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QUANTIFICATION OF ROOT ASSOCIATED NITROGEN FIXATION IN KALLAR GRASS AS ESTIMATED BY ^{15}N ISOTOPE DILUTION

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Key words ARA Atom excess Azospirillum BNF Inoculation Isotopic dilution Leptochloa fusca ^{15}N Yield

Summary Biological nitrogen fixation associated with the roots of Kallar grass (Leptochloa fusca), a salt tolerant grass, has earlier been demonstrated by using acetylene reduction technique with excised roots. Present investigations were made by using ^{15}N isotope dilution technique to quantitatively estimate BNF in Kallar grass when grown under controlled conditions in nutrient solution and inoculated with N_2-fixing bacteria. Azospirillum brasilense and two isolates from the rhizosphere of Kallar grass were used as inoculant. All the treatments received $^{15}(NH_4)_2SO_4$ 15% at ex. at the rate of 0.5 mg, 50 ml nut. solution^{-1}. After harvest acetylene reduction of roots, total yield, total N and ^{15}N analysis were made. Total-N in inoculated treatments was 2-3 times higher than in control and so were the fresh and dry weight yields. The estimates based on isotopic dilution indicated that 50-70% N in the plant was derived from BNF in case of inoculated treatment. The results based on N balance gave relatively lower values of 40-60% of total N derived from fixation. The data revealed that in Kallar grass a substantial amount of plant N is derived from BNF.

INTRODUCTION

Nitrogen fixation associated with the roots of Kallar grass (Leptochloa fusca (L) Kunth., Syn: (Diplachne fusca) was reported by Malik et al[18,19] and further investigated by Bors et al[3]. All these studies have been performed using acetylene reduction technique which at best can be used for qualitative evaluation[15].

Quantification of biologically fixed nitrogen in legumes and in grasses has been the most important factor in determining the role of such fixation in plant nutrition. Nitrogen balance studies have most widely been used as an indication for the extent of nitrogen fixation[5,25]. However, the use of $^{15}N_2$ has given values of direct incorporation of fixed N into the plants[9, 27]. This technique can only be used for short period

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during which plants are grown under enclosed atmosphere and can not be used in the field. These limitations have made this technique in appropriate for routine use. Technique based on ^{15}N dilution are however more versatile and can be adopted to various experimental conditions[26]. ^{15}N isotope dilution techniques have earlier been used for quantifying associative N_2-fixation in wheat[14,27], maize[24], rice[35], sugarcane[29] and in grasses[2].

This paper reports the use of ^{15}N-isotope dilution technique to determine the amount of plant-N derived from N_2-fixation when Kallar grass was grown in vermiculite and inoculated separately with either Azospirillum brasilense (DSM 1691) or two isolates (NIAB 1; Iso-2) earlier isolated from the Kallar grass roots.

MATERIALS AND METHODS

Bacterial preparation

Azospirillum brasilense (DSM 1691) was obtained from DSM, Gottingen. Two bacterial isolates (NIAB-I and Iso-2) were isolated from the rhizosphere of Kallar grass. Bacteria were grown in 500 ml nutrient broth in one liter flask on a rotary shaker. After 48 h of growth, bacterial cells were harvested by centrifugation at 10,000 rpm for 30 min. Pellet was washed thrice with phosphate buffer (0.1 M, pH 6.8) and resuspended in the same buffer to obtain concentrated bacterial suspension. One ml of inoculum (10^8 -10^9 cells ml^{-1}) was provided to one set of tubes while to other set (control) equal amount of heat killed bacterial suspension was given. Total-N in the bacterial suspension was determined by Kjeldahl method.

Plant growth

Seeds of Kallar grass were surface-sterilized by dipping in 50% commercial NaOCl for 30 min. Several washings were done with sterile distilled H_2O. Seeds were then transferred to 1% agar plates and kept in a controlled temperature growth room with 16 h day and 8 h night. Day temperature was 30 $\pm$ 2°C while night temperature was kept at 28 $\pm$ 2°C. Light intensity was 20,000 Lux. After 4 days, seedlings were transferred to glass tubes (20x3 cm) fitted with perforated silicone stoppers (Shinetsu Polymer Inc., Japan). Each tube was filled with gravel (ca. 2mm size) and contained 30 ml of N-free Hoagland nutrient solution. After autoclaving the tubes, seedlings were transplanted onto silicone stoppers by making a narrow slit in such a way that shoots remained above the stopper while the roots were in contact with the gravel inside the tube. The perforated silicone stoppers do not allow any bacterial contamination of the nutrient solution but allow free gaseous exchange. After the establishment of plants in culture tubes (ca. 3-5 days), filter-sterilized 15% at. ex. $^{15}(NH_4)_2SO_4$ solution (500 ug-N tube^{-1}) was given to all tubes. At the same time 10^8 - 10^9 cells of the 3

bacterial strains were inoculated separately. To the uninoculated treatment, the same amount of autoclaved cells were added. Each treatment was replicated six times.

