

Potential for nitrogen fixation in the roots of *Pennisetum purpureum*

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SUMMARY

The potential for nitrogen fixation in the rhizosphere of *Pennisetum purpureum* was studied. Preliminary investigations showed that above-ground and below-ground biomass productions were 4.58 ± 3.84 and 1.65 ± 0.60 kg m⁻². Analysis of roots/rhizomes for carbon substrates showed that the levels of starch and organic acids were very low. Differences also occurred in the values between roots and rhizomes. The levels of reducing sugars and miscellaneous soluble sugars were considerably higher. Rhizosphere soil under stands of this grass had pH of 5.25-5.4 and was found to be sandy loam with high humus content. Highly significant correlation was found to exist between biomass production and total numbers of potential N₂-fixing bacteria ($r = 0.98$) and between counts of *Azospirillum* sp. and titratable acidity. In contrast, significantly very low correlation existed between reducing sugar content of roots/rhizomes and total anaerobic population. This was also observed between aerobic bacteria and miscellaneous soluble carbohydrates. The high biomass production observed could partly be attributed to the N₂-fixing potential of associated *Azospirillum* sp.

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Introduction

In nature, biological fixation of atmospheric nitrogen is widespread among prokaryotes. This process is catalysed by the enzyme nitrogenase. There exists no conclusive evidence of nitrogen fixation by eukaryotes (Dalton, 1974). There has been a great deal of interest in the study of free-living nitrogen-fixing micro-organisms (Mulder, 1975). The role of the process of fixation in global food

RÉSUMÉ

ADOKI, A. & ADOKI, A.B.: *Potentiel pour la fixation de l'azote dans les racines de Pennisetum purpureum*. Le potentiel pour la fixation de l'azote dans la rhizosphère de *Pennisetum purpureum* a été étudié. Des études préliminaires montraient que les productions de la biomasse en sur-sol et sous -sol étaient 4.58 ± 3.84 et 1.675 ± 0.60 kg/m², respectivement. Des analyses des substances carboniques de racines/rhizomes montraient que les niveaux des amidons et des acides organiques étaient très bas. Il y a eu aussi des différences entre les racines et les rhizomes. Les niveaux de sucres réducteurs et d'autres sucres solubles étaient plus hauts. Le sol de rhizosphère qui supporte cet herb a eu un pH de 5.25-5.4 et était limon-sableux avec un taux élevé de l'humus. Il y a une corrélation positive très importante entre la production de la biomasse et le nombre total de bactérie qui ont le potentiel pour fixer N₂ ($r=0.98$) et entre des comptes d'*Azospirillum* spp. et l'acidité titrable. En revanche, la corrélation négative très importante existait entre la teneur en sucre réducteur des racines/rhizomes et la population totale anaérobie. Cette corrélation négative a aussi été observée entre la bactérie aérobique et des diverses hydrates du carbone soluble. L'accroissement de la production de la biomasse observé peut être attribué partiellement au potentiel de fixer N₂ par l' *Azospirillum* spp.

production has been reviewed by Stewart (1975) and Dobereiner (1977).

The bacteria capable of fixing N₂ have been shown to belong to a limited number of families or genera. These include (a) the aerobic azotobacters, (b) the facultatively anaerobic klebsiellas, (c) the facultatively anaerobic bacilli of the *Bacillus polymyxa* and *B. macerans* group, (d) most of the anaerobic saccharolytic clostridia, (e) the anaero-

bic sulphate-reducing bacteria of the genera *Desulphovibrio* and *Desulphotomaculum*, (f) the photosynthetic bacteria and (g) some members of the Spirillaceae (Dalton, 1974; Mulder, 1975; Knowles, 1975). The relationship between the micro-organisms that possess this nitrogenase enzyme and the plants may be a rather close one in which the micro-organisms invade the cortical cells of the roots (Dobereiner, Day & Dart, 1972a; Dobereiner & Day, 1976; Patriquin, 1976).

That dinitrogen fixation of economic importance occurs under tropical grass cover has been suggested for more than a decade (Parker, 1957; Moore, 1963; Jaiyebo & Moore, 1963; Dobereiner, 1961; 1966). These observations have been confirmed with the introduction of the acetylene-reduction method and has shown the close plant-bacteria associations on which fixation depends (Rinaudo, 1970; Rinaudo, Balandreau & Dommergues, 1971; Yoshida & Ancajas, 1971; Dommergues *et al.*, 1973; Dobereiner, 1977; Dobereiner, Day & Dart, 1972a, b).

