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# Short-term measurements of uptake of nitrogen fixed in the rhizospheres of sorghum (*Sorghum bicolor*) and millet (*Pennisetum americanum*)*

K.E. Giller**, S.P. Wani, J.M. Day, and P.J. Dart***

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, UK and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P.O., Andhra Pradesh 502324, India

**Summary.** In a series of short-term experiments root systems of young sorghum and millet plants inoculated with $N_2$-fixing bacteria were exposed to $^{15}N_2$-enriched atmospheres for 72 h. The plants were grown in a normal atmosphere for up to 22 days after the end of the exposure to allow them to take up the fixed $N_2$. Environmental conditions and genotypes of sorghum and millet were selected to maximise $N_2$-fixation in the rhizosphere. Detectable amounts of fixed N (>16 μg/plant) were rapidly incorporated into sorghum plants grown in a sand/farmyard manure medium, but measurable fixation was found on only one occasion in plants grown in soil. $N_2$ fixation was detectable in some experiments with soil-grown millet plants but the amounts were small (2–4 μg/plant) and represented less than 1% of plant N accumulated over the same period. In many cases there was no detectable $^{15}N_2$ incorporation despite measurable increases in ethylene concentration found during an acetylene reduction assay.

**Key words:** $^{15}N$ – $N_2$ fixation – Rhizosphere – *Sorghum bicolor* – *Pennisetum americanum* – Acetylene reduction assay (ARA)

The potential for increased production of cereals and grasses by means of $N_2$ fixed by bacteria associated with their roots has stimulated much research interest (reviewed by van Berkum 1984; Giller and Day 1985; Dart 1986). Enhancement of $N_2$ fixation in the rhizosphere may be possible by inoculation with large populations of $N_2$-fixing bacteria, or by selection of plant genotypes which support greater amounts of bacterial $N_2$ fixation. In crops that are generally grown on soils with a small N content, such as sorghum [*Sorghum bicolor* (L.) Moench] and millet (*Pennisetum americanum* L.), the evolution of such bacterial-root associations may have been favoured; differences have been found between genotypes of these species in root-associated acetylene reduction activity (Bouton and Brooks 1982; Wani et al. 1984). Measurements of acetylene reduction activity, however, may not represent in situ $N_2$ fixation, for a number of reasons (Witty 1979; van Berkum and Bohlool 1980; Giller 1987).

Direct measurement of $N_2$ fixation is possible using $^{15}N$-labelled $N_2$ gas. Incorporation of fixed N into a variety of crops and tropical grasses has been demonstrated using this method (Ruschel et al. 1975; De Polli et al. 1977; Eskew et al. 1981; Giller et al. 1984). Here we report a series of experiments carried out with young sorghum and millet plants in which we used $^{15}N_2$ to examine the rates of $N_2$ fixation and the possible differences in root-associated $N_2$ fixation between genotypes of these crops.

## Materials and methods

*Experimental conditions.* The plants were grown from seed in incubation chambers in a greenhouse at the ICRISAT Center, Patancheru, India, which allowed the intact root/soil system to be enclosed. The chambers were attached to a closed circulating system and gas mixtures containing $^{15}N_2$ introduced. Incubations were carried out for 72 h in all experiments. At regular intervals $O_2$ was replenished by injection into the loop and gas mixtures were circulated over soda-lime to remove accumulating $CO_2$. The methods are described in detail elsewhere (Giller et al. 1984).

