

## Characterization of Diazotrophs Associated with Roots of *Leptochloa fusca* (L) Kunth

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### ABSTRAK

Sekumpulan sembilan diazotroph diasingkan daripada rumput kallar rizosfera dan dikultur di atas nitrogen bebas sederhana.  $K_5, K_9, K_{10}, K_{11}, K_{12}, K_{13}$ , menunjukkan keaktifan nitrogenase yang tinggi ( $718 \text{ n mol C}_2\text{H}_4 \text{ h}^{-1} \text{ rial}^{-1}$ ). Sebaliknya, dibandingkan dengan  $K_7$  dan  $K_{14}$ , ia adalah lebih rendah ( $< 5 \text{ n mol C}_2\text{H}_4 \text{ h}^{-1} \text{ rial}^{-1}$ ). Keaktifan maksimum nitrogenase didapati dalam kultur muda (24 jam selepas pengeraman) kecuali untuk  $K_9$  dan  $K_{13}$  di mana maksimum keaktifan masing-masing ialah selepas 36 dan 48 jam. Semua terikan Gram-negatif, mengeluarkan koloni licin dan pelikel di atas media semi-pepejal. Sel  $K_5, K_7, K_{10}, K_{11}, K_{12}$  dan  $K_{14}$  adalah pleimortik;  $K_8$  berbentuk rod-rod yang panjang;  $K_9$  kecil dan berbentuk bulat ataupun bujur; manakala  $K_{14}$  berbentuk rod-rod yang berlubang. Hanya  $K_8, K_{13}$  dan  $K_{14}$  didapati dalam bentuk motil. Kesemua pencilan ini berupaya mengecilkkan nitrat dan positif untuk oksidase dan katalase. Tiada satupun pencilan-pencilan boleh dinitrifi atau mempunyai keaktifan urase kecuali  $K_{14}$  yang positif urase.  $K_5, K_{13}$  dan  $K_{14}$  difermentasi dan mengeluarkan pigmen merah. Pencilan-pencilan  $K_5, K_{10}$  dan  $K_{12}$  telah dikaitkan dengan genus *Azotobacter* sementara yang lainnya masih tidak dikenalpasti.

### ABSTRACT

A group of nine diazotrophs were isolated from the rhizosphere of kallar grass and cultured on nitrogen-free medium.  $K_5, K_9, K_{10}, K_{11}, K_{12}, K_{13}$  showed high nitrogenase activity ( $> 18 \text{ n mol C}_2\text{H}_4 \text{ h}^{-1} \text{ vial}^{-1}$ ) whereas in  $K_7$  and  $K_{14}$  it was comparatively low ( $< 5 \text{ n mol C}_2\text{H}_4 \text{ h}^{-1} \text{ vial}^{-1}$ ). Maximum nitrogenase activity was found in young cultures (after 24 hours of incubation) except for  $K_9$  and  $K_{13}$  where it was maximum after 36 and 48 h, respectively. All strains were Gram-negat, produced smooth colonies and pellicles on semi-solid media. Cells of  $K_5, K_7, K_{10}, K_{11}, K_{12}$  and  $K_{14}$  were pleiomorphic;  $K_8$  formed long fine rods;  $K_9$  was small, round or oval shaped; while  $K_{14}$  formed beaded rods. Only  $K_8, K_{13}$  and  $K_{14}$  were found to be motile. All isolates were able to reduce nitrate, and were positive for oxidase and catalase. None of them could denitrify or had urease activity except for  $K_{14}$  which was urease positive.  $K_5, K_{13}$  and  $K_{14}$  were fermentative and produced red pigments. The isolates  $K_5, K_{10}$  and  $K_{12}$  are assigned to the genus *Azotobacter* while others remained unidentified.

