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Studies on N_2-fixing bacteria associated with the salt-tolerant grass, *Leptochloa fusca* (L.) Kunth

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Introduction

Numerous reports on nitrogen fixation in grasses and cereals have recently been reviewed by Hubbell & Gaskins (1984) and Dart (1986). There have also been many attempts to isolate and describe nitrogen-fixing micro-organisms with relevant eco-physiological characteristics from the rhizosphere of different plants. (Balandreau 1983; Lindberg & Granhall 1984).

Leptochloa fusca (L.) Kunth is a primary colonizer of salt-affected soils in Pakistan. Several studies so far carried out on this grass have revealed the presence of high nitrogenase activity associated with its roots (Malik *et al.* 1980, 1982; Bors *et al.* 1982; Malik & Zafar 1985). A recent detailed study (Zafar *et al.* 1986) pertaining to seasonal variations in the nitrogenase activity of excised roots and the estimation of diazotrophs in the various fractions of the roots, resulted in the collection of many N_2-fixing bacteria. In this work, morphological, biochemical and some physiological properties of three N_2-fixing isolates from the roots are described.

Materials and methods

Bacterial isolates

Isolations were made from the root segments of *L. fusca* which exhibited acetylene reduction activity (ARA) as described by Von Bulow & Döbereiner (1975). Small root pieces (2 cm long) were inoculated on N-free semi-solid medium (5 ml in 17 ml serum vials) of combined carbon (Rennie 1981) and incubated for two days at 30°C. Acetylene reduction assay (ARA) was performed after the incubation period. Micro-organisms responsible for N_2-fixation were isolated from positive ARA vials by streaking a loopful on Nutrient Agar (Difco) plates. Nitrogen-fixing bacteria were also obtained by streaking from those vials which were used earlier for the enumeration of

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diazotrophs from different fractions of roots (Zafar *et al.* 1986). Single colonies were rechecked for nitrogenase activity by ARA. Cultures were given code numbers and maintained on nutrient agar slants at 5°C and recultured after every three months.

Characteristics of bacterial isolates

Bacteria were subcultured from the maintenance medium and grown for 24–48 h in nutrient broth; inoculum from this was used for common identification tests. Standard morphological, biochemical and nutritional tests were performed as described by Claus (1979). Additionally, commercial identification kits, API 20E and API 50CH (API System, S.A. La Balme les Grottes, 38390 Montalieu, France) were employed. Base composition of DNA of the isolates was measured by the thermal denaturation (Tm) method of Mandel & Marmur (1968), *Escherichia coli* K 12 with a known guanine + cytosine (G+C) content of 51.7 being used as a standard. The results were evaluated by comparison with reference strains, standard literature and the API analytical profile index.

^{15}N enrichment studies

Three isolates and *Azospirillum brasilense* sp. 7 (a positive check) were grown for 36 h in semi-solid CCM. In the culture flask an atmosphere comprising 30% $^{15}N_2$ (17% a.e.), 1% O_2 and 69% Ar was prepared. The inoculated culture flasks were incubated at 30 ± 2°C for two days. N-analysis was carried out by the Kjeldahl method. ^{15}N analysis was done by ^{15}N emission spectrometry (^{15}N. Analysator NOI-5. Statron, E. Germany).

Physiological studies

Effects of combined nitrogen (NO_3^- and NH_4^+), pH and salt (NaCl) on nitrogenase activity of three isolates were examined. Conditions were similar for all the three isolates if not mentioned otherwise.

A loopful of bacteria was taken from a slant and suspended in 30 ml nutrient broth in a 150 ml Erlenmeyer flask. After 24 h of growth, 1 ml of this suspension was added to 30 ml phosphate buffer (0.1 mol/l, pH 7.0) and mixed thoroughly. This suspension was used as inoculum for further studies. Three ml of inoculum was added to 200 ml semi-solid CCM (0.2% agar) in a 500 ml Erlenmeyer flask and swirled thoroughly. Twenty ml amounts of the inoculated CCM were then distributed in 50 ml narrow-mouthed Erlenmeyer flasks and incubated at 35°C. Duplicate samples were taken after 24, 48, 72 and 96 h, for ARA.

NH_4Cl was used to study the effect of ammonium (NH_4^+), and $NaNO_3$ for the effect of nitrate (NO_3^-). Three treatments of 1, 2 and 5 mmol/l were used and the control was without N-source.

