Journal of Microbiological Methods 2 (1984) 307–316
Elsevier

JMM 00067

A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$

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(Received 25 October 1983) (Revised version received 15 March 1984) (Accepted 16 March 1984)

Summary

An apparatus is described in which the root systems of up to ten plants can be simultaneously incubated with $^{15}N_2$ gas mixtures, while the aerial environment remains undisturbed. The size of incubation chamber and the number of chambers can be adjusted depending on the size and growth stage of the plants being tested. The apparatus has the facility for the recovery of gas mixtures, sampling and adjustment of gas concentrations, and control of soil moisture status within a sealed system. Possible experimental applications are suggested and some preliminary results presented which demonstrate bacterial nitrogen fixation and uptake into *Sorghum* seedlings.

Key words: *Cereals – Grasses – Nitrogen-15 fixation – Nitrogen fixation (associative)* – Sorghum

Introduction

Uptake of fixed nitrogen into shoots of sugar cane, tropical grasses and rice has been demonstrated using $^{15}N_2$-enriched atmospheres [1–3]. Experimentally this necessitates enclosure of the soil-bacteria-root system within sealed growth chambers which should match the natural environment as closely as possible. Published methods have involved the enclosure of the whole plants and growth media in incubation chambers in which light, carbon dioxide levels and humidity are difficult to control. If these parameters are not controlled, rates of photosynthesis and evapotranspiration are likely to be altered and unusual plant growth can occur [3].

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Incubation chambers can be evacuated to remove the air before introduction of gas mixtures [1, 2, 4], but the pressure changes may lead to disturbance of the integrity of the rhizosphere and unrepresentative uptake of nitrogen by the plant. Witty and Day [5] proposed that the atmosphere be purged with a carbon dioxide/oxygen mixture, followed by absorption of the carbon dioxide with soda-lime (fused $Ca(OH)_2$ and NaOH). This enables the experimental atmosphere to be drawn into the rooting medium and avoids the need for evacuation.

In the apparatus described here only the growth medium and plant root system is enclosed in an incubation chamber and a carbon dioxide purging step can be used to introduce gas mixtures.

Materials and Methods

Apparatus

The apparatus consisted of two main parts: the incubation chambers and a gas supply manifold, and was made from commercial plumbing and compressed air piping, connectors and valves. Details of components and their suppliers used in the construction of the apparatus are given in Table 1. The chambers (Fig. 1) were sealed around the base of the plant stem using a cotton wool plug and silicone rubber sealant (No 790, Dow Corning, Australia Ptg. Ltd., NSW 2148) which was

TABLE 1

DETAILS OF PARTS AND SUPPLIERS USED IN THE CONSTRUCTION OF THE APPARATUS

Part	Part No.	Supplier
Incubation chambers		
1.25″ ABS class 'C' pipe	01 511 105	Wilford Plastics Ltd.,
1.25″–1 ABS plain reducing sockets	114 125	Cosgrove Way, Luton,
1.25″ plain end caps	140 105	Bedfordshire, England
0.5″ plain – 0.375″ BSP thread reducing sockets	111 121	
Gas supply manifold		
0.25″ O.D. nylon tubing	NYF8	Anglia Pneumatics, Luton, Beds, England
0.25″ O.D. copper tubing	B43003LS	Darenth Automation,
Two-way poppet flow switches		Ox Lane, Harpenden,
Needle valves (1/4-1/4″ O.D.)	WVA1/B/16	Herts., England
3/8″ BSP taper–1/4″ O.D. couplings	7063/B	
1/4″ O.D. elbow couplings	L20/B	Drallim Tube Couplings,
1/4″ O.D. equal tee couplings	L30/B	Bexhill-on-Sea, Sussex,
1/4″ O.D. straight couplings	L10/B	England
Steel bellows electric pump	MB-21	Metal Bellows Corp., Sharon, MA, U.S.A.