**Keywords:** Diazotrophs, nitrogenase activity, physiological and biochemical characteristics, kallar grass, *Leptochloa fusca*, *Azotobacter*

### INTRODUCTION

A number of nitrogen fixing bacteria have been isolated from the roots of different plants (Ahmad, 1979; Cappone and Budin, 1982; Malik and Zafar, 1985; Bilal and Malik, 1987; Reinhold *et al.* 1987; Cavalcante and Dobereiner, 1988; Zafar *et al.* 1988). In Pakistan, attempts have been made to reassess the possible contribution of nitrogen-fixing bacteria on the fertility of saline soil. Attention is being focussed on a salt-

tolerant grass, *Leptochloa fusca* (L), which can grow well on low-fertility saline and sodic soils without the addition of nitrogenous fertilizers. Dinitrogen fixation associated with the roots of kallar grass has been reported Malik *et al.* (1980, 1982). Aerobic nitrogen-fixing bacteria have been reported in the rhizosphere, rhizoplane and histoplane of *Leptochloa fusca*. Bilal and Malik (1987b) have isolated a nitrogen-fixing zoogloea-forming bacterium from the histoplane of kallar

grass while Niemann *et al.* (1985) and Reinhold *et al.* (1986, 1987) have identified diazotrophs associated with the roots of the same grass as *Azospirillum*. Facultative anaerobic diazotrophs *Klebsiella* (Malik and Zafar, 1985) and *Enterobacter* (Zafar *et al.* 1988) from the roots of kallar grass have also been reported. The isolation of nine diazotrophs from the rhizosphere of *Leptochloa fusca* (L.) (Kunth) is reported here.

### MATERIALS AND METHODS

After washing with saline (0.85% NaCl) and sterile, distilled water, soil-free roots of kallar grass were excised into 1 cm long pieces and incubated in 5 ml of semi-solid nitrogen free medium (NFM) which contained (g/l in distilled water)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.02;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0; Malic acid, 5.0; KOH, 4.5; NaCl, 0.1;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.002; Bromothymol blue, 0.5% in 3 ml of ethyl alcohol; Biotin, 0.1; Yeast extract, 0.002; Agar - for semi-solid, 2.0, for solid, 20.0;  $\text{K}_2\text{HPO}_4$ , 0.5;  $\text{Na}_2\text{Fe}$  (EDTA), 1.64% in 4 ml of water; pH, 6.8. Cotton-plugged bottles containing root pieces in NFM medium were incubated at 30°C for 24 - 48 h. Thereafter, fresh vials were inoculated and incubated at 30°C. The cultures were assayed for nitrogenase activity by the acetylene reduction assay (ARA) as per Zafar *et al.* (1988). Cultures from ARA positive vials were streaked on NFM plates and incubated for 48 h. Cultures were then purified by routine streaking methods on nutrient agar plate. Pure cultures were transferred to semi-solid NFM medium and incubated at 30°C. When growth was observed (between 24- 48 h) the acetylene reduction assay was performed and positive cultures were maintained for further study.

Morphological, cultural and biochemical characters of the isolates were studied using standard bacteriological methods (Gerhardt *et al.* 1981) and QTS-20 and cytochrome oxidase strips obtained from DESTO (Defence Science and Technology Organisation) Laboratories, Karachi.

### RESULTS

ARA performed on freshly-grown cultures (24 h) showed that all the vials gave positive results for nitrogenase activity ( $> 18 \text{ n mol C}_2\text{H}_4 \text{ h}^{-1} \text{ vial}^{-1}$ ). These cultures were further streaked to single colonies on plates containing NFM medium. After purifying on nutrient agar plates, twenty single colonies were picked at random

and tested for nitrogenase activity. Of these, eleven isolates gave weak positive results while nine others ( $\text{K}_5, \text{K}_7, \text{K}_8, \text{K}_9, \text{K}_{10}, \text{K}_{11}, \text{K}_{12}, \text{K}_{13}$  and  $\text{K}_{14}$ ) produced rather strong ARA activities and were selected for subsequent studies. Nitrogenase activity of the selected isolate was determined after 24, 36, 48 and 72 h of incubation. ARA was found to be different for each of these isolates (*Fig. 1*). Nitrogenase activity was comparatively low for  $\text{K}_7$  and  $\text{K}_{14}$  and rather strong, with maximum activity after 24 hours of incubation, for  $\text{K}_5, \text{K}_8, \text{K}_{10}, \text{K}_{11}$  and  $\text{K}_{12}$ . In  $\text{K}_9$  and  $\text{K}_{13}$ , maximum nitrogenase activity was observed after 36 and 48 hours of incubation, respectively.

