

# Potential for nitrogen fixation and biomass production in *Panicum maximum*

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## SUMMARY

Investigations were carried out to ascertain the relationship between the potential for biomass production and nitrogen-fixation in the rhizosphere of *Panicum maximum*. With a dry matter production of  $1.85 \pm 1.40$  and  $0.48 \pm 0.33$  kgm<sup>-2</sup>, a positive correlation ( $r=0.62$ ) was found to exist between the numbers of nitrogen-fixing bacteria in the rhizosphere of this grass and total dry matter production. The influence of concentrations of organic carbon substrates in roots/rhizomes on total bacterial populations was also investigated. Results showed that increasing concentrations of organic acids, reducing sugars and miscellaneous soluble carbohydrates at naturally-occurring levels were accompanied by reductions in bacterial population. Aerobic nitrogen-fixing bacteria appeared to play the most dominant role as viable counts of these bacteria were about four times higher than those of their anaerobic counterparts. *Azospirillum* sp. contributed approximately 48 per cent of total aerobic counts and was found to be capable of growth under strictly anaerobic conditions where it constituted about 11 per cent of total counts. *Panicum maximum* was found to be capable of growth in soils of clay-loam, sandy-clay or purely loam types with pH 5.0-5.9. The ammonium-nitrogen levels in these soils were relatively low when compared to those of nitrite-nitrogen.

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## Introduction

There has been a great deal of interest in the study of free-living nitrogen-fixing micro-organisms (Mulder, 1975). This interest was stimulated by the isolation of *Clostridium pasteurianum* by Winogradsky in 1893 and the isolation of

## RÉSUMÉ

ADOKI, A. & ADOKI, A. : Potentiel pour la fixation de l'azote et de la production de la biomasse dans *Panicum maximum*. Des études ont été faites pour établir le rapport entre le potentiel pour la production de la biomasse et de la fixation de l'azote dans le rhizosphère de *Panicum maximum*. Les résultats montrent qu'il existe une corrélation positive ( $r = 0.62$ ) entre le nombre des bactéries qui fixent l'azote dans le rhizosphère de cette herbe et la production totale de la matière sèche de  $1.85 \pm 1.40$  et  $0.48 \pm 0.33$  kgm<sup>-2</sup>. L'influence des concentrations des substrats de carbone organique des racines/rhizomes sur les populations bactériennes était aussi étudié. Les résultats montrent encore que les augmentations de concentrations du sucre organiques, du sucre reducteur et des divers hydrates de carbone soluble à des niveaux naturelles étaient accompagnés par la baisse de la population des bactéries. Les bactéries aérobiques qui fixent l'azote apparaissent de jouer un rôle importante étant donné que la population de ces bactéries était quatre fois plus élevée que celle des bactéries anaérobiques. L'*Azospirillum* sp. a contribué environ 48% des bactéries aérobiques totale et était capable de pousser dans des conditions anaérobiques où il a constitué environ 11% de la compte totale. *Panicum maximum* était capable de pousser dans des sols de type limonargilleux, argile-sableuse ou limoneux avec le pH de 5.0-5.9. Les niveaux de l'azote ammoniacal dans ces sols étaient relativement plus petit que le niveau de l'azote nitrite.

*Azotobacter chroococcum* and *A. agilis* by Beijerinck in 1901. This interest was centred on the belief that these organisms contribute substantially to the nitrogen supply of higher plants. In nature, biological fixation of atmospheric nitrogen is widespread among prokaryotes. This process is

catalysed by the enzyme nitrogenase. In contrast, no good evidence exists to support nitrogen fixation by eukaryotes (Dalton, 1974). 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  $N_2$  have been shown to belong to a limited number of families or genera. These include: (a) the aerobic azotobacters; (b) most of the anaerobic saccharophytic *Clostridia*; (c) the facultatively anaerobic *Klebsiella*; (d) the facultatively anaerobic bacilli of the *Bacillus polymyxa* and *B. macerans*; (e) the anaerobic 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, 1972; Dobereiner & Day, 1976; Patriquin, 1976). In some instances, the relationship is specific and a typical example is the association of *Paspalum notatum* and *Azotobacter paspali*. The occurrence of these bacteria is restricted to the distribution of certain ecotypes of this grass (Dobereiner, 1970) and *Pennisetum purpureum* (Dobereiner, 1970; Dobereiner & Day, 1976). However, other associations may show no specificity.

