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Research on Cereal Nitrogen Fixation at ICRISAT

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Summary

The acetylene-reduction assay for nitrogenase activity has shown that the roots of sorghum and pearl millet stimulate N_2 fixation. A soil-core assay used for measuring nitrogenase activity of field-grown plants showed large plant-to-plant variability. An improved core assay (planted core assay) developed at ICRISAT Center showed higher activity than that recorded by regular core assay. In greenhouse assay methods plants with shoots sustained higher activity than the ones whose tops were cut before the assay. Nitrogenase activity varied throughout the day, as well as over the season. Activity was maximum at the late flowering or early grain-filling stage and it was related to the ontogenetic development of the plant. Activity was favored in moist, warm (ca 35° C) soil and decreased with high levels of fertilizer N.

Genotypic variation in sorghum and pearl millet lines for stimulating rhizospheric nitrogenase activity was observed. Out of 284 pearl millet lines tested, 18 lines stimulated high nitrogenase activity (>460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$) in the rhizosphere. Two lines, Gam 73 and J 1407, were consistently active over several seasons. Similarly, 28 of 334 sorghum lines tested had high nitrogenase activity (>460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$).

At ICRISAT Center, pot-culture experiments with sorghum and pearl millet grown in a low-fertility Alfisol, or in unsterilized washed vermiculite, showed substantial positive balances for N. Long-term N balance studies in the field with sorghum and pearl millet cultivars are continuing.

Using $^{15}N_2$ it has been shown that 20-days-old sorghum seedlings fix N_2 in the rhizosphere, and part of it is taken up by the plant within 3 days after exposure. The ^{15}N isotope dilution technique has been evaluated for studying genotypic variation in sorghum and pearl millet cultivars for N_2 fixation.

Many different kinds of bacteria closely associated with the roots of sorghum and pearl millet plants show nitrogenase activity. Responses to inoculation in terms of increased dry-matter production and N uptake have been observed in pot experiments with sorghum and pearl millet grown in Alfisols and vermiculite. In five out of nine field trials with pearl millet cultivars, inoculations with N_2-fixing bacteria significantly increased the grain and plant dry-matter yields in all the cultivars.

Introduction

The semi-arid tropics (SAT) produce 60% of the world's sorghum, and 95% of the world's millets, from a total cropped area of about 70 million ha. Both cereals respond to inputs of N, yet almost all the production is without the use of fertilizer. The soil fertility with respect to N depends on the rate of biological turnover of nitrogen and the amounts of nitrogen added to soil through fertilizer, manure,

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and biological nitrogen fixation.

The overall objectives of the ICRISAT research program that concentrates on biological N_2 fixation associated with sorghum and millet are

1. To quantify the amounts of N_2-fixation and to gauge its importance in relation to other N inputs that sustain cereal crop production in the SAT.
2. To seek ways of enhancing this activity by genetic, agronomic, or microbiological methods.

Development of Acetylene Reduction Assay (ARA) for Measuring Nitrogenase Activity

Central to our research is the development of suitable assay techniques to measure nitrogenase activity.

A soil-core assay method has been standardized for measuring acetylene-reduction activity (ARA) associated with field-grown plants. Soil cores containing plant roots are taken in metal cylinders (15 cm diameter, 22 cm length) with as little soil disturbance as possible, and incubated in sealed 6 L plastic vessels under an atmosphere of ca 15% acetylene in air (Fig. 1). Gas samples are taken after 1 and 6 h and later analyzed for ethylene production by gas chromatography. The activity of such cores is usually linear with little or no lag period prior to the onset of nitrogenase activity. With this assay technique, we encountered large plant-to-plant variability in nitrogenase activity. We have studied the factors responsible for such plant-to-plant variability and modified our techniques accordingly.

Mechanical disturbance. The cores sampled and transported with precautions had three-fold greater activity than the cores sampled and transported normally.

Time lag between severing the plant tops and injecting C_2H_2. The highest activity was recorded when the time lag was least, i.e., at 0.5 h. An increase in the time taken to inject the C_2H_2 significantly reduced the activity recorded, with a significant negative correlation ($r = -0.421$, $P<0.01$).

Temperature. Most activity was recorded when the soil cores were incubated at 35°C (Wani et al. 1983).

Core size. The activity varied from field to field and the cores taken over the crown of the plant contained between 13 and 50% of the total activity of the plant depending on its age (Rao and Dart 1981).

Time of sampling. This significantly affected nitrogenase activity of field-grown pearl millet hybrid NHB 3. Nitrogenase activity increased from 0915 to 1815 h during the photoperiod and then declined during the night until 0715 h the next morning. The activity changed little between 0915 and 1500 h. However, significantly higher nitrogenase activity was recorded for the plants sampled between 1715 and 2115 h than at 1115 h.

Soil moisture. The effect of soil moisture on nitrogenase activity was studied in two ways: (1) by using the line-source irrigation system and (2) by adding water to cores just before assay. Both methods gave significant correlations between soil moisture and nitrogenase activity.

Obviously, it is necessary to control as many of the above variables as possible if an assay of field-grown plants is to work.

The activity recorded with the improved soil-core assay technique was significantly higher than that recorded with the regular soil-core assay technique. Nitrogenase activity of field-grown pearl millet hybrid NHB 3 estimated by both the assay methods indicated that activities of most of the plants estimated by the regular core assay ranged from 0 to 20 nmol C_2H_4 $plant^{-1}$ h^{-1} compared with 100-250 nmol C_2H_4 $plant^{-1}$ h^{-1}, for the improved planted-core assay. Mean activity recorded for planted cores was 167 nmol C_2H_4 $plant^{-1}$ h^{-1}, significantly higher than the regular core activity of 18 nmol C_2H_4 $plant^{-1}$ h^{-1}. As plants aged, activity of the regular cores declined more than that of planted cores (Wani et al. 1983). Similar results were obtained with sorghum lines as shown in Table 1 (ICRISAT 1983).

Because soil-core assays are time consuming and somewhat variable, we have developed alternative assay systems — one where seedlings are grown in test tubes, and another where plants are grown in pots.

