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Associative N_2-fixation in plants growing in saline sodic soils and its relative quantification based on ^{15}N natural abundance

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Abstract

Saline-sodic soils are characterized by a very low nitrogen and organic matter content and thus are practically non fertile. However under these conditions, certain plants have been found to grow luxuriantly. One of such plants, *Leptochloa fusca* (Kallar grass) has exhibited nitrogenase activity associated with its roots as determined by acetylene reduction assay (ARA). Quantification of such nitrogen fixation was also carried out using ^{15}N isotope dilution technique.

In addition to Kallar grass, other plant species growing in saline sodic soils namely *Atriplex amnicola*, *A. lentiformis*, *Sporobolus* sp., *Kochia indica*, *Desmostachya bipinnata*, *Cynodon dactylon*, *Suaeda fruiticosa* and *Polypogon monspilensis* have been screened for the presence of root associated nitrogenase activity. Some of the plant species tested showed high excised root acetylene reduction activity (ERARA). Isolation of diazotrophs from various fractions of the rhizosphere has also been carried out. *Azospirillum* was the dominant organism in niches closer to the roots, whereas there was a preponderance of the members of the family Enterobacteriaceae in general.

In order to have a relative estimate of the nitrogen fixing ability of different plant species screened, the delta ^{15}N values of plant tops were estimated and were correlated with their ARA values. The delta ^{13}C values of these plants were also determined which indicated that all the plants tested except *P. monspilensis* had the C-4 photosynthetic pathway.

Introduction

Associative nitrogen fixation in the roots of non-legumes has been recognized as a possible significant component of the N cycle in a range of ecosystems including several extreme environments (Dart, 1986). Saline sodic soils are characterized by a very low nitrogen and organic matter content and are practically non fertile. However under these conditions certain plants have been found to grow quite well. Since the development of acetylene reduction methodology for detection of nitrogenase activity, many plants have been shown to harbor N_2-fixing bacteria in and around their roots (Jagnow 1983; Patriquin and Döbereiner, 1978). Using such techniques it has been shown that N_2-fixation in the rhizosphere contributes significantly to the N nutrition of plants growing in highly saline sodic low fertility soils (Malik et al., 1988).

A large potentially arable area in Pakistan is afflicted with salinity and sodicity. Extensive studies have been carried out in order to make economic use of these soils by growing salt tolerant plants. One of such plants, Kallar grass (*Leptochloa fusca*) has been highly successful in colonising these soils (Malik et al., 1986). Extensive studies on nitrogen fixation associated with its roots and quantification of such fixation using ^{15}N isotope dilution techniques have previously been carried out (Malik et al., 1987; Malik and Bilal, 1989).

In addition to Kallar grass various other plant species are known to colonize salt affected soils. All such plants have been screened for possible associative nitrogen fixation using acetylene reduction technique, the results of which are being reported here.

Relative quantification of N_2-fixation has been made by estimating the ^{15}N natural abundance of the plant tops. This method is based on the observation that soil N is usually more abundant in ^{15}N than is atmospheric N_2 (Marioti, 1982; Shearer et al., 1978). As a result of this variation, non N_2-fixing plants whose primary source of N is soil derived N, would be expected to be more abundant in ^{15}N than N_2-fixing plants which take N_2 from the atmosphere as well as from the soil. Thus N_2-fixing plants tend to have values of ^{15}N nearer to that of atmospheric N_2.

Since the majority of the grasses in which associative nitrogen fixation was reported possessed the C-4 photosynthetic pathway, it led Dobereiner et al. (1972) to propose a relationship between C-4 plants and N_2-fixation associated with their roots. However, there have been some exceptions to this relationship but no systematic study in this regard has been carried out. With this objective, all the plants screened for associative nitrogen fixation were also analysed for their photosynthetic pathway based on the $^{13}/^{12}C$ ratios. This method is based on the observation that plants discriminate against ^{13}C during photosynthesis in ways which reflect plant metabolism and environment (Benedict, 1978; O'Leary, 1981).

Materials and methods

Study site

The study site was located between longitude 74–75°E and latitude 31–32°N at Biosaline Research Station (BSRS) near Lahore. Average rainfall in this area is about 500 mm. The mean annual air temperature range between 13°–25°C for minimum and 20°–40°C for maximum. Summers and winters are severe as air temperatures may be as high as 47°C in June and as low as 0°C in January. The soils of study plots belong to Khurerianwala soil series and are sandy clay loam in texture. These are calcareous, and highly saline sodic soils having pH 8.5–9.5, EC (of saturation extract) 10–40 mS cm^{-1}, total N is 0.02% and organic carbon is 0.2%.

