Pak. J. Bot., 19(1): 29–41, 1987.

SEASONAL VARIATION IN RHIZOSPHERIC POPULATION OF DIAZOTROPHS AND ROOT ASSOCIATED NITROGENASE ACTIVITY OF SOME WHEAT MUTANTS

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Abstract

An experiment was conducted to study the possible nitrogenase activity associated with wheat roots. Five mutants (M-5, M-25, M-13, M-14, M-46) originating from two different wheat varieties viz., Indus 66 and C-591, were selected on the basis of better germination percentage when grown in an unfertilized field plot. The roots were excised and subjected to acetylene reduction assays (ARA) to assess the comparative nitrogenase activity of different mutants. Enumeration of N_2-fixing microbial population in rhizoplane (RP) and histoplane (HP) was done at two growth stages i.e. tillering and maturity by most probable number (MPN) method, using visual observation and ARA as an index. Results showed that there was a variability as regards root associated N_2-ase activity. Enumerated number of diazotrophs were more in HP than in RP and number of N_2-fixers was more at maturity than at tillering. Preliminary identification tests on the isolated N_2-fixing microorganisms showed that these belong to family Enterobacteriaceae and Azotobacteriaceae.

Introduction

Dinitrogen fixation in the rhizosphere of wheat has been reported by several workers (Nelson *et al.*, 1976, Neyra & Dobereiner, 1977, Rennie & Larson, 1981) and varying amount of nitrogen fixed has been reported (Neyra & Dobereiner, 1977; Dobereiner, 1978; Rennie *et al.*, 1983). Although the importance of rhizospheric (RS) dinitrogen fixation varies, there seems to be a higher potential under favourable conditions.

There is generally an enrichment of diazotrophs in the rhizosphere soil as compared with non rhizospheric soil (Balandreau *et al.*, 1978) this preferential enrichment of diazotrophs might be attributed to C rich, N poor environment, reduced zone in the root region or provision of essential vitamins by plants (Okon *et al.*, 1980; Martin & Glatzle, 1982).

Genotypic differences regarding the ability to support rhizospheric dinitrogen fixation have been reported in wheat (Pederson *et al.*, 1978, Rennie & Larson, 1979, Neal *et al.*, 1973). The inherent ability of various wheat mutants for harbouring nitrogen fixing organisms in their root zones, and their seasonal variations when grown in an unfertilized low fertility soil is described in this paper.

Materials and Methods

Plant growth: Seeds of 14 mutants derived from two wheat cultivars, Indus-66 and C-591, in which mutation was induced by appropriate doses of γ-radiation were supplied by Atomic Energy Agriculture Research Centre (AEARC) Tandojam. Seeds of mutants were sown in a controlled experimental plot at Nuclear Institute for Agriculture & Biology (NIAB), Faisalabad, where no fertilizer N has been added for the last five years, and rotation of wheat with maize has been carried out in the said period. Chemical analysis of wheat rhizosphere soil had a pH 8.0; EC 0.57 mS cm^{-1}; Sodium adsorption ratio (SAR) 1.7; total C 0.506%; total N 0.056%, Olsen Phosphorous 9.4 ppm. The mutants were sown in a 7.5 sq meter plot in 14 rows. Plant to plant and row to row distance was 15 and 50 cm respectively.

Of all the 14 mutants sown five mutant lines M-5, M-13, M-14, M-25 and M-46 showing better germination percentage (above 70%) were chosen for the study. The root sampling was done twice, first after 12 weeks of growth (tillering stage) and then after 24 weeks (maturity). Three plants of each mutant were excavated along with the rhizosphere soil, at each sampling time. The roots were cut off from the plant top, and were gently shaken free of soil clumps. Unwashed (UW) as well as sterile water washed (W) roots were subjected to the determination of associated acetylene reducing activity.

Acetylene reduction assay (ARA): Nitrogenase activity associated with UW and W roots was determined by measuring their acetylene reduction. The excised UW and W roots (0.5 gm fresh weight) were placed in 30 ml McCartney vials, with 10-12 replicates each, and were stoppered with suba-seals. For direct ARA, 10% of the atmosphere in the vials was replaced with C_2H_2 and incubated at 30°C. C_2H_4 production was determined in a time course assay of up to 24 h.