The test tube culture technique has been developed mainly to test the effect of host genotype and bacterial culture on nitrogenase activity. Plants are grown in 25 mm × 200 mm tubes, with a small tube attached to the side near the bottom. The tubes are filled with 20-25 mL of growth medium, soil, sand, sand:FYM, vermiculite, or nitrogen-free agar, covered and painted to keep light from the root medium. Plants are grown inside the tube, which is plugged with cotton wool until the assay, then with a

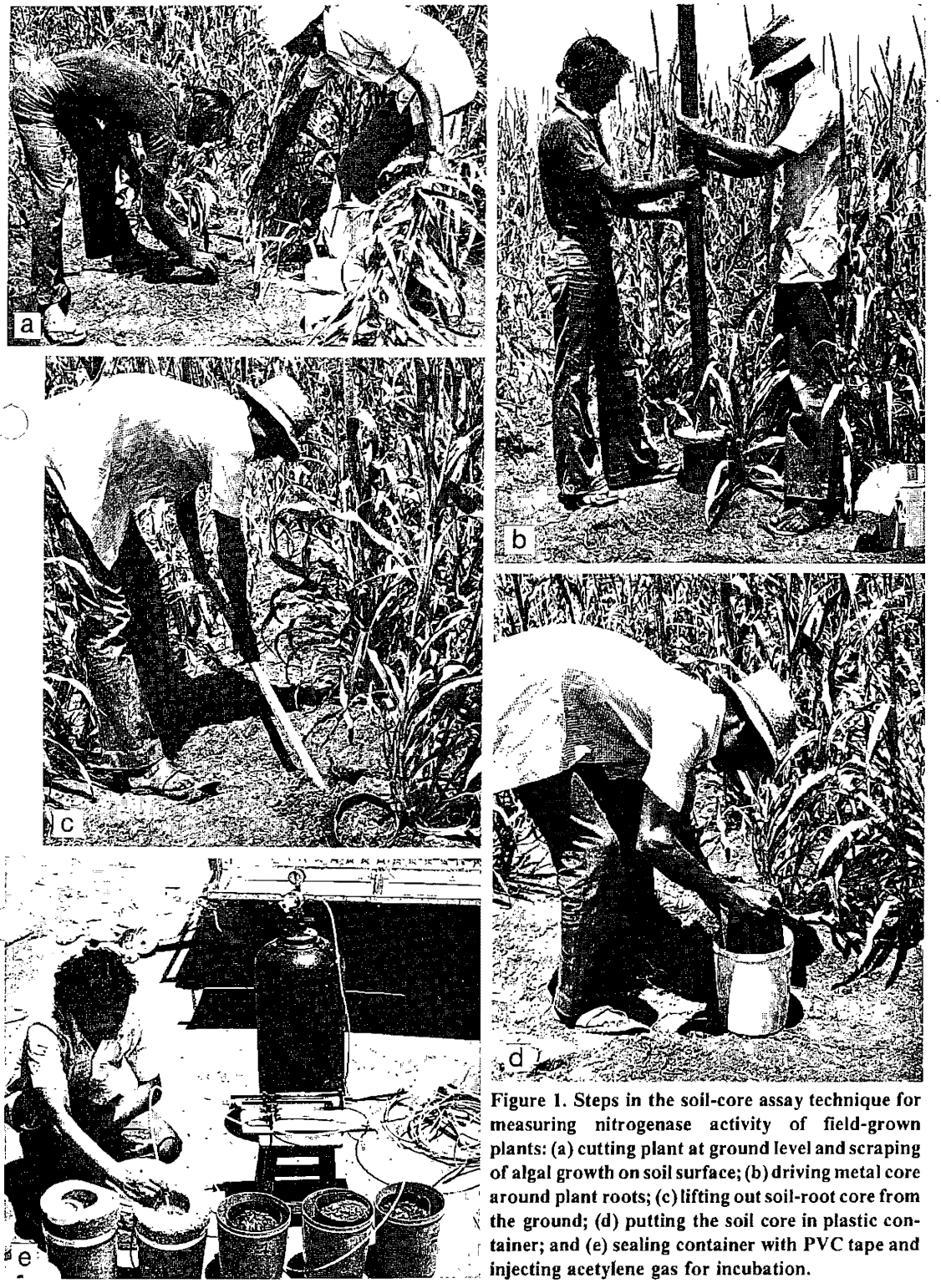

Figure 1. Steps in the soil-core assay technique for measuring nitrogenase activity of field-grown plants: (a) cutting plant at ground level and scraping of algal growth on soil surface; (b) driving metal core around plant roots; (c) lifting out soil-root core from the ground; (d) putting the soil core in plastic container; and (e) sealing container with PVC tape and injecting acetylene gas for incubation.

Table 1. Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1}) of sorghum cultivars as estimated by regular-core and planted-core assay methods at ICRISAT Center.[1]

Cultivar	Regular-core assay	Planted-core assay
IS 1057	24	2101
IS 2207	41	253
IS 9180	33	295
IS 2638	33	316
IS 2391	38	682
IS 3951	37	448
IS 3949	30	267
CSV 5	61	335
Soil	20	119
Mean	35 b	535 c

1. Average of 4 replicated cores. Log transformation (of nmoles C_2H_4+1) used to analyze data. Figures appended with letters vary significantly ($P<0.05$) from each other.

rubber Suba seal; the side tube is also closed with a Suba seal. Acetylene gas, equivalent to 15% of the free volume in the tube, is injected through the bottom Suba seal.

Eleven lines of pearl millet were sown and one control tube without seed inoculated with a rhizospheric extract of field-grown millet to provide a mixed inoculum. Differences between cultivars in stimulating nitrogenase activity were apparent by 20 days after sowing (DAS) and rankings were similar in different growth media. The nitrogenase activity associated with pearl millet seedlings grown in vermiculite test tubes varies with the culture of organisms used. At 14 DAS a maximum activity of 21 nmoles C_2H_4 plant^{-1} d^{-1} was obtained with a culture of *Derxia* sp. The variability among plants in this system was much less than for field-grown plants. It may be possible to screen pearl millet lines more reliably for differences in their ability to stimulate nitrogenase activity in such a system.

Similarly, the activity of sorghum CSH 6 seedlings inoculated with *Azospirillum lipoferum*, *A. brasilense*, *Azotobacter chroococcum*, and *Derxia* spp at 8 DAS was examined. The highest activity of 364 nmol C_2H_4 plant^{-1} d^{-1} was obtained with plants inoculated with *Azotobacter chroococcum*. The activity in the noninoculated control plants was 1.2 nmol C_2H_4 plant^{-1} d^{-1} (ICRISAT 1980).

