	lime	millet
OLDGMC[1/]	N fertilization	corn, millet, guineagrass
BRU[2/]	N fertilization	millet, guineagrass
BRUSHA[2/]	shade	millet
NIFGA[3/]	*nif*⁻ mutant	millet

[1/] Arrendondo fine sand - well drained.

[2/] Wauchaula fine sand - poorly drained.

[3/] Kindrick sand - well drained.

TABLE 4. Statistical analyses of main factors affecting acetylene reduction.

Nursery	Number of Observations	Inocu-lation	Time (days)	Soil Moisture (%)	Soil pH (pH units)	N† fert. (kg/ha)	Shading	Added Lime	Plant Geno-type	P	K
1979											
BRU	71	*	**	ns	**	ns	-	-	-	*	(.07)
CORNMIL	65	ns	**	ns	*	(.09)	-	-	-	ns	**
MILVAR	54	ns	ns	ns	ns	-	-	-	*	-	ns
MILSHA	34	(.10)	**	*	**	ns	ns	-	-	ns	ns
OLDGG	48	ns	ns	ns	*	ns	-	-	-	ns	ns
1980											
BRUSHA	309	ns	**	**	ns	-	*	-	-	-	-
PHMIL	251	-	ns	**	ns	-	-	ns	-	-	-
OLDGMC	140	ns	ns	**	*	*	-	-	-	-	-
NIFGA	84	ns	ns	ns	ns	-	-	-	-	-	-
Total	1056										

† combined N fertilization data was highly significant.

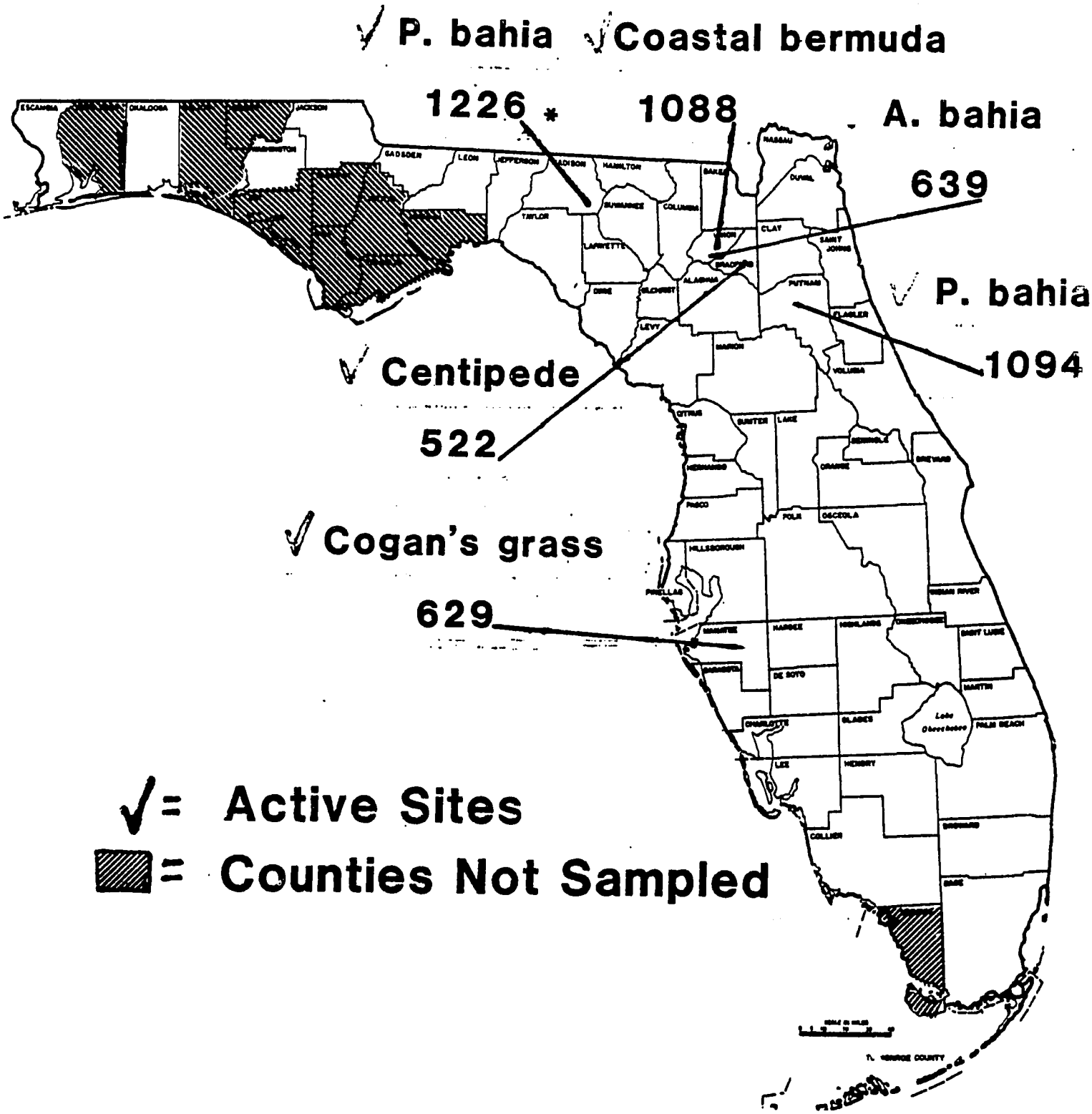
ns not significant, * significant at .05 level, ** significant at .01 level, - not determined.