For preincubation, the gas phase of the vials containing root samples, was replaced by evacuation and flushing with N_2-gas. Ten percent air was injected to the bottles and left overnight at 30°C. After an overnight incubation, 10% of the atmosphere was replaced by C_2H_2 and incubated at 30°C, before running the time course.

Gas samples (100 μl) were analysed on a gas chromatograph (Carlo Erba Model 180) fitted with 1 m x 2 mm steel column filled with Porapak N (180-100 mesh) and H_2 flame ionization detector. Nitrogen was used as a carrier gas at a flow rate of 30 ml min^{-1} and the column temperature was kept at 50°C.

Enumeration: Enumeration of diazotrophs on the root surface (rhizoplane or RP) and histoplane (HP) was done by most probable number (MPN) method (Alexander, 1965) using visual observation and ARA as an index. Five gram of freshly excised roots were taken, and washed throughly, first with tap water and then with distilled water to

remove all adhering soil particles. The roots were then suspended in 45 ml of saline and shaken for half an hour with sharp pebbles to dislodge the bacteria from the root surface. This comprised the RP fraction. Using turbid supernatant as stock, a tenfold dilution series was made in saline. Five replicate vials, containing semi solid combined carbon medium (CCM) (Rennie, 1981) were inoculated from each dilution. All vials were incubated at 30°C for 24 h. After taking visual observation for growth, they were incubated with C_2H_2 for 1 h., after which 100 μl of gas sample was analysed for C_2H_4 production.

For histoplane enumerations, the roots were treated as for RP except that they were soaked in 95% ethanol for 60-90 seconds and rinsed in sterile water, after which they were mechanically macerated in blender at 25,000 rpm for 30 min. The roots were then like a mesh with a very turbid supernatant. Dilution series and inoculations were made as described earlier. This comprised the HP fraction.

Isolation of nitrogen fixing bacteria: Isolation of N_2-fixing bacteria was done by streaking a loopful from highest dilution MPN vial showing ARA, on N-free agar plate. After an incubation of 24–48 h at 30°C the colonies appearing on the Petri dish were picked separately, and transferred to CCM vials containing semi solid medium (CCM). After overnight incubation at 30°C they were subjected to ARA. Streaking from ARA positive vials was done on nutrient agar (NA) plates to check the purity of the culture. Later they were kept on NA slants for further studies.

Preliminary identification tests were performed by Qts-20 strips designed and supplied by DESTO Organization, Karachi, Pakistan. Tests of Qts-20 were often reconfirmed by conventional testing procedures.

Results

Results of MPN enumeration at two growth stages are presented in Fig. 1. Enumerations based on visual observation for growth in N-free medium showed no defined difference in the number of organisms at two growth stages, and in RP and HP. However, visual observation based numbers were always higher as compared to that of ARA.

There was a marked difference in histoplane population at maturity when ARA was used as an index. Number of N_2-fixers increased with growth period in case of histoplane except in M-46, where number of bacteria were recorded low at maturity. The number of RP bacteria was either same or low at two growth stages but HP bacteria in all four mutants were more at maturity than at tillering. The difference was much pronounced in case of mutant M-25, M-13 and M-14. The mutants showing higher values of histoplane bacteria at maturity were chosen for excised root assay, to determine the root associated nitrogenase activity.

