Associative N_2-Fixation in Pearl Millet and Sorghum: Levels and Response to Inoculation[1]

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A study was conducted to assess the agronomic importance of associative N_2-fixation in pearl millet (*Pennisetum glaucum* (L.) R. Br.) and sorghum (*Sorghum bicolor* (L.) Moench). To achieve this objective, the rate of N_2-fixing activity was measured by comparing the magnitude of N_2-fixation between pearl millet or sorghum, and pigeonpea (*Cajanus cajan* (L.) Millsp), chickpea (*Cicer arietinum* L.), or groundnut (*Arachis hypogaea* L.) and by examining the response of the crops to inoculation with N_2-fixing bacteria. The overall mean nitrogenase activity (C_2H_2 reduction) in pearl millet throughout the growing period was less than 0.1% of that in pigeonpea and the activity in sorghum was only 1.3% of that in chickpea. Whether assessed by the determination of the nitrogen (N) balance in pots, using pigeonpea as reference, or by the measurement of the natural abundance of ^{15}N ($\delta^{15}N$) using groundnut as reference, N gain by these cereals, even when they were inoculated with N_2-fixing bacteria was not observed. Inoculation trials were conducted in pots and in the field with *Azospirillum lipoferum* and *Azotobacter chroococcum*. Only one trial with sterilized soil in pot showed a significant response to inoculation. The results obtained indicate that associative N_2-fixing systems in pearl millet and sorghum do not exert an agronomically significant impact through N input on the production of these crops at present.

Key Words: **associative N_2-fixation, pearl millet, sorghum, N balance, natural ^{15}N abundance.**

Since a series of measurements of associative N_2-fixation by the acetylene reduction technique was reported (Döbereiner 1968; Döbereiner et al. 1972), several authors have examined the association of N_2-fixing bacteria in cereal roots. These studies demonstrated that N_2-fixing bacteria are present and exhibit a N_2-fixing activity in association with roots. However, there are discrepancies as to whether associative N_2-fixation makes a significant contribution to N supply to cereals (e.g., Chalk 1991; Christiansen-Weniger and van Veen 1991) and whether artificial inoculation of N_2-fixing bacteria enhances the growth of cereals (e.g., Boddey et al. 1986; Harris et al. 1989).

Many of the studies on associative N_2-fixation used the acetylene reduction technique.

Although acetylene reduction may indicate the presence of putative N_2-fixation, this method may not be suitable for the quantification of the fixed N_2 (Bergersen 1970). It is particularly difficult to assess the agronomic significance of N_2-fixation by measuring the acetylene-reducing activity (ARA). It would be more useful if associative N_2-fixation could be measured by methods in which the agronomic impact of N_2-fixation could be evaluated or compared with other N_2-fixing systems whose impacts on crop production have been recognized.

Inoculation response in terms of increased plant growth is an important criterion for demonstrating that associative N_2-fixation can replace N fertilizer. Increased biomass from inoculation with N_2-fixing bacteria has been reported in various areas of the world (Smith et al. 1976; Sarig et al. 1984; Wani et al. 1985; Boddey et al. 1986). In general, however, not all the inoculation trials have been successful, and the rate of success has tended to vary with the inoculation methods, bacterial strains, and soils (Smith et al. 1984; Alexander and Zuberer 1988).

In semi-arid tropical soils, N fertilizers are generally in short supply. We have therefore been considering the possibility of exploiting associative N_2-fixation to replace N fertilizers. The purpose of this study was to measure the level of N_2-fixation in sorghum and pearl millet and to examine the inoculation response of these crops to *Azospirillum lipoferum* and *Azotobacter chroococcum.*

MATERIALS AND METHODS

General. Plants and soils: The cultivars of pearl millet, sorghum, pigeonpea, chickpea, groundnut, and non-nodulating groundnut used in this study were BJ 104, CSH 5, ICPL 87, K 850, Robut 33-1, and ICGL 1, respectively. All the soils used in this study were Alfisols (USDA soil classification) obtained from four different experimental fields at ICRISAT, Patancheru, Andhra Pradesh, India (17°36′N, 78°16′E, 545 m altitude). Chemical properties of the soils are shown in Table 1. The soil which is referred to as sterilized soil was subjected to autoclaving at 1.41 kg cm^{-2}, 121°C for 1 h on 3 successive days.

