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PROJECT TITLE

# NITROGEN FIXATION BY ASSOCIATIVE GRASS-BACTERIA SYSTEMS

BY

THE UNIVERSITY OF FLORIDA

# INTRODUCTION

This document reports the research accomplished during 1978 for the Contract AID/ta-C-1376 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.

Dr. S. H. West, Coordinator
Dr. K. H. Quesenberry, Agronomy
Dr. Rex L. Smith, Agronomy
Dr. S. C. Schank, Agronomy
Dr. D. H. Hubbell, Soil Science
Dr. M. E. Tyler, Microbiology and Cell Science
Mr. J. R. Milam, Microbiology and Cell Science
Dr. D. E. Duggan, Microbiology and Cell Science
Dr. I. K. Vasil, Botany
Dr. M. H. Gaskins, Agronomy

In addition, the following graduate students have completed theses and dissertations contributing information for this report.

Mercedes Umali-Garcia
Sharon Matthews
Alvin G. Wood
Gary Benzion
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A new project proposal, outlining the continuations of this work through 1981, has been submitted and approved (Ammendment #3 AID/ta-C-1376). It outlines the research we believe will be most productive.

Manuscripts resulting from our research are cited in the Appendix.

I. Evaluation of Azospirillum-Grass Associations and Factors Affecting the Associations.

A. Field Trials

Field trials were conducted in 1978 to study the effectiveness of inoculating grasses with Azospirillum brasilense to increase dry matter and protein yields. In addition, trials were conducted to evaluate the effects of plant genotype, shading, mulching with crop residues, soil moisture, time of planting and nitrogen fertilizer on the Azospirillum grass associations.

Eight inoculated field tests studying the effects of various factors on inoculation response were conducted. Inoculation was accomplished by applying a mixture of two strains of Azospirillum brasilense, strains JM125A2 and $13t^{sr}$ (with the exception of one nursery getting only 13t). Inoculum was prepared from mass cultures of strain $13t^{sr}$ or JM125A2 grown in a New Brunswick Fermenter, using the "SB" medium containing combined nitrogen. Five hundred milliliters of overnight "starter" culture of Azospirillum were added to 10.5 l of medium in the fermenter. The cultures were harvested after 16 h at 36 C, aeration being about one SCFH. Mass cultures of strain $13t^{sr}$ averaged 1 to 10 x $10^8$/ml CFU, JM125A2 averaged 3 to 12 x $10^8$/ml CFU. Half of each batch of cells was sterilized by autoclaving at $121^{\circ}$ C for 30 min for control inoculum.

Live culture was applied at the rate of $10^8$ cells/cm row and autoclaved culture was applied at the same rate to the control plots. Two applications were made, one at planting, the other a week later. Inoculum was injected into the soil in a 4 inch band beneath the seed

or on each side of the row of plants by a specially constructed applicator. In 1977, inoculum was sprinkled over the area which was then irrigated. The new applicator placed the bacteria in moist soil in the root zone and removed the necessity of irrigation.

Four of the field trials measured response to inoculation over three or four nitrogen fertilizer rates. Two of these were with Panicum maximum (guinea grass); one on a well drained site (AGRGG) the other a wet site (BRUGG). The dry site was Arredondo loamy fine sand, a member of the loamy siliceous, hyperthermic, family of Grossarenic Palendult pH 6.3-6.5; the wet site was on Myakka fine sand. One trial was with Pennisetum americanum (pearl millet) on the wet site (BRUMIL), and the last, Zea mays (corn) on the well drained site. The two guinea grass tests produced significant (P= 0.05) dry matter yield increases (14% to 26%) due to inoculation. Significant yield increases were noted in the zero rate of nitrogen fertilizer as well as intermediate rates of 20-80 kg N/ha. Unlike in other tests, the pearl millet showed a negative response to inoculation (P= 0.06) but excess water restricted its yield to about 20% of normal. The corn did not show a yield response.