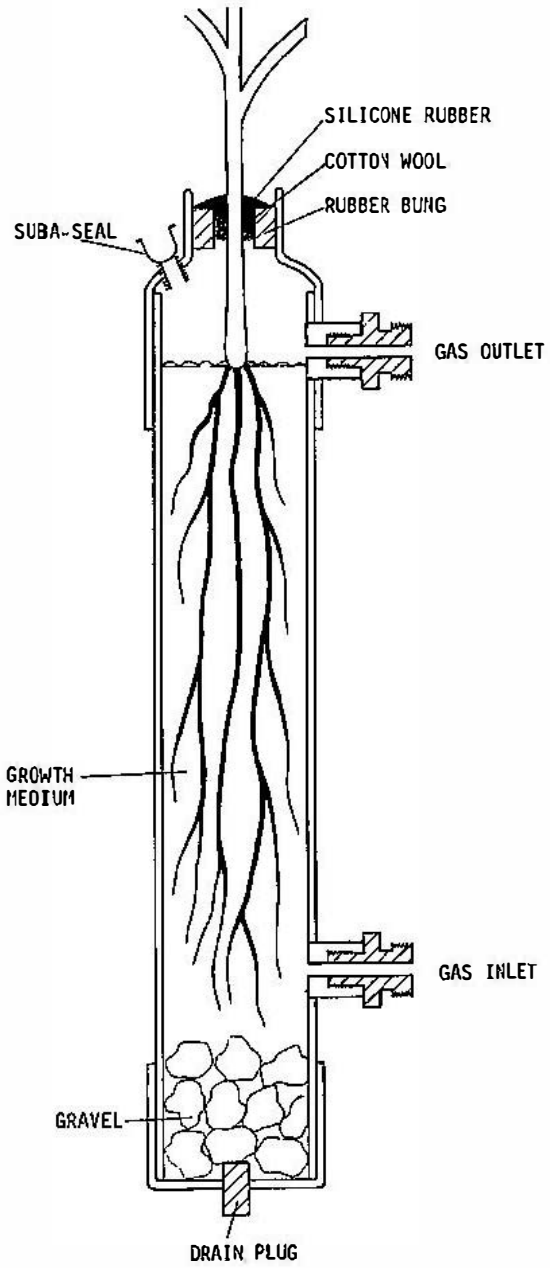

Fig. 1. Chamber for incubation of the intact roots system and growth medium in $^{15}N_2$ enriched gas mixtures.

found not to be toxic to seedlings. Each incubation chamber had a sampling port plugged with a suba-seal (Wm. Freeman and Co. Ltd., Suba-seal Works, Staincross, Barnsley, Yorkshire, England) through which gas samples were taken and water was added using a hypodermic syringe. The chambers were connected to the gas supply manifold with an inlet and outlet for gas mixtures.

The gas supply manifold (Fig. 2) had the capacity for connection of ten incubation chambers. There was a separate needle valve and flow gauge for each chamber to ensure even rates of gas flow for each plant incubated. Hypodermic needles (18 gauge) were used to connect the outlet pipe from the flow gauges to restrict and hence equalize the gas flow through the chambers. The restriction of the flow of gas before it reached the growth medium prevented pressure build up due to flow resistance of the medium. A mercury manometer was connected on the outlet line to allow pressure changes in the chambers to be monitored. Gas was circulated