N_2-fixing bacteria and preparation of inoculants: Cultures of *Azospirillum lipoferum* (ICM 1001) and *Azotobacter chroococcum* (ICM 2001) were isolated from the rhizosphere of sorghum plants inoculated with Napier bajra (a hybrid between *Pennisetum purpureum* × *P. americanum*) root extract and from the rhizosphere of *Cenchrus ciliaris* grown, respectively, in the field at ICRISAT Center (Wani et al. 1985). *Azospirillum lipoferum* and *Azotobacter chroococcum* were multiplied in N-free malate and sucrose broth media, respectively, containing 100 mg L^{-1} yeast extract and peat inoculants were prepared as described by Wani et al. (1985). Culture broth was directly used in inoculation trials with pots. At the time of inoculation, both inoculants contained 10^8 bacterial cells g^{-1} peat or mL^{-1} broth.

Table 1. Chemical properties of the soils (0–15 cm) at ICRISAT Center.

Field	pH (H_2O)	Organic C (g kg^{-1})	Total N (mg kg^{-1})	Olsen P (mg kg^{-1})	Nitrate N (mg kg^{-1})
1	8.3	4.2	558	N.D.[a]	11.6
2	8.4	2.9	573	12.2	1.7
3	7.5	4.1	585	12.9	3.6
4	6.9	3.8	545	6.9	0.0

[a] Not determined in this study.

Experiment I. Measurement of C_2H_2 reduction: ARA of intact sorghum, pearl millet, pigeonpea, and chickpea plants grown in 6 L plastic pots (20.5 cm diameter × 33.5 cm height) with five replications each using a randomized complete block design was measured at five different growth stages as described by Wani et al. (1984). Briefly, the lid with two holes was fitted to the top of the pot and the plant was carefully pulled through one of the two holes. The hole was sealed with silicon rubber 1 d before the ARA measurement. Acetylene gas was injected through a Suba seal fitted to a hole on the side near the bottom of the pot. Gas samples were collected through a Suba seal fitted to the other hole in the lid. Gas samples were analyzed for ethylene evolution with a Pye Unicam 104 Gas Chromatograph (York Street, Cambridge, United Kingdom) fitted with a flame ionization detector. Nitrogenase activities of pearl millet and sorghum were estimated by measuring the amount of ethylene evolved for 6 h from 0900 to 1500. The amount of ethylene evolved from pigeonpea and chickpea was measured for 2 h from 1000 to 1200.

Experiment II. N balance study in pots: Before potting the soil, about 200 kg of soil was sieved through a 5-mm mesh. The sieved soil was thoroughly mixed. Twenty soil samples (10 g each) were subsampled for total N analysis. Sorghum and pigeonpea were grown in plastic pots without drainage holes containing 6 kg soil. Soils planted to sorghum were amended with two N levels, 0 and 62 mg N pot^{-1}, whereas pigeonpea did not receive N. Both crops received 62 mg phosphorus (P) pot^{-1}. Each treatment was replicated 10 times using a randomized complete block design. Sorghum was inoculated with 10 mL broth each of *Azospirillum lipoferum* and *Azotobacter chroococcum* 7 d after germination. Pigeonpea was inoculated with a *Rhizobium* strain (IC 3195) obtained from ICRISAT Rhizobium Germplasm Resources at sowing. During growth, the dead leaves were collected and included in the plant biomass at harvest. When sorghum was harvested at maturity, pigeonpea was also harvested, although pigeonpea was only at the pod-filling stage. After the tops were harvested, the crown roots of both crops were collected. The pots containing the fine roots were kept for 3 months, during which distilled water was ocassionally supplied to maintain the moisture content at approximately 60% water-holding capacity to facilitate the decay of fine roots. This procedure was adopted to avoid the contamination of fine roots in the soil to be sampled, which might affect the total N content of the soil sample. The soils were mixed thoroughly and 10 soil samples (20 g each) were taken from each pot for total N analysis. Total N content in the soil was measured by the Kjeldahl method.

Experiment III. Natural abundance of ^{15}N in plants: From November through January of 1986–1987, pearl millet, sorghum, groundnut, and a non-nodulating groundnut were each planted on ridges 75 cm apart in separate plots of Field 2 which were 9 m wide and 8 m long. Four replications were made using a randomized complete block design. Phosphorus at 17 kg ha^{-1} was applied to all the crops before sowing. Half of the pearl millet and sorghum plots was inoculated with *Azospirillum lipoferum* while the remaining half was not inoculated. Inoculation was conducted as described by Wani et al. (1985), and will be outlined later in Experiment IV.

Above ground plant parts were harvested from an area 6 m wide and 6 m long, oven dried at 60°C for 4 d and analyzed for the natural abundance of ^{15}N ($\delta^{15}N$) with a Finnigan Mat 250 mass spectrometer (Postfach 14 40 62, D-2807, Bremen 14, Germany) as described by Yoneyama et al. (1990). The $\delta^{15}N$ of the plants was expressed as follows:

$$\delta^{15}N(‰) = [(R_{sample}/R_{standard}) - 1] \times 1{,}000,$$

where R is the $^{15}N/^{14}N$ ratio and atmospheric N_2 was used as the standard.