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** *Present address and address for offprint requests:* Department of Biochemistry and Biological Sciences, Wye College, University of London, Wye, Ashford, Kent TN25 5AH

*** *Present address:* Department of Agriculture, University of Queensland, St. Lucia, QLD 4067, Australia

Gas mixtures enriched with $^{15}N_2$ were made by mixing commercially prepared $^{15}N_2$ gas (99 atom%$^{15}N$) or recaptured $^{15}N_2$ gas mixtures from previous experiments with 20% $O_2$. In later experiments 20% argon was substituted for $N_2$ gas in the mixtures to reduce the amount of $^{15}N_2$ required. All gas mixtures were stored over acidified water to remove any contaminating $^{15}N$-labelled ammonia. Air in the circulating loop was removed by purging with $CO_2$ and the $^{15}N_2$ gas mixture allowed to flow into the loop as the $CO_2$ was removed by passing over soda-lime. The initial enrichment of gas mixtures after introduction was generally less than 70 atom% $^{15}N$ due to some residual $^{14}N_2$ in the circulating system and chambers. Gas samples were taken from the incubation chambers immediately after introduction of $^{15}N_2$ and at 24-h intervals, and analysed for $^{15}N$. $^{15}N$ enrichment invariably decreased by approximately 30% over the 3-day incubation period, probably due to gaseous diffusion through the leaf sheaths of the shoots as leakage from the gas circulation system was rarely detected. The amount of $N_2$ fixed was calculated by using the mean gas concentration over the incubation period. The plants were allowed to grow for up to 22 days after the start of the exposure to allow uptake of fixed N before harvest.

The amounts of fixed N incorporated in the plants were calculated as

$$N_2 \text{ fixed} = \text{N in plant parts} \times \frac{\text{R plant part}}{\text{R gas phase}} (\mu g),$$

where R = atom% $^{15}N$ excess, and similarly, the detection limits for incorporation of fixed N were calculated as

$$\text{Detection limit} = \text{N in plant part} \times \frac{0.005}{\text{R gas phase}} (\mu g)$$

using a plant enrichment of 0.005 atom% $^{15}N$ excess as a lower limit of detection due to variation in the natural abundance of $^{15}N$ and analytical precision. Treatments and specific conditions of each experiment are detailed in Tables 1 and 2. Growth media were either a mixture of sand and farmyard manure (97.5:2.5 w/w) or an Alfisol soil (total Kjeldahl N = 0.07%) collected from the surface 0–15 cm of fields at the ICRISAT Center, Patancheru. Both media were used at a rate of 350 g per incubation chamber. Inoculum was pipetted over the seeds at the time of sowing as three different treatments: (1) 5 ml/incubation chamber of a soil suspension of air-dried soil taken from the rhizosphere of field-grown sorghum plants; (2) 5 ml napier bajra root extract (Wani et al. 1984) containing approximately $2\times10^6$ $N_2$-fixing bacteria/ml, counted by the dilution and plate-count technique using N-free sucrose or malate medium, or (3) 3 ml *Azospirillum lipoferum* (ICM 1001) containing $2\times10^7$ cells/ml. Details of genotypes of sorghum and millet used in the experiments are given in Table 1. In total, five $^{15}N_2$ exposures were performed on sorghum genotypes and four on millet genotypes. The plants were watered once a week with an N-free nutrient solution (Broughton and Dilworth 1975) and at other times with deionised

**Table 1** Details of millet (*Pennisetum americanum*) and sorghum (*Sorghum bicolor*) genotypes used in the experiment

| Genotype | Origin | Characteristics in field at ICRISAT | | |
|---|---|---|---|---|
| | | Average days to 50% flowering | Average plant height (cm) | Average days to maturity |
| Millet | | | | |
| BJ104 | Commercial hybrid released in India | 44–46 | 156 | 75–80 |
| ICH107 | ICRISAT test hybrid | 54–55 | 216 | 83–85 |
| Ex Bornu | Population derived from Nigerian land races | 55–58 | 240–250 | 90–95 |
| Sorghum | | | | |
| CSH 5 | Commercial hybrid released in India | 60–70 | 200 | 110–115 |
| IS 3003 | Ethiopian land race | 140 | 390 | 170–180 |

