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Quantification of N_2-fixation and survival of inoculated diazotrophs associated with roots of Kallar grass

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Abstract

Experiments to determine the contribution of biological nitrogen fixation associated with roots of Kallar grass have been carried out both in pot and field using ^{15}N isotopic dilution method. In the absence of any appropriate reference plant, the uninoculated treatment was used as a control in the pot experiment. It was found that as a result of inoculation with *Klebsiella* sp. (NIAB-1) and *Beijerinckia* sp. (Ko-2) 20–26% of the N in the plant was derived from the atmosphere. In the field experiment, a treatment receiving higher application of nitrogenous fertilizer to inhibit nitrogenase activity was kept as a control. Kallar grass was grown in 1 m² microplots for 9 months and harvested thrice. The estimation of N fixed was made by calculating the 'A' value for different treatments at each harvest. It was found that the rate of N fixation increased with the plant growth. At the 2nd harvest, which was after monsoon, it was estimated that 32 kg N ha⁻¹ was fixed. The survival of inoculated bacteria was also studied using fluorescent antibodies prepared against the inoculated bacterium. Survival and proliferation of these bacteria were found only where Kallar grass was growing, thus indicating a beneficial influence of the plant on bacterial growth.

Introduction

During recent years associative nitrogen fixation has been recognized as an important component of a range of ecosystems including several extreme environments (Dart, 1986). However little is known about such plant-bacteria interactions and its contribution to the nitrogen nutrition of the plant. Most of the studies carried out tend to suggest a loose, casual association resulting in some benefits to the plant (Giller and Day, 1985).

The agronomic importance of such associations can only be ascertained if its contribution can be quantified. The acetylene reduction technique can not be used for this purpose for a number of reasons (Lethbridge *et al.*, 1982; Witty, 1979). $^{15}N_2$ has been used to study the incorporation of fixed N into the plants (De Polli *et al.*, 1977). This technique can only be used over short periods during which plants are grown under an enclosed atmosphere and therefore cannot be applied in the field. On the other hand the techniques based on ^{15}N isotope dilution are more versatile and can be adapted to various experimental conditions (Rennie and Rennie, 1983; Fried *et al.*, 1983).

Leptochloa fusca, locally known as Kallar grass, is a highly salt tolerant grass and is being used in Pakistan as a primary colonizer of salt affected waste lands (Malik *et al.*, 1986). Nitrogenase activity, as estimated by the acetylene reduction technique was earlier reported in the excised roots of this grass (Malik *et al.*, 1980, 1982). Detailed investigation into the rhizosphere of this grass have been carried out (Zafar *et al.*, 1986). A number of N_2 fixing bacteria have been isolated from the roots of this grass (Bilal and Malik, 1987; Reinhold *et al.*, 1987; Zafar *et al.*, 1987). The ^{15}N isotope dilution technique has been used to quantify the contribution of the inoculated N_2 fixing bacteria to the nitrogen nutrition of Kallar grass when grown in nutrient solution and soil under controlled conditions (Malik and Zafar, 1985; Malik *et al.*, 1987).

This paper further reports the use of the ^{15}N isotope dilution technique for quantification of N_2 fixation associated with the roots of Kallar grass grown in pots and in the field. The survival of inoculated N_2 fixing bacteria in the rhizosphere has also been studied using the fluorescent antibody (FA) technique.

Materials and methods

Bacterial inoculum

Four diazotrophs namely *Klebsiella* sp. (NIAB-I), *Beijerinckia* sp. (Iso-2), AH_6 and St-16 isolated from Kallar grass roots, were used for quantification and survival studies following inoculation into the Kallar grass rhizosphere. The bacterial cultures for the two experiments were grown overnight in nutrient broth medium. For mixed inoculum treatment, four separately grown strains were mixed in equal volumes.

For survival studies 2.5 ml of inoculum was applied to roots of Kallar grass contained in small beakers (250 gm soil beaker^{-1}). The inoculum of the single culture Iso-2 was also provided in the same manner.

All the four strains used for inoculation reacted with the fluorescent antibody prepared against *Klebsiella* sp. (NIAB-I). *Beijerinckia* sp. (Iso-2) and NIAB-I exhibit strong FA reactions giving +4 fluorescence, whereas AH_6 and St-16 give cross reaction of +2 fluorescence intensity.