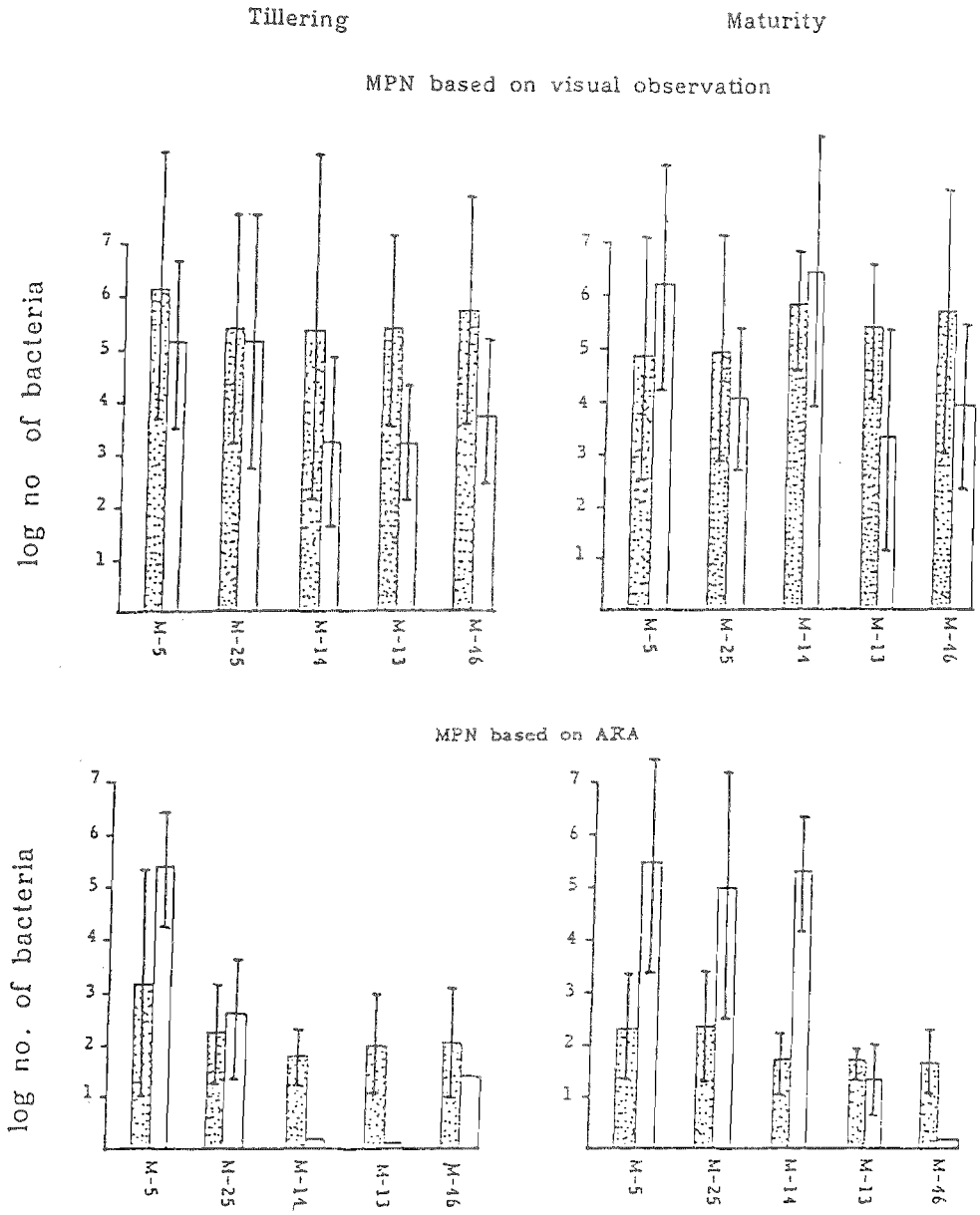

Fig. 1: Most probable numbers of N_2-fixing bacteria associated with various wheat mutants, in two root fractions, rhizoplane (RP) and histoplane (HP), as determined by visual observation for growth in N-free medium and acetylene reduction (ARA), at two growth stages, tillering and maturity. ⊟ Rhizoplane □ Histoplane. Values plotted are mean of three replicates. Bar represents ± Standard error.

Table 1: Ranges of nitrogenase activity (C_2H_2 reduction) of unwashed and washed excised roots of various wheat mutants.

Mutant	Assay/Time (Hrs)	n mol C_2H_4./hour/gm dr wt. root. (Ranges[a]) Unwashed[b] 1	2	3	Washed[c] 1	2
M- 5	Direct[d]	8 – 237 (90)	24 – 395 (114)	18 – 609 (285)	ND	124 – 1,485 (460)
	Preincubated[e]	69 – 5,380 (471)	28 – 4,064 (1,181)	14 – 4,283 (980)	557 – 14,243 (5,101)	194 – 4,478 (3,100)
M-14	Direct	37 – 107 (68)	40 – 207 (117)	36 – 482 (228)	ND	12 – 617 (202)
	Preincubated	10 – 29,219 (6366)	89 – 29,951 (4,285)	67 – 9,582 (2,599)	413 – 8,012 (2821)	34 – 3,541 (2469)
M-25	Direct	19 – 355 (110)	56 – 626 (224)	103 – 2,614 (588)	ND	56 – 636 (294)
	Preincubated	307 – 11,086 (3600)	223 – 10,613 (2,568)	143 – 7,473 (1,924)	112 – 11,094 (3,802)	17 – 8,367 (1,802)

a Ranges given are of 12 replicates. Mean values are given in the brackets.