**Table 2.** Short-term measurements of uptake of fixed N in sorghum plants by exposure of soil/root systems to $^{15}N_2$-enriched atmospheres for 72 h

| Exp number | Genotype | Plant age at (days) | | Growth medium | Inoculum[a] | Mean gas enrichment (atom% $^{15}N$ excess) | Detection limit (μg $N_2$ fixed) | Fixed $N_2$ incorporated (μg/plant)[b] |
|---|---|---|---|---|---|---|---|---|
| | | Exposure | Harvest | | | | | |
| 1a | CSH 5 | 21 | 24 | Sand/FYM[c] | Soil susp | 40.3 | 0.5 | 16 |
| | | 21 | 33 | Sand/FYM | Soil susp | 40.3 | 0.7 | 27 |
| b | CSH 5 | 25 | 41 | Sand/FYM | Soil susp | 16.5 | 1.8 | 46 |
| | | 25 | 47 | Sand/FYM | Soil susp | 16.5 | 2.6 | 31 |
| 2 | CSH 5 | 12 | 26 | Alfisol | Soil susp | 10.7 | 0.9 | 8 |
| | IS 3003 | 12 | 26 | Alfisol | +NBRE | 10.7 | 0.7 | 70 |
| 3a | CSH 5 | 20 | 37 | Alfisol | NBRE | 44.5 | 1.3 | ND |
| | IS 3003 | 20 | 37 | Alfisol | +ICM 1001 | 44.5 | 1.0 | ND |
| b | CSH 5 | 27 | 44 | Alfisol | NBRE | 53.3 | 1.1 | ND |
| | IS 3003 | 27 | 44 | Alfisol | +ICM 1001 | 53.3 | 1.2 | ND |

[a] Soil susp., soil suspension, NBRE, napier bajra root extract (Wani et al. 1984), ICM 1001, *Azospirillum lipoferum* strain ICM 1001

[b] Each figure is mean of five replicate analyses except for the 33-day harvest in exp. 1a where the mean is of four replicates; ND, not detectable

[c] FYM, farmyard manure

water In experiments 2 and 6, 5 ml nutrient solution containing 5 mg N/l (i.e. 25 μg N) was added to each incubation chamber before the incubations were carried out in order to alleviate symptoms of N deficiency in the plants Acetylene reduction activity was measured over a 6-h incubation period on similar plants not exposed to $^{15}N_2$, or at the time of harvest on the plants exposed to $^{15}N_2$ by sealing the chambers and introducing 10% acetylene (Hardy et al 1973)

*Analytical methods* Soil samples were finely ground, sieved (2 mm mesh) and digested by a Kjeldahl procedure (Rothamsted Experimental Station 1978) The N content of the digests was estimated by semi-micro Kjeldahl distillation and titration against a standardised acid, and the samples were dried for $^{15}N$ analysis by a Micromass 602 mass spectrometer. Shoots and roots (plus adhering soil) were analysed separately The plant samples were finely ground and the N content was determined by Kjeldahl digestion and estimation by an automated indophenol blue method (Rothamsted Experimental Station 1978) Ammonium was concentrated by a microdiffusion technique (Conway 1939) and $^{15}N$ enrichment was measured with a Micromass 622 mass spectrometer (VG Isogas, Northwich, Cheshire, U K)

## Results and discussion

### *Incorporation of fixed N in sorghum*

Acetylene reduction activity associated with sorghum roots in growth media amended with farmyard manure is often greater than in soil (Wani et al. 1984). When sorghum plants were grown in a sand/farmyard manure mixture, $N_2$ fixation and its rapid incorporation were easily demonstrated (Table 2; Exp. 1). In a $^{15}N_2$-gas exposure experiment conducted on plants grown in soil, substantial $^{15}N$ enrichment (>0.063 atom% $^{15}N$ excess) was found in all plants of genotype IS 3003, but only small enrichment (>0.010 atom% excess) was found in the plants of the other genotype (CSH 5) examined in the same assay (Table 2; Exp. 2). Genotype IS 3003 generally has low rates of root-associated acetylene reduction activity, and in this experiment, the young plants showed low ethylene production in both genotypes at the time of assay (IS 3003, 23.2 ± 20.4 nm/plant per h; CSH 5, 28.4 ± 3.4 nm/plant per h; $n = 5$). Soils collected from this experiment at harvest were analysed, but enrichment was detected in only one of the soils in which IS 3003 was assayed [0.0046 atom% $^{15}N$ excess; 0.0014 above the natural abundance of 0.0032 (0.00015, $n = 8$) in soil not exposed to $^{15}N_2$] and in one soil in which CSH 5 was grown (0.0006 atom% $^{15}N$ above the value determined for natural abundance on replicate unplanted tubes).