TABLE 5. Ethylene production by *Azospirillum brasilense* strain SR2 cultured on sorghum root powder with cellulase and pectinase.[1/]

Enzyme	C_2H_4 production ($nM \cdot hr^{-1}$) Culture age (days) 4	10	12
Cellulase	523.3±54.7	0	0
Cellulase, denatured	494.7±47.8	0	0
Pectinase	183.2±56.8	0	0
Pectinase, denatured	103.1±22.6	0	0
Control (malate)	12.5± 1.4	40.9±12.0	16.1± 6.6
Control	40.3± 9.3	0.5	0

1/ Enzymes were in citrate-phosphate buffer at pH 7. The root powder concentration was 5 $g \cdot L^{-1}$.

FIG. 1. "ACTIVE" AR SITES IN FLORIDA 1980.



* Nanomoles of ethylene evolved per hour per core (95 cm2 surface area)

Figure 2 EFFECT OF SHADING ON ACETYLENE REDUCTION

(1980 BRUSHA)

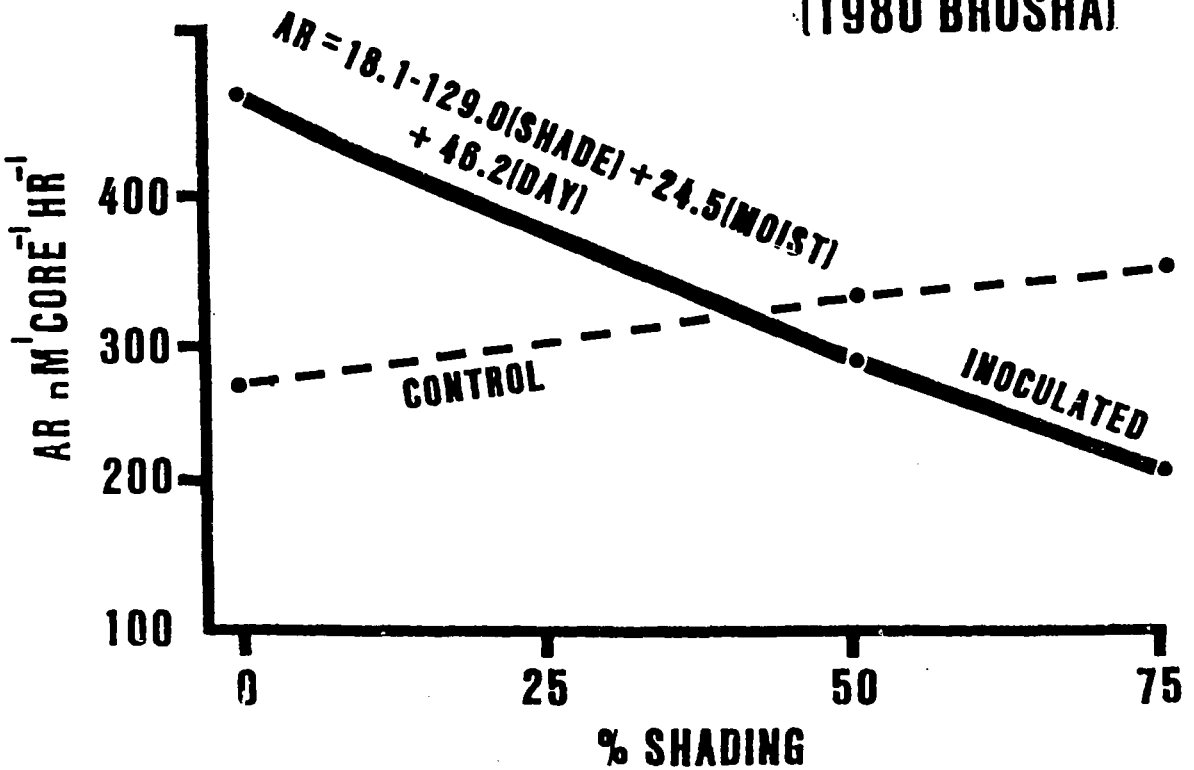
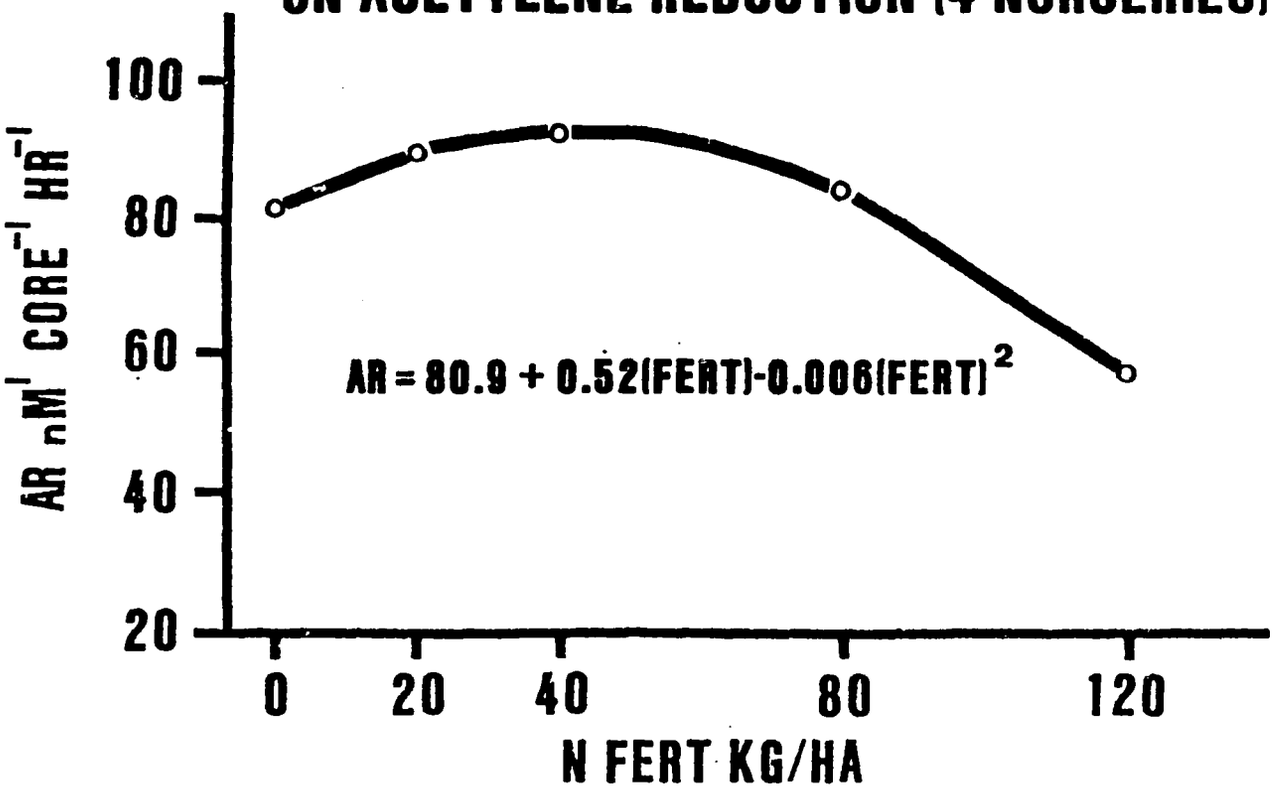