Much of the work done in the tropics in respect of  $N_2$ -fixation by roots of grasses centred on determining or confirming the  $N_2$ -fixing potential of these grasses, and showing how plant, micro-organism or edaphic factors affect this fixation (Mishustin & Shilnikova, 1971). However, not much work, if any, has been done towards showing the relationship between the potential to fix nitrogen, biomass production and levels of root-associated carbon substrates since the amounts and levels of organic substrates in the rhizosphere may, to a great extent, determine the level of nitrogenase activity (Bergersen, 1970). Such studies may reveal the role of the carbon substrates

accumulated in underground tissues in the observed high levels of nitrogenase activity in these roots. This study with *Panicum maximum* focuses attention on direct field investigations and to show possible correlation between dry matter production and potential  $N_2$ -fixing bacteria in the rhizosphere of this grass in some Nigeria soils.

### Materials and methods

#### *Sites, samples and sampling*

During this study, only fully-grown and mature plants of *Panicum maximum* were treated. Plant and soil samples were collected at sites on the campuses of University of Port Harcourt and University of Science and Technology, the Choba-Port Harcourt road and the east-west road all in the Rivers State of Nigeria. Shoot and below-ground portions of the plant were obtained for studies on dry matter production and concentrations of readily available carbon sources. Enumeration of potential nitrogen-fixing bacteria associated with the rhizosphere of *P. maximum* was carried out with the roots and rhizomes which constituted the below-ground tissues. Soil samples were also collected for studies on physico-chemical characteristics of rhizosphere soil.

Samples were collected from 50 cm<sup>2</sup> quadrats laid out within the grass stands. Direct field measurements included plant height and numbers of shoots per quadrat. Shoots were subsequently excised at the soil surface level and the entire root/rhizome system dug up and shaken loose of adhering soil. Soil clumps were further carefully examined for fine roots which were similarly collected. Roots/rhizomes and rhizosphere soils were stored in plastic bags and analysed within 1-2 h after sampling. Sampling for the various studies was carried out during the period between mid-November and April.

#### *Sample analyses*

**Biomass production.** Dry matter production was determined for the above- and below-ground portions by proximate measurements using a beam balance. Prior to drying, samples were cut into shorter lengths and spread on a wire gauze for

drying at 90 °C in a hot air oven (B and T, Searl Company Limited, England) with intermittent weighing until constant weights were achieved. The final weight recorded after drying represented biomass production as kilogram per square metre ( $\text{kg m}^{-2}$ ).

*Soil analysis.* The parameters investigated were soil type, pH and total nitrogen.

- i) Soil pH was determined by the soil-in-water method described by Black (1965) using a pH meter (PYE, UNICAM, Philips, England).
- ii) The levels of organic nitrogen in rhizosphere soils were determined by the traditional Kjeldahl procedure as outlined by Allen *et al.* (1974).
- iii) Ammonium nitrogen ( $\text{NH}_4^+$ -N) content was determined by the method outlined by Allen *et al.* (1974). Absorbance was measured at 635 nm using a spectrophotometer (Hitachi, SEMAC, Japan). Soil extract for the analysis of nitrite-nitrogen ( $\text{NO}_2^-$ -N) was obtained by the method of Allen *et al.* (1974). The concentration of  $\text{NO}_2^-$ -N in the extract was determined by the method of Barnes & Folkhard (1951). Absorbance was read at 543 nm using a spectrophotometer (Hitachi, SEMAC, Japan).

*Carbon sources in underground tissues.* The carbon sources considered were reducing sugars, total soluble carbohydrates, organic acids as titratable acidity and starch.

- i) The levels of reducing sugars in roots/rhizomes were determined by the Hagedorn-Jensen method as outlined by Allen *et al.* (1974).
- ii) Total soluble carbohydrates were determined by proximate analysis using the anthrone procedure outlined by Allen *et al.* (1974). Absorbance was read at 625 nm using a spectrophotometer.
- iii) The organic acids considered were the water-soluble fractions. Titratable acidity was determined as described by Milton & Waters

(1955).