Table 3. Ethylene production (nmol/plant per h) in an acetylene reduction assay of intact soil-root systems of young sorghum plants used in $^{15}N_2$-gas exposure experiment (Exp 3)

| Days after sowing | 21 | 24[a] | 28 | 31 | 38 |
|---|---|---|---|---|---|
| IS 3003 | ND[b] | 38 | 68 | 68 | 9 |
| CSH 5 | ND[b] | 65 | 220 | 94 | 29 |
| SE | – | 5.3 | 74.6 | 19.7 | 8.1 |
| Unplanted tubes | ND[b] | ND[b] | 52 | 97 | 26 |
| SE | – | – | 47.2 | 78.1 | 17.6 |

[a] Statistical analysis indicated significant differences ($P<0.05$) between the genotypes at the 24-day harvest
[b] ND, not detectable

This experiment was repeated in order to examine the differences between genotypes IS 3003 and CSH 5. Both genotypes accumulated dry matter over the course of the second experiment, but took up only small amounts of N (Fig. 1). The N content of the leaves decreased from 2.3% to 0.8% between the 20- and 43-day harvests, indicating that the N available for plant uptake and growth was limited. Easily measurable rates of ethylene accumulation were found at all except the 21-day harvest (Table 3) but despite this, no incorporation of $^{15}N$ was detected in any of the plants examined in two $^{15}N_2$ exposures (Table 2; Exp. 3).

In order to measure the benefits of $N_2$ fixation in more mature plants larger incubation chambers were constructed (3 kg soil) and a plant of genotype CSH 5 was exposed to $^{15}N_2$ after 43 days' growth. A solution containing nitrate was added to these plants on two occasions (total of <10 mg N) in order to alleviate severe N limitation. Despite the good growth and N accumulation (160 mg total N accumulation) in the exposed plant no $^{15}N_2$ incorporation was detected (detection limit 2 μg N incorporated in the plant) over the 7-day growth period after incubation under $^{15}N_2$, nor was enrichment detected in the soil.

### *Incorporation of fixed N in millet*

Small amounts of $^{15}N_2$ incorporation were found in one incubation experiment where Ex-Bornu plants were grown in soil (Table 4). Analysis of the soils in which BJ 104 plants were grown and exposed to $^{15}N_2$ (Table 4; Exp. 6) did not demonstrate any $N_2$ fixation. A further experiment was carried out to examine two genotypes (BJ 104 and ICH 107) grown in an Alfisol soil and inoculated with *Azospirillum lipoferum*. Incorporation of $^{15}N$ was found in all plants examined but the enrichments were again small, and no significant differences were found between the genotypes (Table 5). The initial N content of BJ 104 was 2.2 mg/plant and that of ICH 107 was 3.4 mg/plant after 21 days. The final N content of ICH 107 was 5.12 mg/plant, representing an N uptake of 1.8 mg/plant over 9 days or 200 μg N/plant per day. The subsequent uptake of N fixed during the 3-day incubation period amounted to 2.8 μg/plant in ICH 107 or

0.93 µg/plant per day of exposure, a small amount compared to the N being accumulated. Similar calculations for genotype BJ 104 indicated a fixed-$N_2$ uptake of 0.76 µg/plant per day compared with an N accumulation of 320 µg/plant per day over the same period.