Figure 3 EFFECT OF N FERTILIZER ON ACETYLENE REDUCTION (4 NURSERIES)



CROSS-LINKAGES

A. Domestic

Important cross-linkages with plant-bacterial root association research in the U.S. are maintained by active participation in Regional project S-130. The Florida group was active in the forming of this regional in 1978, when Dr. S. H. West was the administrative advisor, and Dr. D. H. Hubbell was the project chairman. The project title of S-130 is: "Associative Nitrogen Fixation in Grasses." Members of the Florida group continue to contribute and interact in annual meetings and in reports of this cooperative project.

B. Overseas

1. Australian scientists have been especially interested and cooperative in experimentation. Cross-linkages have been established and maintained since 1976, when Dr. Alan Gibson of CSIRO in Canberra visited Florida. 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 of our group took place in Australia in 1980. In 1981, Dr. A. D. Rovira of CSIRO Division of Soils in Adelaide is scheduled as a consultant and visiting scientist to our group.

2. Cross-linkages with ICRISAT in India have been established on sorghum and millet since the visits to Florida of Drs. Nambiar and Subba-Rao in May 1978 and July 1979. In 1980, Drs. S. H. West and Rex L. Smith individually met with Dr. Peter Dart of ICRISAT and have worked on preliminary research cooperation not only at ICRISAT, but with Mali in Western Africa. Dr. Dart visited Florida in March 1981, presented a seminar and continued discussions with our researchers and with International Programs (Dr. Popenoe) to finalize a cooperative agreement between Mali, ICRISAT, USAID and Florida. Current plans are for Dr. S. C. Schank to visit and attend the "Sorghum in the 80's" conference at ICRISAT in November 1981 and to continue these important cross-linkages.

3. CIAT has not shown as much desire to cooperate on grass-bacteria associations. However, Drs. Quesenberry and Hubbell from our group visited CIAT at the Cali meetings in March 1981 and we are ready and willing to cross-react when CIAT indicates an interest.

4. Cross-linkages with Egypt and C.I.D. (Consortium for International Development) have been greatly enhanced by visits of Dr. S. H. West to Egypt in 1980 and 1981. Although their primary interest is in N_2-fixation by legumes, the proposals being studied may also involve grass bacteria systems as well.

5. Cross-linkages with BIFAD are maintained through Dr. Hugh Popenoe, Director of University of Florida's International Programs. He is continually involved in international liaison and we appreciate his support and direction.

6. T.A.D. (Tropical Agricultural Development) grants for the Caribbean, Florida and Hawaii are also in cross-linkage, with Dr. M. H. Gaskins of our group serving in a formal liaison position with TAD. In the past, excellent cooperation has been done on grass bacteria inoculation in the Bahamas and in Puerto Rico.

7. We have had contacts with Dr. Yaacov Okon in Israel, and exchanged information with him. (In addition we are planning to submit a BARD proposal with Dr. Okon).