The above technique was further improved by allowing the shoots to grow outside the test tube. At assay time, Suba seal and silicone rubber were used to seal the gas phase around the root medium, with the shoot remaining free in the air. This method again permitted differences to be detected between pearl millet lines by 20 DAS. Likewise, maximum field activity of nine pearl millet lines recorded with regular soil-core assays over three to six seasons and the activity recorded with seedlings grown in tubes filled with sand:farmyard manure (97:3 w/w) and vermiculite were positively correlated ($r=0.65$, $r=0.67$, $P<0.05$) (ICRISAT 1984).

We have also developed a nitrogenase assay method for intact plants in pots, where only the root system in the pot is exposed to acetylene. Quicker and better diffusion of C_2H_2 and several times greater nitrogenase activity was observed when acetylene was injected at the bottom of the pot than at the top. Intact-plant assays with sorghum and millet showed linear rates of C_2H_4 production up to 1(with a small lag in the beginning (Fig. 2). The time-course assays indicated the feasibility of measuring C_2H_2 reduction by intact sorghum and millet plants grown in pots. After 16 h C_2H_4 production increased slightly, suggesting that the incubation period for estimating ARA by intact-plant assays should be less than 16 h. In our assays we use 6-h incubation for estimating ARA. The initial lag period for C_2H_4 production was possibly because of the time

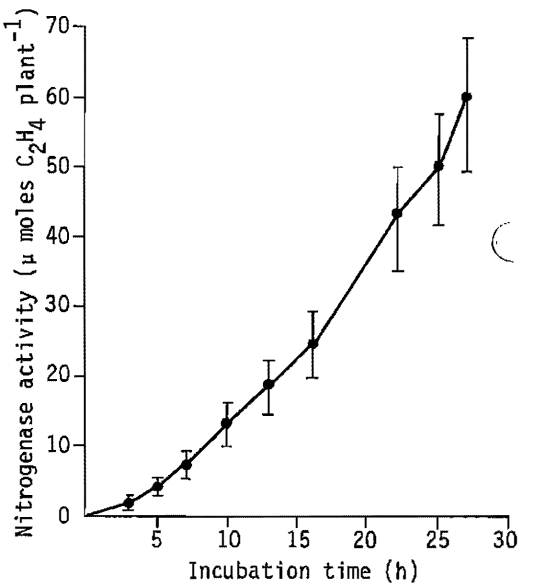

Figure 2. Relationship between ethylene production and incubation time (h) during the assay of intact sorghum plants. Bars represent ±SE.

required for C_2H_2 diffusion throughout the root medium in the pot.

Higher ARA was associated with the intact plants than with decapitated plants. Mean nitrogenase activity of 49-day-old plants across the 15 sorghum cultivars with intact shoots was 625 nmoles C_2H_4 plant^{-1} h^{-1} significantly higher ($P<0.05$) than the activity with decapitated plants (247 nmoles C_2H_4 plant^{-1} h^{-1}). There was no significant interaction between the assay methods and the sorghum cultivars. There was a 26-times reduction in the activity of these cultivars due to decapitation of their shoots with 76-day-old plants as compared with 2.5 times reduction with 49-day-old plants. This higher activity might be due to a continuous supply of photosynthate to the roots from the intact shoot, or the physiological processes of the roots might have been disturbed because of decapitation. Further studies will be required (Wani et al. 1984).

With plants grown in sand, higher nitrogenase activity was observed with increasing FYM concentrations. The increased activity may be due to better plant growth, with increased FYM concentrations providing more root-surface area and more root exudates (derived from an increased root mass) for colonization by N_2-fixing bacteria. Further experimentation is required to understand the role of FYM in stimulating nitrogenase activity associated with plants. Increased activity was recorded with the temperature increasing from 27°C to 33°C. Soil moisture had a large effect on nitrogenase activity of sorghum and millet in the intact-plant assay.

The intact-plant assay was tested for estimating nitrogenase activity associated with 15 sorghum cultivars, and we could differentiate cultivars with high and low associated activity. The variability between plants grown and assayed in pots was much less than between field-grown plants (Wani et al. 1984). This suggested the promise of using this technique for screening sorghum and millet cultivars for their potential to fix atmospheric nitrogen biologically, after studying the relationship between intact-plant assay and field assay methods. It permits a study of the activity at different growth stages of the plant, collection of selfed seed from the plants, and crossing between plants that could be used for producing test hybrids. Maximum expressions of the activity could be obtained by growing plants in 3% FYM mixed with sand or Alfisol soil, by watering the plants to 60-70% water-holding capacity (WHC), by maintaining the temperature of the plant growth medium at around 33°C, and by not decapitating the shoot.

Host Genetic Differences in Promoting N_2-fixation

Different genotypes of both sorghum and pearl millet show differences in stimulating nitrogenase activity. At ICRISAT Center, 135 out of the 284 millet lines tested stimulated nitrogenase activity that was more than twice that of soil without plant roots. Eighteen lines stimulated high nitrogenase activity (>460 nmol C_2H_4 15 cm diam core^{-1} h^{-1}). Two lines, Gam 73 and J 1407, were consistently active over several seasons (ICRISAT 1978, 1980, 1981; Dart and Wani 1982). Large plant-to-plant variability in nitrogenase activity was observed in the Ex-Bornu population, ranging from 0 to 1900 nmol C_2H_4 plant^{-1} h^{-1}. Work on stabilizing the character of high and low nitrogenase activity in this population is under way to study the inheritance of this trait (ICRISAT 1984).

In sorghum, 28 out of 334 field-grown lines tested had high nitrogenase activity (>460 nmol C_2H_4 15 cm diam core^{-1} h^{-1}) associated with their roots. The active lines came from India (12 out of 104 tested), West Africa (6 of 36), East and Central Africa (5 of 63), South Africa (6 of 29), USA (2 of 39), Thailand (1 of 2), and Japan (1 of 3). However, 167 lines stimulated nitrogenase activity of at least 100 nmol C_2H_4 core^{-1} h^{-1}, which was more than twice the mean activity of soil cores without plant roots (range 0-40 nmol C_2H_4 core^{-1} h^{-1}). Fifteen lines have been consistently active in three or more seasons, though they are not consistently active on each assay occasion during the season (ICRISAT 1978, Dart and Wani 1982).