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

*Microbiological analysis.* Samples of roots and rhizomes collected were shaken vigorously to remove adhering soil particles. These were then washed with running tap water to remove residual soil particles and left in a tray for 10 min for water to drain. Twenty gramme (20 g) portions made up of a 1:1 ratio of roots and rhizomes were weighed and surface-sterilized in 1 per cent Chloramine-T solution for 1 h. After sterilization, the tissues were then rinsed three times with sterile distilled water by soaking them in the water for 10 minutes at a time to remove all traces of the chloramine-T. Surface-sterilized tissues were then aseptically macerated using sterile mortar and pestle. The macerated tissues were subsequently transferred aseptically into conical flasks containing 180 ml sterile distilled water. This suspension was then used for further dilutions.

Enumeration of potential  $\text{N}_2$ -fixing bacteria as strict aerobes, microaerophiles, strict and facultative anaerobes was done using the most probable number (MPN) technique of Abd-El-Malek (1971). Aerobes and microaerophilic  $\text{N}_2$ -fixers were enumerated as surface and subsurface pellicle formation respectively. The facultative anaerobes were enumerated by scoring positive for aerobic cultures showing gas production, and strict anaerobes by turbidity and gas production in anaerobic cultures. Microscopic examination of cultures was carried out to confirm the presence of *Azospirilla*.

#### *Culture media*

Two sets of media (Medium A and Medium B) were used for the detection and enumeration of  $\text{N}_2$ -fixing rhizosphere bacteria. Medium A is essentially the same as that outlined by Adoki (1984). It was used for the enumeration of aerobic, microaerophilic

and facultative anaerobic bacteria. Medium B was used for the cultivation of strict and facultative anaerobes. It is a modification of Medium A except that it is liquid. It contained in addition to the other components, 0.2 g sodium thioglycolate and 0.1 g ascorbic acid per litre. After inoculation, the surface of this medium in tubes was layered with a sterile plug of agar (2 per cent) to further enhance anaerobiosis. Cultures were incubated at 37 °C.

#### *Isolation, characterization and identification of potential $N_2$ -fixing bacteria*

Organisms growing in cultures showing positive for the characters mentioned above were isolated on plates of a solid version of Medium A. Criteria used in the identification of isolates included cultural, morphological and biochemical tests. Biochemical tests were grouped as follows:

Group A: (Production of acid and gas in glucose-peptone water) - Methyl red and Voges-Proskauer tests, indole and urease production, lactose fermentation, citrate utilization, hydrolysis of starch, oxidase test and detection of catalase.

Group B: (Production of acid only in 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.

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

## Results

### *Dry matter production*

With a shoot height of approximately 2.7 m, only slight variations were observed between the numbers of shoots per square metre at the different locations sampled. In contrast, seemingly high variations were recorded in dry matter production. Mean shoot dry matter production was  $1.85 \pm 1.40$

compared to  $0.48 \pm 0.33$  for below ground tissues

TABLE 1

*Shoot Number and Height, Water Content and Total Dry Matter Production in Panicum maximum*

Number of shoots per square metre	$188 \pm 2.65$
Shoot height (m)	$1.69 \pm 0.31$
Water content (%)	$30.37 \pm 14.25$
Dry matter production (kg m <sup>-2</sup> )	
Above-ground	$1.85 \pm 1.40$
Below-ground	$0.48 \pm 0.33$

as presented in Table 1.

### *Carbon sources in below-ground tissues*

Marked differences were noticed in the levels of the carbon sources analysed when the values for roots and rhizomes are compared with the exception of the organic acids. Data presented in Table 2 show that the highest levels were recorded for reducing sugars with concentrations of approximately 19.2 and 60.7 mgg<sup>-1</sup> (dry weight basis) in roots and rhizomes respectively. Comparatively, the levels for starch were 0.21 and 1.2 mgg<sup>-1</sup> (dry weight) for roots and rhizomes respectively. Similarly, low levels were recorded for organic acids.

