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YIELD INCREASES OF TROPICAL GRASSES AFTER INOCULATION WITH *SPIRILLUM LIPOFERUM*

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Abstract

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

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

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

Inoculation with *Spirillum lipoferum* has given yield increases for three consecutive years in Florida This indicates

1 response to inoculation can be repeated

2 economically important amounts of nitrogen can be replaced by inoculation

3 a high potential exists for developing grass-bacteria systems

Introduction

New research has shown that some grasses have nitrogen fixing potential when associated with *Spirillum lipoferum* Beijerinck or other bacteria This represents an important breakthrough in this area of agricultural research since the grass family includes most of the world's staple food crops (maize, *Zea mays* L , wheat, *Triticum aestivum* L ; rice, *Oryza sativa* L ; sorghum, *Sorghum bicolor* (L) Moench, millet, *Pennisetum americanum* (L.) K Schum) and forages In a world now operating on costly and dwindling supplies, savings in energy needed to produce chemical nitrogen fertilizers would be very significant

In Brazil, Dobereiner & Day (1976), isolated *Spirillum lipoferum* from the surface of disinfected roots of the tropical grass *Digitaria decumbens* cultivar 'Transvala' which was released and introduced into Brazil by the Florida Experiment Station (Boyd *et al.*, 1973). Since then strains have been isolated from maize, wheat and other grasses (personal communication). We have isolated *Spirillum lipoferum* in Florida from millet and from roots of maize and guinea grass (*Panicum maximum*, Jacq.) in Ecuador and

Venezuela. These isolations have shown that although the bacteria are widely distributed, only certain sites have yielded *Spirillum* (unpublished data)

Nitrogen fixation rate projections of grass-bacteria associations have been based on acetylene reduction activities of roots and soil cores. Rates as high as 2 kg of fixed N $ha^{-1} \cdot day^{-1}$ in maize, 1.7 in elephant grass (*Pennisetum purpureum*), and 90 kg N fixed $ha^{-1} \cdot yr^{-1}$ in bahiagrass were projected (Von Bulow & Dobereiner, 1975, Day *et al.*, 1975, and Dobereiner *et al.*, 1973). Essentially all of the acetylene reduction measurements showing high activity have been associated with a lag period and have undergone a preincubation in near anaerobic conditions. This technique has been criticized as producing unnaturally high rates which needs verification with other types of data such as increased yield, higher N content, or ^{15}N incorporation. The detached root assay with preincubation is valuable, however, in detecting grass root – *Spirillum* associations.

Inoculation studies with *Spirillum lipoferum* were conducted in Florida to determine whether grass-bacteria associations have the potential of increasing yield or decreasing nitrogen fertilizer requirements.

Materials and methods

Replicated grass plots were established for inoculation studies during the past three years. The first year, 1974, 40 tropical forage grass genotypes (five genera) were studied in paired plant comparisons with one of each pair being inoculated and the other left as a control. Inoculum was produced by homogenizing bacterial cultures of *S. lipoferum* (Brazilian strain) grown on semi-solid media without fixed nitrogen with five parts of water. One hundred ml of this suspension was applied to one plant of each pair. An equivalent amount of medium, without bacteria, was used on the uninoculated controls. No nitrogen fertilizer was applied.

In 1975, four species were tested in larger (3.2 m^2) plots replicated eight times. These species were pearl millet, guineagrass, buffelgrass (*Cenchrus ciliaris* L.) and Transvala digitgrass. Four rates of nitrogen fertilizer were superimposed over inoculated and control plots (See Tables 1 – 3 for rates of nitrogen fertilizer). Phosphate and potassium were applied at the rates of 40 and 80 kg ha^{-1} respectively as P_2O_5 and K_2O. Inoculation was accomplished by sprinkling aqueous suspensions of *Spirillum lipoferum* strain 131 (from Brazil), over the plots at a rate of about 10^7 cells per meter of row. This culture was washed into the soil by sprinkler irrigation. The *S. lipoferum* culture was grown in nitrogen-free, mineral salts medium with malate as the energy source (Dobereiner & Day, 1976). Cultures were continuously sparged with a mixture of 5% O_2 – 95% N_2 and were harvested near the end of the logarithmic growth phase. Control plots received medium, without bacteria, at the same rate. About 7 kg ha^{-1} of malate was applied to the control plots.

In 1976, the same four species were tested. Pearl millet and guineagrass were re-established with minimal cultivation. Fertilization rates were similar to those in 1975. Inoculation was accomplished by injecting the bacteria about 7 cm deep into the soil at the rate of about 3×10^9 cells per m^2. Cultures were grown in medium containing nitrogen with aeration. Control plots received autoclaved medium at the same rate and by the same method as the inoculated plots.

