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Association between N_2-fixing bacteria and pearl millet plants: Responses, mechanisms and persistence*

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Abstract

Responses to inoculation with N_2-fixing bacteria were studied in relation to genotypic differences in pearl millet, effect of nitrogen levels, and FYM additions in India. In some experiments, inoculation increased mean grain yield up to 33% over the uninoculated control, whereas in the remaining 11 experiments there was no significant increase. Increased grain yields, > 10% over the uninoculated controls were observed in 46% of the experiments with *Azospirillum lipoferum* (18.7% average increase) and with *Azotobacter chroococcum* (13.6% average increase). Yield increases were nil or reduced in three experiments with *Azos. lipoferum* and four experiments with *Aztb. chroococcum*. In two experiments continued inoculation for two or three years resulted in increased grain, plant biomass yield, and N uptake. Interactions of bacterial cultures with cultivars or years were not observed. The counts of the inoculated strains increased two to three-fold when inoculation was continued for three years. Repeated inoculations increased the mean cumulative N uptake from season 1 to season 3 by 19 kg ha^{-1}. Repeated inoculations with *Aztb. chroococcum* and *Azos. lipoferum* increased mean grain yield of a succeeding crop by 14.4% and 9.8%, respectively, over the uninoculated control. Inoculation increased the efficiency of N-assimilation by pearl millet. Marginal increase in nitrogenase activity, associated with the inoculated plants was observed during later stages of plant growth. Increased leaf nitrate reductase activity (NRA) was observed after inoculation with these bacteria. The responses to inoculation are mainly attributable to increased plant N assimilation which could be the effect of growth promoting substances secreted by the bacteria; and thus the contribution from BNF may be small.

Introduction

Pearl millet (*Pennisetum americanum* (L.) Leeke) is grown on nutritionally poor soils in the semi-arid tropics, often without the addition of fertilizer nitrogen. In many cases increased plant yields and/or increased N accumulation by plants have been observed from inoculations with *Azospirillum* spp. (Boddey and Döbereiner, 1982) *Azotobacter chroococcum* (Fedorov, 1952). Similar responses from inoculation with azospirilla and azotobacters have been reported in cereals (Avivi and Feldman, 1982; Reynders and Vlassak, 1982; Kapulnik *et al.*, 1983; Sarig *et al.*, 1984; Smith *et al.*, 1984; Wani *et al.*, 1985; Boddey *et al.*, 1986; Wani, 1986; Tilak and Subba Rao, 1987; Baldani *et al.*, 1987; Hussain *et al.*, 1987). The positive benefits from inoculation have been attributed to several mechanisms such as biological nitrogen fixation (BNF) (Cohen *et al.*, 1980; Kapulnik *et al.*, 1981a; Sarig *et al.*, 1984) and increased root uptake capacity because of enhanced root development and root hair formation in response to secretion of plant growth hormones (Tien *et al.* 1979, Umali-Garcia *et al.* 1980; Vlassak and Reynders, 1981; Okon, 1985). Other mechanisms such as enhanced uptake of nitrate, phosphate and potassium (Okon, 1982; Kapulnik *et al.*, 1985) and stimulation of NO_3 assimilation due to inocu-

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lation (Villas Boas and Döbereiner, 1981; Boddey *et al.*, 1986), are also believed to increase yields.

There are several reports on positive benefits of inoculation, but information has been scanty on the benefits of continued inoculation on the yields of the main and the succeeding crops and on the survival of the inoculated bacteria. This paper summarizes the results of 25 field inoculation trials with pearl millet at different locations in India. The effects of continued inoculations on yields of main and succeeding millet crops, and also the persistence of inoculated bacteria under field conditions are discussed.

Materials and methods

Bacterial cultures

The *Azospirillum brasilense* (SL 33) culture was obtained from Dr. F. V. MacHardy, University of Alberta, Edmonton, Canada. *Azospirillum lipoferum* (ICM 1001) and *Azotobacter chroococcum* (ICM 2001) were isolated at the ICRISAT Center from the rhizosphere of sorghum cv. CSH 1 and *Cenchrus ciliaris* as reported earlier (Wani *et al.*, 1985).

Preparation of inoculants

Peat inoculants of azospirilla and *Azotobacter* were prepared by injecting 30 ml of culture broth into a packet containing 40 g γ-irradiated peat, (Agriculture Laboratories Pty. Ltd., Australia: Wani *et al.*, 1985). At the time of field inoculation all the inoculants had 10^8 bacterial cells g^{-1} of peat and were free from contamination at the 10^{-5} dilution. For uninoculated control treatments the peat packets were inoculated with sterile N-free sucrose medium.

Experiment details

The detailed experiments were conducted during the rainy season on alfisols (Table 1) at ICRISAT Center, Patancheru, India (17°36'N, 78°16'E, 545 m altitude).

Liquid inoculum was prepared by thoroughly mixing the peat inoculum in unchlorinated tap water (1 g l^{-1}).

All the experiments were sown on ridges spaced 75 cm apart. Plant-to-plant spacing of 10 cm was maintained by thinning the plants 12–16 days after sowing (DAS). Top dressing with N fertilizer was done 18–20 DAS as required. Weeding and interrow cultivations were carried out as and when required. Plant parts above ground level were harvested from the net plot area. The ears were separated and threshed. The plant matter was then chopped. The chopped plant matter and grain were dried at 70°C in an oven for 3 days and their dry weight recorded. Total nitrogen contents of powdered grain and plant dry matter subsamples from each treatment were estimated by a micro-Kjeldahl digestion method using a Technicon Autoanalyser (for details refer to Industrial method No. 218-72A, II, Technicon, Industrial Systems, Tarry Town, NY 10591, U.S.A.).

