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CONFIRMATION OF NITROGEN FIXATION IN TWO TROPICAL GRASSES BY $^{15}N_2$ INCORPORATION

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Summary—Intact soil cores containing plants of *Paspalum notatum* or *Digitaria decumbens* were selected with the acetylene reduction method, and then exposed to $^{15}N_2$ to confirm nitrogen fixation in tropical grass-bacteria associations. In a preliminary experiment with *P. notatum* $^{15}N_2$ incorporation was slow but progressive during 24 h in roots but translocation to rhizomes and leaves ceased after 17 h. With improved assay chambers, enrichments of 0.151 and 0.563 ^{15}N atom % excess were obtained in roots of *D. decumbens* cv transvala and *P. notatum* systems respectively, after 3 days. Enrichments in rhizomes were similar to those of roots; however in the leaves only 8% of root enrichment was observed. The addition of sucrose to the soil doubled N_2-fixation in roots in both grass species studied, but did not result in increased incorporation into the leaves of *P. notatum*.

INTRODUCTION

Estimates of nitrogen fixation through the acetylene reduction method [N_2-fixation (C_2H_2)] indicate the possible economic importance of various tropical grasses associated with bacteria. *In situ* assays in Ivory Coast savannas indicated that relatively small losses of N in equilibrium systems can be replaced by N_2-fixation in grass associations (Balandreau and Willemin, 1973). Tropical C_4 forage grasses, fertilized with phosphorus and molybdenum, showed potentials of N_2 fixation (C_2H_2) of up to 1 kg N $ha^{-1}.day^{-1}$ during the major growing season in summer (Döbereiner and Day, 1975; Day *et al.*, 1975). Maize and sorghum showed peak N_2-ase (C_2H_2) activities at flowering stage which might be even higher (Bulow and Döbereiner, 1975). N_2-fixation (C_2H_2) in sugar cane was shown by Döbereiner *et al.* (1972a). Ruschel *et al.* (1975) obtained very high $^{15}N_2$ incorporation in one group of seedlings of sugar cane but none in others.

The present paper confirms consistent nitrogen fixation with the use of $^{15}N_2$ in two of the grasses: *Paspalum notatum* and *Digitaria decumbens*.

MATERIALS AND METHODS

Plant material

All plants were collected in the experimental fields of EMBRAPA, Rio de Janeiro. For the first experiment in 1974, cylindrical cores of soil containing long-established plants of *Paspalum notatum* cv batatais were placed in bottles whose bottoms had been removed. The bottle necks were closed with subaseals for exposure to C_2H_2 or $^{15}N_2$. After equilibration for 24 h in air, C_2H_2 reduction rates of such cores were approximately linear with time in the late morning hours. The most active cores were selected (194, 142, 115 and 64 n-moles C_2H_2/core.h^{-1}) and transported to CENA, Piracicaba, where they were placed in a growth chamber.

The remaining experiments were performed with plants collected in 1975 from the fields of EMBRAPA, Rio de Janeiro. Twelve out of 34 steel cores of 10 cm dia and 16.5 cm depth were selected after exposure to C_2H_2 in plastic bags. The mean N_2-ase activity of these selected cores was much higher: 1465 ± 85, 1326 and 1200 ± 82 n-moles C_2H_4/core.h^{-1} for *D. decumbens* cv transvala and slenderstem (one only) and *P. notatum* respectively. After transportation to Piracicaba, the plants from the steel cores were transferred with soil into small (4 × 7 cm) jars which were used for $^{15}N_2$ incubations three days later in experiment 3 and after 15 days in experiment 4.