Experiment IV. Inoculation trial: Pot and field trials were conducted in the 1985-1987 rainy (mid-June through October) and postrainy (mid-October through January) seasons with four replications using a randomized complete block design. In the pot trials, the soil from Field 1 was used for Trials 4, 5, and 6; the soil from Field 2 for Trials 1 and 3; and the soil from Field 3 for Trial 2 (Table 2). In the field trials, the soil from Field 1 was used for Trials 1, 2, and 3; the soil from Field 2 for Trials 4 and 5; the soil from Field 3 for Trials 6 and 7; and the soil from Field 4 for Trials 8 and 9, respectively. Before sowing, all the pot trials received P fertilizer at 2 mg P kg^{-1} soil in pots containing 6 kg soil (Trials 1 and 3) or 15 kg soil (Trials 2, 4, 5, and 6), and all the field trials at 17 kg P ha^{-1}, respectively. Fertilizer N treatments were added in four pot trials and in four field trials as shown in Table 2. In one field trial, pearl millet crop residues were incorporated in the soil at a rate of 4 t ha^{-1}.

For the pot trials, culture broth containing 10^8 cells mL^{-1} suspension was used as the inoculum. At 3 d after emergence (DAE) and 10 DAE, 200-mL broth of *Azospirillum*

Table 2. Total biomass production of pearl millet or sorghum inoculated with *Azospirillum lipoferum* and/or with *Azotobacter chroococcum*.

Trial	Crop[a]	Treatment	Total biomass production		SE[b] and statistical difference
			Control	Inoculated	
Pot trial			—g pot^{-1}—		
1	PM	Sterilized soil	13.9	16.3	$\pm 0.53^{**}$
2	PM	0 N	36.9	33.6	$\pm 1.19^{ns}$
		4 mg N kg^{-1} soil	47.6	44.9	
		8 mg N kg^{-1} soil	55.3	54.9	
3	PM	0 N	14.6	13.2	$\pm 0.81^{ns}$
		4 mg N kg^{-1} soil	21.3	22.4	
		8 mg N kg^{-1} soil	27.4	27.3	
4	PM	0 N	77.6	85.8	$\pm 2.85^{ns}$
		4 mg N kg^{-1} soil	165.3	165.3	
		8 mg N kg^{-1} soil	169.9	164.5	
5	PM	0 N	73.5	74.5	$\pm 1.55^{ns}$
		4 mg N kg^{-1} soil	89.7	93.5	
		8 mg N kg^{-1} soil	109.5	104.2	
6	PM		84.7	89.9	$\pm 5.79^{ns}$
7	S		89.0	77.1	$\pm 3.11^{ns}$
Field trial			—kg ha^{-1}—		
1	PM	0 N	3,400	3,170	$\pm 188^{ns}$
		30 kg N ha^{-1}	4,570	4,610	
		4 t straw ha^{-1}	3,960	4,130	
2	PM	20 kg N ha^{-1}	2,470	2,730	$\pm 144^{ns}$
3	S		4,330	4,100	$\pm 234^{ns}$
4	PM		1,750	1,560	$\pm 210^{ns}$
5	PM		1,590	1,680	$\pm 194^{ns}$
6	PM	0 N	2,000	1,560	$\pm 148^{ns}$
		20 kg N ha^{-1}	1,850	1,740	
7	S	0 N	2,810	2,640	$\pm 158^{ns}$
		40 kg N ha^{-1}	2,870	3,190	
8	S		918	980	$\pm 36^{ns}$
9	S		801	820	$\pm 74^{ns}$

[a] PM, pearl millet; S, sorghum. [b] To compare control and inoculate treatment. ** Significant at the $p=0.01$ level of probability; ^{ns} Not significant at $p \geq 0.05$.

lipoferum was inoculated onto each seedling in Trial 1, and 2 mL of the same broth in Trial 2. In Trials 3, 4, and 5, 1-mL broth of *Azospirillum lipoferum* and 1-mL broth of *Azotobacter chroococcum* were simultaneously inoculated at 3 DAE. In Trial 6, 100-mL broth of *Azospirillum lipoferum* and 100-mL broth of *Azotobater chroococcum* were simultaneously inoculated at 3 DAE, and in Trial 7 the same amount was inoculated every fortnight until 73 DAE. Sets of seedlings inoculated with heat-killed *Azospirillum lipoferum* and/or heat-killed *Azotobacter chroococcum* are referred to as noninoculated control.