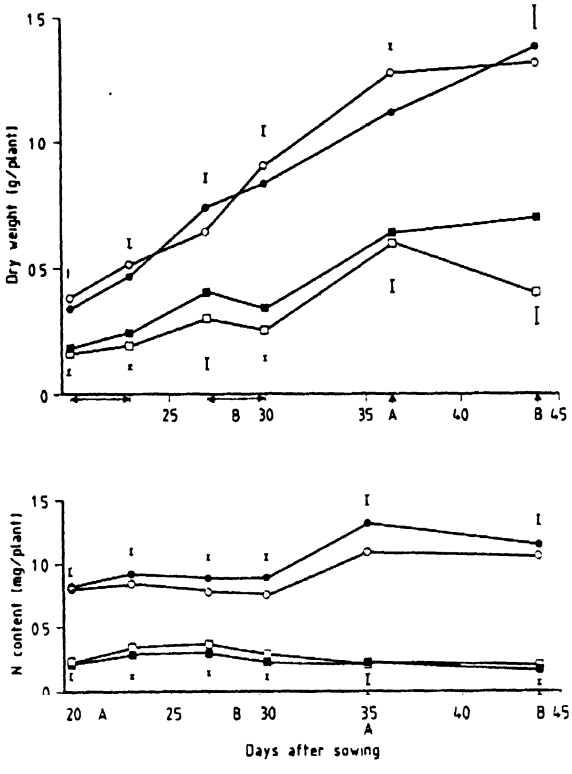


Fig. 1. Dry-matter and N uptake in shoots (*circles*) and roots (*squares*) of sorghum genotypes IS 3003 (*closed symbols*) and CSH 5 (*open symbols*). Exposure periods to $^{15}N_2$ are indicated (*A, B*) as are relevant harvest dates of plants exposed. *Vertical bars* represent standard errors of the means

### *Amounts of $N_2$ fixation associated with young plants of sorghum and millet*

The experimental conditions were manipulated to ensure an optimal environment for $N_2$ fixation; low soil N, high temperatures (30 °C) and a high soil-moisture content (70% water-holding capacity of the medium) as well as a large bacterial inoculum and suitable plant genotypes shown to support substantial acetylene reduction activity (Wani et al. 1984). Despite this, only small amounts of $N_2$ fixation were found for both sorghum and millet. Indeed, in some cases $N_2$ fixation was not detectable even where measurable amounts of ethylene production were found in an acetylene reduction assay.

Where small amounts of $N_2$ fixation were demonstrated for sorghum grown in a sand/farmyard manure medium, rapid plant uptake of fixed N was detected immediately after the 3-day exposure (Table 2). It is likely that bacterial $N_2$ fixation in this medium was favoured by the presence of a large amount of organic matter. Small amounts of incorporated fixed $^{15}N$ were also found immediately after a 24-h incubation of grass-soil cores in Texas (Morris et al. 1985) although fixed-N uptake by rice was slow (Ito et al. 1980; Eskew et al. 1981). Our analyses did not indicate significant enrichment of soil N in the incubation chambers where no $^{15}N$-incorporation was found in the plants, suggesting that the absence of detectable $N_2$-fixation was not due to slow release of fixed N from bacteria.

Incorporation of much larger amounts of fixed N have been demonstrated in sugar cane (Ruschel et al. 1975) and in the tropical grasses *Paspalum notatum* and *Digitaria decumbens* (De Polli et al. 1977) using $^{15}N_2$ incubation experiments. Okon et al. (1983) also