All isolates produced round colonies with entire margins (with some variation in size) on NFM and nutrient agar media. The sizes were 1.5 mm for  $\text{K}_5, \text{K}_7, \text{K}_9, \text{K}_{10}$  and  $\text{K}_{12}$ ; 2.5 mm for  $\text{K}_{11}, \text{K}_{13}$  and  $\text{K}_{14}$ ; and 5.0 mm for  $\text{K}_8$  after 24 hours of incubation. All strains formed pellicles in semi solid media. Colony colour variation was observed on different media by different isolates. Colour of all isolates grown on NFM was cream except for  $\text{K}_{14}$  which was white; on nutrient agar it was yellowish for  $\text{K}_5$ , offwhite for  $\text{K}_8$  and  $\text{K}_{10}$ , and cream for  $\text{K}_7, \text{K}_9, \text{K}_{11}, \text{K}_{12}, \text{K}_{13}$  and  $\text{K}_{14}$ . Colour of colonies cultured on potato extract medium was cream for  $\text{K}_8, \text{K}_{11}$  and  $\text{K}_{14}$ , and brown to dark brown for  $\text{K}_5, \text{K}_7, \text{K}_9, \text{K}_{12}$ , and  $\text{K}_{13}$ . On MacConkey's agar, weak positive results were observed only for  $\text{K}_8, \text{K}_{13}$  and  $\text{K}_{14}$ . Variation in the cell shape of the different isolates was also recorded.  $\text{K}_5, \text{K}_7, \text{K}_{10}, \text{K}_{11}, \text{K}_{12}$  and  $\text{K}_{13}$  were irregular or pleiomorphic.  $\text{K}_8$  formed long fine rods;  $\text{K}_9$  was small, rounded or oval;  $\text{K}_{14}$  formed beaded rod (*Fig. 2*). Only  $\text{K}_8, \text{K}_{13}$  and  $\text{K}_{14}$  were motile. All strains were Gram-negative.

All isolates, except  $\text{K}_5$ , showed positive  $\text{NO}_3^-$  reduction test (Table 1). Denitrification test was negative for all strains except for  $\text{K}_9$  which was positive. Urease activity was negative (except for  $\text{K}_{14}$ ) while oxidase and catalase tests were positive for all the isolates. Only  $\text{K}_8, \text{K}_{13}$  and  $\text{K}_{14}$  were able to ferment glucose and manitol (with acid and gas production), and they also produced red pigment. Results on identification strip using QTS-20 reflect great variation in the biochemical characters of the isolates (Table 2).

### DISCUSSION

NFM media, which contained nitrogen-free malate compound, were used for the isolation of diazotrophs. Use of N-free semi-solid isolation