STUDENT AND POST DOCTORAL TRAINING PROGRAMS

AID/ta-C-1376

NAME	Country of Origin	Duration of Training	MS	PhD	Post Doc	Other
Mercedes Umali-Garcia	Philippines	4 years		X		
Sharon Matthews	U.S.	3 years		X		
Alvin G. Wood	U.S.	5 years		X		
Gary Benzion	U.S.	2 years	X			
Christine Carr Dykstra	U.S.	2 years	X			
Howard Berg	U.S.	2 years			X	
Robert Green	U.S.	3 years		X		
Wai-Jane Ho	Taiwan	2 years		X		
Elza Menezes	Brazil	4 years		X		
Norman Novick	U.S.	4 years		X		
Valerie Pence	U.S.	1 year			X	
Tran Minh Tien	Viet Nam	4 years		X	X	
Glen Weiser	U.S.	4 years	X	X		
Arden A. Baltensperger	U.S.	4 months				X
M. E. Mitchell	U.S.	1 year			X	
Mario Soter	Brazil	1 year		X		
Chiu-Chung Young	Taiwan	3 weeks				X
H. G. Diem	Senegal	3 months				X
J. H. Bouton	U.S.	4 years		X		
Joe Salvo	U.S.	2 years	X			
Prem Patel	India	1 year		X		
Pushpam Shankar	India	6 months		X		
Peggy Akins	U.S.	1 year			X	
Vilma Vasil	India	2 years			X	
Hart Spiller	Germany	1 year			X	

WORKSHOPS, SEMINARS AND SCIENTIFIC
TRAINING AT UNIVERSITY OF FLORIDA

NAME	Home Institution	Workshop	Seminar	On-site Visit	Consultant
Dr. Art Newman	CSRS, USDA	X		X	
Dr. Robert Klucas	USDA & Univ. of Nebraska	X		X	
Dr. Charles Sloger	USDA, Beltsville	X		X	
Dr. Robert Taylor	The Bahamas			X	
Dr. Alden Baltensperger	New Mexico State Univ.	X		X	
Dr. Ed Dunigan	Louisiana State Univ.	X		X	
Mr. Sam Young	Louisiana State Univ.	X		X	
Dr. Rick Weaver	Texas A & M Univ.	X		X	
Ms. Sarah Wright	Texas A & M Univ.	X		X	
Dr. Noel R. Kreig	Virginia Polytechnic		X	X	
Dr. Warren Silver	Univ. of S. Florida		X	X	X
Dr. Yaccov Okon	Univ. of Wisc. Dupont, & Israel		X		
Dr. Robert Burris	Univ. of Wisconsin	X		X	
Dr. Clanton Black	Univ. of Georgia	X		X	
Dr. Alan Gibson	CSIRO, Canberra, Australia			X	
Dr. Johanna Dobereiner	EMBRAPA, Km 47, Brasil		X		
Dr. William Judy	AID - Africa			X	
Dr. Tony Cunha	Calif. Poly, San Luis Obispo			X	
Dr. Christine Kennedy	ARC, Unit, Sussex., England		X	X	X
Dr. Andy Johnston	John Innes Institute, England		X		X
Dr. Dale Moss	Oregan State Univ.			X	
Dr. A. D. Rovira	CSIRO, Adelaide Australia		X	X	X
Dr. Ed L. Schmidt	Univ. of Minnesota		X		X
Dr. H. L. Diem	Senegal		X		
Dr. J. D. Tjepkema	Brazil & Oregon State	X		X	
Dr. H. J. Evans	Cregon State Univ.	X		X	
Dr. Peter Van Berkum	USDA Beltsville, MD	X		X	
Dr. Glenn W. Burton	USDA Tifton, GA	X		X	
Dr. Fenton MacHardy	Soil Sci., Univ. of Albeta Canada			X	
Dr. Peter Dart	ICRISAT, Hyderbad India		X	X	
Dr. John Day	Rothamstad, England	X			
Dr. Felix Albani	FAO Rome, Italy			X	
Dr. E. F. Henzell	Chief, CSIRO, Brisbane, Australia			X	