Much of the work done in the tropics all over the world in respect of N_2 -fixation by roots of grasses centred on determining or confirming the N_2 -fixing potential of tropical grasses, and showing how plant, micro-organism or edaphic factors affect this fixation (Mishustin & Shilnikova, 1971). The present study, along with a series of others in other grasses, seeks to show the relationship between biomass production, carbon-substrate levels in roots/rhizomes and densities of potential N_2 -fixing bacteria.

Materials and methods

Sites, samples and sampling

Samples of the grass studied were all collected at sites along the Choba-Port Harcourt Road, the University of Science and Technology Campus and the East-West Road up to Eleme in the Rivers State, Nigeria. These locations are within a 20-km radius of the city of Port Harcourt.

The above-ground and below-ground tissues of the plant were obtained for studies on biomass production while the carbon substrate content was

determined on the below-ground tissues. Potential nitrogen-fixing bacterial populations were determined on the below-ground tissues. Rhizosphere soil samples were taken for the determination of physico-chemical characteristics.

Samples were collected from within 50 cm² quadrats laid out within the grass vegetation. For biomass production studies, plant height and numbers of shoots within the quadrat were determined after which the shoots were excised at the soil surface level. The entire root/rhizome system from within each quadrat was dug up and shaken loose of adhering soil. Soil clumps were further carefully examined for fine roots which were also collected. Below-ground tissues and rhizosphere soils were stored in plastic bags and analysed within 1-2h after collection.

Sampling was commenced during mid-November and completed by the beginning of April.

Sample analysis

Biomass production. For this parameter, both the above-ground and below-ground tissues at each site were analysed by proximate measurements using a beam balance. Pre-drying treatments included cutting samples into shorter lengths for easy handling. These were spread on a wire gauze in smaller portions and dried at 90 °C for several days in a drying oven (B and T Model, Searl Company Limited, England) with intermittent weighing until constant weights were achieved. The final weight taken after drying represented biomass production calculated as kilogram per square metre (kgm⁻²). The difference between the fresh and dry weights represented the water content of the tissues.

Determination of carbon-source content in under-ground tissues. The carbon substrates determined were reducing sugars, miscellaneous soluble carbohydrates, organic acids as titratable acidity and starch.

For the quantitative determination of reducing sugars, the Hagedorn-Jensen method, which is based on the quantitative oxidation by potassium ferricyanide, was adopted as outlined by Allen *et*

al. (1974).

Miscellaneous soluble carbohydrates were determined by proximate analysis using a spectrophotometer (Hitachi Model, SEMAC, Japan). The method adopted was the anthrone procedure outlined by Allen *et al.* (1974).

For the determination of organic acids, only the water-soluble acids were considered. Titratable acidity was determined as described by Milton & Waters (1955).

Starch was extracted with perchloric acid and determined by the colorimetric modification procedure involving formation of a blue complex with iodine as outlined by Allen *et al.* (1974).

Soil analysis

In the soil analysis, the following were determined: Soil pH, organic nitrogen, inorganic nitrogen as ammonium nitrogen ($\text{NH}_4^+\text{-N}$) and nitrite nitrogen ($\text{NO}_2^-\text{-N}$).

Soil pH was determined by the soil-in-water method as described by Black (1965) using a pH meter (Model PYE UNICAM, Philips, England).

Organic nitrogen was determined by a digestion system using the traditional Kjeldahl procedure as outlined by Allen *et al.* (1974).

Inorganic nitrogen: $\text{NH}_4^+\text{-N}$ was determined in rhizosphere soil samples by the procedure outlined by Allen *et al.* (1974). Absorbance was measured at 635 nm using a spectrophotometer (Hitachi Model, SEMAC, Japan). Soil extract for $\text{NO}_2^-\text{-N}$ analysis was obtained in the same manner as described for $\text{NH}_4^+\text{-N}$ by Allen *et al.* (1974). For the determination of $\text{NO}_2^-\text{-N}$ in the extract, the method of Barnes & Folkhard (1951) was used. Absorbance was read at 543 nm using a spectrophotometer (Hitachi Model, SEMAC, Japan).