### *Physico-chemical characteristics of rhizosphere soil*

Rhizosphere soils under *Panicum maximum*

TABLE 2

*Concentrations of Soluble Carbon Substrates in Below-ground Tissues (mgg<sup>-1</sup>, dry weight)*

Substrate	Root	Rhizome
Total reducing sugars	$19.19 \pm 7.99$	$60.69 \pm 36.56$
Starch	$0.21 \pm 0.21$	$1.2 \pm 0.93$
Miscellaneous carbohydrates	$3.67 \pm 3.23$	$11.16 \pm 6.46$
Total organic acids (as titratable acidity)	$0.18 \pm 0.15$	$0.14 \pm 0.07$

were either clay-loam, sandy-clay or loam with pH 5.0-5.9 (Table 3). Corresponding differences were also recorded in the organic and inorganic nitrogen levels. The organic nitrogen contents of the soils ranged from 1.26 to 3.26 mgg<sup>-1</sup> soil. The concentration of nitrite-nitrogen was relatively very high compared to the levels recorded for ammonium-

nitrogen (Table 3). Very marked differences were recorded in the levels for ammonium-nitrogen as values obtained ranged from 0.001 to 0.01 mgg<sup>-1</sup>.

#### Enumeration and identification of *N<sub>2</sub>*-fixing bacteria in rhizosphere

Viable counts of potential *N<sub>2</sub>*-fixing bacteria associated with the rhizosphere of *P. maximum* are presented in Table 4. Data presented show that with a count of  $58.41 \times 10^6$  cfu/g, the strict aerobic bacteria were more numerous than the other groups considered. Marked differences were observed in the counts of *Azospirillum* sp. when pellicle formation and microscopy are considered. As subsurface pellicle formers, *Azospirillum* sp., constituted only approximately 29 per cent whereas by microscopy this figure rose to 48 per cent of total aerobic counts. Furthermore, microscopic examination of anaerobic cultures showed that the medium used was capable of sustaining *Azospirillum* where, with a count of  $1.75 \times 10^6$  cfu/g, they

TABLE 3

#### Physico-chemical Characteristics of Rhizosphere Soil

Soil type	Clay loam, sandy-clay, loam
pH (soil in water)	5.0 - 5.9 (5.52 ± 0.29)
Total nitrogen (mgg <sup>-1</sup> )	
Organic N	1.26 - 3.26
Inorganic N	
NO <sub>2</sub> -N	0.67 - 1.25
NH <sub>4</sub> <sup>+</sup> - N	0.001 - 0.1

TABLE 4

#### Viable Counts (MPN) of Nitrogen-fixing Bacteria in Rhizosphere of *Panicum maximum* ( $\times 10^6$ cfu/g<sup>1</sup> root/rhizome fresh weight)

Aerobic	Count	Anaerobic	Count
Strict aerobes (as surface pellicle and turbidity)	58.14 ± 75.03	Strict facultative anaerobes (gas producers in anaerobic cultures)	16.11 ± 17.72
<i>Azospirillum</i> sp. (as subsurface pellicle)	16.75 ± 14.19	Facultative anaerobes (gas producers in aerobic cultures)	3.88 ± 3.81
Percentage of total aerobes as subsurface pellicle	28.68		
Percentage of total aerobes as <i>Azospirillum</i> from microscopy	38.07		
<i>Azospirillum</i> (viable counts from microscopic examination)	28.06 ± 21.95	<i>Azospirillum</i> (microscopic examination)	1.75 ± 1.96
		Percentage of total anaerobes as <i>Azospirillum</i>	10.86

constituted about 11 per cent of total anaerobic counts.

From results of cultural, morphological and biochemical tests it was found that the bacteria associated with the rhizosphere of the grass belonged to the following genera: *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Azospirillum* and related bacteria, and a group of unidentified genera. Based on the percentage of total isolates, bacteria in the family Enterobacteriaceae were the single most important group responsible for nitrogen fixation in the rhizosphere of *P. maximum* as shown in Table 5. Comparatively, *Azospirillum* constituted only a small proportion of the total.

#### Statistical analysis

Results of statistical analysis of data obtained from field samples are presented in Fig. 1-4. There

TABLE 5

Percentage Occurrence of Isolates from Rhizosphere of *Panicum maximum*

Organism (genus)	No. of isolates	% of isolates
<i>Enterobacter</i>	6	37.5
<i>Klebsiella</i>	4	25
<i>Pseudomonas</i>	3	18.75
<i>Azospirillum</i> and related bacteria	1	6.25
Others	2	12.5

was a positive correlation between the total numbers of potential  $N_2$ -fixing bacteria in the rhizosphere of *P. maximum* and total dry matter production (Fig. 1). A similar but negative correlation existed between the viable counts of *Azospirillum* sp. and levels of organic acids in the roots/rhizomes as shown in Fig. 2. In contrast, a very low (negative) correlation ( $r=0.13$ ) existed between the viable counts of total aerobic bacteria and levels of miscellaneous soluble carbohydrates (Fig. 3). The existence of any correlation between the levels of