Multilocational trials

During 1982–86, 25 field experiments were conducted at the ICRISAT Center and other locations in India, using different millet cultivars, N doses,

Table 1. Details of pearl millet inoculation trials on Alfisols at ICRISAT Center, rainy seasons[a]

Experiment	Year		Soil pH	EC (m.mhos cm^{-1})	Organic Carbon (g kg^{-1})	Total N (mg kg^{-1})	$NH_4 + NO_3 - N$ (mg kg^{-1})	Gross plot size (m^2)	Harvest area (m^2)
I	1983	ZF[b]	6.8	0.16	3.45	635	7.0		
		LF	6.8	0.18	3.60	728	13.5	18	9
		HF	7.3	0.20	4.20	755	15.2		
III	1985		7.9	0.35	4.10	585	33.1	27	12

[a] Presowing soil samples to 60 cm depth were collected only from Expts I and III before imposing the treatments.
[b] ZF, Zero fertility (no N or P added); LF, 20 kg N and 16 kg P_2O_5 ha^{-1}; HF, 56 kg P_2O_5 and 100 kg N ha^{-1}.

and FYM additions to study the responses to inoculation with N_2-fixing bacteria. Results are summarised, but full details are available from the authors on request.

Experiment I. The experiment was laid out in a split-split plot design. Nitrogen levels (0, 20 and 100 kg N ha^{-1}) were in the main plot, cultures *Azospirillum lipoferum* (ICM 1001), *A. brasilense* (SL 33), *Azotobacter chroococcum* (ICM 2001) were in sub-plots, with an uninoculated sub-plot as control, and cultivars in sub-sub plots.

For the 100 kg N ha^{-1} treatment a basal dose of 56 kg N and 56 kg P_2O_5 ha^{-1} treatment was applied mixed fertilizer (28:28:0), and for the 20 kg N ha^{-1} treatment, 16 kg P_2O_5 ha^{-1} was applied as single superphosphate. The crop was machine sown on 28 June 1983, 16 June 1984, and 22 June 1985. Each treatment was replicated four times. First inoculation was done at 7 DAS. For inoculation, a small furrow was opened by the side of the seedlings, inoculum added at 100 ml m^{-1} row length, and the furrow then closed. Similarly, a second inoculation was done 20 DAS. The remaining dose of N as urea was applied after thinning.

Experiment II. This experiment was conducted in the plot of Experiment I to study the effects of continued inoculations with N_2-fixing bacteria for 3 years on the yield of a cover crop during the 4th year. A basal dressing with N and P and a top dressing with N treatments was given as mentioned above for Experiment I. A uniform cover crop of millet cv. ICMV 1 was machine sown on 17 June 1986. This experiment did not include inoculation with N_2-fixing bacteria.

Experiment III. This experiment was laid out in a split-split plot design. Farmyard manure (FYM) levels (0 and 5 t ha^{-1}) served as the main plot, N levels (0 and 20 kg ha^{-1}) were the sub-plots, and cultures *Azospirillum lipoferum* (ICM 1001), *Azotobacter chroococcum* (ICM 2001) and the uninoculated control served as sub-sub plots.

The treatment plots received FYM, containing 10% moisture, organic carbon (86 g kg^{-1}) total N (12.4 g kg^{-1}) NO_3-N (51 mg kg^{-1}), NH_4-N, (22 mg kg^{-1}) and available P (575 mg kg^{-1}) (pH 7.1), 15 days before sowing. The manure was mixed in the soil with a rotovator. Before sowing, 20 kg P_2O_5 ha^{-1} as single superphosphate was applied as a basal dose and 20 kg N ha^{-1} was applied as urea in the plots receiving N. Each treatment was replicated six times. Millet cv. BJ 104 was dibbled manually on 26 June 1985 and 17 June 1986. At the time of sowing, a furrow was opened on the ridge and 100–120 ml peat suspension m^{-1} row length (1 g peat inoculant L^{-1}) was applied; a second inoculation was done 20 DAS by opening a small furrow by the side of the seedlings.

Nitrogenase activity

Nitrogenase activity associated with the roots was measured 50 and 75 DAS at the flowering and grain filling stages in Experiment I in the 1984 and 1985 seasons following the improved soil-core assay technique (Wani *et al.*, 1983). Three-ml gas samples were taken from each container 1 and 6 h after incubation and were analyzed for C_2H_2 and C_2H_4 concentrations by gas chromatography (Wani *et al.*, 1984).

Most probable number (MPN) of N_2-fixing bacteria

Counts for MPN of N_2-fixing bacteria associated with the rhizosphere soil and roots of millet plants were done from each plot at 74 DAS in Experiment II and at harvest in Experiment III. Four plants from each plot were randomly selected and pulled out by hand or dug out. Roots and rhizosphere soil from the same plot were pooled, and the fresh weight of roots and soil recorded and subsampled. The same samples were also used for ELISA studies. The subsamples of roots and rhizosphere soil were kept at 80°C for moisture content determinations. Tenfold serial dilutions were prepared from each sample, and 0.1 ml from each dilution was added to semi-solid N-free medium in tubes. For the azospirilla and *Azotobacter* treatments, N-free malate medium and N-free sucrose medium enriched with 100 ml L^{-1} yeast extract, respectively, were used. The MPN counts from the uninoculated control plots were made with N-free sucrose medium. Soon after inoculation, the cotton plugs of tubes were replaced with sterilized Subaseals and 1% C_2H_2 injected (Balandreau, 1983; Wani, 1986).

The inoculated tubes were incubated at 33°C under 1% C_2H_2 for 48 h. Gas samples from the tubes were collected in 1 ml syringes and analyzed for C_2H_2 and C_2H_4.