Microbiological analysis

Roots/rhizomes collected during the sampling exercise were shaken vigorously to remove adhering soil after which they were washed with running tap water to remove all residual soil. After washing, samples were left in a tray for about 10-20 min for water to drain. Twenty gramme portions, made up

of a 1:1 ratio of roots and rhizomes, were weighed and then surface-sterilized in 1 per cent chloramine-T solution for 1 h. After sterilization, tissues were then rinsed with sterile distilled water 3 times, by soaking them in the water for 10 min, at a time to remove all trace of the Chloramine-T. Surface-sterilized tissues were aseptically macerated using sterile mortar and pestle. The macerated tissues were then transferred aseptically into conical flasks containing 180 ml sterile distilled water to give a 1-in-10 dilution from which subsequent dilutions were made.

Enumeration of potential N_2 -fixers such as aerobes, microaerophiles, strict and facultative anaerobes was done using the MPN count method (Abd-El Malek, 1971). For the estimation of aerobic and microaerophilic N_2 fixers, growth with the formation of surface and subsurface pellicles, respectively, were regarded as positive. In addition to the microaerophilic and aerobic N_2 -fixers, facultative anaerobes were also enumerated in aerobic cultures since gas bubble formation indicative of nitrogen-fixing strict/facultative anaerobes was observed. Anaerobic growth was indicated by turbidity and gas formation in cultures. For the spirilla, actual microscopic examination of all aerobic tubes was carried out to ascertain the presence of these micro-organisms, since pellicle formation had always been associated with the occurrence of *Spirillum* sp. In addition, microscopic examination of the anaerobically-incubated tubes was also carried out to ascertain the possibility of spirilla thriving in anaerobic cultures.

Culture media for the estimation of numbers of N_2 -fixing bacteria. Two culture media were used:

Medium A, which is essentially the same as that outlined by Adoki (1984), was used for the enumeration of aerobic, microaerophilic and facultative anaerobic N_2 -fixing bacteria and contained per litre of distilled water, the following: Mannitol, 5 g; sucrose, 5 g; sodium malate, 0.5 g; KH_2PO_4 , 0.80 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.20 g; CaCl_2 , 0.06 g; NaCl, 0.10 g; Yeast extract, 100 mg; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 25 mg; Na_2FeEDTA , 28 mg (27 mg Na_2EDTA + 1 mg FeCl_3); Biotin, 5 mg; PABA, 10 mg; Difco agar, 5 g; pH 7.0.

PABA and biotin were sterilized by membrane filtration in 100 ml of distilled water and added to 900 ml of cooled incomplete medium containing the other components. The complete medium was then dispensed aseptically in 9 ml portions into sterile universal culture bottles.

Medium B was used for the cultivation of strict and facultative anaerobes. It was essentially a modification of *Medium A*, except that it contained no agar, but contained 0.2 g sodium thioglycate and 0.1 g ascorbic acid added to the autoclaved portion before sterilization to enhance anaerobiosis. Sterilization and dispensing were done as for *Medium A* in rubber cap-stoppered culture test tubes. After inoculation, the surface of the medium in the tube was layered with a sterile plug of agar (2%) to further enhance anaerobiosis. All cultures were incubated at 37 °C.

Number of N_2 -fixing bacteria was computed by the MPN technique described by Abd-El-Malek (1971).

Isolation, characterization and identification of potential N_2 -fixing bacteria. For isolation, *Medium A* was made solid by adding Difco agar (1.5%). Isolation was done by first streaking from cultures, from the highest dilutions indicating positive growth of N_2 -fixers. Pure cultures were obtained by streaking out on nutrient agar plates. Isolates thus obtained were transferred to *Medium A* (solid agar slants) for subsequent use in identification tests.

Identification of isolates. Identification criteria included cultural, morphological and biochemical tests. Biochemical tests were grouped as follows:

Group A (production of acid and gas from glucose-peptone water) - MRVP, indole and urease production, lactose fermentation, citrate utilization, starch hydrolysis, oxidase test and detection of catalase.

Group B (production of acid only from glucose-peptone water) - Same as for *Group A*.

Group C (no change in glucose-peptone water) - requirement of biotin, malate and mannitol utilization, and hydrolysis of starch.

Cultural and morphological tests were performed

for each group and included colony characters such as pigment production on solid agar plates, slime production, motility and cell shape.

For *Groups A* and *B*, biochemical, cultural and morphological tests were essentially those outlined by Cruickshank *et al.* (1975). For *Group C*, a modified semi-solid version of *Medium A* was used. Three sets of this modified medium were prepared, each set having malate, mannitol or starch as sole carbon source.

