Determination of Increase in Yield of Tropical Grasses Inoculated with *Spirillum lipoferum*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NNN and GCV designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors LOA, NNN and GCV managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Increase in yield of tropical grasses viz. digit grass (*Digitaria decumbens*), guinea grass (*Panicum maximum*) and pearl millet (*Penisetum americanum*) inoculated with tropical nitrogen-fixing bacterium, *Spirillum lipoferum* was investigated. The study was carried out for three consecutive years (2016-2018). Dry matter yields and protein content of the three tropical grasses were used for the assessment. In 2017, pearl millet (*Penisetum americanum*) and guinea grass (*Panicum maximum*) produced significantly higher protein content and dry matter yields. Projected yields using regression analysis of both pearl millet and guinea grass indicated that about 40kgN ha^{-1} yr^{-1} were replaced by inoculation. Although, protein production of guinea grass was lower during 2018, dry matter yield responses were similar to those of 2017. This research has shown that inoculation with *Spirillum lipoferum*, a tropical nitrogen-fixing bacterium reduced acetylene and increased yields or reduced nitrogen fertilizer requirement of the tropical grasses as well as replacement of up to 40KgN ha^{-1}. This amount is agro-economically important and suggests the viability and potential for grass-bacteria systems.

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1. INTRODUCTION

Several bacteria have the capacity to co-exist with others in the rhizosphere of plants in the terrestrial ecosystem. Recently, some investigators have shown that some grasses have nitrogen fixing potential when associated with *Spirillum lipoferum* or other nitrogen fixing bacteria (NFB) [1,2,3]. This represents an important breakthrough in the area of agricultural research since the grass family includes most of the world's staple food crops: maize (*Zea mays),* wheat (*Triticum aestivum*), rice (*Oryza sativa*), Sorghum (*Sorghum bicolor*), millet (*Pennisetum americanum)* and forages. In a world now operating on costly and dwindling supplies in energy, dependence/application of biological nitrogen fixation in agriculture would be more ecofriendly and economical than chemical nitrogen or artificially synthesized nitrogen fertilizers.

Literature reports that, in Brazil, Quesenberry and Day [4] isolated *Spirillum lipoferum* from surface of disinfected roots of tropical grass, digit grass (*Digitaria decumbens*) which was introduced into Brazil by the Florida Experiment Station [5]. Since then strains have been isolated from maize, wheat and other grasses [1,2]. Nitrogen fixation rate projections of grass bacteria associations have been based on acetylene reduction activities of roots and soil cores. Rates as high as 2kg of fixed N. $ha^{-1} day^{-1}$ in maize, 1.7 in elephant grass (*Pennisetum purpureum*), and 90kg N fixed $ha^{-1} yr^{-1}$ in bahia grass were projected [6,7,8]. Essentially all of the acetylene reduction measurements showing high activity have been associated with a lag period and have undergone a pre-incubation 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 pre-incubation is valuable, however, in detecting grass root-*Spirillum* association.

2. MATERIALS AND METHODS

2.1 Study Plots and Location

Replicated grass plots were established at Nyande town farmland, Aleto in Eleme Local Government Area of Rivers State, Nigeria. These replicated grass plots were inoculated for studies during the past three years.

2.2 Collection of Plant Samples

A total of 40 tropical forage grass genotypes (five genera) were collected in 2016, and studied as paired plant, comparisons with one of each pair inoculated and the other left as a control. Inoculum was produced by homogenizing bacterial cultures of *Spirillum lipoferum* 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 without the application of nitrogen fertilizer.

In 2017, pearl millet, guinea grass, digit grass and buffel grass (*Cenchrus ciliaris*) were tested in larger ($3.2m^2$) plots replicated eight times. Four rates of nitrogen fertilizer were super imposed over inoculated and control plots. 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 suspension of *Spirillium lipoferum* 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 [7]. 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 7Kgha^{-1} of malate was applied to the control plots.

In 2018, the same species were tested. Pear millet and guinea grass were re-established with minimal cultivation. Inoculation was accomplished by injecting the bacteria about 7cm deep into the soil at the rate of about $3x10^9$ cells per m^2. Control plots received autoclaved medium at the same rate and by the same method as the inoculated plots.

2.3 Determination of Dry Matter and Nitrogen Content

Technicon autoanalyzer was used to measure dry matter yields and forage nitrogen content and was converted to protein by multiplying by 6.25 [1]. Yield and protein data were analyzed statistically. Acetylene reduction was

accomplished by a modification of Dobereiner and Day [8]. Indirect fluorescent antibody (FA) techniques as described by Schmidt [9] were used to specifically identify and count *S. lipoferum* in the soil. Washed roots were sealed in a plastic bag and then pre-incubated overnight in an atmosphere of 5% - 95% N_2. After pre-incubation, the atmosphere was replaced with fresh O_2-N_2 mixture as above, then 10% acetylene added. Gas samples were collected after a 3h incubation and placed in evacuated tubes. These were stored several days before analyzed for ethylene on a gas chromatograph.

3. RESULTS AND DISCUSSION

The protein and dry material yields of pearl millet, guinea grass and buffel grass for 2017 are presented in Tables 1, 2 and 3 respectively. The first cut of guinea grass response in terms of dry matter yields in 2018 is presented in Table 4.