Dry matter yields were taken and forage nitrogen content was measured by a Technicon autoanalyzer and was converted to crude protein by multiplying by 6.25. Yield and protein data were analyzed statistically.

Acetylene reduction was accomplished by a modification of Dobereiner & Day (1974). Washed roots were sealed in a plastic bag, then preincubated overnight in an atmosphere of 5% O_2 – 95% N_2. After preincubation, the atmosphere was replaced with a fresh O_2 – N_2 mixture as above, then 10% acetylene added. Gas samples were collected after a 3 h incubation and placed in evacuated tubes. These were stored several days before being analyzed for ethylene on a gas chromatograph.

An alternative acetylene reduction technique was developed to avoid preincubation. Cores were cut with the sharp end of steel soft drink cans that had both ends removed. The cans, containing the cores with relatively undisturbed roots, were sealed in pint, wide mouth mason jars. The atmosphere was replaced with gas containing partial pressures of 0.864 atm N_2, 0.045 atm O_2 and 0.091 atm acetylene. After a 4 h incubation period, a gas sample was removed, stored, and assayed as described above.

Indirect fluorescent antibody (FA) techniques as described by Schmidt (1974) were used to specifically identify and count *S. lipoferum* in the soil. These techniques were used to study bacteria populations following inoculation.

Results and discussion

Higher dry matter and protein yields have been obtained from inoculated grasses compared to uninoculated counterparts in each of the three years of experimentation. In the first year, inoculated 'Transvala' digitgrass and *P. maximum* produced 61% and 80% more protein than the uninoculated controls. Dry matter yields for the other 38 genotypes were not statistically different. Buffelgrass produced acetylene reduction values up to 200 n moles · g dry root^{-1} · h^{-1}. The data of this first year's work provided a basis for selecting grass genotypes for the second year's experiments.

In 1975, higher protein and dry matter yields were obtained from inoculated versus control plots of pearl millet, guineagrass and buffelgrass (Tables 1, 2 and 3, respectively). 'Transvala' digitgrass did not respond. Protein content was not statistically different over treatments so results are given as dry matter yields. In each case, some fertilizer nitrogen was required to stimulate inoculation response. Both inoculated and uninoculated regressions of yield upon nitrogen rate in pearl millet were linear but in the guineagrass and buffelgrass the inoculated plots were curvilinear and the control linear. Significantly higher yields were obtained due to inoculation at the 40 and 80 kg fertilizer nitrogen rates for pearl millet and in the guineagrass at the 30 and 60 kg nitrogen rates. Using the regression equations, we calculated that up to 40 kg N ha^{-1} in the millet and 38 kg N ha^{-1} in the guineagrass were replaced by inoculation. Table 2 summarizes two cuts of guineagrass. See also Smith *et al.*, 1976.

Table 1. Pearl millet dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (1975)

Fertilizer N Rate	Dry Matter Yield kg ha^{-1}		Increase from Inoculation
	Inoculated	Uninoculated	
0	4,750	5,300	− 550
20	5,820	6,070	− 250
40	7,350	6,040	1,310*
80	9,140	7,880	1,260*

Repression equations from inoculated and uninoculated plots were $\hat{Y}_I = 4{,}820 + 56X$ and $\hat{Y}_C = 5{,}240 + 31X$, respectively.

Asterisks indicate significance at 0.05 level.

Table 2. Guineagrass dry matter forage yields inoculated with *Spirillum lipoferum* compared with uninoculated (1975)

Fertilizer N Rate	Dry Matter Yield kg ha^{-1}		Increase from Inoculation
	Inoculated	Uninoculated	
0	9,960	10,750	− 790
30	15,550	13,460	2,090*
60	17,610	15,560	2,050*
120	20,020	19,470	550

Regression equations from inoculated and uninoculated plots were $\hat{Y}_I = 9970 + 189X - 0.9X^2$ and $\hat{Y}_C = 10700 + 80X$, respectively.

Asterisks indicate significance at 0.05 level.

Table 3 shows the yield responses of inoculated and control buffelgrass over four nitrogen fertilizer rates. These responses are similar to those of guineagrass, that is, regression analysis gave curvilinear inoculation and linear control responses. Again some fertilizer was required for inoculation response which decreased at the higher rates of nitrogen fertilization.