The effects of plant genotype, shading, mulch and time of planting were studied in a large nursery, on the well-drained soil, in four replicated experiments designated MILVAR, MILSHADE, MILMUL and MILTIME, respectively. Four pearl millet genotypes were critically compared for the third year. Inoculated Gahi-3 produced 5% more dry matter than the uninoculated control. The inbred pollen parent (Tift 186), the male sterile, inbred maternal parent (Tift 23DA), and its maintainer line (Tift 23DB), gave very little or no response. Even though response was too low for statistical significance,

the trends were similar to previous results where Gahi-3 responded and the other genotypes did not.

Shading was superimposed on inoculated and control field plots of Gahi-3 by shade screens. The use of two different screens which blocked 75% or 50% of the light, along with no shading, permitted three treatments. The 75% and 50% shading reduced dry matter yields by 58% and 40%, respectively. No statistically significant responses to inoculation were observed.

Mulching inoculated and control plots with bermudagrass straw increased yields of Gahi-3 by 5% ( significant at P= 0.05). Inoculated non-mulched Gahi-3 produced 6% more dry matter than unmulched control but this was not statistically significant. Late planting of Gahi-3 reduced yields but again no statistically significant responses to inoculation were noted.

The lack of meaningful inoculation responses in these four tests points out a most serious problem for research and application of these associations. Inoculation was done with a mixture containing the double marked mutant strain $13t^{sr}$ to facilitate monitoring bacterial populations over the season (see Section B Bacterial Persistence).

## Field Acetylene Reduction

Nitrogenase activity was estimated over the growing season by means of acetylene reduction assays conducted using cores 7.5 cm dia x 17 cm long. Cores were taken from 10 a.m. until noon on each sampling date by first cutting the plants one inch above the soil level then cutting the core to a depth of 17 cm with the tube centered over the plants. Core tubes were immediately sealed, the free space flushed with argon, then 10% acetylene added. This treatment lowered

the $O_2$ concentration to about 0.10 atm but still gave linear acetylene reduction rates over the 18-20 hr. inoculation period.

Acetylene reduction rates were generally low and variable. Two nurseries BRUMIL (Beef Research Unit-millet) and the corn nursery had much higher activities than the other nurseries. BRUMIL was a wet site and we suspect the wet conditions contributed to the higher activity. Other species on the well drained site had low nitrogenase activity so the increased activity of the corn was probably due to that specific plant-bacteria interaction. Bare inoculated soil was assayed at each sampling date and in no case was the acetylene reduction higher than 4 n moles/core/hr, a very low value. This suggests that plant roots are necessary for elevated acetylene reduction rates in our systems.

Interactions of acetylene reduction and other variables were studied through the use of multiple correlations. In no case was acetylene reduction correlated with inoculation treatment. This indicates that either Azospirillum are highly mobile and spread rapidly to control plots or that indigenous microorganisms are contributing the bulk of the nitrogenase activity. We tend to believe the latter is occuring.

The following significant correlations were noted. The millet shade experiment showed a significant positive correlation between nitrogenase activity and plant wet weight and dry weight of $r^2$= 0.24 (P= 0.04) and $r^2$= 0.26 (P= 0.024), respectively; and a negative correlation between nitrogenase with shading of $r^2$=0.21 (P= 0.08). The millet variety test gave a negative correlation between total

wet and dry matter yields and nitrogenase, activity $r^2 = -0.24$ (P= 0.07). The three genotypes responded differently with Tift 23DA having the most active nitrogenase, Gahi-3 was intermediate and Tift 186 least active. The corn nursery also showed a negative correlation of nitrogenase activity with dry matter yield, $r^2 = -0.69$ (P= 0.059). Shading probably decreases nitrogenase activity. Both positive and negative yield-nitrogenase correlations were noted. Tift 23DA, with the highest nitrogenase activity was also lowest yielding, which would account for the negative yield-nitrogenase correlation. The non-shaded pearl millet had a higher yield than the shaded; unshaded also had higher nitrogenase. These relationships would give positive yield-nitrogenase correlations without nitrogenase affecting yield. Other correlations with nitrogenase and variables, wet and dry matter yield, percent dry matter, treatment (N fertilizer, time of planting and mulch) were not significant.