WORKSHOPS, SEMINARS AND SCIENTIFIC
TRAINING AT UNIVERISTY OF FLORIDA

NAME	Home Institution	Work-shop	Seminar	On-site Visit	Consultant
Dr. Dave Zuberer	Texas, A & M Univ.	X		X	
Dr. Lowell D. Owens	USDA Beltsville, MD	X		X	
Dr. Gordon Lethbridge	Macaulay Inst. Scotland		X	X	
Mr. Tom McDonald	E.M. Univ. of Florida		X		X
Dr. Bob Barnes	USDA SEA Beltsville			X	
Dr. John Torrey	Harvard Univ.		X	X	
Dr. Mike Budden	Rothwell Plant Breeders, England			X	
Dr. Josef Guggenhein	Agric. Research, Israel			X	
Dr. Antonio Sotomayor-Rios	USDA, Puerto Rico	X		X	
Dr. Jake Halliday	CIAT, Colombia (Niftal Hawaii - now)			X	
Dr. W. L. Fuglie	UFR/RA/AID Africa	X		X	
Dr. Aubrey Ventor	Ag Counselor, South Africa			X	
Dr. James Starling	Pennsylvania State Univ.			X	
Dr. Dan Knievel	Pennsylvania State Univ.			X	
Dr. D. E. Stevens	Pennsylvania State Univ.			X	
Dr. N. Nambiar	ICRISAT, INDIA			X	
Dr. Subba-Rao	ICRISAT, INDIA		X	X	
Dr. Arnold Anthony Dorsett	Grass Researcher, Bahamas			X	
Dr. Raymond Jones	CSIRO, Townsville, Australia			X	
Dr. John Wilson	CSIRO, Brisbane, Australia			X	
Mr. Wong Choi Chee	MARDI, Kuala Lumpor, Malaysia			X	
Dr. Carlos Neyra	Brasil & Rutgers Univ.	X		X	
Dr. Dan Raveed	Kettering Inst., Ohio		X	X	
Dr. Tomio Yuswida Yoshida	Univ. of Tsukuba, Japan		X	X	
Dr. Cam Gilmour	Univ. of Idaho	X		X	
Dr. John T. Gilmour	Univ. of Arkansas	X		X	
Dr. ISAO Tarumoto	Grasslands Inst., Tochigi, Japan			X	
Dr. Luiz A. Brasil	Intl. Coop., Brasilia Brasil			X	
Dr. Emil Q. Javier	Director, Inst, of P.B. Philippines			X	
Dr. Ian Staples	Walkamin, Queensland, Australia			X	
Dr. Allan Jones	CIAT, Colombia			X	
Dr. G. Oblisami	Coimbatore, India			X	

WORKSHOPS, SEMINARS AND SCIENTIFIC
TRAINING AT UNIVERSITY OF FLORIDA

NAME	Home Institution	Work-shop	Seminar	On-site Visit	Consultant
Dr. Ralph F. W. Hardy	Dupont, Wilmington, Delaware	X		X	
Dr. Peter Heytler	Dupont, Wilmington, Delaware	X		X	
Dr. G. O. Throneberry	New Mexico State University	X		X	
Dr. N. M. De Sousa Costa	EPAMIG - Minas Gerais Brasil			X	
Dr. Lourival Do Carmo Monaco	Inst. Biologico, Campinas, Brasil			X	

AID/ta-C-1376-EQUIPMENT

Item	Date of Order	Date of Receipt	Price Paid	Location
Gas Chromatograph #1	3/25/77	5/09/77	$3,997.00	516 Bartram E
Top Load Balance	9/08/77	10/15/77	$1,140.45	PSL
Tran m tted Halogen Light Source for microscope	11/13/77	12/08/77	$2,269.00	McC 3197
Phase contrast and Dark Field Condenser	11/13/77	12/08/77	$1,530.00	McC 3197
Double Unit Microferm Fermentor	3/09/77	4/29/77	$8,688.00	516 Bartram 747 E
Gas Chromatograph #2	1/08/80	2/06/80	$6,960.00	PSL
Incubators (2)	1/07/80	2/08/80	$ 641.35	PSL
Water Purifier	1/07/80	2/05/80	$ 825.00	McC 3197
Water Bath Shaker	3/12/80	6/10/80	$1,221.50	PSL
Laminar Flow Hood	11/14/80	12/10/79	$2,154.00	PSL
Hygrothermograph	3/12/80	4/02/80	$ 550.40	PSL
Convection Oven	1/07/80	2/12/80	$ 769.00	McC 3197
Balance	3/12/80	4/01/80	$1,819.50	McC 2164
Dissolved oxygen controller & recorder	3/12/80	4/01/80	$ 824.65	516 Bartram E
Shaking Water Bath	3/12/80	4/02/80	$1,221.50	120 ME