Results

Biomass production

The range of biomass production of different stands of *Pennisetum purpureum* is presented in Table 1. Shoot biomass production ranged between 0.74 and 8.42 kg m⁻² dry weight with a mean value of 4.58 ± 3.84 kg m⁻². Similarly, production in roots/rhizomes ranged between 1.05 and 2.25 kg m⁻² dry weight with a mean value of 1.65 ± 0.60 kg m⁻². Shoot height, number and water content are also presented in Table 1.

TABLE 1

Shoot Number, Shoot Height, Water Content and Total Dry Matter Production in Pennisetum purpureum

Number of shoots per square metre	44.5 ± 17.82
Shoot height (m)	3.73 ± 0.19
Water content (%)	56.1 ± 18.28
Mean biomass production (Dry wt kg m ⁻²)	
Above-ground	4.58 ± 3.84
Below-ground	1.65 ± 0.60

Soluble carbon substrate content in below-ground tissues

Results of studies involving the determination of levels of different soluble carbon substrates in below-ground tissues of the plant are outlined in Table 2. Data presented show that marked differences occurred in the concentrations of these carbon sources. Reducing sugars were of higher

concentrations (18.13 ± 5.39 in roots and 46.98 ± 21.87 in rhizomes) when compared with the concentrations of the other carbon sources analysed.

A comparison of concentrations of carbon substrates showed that appreciably wide differences existed when roots and rhizomes are considered except for starch and organic acids. Higher con-

TABLE 2

Concentration of Soluble Carbon Substrates in Below-ground Tissues (mg g^{-1} , dry weight)

Substrate	Roots	Rhizomes
Total reducing sugars	18.13 ± 5.39	46.98 ± 21.87
Miscellaneous soluble carbohydrates	3.05 ± 1.81	8.46 ± 4.22
Total organic acids (as titratable acidity)	0.15 ± 0.04	0.18 ± 0.15
Starch	0.36 ± 0.15	0.35 ± 0.14

centrations generally occurred in the rhizomes.

Physico-chemical characteristics of rhizosphere soil

Analyses of rhizosphere soil samples revealed that soils under stands of plants of *Pennisetum purpureum* were generally sandy loam or sandy loam with high humus content with pH values ranging between 5.25 and 5.4 (5.34 ± 0.05).

Variations were observed in the nitrogen contents of rhizosphere soil samples. The mean organic nitrogen level was $2.97 \pm 1.47 \text{ mg g}^{-1}$. Similar variations also existed between inorganic nitrogen levels as shown in Table 3. Nitrite-nitrogen ($\text{NO}_2^- - \text{N}$) levels were much higher than those of ammonium-nitrogen ($\text{NH}_4^+ - \text{N}$).

Microbiological analyses

Counts of aerobic, microaerophilic and strict/facultative potential N_2 -fixing bacteria associated with rhizosphere of *P. purpureum* are presented in Table 4. Results obtained show that there were very wide variations in counts at the different

TABLE 3

Physico-chemical Characteristics of Rhizosphere Soil

Soil type	Sandy loam, sandy loam with high humus content
pH	5.25-5.4 (5.34 ± 0.05)
Mean nitrogen (mg g^{-1})	
Organic	2.97 ± 1.47
Inorganic	
$\text{NO}_2^- - \text{N}$	0.70 ± 0.61
$\text{NH}_4^+ - \text{N}$	0.007 ± 0.003

quadrats sampled. The mean count for aerobic N_2 -fixing bacteria was $14.37 \pm 26.42 \times 10^6 \text{ cfu/g}$. The formation of subsurface pellicle in semi-solid agar cultures has frequently been associated with microaerophilic organisms and in this case *Azospirillum*. Counts of *Azospirillum* as subsurface pellicle were $8.43 \pm 9.12 \times 10^6 \text{ cfu/g}$. Comparatively, microscopic examination of tubes showing growth of microaerophilic organisms revealed that actual counts of *Azospirillum* were $0.34 \pm 0.483 \times 10^6 \text{ cfu/g}$. Surface pellicle formers (strict aerobes) constituted 58.7 per cent of growth in aerobic cultures as seen also from Table 4. Similarly, azospirilla when enumerated as subsurface pellicle formers, constituted 26.11 per cent. Microscopic examination showed that these constituted only 2.37 per cent.