## Greenhouse Trials

An experiment was conducted in the growth room at CSIRO's Cunningham Laboratory in Brisbane, Australia, using two soil types. One from Narayen a heavy clay, pH 6.8, 0.299% N, and the other from Beerwah a sand, pH 6.2, 0.042% N. Hybrid digitgrass, X46-2 (USDA PI 421785), was established in cores 12 cm in diameter and 16.5 cm in length. Nutrient solutions, without nitrogen, were added at the beginning and 4 weeks after seedling emergence. Inoculation with live bacterial suspensions of both Australian and Brazilian isolates of *Azospirillum brasilense* and autoclaved bacterial cultures for controls were accomplished when seedlings were 10 days of age and again at 35 days. Acetylene reduction of intact cores was measured 5 times during the experiment. For this assay, cores were incubated

in acrylic plastic chambers under a 10% acetylene-air atmosphere for 24 hrs. At the end of the experiment, dry matter and N content were measured. Contact microscopic slides for Azospirillum population studies were also obtained at intervals during the experiment.

Dry matter yields of the inoculated digitgrass on sandy soil were 23% higher than the controls. On the clay soil, the yield increases due to inoculation were 8.5%. Higher yielding plants had lower nitrogen content. Nitrogenase activity as estimated by acetyline reduction was variable and ranged from 4 to 82 g $ha^{-1}$ $day^{-1}$ in projected nitrogen fixed. These values were somewhat lower than other field grasses from field cores under similar conditions in Australia. In this experiment, no significant inoculation differences in nitrogenase activity were observed. Mean nitrogenase values of Beerwah sandy soils (40 g N $ha^{-1}$ $day^{-1}$) and Narayen clay soils (28 g N $ha^{-1}$ $day^{-1}$) were significantly different. Nitrogenase activity was inversely correlated with plant yield increases (P= 0.02). Root mass, as well as top growth, was significantly greater in the inoculated cores. Inoculated plants had a lower nitrogen and higher dry matter content. When the total nitrogen of the tops was calculated, the inoculated cores clearly outyielded the controls (P= .04). Observations using the fluorescent antibody (FA) technique established that the inoculated bacteria were present at the end of the experiment in the inoculated cores. Acetylene reduction did not correlate well with increased yield. We suggest that a stimulation of root development particularly the fine filamentous roots has caused the inoculated grasses to respond with yield increases, earlier maturity, higher percent dry matter and higher total N in the tops but lower percent nitrogen.

## $^{15}N$ Dilution Studies

Second year $^{15}N$ dilution studies were conducted. Field plots 60 cm dia were labeled by adding ammonium sulfate containing 5% atom excess $^{15}N$ at the rate of 25 g/plot. Incorporation was accomplished by mixing in 100 g sucrose at three weekly intervals (this left less than 10 ppm as soluble N). Guinea grass plants were transplanted, one per plot, and inoculum of either 30 ml live *Azospirillum* culture or 30 ml autoclaved culture was applied at planting and again one week later. Adequate water and other nutrients were applied for good growth. Dry matter yield and nitrogen content of tops, crowns and roots were measured. No inoculation response was observed. Mass spectrometer analysis has not been done for $^{15}N$ to $^{14}N$ ratio. We have had difficulty finding a place to have these analyzed.