Soils used in the cores were classified as Planosol ser. Ecologia (Mendes *et al.*, 1954) which are sandy with very low fertility. A basic fertilizer treatment was applied, consisting in parts/10^6 of 40 P (KH_2PO_4), 50 K (KH_2PO_4), 47 Ca ($CaCl_2.H_2O$), 15 Mg ($MgSO_4$), 0.05 B (H_3BO_3), 4 Cu ($CuSO_4.5H_2O$), 5 Fe ($FeSO_4.7H_2O$), 0.2 Mo ($Na_2MoO_4.2H_2O$) and 2.4 Zn ($ZnSO_4.7H_2O$). Manganese was not added because these soils contain excessive amounts of it (Döbereiner and Alvahydo, 1966).

Assemblies for $^{15}N_2$ exposures

Soil cores with plants, in the first experiment were incubated in the bottomless bottles of 1 l. capacity, the bottoms being closed with paraffin wax. The soil volume in these jars was 8 cm dia and 11 cm deep. In the second and third experiments, 3 l. Pyrex glass incubation vessels were used and in the fourth a 4 l. vessel of lucite. This last vessel had the disadvantage of liberating gases with a lucite smell. In the 3 l. vessel, four small soil/plant cores (4 cm width and 7 cm depth) could be accommodated, while the 4 l. lucite vessel has space for seven small cores.

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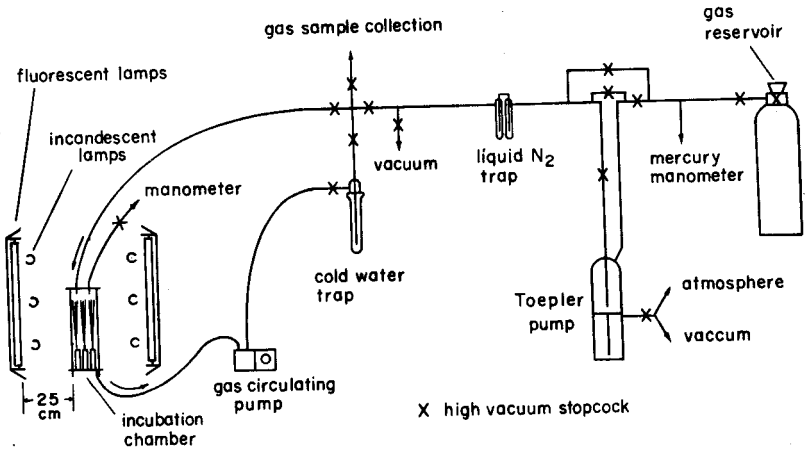

Fig. 1. Schematic diagram of apparatus used for exposure of plants to $^{15}N_2$ for experiments presented in Tables 1–3.

$^{15}N_2$ was prepared from $(^{15}NH_4)_2SO_4$ by addition of alkaline hypobromite *in vacuo*. The liberated N_2 gas was transferred to a storage vessel, passing through a liquid N_2 trap to remove traces of N_2O, NO or NH_3. The gas line assemblies are schematized in Fig. 1. The cold water trap eliminated excess water from the vessels. The assay vessels were evacuated to 133–655 Pa and refilled with a prepared gas mixture of $^{15}N_2$, CO_2, O_2 and Ar, whose composition is given in the tables for each experiment and in Fig. 2. To simulate soil conditions the ratio CO_2/O_2 in the assay vessels was higher than that of air. In the first experiment a gas mixture similar to air was used and the low rates of N_2-fixation observed were attributed to the direct access of air to the N_2-fixation sites after evacuation. For this reason in the following experiments lower pO_2 and higher pCO_2 were used although this might have affected normal plant metabolism.