b Time 1, 2, 3 represents 6, 10 and 20 hrs of incubation for direct and 3, 7 and 24 hrs for preincubated, unwashed samples.

c Time 1, 2 represents 10, 20 hrs for direct and 6, 24 hrs for preincubated.

d Roots were incubated directly with 10% v/v C_2H_2, after excision.

e Roots were preincubated under N_2 gas for 24 hrs before C_2H_2 was introduced for ARA.

ND Not detected.

Excised root assay (ARA): The results of excised root ARA of 3 wheat mutants namely, M-5, M-14 and M-25 are presented in Fig. 2 and Table 1. In case of unwashed excised roots (direct assay) a lag period of 3-6 hours was observed (Fig. 2) before the production of C_2H_4 started and subsequently proceeded linearly up to 21 h of incubation. The ARA rates ranged from 246-588 n moles C_2H_4 g^{-1} weight of roots in case of unwashed direct assays. Maximum ARA was observed in the roots of M-25.

The results of unwashed and washed excised roots preincubated with N_2 showed a reduced lag and much higher rates of ARA (Fig. 3a & b). However in this case, the rates of C_2H_4 production declined with the time course. Maximum values of up to 6.3 μ moles g^{-1} dry root were obtained in case of washed roots of M-14 (Fig. 3b). The ARA of the preincubated washed excised roots gave much higher activities (Fig. 3a & b) when compared to excised washed roots incubated directly with C_2H_4. The lag period was more than 10 hours and after 21 hours, activity was 460, 207 and 294 n moles g^{-1} dry weight for M-5, M-14 and M-25 respectively (Table 1).

Isolation of N_2-fixing bacteria: A number of N_2-fixing bacteria were isolated from various root fractions. Mainly two types of bacteria seemed dominant which have been

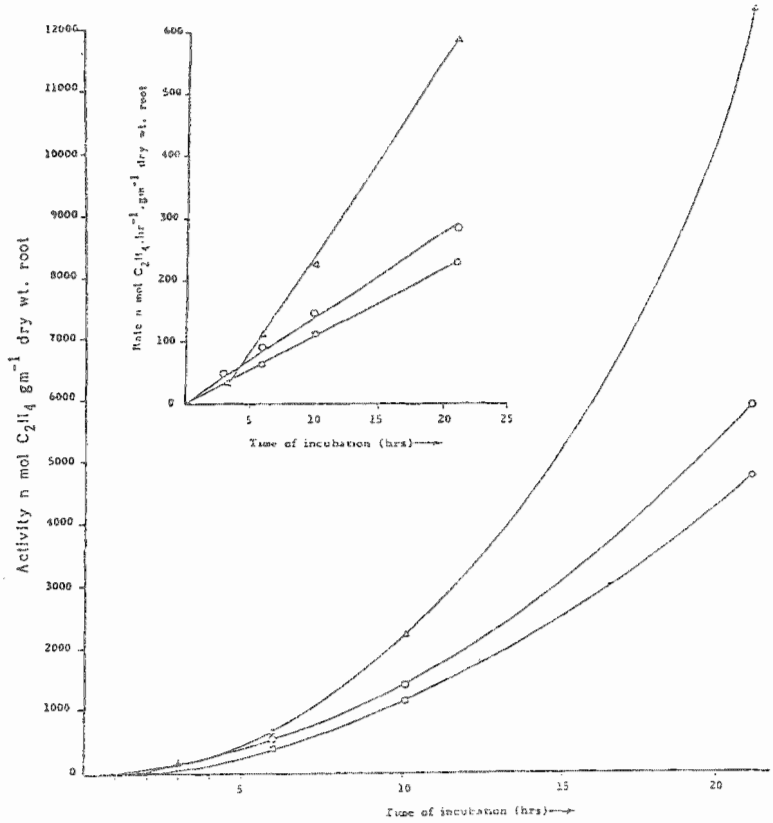

Fig. 2: Time course measurement of acetylene reduction with unwashed excised roots of wheat mutants. Roots were excised from the plant, and placed in 30 ml McCartney vials, acetylene (10% v/v) was injected directly and time course analysis started by taking first reading at zero hours. M-25, △–△ M-5, ○–○ M-14, □–□.