B. Evaluation of Persistence of *Azospirillum brasilense*.

### Double Marked Strains

Double marked strains were made to facilitate the recovery of inoculum and the evaluation of factors affecting survival in the soil. Strains of *Azospirillum brasilense* 13t were made resistant to various combinations of two microbial growth inhibitors. One was chosen as having advantages over the others and a selective medium was devised to allow preferential recovery of this strain from the mixture of microbes in soil. Factors affecting survival in soil were evaluated. Survival was poor in frozen soil (-10$^{o}$ C), in soil at high temperature (42$^{o}$ C) in acid soil (pH 5) and in very dry soil (less than 2% moisture, w/w). The numbers of organisms recoverable declined in nonsterile soil, but increased in sterilized soil. Some evidence was obtained that implicated slime molds in loss of the organisms in unsterilized soil.

Recovery of bacteria from field cores and roots

Roots and soil from field cores were sampled periodically for plate counts on ARM medium (containing 100 mg/ml streptomycin and 100 mg/ml rifampicin). When colonies resembling *Azospirillum* were found, some or all of them were tested for acetylene reduction by picking into N-free agar deeps. Upon the first sampling, 5 days after inoculation of the plots, each core from inoculated plots showed from 1 to 4 x $10^4$ *Azospirillum* per g. Control plots were negative. The second set of core samples, one month later contained few colonies, and only an occasional colony gave positive acetylene reduction. Subsequent core samples were all negative for *Azospirillum*. In all, 376 cores were assayed by plate count, and selected colonies from all were tested for acetylene reduction. It was concluded that *Azospirillum brasilense* double marked strain $13t^{sr}$ survived in the soil of these nurseries for only a few days. The data of this study are not consistent with the immunological data on nonmutated *Azospirillum* reported in section II C.

The double marked *Azospirillum brasilense* strain $13t^{sr}$ used for inoculation was not the only bacteria recovered from plant roots that was capable of growing on the double antibiotic containing ARM medium. This greatly reduced its usefulness in monitoring populations. Immunofluorescence was perhaps the simplest positive method of identifying *Azospirillum brasilense* strains isolated from roots. Both JM125A2 and 13t (or $13t^{sr}$) can be positively identified.

Immunofluorescent analysis of root portions supported the double marked strain recovery data indicating that survival of

*Azospirillum brasilense* was poor. This low survival rate could account for the lack of response in several of the field nurseries.

C. Definition of the Biological Aspects of the Association

Electron Microscopy

Two electron microscopy investigations into the mechanism of infection were completed. In the first, association between grass roots and *Azospirillum brasilense* Sp 7 was investigated by the Fahraeus slide technique using nitrogen-free medium and other axenically grown plants. Young inoculated roots of pearl millet and guinea grass produced more mucigel, root hairs, and lateral roots than uninoculated controls. The bacteria were found within the mucilaginous sheath ( mucigel ) that accumulated on the root caps and along the root axes, and they adsorbed firmly to root hairs and undifferentiated epidermal cells. Some of the bacteria adsorbed in a polar fashion on root hair cell walls. Addition of fixed nitrogen to the medium suppressed this adsorption. Aseptically collected root exudate from pearl millet contained substances which bound to cells of *Azospirillum* and promoted their firm adsorption to the root hairs. These substances were nondialyzable and protease-sensitive. Pectolytic activities, including that of pectin trans-eliminase and endopoly-galacturonase, were detected in pure cultures of *A. brasilense* when induced with purified pectin. *Azospirillum* cells were regularly observed in spaces between cells in considerable numbers but were not observed within living intact cells. The pectinase activity of the bacteria may participate actively in the invasion of intercellular spaces but this was not conclusively demonstrated.

The second EM study was directed toward studying the Azospirillum-root association in natural field conditions with guinea grass roots. This required the use of a method to specifically label Azospirillum on the EM. The immunological technique peroxidase-antiperoxidase (PAP) was adapted for this purpose. It produced an observable electron dense layer of osmium specifically deposited on the perixodase reaction products bound serologically to Azospirillum cells. This study supported the results obtained previously with axenically cultured plants that indicate that bacteria abundantly colonize the mucigel and free spaces within the cortex. Breaks in epidermis and cortex layers were particularly favored sites for bacterial colonization. Small roots usually had intact cortical regions with few microorganisms while large roots contained many microorganisms in cortical regions where cells were often broken and sloughed.