For the studies on the effect of pH on the nitrogenase activity of the three isolates, the same CCM medium was used but with lower concentrations of phosphate buffer (K_2HPO_4 – 0.4 g/l and KH_2PO_4 = 0.1 g/l) and supplemented with yeast extract (100 mg/l). pH was adjusted with NaOH or HCl and rechecked after autoclaving.

The effects of five levels of salt (NaCl) were studied at two pH levels (7.5 and 9.5). The isolates were inoculated on semi-solid CCM as described for the pH experiment except that 1, 2, 3, 4 or 5% NaCl was added. A control sample having 0.01% NaCl at each pH was also included. ARA was performed as described earlier.

The data were subjected to analysis of variance followed by a Least Significant Difference (L.S.D.) test for planned comparisons of paired means.

Acetylene reduction assay (ARA) was performed as described by Hardy *et al.* (1968).

Results

An attempt was made to identify all the N_2-fixing isolates from the roots of *L. fusca*. It was observed that a broad spectrum of diazotrophs was present around the roots of this grass. The most readily isolated bacteria was found to be NIAB-1. These isolates were thoroughly characterized and results are presented in Table 1. API-20E and 50CH system reconfirmed the results obtained earlier by conventional techniques. ^{15}N enrichment was observed in all bacterial isolates (Table 2).

Table 1 Biochemical and physiological characteristics of the three bacterial isolates from the rhizosphere of *L. fusca*

Characteristic	Reaction		
	NIAB-1	C-2	Isolate-2
Catalase production	−	+	+
Oxidase production	−	(+)w	−
Indole production	−	−	−
Voges–Proskauer reaction	+	+	+
Citrate utilization	+	+	+
Urea hydrolysis	+	−	+
$NO_3^- \rightarrow NO_2^-$	+	+	+
Denitrification	−	−	−
Gelatin hydrolysis	−	−	−
Starch hydrolysis	−	−	−
Tween hydrolysis			
20	−	−	−
60	−	−	−
80	−	−	−
pHB hydrolysis	−	−	−
Arginine dihydrolase	−	+	−
Lysine decarboxylase	−	(+)w	−
Ornithine decarboxylase	−	+	−
β-galactosidase	+	+	+
H_2S production	−	−	−
Pigment production	−	−	−
mol % G+C content	56.9	63.6	53.2

+, positive; (+)w, weak; −, negative.

Results on the effect of NH_4^+ and NO_3^- on nitrogenase activity of the three isolates are shown in Fig. 1. Control samples (in N-free medium) of all the isolates exhibited lower ARA values as compared to other treatments. These isolates showed substantial differences however, activity being found to be less in NH_4^+− grown cultures than in NO_3^-− treated cultures. ARA of all isolates was found to be in the increasing order of 1 <5 <2 mmol/l in 24 h grown cultures, except for isolate C−2 in which medium activity was observed in 1 mmol/l NH_4^+ instead of 5 mmol/l NH_4^+.