Table 4. Short-term measurements of uptake of fixed N in millet plants by exposure of soil-root systems to $^{15}N_2$-enriched atmospheres for 72 h

| Exp no. | Genotype | Plant age at (days) | | Growth medium | Inoculum[a] | Mean gas enrichment (atom % $^{15}N$ excess) | Detection limit (µg $N_2$ fixed) | Fixed $N_2$ incorporated (µg/plant)[b] |
|---|---|---|---|---|---|---|---|---|
| | | Exposure | Harvest | | | | | |
| 4 | Ex-Bornu | 24 | 27 | Sand/FYM | Soil susp. | 28.4 | 0.4 | ND |
| | Ex-Bornu | 24 | 34 | Sand/FYM | Soil susp. | 28.4 | 0.5 | ND |
| | Ex-Bornu | 24 | 40 | Sand/FYM | Soil susp. | 28.4 | 0.7 | ND |
| 5 | Ex-Bornu | 29 | 32 | Alfisol | Soil susp. | 26.7 | 0.2 | 2.4 |
| | | 29 | 37 | Alfisol | +NBRE | 26.7 | 0.5 | 3.5 |
| 6 | BJ 104 | 16 | 29 | Alfisol | Soil susp. +NBRE | 37.5 | 0 4 | ND |
| 7 | BJ 104 | 21 | 30 | Alfisol | Soil susp. | 30.3 | 0.8 | 2.7 |
| | ICH 107 | 21 | 30 | Alfisol | +NBRE | 30.3 | 1.3 | 2.8 |

[a] Soil susp., soil suspension; NBRE, napier bajra root extract (Wani et al. 1984)
[b] No. of replicates at each harvest: Exp. 4 = 3, Exp. 5 = 5, Exp. 6 = 10, Exp. 7 = 5. ND, not detectable

**Table 5.** Dry weight, N accumulation and uptake of fixed $N_2$ in young plants of two millet genotypes exposed to $^{15}N_2$ for 21 – 24 days and harvested after 30 days (Exp. 7)

| Genotype | Dry weight (mg/plant) | | N content (mg/plant) | | Atom % N excess | |
|---|---|---|---|---|---|---|
| | Shoot | Root | Shoot | Root | Shoot | Root |
| BJ 104 | 235 | 135 | 4.16 | 0.92 | 0.013 | 0.017 |
| ICH 107 | 234 | 108 | 4.35 | 0.82 | 0.013 | 0.016 |
| SE | 10.6 | 19.1 | 0.174 | 0.137 | 0.0037 | 0.0038 |

| | Fixed N incorporated (µg/plant) | | |
|---|---|---|---|
| | Shoot | Root | Total |
| BJ 104 | 1.77 | 0.51 | 2.27 |
| ICH 107 | 2.39 | 0.41 | 2.80 |
| SE | 0.559 | 0.053 | 0.494 |

demonstrated significant enrichment in *Setaria italica* inoculated with *Azospirillum brasilense*, although the plants largely assimilated only 5% of the fixed N as estimated by acetylene reduction assay. In the work reported here we did not find consistent or significant inputs of N from $N_2$-fixation in the rhizosphere to the growth of young sorghum and millet plants. Rates of acetylene reduction activity are often small for young sorghum and millet plants, increasing to maximal values around the time of flowering (Wani et al. 1984). Examination of the root systems of mature plants using $^{15}N_2$ exposures is difficult, not least because of the large volume of $^{15}N_2$ gas required. In mature plants the large amount of N reduces the sensitivity of detection of $N_2$ fixation due to dilution of the isotope.

Much of the evidence indicating a substantial uptake of N by cereal plants from $N_2$ fixation in the rhizosphere is indirect and subject to other interpretation (van Berkum and Sloger 1985; Giller and Day 1985). Isotope-dilution estimates of $N_2$ fixation with species of *Paspalum* (Boddey et al. 1983) and with sugar cane (Lima et al. 1987) indicate substantial gains from $N_2$ fixation in the rhizosphere. However, despite many claims, it has not yet been substantiated that increases in growth of cereal crops following inoculation with free-living $N_2$-fixing bacteria are due to enhanced fixation of $N_2$ in the rhizosphere (Giller and Day 1985).

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