Table 4 shows the 1976 first cut guineagrass responses. These data resemble the 1975 plots except the yields were lower (Table 3 reports two cuts, Table 4 only the first cut). Only the intermediate fertilizer rates (20 and 40 kg N ha^{-1}) showed significantly ($P = 0.05$) higher yields for the inoculated plots.

Fertilizer nitrogen was required for inoculation response in every case we report. We believe that this nitrogen is necessary to produce a good level of plant growth and metabolism, especially photosynthesis, in our sandy, nitrogen deficient soil.

Acetylene reduction assays of nitrogenase activity using the washed root, preincubation procedure gave good nitrogenase activities in 1975. These values, however, did not correlate well with the yield responses. Only two samples per plot, taken one week apart, were assayed in late summer. This may not be adequate sampling to get correlation. In 1976, the core technique without preincubation was used. We don't believe that either of these acetylene reduction techniques adequately monitored the yield responses from inoculation.

Table 3. Buffelgrass dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (1975).

Fertilizer N Rate	Dry Matter Yield (kg ha^{-1})		Increase From Inoculation
	Inoculated	Uninoculated	
0	1640	2030	−390
20	2938	2642	292
40	3097	2549	548
80	3625	2974	651

Regression equations from inoculated and uninoculated plots were $\hat{Y}_I = 1700 + 55X - 0.4X^2$ and $\hat{Y}_C = 220 + 10N$, respectively.

In this test, significant inoculation effects were based on a significant inoculation X nitrogen interaction at 0.5 level.

Table 4. Guineagrass dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (cut 1, 1976).

Fertilizer N Rate	Dry Matter Yield (kg ha^{-1})		Increase From Inoculation
	Inoculated	Uninoculated	
0	2920	2440	480
20	4007	2986	1021*
40	4848	3158	1690*
80	4901	4708	193

Regression equations from inoculated and uninoculated plots were $\hat{Y}_I = 2896 + 69.9X - 0.56X^2$ and $\hat{Y}_C = 2348 + 27.8X$.

Asterisks indicate significance at 0.05 level.

We have conducted several experiments where yield responses due to inoculation were not obtained Therefore, we questioned the effectiveness of our inoculation and tried methods to improve it In 1976, about 100 times more bacterial cells were applied to the plots than in 1975 and they were placed into the root zone

To monitor soil population changes of *S lipoferum*, fluorescent immunological studies were made. Table 5 gives bacterial counts at about 10 months after the 1975 inoculation Inoculated plots gave significantly higher counts than the control plots but the control plots had a considerable number of bacteria The bacterial populations in uninoculated plots were relatively consistent over the fertilizer treatments, whereas, in inoculated plots higher counts were obtained as more nitrogen was applied These counts correlate well with the yield responses (see Table 1).

This research has shown that inoculation with *S lipoferum*, a tropical nitrogen-fixing bacterium, can increase yields or reduce nitrogen fertilizer requirement of pearl millet, guineagrass and buffelgrass. We have also shown that these increased yield responses can be repeated and that some nitrogen fertilizer was required to enhance them. We have shown that up to 40 kg N ha^{-1} can be replaced by inoculation This amount is economically important and indicates a high potential for grass-bacteria systems

Table 5. Bacterial populations Number of bacteria per gram of soil using the indirect FA technique

Treatment kg N ha^{-1}	Inoculated ($\times 10^2$)	Uninoculated ($\times 10^2$)
0	126	105
20	155	114
40	190	112
80	212	105
Mean	171	109

Inoculated means are significantly higher than uninoculated at 0 05 level

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DISCUSSION

E. A. Paul. Your data was based on dry weight. Do you have nitrogen contents for this material?

R. L. Smith. In 1974 nitrogen contents were reported. In 1975 no nitrogen differences were found over treatments so protein was proportional to dry matter yield. In 1976 analyses are not completed at this time.

E. A. Paul. Did you analyse the root systems for differences in yield and nitrogen content?

R. L. Smith. We did not harvest roots. Guineagrass and buffelgrass are perennial, therefore, we did not want to destroy the plots.

R. L. Todd. I might caution you about any conclusion concerning cell penetration by examining the 'cut-surface' of a specimen. Contamination is very possible.

R. L. Smith. We are aware of this problem and are going to use E. M. with plastic embedding to avoid the problem.

J. E. Beringer. The control is not a control as you are using killed bacteria. Therefore your inoculum may contribute nitrogen or some metabolic by product such as cytokinins and auxins. Why not use non-fixing isogenic bacteria as control?

R. L. Smith. Isogenic control bacteria are not available at this time. These will have to be made by mutation which may also disturb associating or N_2-fixing ability of the strain.