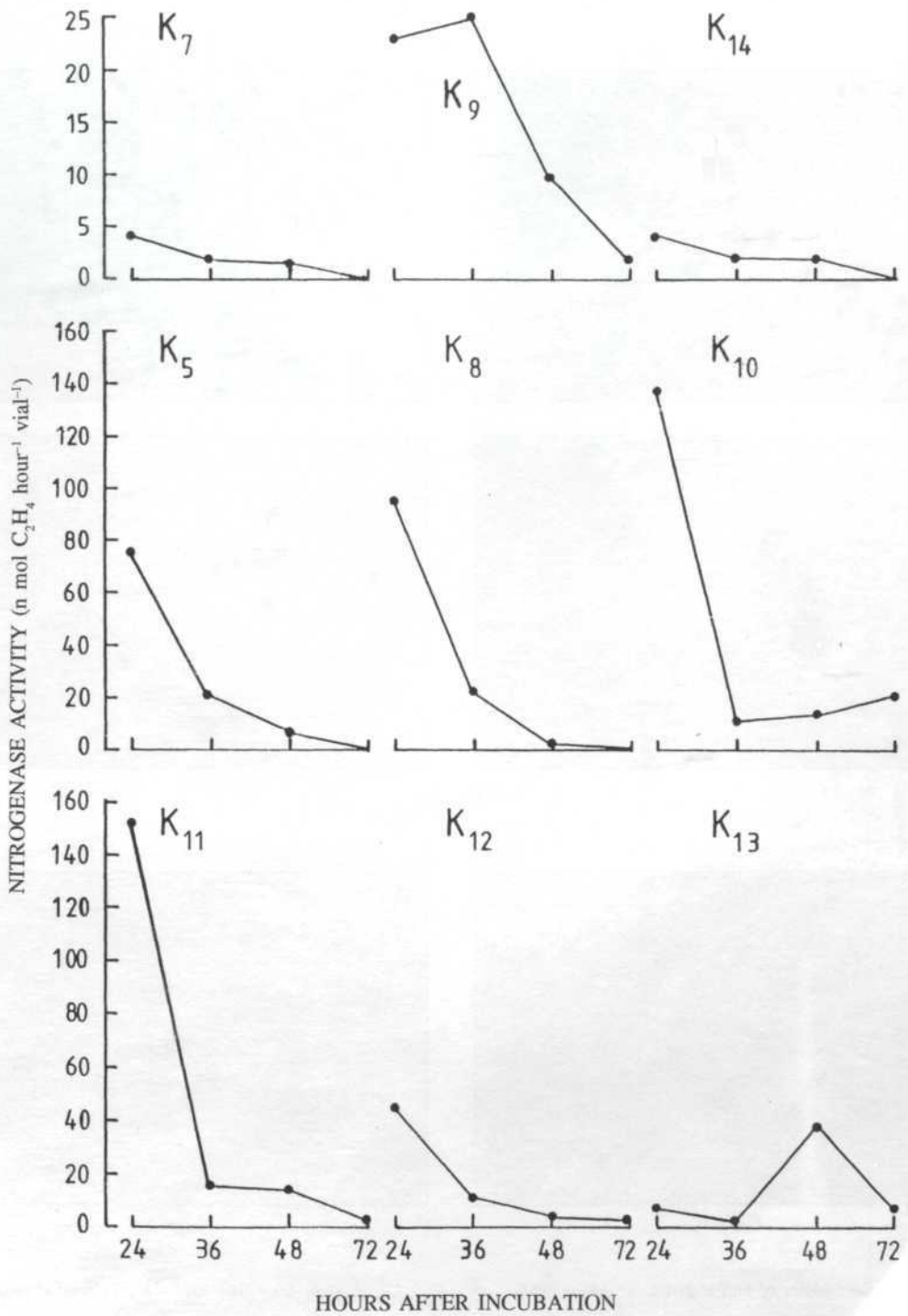


Fig. 1: Nitrogenase activity of the isolates after 24, 36, 48 and 72 hours of incubation

