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9. ABSTRACT

Reports on research concerned with the biology of Azospirillum and grass bacteria associations in relation to nitrogen fixation. Biological characterization has resulted in the reclassification of Spirillum lipoferum into two species, Azospirillum brasilense and A. lipoferum. Associations of Azospirillum with plant roots consist of bacteria in the rhizosphere, on the root surface, in the mucigel layer and between cortex cells. Azospirillum have been found to produce growth regulator-like substances that may affect plant growth. Nitrogenase activity of the associations is $O_2$ sensitive and protection of nitrogenase from $O_2$ appears to be a problem. The association is influenced by soil nitrogen with low and high amounts preventing increases in plant growth due to inoculation. The inconsistency of inoculation to produce beneficial associations with increased plant growth continues to be the greatest deterrent to definitive research and application to commercial agriculture.

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NITROGEN FIXATION

Project No. 931-1004A

by


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# INTRODUCTION

In compiling this annual report on nitrogen fixation, we have followed Article 1, Statement of Work, and outlined our progress on each of the objectives in the same order that they were outlined therein. In some cases it was necessary to deviate from the outline as, for example, when new or improved procedures were discovered or developed. However, the researchers have clearly indicated such departures from the outlined program of work, and have given their progress using the newly developed techniques.

In order to coordinate $N_2$-fixation research on grass-bacterial systems two special workshops were held at the University of Florida during 1977. Dates of these workshops were February 2, 1977 and August 27, 1977. In attendance were all of the researchers from the University of Florida named on the cover of the annual report, plus

Dr. Lloyd Frederick, USAID technical advisor;

Dr. Art Newman, CSRS-USDA;

Dr. Robert Klucas, USDA and University of Nebraska;

Dr. Charles Sloger, USDA, Beltsville, Maryland;

Dr. Robert Taylor, the Bahamas;

Dr. Arden Baltensperger, New Mexico State University;

Dr. Ed Dunigan and Mr. Sam Young, Louisiana State University;

Dr. Rick Weaver and Ms. Sarah Wright, Texas A & M University.

Graduate students working on the grass-bacterial system from the University of Florida who also attended these meetings include Al Wood, Mercedes Garcia, Gary Benzion, Sharon Matthews, Elza Menezes, Chris Carr, Joe Bouton, Norman Novick, and Glen Weiser. These meetings were coordinated by Dr. S. H. West.

OBJECTIVE 1: Identify and define an effective system of grass-bacterial $N_2$-fixation and characterize the environmental conditions necessary for establishment and maintenance of a system. This objective includes the identification of field systems that improve yield and/or protein and a quantitative evaluation of the amount of $N_2$ fixed.

Field Experiments to Study Plant-Bacteria Associations

Previous work (AID contract ta-C-1262) reported in our annual report to AID dated February 22, 1977 outlined replicated field tests conducted in 1975 and 1976. In these tests, pearl millet (*Pennisetum americanum* (L.) K. Shum), guineagrass (*Panicum maximum* Jacq.), buffelgrass (*Cenchrus ciliaris* L.), Transvala digitgrass (*Digitaria decumbens* Stent.), sorghum (*Sorghum vulgare* L. Moench), and corn (*Zea mays* L.) had been evaluated for their response to inoculation with *Azospirillum brasilense* (formerly *Spirillum lipoferum*) [1/]. Inoculated pearl millet, guineagrass, and buffelgrass gave significantly highly dry matter yields than uninoculated plots. These responses were inconsistent with about half of the experiments not responding. We have not been able to establish

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[1/] Kreig, N. R. 1977. Taxonomic Studies of *Spriillum lipoferum*. Genetic engineering for nitrogen fixation (Ed.) A. Hollaender, Plenum Press, N. Y.

the mechanism of these yield increases. Our 1977 experiments were designed to study the causes of the inconsistent responses and to evaluate $N_2$-fixation.

Field experiments were reestablished on sites that responded in the past (Arredondo loamy fine sand, a member of the loamy siliceous, hyperthermic family of Grossarenic Paleudults) in order to evaluate repeatability. In addition, new inoculation experiments were established using 'Transvala' digitgrass, Gahi-3 pearl millet and PM199 guineagrass on a poorly drained site (Leon fine sand, a member of the sandy silicous, thermic family of Aeric Haplaquods) where root zone $O_2$ would be lower and root moisture would be high. The experimental design was a split plot with four levels of supplemental nitrogen as main plots and inoculation with live or autoclaved cultures as subplots. Dry matter yield, protein, acetylene reduction and soil moisture were measured. Soil temperatures at 1 in and 4 in depth, air temperature, relative humidity, and rainfall were obtained for correlation with nitrogenase activity.

A period of extreme drought was experienced during the growth period of all field experiments in 1977. Although the plots were irrigated, a different rhizosphere environment was present with the high evapotranspiration rates experienced in 1977 as compared to previous years. The experiment involving guineagrass (PM199) reestablished in the same area for the third year showed similar trends from inoculation as previous years. Regression analysis shows a significant quadratic effect from inoculation over the four fertility rates and only a linear effect from increasing fertilizer without inoculation. However, the mean differences in dry matter yield between inoculated and uninoculated plots were not significant at any fertilizer level. It is possible that

these results were due to transpiration stress and that decreased plant vigor may have prevented a beneficial plant response to inoculation. Nevertheless, yield response from this experiment coupled with the study of bacteria counts as reported in objective 2 suggest that a grass-bacteria association can be reestablished.