Pleiomorphic forms of Azospirillum were observed that were serologically identical when observed with PAP. The encapsulated form may have a special significance as discussed below (Section II A) where they are referred to as bacteroids.

The continued occurrence of Azospirillum brasilense strains 13t and JM125A2 in field control plots indicated some movement and persistence of the strains. Only strain 13t had been applied, JM125A2 is naturally occurring and was originally isolated from this nursery in 1975.

Although bacteria were observed within intact cortical cells, they were found very infrequently. From these EM studies we have concluded that colonization is external in cortex free spaces, in

the mucigel layer and on root surfaces. This raises questions about efficiency of energy transfer from root to bacteria and fixed nitrogen transfer to the plant. For detailed information of these EM studies see references 6, 11, and 12.

## Plant Growth Substances

Various separation and identification techniques were used to identify plant growth substances excreted by *Azospirillum brasilense* in liquid culture. Indole acetic acid (IAA) and indole lactic acid were produced by *Azospirillum* from tryptophan. IAA production increased with increasing tryptophan concentration from 1 to 100 ug/ml. IAA concentration also increased with the age of the culture until bacteria reached the stationary phase. Shaking favored the production of IAA, 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 ug/ml kinetin, 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 IAA, gibberellin, and kinetin produced changes in root morphology of pearl millet similar to those produced by inoculation with *Azospirillum* (for more details see publication 8.

## II. Characterization of $N_2$-Fixing Microorganisms

### A. Morphological and Biochemical Characterizations

"Fat globules", typically present in *Azospirillum* cells, were identified as poly-B-hydroxbutyrate by isolation and spectrophotometry. One strain each of *A. brasilense* (strain 7) and *A. lipoferum* (strain 59b) gave qualitatively identical results. To our knowledge, this is the first chemically unequivocal demonstration of PHB in *Azospirillum*.

Isolate M82, from a Brazilian maize root, gave differing phenotypic characteristics in two laboratories (University of Florida and that of N. Kreig, Virginia Polytechnic Institute). Subsequent studies showed that the culture, as received, consisted of several colonial variants. From these, two physiological strains were separated and purified: one corresponded to *A. brasilense* and the other to *A. lipoferum*.

In previous years, mass cultures of *Azospirillum* in N-enriched media developed a high pH (ca. 8.0 to 8.4) at harvest (1 to 10 x $10^8$/ml). Experiments were run to attempt control of alkalinity, using equimolar concentrations of succinic acid and fructose as principal carbon-energy sources. Results demonstrated that both strains 13t and $13t^{sr}$ grew equally well in the conventional succinate medium and in the succinate-fructose medium (substrate buffered = SB). Generation times were approximately the same (2 h) as was the total crop after 12 to 14 h. However the terminal alkalinities were very different for the two media: succinate medium at termination of exponential growth, pH 7.8; SB, pH 7.1. As a consequence, the SB medium was used in 1978 for production of all mass cultures for field tests.

In the buffering experiments, mass cultures of strain $13t^{sr}$ were enumerated periodically by spread plate count on both trypticase succinate salts (TSS) agar and on the differential antibiotic-containing medium (ARM); optical densities were performed in parallel. Colony forming units (CFU) were approximately equal on the TSS and ARM, media; however, in older cultures, CFU on the ARM medium varied appreciably, and were sometimes much lower than on TSS. In both cases, CFU did not parallel optical density, probably due to the substantial clumping of bacteria which was routinely found in older cultures by phase contrast microscopic observation.