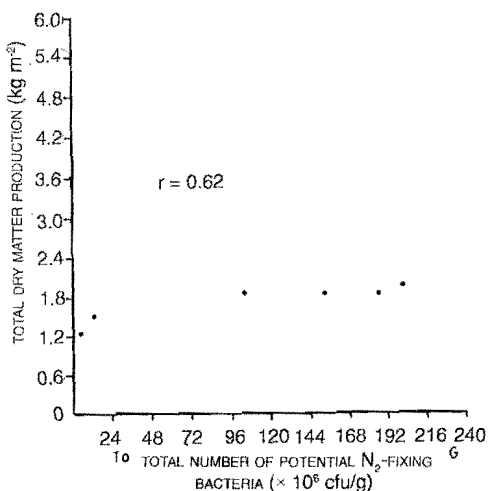


Fig. 1. Relationship between total viable counts of potential  $N_2$ -fixing rhizosphere bacteria and dry matter production in *P. maximum*.

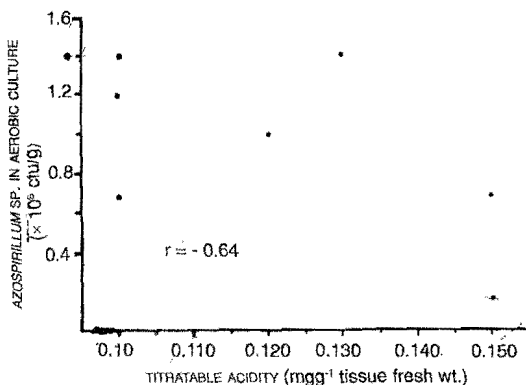


Fig. 2. Relationship between titratable acidity content in roots/rhizomes and viable counts of *Azospirillum* sp. in rhizosphere of *P. maximum*.

reducing sugars and total anaerobic bacteria was similarly ascertained. Data presented in Fig. 4 show that there was a negative correlation ( $r=0.77$ ) between the levels of reducing sugars in the roots/rhizomes of this grass and the viable counts of total anaerobic bacteria in the rhizosphere.

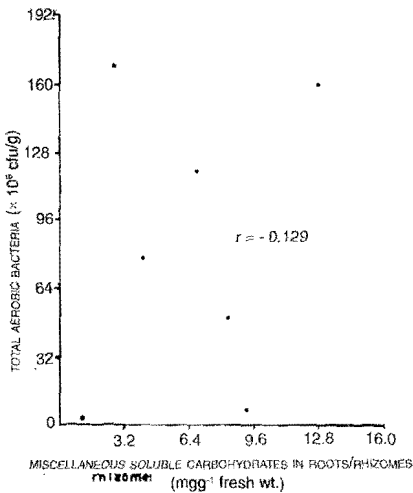


Fig. 3. Relationship between miscellaneous soluble carbohydrates in roots/rhizomes and aerobic  $N_2$ -fixing rhizosphere bacteria in *P. maximum*.

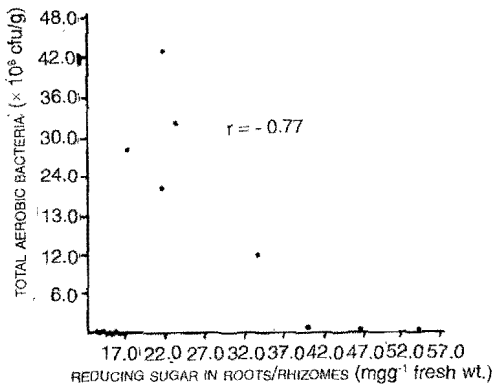


Fig. 4. Influence of reducing sugar in roots/rhizomes on distribution of anaerobic  $N_2$ -fixing rhizosphere bacteria in *P. maximum*.