The new 'Transvala' experiment, where inoculation was superimposed over four levels of fertilizer, gave significant yield increases for inoculation at the 40 kg/ha rate at the first harvest. The response curve from inoculation was very similar to previous experiments with pearl millet and guineagrass. Second harvest data were similar in inoculated plots, but the uninoculated plots also showed a quadratic effect. This suggests that bacteria were translocated from inoculated to uninoculated plots.

New field experiments of Gahi3 and PM199 showed no statistically significant differences in yield but a trend existed in the pearl millet for higher yields ($p = 0.17$) for the inoculated plots at the 40 and 80 kgN/ha rates. Intensive acetylene reduction assays were conducted on these experiments; activities are reported later

Nitrogen Balance Greenhouse Experiment

A nitrogen balance experiment was conducted to obtain an evaluation of the $N_2$ fixed by grass-bacteria systems that produce yield responses to inoculation. 'Gahi-3' pearl millet was selected for this greenhouse study because of its positive yield responses to inoculation (32%) with *A. brasilense* in the field. Twelve liter, glazed, ceramic containers with a bottom drain for catching leachates were filled with methyl bromide treated soil. Fertilizer was mixed in the soil at rates of 60 kg N, 20 kg P, 79 kg K and 4 kg fritted trace elements per hectare. Two

uniform pearl millet seedlings were established in each container. Half the containers were inoculated with $10^8$ cells of A. brasilense strain 13th while the other half received a similar amount of autoclaved inoculum. Treatments were paired into 18 reps which were rotated weekly on the greenhouse bench. Near optimum soil moisture was maintained for plant growth and any leachate retrieved was returned to that container. Soil N content was measured before and after the experiment. Plant shoots and roots were harvested at 42 days when dry weight and nitrogen content were determined (see pages 24 and 25 for the data). The plants grew well during much of the experiment but at the time of harvest growth was slow and nitrogen deficiency symptoms were severe. No visual differences were observed between the inoculation treatments. The inoculated plants yielded only about 4% more dry weight and nitrogen was not significantly different from the controls. Since the phenomenon of yield increase was not obtained, the experiment was inconclusive in evaluating $N_2$-fixation as a cause of the yield increases we have noted in the field. Acetylene reduction values measured in this study were also low but inconclusive as a productive grass-bacteria association was not believed to have been established.

## $^{15}N$ Fertilizer Isotope Dilution Study

A $^{15}N$ fertilizer isotope dilution study was conducted to measure the nitrogenase activity in newly established plots of PM199 guineagrass. This experiment was based on the theory that if biologically fixed nitrogen were incorporated into the plant it would dilute the nitrogen taken up from the soil. If the soil nitrogen were labeled with $^{15}N$, then it may be possible to quantitatively evaluate the effectiveness

of the grass-Azospirillum association in fixing nitrogen under field conditions. Concrete cylinders 2 ft. in diameter and 4 ft. long were installed vertically in the soil and the profile restored inside. Nitrogen was applied as ammonium sulfate at the rate of 162 kg/ha; of this, about 10 kg/ha was $^{15}N$. Fertilizer was applied at the rates of 20 kg P, 80 kg K and 4 kg fritted trace elements (FTE 501). Sucrose at the rate of 100g/cylinder was also applied initially. The soil was dampened (not saturated) and covered with plastic to maintain moisture. Three additional sucrose applications of 50 g/cylinder were applied at weekly intervals with the moisture being maintained to keep biological activity of the soil high. This treatment was intended to incorporate the nitrogen fertilizer into the organic fraction of the soil. At the end of this treatment all cylinders had less than 9 ppm 0.5 N KCl extractable N. This procedure was used to make the isotope level of the avialable nitrogen more stable over the season and to reduce leaching losses of the $^{15}N$. Single guinea-grass plants were transplanted into the cylinders. Sixty militers of live or autoclaved A. brasilense strains 13t and JM125A2 were used in inoculating the seven replicated pairs of cylinders. Bordering plants of the same type were established between and around the cylinders. Soil samples were taken at intervals for nitrogen and $^{15}N$ analysis and a moist nitrogen mineralization control was maintained without plant uptake or leaching. Harvest of top growth was made at 60 days. Mean yield of inoculated plots and control plots were 530g and 523g respectively, which were not statistically different. Since this experiment did not give a yield response, evaluation of nitrogen fixation as a mechanism of producing yield increases was not definitive. Analysis of $^{15}N$ in plant material and soil has not been completed.