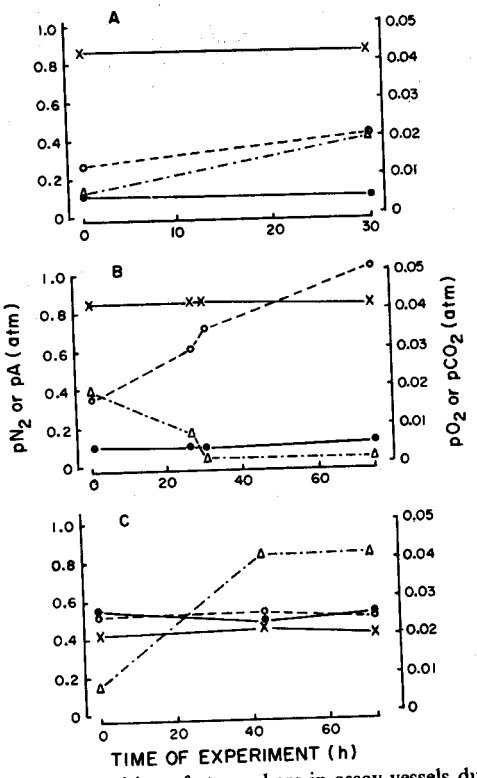

Fig. 2. Composition of atmosphere in assay vessels during the three experiments presented in (A) Table 1, (B) Table 2 and (C) Table 3. (×) N_2; (●) Ar; (○) O_2; (△) CO_2.

In the first experiment, the bottles were incubated under overhead fluorescent lights with daylight supplement (30,000 lx). The illumination cycle was 9 h light, 6 h dark, and 11 h light. In the remaining experiments, illumination was from both sides (Fig. 1) (40,000 lx) with eight fluorescent lamps, supplemented with six incandescent bulbs. Ambient temperatures varied between 30 and 36°C during the light periods and between 25 and 28°C during the dark periods. Day/night cycles were 15/9 hours and the duration of each experiment is given in the tables.

Preparation of plant material and isotopic analysis

Plants were separated from the soil, washed carefully, dried at 65°C, ground and the nitrogen liberated by the Dumas method (Proksch, 1969). Isotopic analysis was performed in a Varian-Mat model CH-4 mass spectrometer. During mass spectrometer analysis precautions were taken against the presence of compounds interfering in the isotopic ratio measurements. In cases of doubt, the samples were frozen with solid CO_2 or liquid N_2 on the inlet system of the mass spectrometer.

RESULTS AND DISCUSSION

In the first experiment (Fig. 3), (without replication) there was low but progressive $^{15}N_2$ incorporation in *Paspalum* plants exposed in intact soil/plant cores. Incorporation into the leaves appeared to cease after 17 h. In this experiment, O_2 concentrations close to those of air were used to fill the evacuated bottles, but by the end of the experiment most of the O_2 had been used up and the CO_2 concentration was more than ten times the initial, which was already high (0.6%).

In a second experiment (Table 1) *P. notatum* roots were removed from the soil and treated with sucrose solution. Enrichments obtained during subsequent exposure to $^{15}N_2$ showed highest N_2-fixation in roots

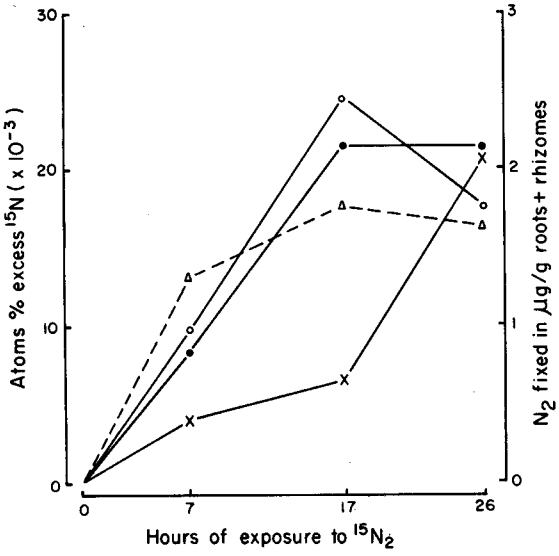

Fig. 3. N_2 fixation and incorporation in *Paspalum notatum* cores exposed to a gas mixture of 50% $^{15}N_2$ (96% atoms %^{15}N) 20% O_2 and 30% Ar for a 9 h light, 6 dark and 11 h light period. Atoms % excess $^{15}N_2$ in (×) roots (○) rhizomes (●) leaves; µg N_2 fixed per g roots + rhizomes (△---△).

remaining attached to the rhizomes but with leaves removed, while isolated roots were least active. C_2H_2 reduction tests had shown similar results with *P. notatum* (Day and Döbereiner, unpublished).