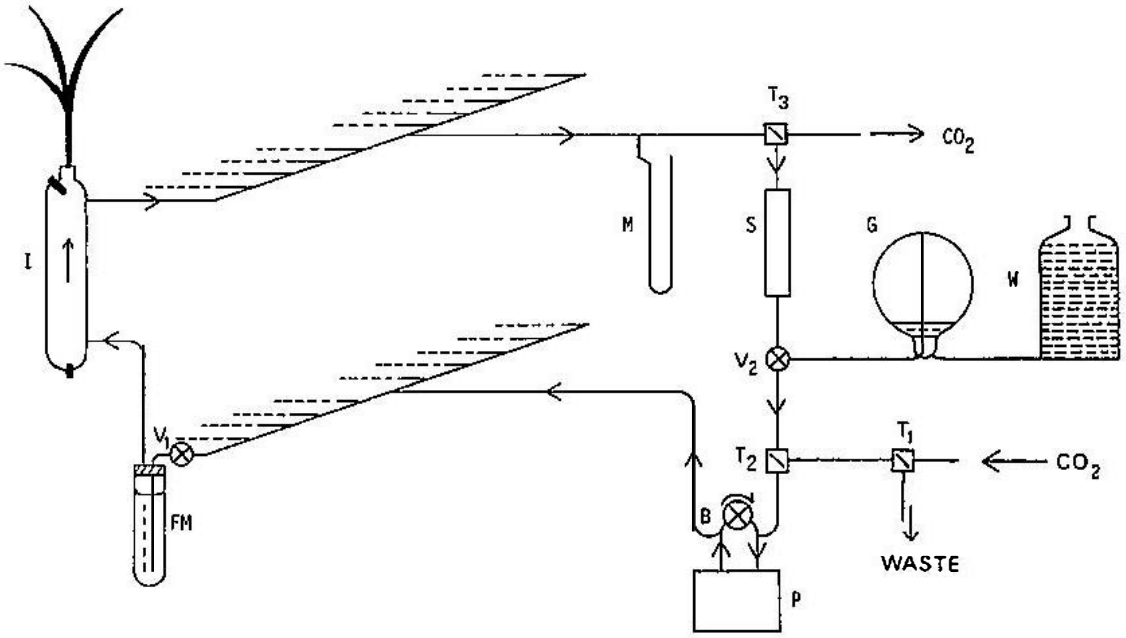

Fig. 2. The gas supply manifold. (I, incubation chamber; M, manometer; B, valve on bypass loop; P, pump; G, gas store; W, water tank; S, soda-lime trap; FM, flow monitors; V_1, V_2, needle valves, T_1, T_2, T_3, flow switches.)

using an electric bellows pump (4.5 l/min capacity) which had a bypass loop including a needle valve which could be used to govern the overall rate of flow through the apparatus. The gas circulation loop included a soda-lime trap and a 2 liter round-bottomed flask which acted as a gas store.

Operation

Plants were grown in the chambers to suitable size (20–30 days after planting for sorghum (*Sorghum bicolor* (L.) Moench) ± 20 cm tall). The chambers were closed around the plant stem with silicone rubber sealant and connected to the gas supply manifold. A layer of water can be maintained above the sealant to help prevent leakage. The complete system (except the electric pump) was submerged in a large water tank and tested for leaks by applying a slight positive pressure (< 10 mmHg). The overall flow rate was adjusted using the needle valve on the pump bypass loop. Uniform flow through each incubation chamber was established by adjustment of the needle valves attached to the inlet line of each chamber. The system was then purged with carbon dioxide for 15 min to ensure complete flushing. The loop was closed and a tap connection to the gas store opened so that as the carbon dioxide was circulated and absorbed by the soda-lime, the $^{15}N_2/O_2$ mixture was drawn in. Water was introduced into the reservoir as the $^{15}N_2/O_2$ mixture was withdrawn to maintain a constant pressure. $^{15}N_2$ gas can either be made from ^{15}N compounds [6] or purchased directly (Prochem, BOC, London, U.K.).

Gas replacement occurred rapidly (Fig. 3) when tested using a sand and dried, ground farmyard manure mixture (sand:FYM, 97.5:2.5 w:w) as the growth medium (CO_2 levels were less than 1% within 3 min). Recent tests have indicated that removal of the carbon dioxide requires a longer time (10 min) when soil is used as a growth medium. The gas was circulated over the soda-lime for a further 30 min to