Acetylene Reduction of Field Plots

Acetylene reduction assays were conducted on field plots using two methods, soil root cores and washed roots without preincubation. For the core assays, coring tubes were developed using 7.6 cm thin wall steel conduit 36 cm long with gas sampling ports in the top. The gas sampling port was closed with a bottle septum and the bottom was closed with a rubber "Jim-Cap" which provides a positive seal, quickly and easily. These coring tubes were gently pushed 18 cm into the soil using a T-handle. Washed root assays were conducted in stoppered 80 ml test tubes with a septum covered port. The oxygen level was lowered to about 2% by replacing air with argon before adding the usual 0.1 atm of acetylene. The washed roots gave curvilinear responses increasing with time while the soil-root cores gave straight line responses. Both methods were characterized by a lag of approximately 4 hours. The effect of $O_2$ tension was studied in soil-root cores. Mean n moles acetylene reduced per core were 4464, 2802 and 914 for $O_2$ levels of 2%, 6% and 20%, respectively. This indicates that high $O_2$ in the root zone restricts nitrogenase activity. The very sandy, well drained soils, such as an Arredondo fine sand, offer little resistance to diffusion of gasses through them. Projected nitrogen fixation rates of 120, 74, and 25 g $N/ha^{-1}$ $day^{-1}$, respectively, were obtained for the three $O_2$ levels of 2%, 6% and 20%. From this data it would appear that reduced $O_2$ availability would be necessary before $N_2$-fixation of practical importance could take place.

A greenhouse experiment was initiated in January, 1978 to study and define the effect of $O_2$ tension in the root zone on Azospirillum association and $N_2$-fixation. Results of this experiment should be useful in planning 1978 field studies.

Biological Aspects of the Association

In studying the biological aspects of the association we are culturing A. brasilense strain 7 in three different media. The first medium is trypticase soy broth (TSB), a rich maintenance medium; the second is a soil extract medium and the third is a root exudate medium. Transmission electron microscope (TEM) studies are in progress comparing cells grown in each of the three media, in order to determine if this organism produces unusual morphological forms, such as the "swarmers" of Rhizobium or the resistant "cysts" of Azotobacter, which may influence growth, survival and associative traits in nature. Pearl millet cv 'Gahi 3' is being used as the source of root exudate because of its genetic uniformity and significant response to inoculation as previously reported. The organism grows more rapidly in root exudate than in soil extract and forms multi-cell aggregates having a distinct electron-dense outer boundary when observed by TEM. These structures appear identical to the aggregates formed by Rhizobium which have a specific influence on the mechanism of plant infection. Study of the possible physiological significance of these aggregates is in progress.

Fahraeus assemblies (plant slide cultures) are being used to study the plant-bacteria interaction in axenic culture. Seeds of guineagrass PM285, a breeding line, are being used because of their small size and demonstrated response of this plant to the bacteria. Inoculated plants grown in +N and -N nutrient solution in these assemblies support equivalent growth of the organism in the rhizosphere, as evidenced by the turbidity of the solutions. Examination of the cultures by light microscopy has shown that the bacteria colonize and adhere predominantly on older plant tissue, including lysed root hairs and sloughed cells. Relatively

few cells were found attached to young root surfaces. The incidence of collapsed root hairs appears to be greatest in inoculated cultures.

Electron microscopic examination of plants in Nfree culture, 1, 2, 3, and 4 weeks after inoculation confirmed that colonization occurred in older parts of the roots (e.g., back of the region of maturation). In examination of colonization sites, root cell walls were observed to vary substantially in thickness. Bacteria were closely associated with thin areas of epidermal walls that were beginning to slough off. The bacteria gained entrance at the point of emergence of lateral roots. In older cultures (e.g., 4 weeks), the bacteria were observed to penetrate the middle lamellae of the second layer of the underlying cortical tissue. No bacteria were observed inside the root tissues of plants grown in the presence of added N.

Lysis of root hairs of inoculated plants (-N) was followed by the degradation of cytoplasm after the first week of inoculation. Three weeks after inoculation, only a few plant cells located on the outer part of the cortex showed signs of degradation. Some dark staining bodies and osmiophilic granules could also be seen in the cytoplasm. No bacteria were observed inside the root tissue of 10 root pieces picked at random from +N plants. Few changes in the cytoplasmic organelles were noted. Examination of root cells of one-month old +N and -N inoculated plants showed normal cytoplasm. The cells adjacent to colonized areas remained alive as shown by the intact plasmalemma and active cytoplasm.

## Forced Associations with Tissue Culture

Experiments designed to force or "engineer" symbiotic association of *A. brasilense* strain with callus tissue cultures of cereals/grasses were initiated in 1977. Callus cultures of sugarcane, pearl millet, rice, and

sorghum were established from explanted seedling, young leaf, or meristematic tissues. Since our early experiments showed more promising results with sugarcane tissue cultures, we have concentrated our efforts on this species. A. brasilense continues to grow on chemically defined media which are either completely devoid of added nitrogen or are supplied with very low levels of nitrogen in the form of glutamine. Such tissue cultures exhibit acetylene reduction activity, showing that the bacteria are active in nitrogen fixation. The bacteria, as shown by electron microscopy, continue to grow in the inter-cellular spaces of the callus tissue, and do not invade living cells. Such associations have now been maintained for about eight months in culture, during several subcultures, and with continued demonstration of acetylene reduction activity by the bacteria.