Pot experiment

The soil used in this study was obtained from Kallar grass fields at the Biosaline Research Station (BSRS) of NIAB near Lahore. It was a saline-sodic soil having pH 9.5 and electrical conductivity of 8.5 mScm^{-1}. The soil was labelled with ^{15}N by adding 1% cellulose powder and 100 ppm ^{15}N ammonium sulphate (4.75 at. % ^{15}N excess). The soil was brought to 60% water holding capacity (WHC) and incubated at 30°C for 4 weeks. During this period $(NH_4^+ + NO_3^-)$-N was estimated at weekly intervals. The incubation was terminated when nitrogen was completely immobilized. The labelled soil was used for an earlier reported experiment (Malik *et al.*, 1987) where Kallar grass was grown and harvested after 10 weeks.

After harvesting Kallar grass shoots and roots the soil was again bulked and thoroughly mixed. Portions of 525 g of soil were added to 500 ml plastic beakers. Triplicate pots were kept for each treatment which included 1) Inoculated with strain Iso-2; 2) inoculated with a mixed inoculum; 3) uninoculated; 4) uninoculated and not planted.

Kallar grass cuttings (1.5 cm) having one node were washed and surface sterilized by dipping them in 50% NaOCl solution for 30 min. and then they were sown in acid washed autoclaved moist sand. After rooting, the cuttings were washed with sterile distilled water and then for the inoculated treatment these were dipped in the appropriate inoculum mixture for 1 min. Three cuttings were planted in each beaker. After 3 d of growth 1 ml inoculum was applied to the soil around each cutting in the inoculated treatment. The beakers were kept in a controlled temperature growth room with 16 h day and 8 h night. The temperature was maintained at 30 ± 2°C. Light intensity during the daytime was 20000 Lux.

The plants were harvested after 8 weeks of growth. The dry weight of shoots and roots were recorded. Total N was determined by the same micro-Kjeldahl method (Bremner, 1965). Distillates were collected and concentrated for ^{15}N analysis. Samples were analysed by the Rittenburg method (Fiedler and Proksch, 1975) on a mass spectrometer fitted with a double inlet system (Varian Mat GD-150). Sodium hypobromite was used for releasing ^{15}N.

Field experiment

The field experiment was carried out at BSRS, Lahore in a highly saline sodic field. The same soil was used for the above described pot experiment. Microplots of 1 m^2 size were prepared and were lined with thick polythene sheet at a depth of 30 cms; the soil that had been removed was then replaced. Following treatments were run in triplicate:

T_1. Ammonium sulphate (AS) ^{15}N 2.5% a.e. 6.
30 kg N ha^{-1}-Inoculated. Split dose 10, 10, 10.

T_2. AS ^{15}N 2.5% a.e. @ 30 kg N ha^{-1}-Uninoculated. Split dose 10, 10, 10.

T_3. AS ^{15}N 1.0% a.e. @ 60 kg N ha^{-1}-Uninoculated. Split dose 20, 20, 20.

The first dose of ^{15}N fertilizer was sprayed in solution form to the top 15 cm and was thoroughly mixed before sowing the Kallar grass cuttings. Subsequently two doses were added after the first harvest. Nine uniform sized root cuttings of Kallar grass were planted in each microplot. In the inoculated treatment, the roots were dipped in a mixed inoculum and were shaken for 5 min before planting. Subsequently 5 ml of inoculum was given to each plant after two weeks of growth. The plants were sown on 4th May 1985. Three harvests of biomass were taken on 15th June, 2nd Sept. and 1st Dec. 1985. Dry weights of the shoots were recorded and N and ^{15}N analyses were performed as described above.

Survival and colonization studies

To supplement the ^{15}N experiment, a parallel experiment was carried out to study the survival and colonization of inoculated bacteria in Kallar grass rhizospheres. All the experimental conditions were the same as for ^{15}N experiment except that the soil was not labelled with ^{15}N. Portions of 250 g of soil were weighed in 300 ml plastic beakers. The soil was moistened to 60% WHC. Three Kallar grass cuttings were transferred to each beaker. A set of three glass contact slides was buried in each replicate. Six replicate beakers were inoculated with Iso-2 and mixed inoculum as mentioned earlier. Three treatments were kept: 1) Inoculated and planted; 2) Uninoculated planted; 3) Uninoculated unplanted. The survival of inoculated bacteria was ascertained by staining the soil contact slides and the roots in the first, third and fifth week of the experiment using the staining procedure of Schmidt (1974). For roots the staining procedure was the same as for soil contact slides except that after FA staining, the roots were counterstained in 0.01% w/v crystal violet solution for 2–5 min and then were washed overnight in phosphate buffer saline (pH 7.2) to remove extra stain.