Acetylene reduction assay (ARA)

A large soil core of ca. 30 cm dia containing a plant was dug up to ca. 20 cm. Three such plant-soil samples for each species were collected from three different locations and assayed for excised root acetylene reduction (ERARA). The roots were separated and soil adhering to them was removed gently. The roots were then subjected to thorough washing with distilled sterile water. Approximately 5 g portions of washed roots were randomly taken in 30 ml capacity vials, sealed with serum caps and 10% v/v C_2H_2 atmosphere was provided. For preincubation, the atmosphere in the vials containing roots was replaced with nitrogen gas and incubated for 24 hours before providing 10% C_2H_2 atmosphere. Ten replicate samples were taken for both direct and preincubated roots for each plant and incubated at 30°C. The gas samples were analysed for C_2H_4 at various intervals using a gas chromatograph (Carlo Erba Fractovap Series 2150) fitted with 0.75 m × 2 mm stainless steel column packed with Porapak N (80–100 mesh, Water Associates Inc. USA), using flame ionization detector (FID). Gas sample (usually 100 μl) was injected by gas tight syringe (Hamilton, USA). The nitrogenase activity was expressed as nmol C_2H_4 g^{-1} root dry wt. 30 mL capacity serum capped vials with 10% v/v C_2H_2 without any roots were used as control.

Isolation of diazotrophic bacteria

Excess soil was removed by placing the rhizosphere under a gentle stream of water. When the roots were free of adhering soil, these were thoroughly washed in several changes of sterile distilled water. For the isolation of bacteria from the root interior, the roots were immersed in 5% NaOCl for 30 min, followed by washing in several changes of sterile distilled water. The roots were excised to 2 cm small pieces and were inoculated in sterile semi-solid nitrogen-free malate medium and combined carbon medium of

Rennie (1981). After 3 to 4 days of incubation at 30°C, a loopful of bacterial growth was transferred into a second vial of nitrogen free medium and was incubated further. The vials were observed daily for growth. The screw caps were replaced by serum stoppers and 10% v/v acetylene was added. The cultures were incubated at 30°C for 1 h, 100 µl of the gas sample was removed and analysed for ethylene by gas chromatography (Bilal and Malik, 1987). For isolation of diazotrophs the cultures yielding more than 100 nmol ethylene/h/vial were streaked on nitrogen-free medium plates supplemented with 0.01% yeast extract. Individual colonies were picked and reinoculated in semi-solid nitrogen-free medium for determining the nitrogenase activity. Various purified cultures giving positive acetylene reduction were retained, after being checked for purity on potato-dextrose or nutrient agar plates.

Estimation of ^{15}N natural abundance

The plant material was collected from the experimental site in the month of February. The fresh material was dried at 60°C for 2 days and was then ground in a Wiley mill to pass a 20 mesh screen. Total N was determined by the Kjeldahl method including steam distillation of the NH_4^+ into boric acid. 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 GD150). Sodium hypobromite was used for releasing ^{15}N.

Estimation of carbon isotope ratios

Leaf tissue was collected from the field and dried in a forced air oven at 80°C for 24 hrs. The dried tissue (5–10 mg) was combusted at 750°C in an excess of oxygen and isotopic ratio ($^{13}C/^{12}C$) of the CO_2 evolved was measured on a mass spectrometer as described by Osmond et al. (1978). Atmospheric CO_2 contains about 1.1% of the heavier isotope ^{13}C and 98.9% of the lighter isotope ^{12}C. The discrimination of ^{13}C in favour of ^{12}C has been highly correlated with the C_3 and C_4 pathways of photosynthetic metabolism. This characteristic when considered in relation to leaf anatomy, provides the most reliable criterion for distinguishing these two photosynthetic pathways (Smith and Brown, 1973).