Several lines of minor millets including *Eleusine coracana*, *Panicum* sp, *P. miliaceum*, and *Setaria italica* have been screened for nitrogenase activity. The proportion of active lines in minor millets was more than that with pearl millet lines (ICRISAT 1979, 1980). Several tropical grasses belonging to the genera *Brachiaria*, *Cenchrus*, *Chloris*, *Cymbopogon*, *Dicanthium*, *Euchlaena*, *Panicum*, *Pennisetum*, *Setaria*, and *Sorghum* have been grown without addition of any nitrogen fertilizer over 7 years in an observation plot. Thirty-four out of 48 entries were very active in stimulating N_2-fixation, as observed from the data obtained from soil-core assays with these grasses. Some of the entries, e.g., *Pennisetum purpureum* and a cross between *P. purpureum* and *P. americanum* (Napier bajra), had remarkably high nitrogenase activity, reaching as much as 300 g N ha^{-1} d^{-1} (ICRISAT 1978).

Measurement of N_2-fixation

Use of ^{15}N techniques

$^{15}N_2$ incorporation. In the $^{15}N_2$ incorporation technique a plant enclosed in a container is exposed to $^{15}N_2$, and after incubation, plant tissues are examined for above-normal concentrations of the heavy isotope. Sorghum CSH 5 seedlings grown in tubes in sand: FYM (97:3 w/w) mixture using an intact-tube assay system were exposed to $^{15}N_2$, by exchanging the gas in the root medium in the test tubes by water displacement (ICRISAT 1983). The oxygen content of the root zone was monitored and maintained at 20%. After exposing 20-day-old seedlings to labelled $^{15}N_2$ in this fashion for 3 days, ^{15}N was detected in the growth medium (0.005 ^{15}N atom % excess). Seven days after the labelled gas was removed, the ^{15}N atom % excess in the plants had increased considerably with 0.029 ^{15}N atom % excess in the roots and 0.019 ^{15}N atom % excess in shoots (ICRISAT 1983). Variation in ^{15}N due to differences in both natural abundance and analytical errors can account for at the most ± 0.010 atom % ^{15}N excess (Bergersen 1970).

An apparatus has been developed to introduce gas mixture into the root-growth chamber by purging with CO_2 followed by absorption of the CO_2 with soda lime (Giller et al. 1984) as suggested by Witty and Day (1978), thus avoiding flooding the root zone with water to transfer the gas used in the previous experiment. Using this device 24-day-old sorghum seedlings were exposed to $^{15}N_2$. The results clearly demonstrated that (1) detectable amounts of $^{15}N_2$ were fixed in the rhizosphere of sorghum seedlings and that (2) fixed ^{15}N was incorporated into the plant roots and shoots within 3 days of initial exposure to the gas (Table 2). After a further week of growth the incorporation of ^{15}N in the roots and in the shoots had almost doubled. The actual amount of $^{15}N_2$ fixed during the exposure period and the amount which was incorporated into the plant roots and shoots can be estimated by converting the results into μg N fixed, using the following formula:

$$\text{N fixed } (\mu g) = \text{Total plant N } (\mu g) \times \frac{^{15}\text{N atom \% excess in plant}}{^{15}\text{N atom \% excess in gas phase}}$$

^{15}N dilution. The isotope dilution principle has been used in pot experiments for differentiating lines of sorghum and millet for their potential to fix atmospheric nitrogen. Six lines each of sorghum and millet were grown in pots in a washed vermiculite:sand mixture (1:1 w/w) and watered with 10 ppm N solution with 10 ^{15}N atom % excess daily as required. The percentage of N_2 fixed by an individual line was calculated by comparing it to the line showing the highest ^{15}N atom % content as control (ICRISAT 1984, Giller et al. 1986). Sorghum line IS 801 derived 27%, and millet line D 180 17%, of total plant N from BNF, in comparison with low nitrogenase stimulating sorghum line IS 3003 and pearl millet ICH 107. However, in these experiments considerable dilution of added ^{15}N was observed in all the lines in comparison with the ^{15}N content of the solution added, suggesting that a source of ^{14}N other than fixation may be present in the system (seed or vermiculite). It was noted that ^{14}N released from vermiculite diluted

Table 2. ^{15}N incorporated into 21-day-old sorghum CSH 5 plants after exposure of root systems to $^{15}N_2$[1] for 72 h.

Plant age at harvest (days)	Dry mass (mg plant^{-1})	Total N (mg plant^{-1})	Atom% ^{15}N excess	Fixed N[2] incorporated (μg plant^{-1})
24				
Shoot	264 ± 7.2[3]	7.0 ± 0.28	0.056 ± 0.006	10 ± 1.2
Root	246 ± 17.4	4.2 ± 0.26	0.056 ± 0.003	6 ± 0.5
Total	510 ± 19.1	11.2 ± 0.33		16 ± 1.5
33				
Shoot	400 ± 13.4	5.9 ± 0.54	0.102 ± 0.022	16 ± 4.7
Root	673 ± 56.5	5.8 ± 0.52	0.073 ± 0.016	11 ± 3.2
Total	1073 ± 62.7	11.7 ± 0.96		27 ± 7.9

1. Mean enrichment 40.3 atom % ^{15}N excess.
2. Calculated as total N × atom % ^{15}N excess in the plant divided by atom % ^{15}N excess in gas.
3. Values are means of five and four replicates for the 24- and 33-day harvests, respectively, ± S.E.

^{15}N added. Therefore, the comparison was made to the line with the highest ^{15}N content. In such experiments extra care has to be taken to prevent the systems getting contaminated with ^{14}N from other sources such as water or the growth medium. An alternative approach is to use uniformly ^{15}N labelled soil in pots for growing lines to screen for their potential to fix atmospheric nitrogen (ICRISAT 1983).

Nitrogen-balance Studies

Another aspect of our work deals with measurement of N_2 fixation associated with sorghum, millet, and related species, and transferred to the crop. We are approaching this by experiments using ^{15}N enrichment, by nitrogen-balance experiments in pot culture, and by the much more difficult, long-term field experiments.

Pot Experiments

We have concentrated so far on establishing nitrogen balances associated with sorghum, millet, and Napier bajra (*Pennisetum purpureum* × *P. americanum*) grown in pot culture in vermiculite media with and without added N fertilizer, and in soil low in nitrogen. These experiments have established that there is a small but measurable N_2 fixation by bacteria associated with the roots of these plants, which is taken up by the plant during the growing season, and some of which also remains in the rooting medium.