In the field trials, pearl millet or sorghum was planted on ridges 75 cm apart at 10 cm spacing. The plot size varied with the trials, but the smallest plot was 9 m wide and 8 m long. Liquid inoculum, prepared by mixing peat inoculum in unchlorinated tap water, was applied at sowing and 14 DAE in each plot so that a 8-m row was inoculated with 10^8 cells of *Azospirillum lipoferum* in Trials 1, 2, 4, 6, 7, and 8 or *Azotobacter chroococcum* in Trials 3, 5, and 9 at each inoculation time. Plant parts above ground were harvested from net plot area (smallest, 6 m wide and 6 m long), dried at 60°C for 4 d and weighed for the determination of the total plant dry matter.

RESULTS

Experiment I

The values of ARA in pearl millet fluctuated considerably over the growing period, whereas the values of ARA in pigeonpea increased continuously from 21 d after sowing (DAS) to 56 DAS (flowering stage), and then decreased (Table 3). Pigeonpea still showed a high activity at 71 DAS (flowering and pod-filling stage), but the measurement was terminated because pearl millet was at the grain-filling stage. The overall mean value of ARA in pearl millet was less than 0.1% of that in pigeonpea. Sorghum also showed a high degree of variability in ARA over the growth period. On the other hand, chickpea showed a sharp increase in ARA from 23 DAS to 55 DAS (flowering stage), and then the values

Table 3. Nitrogen fixation (C_2H_2 reduction) by pearl millet and pigeonpea grown in pots, greenhouse, ICRISAT Center, 1986.

Crop	Nitrogenase activity (nmol C_2H_2 plant^{-1} h^{-1})					
	Days after sowing					Mean
	21	37	45	56	71	
Pearl millet	109	2	63	10	107	58
SE	±11.4	±1.0	±8.5	±3.0	±42.1	±23.0
Pigeonpea	961	39,400	90,200	184,000	65,300	75,800
SE	±54.4	±6,586	±12,930	±9,978	±11,900	±30,780

Table 4. Nitrogen fixation (C_2H_2 reduction) by sorghum and chickpea grown in pots, greenhouse, ICRISAT Center, 1986.

Crop	Nitrogenase activity (nmol C_2H_2 plant^{-1} h^{-1})					
	Days after sowing					Mean
	23	41	55	69	84	
Sorghum	104	60	47	632	398	248
SE	±26.3	±21.8	±6.0	±276.6	±111.6	±134.3
Chickpea	21	5,780	68,400	1,570	1,190	16,023
SE	±6.9	±1,440	±8,867	±662.2	±686.2	±4,634

Table 5. Nitrogen balance sheet for soils in pots planted to sorghum and pigeonpea.

Crop	Nitrogen ± SE (mg pot^{-1})				
	Fertilizer N added (A)	Soil[a] before sowing (B)	Soil after harvest (C)	Crop (D)	N balance [(C+D)−(A+B)]
Sorghum	0	2,862±46	2,582±41	153±5	−127
Sorghum	62	2,862±46	2,611±47	184±5	−129
Pigeonpea	0	2,862±46	2,747±34	526±23	+411

[a] The soil was distributed to each pot from the same soil heap. The N content of this soil was measured before potting the soil.

Table 6. $\delta^{15}N$ in pearl millet sorghum, groundnut, and non-nodulating groundnut.

Crop	Inoculation	$\delta^{15}N$ (%)
Pearl millet	Noninoculated	+ 9.8
	Inoculated	+10.2
Sorghum	Noninoculated	+10.9
	Inoculated	+10.7
Groundnut		+ 1.8
Non-nodulating groundnut line		+ 7.9
SE		± 0.44
CV (%)		13

decreased (Table 4). The overall mean value of ARA in sorghum was about 1.3% of that in chickpea.

Experiment II

As shown in Table 5, the N balance was negative for the two soils planted to sorghum. Growth of pigeonpea resulted in a positive balance, which was due to a larger accumulation of N in the crop. This positive balance undoubtedly resulted from atmospheric N_2-fixation by the nodules.