Counts of strict/facultative anaerobes (gas producers in anaerobic culture) showed a mean value of $12.04 \pm 7.93 \times 10^6 \text{ cells g}^{-1}$ dry weight (Table 4). Slightly lower counts were recorded for facultative anaerobes (gas producers in anaerobic cultures). Microscopic examination of anaerobic cultures showed the presence of spirilla and constituted 2.5 per cent of anaerobic cultures.

A common feature of *Azospirillum* sp. and related organisms is that being microaerophilic, their growth is characterized by the formation of a pellicle beneath the surface of semi-solid nutrient medium in tubes. This characteristic growth is often used to estimate the numbers of these

TABLE 4

Viable Counts of Nitrogen-fixing in Rhizosphere of Pennisetum purpureum ($\times 10^6$ cells g^{-1} roots/rhizomes, fresh weight)

<i>Aerobic</i>	<i>Count</i>	<i>Percentage of total aerobes</i>	<i>Anaerobic</i>	<i>Count</i>
Strict aerobes (counted as surface pellicle and turbidity)	14.37 \pm 26.42	58.66	Strict/facultative anaerobes (gas producers in anaerobic culture)	12.04 \pm 7.93
<i>Azospirillum</i> sp. as subsurface	8.43 \pm 9.12	26.11	Facultative anaerobes (gas producers in aerobic culture)	8.40 \pm 9.37
By microscopy	0.34 \pm 0.48	2.37	<i>Azospirillum</i> (microscopic examination)	0.31 \pm 0.42
			Percentage of anaerobic spirilla cultures	2.57

organisms in cultures. The actual presence of these organisms was confirmed in this study by microscopic examination of tubes showing pellicle formation. A comparison of counts obtained by the above-mentioned criteria showed that by pellicle formation only, *Azospirillum* sp. and related organisms were as high as 24.0×10^6 cells per gram of tissue. By contrast, no counts were recorded when actual microscopic examination of such cultures was carried out. Similarly, a culture with a count of 54.0×10^6 cells by subsurface pellicle formation had by microscopic examination, 0.68×10^6 of *Azospirillum* sp.

Morphological, physiological and biochemical tests of isolates from all cultures showing surface and subsurface pellicles, gas production in aerobic and anaerobic cultures showed that *Enterobacter* sp. constituted 50 per cent, *Pseudomonas*, 12.5 per cent and *Azospirillum* and related organisms, 12.5 per cent of total isolates. Other unidentified organisms also constituted 12.5 per cent. Organisms of the genus *Klebsiella* (*Aerobacter*) were conspicuously absent as presented in Table 5.

Statistical analysis

Statistical analysis was carried out to determine possible negative or positive correlation between different parameters studied. Fig. 1 shows that

TABLE 5

Percentage Occurrence of Isolates from Rhizosphere of Pennisetum purpureum

<i>Organism</i>	<i>No. of isolates</i>	<i>% of total isolates</i>
<i>Enterobacter</i>	4	50
<i>Klebsiella</i> (<i>Aerobacter</i>)	0	0
<i>Pseudomonas</i>	1	12.5
<i>Azospirillum</i> sp. and related organisms	2	25.0
Others	1	12.5

there was a very high positive correlation between the number of potential N_2 -fixing bacteria in the rhizosphere of *Pennisetum purpureum* and the amount of total dry matter produced by this plant ($r = 0.98$). An identical trend was obtained when concentrations of organic acids (as titratable acidity) in roots/rhizomes are compared with the corresponding counts of *Azospirillum* sp. in aerobic cultures, in (Fig. 2.) At a concentration of $0.125 \text{ mg } g^{-1}$ titratable acidity, the bacterial count was zero. A similar attempt at demonstrating possible correlation shows that a very low positive correlation ($r=0.34$) which was not significant at the 95 per cent

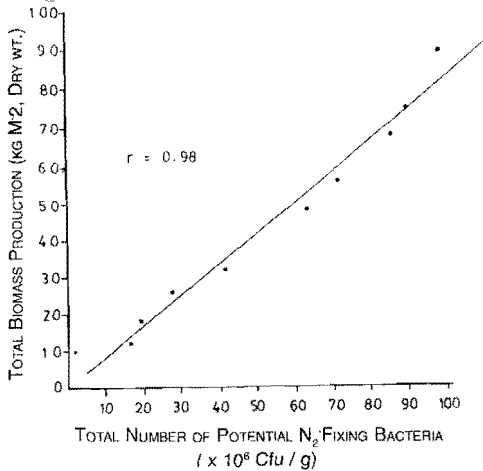


Fig. 1. Relationship between dry matter production and numbers of potential N₂-fixing rhizosphere bacteria in *P. purpureum*.