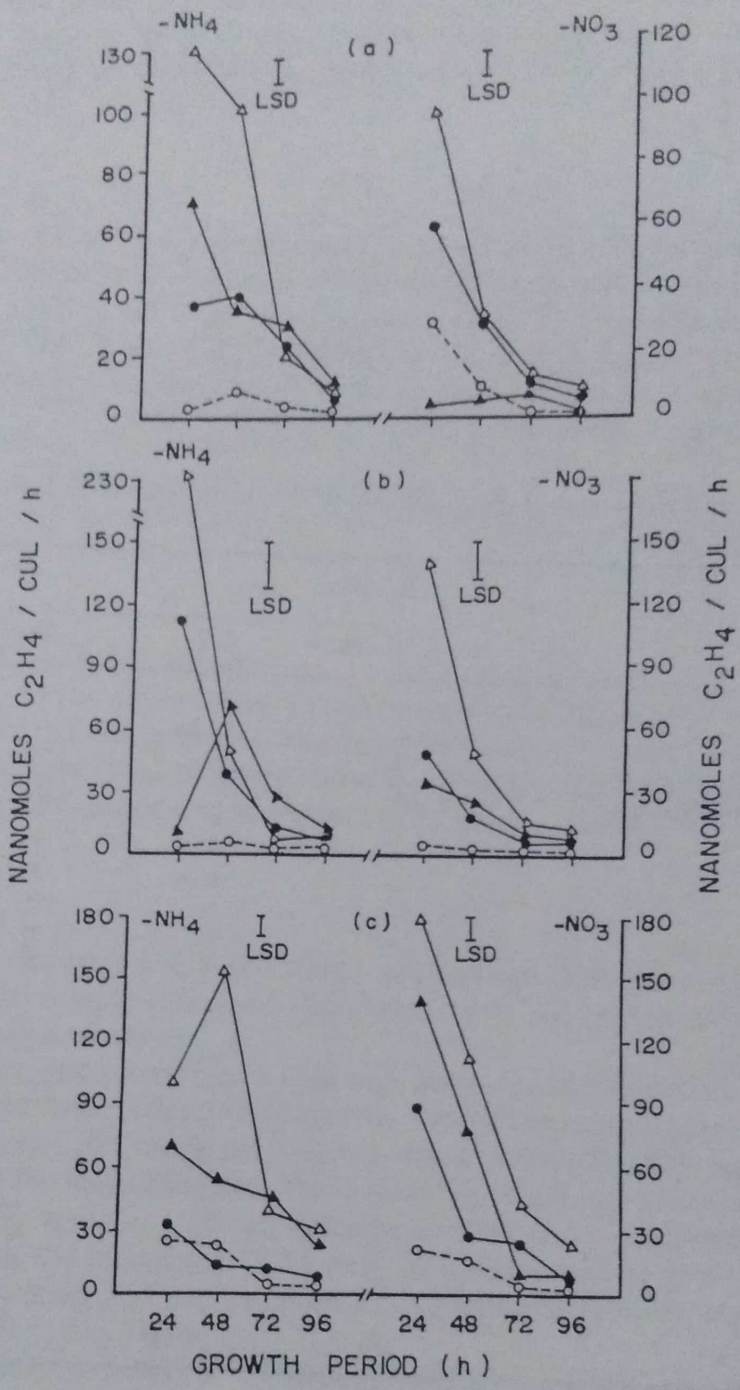

Fig. 1 Nitrogenase activity (nmoles C_2H_4/culture/h) of N_2-fixing isolates from *L. fusca* grown in semi-solid CCM medium with different levels of NH_4^+ or NO_3^-. LSD ($P<0.05$) is for the overall comparison (treatments × time period) for each isolate. (a) NIAB-1; (b) C-2; (c) Iso-2. ○--○, No nitrogen control; ●—●, 1 mmol/l (NH_4^+ or NO_3^-); △—△, 2 mmol/l; ▲—▲, 5 mmol/l.

The three isolates exhibited nitrogenase activity at five different pH values, (5.5 to 9.0) but activity was highest in alkaline conditions (Fig. 2). After 24 h of growth in modified CCM, NIAB-1 exhibited maximum nitrogenase activity (195 nanomoles C_2H_4/culture/h) at pH 9. This isolate had nearly the same activity at pH 7, 8 and 9 and