### Discussion and conclusion

There has been great interest in the study of organisms associated with the rhizosphere of tropical grasses because of their reported potential to fix nitrogen (Dobereiner & Day, 1976; Dobereiner *et al.*, 1976; De-Polli *et al.*, 1977). In this study, it

was found that the major  $N_2$ -fixing bacteria in the rhizosphere of *P. maximum* were *Enterobacter* sp., *Klebsiella* sp., *Pseudomonas* sp., *Azospirillum* sp. and a group of other unidentified genera (Table 5). Although constituting only about 6 per cent of total isolates, actual microscopic examination of aerobic cultures revealed that *Azospirillum* constituted about 48 per cent of total counts. It could, therefore, be deduced that *Azospirillum* is the major  $N_2$ -fixing genus in the rhizosphere of *Panicum maximum*. Table 4 shows that the contribution of anaerobic bacteria to  $N_2$ -fixation in the rhizosphere of this grass is small compared to that of the aerobic bacteria.

Several grass-bacteria associations have been reported. Neal & Larson (1976) have described a very specific association of one wheat line with a *Bacillus* sp. and a lower incidence of total bacteria in the rhizosphere. Their report contrasts with that of Burris *et al.* (1977) who carried out controlled experiments with maize and *Azospirillum* sp. in which no significant difference was noted between inoculated and control plants. From results obtained in this study with *P. maximum*, a negative correlation existed between the concentration of organic acids and counts of *Azospirillum*. The absence of any significant difference reported by Burris *et al.* (1977) could be due to the type of system investigated. The grass studied is, in many respects, different from maize. One of such differences is the presence of rhizomes and, therefore, storage materials in *P. maximum*. Starch is stored in grains on cobs at the shoot level in maize.

The amount of nitrogen fixed by plants, including grasses, would in general be determined partly by the number of  $N_2$ -fixing bacteria associated with their roots (Burris, 1976). This number would in turn be determined by other factors including the levels and availability of carbon substrates (Balandreau *et al.*, 1977), soil pH and levels of inorganic ions (Mulder & Brotonegoro, 1974). Since nitrogen is required for the synthesis of tissue proteins, the amount of dry matter produced by plants would be determined by the amount of nitrogen fixed. Statistical analysis of data obtained

as presented on Fig.1 show that a positive correlation existed between dry matter production in *P. maximum* and the counts of total  $N_2$ -fixing bacteria in the rhizosphere.

When free-living  $N_2$ -fixing bacteria are supplied with  $NH_4^+$ , rapid uptake and assimilation of this compound takes place (Mulder, 1975). This was shown for *Azotobacter vinelandii* by Burris & Wilson (1946) and for *Clostridium pasteurianum* by Daesch & Mortensen (1972). Aerobic bacteria of the *Azotobacter* type respond in a different way to added  $NH_4^+$ .

Immediately after the addition of small amounts of an ammonium salt to an  $N_2$ -fixing culture, nitrogenase activity declines and after one or two hours comes to a complete stand still (Brotonegoro, 1974). It could, therefore, be speculated that low amounts of soil  $NH_4^+$  will favour the development for aerobic  $N_2$ -fixing bacteria. Results presented in Tables 1 and 4 show that with relatively very low  $NH_4^+$  levels, the ratio of aerobic to anaerobic bacteria was approximately 3.6:1.

The development of free-living bacteria in soil is favoured by the presence of considerable amounts of available carbon compound and very low amounts of combined nitrogen so that the C/N ratios are high (Mulder, 1975).

Data presented in Tables 2 and 3 show such wide ratios and could have contributed to the relatively high numbers of nitrogen fixers in the rhizosphere of this grass. If the environments are well provided with combined nitrogen, non-nitrogen-fixing micro-organisms will readily develop and successfully compete with the nitrogen fixers for carbon compounds (Macura & Kunc, 1961).

Since the numbers of potential  $N_2$ -fixing bacteria in the rhizosphere of the grass *Panicum maximum* have been shown to be significantly correlated to the levels of available carbon substrates, it could be speculated that a seasonal variation would influence these counts. Patriquin & MacChung (1981) have reported a seasonal pattern of levels of carbon substrates corresponding to fixation rates in the grass *Spartina*. Table 2 shows a relatively high level of reducing sugars but less of starch.

The level of starch, which is a reserve carbohydrate, in grasses that are still in active growth would be drastically reduced. 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 recorded since samples were collected when the grass was still in active growth.

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