## Greenhouse Inoculation Studies

Greenhouse studies with guineagrass, pearl millet, corn, sorghum, and digitgrass have been conducted to study plant responses to inoculation. Enhancement of dry matter production from inoculaton has occurred with all these species. However, acetylene reduction assays with intact plants or root systems consistently fail to indicate correlation between $N_2$ase activity and inoculation. In one experiment in which washed sand served as the potting medium we found that acetylene reduction values decreased with time. Where the soil contained organic matter, a low level of $N_2$ase activity persisted. However, plant vigor declined steadily with time in all experiments in which mineral nitrogen was withheld. These results with washed sand for a potting medium suggest that soils with very low organic matter may affect the $N_2$ase activity of the association.

Results have been similar in experiments using Klebsiella pneumoniae, Enterobacter cloacae and Azotobacter paspali as inoculum.

We have attempted to increase the activity of the diazotrophs by supplying a carbon source to the soil after inoculation. Some increaased growth of Azospirillum strains occurs when dicarboxylic acids (malate, succinate) are added, but in all of the tests $N_2$ase activity by Azospirillum remained low. On the other hand, adding sucrose greatly accelerated growth and $N_2$ase activity of Azotobacter spp., whether or not the containers were inoculated with an Azotobacter. From these tests it is clear that in an aereated, N-deficient soil, Azotobacter spp., are much more aggressive than the Azospirillum strains we have studied.

We have conducted several experiments based on the hypothesis that exudates produced by plant roots serve as primary energy sources for Azospirillum. Carbohydrate compounds, not the most effective substrates for Azospirillum (but readily used as energy sources by various other $N_2$-fixing heterotrophs) constitute the major portion of the organic exudates. Dicarboxylic acids and their amino derivatives, excellent substrates for the various strains of Azospirillum, are not abundant. Their scarcity may impose severe limits on fixation by Azospirillum species.

Various treatments can be applied to roots to increase production of exudates. Sublethal heating or chilling, or anaerobic incubation, induces excretion of organic acids, and a temporary increase in $N_2$-fixation. However, such increases are not sustained, and the evidence suggests such treatments do not alter substantially the conclusions noted.

OBJECTIVE 2: Characterize $N_2$-fixing microorganisms from grass roots by biochemical, morphological, ecological, and genetic methods.

## Isolation of $N_2$-fixing Bacteria

A standard methodology for the isolation of Azospirillum has been established. Using this technique, over 100 plant and soil samples from South Africa and Florida have yielded a number of isolates which resemble the original strains from South America and Florida.

Other organisms capable of reducing atmospheric dinitrogen (as shown by acetylene reduction) have also been isolated from our experimental plots as well as native sites in Florida. The results of these investigations indicate that several types of diazotrophic bacteria can be isolated from a single root sample depending on the enrichment techniques employed. We have isolated Azospirillum from the roots of field grown plants inoculated with Azospirillum brasilense strain 13t. Various other members of the Enterobacteriaceae were also found whether or not the plots were inoculated with Azospirillum.

## Biochemical and Physiological Studies of Azospirillum

Sixty clones isolated by Dobereiner and by us, have been examined morphologically and biochemically; some 120 physiological and biochemical characters, and relevant morphological features of the isolates have been determined. These associative $N_2$-fixing bacteria can be characterized as (a) being aerobic but reducing acetylene best under microaerophilic conditions; (b) having limited ability to metabolize and utilize simple carbohydrates; (c) being able to grow in synthetic medium (in some cases with biotin as a supplement); (d) having generally distinctive cellular and colonial morphology; and (e) having different guanine + cytosine (G + C) ratios in their DNA. There are at least two types, principally differentiated by the ability of one type to grow and produce acid anaerobically from sugars (very slowly), while the other is strictly aerobic.

## Morphology at Various Culture Ages

In colonial morphology most of the isolates are similar on N-free and complex media. Many strains produce carotenoid pigments and cytochromes in varying degrees. A few strains, belonging to the fermentative type, have distinctive colonies on complex medium: colorless (white), highly raised, extremely convoluted, and smaller in diameter; this feature correlates with extreme aggregation of cells in liquid culture.

All isolates are relatively large, gram negative rods (0.5 to 1.0 um diam. by 1.0 to 3.0 um long), sometimes curved, and in older cultures, showing chains which appear as extended helices. All strains contain fat staining granules (chemically identified as poly-β-hydroxybutyric acid in two strains), which increase in prominence as the cultures age. In older cultures (5 to 19 days on agar or in broth), pleomorphic forms appear as spheres or grossly irregular and sometimes elongated cells, along with conventional rods or vibroids. In cultures in exponential phase, the cells often appear tapered at the poles.

## Serological Tests

Tests for inter-relatedness of Azospirillum isolates will be conducted in 1978.

## Quantitative Acetylene Reduction

Studies of differences among isolates in ability to reduce acetylene have not reached a methodologically satisfactory stage, but are continuing. Mixed cultures often give better acetylene reduction rates than pure cultures.

## Taxonomy

In conjunction with Drs. Noel Kreig and Johanna Dobereiner, a new generic epithet, *Azospirillum*, has been proposed. The choice is based on phenotypic differences and G + C distinctions from other genera. Two species, *A. brasilense* and *A. lipoferum*, are described in the new genus. Kreig has demonstrated a close relationship by DNA-DNA reassociation within the strict aerobes (*A. brasilense*) and within the weakly fermentative strains (*A. lipoferum*), and a lesser but still notable cross reassociation between the two physiological types. He has also shown that there is negligible DNA-DNA reassociation between *Azospirillum* and several other genera which have some phenotypic similarity.