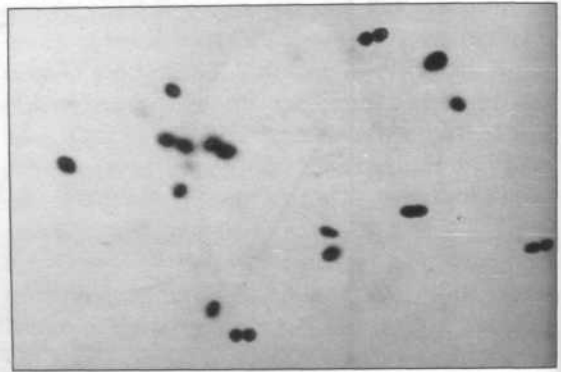
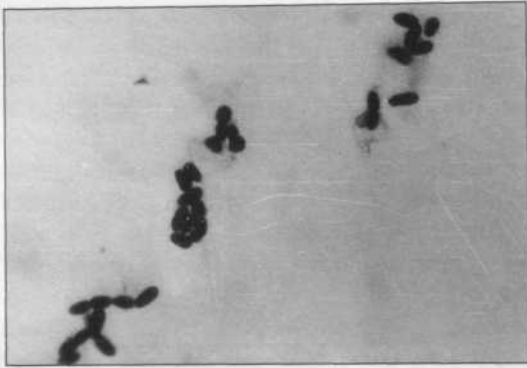
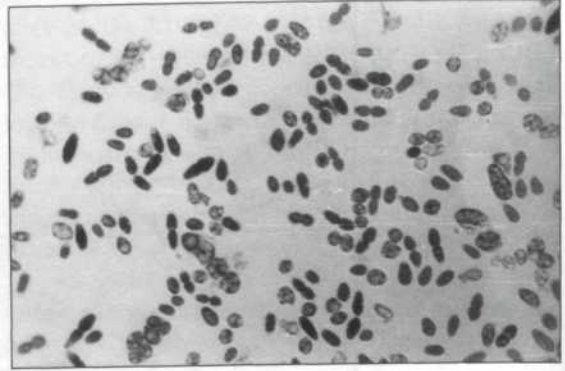
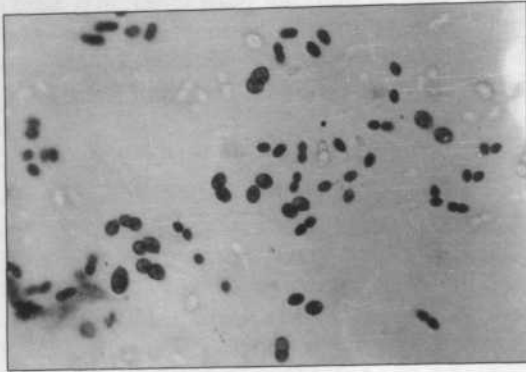


Fig. 2a

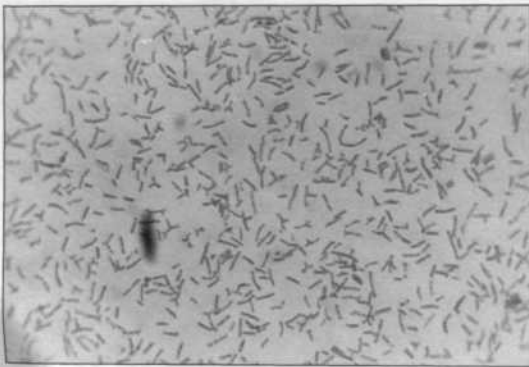


Fig. 2b

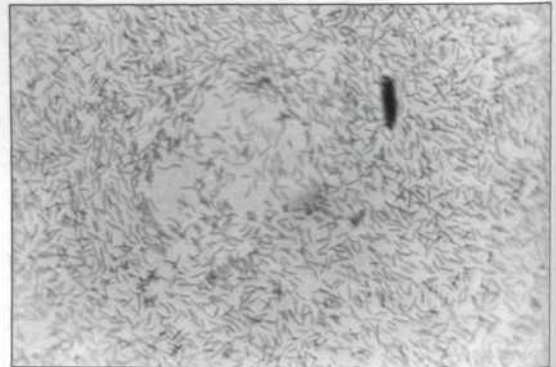


Fig. 2c

Fig. 2: Some isolates of kallar grass, a) pleiomorphic - K<sub>9</sub>, K<sub>10</sub>, K<sub>12</sub>, K<sub>13</sub>; b) long fine rods - K<sub>8</sub>; c) beaded rods - K<sub>14</sub>