Enzyme-linked immunosorbant assay (ELISA) counts

The counts of *Azos. lipoferum* (ICM 1001) and *Azlb. chroococcum* (ICM 2001) from the rhizosphere soil and roots were made from the plots inoculated with *Azospirillum lipoferum* (ICM 1001) and *Azotobacter chroococcum* (ICM 2001), and also from the uninoculated plots in Experiments II and III. Antisera for *A. lipoferum* (ICM 1001) and *A. chroococcum* were prepared by injecting live cells (10^8–10^9 cells ml^{-1} physiological saline) into New Zealand white rabbits. Gamma globulins (antibodies) were collected from the antisera with 1:1600 titre by sodium sulphate precipitation (Van Weeman and Schuurs, 1971). The purified γ-globulins were conjugated with the enzyme alkaline phosphatase, as described by Kishinevsky and Bar-Joseph (1978).

The subsamples from pooled rhizosphere soil and roots from each plot, obtained for MPN counts, were used for counting N_2-fixing bacteria, using ELISA. Ten g of rhizosphere soil was added to 10 ml extraction buffer (Phosphate buffered saline containing: 0.02 mol l^{-1} phosphate, 0.15 mol L^{-1} NaCl, 0.003 mol l^{-1} KCl, pH 7.4, plus 0.05% Tween 20 and 2% polyvinylpyrrolidone, PVP-40T (PBS)) and mixed well. Tenfold serial dilutions were prepared in extraction buffer. Similarly, serial dilutions from 10 g roots macerated in 15 ml extraction buffer in a sterilized mortar were prepared.

For estimating the concentration of antigen in a given sample, the procedure for the direct (double-antibody sandwich) ELISA (Kisihnevsky and Bar-Joseph, 1978) was used wherein alkaline phosphatase enzyme was used to conjugate with γ-globulin, and *p*-nitrophenyl phosphate was used as a substrate. Reactions were stopped at the end of 30 min incubation with 50 μl (per well) of 3 *M* NaOH and the O.D. of *p*-nitrophenol produced in individual wells was read at 410 nm in a Dynatech MR 590 reader. Suitable standards with different concentrations of the standard antigen were included in each experiment along with suitable blanks. The counts of *A. lipoferum* (ICM 1001) and *A. chroococcum* (ICM 2001) were calculated from the standard curves obtained by using varying concentrations of the standard antigen.

Leaf nitrate reductase activity (NRA)

NRA in leaves of millet plants from Experiment I (at 43 and 58 DAS in 1984 and at 45 DAS in 1985) and Experiment III (at 40 DAS in 1986) was estimated. At each sampling, four plants randomly selected from each plot were cut at ground level, transported to the laboratory in polythene bags, and stored in a cold room (4°C). The top fi leaves from each plant were separated and from each leaf three discs of 8 mm diameter were cut, their weight recorded, and NRA measured by the method of Jaworski (1971). The discs from the plants from each treatment were incubated in 15 ml sodium phosphate buffer (0.1 *M* sodium phosphate, pH 7.5, 5% propanol, and 0.02 *M* KNO_3) in 30 ml glass bottles. The discs were subjected to vacuum infiltration for 2 min at 1×10^3 pascals, and incubated at 30°C for 30 min. Ten ml of the incubation mixture was pipetted to a test tube and the nitrite content estimated using Szechrome NIT as described by Hunter *et al.* (1982). The total area of all the green leaves was measured, the leaves were then oven-dried at 60°C for 48–72 h and the total weight was recorded for calculating NRA per plant.

Results

Responses in multilocational trials

Mean grain yields increased significantly (up to 33%) due to inoculation with N_2-fixing bacteria over the respective uninoculated controls in 14 of the 25 experiments. Of the 24 experiments with *Azos. lipoferum* (ICM 1001), in 11 experiments increases in grain yields (average 18.7%) were significantly ($P < 0.05$) high; in 10 experiments the increases in grain yields (9.3%) were not statistically significant; in one experiment no response was observed and in two of them grain yields decreased (2.7%) after inoculation. Similarly, of the 24

experiments with *Aztb. chroococcum* (ICM 2001), in 8 trials mean grain yields across the cultivars/treatments increased significantly ($P < 0.05$) (average increase 13.6%); in 12 experiments grain yield increases (with an average increase of 8.3%) were not statistically significant; in two experiments no response was observed, and in two other experiments grain yields decreased (by 4.5%) after inoculation. *Azospirillum brasilence* (SP 7) caused a reduction in grain yield in the two experiments where this strain was used. In a few other experiments, inoculation with other strains of *Azos. brasilense* resulted in higher grain yields by an average of 8% over the uninoculated control.

Results of continued inoculation experiments

Grain yield

Experiment I. A pooled analysis of three years' data from the experiment revealed that the mean grain yield of pearl millet varied significantly between seasons and cultivars. The results of the pooled analysis in Table 2 show that the mean grain yield of pearl millet cultivars increased significantly after the addition of nitrogen fertilizer, with a maximum grain yield of 2.73 t ha^{-1} with 100 kg N ha^{-1} application. Mean grain yield of cultivars across the years also increased significantly over the uninoculated treatment after inoculation with N_2-fixing bacteria (Table 2). The three inoculants were equally effective in terms of increased grain yield. The interaction between N levels and inocula was not significant.

Experiment II. The results of a uniform crop of millet cv ICMV 1, grown in the plots which were inoculated and treated with nitrogen for three consecutive years previously showed increased grain yield in comparison with the respective control plots (Table 3). A maximum mean grain yield of 2.45 t ha^{-1} (14.4% increase) was observed from the plots inoculated previously with *Aztb. chroococcum.*

Experiment III. The mean grain yield of cv. BJ 104 was significantly ($P < 0.01$) greater in the 1985 season (2.4 t ha^{-1}) than in the 1986 season (1.10 t ha^{-1}). A pooled analysis of the data from both the years showed that mean grain yield increased significantly with FYM addition. Similarly, addition of 20 kg N ha^{-1} increased yield significantly (1.89 t ha^{-1}) over the control (1.59 t ha^{-1}). A higher grain yield was observed with *A. chroococcum* inoculation (1.82 t ha^{-1}), followed by the *A. lipoferum* inoculation (1.77 t ha^{-1}) and the uninoculated control treatments (1.64 t ha^{-1}).