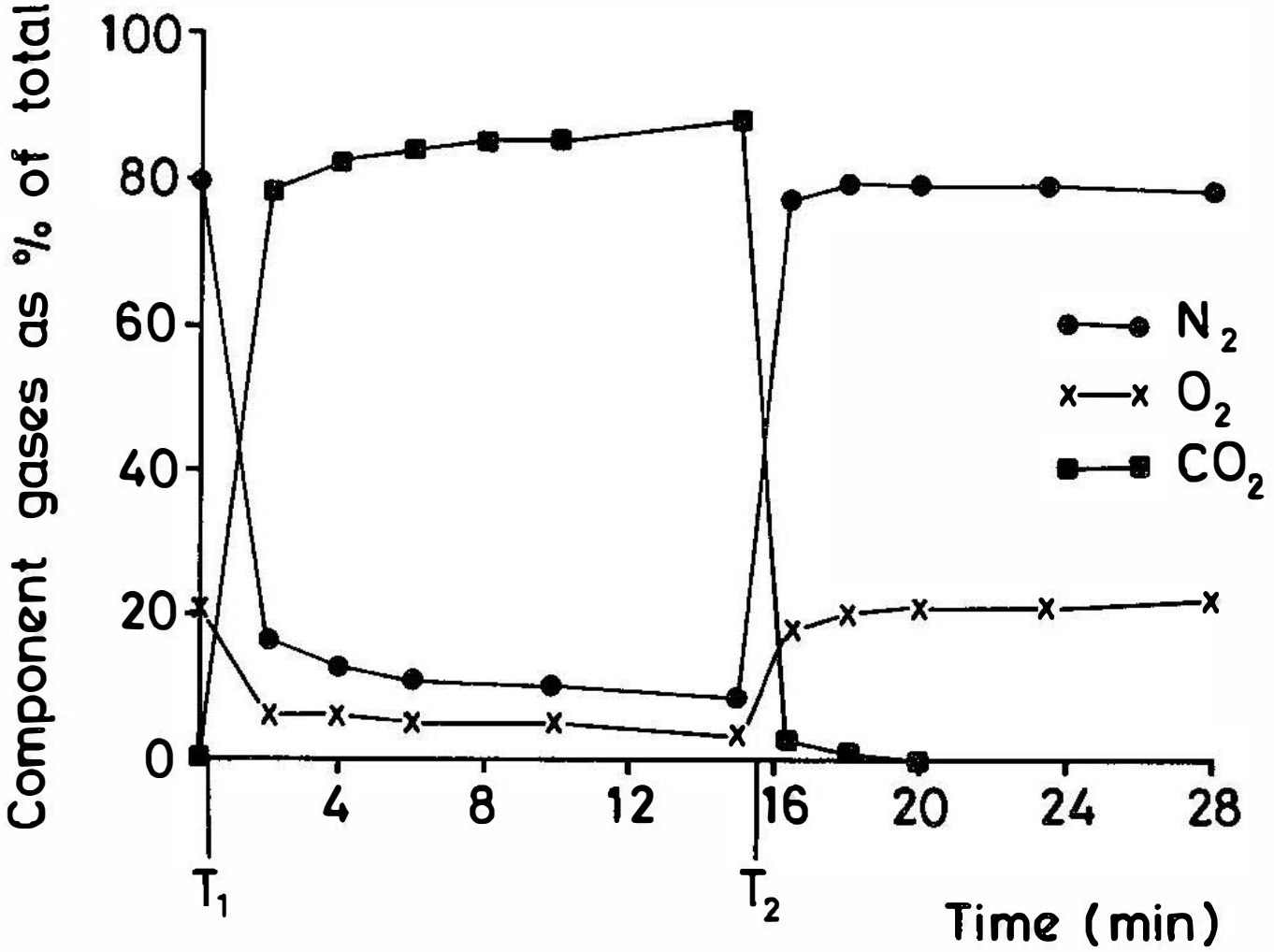

Fig. 3. Changes in gas composition in an incubation chamber during gas replacement. (T_1 = time at which carbon dioxide pinging step started; T_2 = time at which loop closed and tap opened to gas store.)

ensure complete removal of carbon dioxide, and the tap to the gas store was closed. It may be preferable to purge the chambers with an 80% carbon dioxide/ 20% oxygen mixture to prevent anaerobic conditions from developing in the incubation chambers, although there was no evidence of deleterious effects to the plants caused by the 15 min under carbon dioxide. Every 6–8 h gas samples were taken from the incubation chambers and O_2, N_2 and CO_2 levels measured using a Gow-Mac Series 550 thermal-conductivity gas chromatograph (Gow-Mac Instrument Co., Madison, U.S.A.). Oxygen was replenished as necessary and carbon dioxide removed by circulating the gas mixture through the soda-lime trap with the tap to the gas store opened to avoid pressure changes. Because it was not possible to make a completely gas-tight seal around the plant stems (probably due to gas diffusion through leaf sheaths) the ^{15}N enrichment declined slowly. The gas can be reclaimed by either purging with carbon dioxide or flushing with water. After recapture over water, in the first experiment described below, the enrichment of the gas declined from approx. 50% after introduction of the gas to approx. 30% after collection. When flushed with carbon dioxide the $^{15}N_2$ enrichment of the recovered nitrogen gas was too low for it to be used for a further experiment. The dilution was due in this case to $^{14}N_2$ contamination in the carbon dioxide (pure carbon dioxide (<8% nitrogen) was not obtainable when the experiment was car-