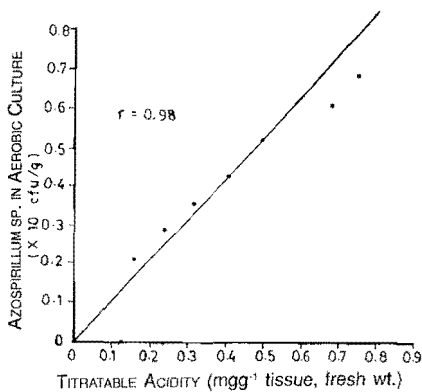


Fig. 2. Correlation between titratable acidity content of roots/rhizomes and viable counts of *Azospirillum* sp. in rhizosphere of *P. purpureum*.

confidence limit, existed between the concentrations of reducing sugars in roots/rhizomes and counts of total anaerobic bacteria. Fig. 3 shows that at a concentration of 24 mg g⁻¹ reducing sugar counts were 16.0 × 10⁶ and 160 × 10⁶ cfu/g. A count of 160 × 10⁶ cells was also recorded at a concentration of 47 mg g⁻¹.

In order to investigate the existence of any correlation between the concentration of readily available and oxidizable carbon substrates in the roots/rhizomes, statistical analyses were carried out. Results showed that there was the absence of

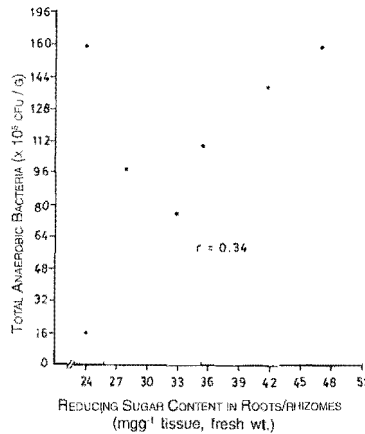


Fig. 3. Relationship between reducing sugar contents of roots/rhizomes and viable counts of anaerobic N₂-fixing rhizosphere bacteria in *P. purpureum*.

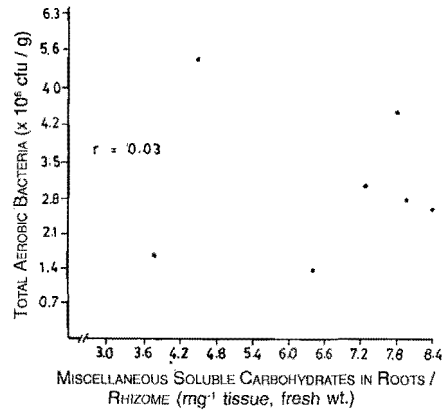


Fig. 4. Relationship between miscellaneous soluble carbohydrate content of roots/rhizomes and populations of total aerobic N₂-fixing rhizosphere bacteria in *P. purpureum*.

any significant correlation between numbers of anaerobic bacteria and concentrations of reducing sugars ($r=0.34$; Fig. 3). Similarly, Fig. 4 shows that there was the near absence of correlation between numbers of aerobic bacteria and concentrations of miscellaneous soluble carbohydrates ($r = 0.03$).

Discussion and conclusion

A number of bacteria have been found to be associated with nitrogen fixation in the roots of some tropical grasses including *Panicum maximum*,

Pennisetum purpureum and *Andropogon tectorum* (Dobereiner & Day, 1976). The bacteria include some members of the Enterobacteriaceae notably *Enterobacter cloacae* and *Klebsiella pneumoniae* which were conspicuously absent in the grass studied, *Azospirillum* sp., the aerobic *Bacillus* spp., and the obligately anaerobic clostridia (Knowles, 1975).

In grasses there is the current belief that the major N_2 -fixing bacteria belong to the genus *Azospirillum* (Bulow & Dobereiner, 1975). Results obtained by comparing the formation of pellicles in semi-solid media and the actual occurrence of spirilla from microscopic examination of culture tubes, indicate that the number of *Azospirillum* sp. may in fact be small compared to that of the aerobic and anaerobic N_2 -fixers. The high numbers obtained by previous workers, for example Bulow & Dobereiner (1975), Dobereiner & Day (1976) and Balandreau *et al.* (1975), may in fact have been due to their associating pellicle formation with the occurrence of spirilla in the roots of *Pennisetum purpureum* and similar grasses. That this may have been the case can be seen more clearly in Table 4 where numbers of spirilla attributable to counts based on observation of pellicle were quite high as compared to the low numbers obtained based on actual microscopic examination. Based on this finding, it is suggested that actual microscopic examination is necessary to positively determine the occurrence of *Azospirillum* in counts of N_2 -fixers.