In one experiment sorghum was grown to maturity without fertilizer N in washed vermiculite. The plants were ratooned twice and grain harvests taken, with total dry-matter production of 195 g pot^{-1} of 10 plants originally sown. In another experiment with sorghum grown in vermiculite for 49 days, the N balance showed a considerable increase in N in planted but not in unplanted pots (Dart and Wani 1982, ICRISAT 1980). The positive N balance across all inoculation treatments was 269 mg pot^{-1} of five plants when no fertilizer N was applied, and 124 mg pot^{-1} when 53 mg N pot^{-1} was applied. A substantial proportion of this N was gained by the root medium (33% of the total N balance for zero-N treatment), although this may result from fine roots missed from the root sample.

In another pot experiment with sorghum CSH 5 grown in an unsterilized Alfisol with three different levels of added nitrogen, a considerable balance for N due to inoculation over the control was observed (Table 3). A maximum mean balance across the nitrogen levels for N over unplanted treatment of 331 mg pot^{-1} was observed in case of inoculation with *A. lipoferum* (ICM 1001). Addition of 20 kg N ha^{-1} equivalent resulted in higher balance for N across the inoculation treatments compared to no N addition and addition of 40 kg N ha^{-1} equivalent (ICRISAT 1984).

Pearl millet grown in vermiculite in pots also attained a positive N balance of 109 mg pot^{-1} containing five plants without added N fertilizer. There was a positive N balance of 96 mg pot^{-1} with nitrogen added equivalent to 20 kg N ha^{-1} (Dart and Wani 1982). Similarly, in a pot trial with millet BJ 104 grown in an unsterilized Alfisol there was a significantly higher positive nitrogen balance (over unplanted treatments) due to inoculation with N_2-fixing bacteria over the noninoculated control. A

Table 3. Nitrogen balance with sorghum CSH 5 inoculated with nitrogen-fixing bacteria.[1]

Culture	Grain mass (g pot^{-1})	Total dry matter (g pot^{-1})	Nitrogen in total dry matter (mg pot^{-1})	Net nitrogen balance (mg pot^{-1})
Azospirillum lipoferum	13.9	79.9	307	331
Azotobacter chroococcum	12.1	75.4	259	226
Napier bajra root extract (NBRE)	14.9	79.9	270	150
Control (noninoculated)	11.6	66.9	223	113
SE ±	0.64	2.52	8.7	56.5
CV %	16.8	11.6	11.8	95

1. Plants were grown in the greenhouse in pots containing nonsterilized Alfisol. Along with inoculation with bacteria, three nitrogen levels (0, 20, and 40 kg N ha^{-1}) were used and each treatment was replicated four times.

positive nitrogen balance of 322 mg pot^{-1} over an unplanted control was recorded in a treatment inoculated with *Azospirillum lipoferum* across the nitrogen levels. A maximum N balance of 240 mg pot^{-1} was observed across the inoculation treatments that received 20 kg N ha^{-1} equivalent. Addition of 40 kg N ha^{-1} reduced the balance significantly (ICRISAT 1984). These findings indicated that addition of nitrogen at lower rates enhanced N_2 fixation that resulted in higher positive balance, and higher rates of N application inhibited N_2 fixation.

Cuttings of the Napier bajra hybrid NB 21 were grown in vermiculite and an Alfisol. The cuttings grew without added N fertilizer to about 150 cm in 72 days before being ratooned. At final harvest at 194 days, the extra N accumulated in the soil amounted to 216 mg N pot^{-1} for a single plant without added N with total positive balance of 539 mg N, and 368 mg N plant^{-1} with 20 kg ha^{-1} added N with the total positive balance of 657 mg N. For the vermiculite rooting medium, N accumulation with zero N treatment was 167 mg N in the medium and 361 mg N positive overall balance (Dart and Wani 1982).

Field Experiments

A long-term field experiment was started in 1978 in collaboration with the Soil Chemistry and Fertility subprogram to measure the N balance in sorghum production in an Alfisol under rainfed conditions. The same eight cultivars with either high nitrogenase activity or high N uptake under low fertility, are grown each year on the same plots. Fertilizer N is added at the rate of 0, 20, and 40 kg ha^{-1}. Mean initial N content of the top 0-15 cm of the soil without fertilizer N was 0.040% N, 0.056% in the 15-30 cm zone, and 0.053% at 30-90 cm. All aboveground plant material is removed at harvest. There were significant differences between the cultivars in grain yield and N uptake from the second season onwards. During the 6th year of this experiment (rainy season 1983), cultivars also varied significantly across the N levels in total dry-matter production. During the 7th year of the experiment (rainy season 1984), a uniform crop of pearl millet was grown. The total dry-matter yield of pearl millet on plots where cultivars CSH 5 and IS 2333 were grown previously was at par across the nitrogen levels.

The cumulative nitrogen uptake through aboveground plant parts from 1978 to 1983 (except 1981) indicated that highest nitrogen uptake amongst the sorghum cultivars across the applied nitrogen levels was in the case of CSH 5 (230 kg ha^{-1}) and the lowest was in the case of IS 2333 (180 kg ha^{-1}) (ICRISAT 1985). These results suggest that sorghum cultivars do vary for their N_2-fixing ability. However, we will have a clearer picture of this when soil-N changes over the first years of the experiment are measured. Samples are currently being processed. Leaching losses and inputs of N through rainfall are likely to be small.

In another long-term N-balance trial at ICRISAT Center, several tropical grasses are grown for 7 years without adding any fertilizer N. The crop receives 40 kg P_2O_5 ha^{-1} a^{-1} and is irrigated during the dry season. The maximum dry-matter production has been obtained with the Napier bajra hybrid NB 21 where an equivalent of 370 t ha^{-1} dry matter, containing an equivalent of 2752 kg N have been harvested in 7 years (Table 4). A preliminary soil sampling up to a depth of 90 cm after 5.5 years showed no difference in N content of the soil from the plots where NB 21 was grown and where the low dry-matter-producing entry was grown. These results suggest that entries like NB 21 are deriving some of their requirement of N through BNF in addition to the soil-N pool. Thorough soil sampling in these plots will allow computation of the exact amounts of N fixed by these entries over the years.