TABLE 2

INSTRUCTIONS FOR THE INTRODUCTION OF $^{15}N_2$ GAS MIXTURES TO THE APPARATUS SHOWN IN FIG. 2

(1) Ensure that the space around the plant stems is sealed using cotton wool and silastic and that the drain plugs and suba-seals are in place.
(2) Connect the chambers to the gas supply manifold clamping the brass unions firmly.
(3) Submerge the apparatus in a large water bath and apply a slight positive pressure (<10 mmHg) to check for leakage.
(4) Switch on the electric pump and adjust the gas flow through each chamber to give uniform rates of flow. The overall flow rate is controlled by the needle valve on the bypass loop (B), and flow through the individual incubation chambers by the needle valves for each chamber (V_1). Flow rates are monitored by the rate of bubbling through the flow gauges.
(5) Check that the flow switch (T_1) is switched to allow the carbon dioxide to flow to waste, turn on the carbon dioxide supply and adjust the rate of flow.
(6) Open the flow switches (T_1, T_2, T_3) to allow the carbon dioxide to flow through the apparatus for 10 minutes. (It may be necessary to readjust the settings of the needle valves slightly.)
(7) Turn the switches (T_1, T_2, T_3) to close the gas supply loop, open the needle valve (V_2) which controls the entry of the $^{15}N_2/O_2$ gas mixture and switch on the electric pump. N.B. It is *essential* that the valve (V_2) is opened before the pump is switched on, otherwise a large negative pressure will develop in the apparatus.
(8) Circulate the gas for 20 min to ensure complete absorption of the carbon dioxide and uniform mixing of the gas through the incubation chambers. The valve to the gas store (V_2) can then be closed.
(9) Every 6–8 h, 0.5 ml gas samples can be taken from the suba-seals of the incubation chambers and analyzed to check the levels of oxygen and carbon dioxide. Oxygen can be replenished as necessary and carbon dioxide removed by circulation over the soda-lime. After sampling or addition of gases the internal pressure can be normalized by opening the valve (V_2) connecting to the gas store.
(10) After incubation period the gas mixture can be reclaimed by purging with carbon dioxide, collecting the gas mixture and absorbing out the carbon dioxide.

ried out). It is probably preferable not to recover the gas mixtures by flooding with water as this is likely to alter the availability of fixed nitrogen for plant uptake. Full instructions for introduction of gas mixtures are given in Table 2.

Applications

The apparatus described here can be used to expose intact plant root systems to gas mixtures grown in various growth media. The apparatus can be set up for use without sophisticated laboratory facilities. It is cheap to construct and requires only a power supply for operation and the facility to check oxygen concentrations which can be carried out simply [5]. If automatic methods of monitoring gas concentrations are available such modifications can be included in the system. The number and size of incubation chambers used can be varied to suit different plant growth stages or species, depending on the availability of $^{15}N_2$ gas and the capacity of the pump.

Experimental Details

Sorghum CSH-5 seedlings were grown in 350 g sand/FYM (97.5:2.5 w:w) in incubation chambers. They were inoculated with a suspension of air-dried soil taken from the rhizosphere of field-grown sorghum plants. The plants were grown in a glasshouse at ICRISAT. After 21 days, when plants were approx. 20 cm tall, nine incubation chambers were sealed around the plant stem bases and connected to the gas supply manifold. Although the apparatus had been tested for use with ten chambers, only nine were used as voltage fluctuations reduced the capacity of the pump. During the incubation period, carbon dioxide and oxygen concentrations were maintained as described above. Mean daily evapotranspiration was measured over the previous week and deionized water added during the experiment to maintain the growth medium at approximately 70% water holding capacity (w.h.c.). After 3 days exposure the gas was recovered by flushing the apparatus with water. The tubes were disconnected from the gas supply manifold; five were harvested immediately and four grown for a further 9 days before harvest to examine uptake and transport of fixed nitrogen. A second set of nine plants was then attached to the manifold, exposed to the gas mixture for 3 days, and then the gas mixtures were removed by flushing with carbon dioxide. Five plants were harvested after 13 days further growth and four plants after 19 days. Roots were collected together with adhering soil to gain an estimate of ^{15}N in the rhizosphere. Dried, ground plant and soil samples were digested using a semi-micro kjeldahl procedure, and nitrogen contents estimated by an automated indo-phenol blue method [7] using a buffer [8]. Nitrogen in the digests was concentrated by a Conway microdiffusion technique and estimated for ^{15}N content using a Micromass 622 mass spectrometer (VG Isogas, Middlewich, Cheshire, England).

Results and Discussion

Results from the first experiment are presented in Table 3. Variation due to differences in both natural abundance and analytical errors can account for, at the most, ± 0.010 atom% ^{15}N excess [9]. There was a clear demonstration that detectable amounts of nitrogen were fixed by bacteria in the growth medium and that fixed nitrogen was incorporated into the plant roots and shoots within 3 days of initial exposure to the gas. After a further week of growth there was a higher enrichment of ^{15}N in the roots and the enrichment in the shoots had almost doubled (an increase from 0.056 to 0.102 atom% ^{15}N excess).