Total plant biomass

Experiment I. Total plant biomass yield of millet cultivars varied significantly across seasons and cultivars. Mean total plant biomass increased significantly with addition of N and also from inoculation with N_2-fixing bacteria (Table 2).

The interaction between millet cultivars and inoculations with N_2-fixing bacteria for plant biomass was significant, however, significant increases were observed only with cv. ICMV 1 inoculated with *Azos. brasilense* (5.9%) and with cv. BJ 104 inoculated with *Aztb. chroococcum* (by 9.4%) and *Azos. lipoferum* (by 8.6%). No significant interactions were observed between N levels and inoculations, cultivars and N levels, and years and inoculations.

Experiment II. Cultivar ICMV 1 yielded similar amounts of total plant biomass from each of the plots that had carried different cultivars over a 3-year period. The mean plant biomass yields of cv. ICMV 1 were significantly higher from the plots supplied with 20 kg N ha^{-1} (6.69 t ha^{-1}) and 100 kg N ha^{-1} (7.48 t ha^{-1}) than from those where no N was applied (5.81 t ha^{-1}). Earlier inoculations with *Azos. lipoferum* and *Aztb. chroococcum* during experiment I resulted in increased plant biomass (6.77 and 6.94 t ha^{-1}, respectively) over that 6.40 t ha^{-1} obtained from the uninoculated control plots (Table 3).

Experiment III. A larger total plant biomass yield was observed in 1985 (5.50 t ha^{-1}) than in 1986 (3.50 t ha^{-1}). Addition of FYM increased plant yield (4.63 t ha^{-1}) over the control yield (4.32 t ha^{-1}). Inoculation with *Azos. lipoferum* and *Aztb. chroococcum* significantly increased plant biomass yield over the uninoculated control (Table 4). A significant interaction between N levels and

Table 2. Mean grain and total plant biomass yield ($t\,ha^{-1}$), mean total plant N uptake ($kg\,ha^{-1}$) and plant dry matter nitrogen percentage of pearl millet cultivars inoculated with N_2-fixing bacteria at three N levels across three years in Experiments I[a]

N applied ($kg\,ha^{-1}$)	Nitrogen fixing bacteria			Uninoculated control	Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azos. brasilense* (SL 33)	*Aztb. chroococcum* (ICM 2001)			
	Grain yield					
0	1.97	1.91	1.92	1.79	1.90	
20	2.50	2.48	2.58	2.43	2.50	0.047*
100	2.66	2.79	2.84	2.62	2.73	
Mean	2.38	2.40	2.45	2.28		0.033*
CV (%)			13.2			
	Total plant biomass					
0	5.68	5.56	5.51	5.42	5.54	
20	6.82	6.81	6.96	6.51	6.78	0.092*
100	7.62	7.75	7.83	7.44	7.66	
Mean	6.71	6.71	6.77	6.46		0.0
CV (%)			11.4			
	Total plant N uptake					
0	37.6	36.4	36.5	32.8	35.8	
20	56.3	54.9	59.1	52.9	55.8	3.05*
100	92.1	90.3	89.7	83.5	88.9	
Mean	62.0	60.6	61.8	56.3		1.18*
CV (%)			19.9			
	Plant dry matter nitrogen (%)					
0	0.31	0.33	0.30	0.26	0.30	
20	0.39	0.36	0.42	0.37	0.39	0.031*
100	0.70	0.63	0.65	0.62	0.65	
Mean	0.47	0.44	0.45	0.42		0.009*
CV (%)			27.2			

[a] Average of 48 replications.
* $P = < 0.05$.

bacterial cultures was observed for mean biomass. Increased ($P < 0.05$) yields of 4.24 and 4.96 $t\,ha^{-1}$ were observed with *Azos. lipoferum* inoculations at zero and 20 $kg\,N\,ha^{-1}$ addition, respectively.

Total plant N uptake

Experiment I. The mean total N uptake of cultivars varied significantly from year to year. A maximum N uptake (68 $kg\,ha^{-1}$) was observed in 1983 followed by 65 $kg\,ha^{-1}$ in 1985 and 48 $kg\,ha^{-1}$ in 1984. The mean nitrogen uptake increased ($P < 0.05$) following inoculation and addition of N (Table 2). There was no interaction between N levels and bacterial cultures for plant N uptake, although there was a significant variety × bacterial culture interaction for total plant N uptake. Inoculation of cv. BJ 104 with *Aztb. chroococcum* and *Azos. lipoferum* increased plant N uptake to 67.6 $kg\,ha^{-1}$ and 65.9 $kg\,ha^{-1}$ respectively, compared with the N uptake of 56.1 $kg\,N\,ha^{-1}$ in the uninoculated control. With any other combinations of variety × bacterial cultures, increase in plant N uptake w not significant.

Experiment II. Previous inoculations in experiment I with N_2-fixing bacteria resulted in increased ($P < 0.05$) N uptake of cv. ICMV 1 (Table 3).

Experiment III. The mean plant N uptake was greater in 1985 (37.3 $kg\,ha^{-1}$) than in 1986 (30.0 $kg\,ha^{-1}$). Increased plant N uptake (30 $kg\,ha^{-1}$) was observed with FYM addition, compared to the zero FYM treatment (27 $kg\,ha^{-1}$).

Nitrogen uptake also increased after application of N and inoculations with N_2-fixing bacteria (Table 4).

Nitrogen content in grain and plant

Experiment I. Nitrogen content in millet grains increased after the addition of 20 and 100 kg N ha^{-1} (1.5 and 2.07%, respectively) over that of the zero N treatment (1.27%).

The mean N content in shoot increased ($P < 0.05$) due to inoculation and the application of N (Table 2).