The heterotrophic viable bacterial counts at about 11 months after the 2017 inoculation using the indirect fluorescent antibody (FA) techniques is presented in Table 5.

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

Higher dry matter and protein yields have been obtained from inoculated grasses compared to uninoculated counterpart in each of the three years of study. In the first year inoculated digit grass and guinea grass produced 62% and 80% more protein than the uninoculated controls. Dry matter yields for the genotypes were not grass genotypes for the second year's experiments.

In 2017, higher protein and dry matter yields were obtained from inoculated plots of pearl

Table 1. Pearl millet dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (2017)

Fertilizer N	Dry Matter Yields (kg ha^{-1})		Increase from
Rate	Inoculated	Uninoculated	Inoculation
0	4,650	5,200	-549
20	5,750	6070	-260
40	7,400	6,050	1,310*
80	9,140	7,880	1,260*

*Regression equations from inoculated and uninoculated plots were $Y_1 = 4,820 + 56x$ and $Y_c = 5,240 + 31X$ respectively. * = indicate significance at 0.05 level*

Table 2. Guinea grass dry matter forage yields inoculated with *Spirillum lipoferum* compared with uninoculated (2017)

Fertilizer N	Dry matter yields (kg ha^{-1})		Increased from
Rate	Inoculated	Uninoculated	Inoculation
0	9,850	9,750	-790
30	16,450	13,450	2,090*
60	18,410	16,460	2,050*
120	19,520	18.570	560

*Regression equations from inoculated and uninoculated plots were $Y_1 = 9970 + 189X - 0.9X^2$ and $Y_c = 10700$ yields + 80X respectively. * = indicate significance at 0.05 level*

Table 3. Buffel grass dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (2017)

Fertilizer N	Dry matter yields (kg ha^{-1})		Increased from
Rate	Inoculated	Uninoculated	Inoculation
0	15540	1950	-390
20	3026	2643	302
40	3098	2629	550
80	3725	3064	701

Regression equations from inoculated and uninoculated plots were $Y_1 = 1700 + 55X - 0.4X^2$ and $Y_c = 220 + 10N$, respectively

Table 4. Guinea grass dry matter forage yields inoculated with *Spirillum lipoferum* compared to uninoculated (Cut1, 2018)

Fertilizer N	Dry matter yields (kg ha^{-1})		Increased from
Rate	Inoculated	Uninoculated	Inoculation
0	3010	2730	480
20	3907	2896	1021*
40	5348	3247	1690*
80	4890	4808	193

*Regression equations from inoculated and uninoculated plots were $Y_1 = 2896 + 69.9X – 0.56X^2$ and $Yc = 2348 + 27.8X$. * = indicate significance at 0.05 level*

millet, guinea grass and buffel grass (Tables 1-3 respectively). Digit grass did not respond, as statistically its protein content was not 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 guinea grass and buffel grass the inoculated plots were curvilinear and the control linear. Significantly higher yields were obtained due to inoculation at 40 and 80 Kg fertilizer nitrogen rates for pearl millet and in the guinea grass at the 30 and 60 kg nitrogen rates. Using the regression equations, we calculated that up to 40kg Nha^{-1} in the millet and 39 Kg Nha^{-1} in the guinea grass were replaced by inoculation which also aggress with the work of Schank et al. [10]. Table 2, summarizes two cuts of guinea grass and it compares favourably with the work of [1].

Table 3 shows the yield response of inoculated and control buffel grass over four nitrogen fertilizer rates. These responses are similar to those of guinea grass, 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. This compares well with the reports of [11].

Table 4 shows 2018 first cut guinea grass responses. These data resemble the 2017 plots except that the yields were lower (Tables 3 and 4). This also aggress with the work of Smith et al. [1]. Only the intermediate fertilizer rates (20 and 40 kg Nha^{-1}) showed significantly (P=0.05) higher yields for the inoculated plots. Acetylene reduction assays of nitrogen activity using the washed root, pre-incubation procedure gave good nitrogen activities in 2017.

To monitor soil population changes of *Spirillum lipoferum*, fluorescent immunological studies

were made. Table 5 gives heterotrophic bacterial counts at about 10 months after the 2017 Inoculation. Inoculated plots gave significantly higher counts than the control plot. 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 (Table 1).

Table 5. Heterotrophic viable bacterial counts per gram (CFU/g) of soil using the indirect FA technique

Treatment Kg Nha^{-1}	Inoculation (x10^2)	Uninoculated (x10^2)
0	127	106
20	166	124
40	195	112
80	215	106
Mean	176	112

Inoculated means are significantly higher than uninoculated at 0.05 level

4. CONCLUSION

This research has demonstrated that inoculation of pearl millet, guinea grass and buffel grass with *Spirillum lipoferum*, a tropical nitrogen-fixing bacterium, can increase yields, reduce nitrogen fertilizer requirement and recovery of up to 40 Kg Nha^{-1} of dry matter and protein. Undoubtedly, this grass-bacterium treatment strategy is agro-economical, safe and potentially viable in comparison with the application of chemical fertilizers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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