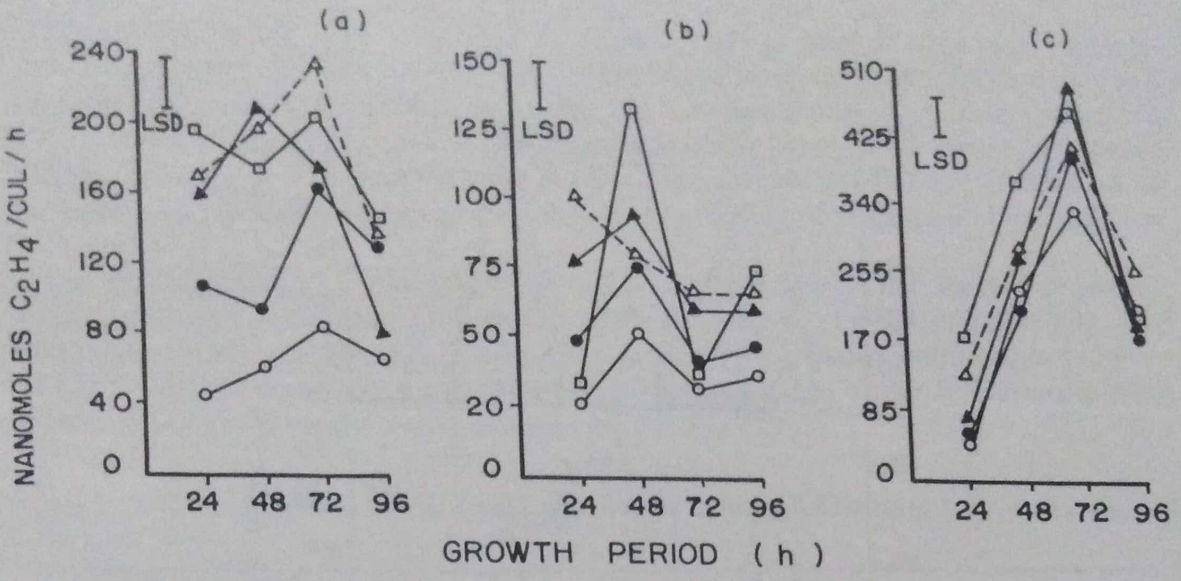

Fig. 2 Nitrogenase activity (nmoles C_2H_4/culture/h) of bacterial isolates measured at different pH values. (a) NIAB-1; (b) C-2; (c) Iso-2. Cultures were grown in semi-solid CCM. LSD ($P<0.05$) is for the overall comparison of each isolate, ○—○, pH 5.5; ●—●, 6.0; △--△, 7.0; ▲—▲, 8.0; □—□, 9.0.

a similar result was given by Iso-2. The third isolate, C–2, was active over a narrow pH range with a maximum mean activity of 103 nanomoles C_2H_4/culture/h at pH 7. At both extremes of pH, low values of ARA were observed. The nitrogenase activity measured after 48 h of growth was found to be increasing. Activity dropped after 72 and 96 h of growth except in the case of the Iso-2 where at 72 h ARA values were higher.

Experiments were also performed to study the effects of 5 levels of salt (NaCl) at two different pH levels. Results of these experiments are presented in Fig. 3. All isolates were unable to grow and fix nitrogen with 5% NaCl at pH 7.5 and 9.5. The ARA values of all isolates with 4% NaCl were low and remained at the same level at the four sampling times (these values are not shown in Fig. 3). Bacterial isolates NIAB-1 and C-2 exhibited higher activity at the 2% NaCl level, while in Iso-2 maximum activity was observed at the 1% NaCl level. The ARA values measured after 48 h of growth were found to be increasing, but they decreased later.

Discussion

Leptochloa fusca is a primary colonizer of salt-affected soils in Pakistan. These wastelands have total N of <0.01% and organic C of <0.5%. In spite of the low fertility the grass grows well and a biomass production of 40 tonnes/ha/year has been estimated. Under these conditions, the N harvest can amount to 180 kg N/ha/year (Malik & Zafar 1985). One possible way in which this may occur is by means of biological nitrogen fixation and several studies have been made to investigate the nitrogen-fixing system of this grass (Bors *et al.* 1982; Kloss *et al.* 1984; Malik & Zafar 1985; Reinhold *et al.* 1985). Many physiological tests have been used to identify bacteria isolated by us and the mol % G+C values of their DNAs have been determined. Isolates NIAB–1, C–2 and Iso–2 have mol % G+C contents of 56.9, 63.6 and 53.2 respectively. There was no possibility of *Azospirillum* spp, being present