Preliminary results of tests with pure cultures of strain 7 showed that growth rates in N-free medium were the same as found by Okon, Albrecht and Burris (generation time = ca. 20 h). Its calculated efficiency of energy utilization for $N_2$ fixation ($C_2H_2$ reduction) was about 40 mg $N_2$ per g malate consumed. Tests are being performed with a select few other strains to compare their efficiencies.

B. Cell Culture

Axenic callus cultures of *Pennisetum*, centipede grass, tobacco, and sugar cane were inoculated with *A. brasilense* strain 7, with and without added fixed nitrogen. The first calli deteriorated quickly with the inoculum used (ca. $4 \times 10^5$ cells per callus). Sugar cane callus survived over the entire period of test (18 months), with or without inoculum or fixed nitrogen. Inoculated sugar cane callus gave appreciable $C_2H_2$ reduction values within a week after inoculation. The bacteria grew prolifically on the callus surface, in intercellular spaces, and in deteriorating cortical cells, but not in healthy cortex. Abberrant morphological forms of bacteria

occurred on the callus surface, consisting of multiple "bacteroids" enclosed in an envelope resembling polysaccharide. Also present were vibroid cells. Pure culture isolates from the callus were compared with the stock strain 7 for reduction in N-free deeps. The callus isolates gave much higher (ca. 4-fold) specific $C_2H_2$ reduction (n moles $C_2H_2$ per mg bacterial protein) than did the stock strain 7.

C. Genetics and molecular biology of *Azospirillum*.

Genetic and Plasmid Analyses

Genetic analysis of bacterial strains required the previous construction of multiple auxotrophic recipient strains with appropriate contraselection markers as well as suitably marked donor strains carrying conjugal fertility factors. During the past year several recipient strains with different markers have been made and donors carrying the P plasmids RP4 and R68 have been shown to transfer antibiotic resistance into the recipient strains. So far donor chromosomal genes have not been transferred into any recipient.

Molecular biology studies on the genetic material of the *Azospirillum* have revealed that these bacteria carry much of their genetic information on plasmids. Each strain carries many plasmids, in the range of 9-13 different sizes; these differ from strain to strain with each carrying a different array of sizes ranging from 28 to 320 megadaltons. At this time, there is no evidence as to what genes are carried on these plasmids. The plasmid DNA has the same G+C content as the total DNA. These organisms seem to carry no R factors or r determinants since they are all sensitive to all

antibiotics tested. All strains are resistant however to trimethoprim. "Curing" agents such as acridine orange and rifampicin did not eliminate the plasmids from strain 13t.

We are currently attempting to separate and characterize plasmids by gel electrophoresis. Only three bands have been seen on 0.7% agarose gels. Improved techniques are being evaluated.

Use of bacterial mutants to characterize the mechanism of stimulation of plant growth

The rationale of this research was to inoculate plants with bacterial mutants unable to either fix nitrogen or to make phytohormones. Ultra-violet light induced and nitrosoguanidine induced mutants unable to reduce acetylene have been isolated on several occasions but such mutants always have been highly unstable. The cultures revert back to wild type in a short time. Other laboratories have found the same. We are continuing to search for the mutants using more selective techniques (ampicillin selection under $N_2$ fixing conditions in a chemostat.)

Phytohormones, that play a role in growth stimulation of certain plants, are excreted into the medium by Azospirillum. The role of such biochemicals in microbial physiology is unknown, a situation that makes the direct selection of phytohormone mutants impossible at this time. Indole acetic acid (IAA) is synthesized in a number of steps from trytophan. However, IAA cannot serve as a sole source of nitrogen so that mutants unable to use IAA cannot be found. Such mutants would have been unable to make IAA. Mutants in a tryptophan auxotroph that will be unable to use IAA to make tryptophan are being sought. Revertants of the latter to tryptophan prototrophy

could then be used as IAA mutants. The use of plant cell culture strains requiring certain phytohormones has been suggested as a method for screening for other phytohormone mutants.