Results

List of plants surveyed for root associated nitrogenase activity is presented in Table 1. Most of the plants screened belonged to the Graminae family while the rest were from the family Chenopodiacae. Out of the 14 plants species screened only five exhibited appreciable nitrogenase activity as determined by ARA whereas two species showed moderate activity while the

Table 1. List of plants surveyed for root associated nitrogenase activity by excised root acetylene reduction assay (ERARA)

Plants species	Location	Family	ERARA*	Remarks
Leptochloa fusca (L.) Kunth	BSRS	Graminae	++	Introduced
Cynodon dactylon (L.) Pers.	BSRS	Graminae	+++	Natural
Desmostachya bipinnata (L.)	BSRS	Graminae	++	Natural
Sporobolus arabicus Boiss.	BSRS	Graminae	−	Natural
Suaeda fruticosa (L.) Forssk.	BSRS	Chenopod	−	Natural
Kochia indica Wight	BSRS	Chenopod	−	Natural
Atriplex amnicola P.G. Wilson	BSRS	Chenopod	++	Introduced
A. lentiformis (Torr.) Wats.	BSRS	Chenopod	+++	Introduced
Panicum distichum L.	BSRS	Graminae	+	Natural
Andropogon gayana	NIAB	Graminae	+	Introduced
Cenchrus ciliaris L.	NIAB	Graminae	−	Natural
Panicum maximum Jacq.	NIAB	Graminae	+	Introduced
Triticum aestivum L. (wheat)	NIAB	Graminae	+++	Cultivated
Oryza sativa L. (rice)	NIAB	Graminae	+++	Cultivated

*, +++, Activity in µmol; ++, more than 500 nmol; +, around 100 nmol g^{-1} dry root h^{-1}.

remaining had either marginal or no activity. Root associated nitrogenase activity in *C. dactylon*, *A. lentiformis* and *D. bipinnata* was further studied from different locations at the experimental site (BSRS). The results of ARA of washed excised roots incubated directly or after preincubation with N_2 for 24 hrs are presented in Table 2. The rates of acetylene reduction varied with different plant species and locations. Maximum nitrogenase activity was exhibited by *A. lentiformis* both in case of direct and preincubation. In the case of *D. bipinnata*, plants sampled from location C showed relatively high nitrogenase activity. The root samples of *C. dactylon* were collected from 4 different locations. Out of these appreciable activity was detected only at one site.

The diazotrophs associated with the roots of grasses were isolated from the root surface (RP) or the root interior (HP). Most of the bacteria were however, isolated from the root surface as presented in Table 3. A total of 57 isolates were obtained using different media. The identification of these isolates were carried out using QTS-20 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan) based on which an identification key was formulated. Some of the organisms which could be identified are listed in Table 4. *Enterobacters* have been shown to be dominant on the root surface whereas *Azospirilla* were exclusively isolated from root interior (HP) of Kallar grass and *Atriplex*. In addition to these *Citrobacter freundi* was also isolated from number of plant species.

All the different plant species growing at BSRS, Lahore were analysed for the $\delta^{15}N$ values. The respective rhizospheric soil (0–9 cms) was also analysed for $\delta^{15}N$ values. The results are summarised in Table 5. The $\delta^{15}N$ varied with plant species. Among the grasses other than Kallar grass, it ranged from +2.48 to +17.99. In case of Kallar grass the values ranged from −3.32 to +9.13. Among the Chenopods, the δ values ranged from +4.05 to +24.43. *Kochia* showed the maximum value whereas *A. amnicola* exhibited the lowest value. In addition, analysis of *Casuarina* and some legumes growing in the same area was carried out. Comparison

Table 2. Comparison of nitrogenase activity (ARA) of excised washed roots of different plants incubated directly or preincubated with N_2 for 24 hours. Activities are described as nmol C_2H_4 g^{-1} dry roots

Plants screened	Location	Direct without N_2		Preincubated with N_2	
		21 h	29 h	2 h	11 h
Cynodon dactylon	A	0–105 (42)	0–262 (87)	132–2376 (1568)	89–11550 (6512)
	B	0–315 (116)	– –	138–582 (402)	209–4950 (1991)
	C	00 –	00 –	38–700 (202)	77–1320 (484)
	D	567–7770 (3591)	609–8845 (4060)	4–1146 (556)	44–5700 (3245)
Atriplex sp.	A	798–4095 (1890)	1392–4872 (2813)	4–2700 (1094)	88–13519 (5577)
	B	1911–7665 (4326)	12813–10498 (5394)	284–2266 (1010)	1078–6138 (3447)
Desmostachya bipinnata	A	21–105 (63)	29–149 (87)	4–1174 (160)	11–5060 (2156)
	B	21–189 (63)	29–174 (73)	0–914 (248)	0–3157 (1248)
	C	21–2940 (987)	29–6119 (1469)	14–1654 (313)	0–17974 (4829)

The values in brackets are averages of ten replicates. h indicates hours of incubation.