## Plant Growth Substances Produced by Diazotrophic Bacteria

Chromatographic and bioassay procedures were used to separate and identify plant growth substances produced by soil bacteria in liquid culture. When ethyl acetate extracts from basic and acidic fractions from five cultures were assayed, indole acetic acid (IAA) was identified in four of the cultures by chromatographic methods. Neither thin layer chromatography (TLC) nor high pressure liquid chromatography (HPLC) was satisfactory for identifying cytokinins or gibberellins. By partitioning the extracts on TLC plates and eluting individual sections from the chromatograms, active components were prepared for bioassay. Lettuce hypocotyls were used to identify cytokinin activity.

IAA was found in amounts from 0.2 ug/ml (*Azotobacter paspali*) to 6.2 ug/ml (*A. brasilense*). Bioassays showed the presence of cytokinin activity in all cultures, but chromatographic data indicated that neither zeatin nor kinetin were present. Experiments to detect gibberellins were

conducted with only one of the bacteria species, A. brasilense. HPLC was unsatisfactory for detecting gibberellins because of interfering compounds. TLC and bioassays indicated a small, but perhaps biologically significant amount of GA was present in the culture.

Fluorescent Antibody Studies with Azospirillum

The fluorescent antibody (FA) technique has been further refined for characterizing Azospirillum. Work has included continued cross-reactivity studies, cryostat sectioning of plant roots and collection of Azospirillum on a membrane filter. During the past year, we have developed an improved FA application which allows us to scan and count fluorescing bacteria on whole root segments.

Direct observation of whole root segments of three tropical grasses was accomplished using FA techniques. Antisera have been prepared which allow identification of isolates of A. brasilense strains 13t from Brazil and JM125A2 from Florida. Root samples collected during the year were assayed for acetylene reduction and examined by FA. The FA tests included bacterial counts immediately prior to, and immediately after the acetylene reduction assay.

The guineagrass plots had a significantly higher number of A. brasilense strain 13t than strain JM125A2, the indigenous strain. These plots had been inoculated with strain 13t for three years, but had not received inoculum of strain JM125A2.

Pearl millet and digitgrass plots inoculated only in 1977 with the mixed culture of A. brasilense strains 13t and JM125A2 showed approximately

equal numbers of the two strains. In sectioned roots using a combinaton of nonspecific and FA staining, we found only 1% of the total bacterial population at the root surface to be FA reactive Azospirillum.

Genetic Studies with Azospirillum

Evidence accumulated is that nitrogenase is genetically stable in Azospirillum. This indicates that it is acceptable to grow Azospirillum in media containing fixed nitrogen for soil inoculum without loss of nitrogenase ability. Rich media support more rapid growth and higher populations of cells.

A search for phage (lytic or temperate) infectious for Azospirillum in Florida and Brazilian soils was unproductive. Dr. Elmerich in Paris and Dr. Drosdowitz in Brazil have also been unable to find phage for Azospirillum.

After our unsucessful search for spontaneous mutants (preferable to induced mutants) and limited success with mild inducing agents, nitrosoquanidine was used to make strains resistant to: (a) streptomycin-rifampicin, (b) streptomycin-spectinomycin, and (c) streptomycin-chloramphenicol. The latter grew at a rate slower than rifampicin revertants and was soon predominantly only streptomycin resistant. The first two doubly marked strains were suitable for recovery studies and were used in combination with a selective medium of the following composition:

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|---|---|
| antibiotic | 100 ug/ml |
| cycloheximide | 200 ug/ml |
| safranin | 0.01% |

The cycloheximide and safranin inhibit or delay the growth of soil fungi until after bacterial colonies are countable.

Mutants unable to fix nitrogen have been isolated in the double marked strain, $str^R$, $rif^R$. This strain will serve as a control in inoculated field studies as a replacement for autoclaved cultures. It will also be used in laboratory studies and is currently in use in greenhouse studies on the role of nitrogen fixation in stimulation of growth in grasses. Mutants derepressed for nitrogen fixation have been sought but have not yet been obtained.

Examination by electron microscopy of the DNA of several strains of Azospirillum has revealed the presence of plasmids. Several different sizes of plasmids have been found in each strain examined. Strains isolated from different locations in Brazil and in Florida have plasmid size classes that differ from each other.

Development of a method for transferring genes between strains of Azospirillum is necessary for genetic analysis of the nitrogenase complex in these organisms. The isolation of multiple auxotrophs, the first step in developing such a system, is progressing with the isolation of several mutants. Demonstration of conjugal transfer of an R plasmid has been accomplished with the transfer of the promiscuous Pseudomonas plasmids $RP_4$ and R 68.45.

OBJECTIVE 3: Develop effective techniques to screen plant genotypes and develop efficient inoculation methods to maximize bacterial persistence and enchance crop productivity.