WORK PLANS AND CURRENT RESEARCH NEEDS

AID/ta-C-1376

1981-82

Based on the project review held May 28-29, 1981 at the University of Florida, we plan to continue work as previously outlined on project No. 931-1004. The same major goal, overall purpose and research objectives are being followed by our multidisciplinary group.

Some of the new work planned under the six research objectives is outlined below:

A. Establishment of axenic plant-bacteria systems.

1. Testing a seedling assay for bacterial stimulation of grasses using inert plant support medium and nutrient solutions, then transplanting to greenhouse and/or field.

2. Search for nif^- bacterial phytohormone mutants and compare them with non-mutated lines under axenic systems.

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

1. Determine whether liquid chromatography separation/detection techniques can be used as a reliable, routine non-destructive method for quantitatively measuring N_2-fixation by root associated organisms.

2. Evaluate a system based on photometric determination of N isotopes as a method for quantitating low levels of N_2-fixation by root associated organisms.

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

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

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

6. Identify field isolates from core samples.

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

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

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

(c) Characterize these mutations biochemically.

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

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

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

C. Examination of bacterial strain and plant genotype specificity.

1. Evaluate various sorghum genotypes in the field for high AR associations.

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

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

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

5. Characterize acetylene reduction on different grass genotypes at BRU field plots.

6. Follow-up plant genotype-bacteria associations in most active soils cores collected from various counties in Florida. (Moisture, OM, micronutrients, etc.).

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

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

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

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

2. Examine and characterize microbial ecology populations of the phyllosphere of monocotyledons by several methods -- isolation, scanning election microscopy, and acetylene reduction.

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

4. Study factors affecting nitrogenase activity in the field.

5. Analyze relationships between pH and nitrogenase activity.

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

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

8. Study hydrolytic enzymes produced by bacteria.

E. Quantification of CO_2 and N_2 fixation.

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

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

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

   (c) Measure root and microorganism metabolism.

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

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

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

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

   (c) Determine the rate of nitrogen transferred from N_2-fixing bacteria to crop plants.

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

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

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

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

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

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

2. Establish suspension culture systems with pearl millet.

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

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

Expenditures (by Outputs) 4/01/80 to 4/01/81	MICRO A89	AGRON A90	SOIL A91	TOTAL
Overhead	$18,673.32	$ 24,773.36	$3,248.43	$ 46,695.11
A. Establishment of axenic plant-bacteria systems.	15,948.86	13,298.32	-0-	29,247.18
B. Collection, characterization and modification of grass rhizosphere N_2-Fixing bacteria.	29,239.56	15,957.98	-0-	45,197.54
C. Examination of bacterial-strain genotype specificity.	2,658.14	19,504.20	-0-	22,162.34
D. Ecological factors that limit or enhance plant-bacteria interactions.	3,721.40	26,596.64	-0-	30,318.04
E. Nitrogen and CO_2 fixation by plants and associated rhizosphere organisms.	-0-	13,298.32	-0-	13,298.32
F. Association of N_2-fixing bacteria with plant tissue culture material.	1,594.89	-0-	6,369.49	7,964.38
Actually Spent	$71,836.17	$113,428.82	$9,617.92	$194,882.91

PROJECT NUMBER
PROCESS
ACTION
INITIALS
CATALOGUE
ABSTRACT
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