Contribution to N Balance by Blue-green Algae

Crusts of blue-green algae develop on the soil surface of many cropped fields during the rainy season and after irrigation (Dart and Wani 1982, ICRISAT 1983). The predominant heterocystous algae, seen forming mats on fields at ICRISAT Center, were two *Anabaena* species and *Nostoc muscorum*. Other *Nostoc* species as well as algae belonging to the genera *Calothrix*, *Aphanothece*, *Microsystis*, *Lyngbya*, and *Oscillatoria* were also observed (ICRISAT 1983). On Alfisols, the growth and N_2-fixing activity of those algae under sorghum and millet was generally low, but under tropical grasses such as *Pennisetum purpureum*, the mats may be very active, depending on the wetness of the soil surface, and the extent of the plant canopy. Activity decreases rapidly as the soil surface dries out, virtually ceasing 3 days after wetting of the soil surface if the radiation levels are high. Nitrogenase activity of these algal

Table 4. Dry-matter production and nitrogen uptake by forage grass spp in long-term nitrogen-balance trial.[1]

Grass	Age (years)	Dry matter cumulative (t ha^{-1})	Production per day (kg ha^{-1})	Nitrogen cumulative (kg ha^{-1})	Uptake per day (kg ha^{-1})
Pennisetum americanum × *P. purpureum* NB 21	6.94	370.7	146.3	2752	1.09
P. purpureum JVM-2	6.94	211.0	83.3	1760	0.69
P. squamulatum	6.94	165.5	65.3	1189	0.47
Cenchrus ciliaris	6.94	164.3	64.9	1235	0.49
Panicum maximum	6.00	152.0	69.0	1080	0.49
Setaria anceps	6.94	119.0	47.0	836	0.33
Pennisetum mezinium	6.94	98.9	39.0	617	0.24
Chloris gayana	6.31	96.3	41.8	706.6	0.31
Panicum antidotale	6.5	43.0	18.1	318	0.13
Pennisetum rupellii	6.5	41.7	17.6	312	0.13

1. Estimated from 6 m^2 net harvest area, harvested 19 times, crop is irrigated during the dry season and 40 kg P_2O_5 ha^{-1} is added every year.

mats when extrapolated to a surface-area basis ranged from 24 to 119 mg N fixed m^{-2} d^{-1}, compared with only 0.5 to 1.6 mg N m^{-2} d^{-1} for surface soil without visible algal growth. The algal mat covered up to 29% of the soil surface (Dart and Wani 1982), at which level this extrapolates to an upper estimate of fixation of 28 kg N ha^{-1} a^{-1}, assuming 80 days at the above level of activity for an irrigated perennial grass crop.

Factors Affecting Associative N_2 Fixation

Nitrogen fixation associated with sorghum and millet is affected by plant genotype, root exudates, seasonal and diurnal variation, soil type, soil moisture, temperature, levels of combined nitrogen, and organic carbon in the soil. Some of these factors have been discussed under various assay techniques and only those not discussed earlier are described below.

Root Exudates

Qualitative differences in the soluble exudates of seedlings of different sorghum genotypes were demonstrated by variation in growth and nitrogenase activity of a given bacterial culture in semisolid synthetic media containing root exudates as the sole carbon source. Only the azospirilla cultures grew well; the other organisms tested grew poorly, with little nitrogenase activity. No correlation was observed between the amount of exudate and root and/or shoot growth of seedlings grown in axenic liquid culture, and the ranking of the cultivars inoculated with *A. lipoferum* for nitrogenase activity did not correlate with the amounts of soluble exudate measured. Similarly, this ranking also differed from that for growth and activity of *A. lipoferum* in synthetic media (ICRISAT 1984).

Combined Nitrogen

The presence of combined N affects the enzyme nitrogenase. With sorghum plants grown in tubes filled with washed sand, nitrogenase activity was drastically reduced when the plants were fed with above 15 ppm N in solution (ICRISAT 1984). Nitrogenase activity of sorghum plants in the field was stimulated due to addition of 20 kg N ha^{-1} over the no-N addition treatment, but 40 kg N ha^{-1} reduced the activity. With millet BJ 104 plants grown in pots filled with Alfisol, mean nitrogenase activity throughout the growth period was significantly higher when the plants were watered with 10 and 25 ppm N in solution daily, than with the plants which received no nitrogen and also at 100 ppm N solution daily. Application of a basal dose of 20, 40, and 80 kg N ha^{-1} equivalent did not affect the activity significantly.

Nitrogen-fixing Bacteria

The marked differences in nitrogenase activity of sorghum and millet between fields may be partly due

to differences in their microbial populations, suggesting that it may be possible to obtain responses to inoculation with bacteria. We compared different methods of estimating bacterial populations and isolating N_2-fixing bacteria from root or soil samples. The N-free media that contained sucrose and malate as the carbon source were used to compare three methods of isolating and quantifying the bacteria. Counts of presumptive nitrogen fixers (4.5×10^8 in sucrose and 2.5×10^8 in malate media g^{-1} of root) were higher under the dilution plate count method than counts estimated by most probable number (MPN) method in semisolid media (5×10^5 in sucrose and 5×10^4 g^{-1} root in malate medium), and higher than axenic plant tubes (5×10^5 g^{-1} root). Recovery of nitrogenase-positive bacterial isolates was 43% higher with sucrose and 30% higher with malate by the MPN method. Recovery of nitrogenase-positive bacterial isolates from MPN with axenic plants was 37% using malate medium (Table 5; ICRISAT 1984).

In the plant enrichment culture technique for isolating N_2 fixers, sterile seedlings of sorghum or millet are grown on a carbon and N-free medium in test tubes, and inoculated with a dilution series of the soil or culture under test. Most probable number estimates of N_2-fixing bacteria are made from the ARA of these tubes, and from direct plating of the highest positive dilutions. A second or third selection from this population can be made by again going through the process of dilution and reinoculation of sterile seedlings. With this technique, with the increasing enrichment during each generation, the number of colony types decreased and the proportion of nitrogenase-positive bacteria increased.

Table 5. Estimation and isolation of nitrogen-fixing bacteria from root samples by different methods.[1]

Method	Total counts	Number of isolates	
		Total	Active
Dilution and plating			
Sucrose medium	4.5×10^8	10	0
Malate medium	2.5×10^8	10	0
MPN in semisolid medium			
Sucrose	5×10^5	7	3
Malate	5×10^4	10	3
MPN with plants	5×10^5		
Isolations on			
Sucrose medium		4	0
		5	0
Malate medium		8	3
		6	1

1. Roots of active Ex-Bornu plants were macerated and serial dilutions were used for plating or inoculating semisolid media in bottles containing 6 mL medium or Ex-Bornu plants grown on Fahraeu's medium. The plants were grown in 6 mL Fahraeu's medium in 25 × 200 mm plant tubes.