Cumulative plant nitrogen uptake

Data on cumulative nitrogen uptake in the above-ground plant biomass in Experiment I during the three seasons showed significant increases ($P < 0.001$) after the addition of 20 and 100 kg N ha^{-1} a^{-1}. In the zero applied N treatments, a mean cumulative N uptake of 107 kg ha^{-1} was recorded; with 20 kg N ha^{-1} a^{-1} it increased to 167 kg N ha^{-1}. A maximum N uptake of 262 kg ha^{-1} was recorded in the 100 kg N ha^{-1} a^{-1} treatment. Similarly, inoculation with N_2-fixing bacteria increased ($P < 0.05$) mean cumulative N uptake. A maximum cumulative plant N uptake of 185 kg ha^{-1} was observed in cultivars inoculated with *Azos. lipoferum* (ICM 1001), followed by 182 kg N ha^{-1} with *Azos. brasilense* (SL 33) and *Aztb. chroococcum* (ICM 2001) inoculated treatments, compared to 166 kg N ha^{-1} in the uninoculated millet cultivars.

Nitrogenase (C_2H_2 reduction) activity

In Experiment I during the 1984 rainy season nitrogenase activity associated with millet cultivars inoculated with N_2-fixing bacteria was greater at 75 DAS than at 50 DAS (Table 5). Inoculation with *Azos. lipoferum*, *Azos. brasilense*, and *Aztb. chroococcum* tended to increase nitrogenase activity although there were some anomalous results (Table 5). Significantly reduced mean activity was obser-

Table 3. Mean grain and total plant biomass yield (t ha^{-1}) and plant nitrogen uptake (kg ha^{-1}) of millet cv. ICMV 1 grown in the plots inoculated earlier with N_2-fixing bacteria, Experiment II during 1986 rainy season

Nitrogen applied (kg ha^{-1})	Nitrogen fixing bacteria			Uninoculated control	Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azos. brasilense* (SL 33)	*Aztb. chroococcum* (ICM 2001)			
	Grain yield					
0	2.13	2.01	1.98	1.67	1.95	
20	2.33	1.99	2.55	2.01	2.22	0.076**
100	2.60	2.82	2.83	2.74	2.75	
Mean	2.35	2.27	2.45	2.14		0.070**
CV (%)			15.1			
	Total plant biomass yield					
0	6.01	5.78	6.01	5.42	5.81	
20	7.00	6.24	7.20	6.31	6.69	0.193**
100	7.28	7.56	7.60	7.48	7.48	
Mean	6.77	6.53	6.94	6.40		0.115**
CV (%)			9.2			
	Total plant nitrogen uptake					
0	41.0	43.1	41.5	34.1	39.9	
20	51.4	45.2	56.7	43.0	49.0	5.43**
100	86.3	87.5	86.0	81.9	85.4	
Mean	59.6	58.6	61.4	53.0		1.68**
CV (%)			13.6			

**$P = < 0.01$

Table 4. Mean grain and total plant biomass yield (t ha^{-1}) and total plant N uptake (kg ha^{-1}) of millet cv. BJ 104 inoculated with N_2-fixing bacteria in experiment III

Nitrogen applied (kg ha^{-1})	Nitrogen fixing bacteria		Uninoculated control	Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azib. chroococcum* (ICM 2001)			
	Grain yield				
0	1.65	1.58	1.54	1.59	
20	1.89	2.06	1.73	1.89	0.042**
Mean	1.77	1.82	1.64		0.034**
CV (%)		13.6			
	Plant biomass yield				
0	4.24	3.94	3.85	4.01	
20	4.96	5.28	4.56	4.93	0.092**
Mean	4.60	4.61	4.20		0.075**
CV (%)		11.6			
	Plant N uptake				
0	26.6	25.5	24.7	25.6	
20	31.5	33.8	29.1	31.5	0.71**
Mean	29.1	29.6	26.9		0.64**
CV (%)		15.6			

[1] A pooled analysis of data from 1985 and 1986 rainy seasons.
**P = < 0.01.

Table 5. Mean nitrogenase (C_2H_2 reduction) activity (nmol C_2H_4 plant^{-1} h^{-1}) associated with millet cultivars inoculated with N_2-fixing bacteria in experiment I during 1984 rainy season

Nitrogen applied (kg ha^{-1})	Nitrogen fixing bacteria			Uninoculated control	Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azos. brasilense* (SL 33)	*Azib. chroococcum* (ICM 2001)			
	50 DAS					
0	71	71	100	40	70	
20	57	22	38	43	40	4.49**
100	41	40	49	11	34	
Mean	56	44	62	31		4.75**
CV %			78			
	75 DAS					
0	257	212	194	190	213	
20	249	191	177	168	196	11.19**
100	115	96	151	131	123	
Mean	207	166	174	163		9.04**
CV %			44			

**P = < 0.01.

ved at 100 kg N ha^{-1} in comparison to the zero N treatment activity at both growth stages.

Leaf nitrate reductase activity (NRA)

Experiment I. The specific mean leaf NRA of cultivars in experiment I during the 1984 rainy season was higher ($P < 0.01$) at 43 DAS (4.1 μmol NO_2 g^{-1} fresh leaf tissue) than at 58 DAS (1.5 μmol NO_2 g^{-1} fresh leaf tissue). Addition of 100 kg N ha^{-1} increased NRA in leaves (3.54 μmol NO_2 g^{-1} fresh leaf) over the zero and 20 kg N ha^{-1} treatments (2.3 and 2.5 μmol NO_2 g^{-1} fresh leaf, respectively). Mean NRA of cultivars varied significantly with cultivars with a maximum specific

NRA (3.3 μmol g^{-1} fresh leaf) in cv. BJ 104 and also with inoculation with N_2-fixing bacteria (Table 6). There was significant interaction between cultivars and bacterial cultures.