Table 3. List of isolated diazotrophs from roots of plants of saline soils

Plant origin	Root	Media	Isolate code	Total
Leptochloa fusca	RP	NFM	K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14	
		CCM	K2, K3, KC11, K1	17
	HP	NFM	KY1	
		CCM	K2HC2	
Atriplex	RP	NFM	AX6, AX7, AX8, AX9, AX10, AX12, AX13, AX11, AX15	17
		CCM	AX1, AX2, AX3, AX4, AX5, AX14	
	HP	NFM	AH1, AH2	
Triticum aestivum	HP	CCM	QH7, ZH2b, AH6	3
Cynodon	RP	NFM	Cd1, Cd2, Cd3, Cd4, Cd5, Cd6	
	HP	NFM	CH1, CH2, CH4, CH6, CH7	13
		CCM	CH3, CH5	
Sporobolus	RP	CCM	SP1	1
Andropogon	RP	CCM	AP1, AP2	2
Kochia	RP	CCM	KO–1	1
Desmostachya	RP	CCM	DS1, DS2, DS3	3
			Total	57

Table 4. List of diazotrophs isolated from roots of plants growing at Biosaline Research Station, Lahore, Pakistan

Plant origin	Identified organisms
Cynodon dactylon	*Enterobacter cloacae*, *E. agglomerans*
Desmostachya bipinnata	*Citrobacter freundi*, *E. agglomerans*
Sporobolus arabicus	*E. agglomerans*
Kochia indica	*C. freundi*
Andropogon gayana	*C. freundi*
Atriplex sp	*E. agglomerans*, *Klebsiella pneumoniae* *E. cloacae* *E. intermedium*
Triticum aestivum	*E. agglomerans*
Kallar grass	*Azospirillum brasilense*, *Azotobacter* sp. *Enterobacter* sp. *Zoogloae* sp.

was also made with nodulating Chickpea and *Phaseolus* which were grown on N free medium in the growth room. The delta values of *Melilotus* and *Sesbanea formosa* were +1.59 and +6.19 respectively.

The results of the soil $\delta\ ^{15}N\%$ are also presented in Table 5. The values ranged from +5 to +7 and did not show much variation.

The results of $\delta\ ^{13}C\%$ are presented in Table 6. All the plants except Polypogon had values between −16 to −14 which fall well within the range of C-4 plants. *Polypogon* however had ^{13}C δ value of 30.49 and thus has a C-3 photosynthetic pathway.

Discussion

Saline-sodic soils constitute an extreme environment in which plants are subjected to number of stresses. However, the plant growth itself exerts beneficial effects on the soil physical and chemical properties thus paving the way for other plant species to colonize (Sandhu and Malik, 1975). Among the plants screened, *D. bipinnata* and *S. fruticosa* were the two dominant species of the experimental site. Kallar grass was introduced and after 3–4 years of its cultivation, other plant species colonized (Mahmood et al., 1989). These essentially include *C. dactylon*, *K. indica*, *Poly-*

Table 5. Delta ^{15}N of rhizospheric soil and leaf/shoot tissues of plants growing at Biosaline Research Station, Lahore, Pakistan

Plant species	Delta ^{15}N(%)		Remarks
	Soil	Leaf/shoot	
Kallar grass			
6 months old	+6.89(0.52)	+3.76(0.18)	green
1 year old	+6.27(0.47)	+2.45(0.25)	green
2 years old	+5.25(0.48)	−3.32(0.16)	green
3 years old	+5.86(0.81)	+4.18(0.85)	green
4 years old	+3.83(0.19)	+9.13(0.15)	green
5 years old	nd	+2.89(0.12)	very young
Other grasses			
Desmostachya bipinnata	+5.09(0.61)	+2.48(0.19)	young
Cynodon dactylon	+5.02(0.29)	+6.30(1.51)	mature
Polypogon monspilensis	+6.86(0.70)	+17.99(0.67)	young
Sporobolus sp.	+7.95(0.88)	+14.49(0.44)	green
Chenopodiaceae			
Sueda fruiticosa	+5.84(2.35)	+9.05(0.42)	green
Kochia indica	+7.33(0.81)	+24.43(0.31)	green
Atriplex lentiformis	+5.61(0.71)	+4.89(0.84)	
A. amnicola	+5.09(1.87)	+4.05(1.33)	
Casuarina sp.	+6.51(0.07)	−1.85 to +5.74	2 yrs old
Legumes			
Chickpea		+0.48(0.09)	−N medium
Phaseolus vulgaris		−1.33(0.17)	−N medium
Melilotus sp.	nd	+1.49(0.54)	young
Sesbanea formosa	nd	+6.19(0.61)	young branches
Acacia Acc. 15771	+4.43(0.19)	+14.12(2.21)	-do-
Acacia Acc. 15762	+5.08(1.24)	+15.49(2.29)	-do-

Figures in parenthesis are standard error.

