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 1.67±0.60 kg m⁻², respectively. 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.

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 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 N₂-fixation by roots of grasses centred on determining or confirming the N₂-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 N₂-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 Hagedarn-Jensen method, which is based on the quantitative oxidation by potassium ferricyanide, was adopted as outlined by Allen et
Nitrogen fixation in the roots of *Pennisetum purpureum* (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 (NH$_4^+$-N) and nitrite nitrogen (NO$_2^-$-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: NH$_4^+$-N was determined in rhizosphere soil samples by the procedure outlined by Allen et al. (1974). Absorbance was measured at 635 mm using a spectrophotometer (Hitachi Model, SEMAC, Japan). Soil extract for NO$_2^-$-N analysis was obtained in the same manner as described for NH$_4^+$-N by Allen et al. (1974). For the determination of NO$_2^-$-N in the extract, the method of Barnes & Folkhard (1951) was used. Absorbance was read at 543 mm 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 percent 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-ElMalek, 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$_2$PO$_4$, 0.80 g; MgSO$_4$, 7H$_2$O, 0.20 g; CaCl$_2$, 0.06 g; NaCl, 0.10 g; Yeast extract, 100 mg; NaN$_3$O$_4$, 2H$_2$O, 25 mg; Na$_2$FeEDTA, 28 mg (27 mg Na$_2$EDTA + 1 mg FeCl$_3$); Biotin, 5 mg; PABA, 10 mg; Difco agar, 5g; 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 thioglyolate 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 N₂-fixing bacteria was computed by the MPN technique described by Abd-El-Malek (1971).

Isolation, characterization and identification of potential N₂-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 N₂-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 outline 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⁻¹ dry weight with a mean value of 1.65 ± 0.60 kg m⁻¹. 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) | 4.58 ± 3.84 |
| Above-ground | 1.65 ± 0.60 |
| Below-ground | |

**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
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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 concentrations generally occurred in the rhizomes.

**TABLE 2**

Concentration of Soluble Carbon Substrates in Belowground 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              | 0.15±0.04         | 0.18±0.15         |
| Starch                           | 0.36±0.15         | 0.35±0.14         |

Physico-chemical characteristics of rhizosphere soil

Analyses of rhizosphere soil samples revealed that soils under stands 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±1.47 mg g⁻¹. Similar variations also existed between inorganic nitrogen levels as shown in Table 3. Nitrite-nitrogen (NO₂⁻-N) levels were much higher than those of ammonium-nitrogen (NH₄⁺-N).

**Microbiological analyses**

Counts of aerobic, microaerophilic and strict/facultative potential N₂-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 quadrats sampled. The mean count for aerobic N₂-fixing bacteria was 14.37±26.42 × 10⁶ 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 ± 9.12 × 10⁶ cfu/g. Comparatively, microscopic examination of tubes showing growth of microaerophilic organisms revealed that actual counts of *Azospirillum* were 0.34± 0.48 × 10⁶ 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 facultative anaerobes (gas producers in anaerobic culture) showed a mean value of 12.04 ± 7.93 × 10⁶ 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 organisms.
There was a very high positive correlation between the number of potential N₂-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 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 percent
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**Fig. 1.** Relationship between dry matter production and numbers of potential N\(_2\)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\(\times\)10\(^6\) and 160\(\times\)10\(^6\) cfu/g. A count of 160\(\times\)10\(^6\) 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 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*,

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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 N₂-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 N₂-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 pellicles 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 N₂-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 N₂-fixers compared to the spirilla. Similarly, numbers of the facultative/strict anaerobic N₂-fixers, including Enterobacter, were relatively high. In a similar study, Balandreau et al. (1975) have shown that numbers of aerobic N₂-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 N₂-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 N₂-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 N₂-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 (Maca & Kunc, 1961) so that wide C/N ratios would favour N₂-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 NH₄⁺-N and NO₃⁻-N. The types of carbon substrates available determine the predominant N₂-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 N₂, 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 NH₄⁺ - 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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