Enumeration of bacteria was done only once, *i.e.*, after one week of inoculation. We employed the modified soil release procedure (MSRP) of Kingsley and Bohlool (1981) using partially hydrolyzed gelatin diluted in ammonium phosphate for effective recovery of bacteria from soil clumps.

Results

Pot experiment

In this experiment, the uninoculated treatment was regarded as a non fixing control. The effect of inoculation on the dry matter yield, N content and ^{15}N abundance in roots and shoots of Kallar grass grown in ^{15}N labelled soil are presented in Table 1. The shoot dry matter increased due to inoculation, whereas root dry weight did not show any such increase. Total N yield was more in inoculated treatment. The results of the ^{15}N enrichment showed a higher ^{15}N abundance in uninoculated treatments as compared to the inoculated ones. This is indicative of isotopic dilution which is the result of the uptake of biologically fixed atmospheric nitrogen.

Quantification of fixation based on isotope dilution has been calculated by the formula of Fried and Middleboe (1977) which is

$$\%\ \text{N fixed} = 1 - \frac{(^{15}\text{N at. \% excess})\text{fs}}{(^{15}\text{N at. \% excess})\text{nfs}} \cdot 100$$

Table 1. Effect of inoculation on the dry matter yield (DMY), N content and ^{15}N excess in root and shoot of Kallar grass grown in ^{15}N-labelled saline-sodic soil

	DMY (g pot^{-1})			N (yield mg pot^{-1})			At % ^{15}N excess		
	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Weighted av.
Uninoculated	0.3106	0.8479	1.1585	1,79	10.86	12.65	1.3454	.4821	1.4627
Inoculated									
Iso-2	0.2363	1.0714	1.3077	1.61	11.58	13.19	1.0874	.1795	1.1682
Mix.	0.2672	1.0874	1.3546	1.96	13.78	15.74	1.2256	.0629	1.0831

Table 2. Estimates of nitrogen fixation based on ^{15}N isotopic dilution in roots of Kallar grass grown in ^{15}N-labelled saline sodic soil

Inoculum		% N fixed	Weighted av.
Iso-2	Shoot	20.42	20.13
	Root	19.12	
Mixed	Shoot	28.28	25.95
	Root	8.90	

Table 3. Nitrogen balance of the pot experiment after harvest (mg pot^{-1})

Treatment	Total N in soil	N harvested through plant	N gain
Unplanted	178.15 ± 1.5		–
Planted + uninoculated.	187.08 ± 3.6	12.65 ± 2.9	21.85
Planted + inoculated.	182.2 ± 5.0	15.74 ± 2.9	19.79

Where fs is fixing system and nfs is non fixing system. Table 2 presents the estimates of % N fixed based on ^{15}N isotopic dilution. Inoculation with *Beijerinckia* sp. (Iso-2) resulted in 20% N fixed, whereas with the use of mixed inoculum 26% of the plant N was derived from biological N fixation. However, in both these treatments the distribution of biologically fixed nitrogen was different in the root and shoot. With the mixed inoculum, only 9% of the root N was derived from fixation, whereas nearly 28% was found in the shoot. In case of *Beijerinckia* sp. (Iso-2) inoculation, nearly the same percent of fixed nitrogen was found in the root and shoot.

As mentioned earlier all these estimates can only be taken as the amount of nitrogen fixed in response to inoculation. After harvesting the Kallar grass roots and shoots, the total N content of the soil was determined. The N balance is presented in Table 3. The N gain in both the inoculated and

Table 4. Record of the survival of inoculated diazotrophic bacteria in presence or absence of Kallar grass growth by using FA staining of contact slides buried in soil

Sampling date	Planted			Unplanted
	Mixed inoculum	Beijerinckia (Iso-2)	Uninoculated	Uninoculated
1) 26.4.85	+ +* Bacteria approx 30/field-present in upper quarter of the slide only.	+ + Bacteria 40/field in upper quarter of slide	+ Few bacteria in upper quarter of the slide.	No bacteria
2) 6.5.85	+ + + More bacteria in upper quarter of the slide – Mostly Iso-2 type	+ + + Lot of bacteria present in clumps, more than 100/field in upper quarter of slide only.	+ Few bacteria/field.	No bacteria
3) 27.5.85	+ + + Bacteria evenly distributed on the slide but in upper quarter only. More than 100/field.	+ + + Lot of bacteria but only in upper quarter of the slide.	+ Few bacteria/field.	No bacteria

* Number of bacteria observed: High + + +; Medium + +; Low +.

uninoculated treatment was nearly the same in spite of the fact that more nitrogen was taken up by the plant from the inoculated treatment. This indicates that the inoculated bacteria are living in the vicinity of the roots, and thus more of the nitrogen fixed is being transported to the plant. This results in more ^{15}N isotopic dilution.