Large populations of bacteria capable of growing in air on N-free media exist in soil. Use of the acetylene-reduction assay indicated nitrogenase activity for about 60% of these presumptive N_2 fixers. The number of colony types and the population sizes depended on the selection of media and varied with the carbon source. About four times as many bacteria grew on a sucrose-based medium than on a malate-based medium. Adding a small amount of yeast extract (100 mg L^{-1}) doubled their number on both the media. The number in the top 40 cm of soil was about 10 times greater than that in the 40-to-60 cm zone. There were over a million Enterobacteriaceae g^{-1} soil, several species of which are known to fix nitrogen anaerobically.

Stimulation of presumptive, aerobic N_2 fixers occurs in the rhizosphere of several plants. The number of easily recognizable types of bacteria from the root surface that grew on N-free media was 10 times greater than the population growing in the soil away from the roots. A selection for particular types of bacteria also occurred in the root zone, resulting in less than half the types found in the bulk soil. Some bacteria were found very closely bound to the root and perhaps even in the root tissues. After shaking the root with glass beads to remove surface-attached bacteria and then thoroughly sterilizing the root surface with 1% chloromine T for 1 h, we recovered more than 400 000 bacteria g^{-1} of fresh root from the root macerate (Dart and Wani 1982).

A survey of 200 sites in the traditional pearl millet growing areas in northwestern India indicated that total population of organisms capable of growing on a N-free sucrose medium supplemented with 50 mg L^{-1} yeast extract (YE), ranged from 10^7 to 10^8 g^{-1} soil (Dart and Wani 1982). Nitrogenase activity was detected in 42% of the 3760 isolates made from the highest-dilution plates. Every soil contained organisms which produced pellicles and reduced C_2H_2 on a malate medium, with a MPN of N_2 fixers varying from 10^3 to 10^5 g^{-1} soil. Some of these soils did not contain *Azospirillum*, and Enterobacteriaceae and *Pseudomonas* were commonly isolated from the malate enrichment cultures. The isolates from the sucrose-based medium could be classified into at

least seven different genera of N_2-fixing bacteria, including types which are still to be identified. *Enterobacter cloacae* was the most-common isolate. Some *Pseudomonas* types were also nitrogenase positive. There were at least 10^6 actimonycete-like organisms g^{-1} soil in every sample and of the 229 isolates, 70 had nitrogenase activity on sucrose + YE medium but on subsequent purification they lost the activity. The isolates obtained from this study could be classified on colony morphology into at least 22 different groups. However, they represent only the most-numerous organisms able to grow on two media (Dart and Wani 1982, ICRISAT 1982).

Some of these cultures lose activity during purification and subculturing. In laboratory studies a synergistic effect on nitrogenase activity was found when two low-fixing pure cultures were mixed together. Cultures of *Erwinia herbicola* and *Enterobacter cloacae* each grew on N-free media, but were inactive when assayed for C_2H_2 reduction activity. When both cultures were grown together, a high activity was measured by C_2H_2 reduction (101 nmoles C_2H_4 bijou bottle^{-1} h^{-1}) (ICRISAT 1982). Similarly, *Azotobacter chroococcum* in pure culture alone had nitrogenase activity of 50 nmoles C_2H_4 bijou^{-1} h^{-1}, but when it was grown with *Erwinia herbicola* the activity increased to 121 nmoles C_2H_4 bijou^{-1} h^{-1} (ICRISAT 1982).

Responses to Inoculation

In pot-culture studies, the response of sorghum and millet to inoculation with N_2-fixing bacteria varied with the growth medium and amount of N fertilizer added. The responses of sorghum grown in pots in nonsterile Alfisol, to inoculation with *Azospirillum lipoferum* and NBRE, a mixed culture, were a grain yield increase of 22% and total dry matter increase of 29%. The yield increase occurred even when the equivalent of 40 kg N ha^{-1} was added (ICRISAT 1982). Nitrogen-balance studies indicated a positive N balance over the control due to inoculations (Table 3). In a field trial on an Alfisol with the three sorghum hybrids CSH 1, CSH 5, and CSH 9 inoculated with liquid peat culture suspension of N_2-fixing bacteria, we observed increased dry-matter production ($P<0.1$) of all three hybrids inoculated with *A. lipoferum* (ICM 1001) and a NBRE culture (ICRISAT 1983).

Another field trial with three sorghum cultivars CSH 5, CSH 9, and SPV 351 and 10 inoculation treatments was conducted during the 1984 rainy season. Inoculation of sorghum cultivars with nitrogen-fixing bacteria resulted in 2-10% increase in grain yields across the cultivars over the noninoculated control. However the increases were not statistically significant. A similar trend was observed for total dry-matter production (ICRISAT 1985).

During 1982-1984, nine field trials were conducted at ICRISAT Center and different locations in India to study the response of millet cultivars to inoculation with nitrogen-fixing bacteria. In all the trials inoculation increased the grain and total dry-matter yields of millet. However significant yield increases due to inoculation were observed in five trials (Table 6). The summary table indicates that up to 30% increase in mean yield over the control was observed in the test cultivars due to inoculation with N_2-fixing bacteria.

In all the experiments, plant dry-matter yields also followed trends similar to that of grain yield. Inoculations increased plant N uptake in all the experiments except where *A. brasilense* (SP 7) was used. In a few cases grain N content was increased due to inoculations. In all the trials, trends for host cultivar and bacterial strain interactions for grain and plant dry-matter yields were observed; however, significant interaction for grain and plant dry-matter yields was observed in one trial each only.

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Table 6. A summary of pearl millet inoculation experiments conducted at different locations during 1982-84.