In Table 2, results from a well replicated experiment with intact soil/plant systems of *D. decumbens* show that with this species also, significant $^{15}N_2$ enrichment can be obtained. The incorporation into roots exposed for 3 days without any amendment, was considerably higher than in the first experiment but there was less translocation into the leaves. The low initial pO_2 which was used to avoid excessive O_2 infiltration after evacuation, may have affected normal plant metabolism, although gas phase analyses during the experiment indicate that the pO_2 increased from 0.015 to 0.05 atm (Fig. 2). It is uncertain whether the ^{15}N recovered in the rhizomes was translocated from the roots or fixed in the rhizomes. Rhizomes of the *Digitaria* plant (also called stem bases) have been observed to reduce C_2H_2 as fast as roots (Döbereiner and Day, 1976).

The results in Table 3 which were obtained with plants well established in the pots (2 weeks) confirm definitively the N_2 fixation in these two grasses which has been shown repeatedly by the indirect C_2H_2 reduction method (Döbereiner *et al.*, 1972b; Day *et*

Table 1. $^{15}N_2$ incorporation into *Paspalum notatum* plants removed from the soil and enriched with sucrose*

System	Part of the plant	Dry weight (mg/pot)	Total N (mg/pot)	Atoms % excess ^{15}N	N_2 fixed µg/pot	N_2 fixed (µg/g roots + rhizomes)
Intact	Roots	955	4.17	0.022	0.97	
	Rhizomes	924	5.03	0.009	0.48	
	Leaves	946	12.59	0.001	0.13	
	Total	2825	21.79		1.58	0.84
Leaves removed	Roots	724	3.45	0.123	4.47	
	Rhizomes	1155	4.95	0.035	1.82	
	Total	1879	8.40		6.29	3.35
Each part separate	Roots	256	1.41	0.001	0.02	
	Rhizomes	1065	5.44	0.020	1.15	
	Leaves	632	7.56	0	0	
	Total	1953	14.41		1.17	0.88

* Each vessel contained parts of several plants which were incubated 30 h under gas mixture containing 85.2% N_2 ($^{15}N_2$ 95.0% enriched), 2.2% O_2, 1.8% CO_2, 10.8% Ar. The roots were immersed in a 0.5% sucrose solution before incubation.

Table 2. N_2 fixation and incorporation in *Digitaria decumbens* cv transvala*

Part of plant	Dry weight (mg/pot)	Total N in plants (mg/pot)	Atoms % excess ^{15}N	N_2 fixed (µg/pot)	N_2 fixed (% of total)	N_2 fixed (µg/g roots + rhizomes)
Roots	382	2.03	0.186 ± 0.018**	3.75 ± 0.50**	46.5	
Rhizomes	557	3.62	0.073 ± 0.016	3.05 ± 1.23	38.0	
Stems	836	4.65	0.018 ± 0.005	0.83 ± 0.23	11.0	
Leaves	735	6.63	0.007 ± 0.0012	0.43 ± 0.08	5.5	
Total	2510	16.93		8.06	100.0	9.10

* Values are means of 4 pots exposed in one jar for 77.5 h (15 h light, 9 h dark periods) to a gas mixture containing 84.5% N_2 (enrichment $^{15}N_2$ 91.7%), 3.7% O_2, 0.6% CO_2 and 11.2% Ar. Each pot was analysed separately and the C.V. for the mass spectrometer analysis was 0.40 – 2.62%.