TABLE 1  
Some physiological and biochemical characteristics of the isolates

Isolates	Catalase	Urease	Oxidase	Pigment	NO <sub>3</sub> <sup>-</sup> red	Denitrification	Sucrose Utilization	OF	Gram staining
K5	+	-	+w	-	+	-	+	-	-
K7	+	-	+	-	+	-	ND	-	-
K8	+	-	++	red	-	-	-	AG	-
K9	+	-	+W	-	+	+	+	-	-
K10	+	-	+W	-	+	-	+	-	-
K11	+	-	+W	-	+	-	ND	-	-
K12	+	-	+W	-	+	-	+	-	-
K13	+	-	++	red	+	-	-	AG	-
K14	+	+	++	red	+	-	-	AG	-

+, positive; +W, weak positive; -, negative; ND, not determined; AG, acid and gas

TABLE 2  
QTS -20 Biochemical characterization of the diazotrophic isolates from the rhizosphere of kallar grass

Isolates Code	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v
K <sub>5</sub>	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	+
K <sub>7</sub>	-	+	+	-	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	+	+
K <sub>8</sub>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
K <sub>9</sub>	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	+
K <sub>10</sub>	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+
K <sub>11</sub>	-	-	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-	-	+	+	-	+
K <sub>12</sub>	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	-	-	-	+
K <sub>13</sub>	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	+	-	-
K <sub>14</sub>	+	-	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	-	+	+	-	-

a, ONPG; b, citrate; c, malonate; d, lysine; e, arginine; f, ornithine; g, H<sub>2</sub>S; h, Urea; i, TDA; j, indole; k, vp; l, gelatine; m, glucose; n, nitrate; o, maltose; p, sucrose; q, mannitol; r, arabinose; s, rhamnose; t, sorbitol; u, inositol; v, oxidase; W+, weak positive.

media provided considerable progress for the isolation of nitrogen fixers (Boddey and Dobreiner, 1984). Species of *Azospirillum* and *Pseudomonas* have been mainly isolated on N-free medium (Barraquio *et al.* 1983; Falk *et al.* 1985; Reinhold *et al.* 1987).  $K_5$ ,  $K_8$ ,  $K_{10}$  and  $K_{11}$  showed reasonable nitrogenase activity ( $> 70$  n mol  $C_2H_4$  h<sup>-1</sup> vial<sup>-1</sup>) whereas in  $K_7$  and  $K_{14}$  nitrogenase activity was rather low ( $< 5$  n mol  $C_2H_4$  h<sup>-1</sup> vial<sup>-1</sup>). The range of activity was between 4 and 168 n mol  $C_2H_4$  h<sup>-1</sup> vial<sup>-1</sup> and it decreased with the age of the culture, and after 48 hours it was very low except for  $K_{13}$  where it was maximum. Tropical grasses show high rates of nitrogenase activity (Ahmad, 1979) and while xeric grasses do show activity, they do so at low rates (Wullstein *et al.* 1979). High nitrogenase activity has been found to be associated with the roots of kallar grass (Malik *et al.* 1982).

Bilal and Malik (1987b) have isolated nitrogen-fixing bacteria from the histoplane of kallar grass, which show very high (500 - 600 n mol  $C_2H_4$  h<sup>-1</sup> culture-bottle<sup>-1</sup>) nitrogenase activity. Low nitrogenase activity of the isolates described here may be attributed to the low temperature of soil (Thompson *et al.* 1984), since samples for this study were collected in December.