The specific leaf NRAs at 43 and 58 DAS were positively correlated with grain yield, total plant biomass yield, grain and plant N uptake, and grain N content of cultivars, and the relationship was stronger at 58 DAS. At 58 DAS, NRA was positively correlated with grain yield ($r = 0.46$), total plant biomass ($r = 0.54$), grain N uptake ($r = 0.74$), plant N uptake ($r = 0.77$), and grain N percentage ($r = 0.75$).

During 1985 the results for mean leaf NRA at 45 DAS were similar with the leaf NRA results observed in 1984 season (Table 6).

Experiment III. The mean leaf NRA of millet cv. BJ 104 during 1986 marginally increased with *Azos. lipoferum* and *Aztb. chroococcum* inoculation up to 3.5 and 3.9 μmol $NO_2\,g^{-1}$ fresh leaf, respectively, as against the uninoculated treatment activity of 3.2 μmol $NO_2\,g^{-1}$ fresh leaf. There was no appreciable effect of N and FYM addition on NRA of cv. BJ 104. The mean leaf NRA on a per plant basis increased significantly ($P < 0.05$) with *Aztb. chroococcum* inoculation (658 μmol NO_2 $plant^{-1}$) as against to NRA with the uninoculated control (478 μmol NO_2^{-1} $plant^{-1}$). Inoculation with *Azos. lipoferum* increased leaf NRA of cv. BJ 104 to 535 μmol NO_2 $plant^{-1}$; this increase was however marginal over the uninoculated control.

Counts of N_2-fixing bacteria

Experiment II. Earlier inoculations with *Azos. lipoferum* and *Aztb. chroococcum* resulted in increased MPN counts over the uninoculated controls; increases, however, were statistically not significant. The mean MPN counts of N_2-fixers in the rhizosphere soil increased to $9.3 \times 10^4\,g^{-1}$ dry soil with *A. chroococcum* inoculation and $6.4 \times 10^4\,g^{-1}$ dry soil with *A. lipoferum*, as against to $4.7 \times 10^4\,g^{-1}$ dry soil from the uninoculated control. The MPN counts in the rhizosphere soil did not change with millet cultivars. Similar results were found for MPN counts on a 'per plant' basis. The mean MPN count of N_2-fixers from macerated roots increased significantly ($P < 0.05$) in plots fertilized with 100 kg N ha^{-1} ($9.8 \times 10^5\,g^{-1}$ dry roots) compared to MPN counts from 20 kg N ha^{-1} and zero N

Table 6. Mean specific leaf nitrate reductase activity (μmole $NO_2\,g^{-1}$ fresh leaf) of millet cultivars inoculated with N_2-fixing bacteria in experiments during the 1984 and 1985 rainy seasons

Cultivar	Nitrogen fixing bacteria				Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azos. brasilense* (SL 33)	*Aztb. chroococcum* (ICM 2001)	Uninoculated control		
	1984					
ICMV 1	2.9	3.0	2.9	2.2	2.7	
ICMV 4	3.2	2.6	2.5	2.1	2.6	
BJ 104	4.4	3.2	3.2	2.5	3.3	0.07**
Ex-Bornu	2.7	3.5	2.6	2.0	2.5	
Mean	3.3	2.8	2.8	2.2		0.08**
CV (%)			33			
	1985					
ICMV 1	2.5	3.0	2.4	2.4	2.6	
ICMV 4	3.3	3.2	2.7	2.7	3.0	
BJ 104	3.3	3.1	3.6	2.7	3.1	0.01**
Ex-Bornu	3.3	3.2	3.1	3.1	3.2	
Mean	3.1	3.1	2.8	2.7		0.10**
CV (%)			25			

Four 1984 season each value is mean of two samplings at 43 and 58 DAS; three N levels, 0, 20, and 100 kg N ha^{-1} and four replications at each sampling. For the 1985 season, sampling was done at 45 DAS and other details are the same as for the 1984 season.
**P = < 0.01.

treatments (4.0×10^5 and $3.8 \times 10^5\,g^{-1}$ dry roots, respectively). Previous inoculations with *Azos. lipoferum* and *Aztb. chroococcum* increased the MPN counts from the roots of cv. ICMV I up to 6.7×10^7 and $6.0 \times 10^7\,g^{-1}$ dry roots, respectively, as against $5 \times 10^7\,g^{-1}$ dry roots in the uninoculated treatment. Similar results for MPN counts from the roots were observed on a per plant basis also.

The counts of *Azos. lipoferum* in the rhizosphere soil and macerated roots of cv. ICMV I grown in the plots inoculated earlier in Experiment I increased significantly (Table 7). The *Azos. lipoferum* counts in the rhizosphere soil of cv. ICMV I on a per plant basis increased ($P < 0.05$) to $3.2 \times 10^6\,plant^{-1}$ where inoculations had been done in Experiment I, as against $2.2 \times 10^6\,plant^{-1}$ in the uninoculated plant rhizosphere soil. Similarly, with addition of 20 and 100 kg N ha^{-1}, *Azos. lipoferum* counts increased to 2.9×10^6 and $3.4 \times 10^6\,plant^{-1}$, respectively, compared to $1.8 \times 10^6\,plant^{-1}$ with zero N treatment.

The ELISA counts of *Aztb. chroococcum* in the rhizosphere soil and macerated roots increased significantly up to $3.9 \times 10^3\,g^{-1}$ dry soil and $9.88 \times 10^5\,g^{-1}$ dry roots, compared to the uninoculated control counts of $2.0 \times 10^3\,g^{-1}$ dry soil and $3.61 \times 10^5\,g^{-1}$ dry roots (Table 7).

Experiment III. The mean MPN counts of N_2-fixing bacteria in the rhizosphere soil of cv. BJ 104 increased significantly in 1986 after inoculation with N_2-fixing bacteria (Table 8). Similarly, MPN counts of N_2-fixing bacteria in the rhizosphere soil on a per plant basis also increased ($P < 0.01$) with *Azos. lipoferum* and *Aztb. chroococcum* inoculation (5.8×10^6 and $5.6 \times 10^6\,plant^{-1}$, respectively), compared to the uninoculated control counts ($1.5 \times 10^6\,plant^{-1}$). The MPN counts from the macerated roots on a per plant basis varied significantly with addition of 20 kg N ha^{-1}, and also after inoculation with N_2-fixing bacteria (Table 8).