Survival and colonization studies

The growth and survival of inoculated bacteria in the Kallar grass rhizosphere and their colonization on root surface was monitored with the help of fluorescent antibody staining procedures for soil contact slides and roots, and enumerations using the soil release procedure of Kingsley and Bohlool (1981).

When contact slides buried in differently treated soil were stained after different intervals, they showed that bacteria were present mostly in the upper 2.5 cm of the slide. These observations have been tabulated in Table 4. Bacterial numbers were higher in inoculated and planted treatments as compared to uninoculated planted controls. Bacteria present on the slides of the control beakers showed the natural population of the homologous bacteria in the soil, since the soil used was unsterilized. No bacteria were found in the soil which was unplanted suggesting that the presence of plants exerts an influence on the natural and inoculated population of the diazotrophs. The presence of a higher number of bacteria on the slides on the 3rd staining (at 5th week) showed that inoculated bacteria survived and proliferated. There was no marked increase in the number of bacteria on contact slides of uninoculated and planted pots indicating that the initial population of the homologous diazotrophs was low and that inoculation is beneficial, as shown by proliferation of inoculated bacteria. This also supplements the results of quantification in which more ^{15}N isotopic dilution was obtained in inoculated than in uninoculated treatments.

The results of enumeration using MSRP are presented in Table 5. The results show a greater number of bacteria in planted soil than in the unplanted soil. The inoculation resulted in increased total soil population as shown by the enumerated values of uninoculated and planted soil, 6.8×10^4 as compared to 26.5×10^4. gm^{-1} soil. There was no difference in the enumerated values of total reactive organisms in the mixed and pure (Iso-2) inoculum treatments.

Table 5. Enumeration of inoculated bacteria in Kallar grass rhizosphere by modified soil release procedure (Kingsley and Bohlool, 1981) using fluorescent antibody staining

Inoculum	Mixed*		Iso 2		Uninoculated	
	P	Up	P	Up	P	Up
No. of fields observed.	49	22	57	22	53	22
No. of positive fields.	41	11	35	16	35	16
% positive fields.	84	50	61	73	66	73
Total No. of bacteria counted.	317	37	366	47	89	47
Average/field	12.8	1.6	11.5	2.1	3.2	2.1
Bacteria g^{-1} soil × 104 (wet wt)	26.5	6.7	24.0	8.8	6.8	8.8

* Mixed inoculum of 4 N-fixing isolates. P = Planted. Up = Unplanted

Field experiment

The grass was harvested thrice during the period of eight months of growth (May–Dec). The results of dry matter yield and N uptake are presented in Table 6. There was no appreciable difference at first harvest between the inoculated and the uninoculated treatments. These differences became pronounced during the subsequent harvests. Maximum biomass and nitrogen yield was obtained at the 2nd harvest (H_2) which covers the whole of the monsoon period. Kallar grass growth is optimum during this period. However during winter months (Oct. to Dec.) the growth of Kallar grass slows down (Malik *et al.*, 1986).

The ^{15}N atom % excess in Kallar grass shoots as a result of inoculation is presented in Table 7. Maximum ^{15}N enrichment was found in the first harvest and ^{15}N concentration decreased in the subsequent harvests. The percent nitrogen derived from fertilizer (% Ndff) and percent fertilizer utilization efficiency (% FUE) is presented in Table 8. Maximum % Ndff was found in the first harvest and it declined during the subsequent harvests.

Table 6. Dry matter yield (DMY) and N uptake of Kallar grass grown in differently treated field microplots

Treatment		DMY (g plot⁻¹)				N uptake (g plot⁻¹)			
		H_1	H_2	H_3	Total	H_1	H_2	H_3	To
T_1	AS 2.5 a.e. 1/at 30 kg N ha⁻¹ inoculated	111.5	387.7	324.0	823.3	1.25	5.82	1.94	9.0
T_2	AS 2.5 a.e. 1/at 30 kg N ha⁻¹ uninoculated	110.5	324.2	257.8	692.5	1.29	3.75	1.57	6.6
T_3	AS* 1.0 a.e. 2/at 60 kg N ha⁻¹	130.9	388.0	420.7	939.9	1.40	5.09	1.59	9