Table 6. Delta ^{13}C values of some plants growing at Biosaline Research Station, Lahore, Pakistan

Plant species	Delta ^{13}C (%)
Leptochloa fusca (Kallar grass)	−15.29
Atriplex amnicola	−14.88
A. lentiformis	−16.09
Sporobolus sp.	−14.06
Kochia indica	−12.63
Desmostachya bipinnata	−14.16
Cynodon dactylon	−14.22
Suaeda fruiticosa	−14.20
Polypogon monspilensis	−30.49

pogon sp., *Sporobolus* sp. In addition, *Atriplex* species have also been introduced from Australia.

All these plant species were subjected to ERARA for associative nitrogen fixation. *D. bipinnata*, *C. dactylon* and *Atriplex* spp gave high acetylene reduction values. Some species showed marginal or no activity. Excised root assay with pre-incubation has been criticised by some workers (Van Berkum 1980; Lethbridge et al., 1982). The ARA of excised roots was therefore performed without preincubation with N_2. There was a large variation in the ARA values which is not uncommon in such studies (Rao and Rao, 1984) and it is usually due to uneven distribution of bacteria on the root surfaces and to the difference between old and young roots (Capone and Buding, 1982).

Several types of diazotrophs can be isolated from the same root depending on the medium used. The use of nitrogen free malate medium ensures the isolation of *Azospirillum* (Rao and Rao, 1984; Reinhold et al., 1986). However, using combined carbon medium (Rennie, 1981) other diazotrophic species were also identified (Bilal et al., 1990). All the isolates obtained from the rhizoplane (root surface) belonged to family Enterobacteriaceae, predominantly *E. agglomerans*, followed by *E. cloacae*, *E. intermedium* and *K. pneumoniae*. In addition, *Citrobacter freundi* was also identified. *Azospirilla* were isolated from histoplane fractions of Kallar grass and *Atriplex* roots (Bilal et al., 1990).

The significance of nitrogen fixation associated with roots of grasses can only be demonstrated if it is properly quantified. ^{15}N isotope dilution methodologies have quite extensively been used in the case of legumes (Chalk, 1985) where enriched ^{15}N fertilizer sources is applied to both fixing and non fixing reference plants. Application of this methodology to quantify nitrogen fixation in grasses has also been made (Malik and Bilal 1989; Urquiaga et al., 1989). However, the problem of finding a good reference plant for saline environments has always made such experiments difficult.

In this study $\delta\ ^{15}N$ values of the plants growing in saline soils have been determined. No efforts has been made to quantify nitrogen fixation but to have a relative picture as to the extent of fixation and correlate it with ARA values. Shearer and Kohl (1986) have reviewed the application of ^{15}N natural abundance methodology to various ecosystems. Based on these methods, the plant using all the nitrogen through fixation should have zero $\delta\ ^{15}N$. Hence it is possible to grade various plants for their ability to support associative nitrogen fixation on the basis of their $\delta\ ^{15}N$. *D. bipinnata* and *Atriplex* sp. gave very high ARA whereas these two also had relatively low $\delta\ ^{15}N$ values.

The data regarding Kallar grass showed variation with respect to the age and location. This is a perennial grass and is being continuously cut. However, the low delta values indicate the extent of nitrogen fixation. These values confirm the estimates of nitrogen fixation obtained earlier by using ^{15}N isotope dilution technique (Malik et al., 1988). In the same area *Melilotus* sp. was also sampled and had $\delta\ ^{15}N$ value of +1.49.

Shearer and Kohl (1986) reported $\delta\ ^{15}N$ values of leaf tissue of number of legumes and non legumes. The mean values for Papilionoideae were +1.80; for Prosopis +8.90; for Acacias +10.60 and for non legumes it was 9.30. The $\delta\ ^{15}N$ of non legumes which show no ERARA are similar to the one reported by Shearer and Kohl (1986) whereas the plants showing high ERARA have values nearer to the legume values.

The studies reported here have indicated possibilities of using $\delta\ ^{15}N$ values of plants growing in an ecosystem, as an indicator for the extent of associative nitrogen fixation or the sources of its nitrogen nutrition.

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