Location and season	Soil type	Cultivars	Inoculation treatment	Percentage increase[1]	Remarks
ICRISAT Center Summer 1982	Alfisol	2. IP 2787 ICMS 7819	4. *A.lipoferum* (1) *A.brasilense* (SP 7) NBRE	6.05 -6.5	For plant dry matter significant interaction ($P = 0.05$) between cultivar and culture was noted.
ICRISAT Center Rainy 1982	Alfisol	3. IP 2787 ICMS 7703 WC C 75	6. *A.lipoferum* (1) *A.lipoferum* (ICM 1001) NBRE *A.chroococcum* (ICM 2001) *A.brasilense* (SP 7)	14.5 21.0[2] 6.0 14.0 -11.8	For grain Nb content, significant interaction between cultivars and cultures was observed.
ICRISAT Center Summer 1983	Alfisol	3. BJ 104 MBH 110 MEBH 23/81	5. *A.lipoferum* (1) *A.lipoferum* (ICM 1001) *A.chroococcum* (ICM 2001) NBRE	5.0 6.0 6.0 12.4	
ICRISAT Center Rainy 1983	Alfisol	3. BJ 104 MBH 110 MEBH 23/81	5. *A.lipoferum* (1) *A.lipoferum* (ICM 1001) *A.chroococcum* (ICM 2001) NBRE	- 5.5 - -	
ICRISAT Center Rainy 1984	Alfisol	4. BJ 104 BK 560 WC C 75 ICMS 7703	7. *A.lipoferum* (ICM 1001) *A.brasilense* (1) *A.brasilense* (2) Ab1 + Ab2 *A.chroococcum* (ICM 2001) NBRE	10.7[2] 0.7 7.6 4.8 11.6[2] -	Significant interaction for grain yield between cultivars and inoculants was noted.
Vaijapur[3] Rainy 1984	Mixed Red and black	4. BJ 104 BK 560 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (2) *A.chroococcum* (ICM 2001) NBRE	25.6[2] 6.1 6.1 17.4	
Bhavanisagar Rainy 1984	Alfisol	4. BJ 104 BK 560 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (SL 33) *A.chroococcum* (ICM 2001) NBRE	18.7[2] 16.5[2] 6.1 -	
Parbhani[4] Rainy 1984	Vertisol	4. BJ 104 MP 21 WC C 75 ICMS 7703	7. *A.lipoferum* (ICM 1001) *A.brasilense* (1) *A.brasilense* (2) Ab1 + Ab2 *A.chroococcum* NBRE	24.5[2] 19.3[2] 29.5[2] 10.5 30.6[2] 27.8[2]	
Rahuri[5] Rainy 1984	Vertisol	4. BK 560 RHR 1 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (SL 33) *A.chroococcum* (ICM 2001) NBRE	9.4 7.1 13.9 6.7	

1. Increase in mean grain yield across the cultivars.
2. Significantly ($P = 0.05$) higher over the control.
3. In cooperation with G.S. Jadhav, Marathwada Agricultural University, Parbhani.
4. In cooperation with N.S. Subba Rao and K.V.B.R. Tilak, IARI, New Delhi.
5. In cooperation with S.D. Ugale, Mahatma Phule Agricultural University, Rahuri.

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Discussion

P.Tauro:
Considering that millets are grown with minimum agronomic practices, what inoculation technique would you recommend?

S.P.Wani:
Seed inoculation is the best, but if we find that slurry inoculation is superior we will have to opt for it. At present we are comparing various methods of inoculation.

P.Tauro:
Is anything known about the N-scavenging ability of different grasses?

S.P.Wani:
No. But with thorough soil sampling we should be in a position to find out the status of N source in the soil.

P.Tauro:
Could N recovery up to 2000 kg/5 yr be more due to scavenging than due to BNF?

S.P.Wani:
No. As the initial soil sampling and sampling after 5 years have not shown N differences to this extent, we can infer that soil N was not the source. Removal of 2000 kg N from the soil will be easily detected by Kjeldahl analysis.

P.Tauro:
Have you noticed any negative response to inoculation?

S.P.Wani:
Yes, in the case of inoculation with *Azospirillum brasilense*.

B.K. Konde:
What added advantages did you observe from slurry inoculum over seed inoculation?

S.P.Wani:
By using slurry inoculation (peat culture suspended in water), more bacterial inoculum can be added in the field as compared to seed inoculation. Another advantage is that in drought-prone areas, this additional moisture helps in the establishment of the plants in the early stage. Such an effect has been observed at Vaijapur where there were no rains at all after sowing, and on the entire station only the inoculation trial where liquid suspension was applied has come up well, whereas all other trials sown in the normal way resulted in patchy plant establishment.

B.K.Konde:
When do you inoculate the plants?

S.P.Wani:
Generally at the time of sowing if the trial is hand sown, otherwise soon after emergence, i.e., 5-6 days after sowing, if the trial is tractor sown.

G. Oblisami:
What is the cost of liquid inoculation of *Azospirillum* to sorghum when compared to seed inoculation?

S.P.Wani:
We have not worked out the exact costs of slurry inoculation, but if sowing is done by a bullock-drawn implement, the cost will be about Rs. 30-40. A simple, inexpensive device has been developed by our Farm Power and Equipment scientists for this purpose in groundnut, and it can be used for other crops also.

O.P. Rupela:
In continuation of Dr. Tauro's questions, was the negative effect statistically significant?

S.P.Wani:
The negative effects with *A. brasilense* (SP 7) inoculation were consistent in two trials where 4 cultivars were tried, and in one trial the reduction was statistically significant.

G.S. Murthy:
When there is no increase in the grain yield but there is an increase in total biomass yield, perhaps other nutrients such as phosphorus and zinc are becoming limiting factors.

S.P.Wani:
I don't think so, as generally basal dressings with P_2O_5 are done in all the trials.

Joseph Thomas:
I am impressed by the painstaking experiments with improved field techniques. The quantity of nitrogen recovered by Napier grass works out to 465 kg ha^{-1}. Do you have any ^{15}N data to substantiate this?

S.P.Wani:
No.

S.V. Hegde:
Did you have an unplanted soil system for N-balance study? What was the N gain due to planting per se?

S.P.Wani:
Yes, we do maintain an unplanted control in all N-balance trials. The N gain due to planting alone was 113 mg N pot^{-1} sown with sorghum, and 27 mg N pot^{-1} sown with millet. These gains are across the 3 N levels used.

S.V.Hegde:
Is it appropriate scientifically to use Napier bajra root extract (NBRE), which is a mixture of several microorganisms and which could also change every time the culture is obtained from bajra root? Instead can we not isolate the organism or organisms responsible in pure culture and use them?

S.P.Wani:
I agree with you that NBRE is a complex culture and there will be problems in producing and maintaining the quality of such a complex inoculum. I don't think NBRE can be recommended for general use. We started using NBRE as a test mixture and as it gave positive responses we continued with it. It cannot be used unless known bacteria from NBRE mixed together perform equally well. We are studying the composition of NBRE in terms of microbial components and there are at least 14-15 different bacteria involved which should be equally good in performance. This will take more time and I am not sure whether in the near future it will be possible or not. Coming to the question of maintaining the same composition, we always use the starting material for preparing NBRE from our long-term N-balance trial and till now we have observed positive responses with NBRE prepared at different times.