** Standard deviation of the mean.

Table 3. N_2 fixation and incorporation in *Digitaria decumbens* and *Paspalum notatum* grown in pots for 2 weeks*

Plant species and amendment	Part of plant	Dry weight (mg/pot)	Total N in plants (mg/pot)	Atoms % excess ^{15}N	N_2 fixed		
					(μg/pot)	(% of total)	(μg/g roots + rhizomes)
D. decumbens	Roots	340	1.78	0.151	2.69	33.1	
cv. transvala	Rhizomes	416	2.74	0.146	4.00	49.3	
without	Stems	1107	4.32	0.021	0.91	11.2	
sucrose	Leaves	911	7.34	0.007	0.51	6.3	
	Total	2774	16.18		8.11	99.9	10.73
D. decumbens	Roots	369	1.79	0.276	4.94	32.0	
cv. transvala	Rhizomes	862	5.68	0.154	8.75	56.7	
with 0.5%	Stems	914	4.06	0.012	0.49	3.2	
sucrose	Leaves	1051	7.77	0.016	1.24	8.1	
	Total	3196	19.30		15.42	100.0	12.52
D. decumbens	Roots	432	1.40	0.582	8.15	21.0	
cv. slenderstem	Rhizomes	719	4.06	0.709	28.79	74.3	
with 0.5%	Stems	496	1.80	0.073	1.31	3.4	
sucrose	Leaves	678	4.74	0.010	0.47	1.2	
	Total	2325	12.00		38.72	99.9	33.64
P. notatum	Roots	659	2.89	0.563	16.34	47.5	
cv. batatais	Rhizomes	747	2.62	0.703	15.28	43.8	
without	Leaves	692	6.03	0.070	2.97	8.6	
sucrose	Total	2098	11.54		34.59	99.9	25.39
P. notatum	Roots	585	2.71	1.021	27.56	38.8	
cv. batatais	Rhizomes	805	3.25	1.392	44.39	58.5	
with 0.5%	Leaves	501	3.85	0.053	2.06	3.1	
sucrose	Total	1891	9.81		74.01	100.4	43.33

* Values from *D. decumbens* are from single pots, but those from *P. notatum* from duplicate pots. All pots were incubated in the same jar for 72 h (15 h light and 9 h dark periods) in a gas mixture containing an average 42.8% N_2 (enrichment $^{15}N_2$ 85.5%), 2.6% O_2, 3.2% CO_2 and 51.4% Ar. The C.V. for the mass spectrometer analysis was 0.40 – 2.62%.

al., 1975). The presence in the assay vessels of pots with sucrose amendment resulted in high CO_2 concentrations (Fig. 1). It is unlikely that this would cause drastic changes such as those observed in soybeans (Havelka and Hardy, 1974) because in the C_4 grasses, photosynthesis should not respond in the same way to increased CO_2 concentrations.

In the *Paspalum* pots the N_2-fixation was so high that considerable amounts were translocated to the leaves. The effect of added sugar was pronounced especially in *P. notatum* roots and rhizomes but did not result in increased incorporation of $^{15}N_2$ in leaves and stems. This indicates fixation of N_2 by rhizosphere organisms on the root surface, which was not readily available.

The results presented confirm N_2 fixation in two species of tropical grasses transplanted from the field. The isotope test also showed that the fixed N was incorporated in the plant tissue. To our knowledge this is the first direct evidence of N_2 fixation in tropical forage grasses in amounts to be measured far beyond the analytical and experimental error. The system used permits reproducible results and could be extended to other plant/bacteria associations. However, it is difficult to extrapolate from these results to rates of N_2 fixation which might be occurring in the field. Experiments where 24 h C_2H_2 reduction rates are compared with $^{15}N_2$ incorporation over an equivalent period seem desirable to determine the N_2:C_2H_2 calibration. In view of the high enrichments obtained, shorter incubation periods (24 h or less) are possible and should yield more reliable results.

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