## Greenhouse Screening Studies

Eight turf bermudagrasses were screened for response to A. brasilense and Azotobacter paspali under two nitrogen fertility levels. Harvests were made of top growth four times at intervals of 14, 28, 42 and 62 days

after inoculation. The cumulative yields of all genotypes are shown in Figure 1 (see page 26). The inoculated treatment produced 17% more top growth than controls at the low fertility level. Nitrogen analyses were performed on the composite top growth samples and crown and root samples from each pot. Although percent nitrogen was not significantly higher, the total nitrogen accumulated in the top growth increased by 21% over controls. Mean inoculation responses of the eight genotypes ranged from +43% to -4%, but no genotypic differences could be statistically demonstrated. The performance of each of the bermudagrass genotypes is shown in Figure 2 (see page 27). Although a genotype x inoculation interaction was not found in this experiment, we believe it may be possible to select bermudagrass plants that are more responsive to Azospirillum.

Six genotypes of Paspalum notatum were studied in a dinitrogen fixation greenhouse study during the summer and fall of 1977. Two bacteria, A. brasilense and Azotobacter paspali were applied as treatments, both alive and autoclaved during one inoculation in a split-split plot design. The experiment was planned to study the effects of the bacteria-plant genotype interaction by the analysis of total dry matter, total nitrogen, acetylene reduction and fluorescent antibody reaction as an indication of dinitrogen fixation. Two top growth harvests at predetermined intervals and a final total harvest partitioned into above and below ground components were taken to determine total dry matter increase. Preliminary analysis of this data showed a definite statistical interaction of bacteria-plant genotype. The remaining data has not been completely analyzed.

## Field Screening Studies

Second year data were obtained on responses of pearl millet genotypes to inoculation with Azospirillum. The two year data indicated that genetic variability exists in this response. Gahi-3, an $F_1$ hybrid of Tift 23DA X Tift 186, responded significantly with higher yields due to inoculation while neither of the parental inbreds responded to inoculation. Another inbred, Bil 3B, showed an indication of response. Hybridization of Bil 3B and several other lines is being planned in an attempt to enhance response through genetics.

## Soil Conditions Affecting Inoculum Persistence

Double-marked strains and selective medium were used to study effects of environmental factors on persistence of Azospirillum in soils. Two experiments have been carried out showing that the cells die very rapidly in very dry soil (2-3% moisture), have a short lifespan in 3-4% moisture and survive well in 15-30% and 50% moisture. There were only minor differences in survival in 5, 10, and 15% moisture. Cells do not survive in soil at -20 C or at 46 C but survival differed only slightly at 8 C, 22 C and 32 C.

Azospirillum survived poorly in acid soil (about pH 5) but differed little in neutral (about pH 6.5) or in alkaline (about pH 8) soils.

The addition of a carbon and energy source (succinate 1%) to soil resulted in an initial drop of about one log cycle in surviving numbers, followed by an increase in the population level. This drop and rise occurred in the three experimental conditions that included succinate. Survival studies under these experimental conditions are being repeated.

The addition of minerals as fritted trace elements (FTE501) or as salts of Dobereiner's medium produced mixed effects. Survival with FTE alone decreased steadily, FTE plus succinate dropped greatly and then rose dramatically. Survival with added Dobereiner's salts was nearly 70% after 6 weeks and over 100% with Dobereiner's salts plus succinate. The numbers of Azospirillum in sterile soil increased 150% for 4 weeks before leveling or dropping somewhat.

## SUMMARY & CONCLUSIONS

We have taken a broad research approach and have made considerable progress in understanding the biology of Azospirillum and grass-bacteria associations. Methodology for studying the complex associations has also been expanded. Biological characterization has resulted in the reclassification of Spirillum lipoferum into two species Azospirillum brasilense and A. lipoferum. Associations of Azospirillum with plant roots consist of bacteria in the rhizosphere, on the root surface, in the mucigel layer and between cortex cells; to date few bacteria have been found in intact root cells. Azospirillum have been found to produce growth regulator-like substances that may affect plant growth. Nitrogenase activity of the associations is $O_2$ sensitive and protection of nitrogenase from $O_2$ appears to be a problem. The association is influenced by soil nitrogen with low and high amounts preventing increases in plant growth due to inoculation. New tools have been utilized such as FA for observing and identifying bacteria on plant roots. Double marked strains have been produced as a tool to study population changes in field and controlled environment studies. $Nif^-$ mutants are ready for

inoculation experiments that may help define the relative role growth regulators play in the growth response to Azospirillum.

The inconsistency of inoculation to produce beneficial associations with increased plant growth continues to be the greatest deterrent to definitive research and application to commercial agriculture. Many important questions, such as the relative contribution of nitrogen fixed by productive grass-bacteria associations to the crop, remain unanswered. Our research in 1978 will be designed to determine some of the causes for inconsistent results.

Abstracts & Manuscripts Submitted Under

Contract No. AID/ta-C-1376

Baltensperger, A.A., S. C. Schank, Rex L. Smith, R. C. Littell J. H. Bouton and A. E. Dudeck. Effect of inoculation with Azospirillum and Azotobacter on turf type bermudagrass genotypes. Crop Science, submitted for publication.