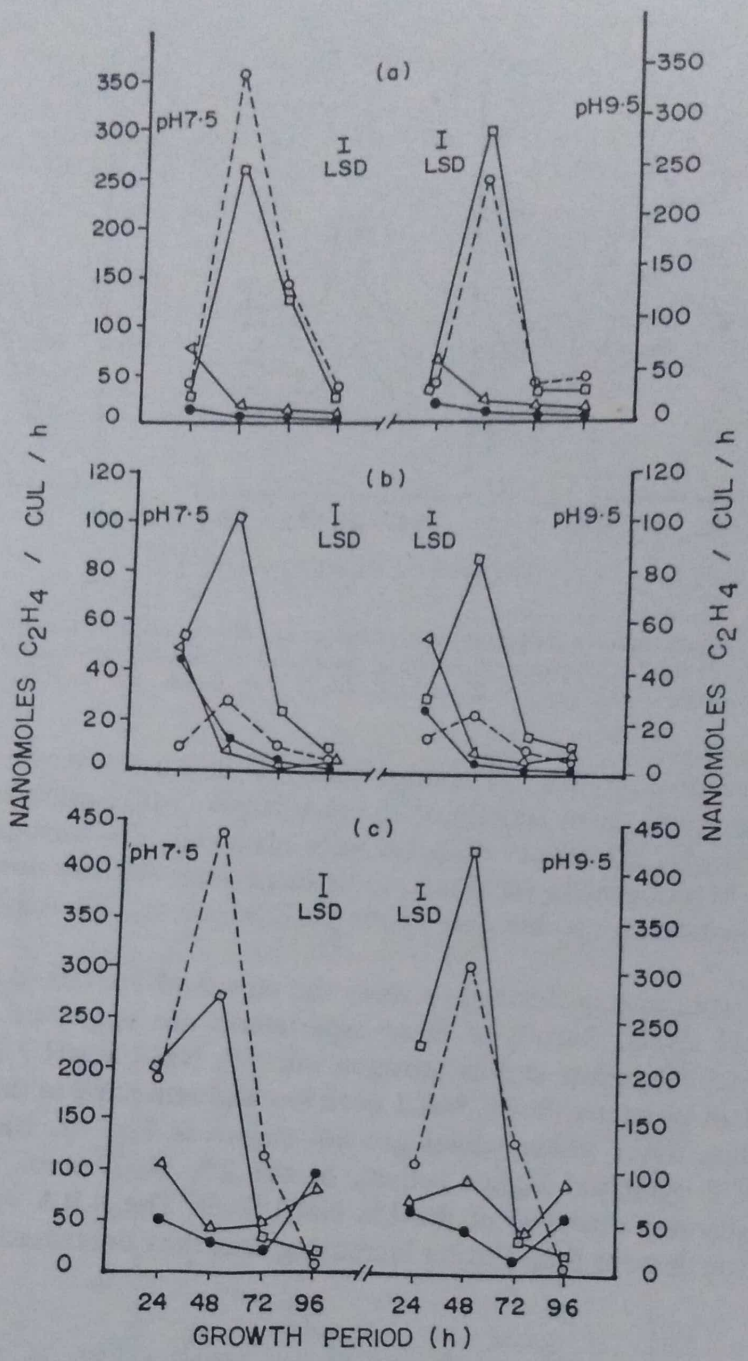

Fig. 3 Combined effect of pH and salt (NaCl) concentration on nitrogenase activity of bacterial isolates grown in semi-solid CCM with pH adjusted to 7.5 and 9.5. (a) NIAB-1; (b) C-2; (c) Iso-2. ARAs were performed at various time intervals. LSD ($P<0.05$) is for the overall comparison of each isolate. ○—○, 0.01% NaCl (control); □—□, 1% NaCl; △—△, 2% NaCl; ●—●, 3% NaCl.

because the mol % G+C of this genus is in the range of 68–70 (Krieg & Döbereiner 1984). In another study on kallar grass roots, Bors *et al.* (1982) were able to identify one of their strains (SST 22) as *A. brasilense* and recently Reinhold *et al.* (1985a) reported the isolation of *A. halopraeferans*, a new species of the genus *Azospirillum* from naturally growing *L. fusca* in Pakistan. They also observed that the genus *Azospirillum* was not dominant in the rhizosphere and endorhizosphere but on the

rhizoplane (Reinhold *et al.* 1985b). It is known that the type of organisms isolated from rhizosphere depends on the media and conditions used for enrichment and isolation (Balandreau 1983). In our study, the combined carbon medium of Rennie (1981) was employed because all commonly isolated genera of dinitrogen fixing bacteria grow on it. ^{15}N incorporation studies were carried out and enrichment by all the isolated strains was observed, which clearly indicates that these isolates are true N_2-fixers.

The characteristics of these isolates were compared with the known N_2-fixers, *Azotobacter* and *Azomonas* (Johnstone 1974; Becking 1981), *Beijerinckia* (Becking 1981), and *Klebsiella* (Seidler 1981). The summary of major similarities between NIAB-1 and *K. pneumoniae* and *K. oxytoca* is presented in Table 3. The occurrence of this genus has recently been reviewed by Seidler (1981).

Table 2 ^{15}N incorporation by bacterial isolates from kallar grass after 48 h of incubation with 17% a.e. $^{15}N_2$.