Plant analysis

Plants were harvested after 5 weeks of growth. Fresh and dry weights were taken. Total-N was determined by semimicro Kjeldahl method[4]. Distillates were collected for ^{15}N analysis. Samples were analysed by Rittenburg method[10] on a Varian Mat GD 150 mass spectrometer fitted with double inlet system.

Acetylene reduction assay

Acetylene reduction assays were performed at the end of experiment. Whole root system from each culture tubes was placed in 30 ml screw capped vial and flushed with N_2 gas . After repeating this process for four times, 12% (v/v) acetylene and 1% (v/v) air was added to vials after replacing the same amount of N_2 gas. Gas samples were analysed after 24 h of incubation on a gas chromatograph (Carlo-Erba, Fracto Vap series 2150) fitted with 0.75 m x 2 mm stainless steel column packed with porapak-N (80-100 mesh, Water Associates Inc. Mass. USA) with the flame ionization detector.

TTC reduction test

TTC (2,3,5 triphenyl tetrazolium chloride) reduction test of roots was performed at the harvesting stage as described by Patriquin & Dobereiner [22]. Washed root segments were incubated overnight in tubes half filled with sterilized tetrazolium-phosphate buffer solution (0.05 M potassium phosphate buffer pH, 7; 0.625 g L^{-1} sodium malate and 1.5 g L^{-1} TTC. Latter was added by filter-sterilization). Whole root segments or hand-cut sections were examined and photographed with a Microlux-11 microscope equipped with Pentax-ME camera.

RESULTS AND DISCUSSION

Inoculation resulted in higher yield in all treatments as shown in Table-1. Yields were significantly higher (P 0.01) in inoculated treatments as compared to control. One to two fold increase in yield was obtained when plants were supplied with N_2-fixing bacteria. Maximum dry weight (153 mg plant $^{-1}$) was observed in plants inoculated with bacterial isolate, Iso-2 while inoculation with A. brasilense gave lowest yield (103 mg plant $^{-1}$). Several other workers have also found that plants inoculated with N_2 -fixing microorganisms produced yield increase [12, 21, 24]. It is intresting to note that inoculation with bacterial isolates of its own rhizosphere gave maximum yield. It was suggested by Dobereiner & De Polli [8] that interaction of specific strains with different genotypes of the host is a crucial aspect to be understood for better use of associative system. Unlike many other studies [11,21,24] no external carbon source was

Table 1. Fresh and dry matter yield and N-contents of upper parts Leptochloa fusca. Readings are averages of 6 replicates. Figures followed by same letter are not significantly different at 1% level as determined by DMR test

Treatment	Fresh wt. mg,plant^{-1}	Dry wt. mg,plant^{-1}	Total-N* mg,plant^{-1}	% N
Control	130 c	62 c	0.63 c	1.03 c
Azospirillum brasilense	223 b	103 a	1.31 a	1.34 a
NIAB-I	355 a	128 a	1.01 b	0.80 c
Iso-2	337 a	153 a	1.53 a	1.00 b

*Inputs of N include 500 ug $^{15}(NH_4)_2SO_4$-N + 148 ug N in the inoculum + 7 ug N in the seed-Total 655ug N in each tube.

added in the growth medium. It was observed that nutrient secretions from roots of L. fusca were probably enough to sustain microbial growth in this short term experiment. Kloss et al[13] has carried out a detailed study of organic acids of the root exudates of L. fusca in sterile and inoculated conditions. They observed that 95% of the acids can be directly used by the diazotroph and found correlation in increase of biomass of the inoculated bacteria with a decrease of exudates in the nutrient solution. Earlier Lethbridge & Davidson[14] were unable to detect nitrogen fixing activity in the unamended treatments. In contrast to this observation, Rennie[24] reported that unamended plants were also able to derive 12.6% N from the atmosphere.