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DRY WEIGHT AND NITROGEN RESPONSE OF 'GAHI-3' PEARL MILLET PLANTS TO INOCULATION WITH *A. BRASILENSE* STRAIN 13t IN THE NITROGEN BALANCE EXPERIMENT

| Treatment | Dry Weight (g) | | | %N | | g N | | |
|---|---|---|---|---|---|---|---|---|
| | Top | Root | Total | Top | Root | Top | Root | Total |
| Live Sp 13t | 39.67 | 16.89 | 56.55 | 0.702 | 0.717 | 0.2784 | 0.1193 | 0.3977 |
| Autoclaved Sp 13t | 38.67 | 16.11 | 54.78 | 0.698 | 0.704 | 0.2675 | 0.1139 | 0.3814 |
| % increase due to inoc. | 2.5 | 4.9 | 3.2 | 0.5 | 1.8 | 4.1 | 4.7 | 4.3 |

NITROGEN CONTENT OF 'GAHI-3' SOIL SYSTEM
IN THE NITROGEN BALANCE EXPERIMENT

| Treatment | Soil N (g) | | | Plant N (g) | Balance (g N) Plant N - Soil N Lost |
|---|---|---|---|---|---|
| | Initial | Terminal | Lost (Initial-Terminal) | | |
| Live Sp 13t | 7.5693 | 7.4047 | 0.1645 | 0.3977 | +0.2332 |
| Autoclaved Sp 13t | 7.5228 | 7.4339 | 0.0889 | 0.3814 | +0.2925 |
| $\bar{X}$ For Combined Treatments | 7.5460 | 7.4194 | 0.1267 | 0.3895 | +0.2628 |