III. Screening plant genotypes and developing inoculation techniques

A. Screening plant genotypes

Six genotypes of bahiagrass, *Paspalum notatum* Fluge, three diploids and three tetraploids, were studied in a dinitrogen fixation greenhouse experiment. Two $N_2$-fixing bacteria, *Azospirillum brasilense* (JM125A2) and *Azotobacter paspali* (ATCC=23833), were applied as separate treatments, both live and autoclaved, in liquid inoculum at the start of the experiment. Two top growth harvests and a final harvest partitioned into above-and belowground components were analyzed to measure a genetic or ploidy interaction between bacteria and plant genotypes. This interaction was studied by measuring increases in dry weight, percent nitrogen and total nitrogen due to the treatments imposed.

When compared to autoclaved controls, a significant (P 0.01) increase of 18 percent in shoot dry weight was measured in the diploid P8 inoculated with live *A. brasilense*. At a later harvest date, a P8 tetraploid isogenic line, PT8, inoculated with live *A. paspali*, and the tetraploid batatais inoculated with live *A. brasilense* showed a significant (P=0.01) shoot dry weight increase of 22.9 and 19.5 percent respectively over autoclaved controls. Batatais also showed a significant (P=0.05) increase in dry root weight of 16.5 percent over autoclaved controls when inoculated with live *A. brasilense* . The diploid 'Argentine', when inoculated with

live A. paspali, showed a significant (P=0.05) root weight decrease of 8 percent compared to the autoclaved controls.

Combining total shoot and root weight, the diploid P8 and the tetraploid batatais when inoculated with live A. brasilense, showed a significant (P=0.05) total weight increase of 11.7 and 15.7 percent, respectively, over autoclaved controls. No similar response was found for the P8 isogenic tetraploid, PT8.

Analysis of batatais shoots and roots and 'Argentine' roots for percent nitrogen and total nitrogen showed no significant increase due to inoculation.

A second greenhouse study was conducted to determine the effect of limiting the photosynthate supply on dinitrogen fixation. Batatais was subjected to either 67 percent shading or complete removal of green shoots. Acetylene reduction assay and contact slide bacteria counts by the fluorescent antibody methods were used to measure treatment effects. Although not significant, trends toward lower bacteria numbers and lower acetylene reduction values were noted under shading.

Dry weight increases showed a definite plant genotype-bacterial strain interaction that appeared to depend on plant genotype and the ploidy level at which the genotype was expressed. Limitation of photosynthate supply did not significantly affect the acetylene reduction activity or the bacterial counts on the contact slides. The test bacteria, however, were found in close proximity to fungal hyphae. This suggests that a more complex interaction than just bacteria and plant may be involved in bahiagrass associated dinitrogen fixation.

For more detail see reference 2.

B. Inoculation Methodology

Survival of *Azospirillum* in peat was tested using strain $13t^{sr}$ in two peat types kindly supplied by Dr. J. C. Burton of the Nitragin Co. Survival was best in peat neutralized with $CaCO_3$ (ca. pH 6.8) but otherwise untreated. At about 30% moisture (by weight) the plate counts on ARM medium dropped to about 0.1 to 1% of original ($10^8$/g) in 4 weeks; after 14 weeks there was a slight additional decrease. The poor precision of the plate count technique must be re-emphasized; the recoveries found may be considered minimal by the method used. Recoveries from peats "sterilized" at 80 C for 4 h, with or without the addition of Pelgel (used for adherence of Rhizobium to seeds) were much lower than from the peat samples which were merely neutralized with $CaCO_3$. Numerous fungus and some actinomycete colonies developed on the culture plates. Sample colonies from the ARM plates used for determining CFU were inoculated into N-free deeps and were found to reduce $C_2H_2$ actively; they also had the typical colonial and microscopic morphology of *Azospirillum*.

Appendix I

Manuscripts written, submitted, accepted for publication or published in 1978.

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