The mean ELISA counts of *Azos. lipoferum* in the rhizosphere soil of cv. BJ 104 increased significantly ($P < 0.01$) with inoculation ($9.6 \times 10^3\,g^{-1}$ dry soil, compared to $5.8 \times 10^3\,g^{-1}$ dry soil with the uninoculated control plants) (Table 9). Similarly, ELISA counts of *Azos. lipoferum* with roots increased twofold over the uninoculated control after inoculation. Similarly, increased counts of *Azos. lipoferum* in the rhizosphere soil and from the plant roots were observed on a per plant basis.

The ELISA counts of *Aztb. chroococcum* associated with the rhizosphere soil and roots of cv. BJ 104 increased significantly ($P < 0.01$) after inoculation (Table 10). Addition of FYM had no effect on the population of *Aztb. chroococcum*, and 20 kg N addition reduced *Aztb. chroococcum* compared to the zero N treatment (Table 10). Similar results were observed for *Aztb. chroococ-*

Table 7. Number of *A. lipoferum* and *A. chroococcum* in experiment II using ELISA ($\times 10^3\,g^{-1}$ dry rhizospheric soil/dry root) associated with millet cv. ICMV I grown in the plots which were inoculated earlier in experiment I[a]

Nitrogen applied (kg ha^{-1})	Rhizosphere soil			Root macerate		
	Azos. lipoferum	Control	Mean	*Azos. lipoferum*	Control	Mean
0	7.3	5.1	6.2	31.6	20.5	26.1
20	9.0	7.4	8.2	36.2	29.2	32.7
100	8.3	6.5	7.4	49.6	40.1	44.8
Mean	8.2[a]	6.3[b]		39.2[a]	29.9[b]	
CV (%)	2			3		
Nitrogen applied (kg ha^{-1})	Rhizosphere soil			Root macerate		
	Aztb. chroococcum	Control	Mean	*Aztb. chroococcum*	Control	Mean
0	2.9	0.6	1.8	712	452	378
20	4.5	1.5	3.0	1050	416	733
100	4.4	4.0	4.2	1202	622	912
Mean	3.9[a]	2.0[b]		988[a]	361[b]	
CV (%)	10			12		

[a] Average of eight replications, mean across the cultivars. Log transformations of data used for analysis and figures with different letters vary significantly ($P = < 0.05$) from each other.

Table 8. Most probable number of N_2-fixing bacteria associated with the rhizospheric soil and roots of millet cv. BJ 104 in experiment III during the 1986 rainy season

Nitrogen applied ($kg\,ha^{-1}$)	Nitrogen fixing bacteria		Uninoculated control	Mean	SE ±
	Azos. lipoferum (ICM 1001)	*Azotb. chroococcum* (ICM 2001)			
	Rhizosphere soil				
			($\times 10^4\,g^{-1}$ dry soil)		
0	2.4	2.2	0.6	1.7	
20	2.5	2.0	0.9	1.8	0.381
Mean	2.5	2.1	0.7		0.315***
			($\times 10^6$ plant^{-1})		
0	5.5	5.6	1.1	4.1	
20	6.2	5.6	1.9	4.6	0.943
Mean	5.8	5.6	1.5		0.792
	Macerated roots				
			($\times 10^4\,g^{-1}$ dry roots)		
0	9.9	4.8	1.3	5.4	
20	9.9	6.3	2.3	6.2	1.48
Mean	9.9	5.6	1.8		1.58**
			($\times 10^5$ plant^{-1})		
0	7.0	4.0	0.9	4.0	
20	11.5	8.3	2.6	7.5	1.06*
Mean	9.3	6.2	1.7		1.33**

*P = <0.05; **P = <0.01; ***P = <0.001.

cum counts in the rhizosphere soil and with roots on a per plant basis.

Discussion

The results from multilocation experiments conducted in fields where millet had been grown several times before under different environmental and soil conditions, indicated a higher success rate and more increases with *Azos. lipoferum* than with *Azotb. chroococcum*. In the USSR, from a comprehensive survey of the data obtained with *Azotb. chroococcum* inoculation experiments, increased yields of cereal and vegetable crops were obtained in 890 out of 1095 trials and the increase in yield

Table 9. Mean population of *Azos. lipoferum* associated with the rhizospheric soil ($\times 10^3\,g^{-1}$ dry soil) and roots ($\times 10^4\,g^{-1}$ dry roots) of millet cv. BJ 104 in experiment III using ELISA, during the 1986 rainy season

Nitrogen applied ($kg\,ha^{-1}$)	*Azos. lipoferum* (ICM 1001)	Uninoculated control	Mean	SE ±
	Rhizosphere soil			
0	9.4	5.0	7.2	
20	9.9	6.6	8.3	0.53
Mean	9.6	5.8		0.72**
	Macerated roots			
0	2.5	1.5	2.0	
20	3.5	1.6	2.5	0.15*
Mean	3.0	1.5		0.18**

* P = <0.05; **P = <0.01.

Table 10. Population of *Azotb. chroococcum* associated with the rhizospheric soil ($\times 10^2\,g^{-1}$ dry soil) and roots ($\times 10^3\,g^{-1}$ dry roots) of millet cv. BJ 104 in experiment III using ELISA, during the 1986 rainy season

Nitrogen applied ($kg\,ha^{-1}$)	*Azotb. chroococcum*	Uninoculated control	Mean	SE ±
	Rhizosphere soil			
0	2.2	1.5	1.8	
20	2.4	0.8	1.6	0.13**
Mean	2.3	1.2		0.17**
	Macerated roots			
0	1.9	1.0	1.4	
20	1.2	0.4	0.8	0.13**
Mean	1.5	0.7		0.12**

**P = <0.01.

amounted to > 10% in 514 experiments (47%) (Fedorov, 1952). In a few experiments, no increases or small reductions in yields were observed in our studies. Such non-significant effects and a small reduction in plant yield and total N uptake were observed in earlier studies also (see Boddey and Dōbereiner, 1982; Fedorov, 1952; Bouton *et al.*, 1979; Ruschel *et al.*, 1982).