Acetylene reduction assays were performed at the end of experiments to confirm the presence of N_2-fixing microorganisms. No activity was observed in non-inoculated plants which indicated the absence of contamination with N_2-fixing bacteria. Nitrogenase activity was found to be low (Table 2). This low activity may be due to limited supply of available C and energy source. Maximum AR value was observed in plants inoculated with bacterial isolate Iso-2. Maximum yield was also observed in the same treatment.

Evidence for the presence of asymbiotic nitrogen fixing bacteria inside the roots of the host is usually inferred by using the vital TTC staining technique[7]. In the present study, inoculated roots turned intense red while uninoculated roots were unable to reduce the compound. Hand-cut sections showed the crystals of formazan in the cortical as

Table 2. Nitrogenase activity of roots of L. fusca at the end of experiments. Roots from each plant were placed in McCartney vials, flushed with N_2 gas and 1% air (v/v) and 12% (v/v) acetylene was added after replacing same amount of nitrogen gas. Vials were incubated at 35°C for 24 hrs. Gas samples were analysed on a Carlo-Erba gas chromatograph Model 180. Readings are averages of 6 replicates and ± is the standard deviation from the mean

Inoculum	Nitrogenase activity nmoles C_2H_4, g wt^{-1} 24 h^{-1}
Uninoculated	Nil
Azospirillum brasilense NIAB-I	190 ± 101
Iso-2	81 ± 32
	29 ± 115

well as in the steler regions (Fig.1). TTC-reducing bacteria inside the roots have also been observed in maize[17,22], sugarcane[23] and in the roots of orchard plants[33]. Colonization of the inoculated bacteria was clearly demonstrated by this method however it is not clear whether these bacteria fix N_2 in-situ or contribute in any manner towards plant growth.

Total nitrogen content of the plant tops is presented in Table 1. Higher total-N was present in inoculated plants as compared to control. Statistically significant effects of inocualtion on plant nitrogen have earlier been obtained. In phytotron and field conditions, cultures of Bacillus sp. were shown to increase total-N in wheat plants[25]; peat inoculant was shown to increase sorghum nitrogen[32]. Azospirillum brasilense in phytotron conditions increased nitrogen yield of maize as well as of Panicum[12]. Percent N was found to be in the range of 0.84-1.34, which was same or even less than the control (1.03%) except in case of plants inoculated with A. brasilense.

^{15}N analysis and quantification of N_2-fixation

The ^{15}N abundance of the plant tops of both inoculated and uninoculated treatments is presented in Table 3. Statistically significant differences in the ^{15}N enrichment occurred due to inoculation. Most dilution of the ^{15}N abundance was detected in case of Iso-2 inoculations indicating maximum

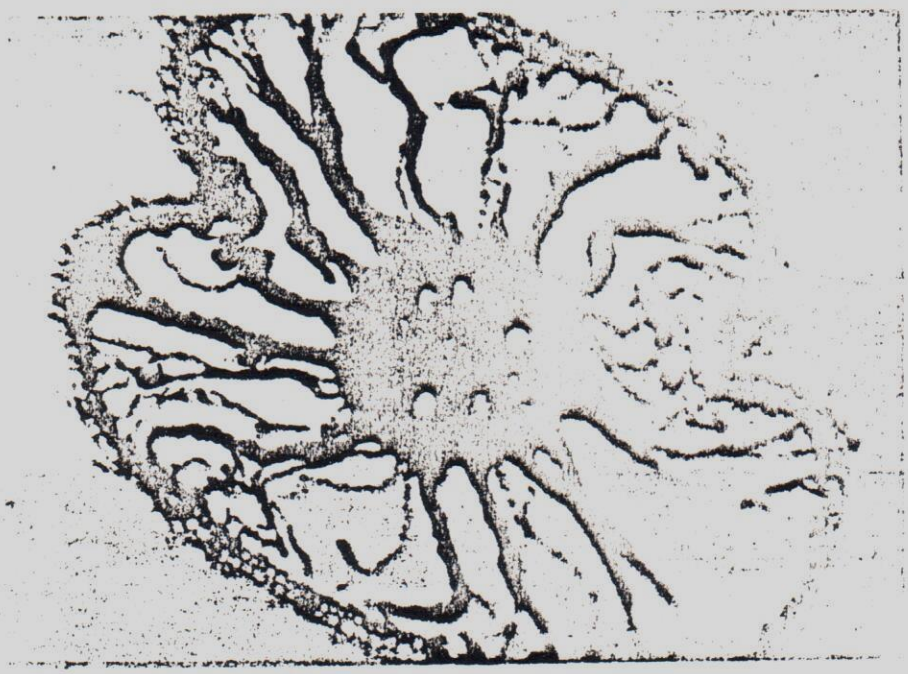

Fig.1. Transverse section of inoculated roots of L. fusca showing dark zones of TTF (x 25).