AS = Ammonium sulphate, $H_1 H_2$, H_3 = Harvest 1, 2, 3; Split doses 1/ = 10, 10, 10; 2/ = 20, 20, 20

From these data, the 'A' value for all the treatments at three harvests has been calculated. The quantification of the nitrogen fixed has been estimated by the formula (Fried and Broeshart, 1975).

$$\text{N fixed (kg ha}^{-1}) = (A_{fs} - A_{nfs}) \cdot \frac{\%\,\text{FUE}}{100}$$

In this field experiment, in T_3 where AS @ 60 kg N ha⁻¹ was added, has been taken as nfs with the assumption that higher rates of nitrogenous fertilizer application inhibit nitrogenase activity of the rhizospheric bacteria. This treatment gave the lowest 'A' values at all harvests. Calculations based on the differences in 'A' value, showed maximum uptake of biological nitrogen at the second harvest. It ranged from 32 kg ha⁻¹ in the inoculated treatment and 15 kg ha⁻¹ in the uninoculated treatment. The amount of nitrogen fixed decreased at the 3rd harvest and was calcuated to be 6.54 kg ha⁻¹ and 3.76 kg ha⁻¹ in the inoculated and uninoculated treatments, respectively.

Table 7. Atom % ¹⁵N excess in Kallar grass shoots at 3 harvests (H_1, H_2, H_3)

Treatments		¹⁵N atom % excess		
		H_1 (June)	H_2 (September)	H_3 (December)
T_1	AS* (2.5 a.e.) 1/at 30 kg N/ha, Inoculated	0.5286 ± 0.0090	0.0949 ± 0.0028	0.0612 ± 0.02
T_2	AS* (2.5 a.e.) 1/at 30 kg N/ha, Uninoculated	0.5867 ± 0.009	0.1223 ± 0.0020	0.0670 ± 0.009
T_3	AS† (1.0 a.e.) 2/at 60 kg N/ha,	0.2634 ± 0.0045	0.1164 ± 0.0230	0.535 ± 0.002

Estimated ¹⁵N at % excess * 2.2931 ± 0.6921
Split doses: 1/ = 10, 10, 10; 2/ = 20, 20, 20

The comparison of the treatments 1 and 2 showed relatively lesser isotopic dilution in the uninoculated treatment (Table 7). This indicates that some contribution to the biologically fixed nitrogen is made by the inoculated organisms. This contribution can be quantified if T_2 (uninoculated) is taken as non fixing control. Based on this assumption, precent nitrogen derived from fixation for the inoculated treatment can be calculated as 9.5, 22.4 and 8.7 for harvests 1, 2 and 3 respectively (Table 8). This estimation essentially measures the response to inoculation, since nitrogen fixation in uninoculated treatment (T_2) by indigenous microflora is also occurring. It is also clear from the 'A' values of T_2 and T_3.

Discussion

The ¹⁵N dilution technique has been used widely for quantification of biologically fixed nitrogen in legumes (Chalk, 1985). Reviews on the methodology of measurement of notrogen fixation associated with non-legumes agree that isotope dilution methods are suitable for field measurements and these are the best methods for demonstrating amounts of fixed N taken up by the total plant (Fried *et al.*, 1983; Rennie and Rennie, 1983). Both

Table 8. Calculation of fertilizer nitrogen uptake and N_2-fixed by Kallar grass based on the 'A' value of soil

		%Ndff	%FUE	'A'	T_3 as control		T_2 as control	
					N fixed kg ha^{-1}	% N fixed	N fixed kg ha^{-1}	% N fixed
T_1	H_1	23.05	29.74	33	0.3	2.4	1.2	10.0
	H_2	4.14	8.03	695	31.56	54.2	13.0	22.4
	H_3	2.67	1.73	1094	6.54	33.7	1.7	8.7
T_2	H_1	25.58	31.97	29	–	–		
	H_2	5.33	6.66	533	15.38	41.0		
	H_3	2.92	1.34	997	3.76	2.4		
T_3	H_1	38.05	26.63	32				
	H_2	16.56	14.05	302				
	H_3	7.73	3.34	716				

Ndff = Nitrogen drived from fertilizer
FUE = Fertilizer use efficiency
'A' = 'A' value

of these reviews acknowledge the mthodological problems involved in selection of suitable control plants for isotope dilution experiments in the field; the problems also have been discussed by Witty (1983) for the legume system.