coded as isolate AH_6 & QH_7. These grew well on N-deficient media and also gave comparatively high ARA. Their number was higher than other bacteria, since all the isolations were done by streaking from the highest dilution ARA positive vials. Isolate AH_6 was from 10^{-6} histoplane dilution vial of mutant M-5, whereas the isolate QH_7 was from histoplane of M-25. QH_7 type isolates were usually obtained from all mutants. The colony of this isolate on agar as well as in semi solid media was very gummy. In the broth culture, the slime production was so much that it was difficult to take the culture sample with a loop.

Colony of isolate AH_6 was smooth, soft, translucent, and easy to pick. The growth from agar, unlike QH_7, goes into suspension easily and is capable of growth and N_2-fixation both aerobically and anaerobically (facultative anaerobe). Both isolates are

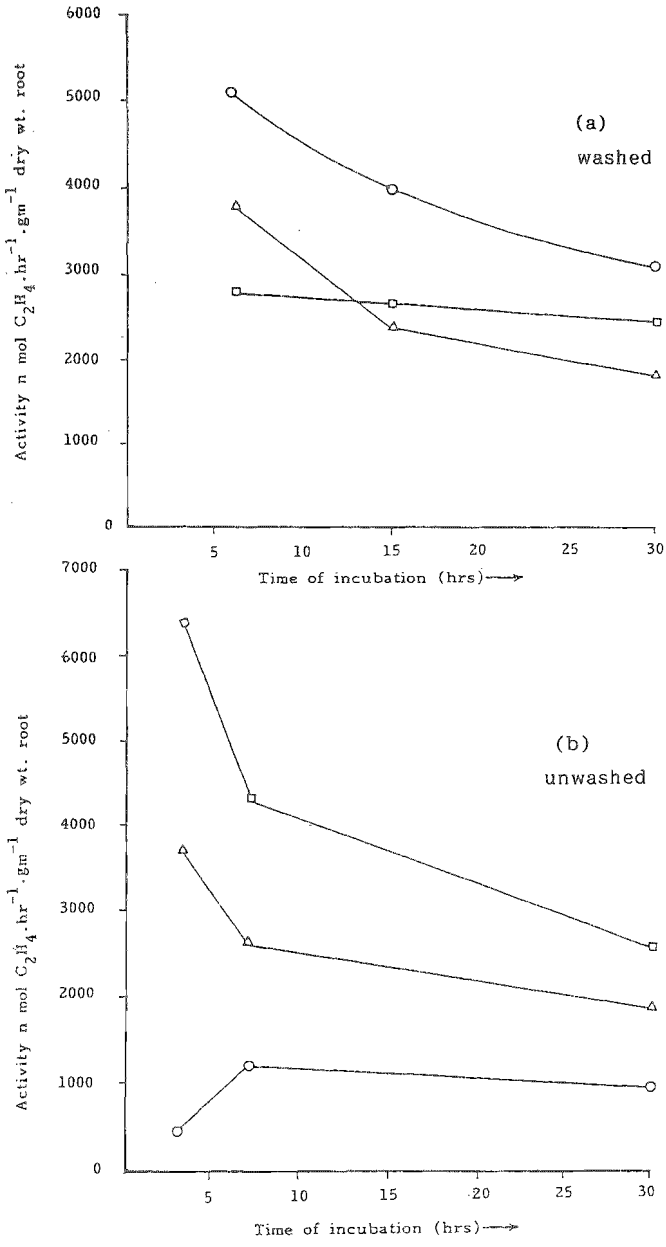

Fig. 3: Time course measurement of acetylene reduction with unwashed and washed roots of wheat mutants. The determinations were made after excision of roots and a preincubation of 24 hours under N_2 before 10% v/v C_2H_2 was introduced and a time course analysis performed. ○–○–○ M-5, △–△ M-25, □–□ M-14.