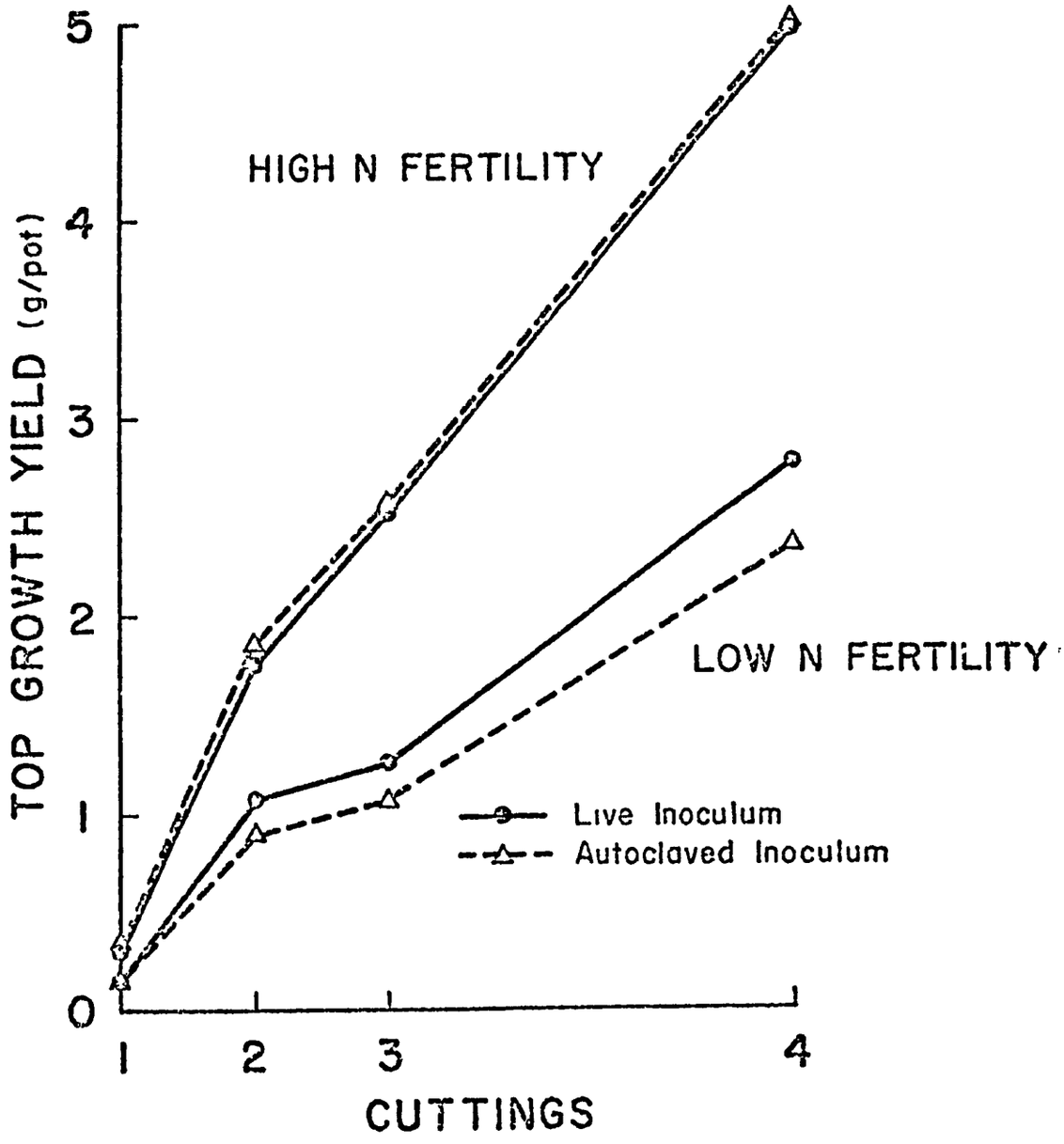


Fig. 1 MEAN CUMULATIVE TOP GROWTH DRY MATTER YIELD OF EIGHT BERMUDA-GRASS GENOTYPES TREATED WITH LIVE AND AUTOCLAVED INOCULUM AT LOW AND HIGH NITROGEN FERTILITY LEVELS IN A GREENHOUSE EXPERIMENT. CUTTINGS 1, 2, 3, AND 4 WERE AT 14, 28, 42, AND 62 DAYS FOLLOWING INITIAL INOCULATION.
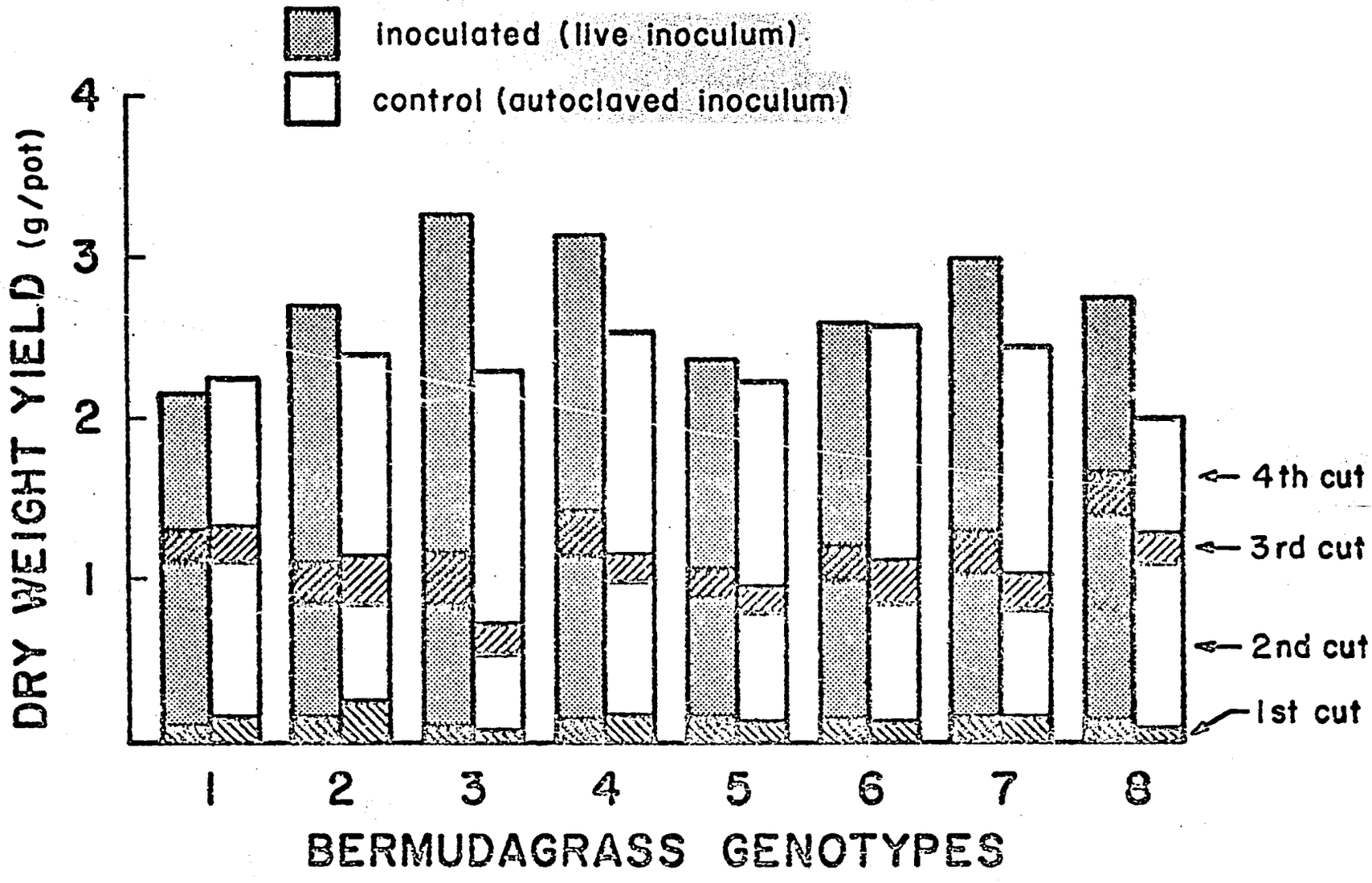
Fig. 2 MEAN TOP GROWTH DRY WEIGHT YIELDS OF EIGHT BERMUDAGRASS GENOTYPES COMPARING INOCULATED GRASSES TO CONTROLS FOR 4 HARVESTS AT LOW NITROGEN FERTILITY LEVEL; GREENHOUSE EXPERIMENT.