In some grasses, such as wheat, other grass-bacteria associations have been found. Neal & Larson (1976) described a very specific association of one wheat line with a *Bacillus* sp. and a lower incidence of total bacteria in the rhizosphere. In the present study, the results obtained showed greater number of aerobic N_2 -fixers compared to the spirilla. Similarly, numbers of the facultative/strict anaerobic N_2 -fixers, including *Enterobacter*, were relatively high. In a similar study, Balandreau *et al.* (1975) have shown that numbers of aerobic N_2 -fixers were higher than those of the anaerobic species in the rice plant. A comparison of counts

presented in Table 4 shows a striking relationship between horizontal parameters under aerobic and anaerobic considerations.

The amount of nitrogen fixed by plants would in general be determined partly by the number of N_2 -fixing heterotrophic bacteria on their roots. This number in turn is determined by other factors including the levels and availability of carbon substrates, soil pH and levels of inorganic ions (Paul, Meyer & Rice, 1971; Mulder & Brotonegoro, 1974). The amount of nitrogen thus fixed would determine biomass production since nitrogen is essential for the build-up of tissue proteins. Fig. 1 shows more or less linear correlation ($r=0.98$; $P=0.05$) between total numbers of potential N_2 -fixers and total dry matter production.

Assuming the nutrient conversion rate of a plant is low, it could be speculated that there would be a build-up of absorbed nutrients in the plant tissues to such an extent that they become actually inhibitory to the plant's development. The nutrient conversion rate would be indirectly related to the amount of root system developed. In plants with a high nutrient conversion rate and high biomass production, there would be the need to develop a corresponding efficient root system where shoot and root/rhizome dry matter production levels are positively correlated (Adoki, 1984).

The numbers as well as development of potential N_2 -fixing bacteria on grass roots are determined to great extent by the presence and availability of carbon compounds (Dobereiner, Day & Dart, 1972) and very low amounts of combined nitrogen (Macura & Kunc, 1961) so that wide C/N ratios would favour N_2 -fixation (Jensen, 1965; Abd-El-Malek, 1971). In the grass studied, wide C/N ratios were observed between levels of total soluble carbohydrates, reducing sugars and NH_4^+ -N and NO_2^- -N. The types of carbon substrates available determine the predominant N_2 -fixing group (Brouzes, Mayfield & Knowles, 1971; Paul, Meyers & Rice, 1971). That this is the case can be seen in the association of *Azospirillum* with organic acids where most strains do not use sugars but commonly grow on malate (Dobereiner & Day, 1976).

Generally, low levels of organic acids and high levels of total soluble carbohydrates and reducing sugars occurred in the roots/rhizomes of this grass. The latter forms, not effectively utilized by the *Azospirillum* group, could be responsible for their rather low numbers in the grass.

It has been observed (Dobereiner & Day, 1976) that although *Azospirillum lipoferum* is quite capable of fixing N_2 , it can grow more rapidly when supplied with a source of combined nitrogen, especially ammonia (Burris, Okon & Albrecht, 1976). Table 3 shows a very low level of soil $NH_4^+ - N$, which could also have contributed to the low numbers observed for the *Azospirillum* group. If similar conditions exist in soils under maize cultivation, this result could partially explain the observed low level of N input in maize roots associated with *Azospirillum* by (Burris, 1976; Burris, Albrecht & Okon, 1977).

On the other hand, these findings may be explained if the levels of these carbon substrates are determined seasonally, for example in the temperate grass *Spartina* where these have been reported to show a seasonal pattern corresponding to that of fixation rates (Patriquin & McClung, 1981). Table 2 shows a relatively high level of reducing sugars but less of starch. Starch is a reserve or storage carbohydrate, therefore, its level would be drastically reduced in grasses that are still in active growth. Since the grass studied has a seasonal pattern of growth, the levels of storage carbohydrates would show a corresponding variation. Starch, like other reserve carbohydrates, would be hydrolysed for use during the growing season. This could partially explain the very low levels of starch, since samples were collected when the grass was still in active growth.

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