In Experiments I (Table 2) and III (Table 4) inoculation caused increases in mean grain and total plant biomass yields and the increases with different cultures were similar, indicating no specific affinity between the cultures and millet cultivars tested. Strains isolated from the roots of the same crop into which they were subsequently inoculated, have been termed 'homologous' (Boddey and Dōbereiner, 1982). The strains used in the present studies were not homologous and except for *Azos. brasilense* (SP 7), in general, inoculations with all the strains increased the yields. The MPN counts of N_2-fixers in the pre-sowing soil samples from Experiments I and III were 10^2 and $10^3\,g^{-1}$ dry soil, respectively. Boddey *et al.* (1986) suggested that when azospirilla populations are low, *Azospirillum* strains of diverse origin may cause significant response, but in the areas where these bacteria are abundant, 'homologous' strains are more likely to stimulate yield increases. The lack of interaction between inoculations and years in experiments I and III suggest that subsequent inoculations in the same plot increased the yields. The MPN and ELISA counts in Experiments II (Table 7) and III (Tables 8–10) revealed that when the same plots were inoculated thrice and twice, respectively, the counts of the inoculated strains showed only a 1.8–3.0 fold increase over the uninoculated control. In other studies inoculating once resulted in a 2-3-fold increase in the MPN of N_2-fixers (Rao and Venkateswarlu, 1987). Smith *et al.* (1984) reported a continued decline in the population of *Azos. brasilense* to less than 10^2 by the 5th week after inoculation. These results reveal the inability of these bacteria to establish in the rhizosphere in large numbers and it may be a reason for lack of interaction between cultures and seasons.

Application of combined N significantly increased grain, plant biomass, and N content in grains and plant tissues. Inoculation did not increase grain N content in any experiment. However, plant N content increased in Experiment I after inoculation (Table 2). Inoculation of *Azospirillum* often causes increases in plant dry matter with decreases or no increases in N concentrations (Avivi and Feldman, 1982; de Freitas *et al.*, 1982; Millet and Feldman, 1984), and these responses have, therefore, been attributed to effects of plant growth substances. In other experiments, increased plant N concentration with *Azospirillum* inoculation indicated effects of inoculation on N_2 fixation or more nitrogen assimilation by plants (Kapulnik *et al.*, 1981a, b; Baldani *et al.*, 1983; Hegazi *et al.*, 1983; Pacovsky *et al.*, 1985; Wani *et al.*, 1985).

Azospirillum lipoferum inoculation increased mean cumulative plant N uptake ($185\,kg\,ha^{-1}$) by $19\,kg\,N\,ha^{-1}$ more than the uninoculated control plant N uptake during three seasons. The mean cumulative total N uptake by three millet crops in Experiment I with $20\,kg\,N\,ha^{-1}$ treatment was $167\,kg\,N\,ha^{-1}$, as against $107\,kg\,N\,ha^{-1}$ from the zero N plots. These figures showed 100% recovery of added combined N during three years at $20\,kg\,N\,ha^{-1}$ which is a remarkably high value for N recovery studies. With $100\,kg\,N\,ha^{-1}$, however, the N recovery value was just 52%. These results indicated efficient N assimilation by the inoculated plants over the uninoculated control at a low level ($20\,kg\,N\,ha^{-1}$) of combined N. In all, three years of continued inoculation enabled the crops (3 main crops and one succeeding crop) to assimilate $25.6\,kg$ extra $N\,ha^{-1}$ over the uninoculated control plots. These increases were observed along with a 2–3 fold increase in the MPN and ELISA counts of *Azos. lipoferum* and *Aztb. chroococcum* (Table 7) associated with the succeeding crop. The lack of significant interaction between the cultures and seasons in both the experiments and only a 2–3 fold increase in the number of inoculated bacteria after three years of repeated inoculations suggest that these bacteria do not establish well in the soil and continued inoculation may be necessary for obtaining increased yields.

Such positive benefits in terms of increased grain, plant biomass, and N uptake could be attributed to a small increase in N input from BNF (Cohen *et al.*, 1980), development and branching of roots (Umali-Garcia *et al.*, 1980; Tilak and Subba Rao, 1987), production of plant growth hormones (Tien *et al.*, 1979; Vlassak and Reynders, 1981; Brown, 1974); and increased uptake of NO_3^-, K^+, and H_2PO_4

(Lin *et al.*, 1983; Boddey *et al.*, 1986). In the present studies nitrogenase (C_2H_2 reduction) activity associated with the inoculated plants was increased (Table 5), but such increased activity was observed only during later stages of plant growth (Table 5, Wani *et al.*, 1983; Wani, 1988) for a shorter period. As most of the N required for plant growth in millet is taken up before flowering (45–50 DAS) (S.P. Wani, unpublished data) and increased nitrogenase activity was observed after flowering for a short period, the nitrogenase activity may not account for the increased N uptake observed in these studies. Inoculation always increased leaf NRA, suggesting a greater supply of NO_3 to the leaves over the uninoculated control (Table 6) and the increased NO_3 uptake may relate to increased root development in response to the production of hormones by these bacteria (Tien *et al.*, 1979; Umali-Garcia *et al.*, 1980; Brown, 1974; Tilak and Subba Rao, 1987). Further physiological, morphological, and biochemical studies on the plant-bacteria interaction should provide a better understanding of the increased N uptake mechanism.

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