Diazotrophs isolated in this study are Gram-negative, and with the exception of some bacilli (Seldin *et al.* 1984) the majority of N<sub>2</sub> fixers are Gram-negative (Oken, 1982; Reinhold *et al.* 1987; Bilal and Malik, 1987b; Zafar *et al.* 1988). On the basis of cytochrome oxidase and fermenting abilities,  $K_8$ ,  $K_{13}$  and  $K_{14}$  have been included in Gram-negative, facultative anaerobic rods, while the rest of the isolates were Gram-negative aerobic rods (Krieg and Holt, 1984).  $K_8$ ,  $K_{13}$  and  $K_{14}$  were compared with all genera of Enterobacteriaceae but they remained unidentified. Previously, nitrogen-fixing *Klebsiella pneumoniae* have been reported from the rhizosphere of kallar grass (Malik and Zafar, 1985) while aerobic diazotrophic *Zoogloea* (Bilal and Malik, 1987b) and *Azospirillum* (Niemann *et al.* 1985; Reinhold *et al.* 1987) have been isolated from the roots of kallar grass. *Zoogloea*-forming bacteria were isolated on CCM (Carbon Combined Medium) and *Azospirillum* on NFM. *Zooglae* was pleiomorphic while *Azospirillum* formed vibroid to S-shaped cells. Aerobic rods of  $K_5$ ,  $K_7$ ,  $K_{10}$ ,  $K_{11}$  and  $K_{12}$  showed pleiomorphic morphology while while  $K_9$  formed small oval rods. Those which had 2 m m dia 3 3 m m length

were taken as presumptive *Azotobacter* and *Azomonas*. Considering other biochemical and morphological characters  $K_5$ ,  $K_{10}$  and  $K_{12}$  can initially be placed in the genus *Azotobacter*. These colonies were smooth, glistening, opaque and low convexed. All of them were non-motile, catalase-positive and had ovoid cells which occur singly, in pairs, in irregular clumps or small chains (Fig. 2a). Slime formation was also observed. Cysts were also formed in old cultures. All of them could reduce nitrate but none denitrified. All of them can utilize sucrose as the sole carbon source.  $K_5$  and  $K_{10}$  utilize rhamnose while  $K_{12}$  did not. Table 3 shows the comparison of some biochemical characters of  $K_5$ ,  $K_{10}$  and  $K_{12}$  with six species of *Azotobacter* from Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Ni % G + C or DNA/RNA or DNA/DNA homology studies were performed with these strains which can ascertain the taxonomics position of these isolates. Both *Azotobacter* and *Azimonas* are included in RNA super family II (De Smedt *et al.* 1980) and both of them are able to fix N<sub>2</sub> under atm pO<sub>2</sub>.

From the above account it can be surmised that three *Azotobacter* strains ( $K_5$ ,  $K_{10}$ ,  $K_{12}$ ) with comparatively high nitrogenase activity were obtained from the rhizosphere of *Leptochloa fusca*. Two other strains ( $K_8$ ,  $K_{11}$ ) also showed high nitrogenase activity but their affinities remain to be established. The extent of nitrogenase activity depends upon the incubation period. Generally, it is highest after 24 hours of incubation but was reduced with the prolonged incubation period.

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TABLE 3  
Some biochemical characteristics of  $K_5$ ,  $K_{10}$  and  $K_{12}$  along with selected strains of *Azotobacter*  
from Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984)

	<i>A. chroococcum</i>	<i>A. Vinelandii</i>	<i>A. beijerinckii</i>	<i>A. nitrican</i>	<i>A. armeniacus</i>	<i>A. paspali</i>	$K_5$	$K_{10}$	$K_{12}$
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	d	+	+	+	+
ND <sub>5</sub> reduction	+	+	+	+	-	-	+	+	+
Denitrification	-	-	-	-	-	-	-	-	-
Rhamnose	-	+	-	-	-	-	+	+	-
Sorbitol	+	+	d	d	+	-	-	+	-
Inositol	-	+	d	-	d	-	-	-	-
Mannitol	+	+	d	d	+	-	-	+	-
Malonate	d	+	+	d	-	-	+	-	+
Sucrose	+	+	+	+	+	+	+	+	+
H <sub>2</sub> S production	d	+	d	d	-	+	-	-	-
Pigment	+	-	+	+	-	-	-	-	-
Motility	+	+	-	-	+	+	-	-	-

+. all strains positive; -, all strains negative; d, 11-8 stains are positive