If the results are converted into μg N fixed using the formula

$$\text{N fixed } (\mu g) = \text{Total plant N } (\mu g) \times \frac{R \text{ plant}}{R \text{ gas phase}}$$

Where R = atom % ^{15}N excess

then we can estimate the actual amount of nitrogen, fixed during the 3 day exposure period, which was incorporated into the plant roots and shoots.

TABLE 3

^{15}N INCORPORATED INTO 21-DAY-OLD PLANTS OF SORGHUM CSH-5 AFTER EXPOSURE OF ROOT SYSTEMS TO $^{15}N_2$[a] FOR 72 h

Plant age at harvest (days)	Dry weight (mg/plant)	Total N (mg/plant)	Atom% ^{15}N excess	Fixed N[b] incorporated (μg/plant)
24				
Shoot	264[c] ± 7.2	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

[a] Mean enrichment 40.3 atom% ^{15}N excess.
[b] Calculated as Total N × atom% ^{15}N excess plant/atom% ^{15}N excess gas.
[c] Values are means of five and four replicates for the 24 and 33 day harvests, respectively, ± S.E.

It is obviously not possible to extrapolate amounts of nitrogen fixed by using $^{15}N_2$ incorporation over such a short time, to amounts fixed per plant in a season, with any accuracy. The amount fixed and incorporated by the 21-day-old seedlings is small (27 μg/plant) but the seedlings were exposed to $^{15}N_2$ gas for only 72 h. In a second experiment (Table 4) with slightly older plants grown for 13 days after exposure, there was more fixed N_2 incorporated (46 μg N/plant). In plants grown for a further 6 days the amount of N_2 incorporated was slightly but not significantly lower. It should be noted that rates of acetylene reduction activity are normally lower in soil than in the sand/FYM medium used in this experiment.

TABLE 4

^{15}N INCORPORATED INTO 25-DAY-OLD PLANTS OF SORGHUM CSH-5 AFTER EXPOSURE OF ROOT SYSTEMS TO $^{15}N_2$[a] FOR 72 h

Plant age at harvest (days)	Dry weight (mg/plant)	Total N (mg/plant)	Atom% ^{15}N excess	Fixed N[b] incorporated (μg/plant)
41				
Shoot	526[c] ± 21.5	8.1 ± 0.52	0.067 ± 0.019	33 ± 8.2
Root	580 ± 42.6	5.5 ± 0.28	0.040 ± 0.005	13 ± 2.4
Total	1106 ± 51.8	13.6 ± 0.71		46 ± 8.4
47				
Shoot	634 ± 19.7	9.1 ± 0.93	0.033 ± 0.003	19 ± 2.7
Root	1021 ± 54.9	8.7 ± 0.49	0.024 ± 0.003	13 ± 1.6
Total	1655 ± 62.8	17.8 ± 1.14		31 ± 3.5

[a] Mean enrichment 16.5 atom% ^{15}N excess.
[b] Calculated as total N × atom% ^{15}N excess plant/atom% ^{15}N excess gas.
[c] Values are means of five and four replicates for 41 and 47 day harvests, respectively, ± S.E.

TABLE 5

INDIVIDUAL REPLICATE ANALYSES OF PLANTS FROM THE 24 DAY HARVEST

Shoot weight (mg)	Root[a] weight (mg)	Shoot nitrogen (mg)	Root nitrogen (mg)	Atom% ^{15}N excess shoot	Atom% ^{15}N excess root
280	254	6.7	3.9	0.053	0 065
278	216	7.9	3.7	0.049	0.061
266	263	7.4	4.9	0.077	0.063
252	298	6.3	4.8	0.042	0.054
243	200	6.7	3.8	0.061	0.051

[a] 'Root' includes closely adhering soil — see text.

This was the first demonstration of plant uptake of nitrogen fixed in a growth medium by sorghum roots and incorporation into the plant using $^{15}N_2$ gas. The results demonstrate that nitrogen fixation was taking place and that fixed nitrogen was freely available for uptake by the plants. Incorporation was similar in all of the plants examined (Table 5) and there was not as much heterogeneity between plants as is commonly found when sorghum plants are assayed for acetylene reduction activity. The apparatus described is simple to operate and works well. Further work is continuing using this technique to investigate uptake of fixed nitrogen by sorghum and millet.

Acknowledgements

We thank Angela Davitt and M. Babu Rao for technical assistance and Dr. J.F. Witty for helpful discussions. This work was funded through Research Scheme R3648 of the Overseas Development Administration.

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