Experiment III

Values of $\delta^{15}N$ in field-grown crops are shown in Table 6. By using a non-nodulating groundnut as a reference plant, the amount of atmosphere-derived N in nodulating groundnut was calculated as described by Yoneyama et al. (1990). The rate of fixed N was estimated at 68% of plant N in nodulating groundnut. The $\delta^{15}N$ values of pearl millet and sorghum were higher than those of non-nodulating groundnut. In ICRISAT fields, the root system of pearl millet or sorghum penetrates into deeper soil layers than that of non-nodulating groundnut, and the analysis of $\delta^{15}N$ in our soil before sowing showed that the values of $\delta^{15}N$ decreased with depth (data not shown). Therefore, these higher values cannot be attributed to the penetration of the root systems of pearl millet and sorghum to deeper soil layers and uptake of N with a higher $\delta^{15}N$ value from deeper layers. The $\delta^{15}N$ values for pearl millet and sorghum, regardless of inoculation, were significantly higher than that for the non-nodulating groundnut. These results suggest that there is no significant incorporation of atmospheric N_2 into pearl millet and sorghum.

Experiment IV

In the pot trials, only one trial (Trial 1) with sterilized soil showed a significant inoculation effect (Table 2), and the addition of fertilizer N did not affect the inoculation effect. No significant increase in total biomass production due to inoculation was observed in any of the field trials, and the treatment with a low level of fertilizer N or pearl millet crop residue did not affect the inoculation effect.

DISCUSSION

Several authors have examined the N_2-fixing activity in non-leguminous plants. However, most of these studies have dealt with cereals or grasses alone, without comparison with leguminous plants where N_2-fixation significantly contributes to N input into the plant-soil system. Very few authors have compared the N_2-fixing activity of cereals and legumes under identical experimental conditions. Bouton (1988) reported that the average N_2-fixing activity of all the pearl millet lines tested was about 2% of that of alfalfa grown in the same soil. In our study, the overall average ARA value of sorghum and pearl millet throughout the duration of growth did not exceed that reported by Bouton. Pigeonpea and chickpea have been reported to fix about 80 and 40 kg N ha^{-1}, respectively (Kumar Rao et al. 1987; Giller et al. 1988). Assuming that the acetylene reduction assay technique measures an equal proportion or all of nitrogenase activities of cereals and legumes under the same assay conditions, the levels of N_2-fixation by sorghum and pearl millet are very low (<0.5 kg N ha^{-1}) and are not agronomically significant in terms of N input into the plant-soil system.

The above conclusion is also supported by the results from studies on the N balance and the natural abundance of ^{15}N. Both studies clearly show that the growth of sorghum or pearl millet, even when the plants were inoculated with N_2-fixing bacteria, resulted in N loss rather than N gain in the plant-soil system. At present, the reason for the N loss which led to a negative N balance in the pot study or higher ^{15}N value in sorghum and pearl millet than in non-nodulating groundnut cannot be determined. One may argue that the rate of associative N_2-fixation was substantial but that the rate of denitrification was much higher than the N_2-fixation. However, this assumption is unlikely in that the conditions conducive to a high rate of denitrification inhibit N_2-fixation due to the presence of a high level of substrate for denitrification, combined N.

Boddey and Döbereiner (1982) quoted results from consistently positive inoculation response in which their noninoculated control was always free of *Azospirillum* and did not exhibit any (zero) nitrogenase activity. *Azospirillum* and *Azotobacter* are known to be ubiquitous in agricultural soils (Hubbell and Gaskins 1984). We also isolated *Azospirillum* and *Azotobacter* in the order of 10^2 to 10^4 g^{-1} soil from the soils used in this study. It is likely that since the noninoculated soils in this study contained indigenous *Azospirillum* and *Azotobacter* at high levels, the addition of exogenous *Azospirillum* and *Azotobacter* by inoculation was not effective enough to increase crop biomass production. This assumption is also supported by the fact that the response to inoculation was observed only with sterilized soil (Table 2). Earlier reports (Wani et al. 1985) showed that a pearl millet genotype, BJ 104, responded to the inoculation of strains, *Azospirillum lipoferum* (ICM 1001) and *Azotobacter chroococcum* (ICM 2001). The same pearl millet genotype and bacterial strains were used in this study to evaluate their response to inoculation over different growing seasons and fields. Based on the results of previous reports and this study, the yield increase due to inoculation does not seem to be consistent. In order to identify the factors

controlling the effectiveness of inoculation, factors that affect the establishment of the bacterial strains in the rhizosphere of pearl millet or sorghum should be analyzed.

In conclusion, it appears that the levels of associative N_2-fixation in pearl millet and sorghum are so low that the contribution of fixed N_2 to the N supply of these cereals is negligible, which may pose a constraint on the replacement of N fertilizer by associative N_2-fixing system in pearl millet and sorghum. Response of pearl millet and sorghum to the inoculation with N_2-fixing bacteria was not observed unless the soil was sterilized. This observation indicates that a positive response to inoculation may depend on the management of the indigenous N_2-fixing bacteria.

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