Isolate	Nitrogen fixed* (mg/20 ml medium)	Atom % excess† ^{15}N at the end of incubation
Azospirillium brasilense	0.56 c	0.15±0.01
C-2	0.67 c	0.17±0.03
NIAB-1	1.43 b	0.72±0.13
Isolate-2	1.71 a	1.18±0.16

* Calculated from total N determination by Kjeldahl assay. Data followed by same letter are not significantly different at 1% level.

† Values are the mean ± standard deviation of the mean (triplicate samples for ^{15}N analysis).

Table 3 Comparison of NIAB-1 isolate with *Klebsiella* species.

Characteristic	NIAB-1	*K. pneumoniae*	*K. oxytoca*
Moles % G+C	56.9	53.9–59.2	53.9–59.2
Motility	−	−	−
Capsule	+	+	+
Spore	−	−	−
Oxidase	−	−	−
Catalase	−	−	−
Indole	−	−	+
VP*	+	+	+
ADH**	−	−	−
ODC***	−	−	−
Urease	+	d mostly +	d mostly +
$NO_3^- \rightarrow NO_2^-$	+	+	+
Citrate utilization	+	+	+
Starch hydrolysis	−	−	−
Acidification of:			
glucose, sucrose, mannitol, xylose, trehalose	+	+	+

* Voges–Proskauer reaction; ** arginine dihydrolase; *** ornithine decarboxylase; d, differential; +, Positive; −, Negative.

Data for *K. pneumoniae* and *K. oxytoca* are taken from Buchanan & Gibbons (1974) and Seidler (1981).

Table 4 Comparison of the bacterial isolates with other Gram negative aerobic N_2-fixing bacteria

Characteristic	C-2	Iso-2	*Azotobacter*	*Azomonas*	*Beijerinckia*	*Mycobacterium flavus* 301	*Derxia*
DNA (G+C mole %)	63.7	53.4	63–66	53–59	54–59	69	70
Cell size	Large	Small	Large	Large	Small	Small	Large
Motility	+	+	±	+	±	−	+
Pigment (non-white)	Off-white yellowish	Off-white	+ (Also fluorescent)	(+ with age)	± (+ with age)	+	±(+ with age)
Slime	+ (less)	+	±	±	+	±	++
Catalase	+	+	+	+	±	−	
Growth on peptone	+	+	NA	−	+	+	NA
Malate	+	+	NA	NA	±	NA	NA
Glucose	+	+	+	+	+	−	+
Starch hydrolysis	−	−	±	−	NA	NA	−

NA, not available
Data for all bacteria except C-2 and Iso-2 are taken from Buchanan & Gibbons (1974) and Becking (1981).

Comparison of different characteristics of the remaining two isolates (Iso-2 and C-2) with the possibly closer genera is presented in Table 4. Based on the majority of the tests performed, Iso-2 could be placed in the genus *Beijerinckia*. The actual position of C-2 is uncertain although it appears to be related to the Azotobacteriaceae. Recently, some new N_2-fixing micro-organisms have been reported and described precisely, use having been made of DNA/RNA hybridization and immunological reactions (Balandreau 1983).

Salinity, together with drought, involve changes in water potential. The situation is further complicated by the type of salinity (ionic species) and whether it is coupled to pH changes (Sprent 1984). The problem of high pH (8–10) in soils where kallar grass is grown is linked with the preponderance of sodium salts (50–350 mmol/l). As the isolates were obtained from such an unusual environment, some physiological experiments were carried out to determine whether they had any special characteristics. Studies revealed that all isolates had relatively high tolerance limits of pH (8–9) and sodium chloride (up to 3%).

There is a renewed interest in the use of nitrogen fixing bacteria as inoculants for cereals and grasses (Okon 1984). Thus, information regarding the effect of NH_4^+ and NO_3^- on the nitrogenase activity of these isolates could be useful. All bacterial isolates tested exhibited nitrogenase activity in the semi-solid static cultures amended with as high as 5 mmol/l nitrate or ammonium. In the present study poor growth and low activity was observed in isolates grown on completely N-free medium, which indicated the requirement of mineral or organic-N in the medium for these isolates.