Table 3. ^{15}N abundance (at % ^{15}N) in the aerial parts of L. fusca. Samples were analysed by Rittenburgh method after spiking with known amount(2 mg-N of unenriched $(NH_4)_2SO_4$ on a Varian MAT GD 150 mass spectrometer

Replicates	Control	Azospirillum brasilense	NIAB-I	Iso-2
1.	9.251	3.855	4.721	1.095
2.	10.040	3.647	3.494	4.094
3.	10.072	6.621	6.578	2.535
4.	10.287	5.530	2.888	3.723
5.	8.451	4.960	5.997	2.753
6.	10.973	5.148	3.719	1.748
Average	9.845 a	4.960 b	4.566 b	2.658 c

Readings followed by same letter are not significantly different at 1% level as determined by DMR test.

N_2-fixation. From the extent of isotope dilution, amount of nitrogen fixed and taken up by the Kallar grass tops could be determined by the equation.

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

Where fs = fixing system (inoculated treatment) and nfs = non-fixing system (uninoculated treatment). The results thus estimated are presented in Table 4. The values obtained can be compared with those obtained by classical difference method based on total Kjeldahl determinations as all the inputs are known and the plants were grown under aseptic condition. The formula used for these estimations is as follows:-

$$\% \text{ N fixed} = \frac{\text{yield of N (fs)} - \text{yield of N (nfs)}}{\text{yield of N (fs)}} \times 100$$

Higher values of % N_2-fixation based on isotope dilution were obtained except in case of A. brasilense inoculation where both estimations gave quite similar values. However, N difference method is yield dependent and is liable to many errors.

Percent nitrogen derived from fixation in Kallar grass ranged from 50-70% of the total nitrogen in plant tops. It was reported[24] that 38% atmospheric nitrogen derived by maize when inoculated with Azospirillum in the presence of succinate as carbon source. In the present investigations no extra carbon source was added to the nutrient solution. All the carbon energy must have been derived by the inoculated bacteria from the root exudates.

Table 4. Estimates of biologically fixed nitrogen by analysing yield data and ^{15}N abundance in aerial parts of L. fusca

Treatments	% N fixed (yield based)	% N fixed based on ^{15}N dilution
Azospirillum brasilense	51.91 a	49.62 b
NIAB-I	37.62 b	53.62 b
Iso-2	58.22 a	73.00 a

Readings are averages of six replicates. Figures followed by same letter are not significantly different at 1% level as determined by DMR test.

The values of N_2-fixation obtained in the laboratory under controlled conditions may not hold true in the field. But in case of Kallar grass, which grows luxuriantly under highly salt affected and low fertility soil the value obtained in the laboratory do not seem to be very high. A biomass production of 40 tonnes ha^{-1}, $year^{-1}$ (amounts to 18 tonnes dry matter) has been estimated for this grass when grown on highly saline sodic soil having total-N of 0.01% and organic C of 0.05%. Negligble NH_4^+ & NO_3^- are detected in the rhizosphere of the grass. Under these conditions the N harvest amounts to 180 kg N, ha^{-1}, $year^{-1}$.

Inoculation of various agricualtural crops with N_2-fixing bacteria has been frequently reported to result in increased yield[1,28,30,32]. Such effect may not necessarily be due to N_2-fixation[16,31]. Plant growth promoting substances have been reported to be secreted by many of these microorganisms[34]. However, the use of ^{15}N dilution technique affords a conclusive proof that the grass can derive some of their nitrogen requirement from atmosphere through the courtesy of N_2-fixing microorganisms.

The conditions of plant growth during present investigation were kept sterile and therefore any dilution of ^{15}N abundance in the Kallar grass tops is the result of N_2-fixation around or inside the roots and then transport of this fixed nitrogen to the other parts. The form and mechanism of this transport is still unknown and needs further investigation.

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