Gram -ve, non-motile, short rods. Some biochemical tests of these isolates are given in Table 2. Preliminary identification suggests AH_6 being member of Enterobacteriaceae and QH_7 resembling *Beijerinckia* spp of family Azotobacteriaceae.

Discussion

Difference in the ability to stimulate N_2-ase activity in the roots have been found among different varieties of grasses. Excised root assays (ARA) have been used to determine the genotypic difference in corn, *Paspalum notatum, P. purpureum* and wheat (Von Bulow & Dobereiner 1975; Day *et al.*, 1975; Dobereiner & Day 1975; Dobereiner, 1977). Previously, Pederson *et al.* (1978) surveyed various genotypes of winter wheat growing in Nebraska, USA, for root associated nitrogenase activity, estimated by ARA. Nearly all the varieties had very low ARA ranging from 0 to 3.1 n mol C_2H_4 hr^{-1} g^{-1} roots with a lag of 18–24 hour before any nitrogenase activity was detectable. Even the inoculation of N_2-fixing bacteria did not have any effect on ARA. Cakmacki et al. (1981) isolated *Klebsiella oxytoca* from wheat roots but when reinoculated, it did not produce any significant increase in the plant growth. However under different environmental conditions, inoculation of N_2-fixing bacteria (commonly *Azospirillum* spp.) did gave a response resulting in increased nitrogen uptake (Kapulnik et al., 1983; Reynder & Vlassak, 1982).

The evolution of high yielding, fertilizer responsive wheat varieties has resulted in ever increasing dependence on chemical fertilizers. The nitrogenase activity associated with roots becomes operational essentially under low input and low soil fertility conditions. The soil used in the present study was also very low in fertility and depleted of nutrients as it was subjected to wheat maize rotation for the last five years, without addition of any nitrogenous fertilizers. Such conditions seemed to be favourable for realizing the potential of dinitrogen fixation.

Lee et al. (1977) reported seasonal and varietal difference of rice, and a number of other workers, using a variety of techniques, have shown that nitrogenase activity was absent or low during early vegetative growth and was maximum at some stage during reproductive development (Patriquin et al., 1983; Torbjorn & Granhall 1984; Von Bulow & Dobereiner, 1975). In four mutants out of five tested, the number of N_2-fixing bacteria in HP was high at maturity, except in case of M-46 where the number was recorded low. The increase in number of bacteria can very well be correlated with the above reported seasonal variation of grass N_2-fixation. Our study not only shows the seasonal variation but difference in the inherent ability of mutants to support N_2-fixation which was indicated by number of N_2-fixers, as well as by excised root assays.

The presence of N_2-fixing bacteria on the root surface (RP) and inside the root cells (HP) is an indication of their probable role in providing reduced nitrogen to the plants.

The number of N_2-fixers in the RP and HP fractions of wheat varied in different mutants at both stages (Fig. 1). The variation pattern is much marked when MPN estimations were based on ARA, and the number of histoplane bacteria was much higher at maturity except in mutant M-46. MPN estimation based on ARA are more reliable than visual observation for growth on N-free medium, since all the organisms appearing on N-free media are not necessarily N_2-fixers.

During early stages of plant growth high photosynthetic activity and exudation from the roots occur, which could be diffusable water-soluble or diffusable-volatile, (Dommergues & Rinaudo 1979), or non diffusable mucigel which provides energy substrates for the rhizosphere and rhizoplane bacteria. This could account for high number of rhizospheric N_2-fixing population. The higher number of histoplane N_2-fixing bacteria at maturity, can be attributed to root lysis and migration of bacteria to closer niches of the roots in search of diminished carbon supplies due to decreased plant photosynthetic activity.