All physiological studies reported here were done in enclosed vessels where growth conditions alter rapidly. Studies were therefore initiated to investigate the behaviour of rhizospheric isolates of kallar grass in a continuous chemostat culture (Niemann *et al.* 1985) and in batch culture in our laboratory. Some of the preliminary reports confirmed our earlier observations on the upper tolerance limits of NaCl by NIAB-1. Comparison was made with a wild strain 5AL (obtained from Dr. Lowe, AFRC Unit of Nitrogen Fixation University of Sussex, UK) and it was observed that the pH and salt optima for NIAB-1 was 8 and 100 mmol/l, respectively. Further studies on its different physiological characteristics, on electron transport system, isolation of different proteins of nitrogenase complex and compounds responsible for osmotolerance are under current investigations.

Acknowledgement

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Summary

N_2-fixing bacteria were isolated from the rhizosphere of naturally grown salt tolerant grass (*Leptochloa fusca*). A broad spectrum of diazotrophs was found to be associated with the roots of *L. fusca*. the systematic position of the three isolates, NIAB-1, C-2 and Iso-2 was determined by morphological, biochemical and mol % (G+C) DNA contents. Two isolates were identified as *Klebsiella pneumoniae* (NIAB-1) and *Beijerinckia* sp. (Iso-2). ^{15}N enrichment studies confirmed the nitrogen fixing ability of the isolates. The effects of different levels of combined nitrogen (NO_3^- & NH_4^+), pH (5.5–9.0) and salt (NaCl) on nitrogenase activity of the isolates were determined at various time intervals. All isolates exhibited nitrogenase activity even in the presence of 5 mmol/l NO_3^- or NH_4^+ in a semi-solid medium after 24 h of growth. Maximum nitrogenase activity was observed at alkaline pH and all isolates were able to tolerate up to 3% NaCl in the medium.

Résumé

Etudes sur les bactéries fixatrices de l'azote associées à l'herbe halo-tolérante Leptochloa fusca *(L.) Kunth*

Des bactéries fixatrices de l'azote ont été isolées à partir de la rhizosphère de l'herbe halo-tolérante *Leptochloa fusca* développée dans les conditions naturelles. Il a été constaté qu'un large spectre de diazotrophes est associé aux racines de la plante. La position taxonomique de 3 souches isolées, NIAB-1, C-2 et Iso-2, a été déterminée par des critères morphologiques et biochimiques et par le pourcentage de (G + C) de l'ADN. Deux souches on été identifiées comme *Klebsiella pneumoniae* (NIAB-1) et *Beijerinckia* sp. (Iso-2). Les études d'enrichissement en ^{15}N ont confirmé l'aptitude des souches à fixer l'azote. les effets de différents niveaux d'azote combiné (NO_3^- et NH_4^+), de pH (5.5–9.0) et de sel (NaCl) sur l'activité nitrogénasique des souches ont été déterminés à divers intervalles de temps. Toutes les souches présentent une activité nitrogènase après 24 h de croissance en milieu semi-solide, et cela même en présence de 5 mmol/l de NO_3^- ou NH_4^+. L'activité nitrogènase maximum est observée à pH alcalin, et toutes les souches tolèrent jusqu'à 3% de NaCl dans le milieu.

Resumen

Estudios sobre bacterias fijadoras de N_2 asociadas con una graminea halófila: Leptochloa fusca *(L.) kunth*

Se han aislado bacterias fijadoras de N_2 en la rizosfera del hábitat natural de la graminea halófila *Leptochloa fusca*. Un amplio espectro de diazotrofos se encontró asociado con las raíces de *L. fusca*. La posición sistemática de tres aislados: NIAB-1, C-2 y Iso-2 se determinó utilizando sus características morfológicas, bioquímicas y el % (G+C) molar del ADN. El aislado NIAB-1 se identificó como *Klebsiella pneumoniae* y el aislado Iso-2 como *Beijerinckia*. sp. Estudios mediante ^{15}N confirmaron la habilidad fijadora de N_2 de los aislados. Se determinaron periodicamente los efectos de distintos niveles de nitrógeno combinado (NO_3^- y NH_4^+), pH (5.5–9.0) y sal (NaCl) en la actividad nitrogenásica de los aislados. Todas las cepas aisladas mostraron actividad nitrogenásica incluso en presencia de 5mmol/l de NO_3^- y NH_4^+ en un medio semisólido después de 24 h. de crecimiento. La actividad nitrogenásica máxima se observó a pH alcalino y todos loa aislados eran capaces de tolerar hasta 3% de NaCl en el medio.