The problem of selecting a control for Kallar grass is further compounded by the fact that not many plants are able to grow at the extreme conditions of saline-sodic soils. However, in the field experiment reported, efforts wree made to grow nonfixing reference plants, namely *Typha* sp. and *Desmostachya bipinnata*. Though these are weeds naturally growing in the saline sodic areas, we were unable to grow them in the experimental fields alongside Kallar grass in the ^{15}N microplots. Therefore, in order to overcome this non availability of proper reference plants, plants from the treatment receiving the higher ^{15}N application (60 kg N ha^{-1}) were taken as controls with the assumption that nitrogenase activity would have been inhibited.

The method of inhibition of rhizospheric nitrogenase activity as a means of obtaining a nonfixing control has not received due attention (Chalk, 1985). Rennie *et al.* (1978) suggested that a high rate of ^{15}N labelled fertilizer could be applied to the reference plant to inhibit nitrogenase and the 'A' value modification used to estimate N_2-fixation. Specific inhibitors of nitrogenase have also been reported (Nohrstedt, 1984; Vlassak *et al.*, 1976). Their usefulness is however, dependent on their extent of phytotoxicity and sustained bioactivity of the plants and microorganisms.

The results of the field experiment reported here are based on the estimation of 'A' values at different harvesting times. The advantage of the 'A' value is that the fixing system (fs) and non fixing system (nfs) may be given different rates of fertilizer N. An important condition is that the magnitude of the 'A' value must be independent of the fertilizer rate (Rennie and Rennie, 1983). Gauthier *et al.* (1985) while studying actinorhizal associations in a methylbromide fumigated soil, found that the 'A' value for the reference plant increased with increasing rates of N application. Other studies also have shown that 'A' values for reference plants do vary with increasing rates of N addition but the results have not been consistent (Diebert *et al.*, 1979; Rennie, 1979).

In the present field study, 'A' values decreased with an increase in fertilizer N application. The higher 'A' value is attributed to the occurrence of nitrogen fixation in the inoculated and uninoculated treatments having lower rate of N fertilization. Based on this difference in 'A' value, the amount of N fixed (kg ha^{-1}) has been calculated. The results have indicated that nearly 32 kg N ha^{-1} was fixed by the 2nd harvest which covered the period of high photosynthetic activity and maximum biomass production. The amount of N fixed decreased at the 3rd harvest which was during winter when the growth of Kallar grass is relatively slow. The differences in the amount of N fixed between inoculated and uninoculated treatments gives an indication of the extent of response to inoculation with N_2 fixing bacteria. This has also been quantified by using uninoculated treatment as non fixing control. However, either approach for estimating fixation shows the values to be substantive.

In the pot experiment, the uninoculated treatment was kept as the non fixing control. Based on this reference, it was estimated that 20–26% of the N in Kallar grass was derived from the atmosphere through nitrogen fixation. Since the soil was non sterile, any estimation of notrogen fixation in this case will reflect a response to inoculation and therefore can be regarded as an under estimation of actual total nitrogen fixed. Uninoculated treatments have been used by other workers to assess N_2 fixation associated with wheat (Davidson, 1983; Kapulnik *et al.*, 1985; Rennie *et al.*, 1983) and rice (Watanabe and Lin, 1984).

Our earlier results using the ^{15}N isotopic dilution technique in kallar grass grown in sterile nutrient solution revealed that 60–80% of the N in aerial parts of Kallar grass was derived from fixation by the added bacteria (Malik *et al.*, 1987). In these experiments no additional carbon source was added indicating that the plant growth was able to sustain the proliferation of diazotrophs.

There are few studies carried out on the fate of inoculated N_2 fixing bacteria in the rhizosphere of grasses (Schank *et al.*, 1979). During the present study, the fate of inoculated bacteria in the soil was studied using the immunofluorescence technique. The results indicated that Kallar grass growth exerted a beneficial effect on the survival and proliferation of bacteria inoculated into the rhizosphere. This was demonstrated by fluorescent antibody staining of soil contact slides and roots, and by enumeration using modified soil release procedures of Kingsley and Bohlool (1981).

In conclusion, it can be stated that, we have reported further evidence regarding the contribution of associative nitrogen fixation to the nitrogen nutrition of Kallar grass. Earlier experiments using ^{15}N isotopic dilution technique, carried out in nutrient solution had indicated a high potential of nitrogen fixation in association with roots of Kallar grass (Malik *et al.*, 1987; Malik and Zafar, 1985). The studies reported here have been carried out in pots and in the field, and the results have confirmed the contribution of associative N_2-fixation, though the estimates appear on the conservative side due to experimental limitations.

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