The major limitation of ARA based data is the criticism over the rates of C_2H_2 reduction, especially after preincubation, where the ability of some roots to support growth and subsequent nitrogenase activity by bacteria is accentuated (Van Berkum & Sloger, 1984). The results presented in our study show that after 3-6 hr of incubation directly with C_2H_2, appreciable C_2H_4 production, ranging from 123-540 n mol, g^{-1} dry root was obtained. Some workers have reported far lower values for much longer incubation periods (Ichio Nioh, 1979). Van Berkum & Sloger (1984) reported immediate acetylene reduction with roots of grasses, but the activity was in the range of 10-100 n mol g^{-1} for *Zizania aquatica* and *Spartina alterniflora.* The higher values of more than 2 μ mol g^{-1} dry weight root at 16 h of incubation reported by them for *Sorghum vulgare* are also comparable to values obtained for wheat mutants, especially M-25. There is a large variation in ARA values of the replicate samples of excised roots (Table 1). This may be due to the heterogenous root samples and the non uniform colonization or association of the rhizospheric N_2-fixing bacteria with the various root parts. With such limitations, ARA can at best be used for qualitative analysis of N_2-fixing systems.

Isolation of wheat root associated bacteria has been made by various workers, and this includes *Azospirillum lipoferum* besides a number of Enterobacteriaceae, mainly *Klebsiella pneumoneae, K. oxytoca, Enterobacter cloaceae, Erwinia herbicola* (Klucas *et al.,* 1979. Cakmacki *et al.,* 1981), Nelson *et al.* (1976) found *Bacillus polymyxa, B. macerans* and *Enterobacter cloaceae.* Since the enrichment of the organism depends upon media employed and other cultural conditions, combined carbon medium (CCM) was used in our studies, which is known to allow proliferation of a number of bacteria belonging to different genera (Rennie, 1981). Dinitrogen fixing Bacilli may also be important bacteria in the study of N_2-fixation associated with wheat (Rennie & Larson, 1981). The characteristics of bacterial isolates from various wheat mutants along with

Table 2: Physiological and biochemical characteristics of isolates from wheat mutants, alongwith comparison with selected strains of *Enterobacter* and *Klebsiella*.

Organism Test	Isolate AH_6	Enterobacter cloacae	Klebsiella oxytoca	Isolate QH_7
ONPG	+	+	*	+
Na. Cit.	–	+	*	W+
Na. Mal.	+	*	+	+
LDC	–	–	+	W+
ADH	–	+	–	+
ODC	W+	–	–	+
H_2S	+	–	–	–
Urea	+	*	+	–
TDA	W+	*	–	–
Indole	–	–	+	–
VP	+	+	+	+
Gelatin	–	–	–	–
Glucose	+	+	+	+
Nitrate	+	+	+	+
Maltose	+	+	*	W+
Sucrose	+	+	+	W+
Mannitol	+	+	+	W+
Arabinose	+	+	+	+
Rhamnose	+	+	+	W+
Sorbitol	+	+	+	W+
Inositol	+	[–]	*	+
Denitrification	–	*	*	–
Catalase	+	*	*	+
Oxidase	*	–	–	–
Slime	–	*	*	+
Motility	–	+	–	–
Growth and N_2 fixation anaerobically	+	+	*	*
Resistant to:				
Ampicillin	+	+	*	*
Chloramphinicol	+	*	*	*
Tetracycline	–	*	*	*

Legend: + positive, – negative, w, weak positive [–] Majority of strains giving the reaction. Generally more than 89%. * not mentioned in Bergey's manual LDC, Lysine decarboxylase, ADH, Arginine dihydrolase. ODC, Ornithine decarboxylase. TDA, Tryptophane deaminanse, VP, voges proskauer. ONPG, ortho nitrophenyl glucosidase.

comparison with known strains are presented in Table 2. Based on identification tests done, isolate AH_6 can be placed in family Enterobacteriaceae, because it is capable of growth and N_2-fixation under aerobic as well as anaerobic conditions. Most of its characteristics resemble *E. cloaceae* but is non motile. However, QH_7 resembles *Beijerinckia* spp. in colonial and morphological characteristics.

Though higher rates of excised root ARA were found in case of M-25, the number of diazotrophs in HP fraction of all three mutants M-5, M-25 and M-14 were approximately the same. The MPN results have shown a variability as regards N_2-ase activity among various mutants. Moreover the number of N_2-fixing bacteria in the HP fraction were found to be more at maturity than at tillering. This indicates that nitrogen fixing bacteria are closely associated with the wheat roots and the extent of this association is dependent on the energy supply for the diazotrophs in the form of root exudation.

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(Received for publication 24 May, 1986)